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# Effect of a novel piperazine compound on cancer cells

So Hyun Jeon and Cha-Gyun Shin\*

## Abstract

Many drugs have been developed for anticancer chemotherapy. However, more anti-cancer drugs should be developed from potential chemicals to circumvent the disadvantages of existing drugs. Most anti-cancer chemicals induce apoptosis in cancer cells. This study tested the efficiency of a new chemical, the piperazine derivative 1-[2-(Allylthio)benzoyl]-4-(4-methoxyphenyl) piperazine (CB01), on glioblastoma (U87) and cervix cancer (HeLa) cells. CB01 was highly cytotoxic to these cells ( $IC_{50S} < 50$  nM) and induced the traditional apoptotic symptoms of DNA fragmentation and nuclear condensation at 40 nM. Western-blot analysis of the cell lysates revealed that the intracellular apoptotic marker proteins, such as cleaved caspase-3, cytochrome c, and Bax, were highly upregulated in the CB01-treated cells. Furthermore, increased activities of caspase-3 and -9, but not caspase-8, were observed. Therefore, these results suggest that CB01 can act as an anticancer chemotherapeutic by stimulating the intrinsic mitochondrial signaling pathway to induce cytotoxicity and apoptosis in cancer cells.

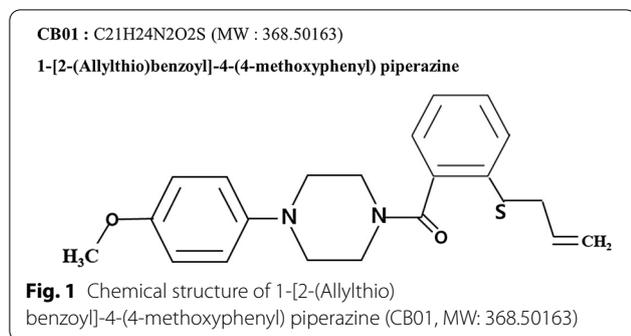
**Keywords:** Apoptosis, Human cancer cells, Piperazine derivative

## Introduction

Piperazine is a chemical compound in which two of the six carbons facing each other in a hexagon ring were replaced with nitrogen. It is an important compound widely used to develop therapeutics of interest against various diseases due to its availability, simple processing, and high yield [1–3]. Currently, there are several piperazine-based therapeutics, such as anti-depressants [4, 5] as well as anti-viral [6, 7] anti-inflammatory [8–11], and antioxidant [12, 13] agents. Given this high performance of piperazine, several academic and industrial research institutes have developed new piperazine derivatives as therapeutic agents [14, 15]. Recently, piperazine backbones synthesized via rational drug designing such as piperazine-linked bisanthrapyrazole, 5-hydroxychromenone piperazine, quinazoline linked substituted-piperazine have shown an excellent performance as anti-cancer drugs [1, 16, 17]. Each of these compounds

induces apoptosis and suppress the proliferation of cancer cells in erythroleukemic A562 cell line, epidermal cervical cancer, and lung cancer cells, respectively [1, 18]. Apoptosis is a natural and necessary process that maintains homeostasis in diverse organisms. Defects in apoptosis cause immunodeficiency, and genetic and autoimmune problems, eventually leading to cancer. Apoptosis is known to occur via two different pathways—the extrinsic pathway (also called the “death receptor pathway”) and the intrinsic pathway (occurs through mitochondria). However, these two pathways are connected, and the factors of one pathway can affect the other. The morphological changes associated with apoptosis in a cell include cell contraction, chromatin condensation, and nuclear and DNA fragmentation. Many currently used chemotherapeutics suppress cancer-cell survival by inducing apoptosis via the two main signaling pathways mentioned above [19, 20]. This study assessed for the anti-cancer effect of a recently synthesized piperazine compound, 1-[2-(Allylthio)benzoyl]-4-(methoxyphenyl) piperazine (CB01) (Fig. 1). This compound was identified as toxic to non-small cell lung cancer by Dr. SH Hong of

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the Korea Institute of Radiological Medical Sciences and reported as a potential anticancer compound and was selected from a chemical library obtained from ChemBridge (San Diego, CA, USA).

