〈Research Article〉

Violacein induces various types of cell death in highly differentiated human hepatocellular carcinoma and hepatitis C virus replicon cell lines

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Summary The pigment violacein is a natural product of the bacterium *Chromobacterium violaceum* and has promising pharmacological activity. In this study, violacein was purified from *C. violaceum* and deoxyviolacein was obtained as a by-product. The antiviral effect of violacein and deoxyviolacein on hepatitis C virus (HCV) has not been reported; thus, we examined their effects on the well-differentiated human hepatocellular carcinoma cell line Huh7 and the HCV full-genome replicon cell line NNC#2. Violacein showed cytotoxicity and apoptosis in a dose-dependent manner on Huh7 and NNC#2 cells, although the effect on NNC#2 cells was weaker than that in Huh7 cells. In contrast, deoxyviolacein had no effect on cells at the test concentrations. Based on these results, violacein had an antitumor effect on liver cancer cell lines and may be effective as a therapeutic agent for HCV-infected cells. In addition, high concentrations of violacein appeared to cause rapid changes in the cell membrane, causing various types of cell death, including cell detachment and necrosis. The cell morphology observed after treatment with violacein may indicate other types of cell death. Further research is required in the future.

Key words: Violacein, Deoxyviolacein, Huh7, NNC#2, Apoptosis, Cell Death

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1. Introduction

The blue-violet pigment violacein[1] is a natural product that was discovered in 1882 following its isolation from the bacterium *Chromobacterium violaceum* collected in the Amazon basin[2]. *C. violaceum* is a Gram-negative bacterium found in tropical soil and water samples. Violacein is derived from L-tryptophan (L-Trp) in *C. violaceum*, and it is also found in other bacteria, such as *C. fluviatile* and *Janthinobacterium lividum*[3]. Violacein has a wide range of physiological effects, including antiviral[3,4], antioxidant, antibacterial[3], and antitumour[5] activity. In particular, the antitumour activity of violacein has been reported against breast cancer cells[6] and leukaemia cells[7].

Natural products have long been a cornerstone of drug development. Notable examples include the antibiotics penicillin and streptomycin, and the anticancer drug paclitaxel[8]. In recent years, the natural product shikimic acid has been used to produce influenza neuraminidase inhibitors, such as oseltamivir (sold under the brand name Tamiflu). Also, ivermectin, which is an avermectin derivative produced by actinomycetes, is used as an antiparasitic drug. Biological production of violacein has several advantages, including ambient reaction conditions, low cost, ease of mass production, and regio- and stereoselectivity of biocatalysts for the synthesis of complex and diverse natural products[9]. Against this background, the unique colour of violacein and its wide range of antibacterial and antiviral activity make it an interesting target[10].

Hepatitis C virus (HCV) is a member of the the genus *Hepacivirus* (Flaviviridae). Discovered in 1989, it is an enveloped single-strand plus-strand ribonucleic acid (RNA) virus[11]. Globally, over 115 million people are infected with HCV. It causes more than 700,000 deaths annually[12] and is a major cause of cirrhosis and hepatocellular carcinoma[13,14]. Combination therapy with pegylated interferon alfa-2α (peg-IFNa) and ribavirin (RBV) is the conventional treatment for HCV infection[15]. Direct-acting antivirals (DAAs) have been developed in recent years[16,17] but are still very expensive. Thus, there is hope for the development of a cheap and safe alternative.

In this study, we purified violacein from violacein-producing bacteria (VP) 2 of *C. violaceum*, isolated from soil in Ibaraki Prefecture, Japan and investigated the antitumour effect of violacein on well-differentiated human hepatocellular carcinoma Huh7 cells and the HCV full-genome replicon cell line NNC#2[18,19].

