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# Inhibitory effects of NSAID-conjugated SN-38 on the viability of A549 Non-small cell lung cancer cells

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ABSTRACT

The goal of this paper was to look into the anti-tumor mechanism of Non-Steroidal Anti-Inflammatory Drug (NSAID)-conjugated SN-38 Prodrug in A549 lung cancer cells. We found that Indomethacine–SN–38 (IndoSN-38) and Naproxen–SN–38(NaproSN-38) as a theranostic prodrug targeting cyclooxygenase-2(COX-2) in cancer cells inhibited A549 cell viability in a dose-dependent fashion. IndoSN-38 and NaproSN-38 inhibited A549 cell viability in a dose-dependent fashion. The suppression of A549 cell viability was due to induction of the cell apoptosis by enhancing the activities of Caspase 3 and Caspase 8. The cell cycle arrest of sub-G1 was found in the cells treated with IndoSN-38 or NaproSN-38. Collectively, these data suggested that the anti-proliferative activities of the NSAID-conjugated SN-38 prodrugs were due to promotion of cell death and arresting the cell cycle which was similar with those of SN-38.

## 1. Introduction

Inflammatory diseases and carcinogenesis in the human body are related to COX-2, which has been shown to promote tumor angiogenesis, tissue invasion, and resistance to apoptosis, and is found in most solid tumors such as colon, liver, pancreas, breast, and lung cancers [1,2]. For this reason, NSAIDs are currently used for cancer treatment [3]. It is known that Prostagladin E2 (PGE2) plays an important role in cancer development because it shows remarkably high levels in cancer tissues [1,4]. PGE2 exists inside cells and comes out through cell membranes, and then enters other cell membranes. It enters the interior through the EP1, EP2, EP3, and EP4 receptors [1,4,5]. Introduced PGE2 activates c-Jun N-terminal kinase (JNK), RAS, and AKT signaling mainly through EP2 and EP4 receptors, stimulates COX-2 gene transcription factors to increase COX-2 expression, and more inflammatory substances are produced leading to cancer development [5–7]. Irinotecan is an anticancer drug with a wide range of activities characterized by complex pharmacology [8]. It is mainly active in solid tumors (including brain cancer, stomach cancer, colon cancer, pancreatic cancer, lung cancer and ovarian cancer) [6,8]. Irinotecan is converted to SN-38 in the body and plays a role in anticancer efficacy and cytotoxicity [8–10]. When Irinotecan enters the human body, it is converted to SN-38 by hydrolysis of Carboxylesterase 2(CES2), a carboxylesterase. Irinotecan undergoes cytochrome 3PA4(CYP3A4) oxidation in the liver to produce inactive metabolites APC and NPC [8]. SN-38 is a topoisomerase I inhibitor that binds to DNA Topoisomerase I to form a complex, inhibiting the original role of Topoisomerase I, DNA twist prevention, resulting in double-stranded DNA breakage and cell cycle (M, S phase) arrest and cause cell death [8,11]. SN-38 has 100 to 1000 times stronger anticancer effect and cytotoxicity than Irinotecan [7,9,12]. Despite its strong anticancer effect, SN-38 cannot be used directly because of its solubility and toxicity [7,9,12].

In our previous works, various small-molecule-conjugated SN-38 derivatives have evolved into a promising approach for enhancing anticancer efficacy by facilitating tumor microenvironment-specific activation or targetability [13,14]. Due to the varied physicochemical characteristics of each drug used in cancer treatment, these strategies have been found to address variable pharmacokinetics in the circulatory system [15]. In particular, the use of COX-2 inhibitors, which have shown potential antiangiogenetic effects in combination with anticancer drugs [16]. Besides, as a targeting strategy, COX-2 inhibitors could play a significant role as a targeting moiety, particularly in the case of the

