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## Accepted Manuscript

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1 ATP depletion alters the mode of cell death induced by benzyl isothiocyanate  
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13 *Keywords:* benzyl isothiocyanate; apoptosis; necrosis; HeLa cell; ATP; ROS

14

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19

20 <sup>1</sup>Abbreviations: ITC, isothiocyanate; BITC, benzyl ITC; GSH, glutathione; ROS,  
21 reactive oxygen species; PEITC, phenethyl ITC; 2-DG, 2-deoxyglucose; PI, propidium  
22 iodide; H<sub>2</sub>DCF-DA, 2',7'-dichlorofluorescein diacetate; LDH, lactate dehydrogenase;  
23 ETP, etoposide; CHX, cycloheximide; GPx, glutathione peroxidase; TR, thioredoxin;  
24 GST, glutathione-S-transferase; GCLC, glutamate-cysteine ligase catalytic subunit;  
25 GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AKR1C, aldo-keto reductase  
26 1C; TR, thioredoxin; ATM, ataxia telangiectasia-mutated.

1 **ABSTRACT**

2 Pro-inflammatory death is presumably an undesirable event in cancer  
3 prevention process, thus biochemical comprehension and molecular definition of this  
4 process could have important clinical implications. In the present study, we examined  
5 the cytophysiological conversion of cell death mode by benzyl isothiocyanate (BITC)  
6 in human cervical cancer HeLa cells. The detailed studies using flow cytometric and  
7 morphological analyses demonstrated that the cells treated with appropriate  
8 concentration (25  $\mu\text{M}$ ) of BITC showed apoptotic feature, such as chromatin  
9 condensation, DNA fragmentation, and preserved plasma membrane integrity, whereas  
10 these features were disappeared by treatment with higher concentration (100  $\mu\text{M}$ ).  
11 The treatment with 2-deoxyglucose, an inhibitor of ATP synthesis, drastically increased  
12 in the ratio of necrotic dead cells, while it influence little that of apoptotic cells.  
13 Moreover, an analysis using the mitochondrial DNA deficient HeLa cells demonstrated  
14 that the  $\rho^0$  cells were more susceptible to the BITC-induced necrosis-like cell death  
15 compared to the wild-type ( $\rho^+$ ) cells, whereas the ROS production was significantly  
16 inhibited in the  $\rho^0$  cells. It is likely that the BITC-induced ROS is derived from  
17 mitochondrial respiratory chain and ruled out the contribution to the mechanism of cell  
18 death mode switching. In addition, the BITC treatment resulted in a more rapid  
19 depletion of ATP in the  $\rho^0$  cells than in the  $\rho^+$  cells. Furthermore, a caspase inhibitor,  
20 Z-VAD-fmk counteracted not only apoptosis, but also necrosis-like cell death induced  
21 by BITC, suggesting that increment in this cell death pattern might be due to the  
22 interruption of events downstream of a caspase-dependent pathway. The obtained  
23 data suggested that the decline in the intracellular ATP level plays an important role in  
24 tuning the mode of cell death by BITC.

## 1 **1. Introduction**

2 A number of studies support the idea that certain food phytochemicals protect  
3 against cancer. An important group of food compounds that have a cancer  
4 chemopreventive property are organosulfur compounds including isothiocyanates  
5 (ITCs)<sup>1</sup>. ITCs are compounds that occur as glucosinolates in a variety of cruciferous  
6 vegetables such as *Brussica* species. Naturally occurring ITC compounds are effective  
7 preventive agents against chemical carcinogenesis in the lung, the esophagus, the  
8 mammary gland, the liver, the small intestine, the colon, and the bladder [1].  
9 Epidemiological studies also indicate a significant correlation between the dietary intake  
10 of ITC-containing foods and the reduced risk for several cancers [2,3,4].  
11 Mechanistically, ITCs are capable of inhibiting both the formation and development of a  
12 cancer cell through multiple pathways; i.e., the inhibition of carcinogen-activating  
13 cytochrome P450 monooxygenases, induction of carcinogen-detoxifying enzymes,  
14 induction of apoptosis, and inhibition of the cell cycle progression [5]. We recently  
15 clarified the molecular mechanism underlying the relationship between cell cycle arrest  
16 and apoptosis induced by benzyl-ITC (BITC) [6]. Additionally, we demonstrated that  
17 antioxidant mechanisms also potentially contribute to a BITC-mediated  
18 chemoprevention against inflammation-related carcinogenesis [7].

19 In contrast, there is abundant evidence that ITCs induce cellular stress  
20 themselves [8]. The concentration of ITCs required for cellular stress induction  
21 exceeds or, at least some parts, overlaps those needed for the enhancement of the  
22 cellular antioxidant protein expression or apoptosis induction. The unfavorable effect  
23 of higher concentrations of ITCs has been observed; e.g., the treatment with an  
24 excessive concentration of BITC resulted in severe cytotoxicity with caspase-3  
25 inactivation and no DNA ladder formation [6,9]. Some in vivo experiments also  
26 demonstrated the enhancement of tumorigenesis by ITCs. For instance, both BITC  
27 and phenethyl ITC (PEITC) promote urinary bladder carcinogenesis in rats treated with

1 diethylnitrosamine and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. Since the employed  
2 dose was higher than that required for chemoprevention [10], the promotional effect of  
3 ITCs could be dependent on their concentration. Taken together, it is suggested that  
4 accidental cell death induced by higher concentrations of BITC might be involved in its  
5 ability to promote carcinogenesis [11].

6           The death of a cell can be defined as an irreversible loss of plasma membrane  
7 integrity [12]. Historically, three types of cell death have been distinguished in  
8 mammalian cells by morphological criteria. ‘Apoptosis’ is accompanied by  
9 rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume  
10 (pyknosis), condensation of the chromatin, fragmentation of the nucleus (karyorrhexis),  
11 little or no ultrastructural modification of cytoplasmic organelles, plasma membrane  
12 blebbing, and maintenance of an intact plasma membrane until late stages of the  
13 process. ‘Autophagic cell death’ is characterized by a massive accumulation of  
14 two-membrane autophagic vacuoles in the cytoplasm. ‘Necrosis’ is usually  
15 considered as a type of cell death with no signs of apoptosis or of autophagy, which is  
16 a negative definition [13]. The distinction between cell death type is important,  
17 particularly because necrosis is often associated with unwarranted cell loss in human  
18 pathologies [14,15,16] and can lead to local inflammation, presumably through the  
19 liberation of factors from dead cells that alert the innate immune system [14,17,18].  
20 Although recent some researches suggest a ‘programed’ necrotic cell death for some  
21 reasons [19,20,21] it’s still obscure that molecular mechanism of chemopreventive ITC  
22 induced necrotic cell death.

23           In the present study, we examined the switching mechanism of the cell demise  
24 mode from apoptosis to necrosis-like cell death using different concentrations of BITC.  
25 We also observed that BITC-induced ROS production is closely correlated with  
26 modification of mitochondrial respiration chain but ruled out in the mechanism of cell  
27 death mode alteration. This is the first report that changing the mode of cell death

1 produced by BITC depends on its concentration and intracellular ATP level.