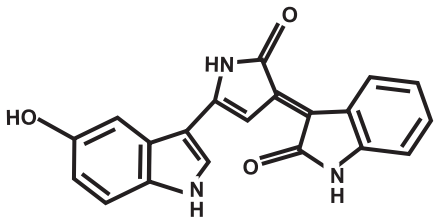
2. Materials and Methods

Isolation of violacein from *C. violaceum*

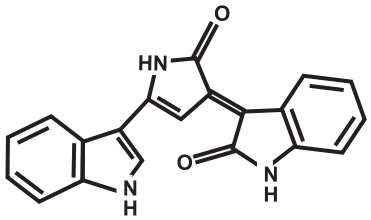
We identified a Gram-negative bacillus isolated from soil collected from Ibaraki Prefecture, Japan as *C. violaceum* VP2 by 16S rRNA sequence analysis. The structure of violacein is shown in Fig. 1A. The violacein produced by *C. violaceum* was quantified at the maximum absorbance wavelength (λ_{max}) of 575 nm (A575). The bacterial cells were obtained by centrifuging the *C. violaceum* culture solution and the pigment was extracted with ethanol and filtered through filter paper No. 5C (ADVANTEC Toyo, Tokyo, Japan). The filtered ethanol extract was purified by gel permeation chromatography (Table 1). Furthermore, the filtered methanol extract was also filtered through a 0.2 μm membrane filter and purified by high performance liquid chromatography (HPLC), as shown in Table 1. The solvent was removed under reduced pressure, and then the products were lyophilised to obtain analytically pure (100%) violacein (Fig. 1A) and deoxyviolacein (Fig. 1A) as blue-violet and reddish-violet solids, respectively. The identity and purity of violacein and deoxyviolacein were confirmed by HPLC and liquid chromatography-mass spectrometry (LC/MS).

Purified violacein was dissolved in dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical Co., Tokyo, Japan) and stored at 4°C in the dark until tested[20]. The final concentration of DMSO was used as 0.1%.

A



Violacein



Deoxyviolacein

B

Figure 1 Structures of violacein and deoxyviolacein and schematic representation of NNC#2 cells. A. Violacein and deoxyviolacein showing either the presence or the lack of the hydroxyl groups. B. HCV replicon cell line NNC#2 cells carries a full-genome replicon[18]. NNC#2 cells is clones 2 of HCV Full length genome replicon cells carrying HCV genotype 1b NN strain (NN/1b/FL)[19].

Table 1 Chromatography conditions

[Analytical Gel Filtration Chromatography conditions]
Column : Toyopearl HW-40F (OSAKA SODA CO., LTD.)
Eluate Buffer : Ethanol
Flow Rate : 200 mL/hours
Column Temp. : Room temperature
Fraction: 13 mL
[Preparative HPLC conditions]
Column : CAPCELL PAK C18 (4.6 I.D.×250 mm) (OSAKA SODA CO., LTD.)
Mobile Phase : 70% Methanol （v/v）
Flow Rate : 500 µL/min
Column Temp. : Room temperature
Injection Vol. : 10 µL
Detection : 575 nm
[Liquid chromatograph mass spectrometer (LC/MS) conditions]
Column : CAPCELL PAK C18 (4.6 I.D.×250 mm) (OSAKA SODA CO., LTD.)
Mobile Phase : 70% Methanol contains 0.1% acetic acid (v/v) (pH4.9)
Flow Rate : 500µL/min
Column Temp. : Room temperature
Ion : ESI (+ / - ion)
Scan range : 150 - 1000

Cell culture and reagents

Huh7 human liver cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical Co.) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin and 100 U/mL nonessential amino acids (FUJIFILM Wako Pure Chemical Co.). NNC#2 cells were grown in DMEM supplemented with 800 μmol/L L-glutamine, 10% FBS, 100 U/mL nonessential amino acids, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 300 μg/mL G418 (Nacalai Tesque, Inc., Kyoto, Japan) at 37°C and 5% CO_2.

Cell viability

Cells (4×10^4 per well) were seeded into 96-well plates in 100 μL of culture medium for 24 h, followed by the addition of increasing concentrations of violacein. The number of viable cells was determined after incubation for 4 and 24 h using a soluble tetrazolium-based MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Dojindo Laboratories, Kumamoto, Japan) at 550 nm, with subtraction of the blank absorbance measured in the wells without cells[21,22,23].