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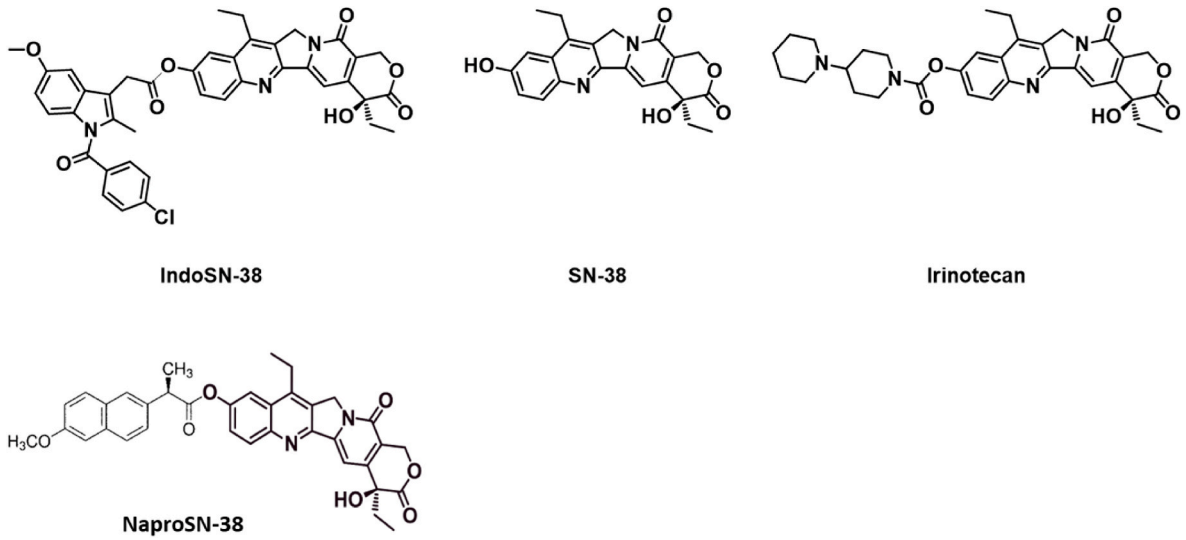
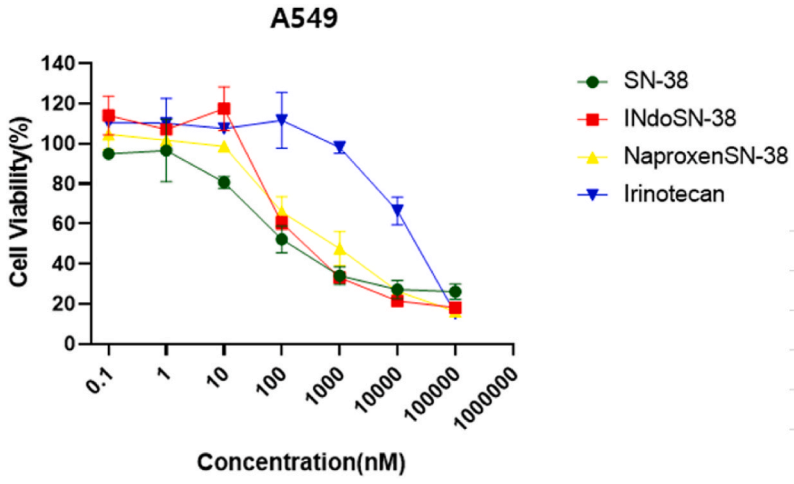



**Fig. 1.** NSAID based Theranostic Prodrugs for targeting COX-2 overexpressed Tumor Cells.




| Sample | IC50(μM) |
|---|---|
| SN-38 | 0.168±0.27 |
| IndoSN38 | 0.201±0.21 |
| naproxenSN38 | 0.206±0.075 |
| Irinotecan | 13.99±7.67 |

**Fig. 2. Inhibitory effect of SN-38, IndoSN-38, NaproSN-38, Irinotecan on A549 cell viability.** The cells were incubated with various concentration of drugs for 48hr, after which cell viability were performed use MTT assay. Data represent Means ± S.D (n = 3). **P < 0.01.

heterogeneous tumor microenvironment [17]. Herein, to this end, NSAID-conjugated SN-38 prodrugs, IndoSN-38 and NaproSN-38, were developed and examined anti-tumor function compared to SN-38 to minimize these side effects as well as to maintain its anti-cancer effect by using the COX-2 biomarker target (Fig. 1).