2

3

## 4 **2. Materials and Methods**

5

### 6 *2.1. Chemicals*

7 BITC and Triton X-100<sup>TM</sup> were obtained from Nacalai Tesque, Inc., Kyoto,  
8 Japan. Trypan Blue and 2-deoxyglucose (2-DG) were purchased from Sigma, St.  
9 Louis, MO. Propidium iodide (PI) and 2',7'-dichlorofluorescein diacetate  
10 (H<sub>2</sub>DCF-DA) were obtained from Molecular Probes, Inc., Eugene, OR. Z-VAD-fmk  
11 was obtained from Peptide Institute (Osaka, Japan). All other chemicals were  
12 purchased from Wako Pure Chemical Industries, Osaka, Japan.

13

### 14 *2.2. Cell culture and preparation of $\rho$ cells*

15 The human cervix adenocarcinoma HeLa cells (provided by Dr. Hideki  
16 Shibata of Nagoya University) were maintained in DMEM (Nissui, Tokyo, Japan)  
17 supplemented with 10% FBS, 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin, and  
18 grown in an atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. The HeLa  $\rho^0$  cells depleted  
19 of mtDNA were generated from the HeLa wild type ( $\rho^+$ ) cells by long-term exposure  
20 to ethidium bromide [22]. Briefly, HeLa cells were cultured in 10 % FBS containing  
21 DMEM supplemented with 0.05  $\mu$ g/ml ethidium bromide, 0.05 mg/ml uridine, 0.11  
22 mg/ml sodium pyruvate, and 3.5 mg/ml glucose for more than one month. The  
23 surviving HeLa  $\rho^0$  cells that formed colonies in the culture dish were harvested and  
24 tested as described below. The depletion of mitochondrial DNA was confirmed by  
25 PCR using the following primer: 5'-GTAGGAGAGTGATATTTGATCAGG-3', and  
26 5'-CCATCTGCCTACGACAAACAGACC-3'.

27

### 1 2.3. Assay for cell viability

2 The MTT assay and Trypan blue dye exclusion assay were carried out for the  
3 quantitative analysis of cell viability. After culturing with BITC at 37°C for 18 h, 10  
4 µl of an MTT solution were added to each well, and the fluorescence was measured  
5 with excitation at 560 nm and emission at 590 nm according to the manufacturer's  
6 instructions after incubation at 37°C for 2-3 h in a humidified CO<sub>2</sub> incubator. The  
7 obtained values were compared with each of the controls incubated with vehicle only.  
8 For the Trypan blue dye exclusion assay, the cells cultured with BITC at 37°C for 24 h  
9 were harvested, collected by centrifugation, and resuspended in PBS. The cell  
10 suspensions were mixed with 0.4% trypan blue stain. The total cells and viable cells  
11 (cell that excluded blue dye) were counted using a hemocytometer under a light  
12 microscope.

### 14 2.4. Detection of nuclear fragmentation

15 For the DNA fragmentation analysis, HeLa cells ( $5 \times 10^5$  cells) were  
16 incubated in culture medium in the presence or absence of BITC or etoposide (ETP).  
17 Both the attached and floating cells were pelleted by centrifugation, and DNA was  
18 isolated from the cell pellets as previously described [6]. The DNA was then  
19 subjected to electrophoresis in 2% agarose gels, stained with ethidium bromide, and  
20 imaged with a FluorImager (Molecular Dynamics, Tokyo, Japan). Apoptotic cell death  
21 was also evaluated by fluorescence microscopy. After incubated with BITC, HeLa cells  
22 staining with Hoechst 33342 and PI were observed under a confocal fluorescence  
23 microscope (LSM 510 META, Carl Zeiss, Inc., Thornwood, NY).

### 25 2.5. Flow cytometric analysis

26 For the analysis of apoptosis/necrosis, we used an Annexin V-FITC Apoptosis  
27 Detection kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) according



1 to the protocol by the manufacturer. Both the attached and floating cells were stained  
 2 with annexin V-FITC and PI. Flow cytometric analyses were performed using BD  
 3 FACSCanto II (BD Bioscience, San Jose, CA). The intracellular oxidative products  
 4 were detected by H<sub>2</sub>DCF-DA as an intracellular fluorescence probe. Briefly, the cells  
 5 were treated with H<sub>2</sub>DCF-DA (50 μM) for 30 min at 37°C. After washing twice with  
 6 PBS, the BITC was added to the complete medium and incubated for another 2 hours.  
 7 Then, flow cytometric analysis was performed to detect DCF formed by the reaction of  
 8 H<sub>2</sub>DCF with the intracellular oxidative products.

#### 10 2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

11 Total RNA was isolated with Isogen reagent (Nippon Gene, Tokyo, Japan)  
 12 and spectrophotometrically quantified. The RT reaction was performed with 1 μg of  
 13 total RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR  
 14 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PCR was  
 15 carried out using TaKaRa Ex Taq (TaKaRa bio, Inc., Tokyo, Japan). The following  
 16 primers were used: SOD1-(F) 5'-CGAGCAGAAGGAAAGTAATGG-3' and (R)  
 17 5'-AAGTCGTTTGGCTTGTGGTGT-3' and SOD2-(F)  
 18 5'-CCTGGAACCTCACATCAACG-3' and (R)  
 19 5'-GAAGGTAGTAAGCGTGCTCC-3' and glutathione peroxidase 1 (GPx1)-(F)  
 20 5'-AGTCGGTGTATGCCTTCTCG-3' and (R) 5'-GAATCTCTTCGTTCTTGGCG-3'  
 21 and GPx4-(F) 5'-GCACATGGTTAACCTGGACA-3' and (R)  
 22 5'-AAATAGTGGGGCAGGTCCTT-3' and thioredoxin (TR)-(F)  
 23 5'-TCGCTTTGGAGTGCGCTGGA-3' and (R)  
 24 5'-GGCCACAAGCACCATATTCCAA-3' and glutathione-S-transferase P1  
 25 (GSTP1)-(F) 5'-CTCACTCAAAGCCTCCTGCCTAT-3' and (R)  
 26 5'-CAGGATGGTATTGGACTGGTACAG-3' and glutamate-cysteine ligase catalytic  
 27 subunit (GCLC)-(F) 5'-TTGATTAAGGCTTCTTTGGTAGG-3' and (R)

1 5'-TTTCAATAAATCAGGTCCCAGG-3' and  $\beta$ -actin-(F)  
2 5'-GTCACCCACACTGTGCCCATCTA-3' and (R)  
3 5'-GCAATGCCAGGGTACATGGTGGT-3' and glyceraldehyde-3-phosphate  
4 dehydrogenase (GAPDH)-(F) 5'-AACCCATCACCATCTTCCAGGAGC-3' and (R)  
5 5'-CACAGTCTTCTGAGTGGCAGTGAT-3'

6

### 7 *2.7. Measurement of intracellular ATP level*

8 Intracellular ATP was measured using an ATP Determination Kit (Molecular  
9 Probes) according to the manufacturer's protocol. Briefly, the attached cells were  
10 harvested with 100  $\mu$ l of 100 mM Tris-HCl and 4 mM EDTA, boiled for 2 min, and  
11 centrifuged at 4000 rpm for 1min. After mixing 10  $\mu$ l from each of the supernatants  
12 or the 0-25  $\mu$ M ATP standard solution with 90  $\mu$ l of the ATP standard reaction  
13 solution, the emitted light was measured. Aliquots from each sample were used for  
14 measuring protein concentration using the BCA protein assay kit (Pierce). The blank  
15 value (no ATP) was subtracted from each sample's raw data, and finally the ATP  
16 concentrations were calculated from the linear part of a standard curve and expressed  
17 as the relative ATP content or n mol per mg protein.