The percentages of viable cells were calculated using the following formula: (A490 KNK437-treated cells/A490-untreated cells) × 100. Data are presented as the mean ± standard deviation from three independent experiments and each experiment was performed in triplicate.

Morphological changes in cells

The Huh7 and NNC#2 cells (4×10^4 cells/well) were seeded into separate 96-well tissue culture plate. After 24 h incubation, stimulation was performed with each concentration of violacein or control DMSO. After 4 and 24 h, cells were analysed using a BZ-X800 fluorescence microscope (Keyence Corporation, Osaka, Japan).

The effect of violacein on cell morphology was observed by double-staining (separate) living and dead cells with propidium iodide (PI; Dojindo Laboratories) and Hoechst 33258 (Hoechst) (Merck KGaA, Darmstadt, Germany). PI fluoresces red when it penetrates the cell membrane of dead cells and binds to nucleic acids[24,25]. Membrane-permeable Hoechst is a bright blue fluorescent stain that stains the nucleus of apoptotic cells and necrotic cells. The cells were incubated with PI (×10,000) and Hoechst (×1,000) at 37°C and 5% CO_2 for 30 min, and then the fluorescence was observed.

An apoptotic/necrotic/healthy cell detection kit (Promoter Cell Inc., Heidelberg, Germany) was used to detect apoptotic and necrotic cells by fluorescence microscopy according to the manufacturer's protocol. The reagent, annexin V (labelled with fluorescein isothiocyanate (FITC; $\lambda_{abs}/\lambda_{em}$ = 492/514 nm)), specifically binds to phosphatidylserine exposed in the outer lobules of apoptotic cells and stains bright green. Ethidium homodimer III (EthD-III) does not penetrate living and apoptotic cells; however, when it penetrates necrotic cells, it strongly fluoresces red ($\lambda_{abs}/\lambda_{em}$ = 528/617 nm). Membrane-permeable Hoechst 33342 (Hoechst in the kit; Hoechst-k) fluoresces bright blue when bound to DNA, staining the nucleus of both apoptotic cells and necrotic cells ($\lambda_{abs}/\lambda_{em}$ = 350/461 nm). Secondary necrotic cells are stained green and blue. Necrotic cells are stained red and blue. Blue-, green-, and red-stained cells are dead cells that progress from an apoptotic cell population. The experiment was done in triplicate.

Caspase-3 detection

We seeded Huh7 and NNC#2 cells (2×10^4 cells / well) in 96-well tissue culture plates to confirm caspase-3 activity in cytomorphologically observed apoptotic cells. After 4 h incubation, each concentration of violacein or control DMSO was stimulated to bind to FITC (FITC-DEVD-FMK), a caspase-3 inhibitor DEVD-FMK (Cleaved Caspase-3 Staining Kit (FITC), Abcam, Cambridge, UK). Caspase 3 by FITC was detected by flow cytometry (FACS Calibur™ Flow Cytometer; BD Biosciences). Data are presented as the mean ± standard deviation from three independent experiments and each experiment was performed in triplicate. Statistical analyses were performed using the Student's *t*-test.

3. Results and Discussion

We discovered a dark blue-violet Gram-negative bacterium in soil collected in Ibaraki Prefecture, and 16S rRNA sequence analysis identified the bacterium as *C. violaceum*.

Spectrum characteristics of the identified *C. violaceum* VP2 and analysis of the dye

The dye produced by *C. violaceum* VP2 was extracted from aggregated cells with ethanol and analysed by HPLC using a reverse-phase Octa Decyl Silyl (ODS) column under the conditions shown in Table 1. The violet pigment was a mixture of a blue-violet solid and a red-violet solid (Fig. 2).

LC/MS showed two ions at *m/z* 342.20 (negative) and 326.30 (negative), which had molecular weights of 343 and 327, respectively. The blue-violet fraction eluted at 8.633 min and was identified as violacein (MW:343.34), and the reddish-violet fraction eluted at 12.542 min and was identified as deoxyviolacein (MW:327.24).

Violacein induced loss of cell viability

The effects of violacein were investigated using the human hepatocellular carcinoma cell line Huh7 and NNC#2 cells (Fig. 1B).