## 2. Materials and methods

### 2.1. Materials

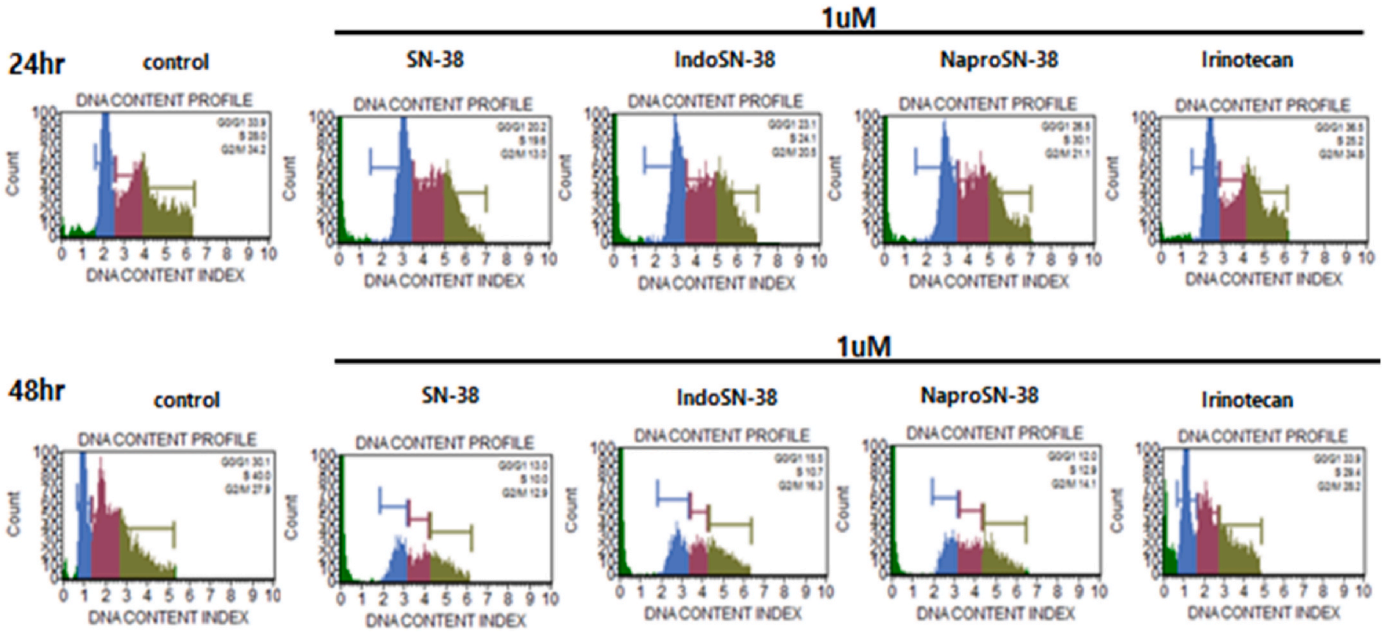
IndoSN-38, NaproSN- 38, Irinotecan, and SN38 were provided by Professor Jong Seung Kim, Department of Chemistry, Korea University, Seoul, Korea. Trypsin-EDTA (Welgene), FBS (Welgene), antibiotic (Welgene), MTT[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (Amresco), Muse™ Annexin V and Dead cell reagent, Muse™ Cell cycle Kit (Merck Milipore, Billerca, MA, USA), and Caspase-3. Caspase-8 (BD biosciences, USA) were purchased. Muse™ Cell Analyzer (Merck Milipore, USA) and Spectra Max iD3 microplate reader (Molecular Devices, USA) were used.

### 2.2. Cell culture

A549 (Human Non-Small Cell Lung Cancer cells) were cultivated in Roswell Park Memorial Institute 1640 (RPMI1640) in the presence of Fetal Bovine Serum (FBS) and antibiotics (Welgene, Daejeon, Korea). The cell cultures were maintained in 5% $CO_2$ and 37 °C under a humidified air.