18

### 19 *2.8. Caspase activity determination*

20 Caspase-3-like activity was monitored by the cleavage of  
21 Ac-Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) according to the protocol outlined  
22 by the manufacturer in a Caspase-3 Colorimetric Protease Assay Kit (Medical and  
23 Biological Laboratories, Nagoya, Japan). The caspase-3-like activity was determined  
24 in the cytosolic extracts of HeLa cells that had been preincubated with or without 12.5  
25 mM 2DG for 12.5 hours followed by treatment with 25  $\mu$ M BITC for 9 hours.

26

### 27 *2.9. Statistical analyses*

1           When applicable, the mean  $\pm$  Standard Deviation (SD) values are shown.  
2 Significant difference among treatments was examined using Student's t-test (two  
3 sided), which assumed unequal variance.

### 6 **3. Results**

#### 7 *3.1. BITC-induced cell death with apoptotic and necrotic feature in HeLa cells*

8           In our and other previous studies, BITC showed a cytotoxic effect in several  
9 cultured cell lines [6,9,23,24,25,26,27,28,29,30,31,32]. Since these cell lines have  
10 individually different cellular features associated with cell death regulatory factors, such  
11 as the intracellular redox status, MAP kinase activities, and cell cycle regulation-related  
12 proteins, the 50% inhibitory concentrations of BITC were diverse in each of the cell  
13 lines. Therefore, we used the human cervix adenocarcinoma HeLa cells widely used  
14 in various investigations of cancer biology. First, we monitored the cytotoxic activity  
15 of BITC in the HeLa cells using the modified MTT assay and Trypan blue exclusion  
16 assay. When HeLa cells were incubated with BITC, the viability was inhibited in a  
17 concentration-dependent manner up to 100  $\mu$ M with an IC<sub>50</sub> value of 42  $\mu$ M (Fig. S1A  
18 in the supplemental data available with this article online). The Trypan blue exclusion  
19 assay also showed that the ratio of dead cells dose-dependently increased (Fig. S1B).  
20 Furthermore, the loss of cell membrane integrity was also measured by release of lactate  
21 dehydrogenase (LDH) into the cell culture medium (Fig. S1C). Although LDH release  
22 was observed with a time- and dose-dependency up to 25  $\mu$ M, no further increase was  
23 detected when the cells were treated with more than 25  $\mu$ M BITC, which might be due  
24 to the direct interference of LDH activity by BITC. We next performed an annexin V/  
25 PI analysis to examine the membrane reconstitution and permeabilization associated  
26 with BITC-induced cell death (Figs. 1A – C, Fig. S1D). As shown in Fig. 1A, the  
27 treatment with 25  $\mu$ M BITC for 3 h resulted in a significant increase in the annexin (+)/

1 PI (-) and annexin (+)/ PI (+) populations (2.6-fold increase compared with control).  
2 The change in these populations [annexin (+)] is time-dependent (Fig. 1A). However,  
3 a greater amount of the annexin V (+)/ PI (+) population was induced by 100  $\mu$ M BITC  
4 although the annexin V (+)/ PI (-) one was slightly induced (Fig. 1A). Besides, 100  
5  $\mu$ M BITC induced a rapid and significant membrane permeabilization, evaluated by the  
6 PI uptake, when compared to treatment with 25  $\mu$ M BITC (Fig. 1B). Furthermore, the  
7 exposure of HeLa cells to 100  $\mu$ M BITC resulted in a rapid cell swelling with a peak at  
8 1 h accompanied by a 1.5-fold cell size change (Fig. 1C). Interestingly, we observed  
9 that the swollen cells still remained their membrane integrity after the 1 h treatment with  
10 100  $\mu$ M BITC, suggesting severe cell swelling occurred prior to the membrane  
11 disruption (Fig. S1D). In contrast, although 25  $\mu$ M BITC induced a weak cell swelling  
12 with a peak at 3 h (1.19-fold increase), it coincided with the cell membrane disruption  
13 (Figs. 1B and C). These morphological distinctions between the 25 and 100  $\mu$ M  
14 BITC-induced cell deaths suggest that obviously different cell death forms were  
15 dose-dependently induced by BITC.

16 To obtain further evidence, the membrane disruption and nuclear  
17 fragmentation were investigated using a fluorescence dye, PI and membrane permeable  
18 Hoechst 33342. As a result, although treatment with 25  $\mu$ M BITC for 2 h showed  
19 little effect on the membrane disruption (stained less than 4 %), many of the cells were  
20 stained by PI after treatment with 100  $\mu$ M BITC (Fig. S1E). Additionally, after  
21 treatment with 25  $\mu$ M BITC for 20 h, we observed the nuclear chromatin condensed  
22 cells (9 of 53 cells in Fig. S1E), some of which still have an intact cell membrane (6 of  
23 9 cells in Fig. S1E). Furthermore, treatment of the cells with 25  $\mu$ M BITC resulted in  
24 a significant DNA fragmentation (Fig. 1D). Interestingly, when the cells were treated  
25 with 50  $\mu$ M BITC, DNA fragmentation was not detected in spite of the severe  
26 cytotoxicity, while the treatment of an excessive concentration of etoposide (ETP, 500  
27  $\mu$ M) resulted in the DNA fragmentation. These results showed that 100  $\mu$ M BITC

1 induces cell death with neither a DNA fragmentation nor early apoptotic feature. Our  
2 previous report demonstrated that caspases-3 and -9 were activated by BITC at lower  
3 concentrations, while their activities were reduced at the higher BITC concentration in  
4 RL34 cells [9]. Taken together, we further confirmed that BITC is able to regulate  
5 cell death mode dependent on its concentration.

### 6 7 *3.2. Involvement of intracellular ATP level to the determination of BITC-induced cell* 8 *death form*

9 It is widely accepted that the intracellular ATP level is one of the important  
10 factors to decide the cell death form when the cells were exposed to a lethal  
11 stimulation [10,11,33]. Therefore, we compared between the intracellular ATP level  
12 and necrotic cell ratio in BITC-treated attached HeLa cells. As shown in Fig. 2A, the  
13 treatment with 50  $\mu$ M of BITC for 3 h significantly reduced the ATP level to 55 % of  
14 the control level coincided with 25% of the necrotic-featured dead cells. These  
15 results suggested that depletion of ATP might be due to not only leak by enhancement  
16 of membrane permeability but also inhibition of ATP synthesis. Next, to examine the  
17 involvement of the intracellular ATP level on the switching of apoptosis/necrosis,  
18 HeLa cells were pretreated with an ATP synthesis inhibitor, 2-deoxyglucose (2DG).  
19 Treatment with 12.5 mM 2DG resulted in an ATP depletion to 50 % of the control level  
20 and a slight cytotoxicity in HeLa cells (Fig. S2). As shown in Fig. 2B, the 2DG  
21 pretreatment enhanced the susceptibility to both apoptosis and necrotic cell death  
22 induced by BITC. Particularly, a higher ratio of necrotic-featured cells was detected  
23 in the ATP-depleted cells treated with 25  $\mu$ M BITC. Consistent with this, caspase-3  
24 activity was significantly reduced by 2DG pretreatment (Fig. 2C). These results  
25 suggested that regulation of the intracellular ATP level by BITC plays an important  
26 role in the determination of the mode of cell death.