An MTT assay revealed that, in comparison with the control, the proliferation of Huh7 cells treated with violacein for 4 h was suppressed by 11.1% at 0.31 μmol/L, and that of NNC#2 cells was suppressed by 9.7% at 0.625 μmol/L (Fig. 3A). After 24 h, the proliferation of Huh7 cells was suppressed by 32.2% at 0.31 μmol/L, and that of NNC#2 cells was suppressed by 14.3% at 0.625 μmol/L (Fig. 3B).

The half maximal effective concentration (EC_{50}) of violacein in Huh7 cells calculated by probit analysis[24] was 1.53 μmol/L at 4 h and 0.47 μmol/L at 24 h, and NNC#2 cells showed approximately EC_{50} viability when exposed to 6.11 μmol/L at 4 h and 2.48 μmol/L at 24 h (Figs. 3A, B). Thus, violacein displayed dose-dependent inhibitory effects on the proliferation of Huh7 and NNC#2 cells. Weaker cytotoxicity was observed in NNC#2 cells than in Huh7 cells (Figs. 3A, B), and thus Huh7 cells were more sensitive to treatment. In contrast, deoxyviolacein was not toxic to Huh7 cells and NNC#2 cells at the concentrations we examined.

These results indicate that violacein has anti-cancer effects against Huh7 liver cancer cells and in HCV-infected NNC#2 cells, although it was weaker than that in Huh7 cells. The MTT assay and cell morphology changes showed that violacein had a weaker effect on NNC#2 cells than on Huh7 cells. These observations indicate that HCV is involved in cell death, resulting in the weaker effect of violacein. Translation of the HCV RNA genome occurs by internal ribosome entry site (IRES) through the 5'

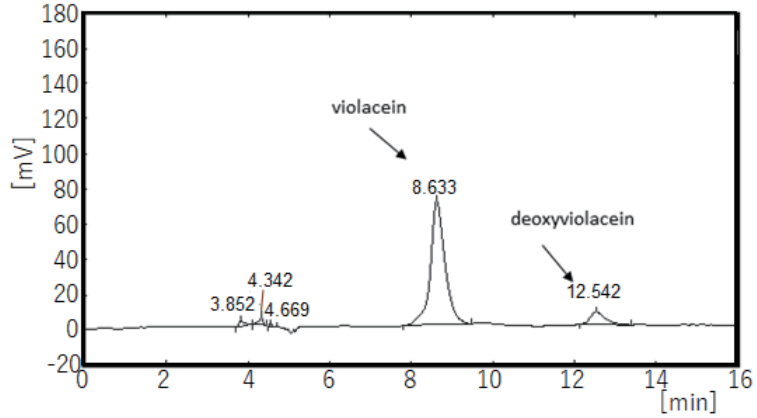


Figure 2 Spectrum of violacein and deoxyviolacein
HPLC of violacein and deoxyviolacein. Violacein eluted at 8.633 min and deoxyviolacein eluted at 12.542 min.