## Materials and methods

### Reagents

CB01 was obtained from ChemBridge (San Diego, CA, USA). Each dose of CB01 was diluted with 40% dimethyl sulfoxide (DMSO). The reagents 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lactate dehydrogenase (LDH) assay kit was purchased from Dongin Biotech (Soul, Korea). Caspase-3, -8, -9 assay kits, and Z-VAD-FMK, a caspase-3 inhibitor, were purchased from Promega (Madison, WI, USA). Antibodies against caspase-3, Bax, and cytochrome c were bought from Santa Cruz (Santa Cruz, CA, USA).

### Cell culture

U87 and HeLa cells (Korean Cell Line Bank, Seoul, Korea) were grown in Dulbecco's modified Eagle's Medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL Penicillin, and 100 g/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Both cells were cultured in an incubator at 37 °C with 5% CO<sub>2</sub>.

### MTT assay

The MTT assay was performed using a common protocol [21, 22]. Briefly, cells were seeded in 96-well plates (50 µl of 4 × 10<sup>4</sup> cells/well). After 4 h, 50 µl of fresh medium containing CB01 at the indicated dose was added. After 48 h, the MTT solution at 0.1 mg/mL was added into each well, and the samples were incubated for 4 h. After removing the supernatant, 200 µl of 100% (w/v) DMSO was added to each well, and the samples were incubated at room temperature (25 °C) for 10 min. Finally, the absorbance of the samples at 595 nm was measured using

a microplate reader. The experiment was repeated three times, each in triplicate.

### LDH cytotoxicity assay

The LDH assay was performed using the D-Plustm LDH Cell Cytotoxicity Assay Kit (Dongin LS Biotech, Seoul, Korea). Briefly, 50 µl of 2.5 × 10<sup>4</sup> cells per well were inoculated into a 96-well plate. After 24 h of culture, 50 µl medium with CB01 at the indicated concentration was added to each well. After 48 h, the cells floating in the culture were precipitated via centrifugation at 600 g for 5 min. The control group was treated with 10 µl of lysis buffer in the kit before the centrifugation, and 10 µl of the supernatant was transferred to a different well in new 96-well plate. Afterward, 100 µl of the LDH reaction mixture, composed of LDH buffer and water-soluble tetrazolium salt (WST) substrate at a 1:50 ratio, was added. The plate was cultured at 20–30 °C for 30 min, and the absorbance of the samples at 450 nm was measured using a microplate reader. The experiment was repeated three times, each in triplicate.

### Apoptotic DNA-fragmentation assay

Total DNA with low molecular mass was extracted from the cells as described previously [23]. First, the cells were grown in 60 mm plates. After 24 h, CB01 was administered at 10 or 40 nM, and the cells were cultured for 48 h. Afterward, they were washed with phosphate-buffered saline (PBS) and lysed using the ice-cold lysis buffer [0.2% Triton X-100, 10 mM Tris (pH 7.5), and 10 mM EDTA]. The lysates were incubated on ice for 40 min. Positive control was conducted using 5 µM camptothecin (CPT). The lysates were centrifuged at 10,000g at 4 °C for 30 min, and the harvested supernatants were treated with buffered phenol and buffered chloroform-isoamyl alcohol (24:1, v/v). DNA was ethanol-precipitated and then dissolved in TE buffer [10 mM Tris (pH 7.5) with 1 mM EDTA] with 50 µg/ml RNase A. The DNA samples were analyzed through electrophoresis on 1.2% agarose gels.

### Evaluation of nuclear morphology

The morphological changes in the nuclei of the cells treated with CB01 were examined through DAPI staining as described before [21]. U87 and HeLa cells were seeded in 35 mm plates (1.6 × 10<sup>5</sup> cells) and treated with 40 nM CB01. After 48 h of culture, the medium was removed, and the cells were washed three times with PBS. Next, they were fixed for 20 min at 25 °C with 4% formaldehyde containing 0.1% Triton X-100. The cells were then stained for 1 h at 37 °C with 300 µM DAPI diluted in PBS (1:100, v/v). The stained cells were observed using a Nikon fluorescence microscope (TE 2000 U; Tokyo, Japan) with ultraviolet (UV) excitation at 300–500 nm.