27

1 3.3. *Effect of BITC on intracellular ROS production and cytotoxicity in HeLa  $\rho^0$  cells*

2 The mitochondrial DNA-deficient  $\rho^0$  cell is widely used in studies of the  
3 mitochondrial function, respiration, and mitochondria-derived ROS. Therefore, we  
4 prepared HeLa  $\rho^0$  cells (Fig. 3A) to investigate the involvement of mitochondrial  
5 function alteration in the BITC-induced change of cell death mode. As shown in Fig.  
6 3B, the intracellular ROS level in the HeLa  $\rho^0$  cells was significantly lower than in the  
7 wild type ( $\rho^+$ ) cells under the normal culture conditions. Moreover, BITC-induced  
8 enhancement of the ROS level was significantly suppressed in the  $\rho^0$  cells, although  
9 the inhibitory effect on the 50  $\mu$ M BITC-induced ROS production was also partly  
10 suppressed. In the meantime, we observed that the ROS production induced by  
11 rotenone, a mitochondrial respiration inhibitor, was completely abolished in the  $\rho^0$   
12 cells whose mitochondrial membrane voltage was dysregulated (Fig. S3). In addition,  
13 no significant differences of various antioxidant enzyme expressions between in the  $\rho^+$   
14 and  $\rho^0$  cells were observed (Fig. 3C). Furthermore, BITC failed to induce the  
15 antioxidant enzyme expression including aldo-keto reductase 1C1/2 (AKR1C1/2),  
16 thioredoxin (TR), and GSTP1 in HeLa  $\rho^+$  cells (Fig. S4). These results indicated that  
17 the BITC-induced ROS mainly originated from mitochondrial respiratory chain but not  
18 from the alteration of antioxidant defense system, consistent with our reports that  
19 BITC can directly modify the mitochondrial function [9,34].

20 Hence, we examined the effect of mitochondria deficiency on the  
21 BITC-induced apoptosis and necrosis in the mitochondria DNA deficient  $\rho^0$  cells. As  
22 shown in Fig. 4A, the ratio of apoptotic cell death induced by BITC in the  $\rho^+$  cells  
23 increased in a dose-dependent manner up to 50  $\mu$ M. Conversely, apoptotic cell death  
24 in the  $\rho^0$  cells were transiently increased with the peak of 23 % apoptosis at 25  $\mu$ M.  
25 In the  $\rho^0$  cells, treatment with 50  $\mu$ M BITC resulted in reduction of the apoptotic cell  
26 ratio and an increase in the cells with features of necrosis (Fig. 4B). Also, the  
27 BITC-induced DNA fragmentation was dose-dependently detected in the HeLa  $\rho^+$  cells,

1 while a smaller amount of fragmented DNA was observed in the 25  $\mu\text{M}$  BITC-treated  
2 HeLa  $\rho^0$  cells (Fig. 4C) despite the flow cytometry data that the annexin V-positive cell  
3 ratio induced by 25  $\mu\text{M}$  BITC was quite similar between these cells (Fig. 4A). These  
4 contradictory observations might be due to the fact that the DNA fragmentation is  
5 downstream of the apoptosis process compared to phosphatidylserine outer membrane  
6 exposure or that the concentration of BITC at 25  $\mu\text{M}$  is nearly a threshold of tuning the  
7 mode between apoptosis and necrosis-like cells.

8 In any case, significant apoptosis/necrosis switching in the  $\rho^0$  cells might be  
9 selective to BITC, because cycloheximide (CHX), an anti-cancer antibiotic inhibiting  
10 protein synthesis, exhibited a more potent cytotoxic effect on the  $\rho^+$  cells than the  $\rho^0$   
11 cells, and apoptosis/necrosis switching was not observed (Figs. 5A and B).

#### 12 13 3.4. Effect of BITC on intracellular ATP level in HeLa $\rho^0$ cells

14 To further explore the regulating mechanism of BITC-induced cell death  
15 mode, we investigated the intracellular ATP level using the  $\rho^0$  cells. As shown in Fig.  
16 6, the ATP level in the  $\rho^+$  cells was increased by 25  $\mu\text{M}$  BITC up to 3 h, and sustained  
17 at the basal level 24 h after the treatment. Conversely, the ATP level in the  $\rho^0$  cells  
18 was significantly decreased 3 h after the BITC treatment, and continuously declined up  
19 to 24 h. Thus, BITC might affect energy production system more severely in the  $\rho^0$   
20 cells than in the  $\rho^+$  cells. Most of the energy production in the  $\rho^0$  cells depends on the  
21 glycolytic system due to the deficiency of mitochondrial electron transport chain.  
22 The GAPDH activities, one of the key enzymes for glycolytic system and it has been  
23 reported that BITC is able to inactivate GAPDH *in vitro* by modulating the cysteine  
24 residue at an active site center, slightly, but significantly, decreased in a BITC  
25 dose-dependent manner in the both of  $\rho^0$  and  $\rho^+$  cells (Fig. S5). Although these  
26 partial inhibitory effects may be insufficient to explain the rapid ATP decline, BITC is  
27 very likely to modify the total activity of glycolytic system.

1

2 *3.5. Involvement of caspase cascade to the apoptosis/necrosis switch by BITC*

3 Our previous report showed that BITC activates caspase-9 and -3 during the  
4 apoptosis induction [9]. Here we examined the effect of caspase inhibitor on the  
5 BITC-induced cell death in the  $\rho^0$  cells. Interestingly, z-VAD-fmk, a caspase inhibitor,  
6 was capable of rescuing the cells not only from apoptosis in the  $\rho^+$  cells, but also from  
7 necrosis-like death in the  $\rho^0$  cells regardless of the ATP deprivation (Fig. 7). These  
8 results suggest that necrotic-featured cell death induced by BITC is placed downstream  
9 of the caspase cascade.

10

11 **4. Discussion**

12 In this study, we examined the molecular mechanism of cell death  
13 morphology alteration induced by the different concentration of BITC, and  
14 demonstrated the involvement of the intracellular ATP level. The complete apoptotic  
15 process involves energy requiring steps such as caspase activation, enzymatic  
16 hydrolysis of macromolecules, chromatin condensation, bleb formation and apoptotic  
17 body formation. A deficiency of ATP in these steps would prevent downstream  
18 processes including caspase-3 activation, because, for example, the failure of  
19 apoptosome formation is unable to not only activate but also amplify the caspase  
20 cascade [35]. Also, low intracellular levels of free ATP might permit apoptotic  
21 stimuli to induce necrosis [33,36,37,38,39,40]. Consistent with the results from  
22 previous studies, the present results showed that a greater amount of cells had their  
23 death form shifted to necrosis in experiments using  $\rho$  cells as well as using 2DG (Figs.  
24 2 and 4). Hence, our results demonstrated here that higher concentration of BITC  
25 itself rapidly reduced the intracellular ATP level (Fig. 2A), which might block the  
26 completion of apoptosis. Also, not only higher concentration of BITC itself [9] but  
27 also 2DG pretreatment significantly inhibited caspase-3 activity (Fig. 2C). Although



1 further study to reveal the detailed mechanism for the ATP decline by BITC is required,  
2 we showed that both the membrane permeability enhancement and inhibition of  
3 glycolytic system by BITC would partially contribute to energy deprivation. Taken  
4 together, it appears certain that the intracellular ATP level is a key determinant to  
5 decide the cell death form.