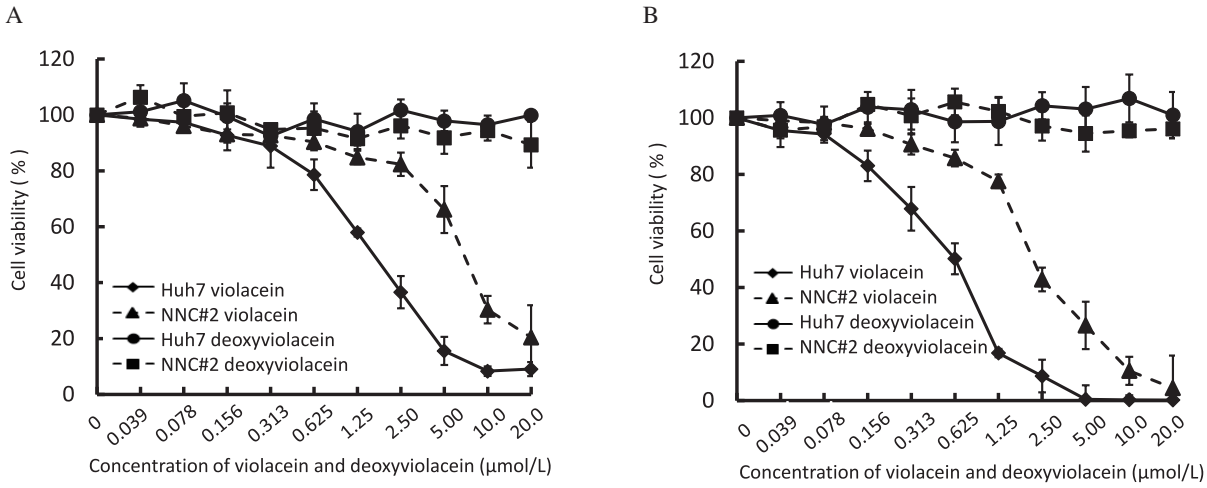


Figure 3 Cell viability. A and B showing Huh7 cells and NNC#2 cells treated with violacein and deoxyviolacein tested at 4 h and 24 h, respectively.

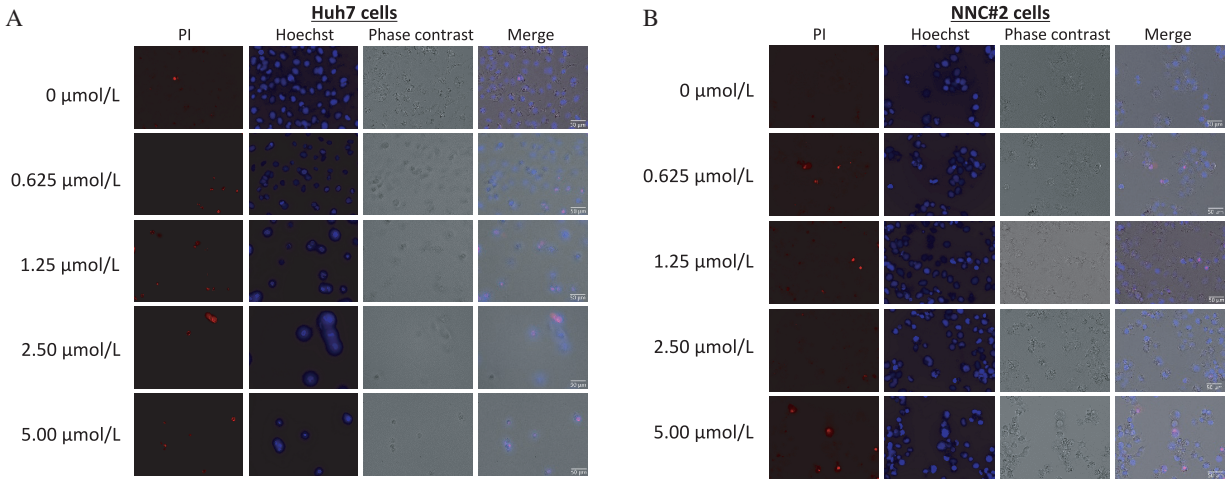


Figure 4 Morphological observations of Huh7 and NNC#2 cell lines. A, B. The Huh7 and NNC#2 cells were seeded into separate 96-well tissue culture plate. Twenty four hours post seeding, cells were inoculated with each concentration of violacein or control DMSO. After 4h, cells were analysed using a fluorescence microscope. The images are phase contrast and merged fluorescence images of cells stained with propidium iodide (PI) and Hoechst 33258 (Hoechst). The number of adherent cells Huh7 and NNC # 2 cells that died and became stained with PI increased with the concentration of violacein; at the same time, the cell morphology changed and the cells peeled off from the plate. Scale bar: 50 µm.

end (5' noncoding region). This RNA gene encodes 10 viral proteins. It was including the structural proteins core (C), envelope (E) 1, and E2 , endogenous integral membrane ion channel protein of p7, as well as the non-structural (NS) proteins, NS2, NS3, NS4A, NS4B, NS5A and NS5B[18,19].

Previous reports have shown that the HCV core and the E1 and E2 structural proteins promote apoptosis and the NS3, NS4A, NS5A, and NS5B nonstructural proteins suppress apoptosis[26,27,28].