**Caspase activity assay**

The activities of caspase-3, -8, and -9 were examined using caspase-3, -8, -9 colorimetric assay kits (Promega, Biovision, USA) as described before [21, 23]. Cells were treated with 40 nM CB01 for 0, 24, or 48 h. They were then lysed using 50 µl lysis buffer, and the supernatants of the lysates were collected by centrifugation at 10,000g for 5 min. Subsequently, the total protein concentration of each supernatant was quantified using the Bradford assay. Then, a sample (3 µl) from each lysate was mixed with the 2 × buffer from the assay kit to reach the total volume of 50 µl, and 4 mM DEVD-pNA substrate from the assay kit was added. After incubating the samples at 37 °C for 1.5 h, their absorbance at 405 nm was measured.

**Western blotting**

Western blotting was performed as described before [24]. Cells seeded in 60 mm plates were treated with 40 nM CB01 and incubated for 48 h. The cells were then lysed using Radioimmunoprecipitation Assay buffer [0.1% SDS, 50 mM Tris–HCl (pH 7.4), 0.5% sodium deoxycholate, and 150 mM NaCl]. The lysates were centrifuged at 20,000g for 15 min at 4 °C, and the total concentration of each supernatant was measured using the Bradford assay. The proteins in each lysate sample were separated via Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) using 12.5% gel at 130 V for 1.5 h and then transferred onto nitrocellulose membranes (GE Healthcare UK Ltd., Hammersmith, UK) at 32 mA for 1.5 h by using semi-dry transfer equipment (Hoefer,

Inc., Holliston, MA, USA). The membranes were blocked with the blocking agent [5% (w/v) non-fat dry milk and 0.1% (w/v) Tween 20 in PBS] for 2 h at 4 °C. Lastly, the membranes were probed overnight with monoclonal antibodies against apoptosis-associated proteins [1: 1,000 dilution in PBS with Tween20 (PBST)]. After washing the membranes three times with PBST, they were incubated for 1 h at 25 °C with goat anti-mouse IgG conjugated to horseradish peroxidase (1: 5,000 dilution in PBST, Abcam). Afterward, the membranes were washed again with PBST and incubated with the developer kit (Bio FACT, Daejeon, Korea). As an internal control β-actin was probed with a mouse monoclonal antibody (1: 5000 dilution, Thermo Fisher Scientific, Waltham, MA, USA).

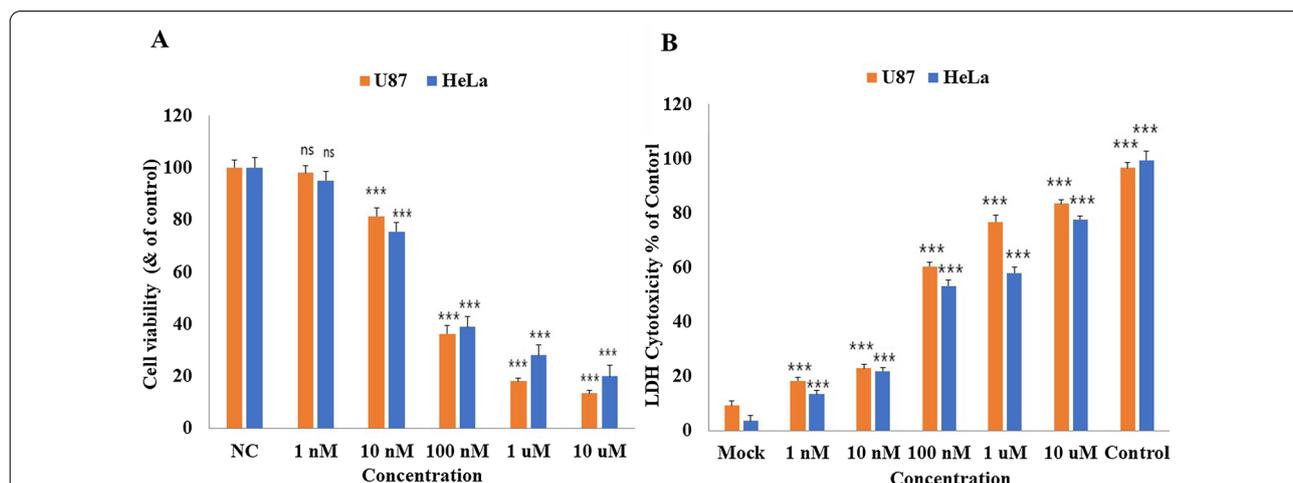
**Statistical analysis**

All the data are presented as mean ± SEM. Statistical significance was assessed using the *t* test with paired samples; \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001.