6 In addition to the significant role of ATP, we demonstrated that not only  
7 apoptosis, but also necrosis were counteracted by treatment with the caspase inhibitor,  
8 Z-VAD-fmk (Fig. 7). These results implied that the BITC-induced necrotic-featured  
9 cell death might be a consecutive event from the apoptosis program incompleteness rather  
10 than simple accidental death by severe physiological damage, although BITC itself has  
11 a weak membrane lytic activity (unpublished observation). It has recently been  
12 reported that cells triggered to undergo apoptosis are instead forced to die by necrosis  
13 when the energy level is rapidly compromised [36,41]. There is other evidence that in  
14 vivo, under pathological conditions, apoptosis and necrosis may often coexist. These  
15 imply that several types of cell death pathway can coexist in the same cell. In contrast  
16 to apoptotic cells, which are cleared by phagocytosis and subsequent intracellular  
17 degradation [42], necrotic cells are thought to promote an inflammatory response  
18 mainly caused by the leakage of nucleic acids prior to phagocytosis [43,44]. Therefore,  
19 reducing the necrotic cell death by regulation of the energy level might be practical for  
20 the prevention of an undesirable phenomenon by cytotoxic agents.

21 We previously demonstrated that the BITC-induced intracellular oxidation of  
22 H<sub>2</sub>DCF-DA is mainly due to the production of hydrogen peroxide derived from  
23 superoxide radical dismutation [9]. Our findings were that BITC directly modifies the  
24 mitochondrial respiratory chain [9,34] and activates ataxia telangiectasia-mutated  
25 (ATM) possibly in response to DNA oxidative damage and ATM-dependent p53  
26 accumulation [24]. Previous reports indicated that ROS produced by ITCs trigger the  
27 activation of antioxidant response element (ARE) following to ARE-driven antioxidant

1 enzyme induction, such as GSTP1, GCLC, TR, and AKR1C1, through the Nrf2/Keap1  
2 pathway [45,46]. Consistent with previous results, an ROS accumulation occurred in  
3 the BITC-treated wild-type  $\rho^+$  HeLa cells, while the  $\rho^0$  cells exhibited a lower  
4 sensitivity (Fig. 3B). Thus the present experiment using  $\rho^0$  HeLa cells further  
5 confirmed that BITC-induced ROS originated from mitochondrial electron transfer  
6 chain with no significant change of various antioxidant enzyme gene expressions. We  
7 observed that the  $\rho^0$  cells are sensitive to BITC cytotoxic stimulus, even though their  
8 apoptosis/necrosis ratio was quite distinct from that of the  $\rho^+$  cells, indicating that the  
9 mode of cell death induced by BITC is independent of the ROS produced, and this will  
10 depend on the reactive preference of BITC against the energy metabolism such as  
11 glycolysis or tricarboxylic acid cycle. It should be pointed out that, experimentally,  
12 hydrogen peroxide failed to induce apoptosis in cells in which the ATP levels are below  
13 roughly 25% of the control ATP levels [47,48]. Therefore, if ROS were attributable to  
14 the BITC-induced cell death, it would also depend on the intracellular ATP level

15 Future studies will examine the molecular targets and pathways that account for  
16 the BITC-induced alteration of the ATP level in both the  $\rho^+$  cells and  $\rho^0$  cells. The  
17 rapid loss of membrane integrity in cells treated with BITC (as evidenced by the PI  
18 uptake) indicates that the BITC-induced necrosis derives in part from the direct attack  
19 on the cell membrane. BITC is known to modify protein sulfhydryls [7,34,49,50] and  
20 thus BITC may not act on membrane lipids, even though BITC-induced ROS can  
21 oxidize them. BITC has been shown to modify and inhibit the activities of various  
22 proteins [7,50]. As well, we observed that GAPDH activity, having a cysteine residue  
23 at the active site, was slightly decreased by BITC in both the  $\rho^0$  and  $\rho^+$  cells (Fig. S5).  
24 The critical task for apoptosis/necrosis switching research will be to identify the  
25 specific molecular targets that initiate ATP deprivation.

26 In conclusion, to the best of our knowledge, the present study is the first  
27 published report to convincingly document the induction of necrosis by an ITC class of

1 dietary chemopreventive agents. The present study also provides biological evidence  
2 of cell death mode switching from apoptosis to necrosis-like cell death by BITC. The  
3 switching was dose-dependently induced by BITC, in which the intracellular ATP level  
4 plays a critical role in it. We preliminarily observed that higher concentration of  
5 BITC exhibits ATP-dependent alteration of cell death mode in several cell lines such as  
6 the renal proximal tubular epithelial LLC-PK<sub>1</sub> cells (Nakamura, Y. et al., unpublished  
7 data), suggesting that these phenomena might not be due to cell-specific effects. The  
8 ITCs are one group of the most promising cancer preventive agents if their amounts are  
9 relevant. However, BITC experimentally possesses both carcinogenesis and  
10 anti-carcinogenesis activity in a dose-dependent manner. Our results support the idea  
11 that the intake of excess amount of ITCs, inducing necrotic cell death, would provoke  
12 an inflammation reaction which could contribute to promoting the carcinogenesis  
13 process. In this study, we examined the concentration of BITC up to 100  $\mu$ M as a  
14 supra-pharmacological but locally achievable dose, since the recent preclinical  
15 evaluation revealed that ITCs concentration in the gastric lumina was temporally  
16 achieved to approximately 600-2000  $\mu$ M, after the consumption of the broccoli extract  
17 [51]. Therefore, more attention should be paid to the dose administered as a  
18 supplement of condensed extracts, and thus carefully designed pharmacokinetics  
19 studies are needed before clinical testing of ITCs.

20

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24 of the Japanese Government.

25

**References**

- [ 1 ] S.S. Hecht, Inhibition of carcinogenesis by isothiocyanates, *Drug. Metab. Rev.* 32 (2000) 395-411.
- [ 2 ] G.J. Kelloff, J.A. Crowell, V.E. Steele, R.A. Lubet, C.W. Boone, W.A. Malone, E.T. Hawk, R. Lieberman, J.A. Lawrence, L. Kopelovich, I. Ali, J.L. Viner, C.C. Sigman, Progress in cancer chemoprevention, *Ann. N Y Acad. Sci.* 889 (1999) 1-13.
- [ 3 ] B. Zhao, A. Seow, E.J. Lee, W.T. Poh, M. Teh, P. Eng, Y.T. Wang, W.C. Tan, M.C. Yu, H.P. Lee, Dietary isothiocyanates, glutathione S-transferase-M1, -T1 polymorphisms and lung cancer risk among Chinese women in Singapore, *Cancer Epidemiol. Biomarkers Prev.* 10 (2001) 1063-1067.
- [ 4 ] H.J. Lin, N.M. Probst-Hensch, A.D. Louie, I.H. Kau, J.S. Witte, S.A. Ingles, H.D. Frankl, E.R. Lee, R.W. Haile, Glutathione transferase null genotype, broccoli, and lower prevalence of colorectal adenomas, *Cancer Epidemiol. Biomarkers Prev.* 7 (1998) 647-652.
- [ 5 ] Y. Zhang, Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action, *Mutat. Res.* 555 (2004) 173-190.
- [ 6 ] N. Miyoshi, K. Uchida, T. Osawa, Y. Nakamura, A link between benzyl isothiocyanate-induced cell cycle arrest and apoptosis: involvement of mitogen-activated protein kinases in the Bcl-2 phosphorylation, *Cancer Res.* 64 (2004) 2134-2142.
- [ 7 ] N. Miyoshi, S. Takabayashi, T. Osawa, Y. Nakamura, Benzyl isothiocyanate inhibits excessive superoxide generation in inflammatory leukocytes: implication for prevention against inflammation-related carcinogenesis, *Carcinogenesis* 25 (2004) 567-575.
- [ 8 ] Y. Nakamura, N. Miyoshi, Cell death induction by isothiocyanates and their

underlying molecular mechanisms, *Biofactors* 26 (2006) 123-134.