Changes in cell morphology due to violacein

The DNA of the cells treated with violacein was stained Hoechst and PI to detect necrosis. Huh7 (Fig. 4A) and NNC#2 (Fig. 4B) viable cells had fewer PI-stained cells at 0.625 µmol/L and 1.25 µmol/L low levels of violacein, and as the concentration of violacein increased, the numbers of PI-stained cells and floating detached cells increased. Therefore, violacein caused cell death in Huh7 and NNC#2 cells.

Next, to determine whether violacein–induced

cell death is related to apoptosis or necrosis, Huh7 and NNC#2 cells treated with violacein was stained with an apoptosis/necrosis/healthy cell detection kit using a fluorescence microscopy. At low violacein levels, apoptotic Huh7 (Fig. 5A) and NNC#2 cells (Fig. 5B) were visible. And, apoptotic cells and necrotic cells were identified as the violacein concentration increased. Thus, the type of cell death that violacein induced depended on concentration. However, some cells treated with violacein appeared to have caused other types of cell death. We speculated that high concentrations of violacein caused rapid changes in the cell membrane, resulting in cell death by necrosis as well as by apoptosis.

The effect of violacein was confirmed by our observations of apoptotic cell state and weaker effect of cell death in NNC#2 cells than in Huh7 cells. Some cytoplasmic vacuolation was also observed in Huh7 cells treated with violacein, suggesting that the cells starved and underwent autophagy-induced cell death.

Violacein induced cell death of Huh7 and NNC#2 cells

We investigated whether the apoptosis-induced inhibition of Huh7 and NNC#2 cell proliferation by violacein was mediated by a caspase-dependent pathway. Caspase-3 has a central role in cell apoptosis and is activated by exogenous and endogenous cell death pathways. Huh7 and NNC#2 cells were treated with 0, 0.625, 1.25, and 2.50 μmol/L violacein for 4 h, and then apoptotic cells with caspase-3 externalisation were detected by FITC staining and flow cytometry.

The percentages of apoptotic cells of caspase 3-positive were 4.90 ± 2.04%, 13.34 ± 1.34%, 12.02 ± 1.79%, and 16.36 ± 3.60% on Huh7 cells, and were 3.10 ± 0.05%, 21.62 ± 2.08%, 19.86 ± 1.36%, and 30.17 ± 4.34% on NNC#2 cells, respectively (Fig. 6A). The proportion of apoptotic cells was approximately 2.45 to 3.3 times higher in Huh7 cells and approximately 6.4 to 9.7 times higher in NNC # 2 cells compared to 0 μmol/L concentrations of violacein (Fig. 6B). Therefore, violacein induced apoptosis via a caspase-dependent pathway in Huh7 and NNC#2 cells. In NNC#2 cells, HCV seemed to suppress apoptosis, as previously reported[25,26,27]. The calculation of the *t*-test showed that this change was statistically significant at the $p < 0.05$.

Cell death was previously classified as apoptosis, autophagy, cornification, and necrosis[22,30]. However, cell death is now classified into 13 types[31], and the molecular signals involved are being elucidated. There are reports that the cancer cell proliferation arises from mutations in genes related to apoptosis that prevent cell death[32]. Our study shows that violacein causes apoptosis and other types of cell death in Huh7 and NNC#2 cell in a concentration-dependent manner, and at higher violacein concentrations cell death may occur via mechanisms other than apoptosis. Thus, violacein may be a useful compound for investigating types of cell death and is also a promising therapeutic agent for HCV-infected hepatocellular carcinoma cells. In addition, high levels of violacein can cause cell death through non-apoptotic mechanisms.