### 2.3. Cancer cell viability assay

A549 cells ($2 \times 10^3$cells/well) were seeded into 96-well plates and incubated for 24 h at 37 °C to adhere. The cells were treated with NaproSN-38, SN-38, Irinotecan, and IndoSN-38 and incubated for 48 h. Then, 50 μl of a MTT solution (1 mg/mL) was put into 96 well plates containing the cells and cultured for 2 h at 37 °C. After removing the supernatant, the remaining formazan crystals were dissolved in 100 μl of Dimethyl sulfoxide (DMSO). The absorbance of 96 well plates was measured in triplicate at a wavelength of 595 nm in an enzyme-linked immunosorbent assay(ELISA) plate reader (Emax, Molecular Devices, USA).




| Time/Sample | | sub-G1(%) | G0/G1(%) | S(%) | G2/M(%) |
|---|---|---|---|---|---|
| 24hr | Control | 3.8 | 48.6 | 29.3 | 18.3 |
| | SN-38 | 47.2 | 20.2 | 19.6 | 13 |
| | IndoSN-38 | 32.3 | 23.1 | 24.1 | 20.5 |
| | NaproSN-38 | 22.3 | 26.5 | 30.1 | 21.1 |
| | Irinotecan | 3.2 | 43.5 | 32.8 | 20.5 |
| 48h | Control | 2 | 30.1 | 40 | 27.9 |
| | SN-38 | 64.1 | 13 | 10 | 12.9 |
| | IndoSN-38 | 57.5 | 15.5 | 10.7 | 16.3 |
| | NaproSN-38 | 61 | 12 | 12.9 | 14.1 |
| | Irinotecan | 8.5 | 33.9 | 29.4 | 28.2 |

**Fig. 3. Cell cycle analysis of A549 cells treated with SN-38, IndoSN-38, NaproSN-38, and Irinotecan.** Cell cycle analysis was performed by Muse™ cell analyzer and by determining percentage of cell stage by different times (24, 48hr). Data represent means ± S.D (n = 3). *P < 0.05, versus control group.

### 2.4. Cell apoptosis and cell cycle analysis

The A549 cells were administered with NaproSN-38, SN-38, Irinotecan, and IndoSN-38 for 24 h or 48 h respectively. Cell apoptosis was examined by using the Muse™ Annexin V and dead cell kit. The cell cycle was analyzed by using Muse™ Cell cycle kit (Merck Millipore, Billerica, MA, USA).

### 2.5. In vitro caspase-3 and -8 activity assay

The A549 cells were harvested by using Trypsin-EDTA after treatment with NaproSN-38, SN-38, Irinotecan, and IndoSN-38 for 24 h or 48 h. Harvested cells were spun down, the supernatant was removed, and the residual cell precipitate was dissolved with lysis-M solution for 15 min on ice. After centrifugation of the lysed cells, protein concentration of the supernatant was determined. 100 μg of the protein was placed into 96-well plate and 1.0 M Dithiothreitol (DTT) was added to each well to a final concentration of 0.1 M. And the plate was incubated for 2 h at 37 °C after adding 5 μL of LEHD-pNA, a substrate of caspase-3 to each well. The optical density of the plate was determined at 405 nm by using an ELISA plate reader (Emax, Molecular Devices, USA).