**Results**

**CB01 is cytotoxic**

CB01 significantly reduced the viabilities of U87 and HeLa cells, as assessed via the MTT and LDH assays (Fig. 2). For U87 cells, treatment with 1 nM, 10 nM, 100 nM, 1 µM, and 10 µM CB01 decreased the cell viability to 97.95 ± 2.9%, 81.38 ± 3.1%, 36.21 ± 3.37%, 18.13 ± 1.09%, and 13.37 ± 1.26%, respectively, of the untreated control cells. The same concentrations of CB01 decreased the viability of HeLa cells to 95.08 ± 3.45%,



**Fig. 2** Cytotoxicity of CB01. **A** U87 and HeLa cells were incubated in 96-well cell-culture plates with no drug, 1 nM, 10 nM, 100 nM, 1 µM, or 10 µM CB01. After 48 h, the cytotoxicity of CB01 was investigated by measuring the number of alive cells via the MTT assay or **B** by measuring the quantity of lactate dehydrogenase (LDH) released into the medium. The cell viability and quantity of released LDH are shown as percentages of the control levels (**A** untreated cells; and **B** complete lysis of untreated cells). Based on the dose–response curve, the IC<sub>50</sub> of CB01 was estimated at approximately 40 nM. All the data are presented as mean ± SEM. Statistical significance was evaluated using the paired sample *t* test; ns = *p* > 0.05, \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001

75.51 ± 3.38%, 38.91 ± 3.96%, 27.98 ± 3.9%, and 19.3 ± 4.13%, respectively, of the untreated control. The IC<sub>50</sub> of CB01 was assessed using dose–response curves and found to be approximately 40 nM in both cells (Fig. 2A). LDH is a cytoplasmic enzyme that exists in various cells and is released into the medium when the cell membrane is disrupted [25]. The LDH assay is a simple colorimetric method used to determine cytotoxicity accurately. For U87 cells, the LDH-based cytotoxicity levels of the cells treated with 0 nM (mock), 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM CB01 were 25.32 ± 1.82%, 33.08 ± 1.31%, 37.06 ± 1.43%, 68.74 ± 1.55%, 82.70 ± 2.28%, and 88.36 ± 1.58%, respectively, of the control, which was lysed untreated cells. In addition, the LDH-based cytotoxicity levels of HeLa cells were 19.62 ± 1.89%, 27.77 ± 1.48%, 34.89 ± 1.41%, 61.38 ± 2.22%, 65.44 ± 2.09%, and 81.61 ± 1.17%, respectively (Fig. 2B). As shown in Fig. 2, the LDH-assay results are in line with the MTT-assay results. Overall, these results show that CB01 is highly cytotoxic to U87 and HeLa cells.

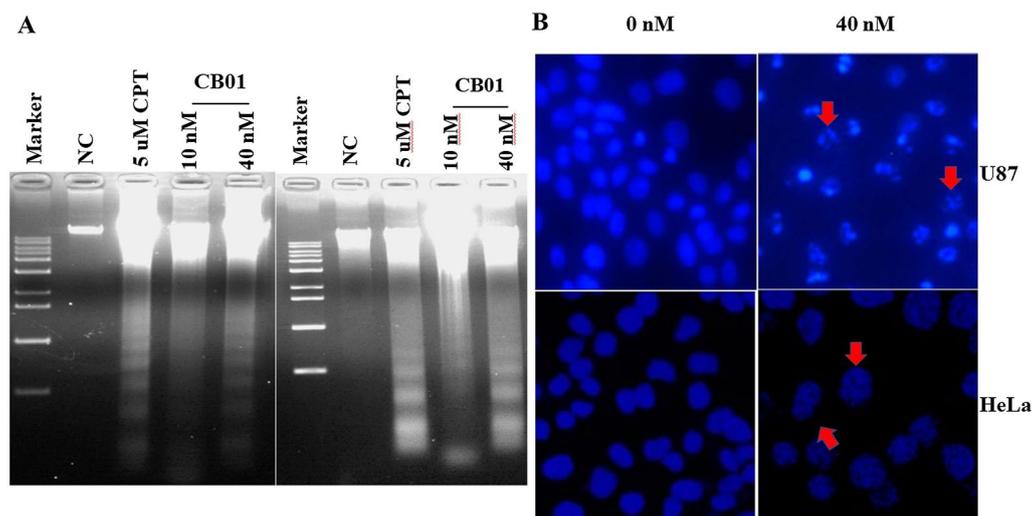
#### CB01 induces the apoptotic symptoms of DNA and nuclear fragmentation