Our study found that violacein causes apoptosis and other types of cell death in Huh7 and NNC#2 cells in a concentration-dependent manner, and that a high concentration of violacein can lead to cell death through mechanisms other than apoptosis.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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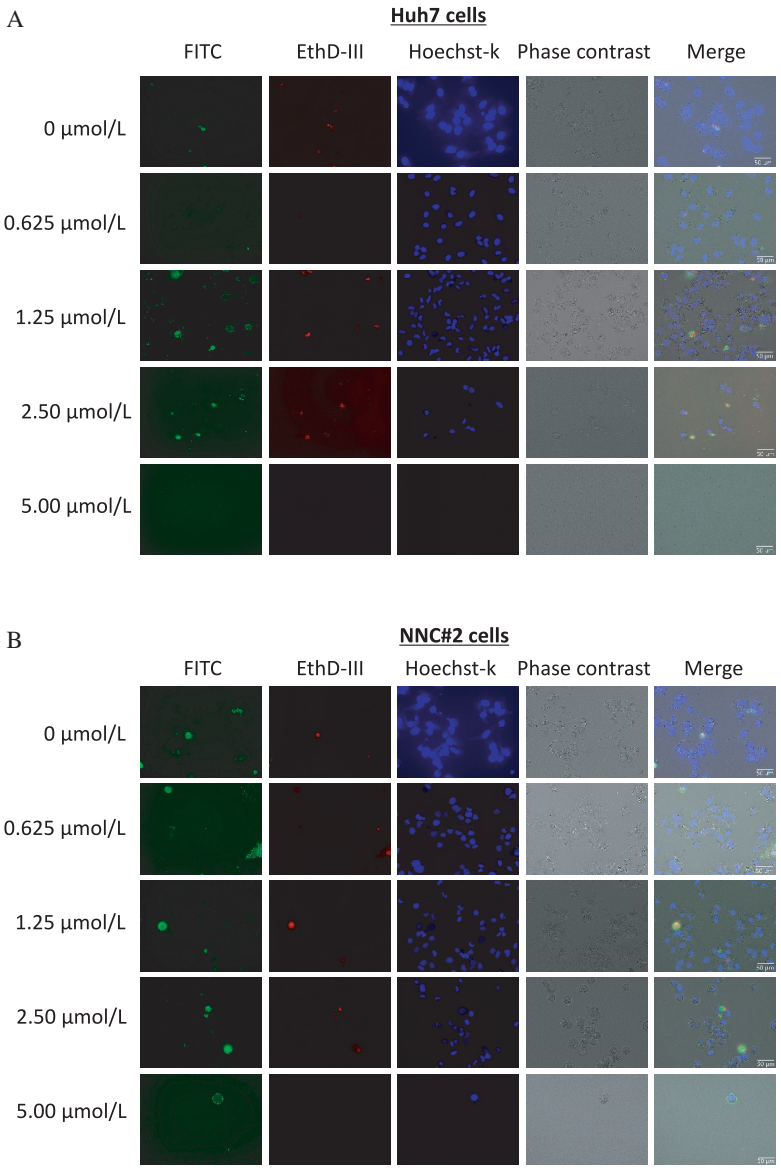

Figure 5 Apoptotic or necrotic effects of the violacein on Huh7 and NNC#2 cells. A,B. The Huh7 and NNC#2 cells were seeded into separate 96-well tissue culture plate. Twenty four hours post seeding, cells were incubated with each concentration of violacein or control DMSO. After 4h, cell lines were stained with an apoptotic/necrotic/healthy cell detection kit. The images are phase contrast and merged fluorescence images of cells stained with fluorescence of FITC (FITC-labelled annexin V), EthD-III, and Hoechst 33342 (Hoechst-k) and investigation using a fluorescence microscopy (green, red, and blue fluorescence indicates early apoptotic cells, late apoptotic cells and necrosis, and viable, respectively). Higher levels of violacein increased the number of apoptotic cells stained with FITC-Annexin V (green), and the cells were stained with EthD-III in a violacein concentration-dependent manner, leading to late apoptosis. However, some cells underwent necrosis in response to EthD-III only, and some cells had abnormal intracellular vacuoles and only the nucleus was stained. Scale bar: 50 μm.