[9] Y. Nakamura, M. Kawakami, A. Yoshihiro, N. Miyoshi, H. Ohigashi, K. Kawai, T. Osawa, K. Uchida, Involvement of the mitochondrial death pathway in chemopreventive benzyl isothiocyanate-induced apoptosis, *J. Biol. Chem.* 277 (2002) 8492-8499.

[10] M. Hirose, T. Yamaguchi, N. Kimoto, K. Ogawa, M. Futakuchi, M. Sano, T. Shirai, Strong promoting activity of phenylethyl isothiocyanate and benzyl isothiocyanate on urinary bladder carcinogenesis in F344 male rats, *Int. J. Cancer* 77 (1998) 773-777.

[11] K. Akagi, M. Sano, K. Ogawa, M. Hirose, H. Goshima, T. Shirai, Involvement of toxicity as an early event in urinary bladder carcinogenesis induced by phenethyl isothiocyanate, benzyl isothiocyanate, and analogues in F344 rats, *Toxicol. Pathol.* 31 (2003) 388-396.

[12] G. Kroemer, W.S. El-Deiry, P. Golstein, M.E. Peter, D. Vaux, P. Vandenabeele, B. Zhivotovsky, M.V. Blagosklonny, W. Malorni, R.A. Knight, M. Piacentini, S. Nagata, G. Melino, Classification of cell death: recommendations of the Nomenclature Committee on Cell Death, *Cell Death Differ.* 12 Suppl 2 (2005) 1463-1467.

[13] G. Denecker, D. Vercammen, W. Declercq, P. Vandenabeele, Apoptotic and necrotic cell death induced by death domain receptors, *Cell. Mol. Life Sci.* 58 (2001) 356-370.

[14] N. Festjens, T. Vanden Berghe, P. Vandenabeele, Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response, *Biochim. Biophys. Acta.* 1757 (2006) 1371-1387.

[15] W.X. Zong, C.B. Thompson, Necrotic death as a cell fate, *Genes Dev.* 20 (2006) 1-15.

[16] J. Yuan, Divergence from a dedicated cellular suicide mechanism: exploring

the evolution of cell death, *Mol. Cell* 23 (2006) 1-12.

[17] A.L. Edinger, C.B. Thompson, Death by design: apoptosis, necrosis and autophagy, *Curr. Opin. Cell Biol.* 16 (2004) 663-669.

[18] L. Zitvogel, N. Casares, M.O. Pequignot, N. Chaput, M.L. Albert, G. Kroemer, Immune response against dying tumor cells, *Adv. Immunol.* 84 (2004) 131-179.

[19] P. Golstein, G. Kroemer, Redundant cell death mechanisms as relics and backups, *Cell Death Differ.* 12 Suppl 2 (2005) 1490-1496.

[20] M. Chautan, G. Chazal, F. Cecconi, P. Gruss, P. Golstein, Interdigital cell death can occur through a necrotic and caspase-independent pathway, *Curr. Biol.* 9 (1999) 967-970.

[21] G. Kroemer, S.J. Martin, Caspase-independent cell death, *Nat. Med.* 11 (2005) 725-730.

[22] J. Hayashi, S. Ohta, A. Kikuchi, M. Takemitsu, Y. Goto, I. Nonaka, Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 10614-10618.

[23] N. Miyoshi, K. Uchida, T. Osawa, Y. Nakamura, Benzyl isothiocyanate modifies expression of the G2/M arrest-related genes, *Biofactors* 21 (2004) 23-26.

[24] N. Miyoshi, K. Uchida, T. Osawa, Y. Nakamura, Selective cytotoxicity of benzyl isothiocyanate in the proliferating fibroblastoid cells, *Int. J. Cancer* 120 (2007) 484-492.

[25] R. Zhang, S. Loganathan, I. Humphreys, S.K. Srivastava, Benzyl isothiocyanate-induced DNA damage causes G2/M cell cycle arrest and apoptosis in human pancreatic cancer cells, *J. Nutr.* 136 (2006) 2728-2734.

[26] S. Kalkunte, N. Swamy, D.S. Dizon, L. Brard, Benzyl isothiocyanate (BITC) induces apoptosis in ovarian cancer cells in vitro, *J. Exp. Ther. Oncol.* 5 (2006) 287-300.

- [27] Y.F. Kuang, Y.H. Chen, Induction of apoptosis in a non-small cell human lung cancer cell line by isothiocyanates is associated with p53 and p21, *Food Chem. Toxicol.* 42 (2004) 1711-1718.
- [28] S.K. Srivastava, S.V. Singh, Cell cycle arrest, apoptosis induction and inhibition of nuclear factor kappa B activation in anti-proliferative activity of benzyl isothiocyanate against human pancreatic cancer cells, *Carcinogenesis* 25 (2004) 1701-1709.
- [29] V.W. Lui, A.L. Wentzel, D. Xiao, K.L. Lew, S.V. Singh, J.R. Grandis, Requirement of a carbon spacer in benzyl isothiocyanate-mediated cytotoxicity and MAPK activation in head and neck squamous cell carcinoma, *Carcinogenesis* 24 (2003) 1705-1712.
- [30] Y.M. Yang, C.C. Conaway, J.W. Chiao, C.X. Wang, S. Amin, J. Whysner, W. Dai, J. Reinhardt, F.L. Chung, Inhibition of benzo(a)pyrene-induced lung tumorigenesis in A/J mice by dietary N-acetylcysteine conjugates of benzyl and phenethyl isothiocyanates during the postinitiation phase is associated with activation of mitogen-activated protein kinases and p53 activity and induction of apoptosis, *Cancer Res.* 62 (2002) 2-7.
- [31] C. Bonnesen, I.M. Eggleston, J.D. Hayes, Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines, *Cancer Res.* 61 (2001) 6120-6130.
- [32] R. Yu, S. Mandlekar, K.J. Harvey, D.S. Ucker, A.N. Kong, Chemopreventive isothiocyanates induce apoptosis and caspase-3-like protease activity, *Cancer Res.* 58 (1998) 402-408.
- [33] N. Miyoshi, H. Oubrahim, P.B. Chock, E.R. Stadtman, Age-dependent cell death and the role of ATP in hydrogen peroxide-induced apoptosis and necrosis, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 1727-1731.