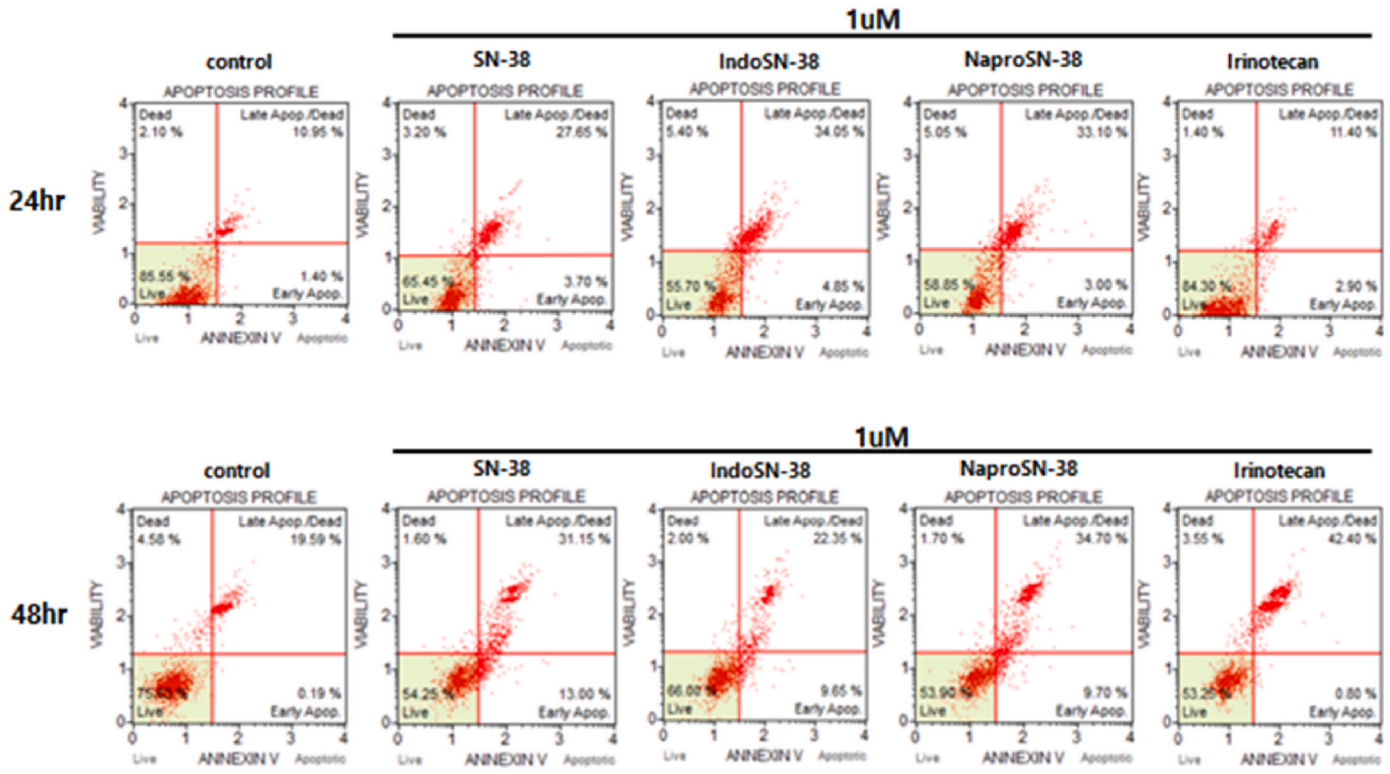
### 2.6. Statistical analysis

All results are analyzed as the mean ± standard deviation (SD) of the mean(SEM). Statistical comparisons were carried out for caspase activity data and ELISA results using one-way ANOVA with post hoc Dunnett's test. Statistical analyses were performed using Microsoft Office Excel version 2007 (Microsoft Corporation, Redmond, WA, USA). Differences were considered significant at a P-value ≤0.05.