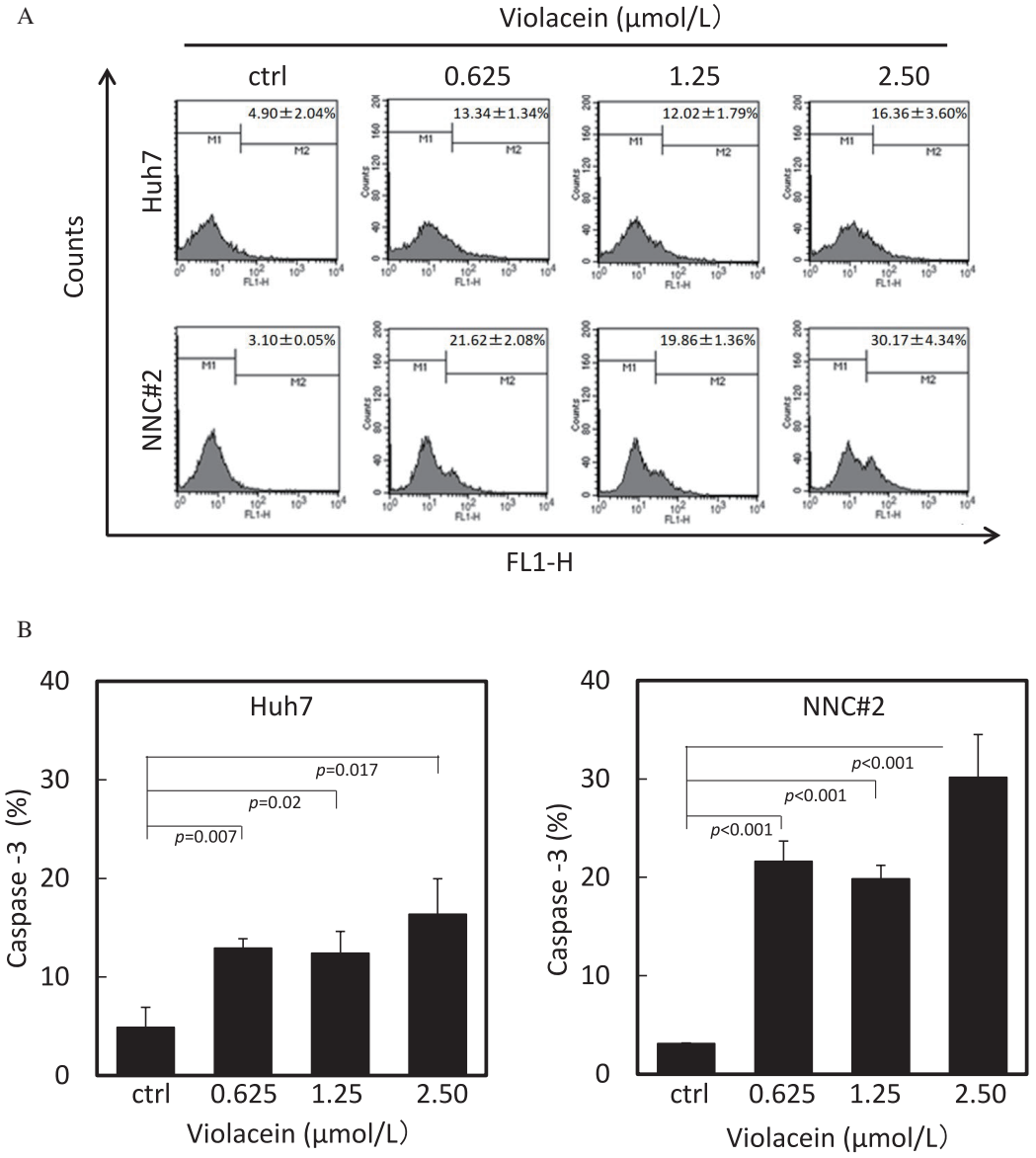

Figure 6 Assessment of Caspase-3-induced apoptosis in Huh7 cells and NNC#2 cells. A. Flow cytometry-based caspase-3 FITC staining for detection of apoptosis. Cells are treated with the indicated concentration of violacein for 4 h each and stained with caspase-3 FITC. B. Quantitative analysis of apoptotic cells of A. $p < 0.05$ control vs. caspase-3 indicates statistical significance. (ctrl:DMSO)

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