- [34] M. Kawakami, N. Harada, M. Hiratsuka, K. Kawai, Y. Nakamura, Dietary isothiocyanates modify mitochondrial functions through their electrophilic reaction, *Biosci. Biotechnol. Biochem.* 69 (2005) 2439-2444.
- [35] X. Wang, The expanding role of mitochondria in apoptosis, *Genes Dev.* 15 (2001) 2922-33.
- [36] M. Leist, B. Single, A.F. Castoldi, S. Kuhnle, P. Nicotera, Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis, *J. Exp. Med.* 185 (1997) 1481-1486.
- [37] P. Nicotera, G. Melino, Regulation of the apoptosis-necrosis switch, *Oncogene* 23 (2004) 2757-2765.
- [38] Z. Fishelson, G. Attali, D. Mevorach, Complement and apoptosis, *Mol. Immunol.* 38 (2001) 207-219.
- [39] D. Kanduc, A. Mittelman, R. Serpico, E. Sinigaglia, A.A. Sinha, C. Natale, R. Santacroce, M.G. Di Corcia, A. Lucchese, L. Dini, P. Pani, S. Santacroce, S. Simone, R. Bucci, E. Farber, Cell death: apoptosis versus necrosis (review), *Int. J. Oncol.* 21 (2002) 165-170.
- [40] L. Vergara, X. Bao, E. Bello-Reuss, L. Reuss, Do connexin 43 gap-junctional hemichannels activate and cause cell damage during ATP depletion of renal-tubule cells?, *Acta. Physiol. Scand.* 179 (2003) 33-38.
- [41] M. Leist, F. Gantner, S. Jilg, A. Wendel, Activation of the 55 kDa TNF receptor is necessary and sufficient for TNF-induced liver failure, hepatocyte apoptosis, and nitrite release, *J Immunol* 154 (1995) 1307-1316.
- [42] H. Yoshida, K. Kawane, M. Koike, Y. Mori, Y. Uchiyama, S. Nagata, Phosphatidylserine-dependent engulfment by macrophages of nuclei from erythroid precursor cells, *Nature* 437 (2005) 754-758.
- [43] K. Kawane, M. Ohtani, K. Miwa, T. Kizawa, Y. Kanbara, Y. Yoshioka, H. Yoshikawa, S. Nagata, Chronic polyarthritis caused by mammalian DNA that escapes



from degradation in macrophages, *Nature* 443 (2006) 998-1002.

[44] S. Nagata, DNA degradation in development and programmed cell death, *Annu. Rev. Immunol.* 23 (2005) 853-875.

[45] Y. Nakamura, H. Ohigashi, S. Masuda, A. Murakami, Y. Morimitsu, Y. Kawamoto, T. Osawa, M. Imagawa, K. Uchida, Redox regulation of glutathione S-transferase induction by benzyl isothiocyanate: correlation of enzyme induction with the formation of reactive oxygen intermediates, *Cancer Res* 60 (2000) 219-225.

[46] X.J. Wang, J.D. Hayes, C.J. Henderson, C.R. Wolf, Identification of retinoic acid as an inhibitor of transcription factor Nrf2 through activation of retinoic acid receptor alpha, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 19589-94.

[47] Y.J. Lee, E. Shacter, Oxidative stress inhibits apoptosis in human lymphoma cells, *J. Biol. Chem.* 274 (1999) 19792-19798.

[48] Y.J. Lee, E. Shacter, Hydrogen peroxide inhibits activation, not activity, of cellular caspase-3 in vivo, *Free Radic. Biol. Med.* 29 (2000) 684-692.

[49] C.C. Conaway, J. Krzeminski, S. Amin, F.L. Chung, Decomposition rates of isothiocyanate conjugates determine their activity as inhibitors of cytochrome p450 enzymes, *Chem. Res. Toxicol.* 14 (2001) 1170-1176.

[50] C.S. Tang, W.J. Tang, Inhibition of papain by isothiocyanates, *Biochim. Biophys. Acta* 452 (1976) 510-520.

[51] B.S. Cornblatt, L. Ye, A.T. Dinkova-Kostova, M. Erb, J.W. Fahey, N.K. Singh, M.S. Chen, T. Stierer, E. Garrett-Mayer, P. Argani, N.E. Davidson, P. Talalay, T.W. Kensler, K. Visvanathan, Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast, *Carcinogenesis* 28 (2007) 1485-1490.

## 1 **Figure legends**

2

3 Fig. 1. Dose-dependent effect of BITC on the apoptosis and necrosis induction.  
4 HeLa cells were treated with BITC at indicated concentration. (A) Effect of BITC on  
5 the plasma membrane reconstitution. The cell population of annexin V (+)/ PI (-) (□)  
6 and annexin V (+)/ PI (+) (■) in each quarter area of flow cytometric analysis  
7 (Representative data are shown in Supplemental Fig. 1D a - e) were quantified using  
8 BD FACSDiva software. Data are means of three experiments. B and C.  
9 summarized the effect of BITC on plasma membrane disruption (B) and cell swelling  
10 (C), performed quantitative analyses by BD FACSDiva software for the cells treated  
11 with 25 (●), or 100 (■) BITC for indicated times. Statistical significance was  
12 determined by the Student's t test and expressed as: \*, versus counterpart of the cells  
13 treated with 25  $\mu$ M BITC,  $P < 0.05$ . (D) DNA fragmentation in the BITC-treated  
14 HeLa cells. The cells were incubated with BITC or etoposide (ETP; lane 7) at the  
15 indicated concentration for 18 hours.

16

17 Fig. 2. Involvement of intracellular ATP level in BITC-induced apoptosis and  
18 necrosis. (A) Time-dependent effect of BITC on the intracellular ATP level and  
19 necrosis in the attached HeLa cells. Values are mean  $\pm$  SD (n = 3). No error bar  
20 means standard deviation for the experiments was within 5%. Statistical significance  
21 was determined by the Student's t test and is expressed as: \*, versus control (0 h)  
22 treated only with DMSO,  $P < 0.05$ . (B) Effect of an ATP synthesis inhibitor on the  
23 BITC-induced apoptosis and necrosis. The cells pretreated with (open bars) or  
24 without (closed bars) 2-deoxyglucose (2-DG) for 12.5 hours were exposed to BITC at  
25 the indicated concentration for 9 hours. Annexin V/ PI analysis were performed to  
26 identify the apoptotic (annexin V (+)/ PI (-)) and necrotic (annexin V (-)/ PI (+)) cell  
27 death. (C) Effect of an ATP synthesis inhibitor on the BITC-induced caspase-3

1 activation. Statistical significance was determined by the Student's t test and  
2 expressed as: a, versus BITC 0  $\mu\text{M}$  group,  $P < 0.05$ , b, versus control (without 2-DG)  
3 group with the same BITC concentration,  $P < 0.05$ .

4

5 Fig. 3. Effect of BITC on ROS production in HeLa  $\rho$  cells. (A) The depletion of  
6 mitochondrial DNA in HeLa  $\rho^0$  cells. (B) Effect of BITC on the intracellular ROS  
7 production in HeLa  $\rho$  cells. The wild type HeLa  $\rho^+$  (closed bars) and mitochondrial  
8 DNA-deficient  $\rho^0$  (open bars) cells were incubated with  $\text{H}_2\text{DCF-DA}$  (50  $\mu\text{M}$ ) for 30  
9 min and then stimulated by BITC. Values are mean  $\pm$  SD ( $n = 3$ ). Statistical  
10 significance was determined by the Student's t test and expressed as: \*, versus the  $\rho^+$   
11 cells group with the same BITC concentration,  $P < 0.05$ . (C) mRNA expression  
12 profile of antioxidant enzyme genes in the  $\rho^+$  and  $\rho^0$  cells were analyzed by RT-PCR.