## 3. Results and discussion

### 3.1. Inhibition of tumor cell viability by NaproSN-38, SN-38, irinotecan, and IndoSN-38

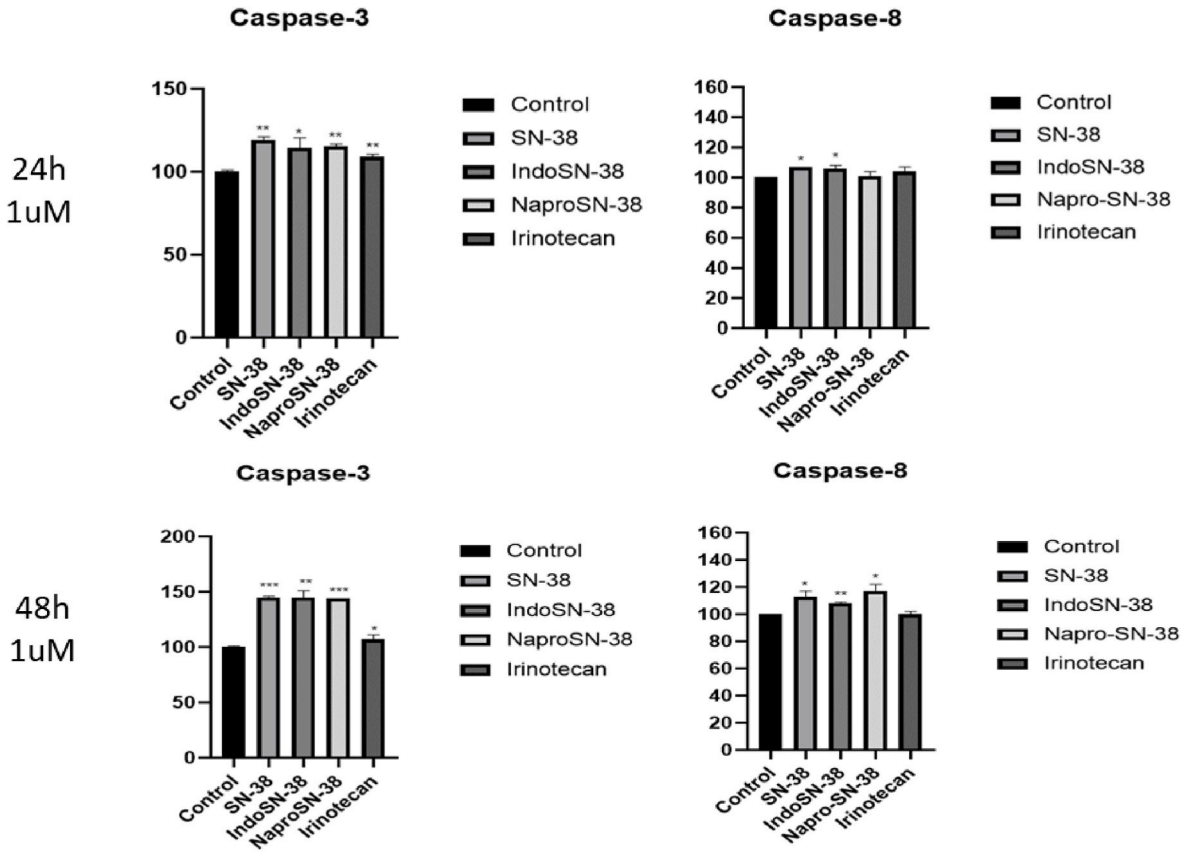
It was investigated that indomethacin and naproxen are nonselective NSAIDs, with 0.7 and 1.9 of COX-1/COX-2 $IC_{50}$ ratios, respectively [18].




| Apoptosis profile(%) | | Late Apoptosis | Early Apoptosis | Live | Dead |
|---|---|---|---|---|---|
| 24h | Control | 10.95 | 1.4 | 85.55 | 2.1 |
| | SN-38 | 27.65 | 3.7 | 65.45 | 3.2 |
| | IndoSN-38 | 34.05 | 4.85 | 55.7 | 5.4 |
| | Napro-38 | 33.1 | 3 | 58.85 | 5.05 |
| | Irinotecan | 11.4 | 2.9 | 84.3 | 1.4 |
| 48h | Control | 19.59 | 0.19 | 75.63 | 4.58 |
| | SN-38 | 31.15 | 13 | 54.25 | 1.6 |
| | IndoSN-38 | 22.35 | 9.65 | 66 | 2 |
| | Napro-38 | 34.7 | 9.7 | 53.9 | 1.7 |
| | Irinotecan | 42.4 | 0.8 | 53.25 | 3.55 |

**Fig. 4. Analysis of apoptosis in A549 Cells treated with SN-38, IndoSN-38, NaproSN-38, and Irinotecan.** A549 Cells were treated with the NSAID-conjugated SN-38 prodrugs to stimulate the cell apoptosis for 24 h and 48 h, respectively. Data represent means ± S.D (n = 3). *P < 0.05, versus empty vehicle-treated control group.

Therefore, we have estimated that both suggested drug conjugates, IndoSN-38 and NaproSN-38, comprising indomethacin and naproxen as a targeting moiety, would not have biochemical selectivity towards COX-1 and/or COX-2. To assess the effects of NaproSN-38, IndoSN-38, SN-38, and Irinotecan on the viability of A549 cells expressing COX-2, we employed *in vitro* cell proliferation assay. NSAID-conjugated SN-38 prodrugs suppressed the proliferation of A549 tumor cells in a concentration-dependent fashion. Half-maximal inhibition of A549 cell proliferation by the administration of SN-38, NaproSN-38, IndoSN-38 was determined at a dose of 0.168 ± 0.27 μM, 0.206 ± 0.075 μM, and 0.201 ± 0.21 μM, respectively, whereas the $IC_{50}$ value of Irinotecan was 13.99 ± 7.67 μM (Fig. 2). This result indicated that anti-proliferative effect of NSAID-conjugated SN-38 prodrugs, NaproSN-38, IndoSN-38 is similar to that of SN-38, but stronger than that of Irinotecan.