To determine whether the cytotoxicities observed in Fig. 2 are related to apoptosis, CB01-treated cells were evaluated for DNA and nuclear fragmentation, which are indicators of apoptosis. Specifically, DNA fragmentation was observed in HeLa and U87 cells treated

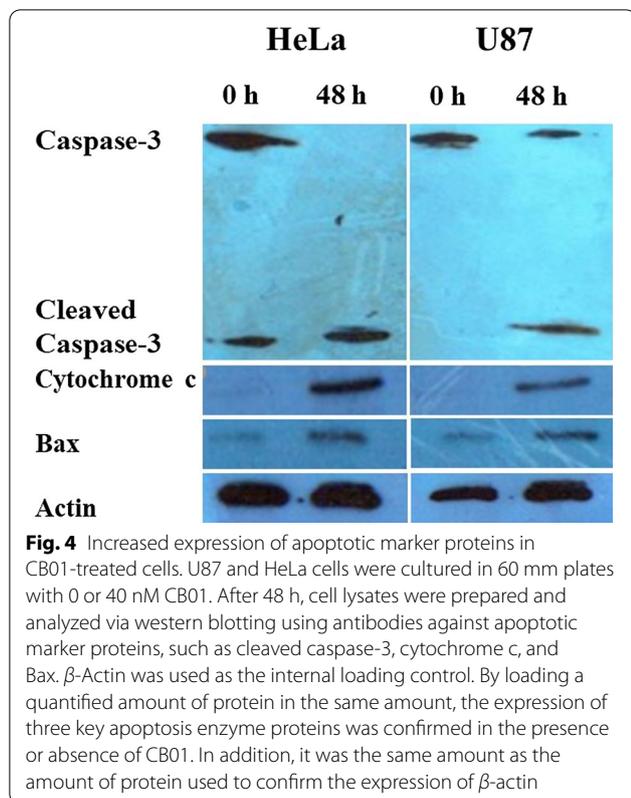
with 40 nM CB01 (Fig. 3A). In this experiment, 5 μM camptothecin (CPT), which is an alkaloid that is a well-known drug that causes apoptosis by selectively inhibiting DNA topoisomerase type 1, was used as a positive control [26]. CB01 induced DNA fragmentation very clearly at 40 nM. In addition, DAPI staining revealed that CB01 caused nuclear fragmentation in U87 and HeLa cells (Fig. 3B).

#### CB01 upregulates the major apoptotic proteins

Caspases are known as the core enzymes of apoptosis [27]. The levels of apoptotic markers in CB01-treated cells were assessed to determine whether the CB01-induced cytotoxicity is associated with caspases. Through western blot analysis, the activities of apoptotic marker proteins in cells treated with 40 nM CB01 for 48 h were assessed. The three proteins play an important role in initiating apoptosis. The activated Bax induces permeability in the mitochondrial outer membrane. The cytochrome c was released from mitochondria. The caspase proteins which are key enzymes for apoptosis, were activated. The activities of apoptosis core proteins, such as cytochrome c, Bax, and cleaved caspase-3, which is active form, were observed in the cells treated with 40 nM CB01 for 48 h (Fig. 4). These data demonstrate that CB01 causes cytotoxicity through induction of apoptosis.



**Fig. 3** DNA fragmentation and nuclear-morphology changes induced by CB01. **A** DNA fragmentation. U87 and HeLa cells were cultured with 10 and 40 nM CB01, respectively, in 60 mm culture plates. After 48 h, the cells were lysed, and the supernatants of the lysates were collected and subjected to phenol–chloroform extraction. DNA was harvested by ethanol-precipitation and then treated with RNase A for 2 h. The positive control for the DNA fragmentation was cells treated with 5 μM camptothecin; a 1 kb DNA size marker was used. **B** Evaluation of nuclear morphology. U87 and HeLa cells were grown in 35 mm culture plates with 0 or 40 nM CB01. The nuclei of the cells were stained with DAPI

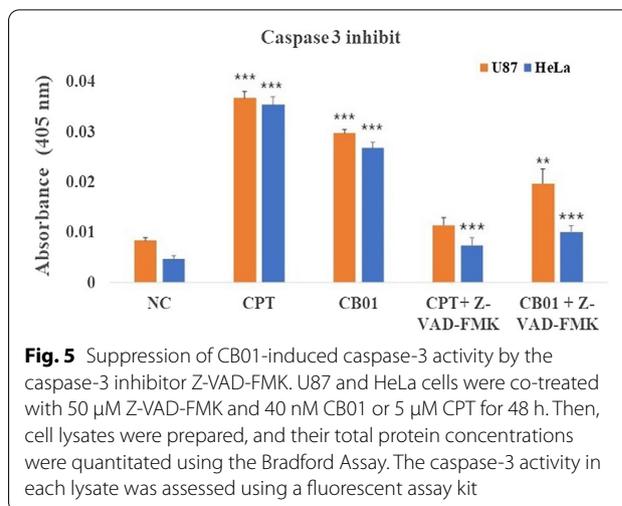


#### The caspase inhibitor Z-VAD-FMK suppressed the CB01-induced apoptosis

The two known apoptosis pathways commonly lead to the activation of caspase-3, and thus caspase-3 activation serves as direct evidence of apoptosis [23]. We observed significantly elevated caspase-3 activities in the lysates of CB01-treated U87 and HeLa cells, compared with the levels in the untreated controls. In addition, these CB01-induced increases in caspase-3 activities were suppressed by the pan-caspase inhibitor Z-VAD-FMK, which irreversibly binds to the cleavage site of a caspase, whereby the caspase cannot be cleaved to be activated [28]. Collectively, our results suggest that CB01 selectively induces the activation of caspase-3 in U87 and HeLa cells (Fig. 5).

#### CB01 induces apoptosis via the intrinsic pathway

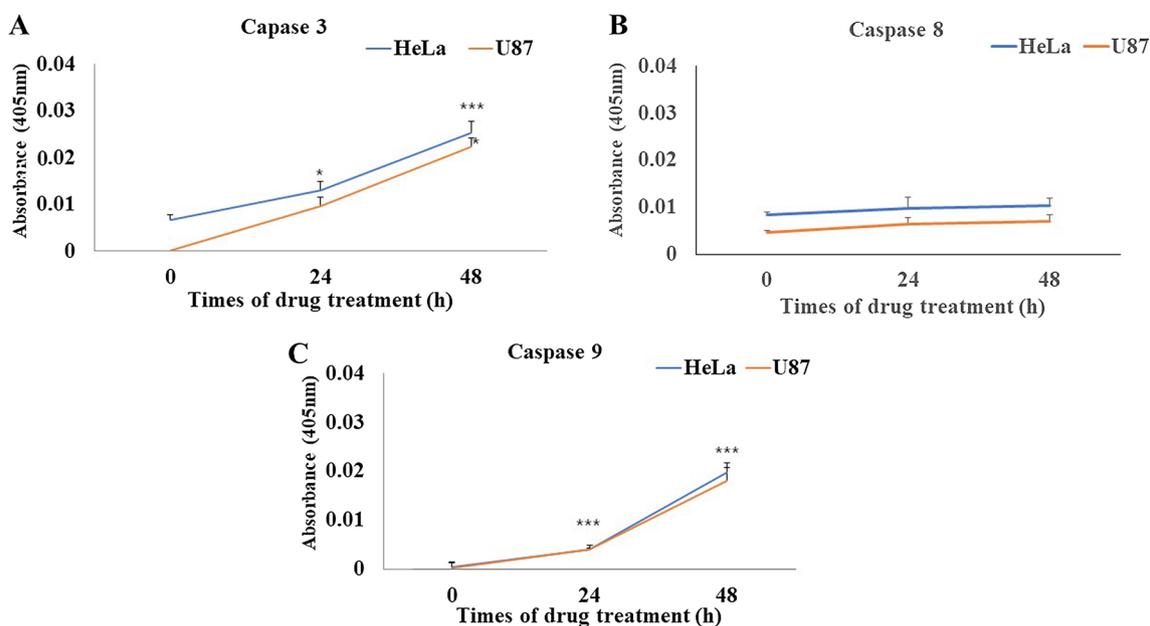
Apoptosis occurs through either an extrinsic or intrinsic pathway. The extrinsic pathway is activated through ligand-binding interactions on extracellular surface receptors, whereas the intrinsic pathway, also called the mitochondrial pathway, is activated through intracellular signals within the mitochondrial inter-membrane space [29]. The caspase-3, -8, and -9 activities were investigated using a colorimetric assay to determine which apoptotic pathway was induced in CB01-treated cells. The activities