(B)

| Caspase activity | | Control (%) | SN38 (%) | IndoSN-38(%) | NaproSN-38 (%) | Irintecan (%) |
|---|---|---|---|---|---|---|
| Caspase-3 | 24h | 100±1.05 | 119±2.1 | 114±6.29 | 115±1.05 | 109±1.05 |
| | 48h | 100±1.17 | 145±1.17 | 145±5.84 | 144 | 107±3.51 |
| Caspase-8 | 24h | 100 | 107 | 106±2 | 101±3.1 | 104±3.1 |
| | 48h | 100 | 113±3.7 | 108±1.2 | 117±4.9 | 100±2.4 |

**Fig. 5. Analysis of Caspase-3, Casapse-8 activities by SN-38, IndoSN-38, and NaproSN-38 in A549 cells.** A549 cell treated with drug for 24 h, 48 h. The activities of Caspases −3, Caspase −8 were represent using (A) bar graphs (B) table data represent means ± S.D (n = 3). *P < 0.05, versus control group.

### *3.2. Analysis of cell cycle and apoptosis in A549 cells administered with NSAID-conjugated SN-38 prodrugs*

To further assess whether the suppression of the cell proliferation by the administration of NSAID-conjugated SN-38 prodrugs on A549 cells is due to the cell cycle changes, we performed analysis of cell cycle by using Muse™ cell analyzer. Forty-eight hour-administration of IndoSN-38, NaproSN-38, SN-38, and Irinotecan in A549 cells showed 64.1% of SN-38, 57.5% of IndoSN-38, 61% of NaproSN-38, 8.5% of Irinotecan, respectively in sub-G1 phase (Fig. 3). Analysis of the cell apoptosis was carried out by using the Muse® Cell cycle kit and Annexin V & Dead cell kit. A549 cells treated with NSAID-conjugated SN-38 prodrugs for 48 h revealed 13% of SN-38, 9.65% of IndoSN-38, 9.7% of NaproSN-38, and 0.8% of Irinotecan in early apoptosis(Fig. 4). This finding indicated that the early apoptosis rate of A549 cells treated with the NSAID-conjugated SN-38 prodrugs was significantly increased compared to Irrinotecan-administered cells and empty vehicle-administered control group. Several lines of these data demonstrated that the suppression of A549 cell proliferation by NSAID-conjugated SN-38 prodrugs was due to promotion of early stage apoptosis in the cells via arresting sub-G1 phase

of the cell cycle.

### 3.3. Activation of caspases −3 and −8 in A549 cells administered with the NSAID-conjugated SN-38 prodrugs

To verify the promotion of apoptosis in A549 cells administered with the NSAID-conjugated SN-38 prodrugs, we performed caspases (caspase-3 and -8) activity assay *in vitro*. Caspase-3 and -8 activities were significantly increased in A549 cell treated with NSAID-conjugated SN-38 prodrugs and SN-38 for 48 h compared to irinotecan-treated cells (Fig. 5). This result suggested that the NSAID-conjugated SN-38 prodrugs and SN-38 revealed to be further effective in increasing the caspase activities compared to Irrinotecan-treated group. Based on these data, it was demonstrated that the mechanism of anti-proliferative activities of NSAID-conjugated SN-38 prodrugs were similar to that of SN-38, but stronger than that of Irinotecan.

## 4. Conclusions

In the current study, NSAID-conjugated SN-38 anti-cancer agent we have developed represents a potential step forward for enhancing chemotherapy of the COX-2 expressed tumor. Indeed, IndoSN-38 and NaproSN-38 demonstrated outstanding anti-cancer efficacy in A549 lung cancer cell line with a statistically significant reduction in cell viability. The present study serves to understand the role that NSAID-conjugated SN-38 may play in overcoming poor therapeutic outcomes in contrast to single-modal anti-cancer agents.

## Authors' contributions

Conceptualization, ICK.; Data curation, HWK and JA; Formal analysis, HWK; Investigation, ICK; Methodology, JA and HWK; Project administration, ICK; Software, HWK; Validation, HWK; Writing—original draft, HWK; Writing—review & editing, JSK and ICK. All authors have read and agreed to the published version of the manuscript.

## Declaration of competing interest

The authors declare that they have no competing interests.

## Data availability

The data that has been used is confidential.

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