of caspase-3 and -9 in cells treated with 40 nM CB01 increased over time; however, the caspase-8 activities in these cells were unaffected (Fig. 6). Thus, CB01 appears to cause apoptosis via the intrinsic pathway.

#### Discussion

Several anticancer drugs are currently used in chemotherapy. Chemotherapy has the benefit of being applicable irrespective of the cancer stage. Over the years, tremendous medical advances have been made in comprehending cancer biology and devising targeted chemotherapy [30]. Novel remedial chemicals and access methods with powerful effects on tumor or healthy tissues are continuously being adopted in clinics [31]. Although the efficacy of existing chemotherapeutics is not without controversy, there is a growing consensus that their anti-cancer effects are partly due to their abilities to induce apoptosis. The clinical use of these drugs is time-consuming and expensive, thus economical, and efficient anti-cancer drugs are needed [32]. Piperazine derivatives are a class of chemical compounds that contain piperazine as the key functional group and possess many pharmacological properties. Several studies on many piperazine derivatives have been reported, and the results of several relevant clinical trials are encouraging [4, 6, 8]. As the piperazine skeleton can easily be combined with various structures, whereby promising economical anticancer drugs may be developed in the future [1]. A recent study found that the synthesized piperazine derivative CB01 is effective in killing U87 and HeLa cells by inducing apoptosis according to mitochondrial changes. This study is the first to explore, discover, and reveal the mechanism of action of CB01, a new anticancer substance, and is expected



**Fig. 6** Comparison of the caspase-3, -8, and -9 activities. U87 and HeLa cells were treated with 40 nM CB01. Cell lysates were prepared after 24 h or 48 h, and their total protein concentrations were quantitated using the Bradford Assay. The caspase-3, -8, and -9 activities of each lysate were measured using fluorescent assay kits. The samples were incubated at 37 °C for 2 h, and their absorbance at 405 nm was measured. All the data are expressed as mean  $\pm$  SEM. Statistical significance was evaluated using the paired sample *t* test; ns =  $p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$

to greatly contribute to the discovery of new anticancer substances and the promotion of cancer research in Korea. Furthermore, it is expected that a new cancer treatment strategy will be established based on the mechanism of DNA damage response by CB01. The strategy of administering CB01 to radiation therapy and chemotherapy, which are treatments that induce DNA damage, can increase the efficiency of chemotherapy. Given the modifiability of piperazine, it is expected that the basic structural properties of piperazine can be used to devise a supplementary alternative, for the treatment of solid tumors, particularly in the breast, pancreas, colon, and lung.

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#### Authors' contributions

Objectification, survey, data analysis and drafting, data collection, and visualization, were all under SHJ, and revision of manuscripts, project management and funding were done by C-GS. All authors have read and approved the final manuscript.

#### Declarations

##### Competing interests

The author of the paper does not declare a competitive fiscal gain.

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