13

14 Fig. 4. Effect of BITC on cytotoxicity in HeLa  $\rho$  cells. (A, B) Apoptosis and necrosis  
15 induction by BITC in HeLa  $\rho$  cells. The HeLa  $\rho^+$  (closed bars) and  $\rho^0$  (open bars)  
16 were treated with BITC at the indicated concentration for 9.5 hours. Statistical  
17 significance was determined by the Student's t test and expressed as: \*, versus the  $\rho^+$   
18 cells group with the same BITC concentration,  $P < 0.05$ . (C) DNA fragmentation in  
19 the BITC-treated HeLa  $\rho$  cells. The HeLa  $\rho$  cells were incubated with BITC at the  
20 indicated concentration for 18 hours.

21

22 Fig. 5. Effect of cycloheximide on cytotoxicity in HeLa  $\rho$  cells. (A, B) Apoptosis and  
23 necrosis induction by cycloheximide (CHX) in HeLa  $\rho$  cells. The HeLa  $\rho^+$  (closed  
24 bars) and  $\rho^0$  (open bars) were treated with CHX at the indicated concentration for 9.5  
25 hours. Values are mean  $\pm$  SD ( $n = 3$ ). Statistical significance was determined by the  
26 Student's t test and expressed as: \*, versus the  $\rho^+$  cells group with the same CHX  
27 concentration,  $P < 0.05$ .

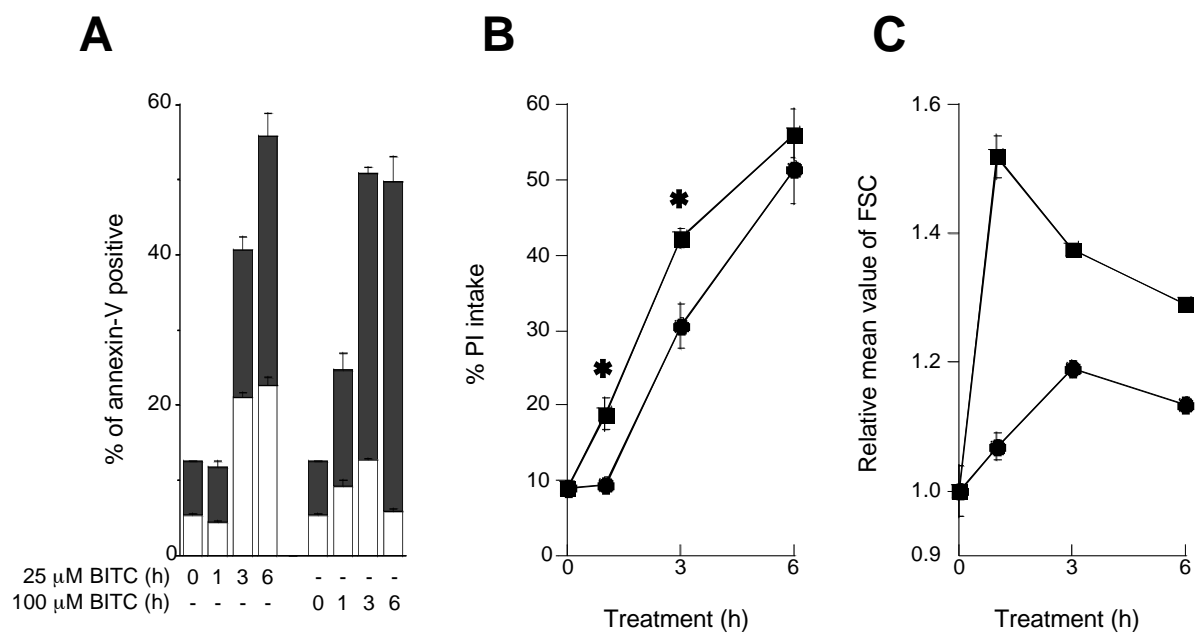
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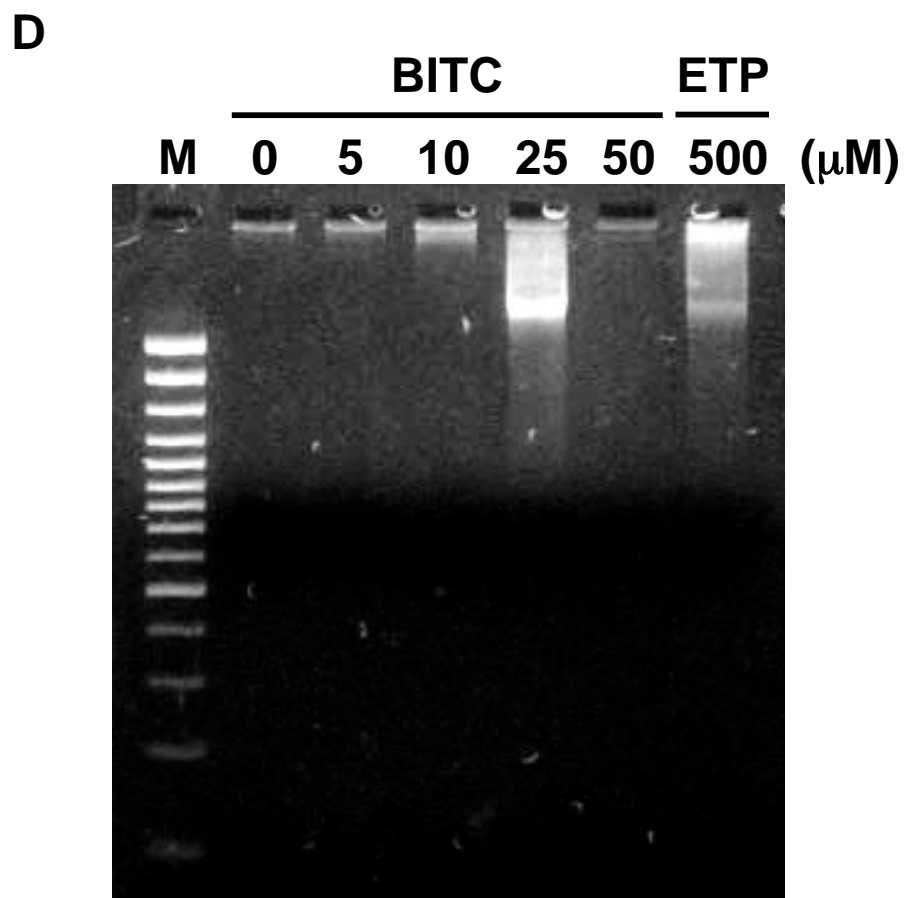
2 Fig. 6. Effect of BITC on intracellular ATP level in HeLa  $\rho^+$   
3 (closed bars) and  $\rho^0$  (open bars) were treated with or without 25  $\mu\text{M}$  BITC for the  
4 indicated times. Values are mean  $\pm$  SD (n = 3). Statistical significance was  
5 determined by the Student's t test and expressed as: \*, versus the  $\rho^+$  cells group with  
6 the same BITC concentration,  $P < 0.05$ .

7

8 Fig. 7. Inhibitory effect of caspase inhibitor on the BITC-induced apoptosis and  
9 necrosis in HeLa  $\rho^+$  and  $\rho^0$  cells. The HeLa  $\rho^+$  and  $\rho^0$  cells were treated with BITC for 9 hours  
10 in the presence or absence of the caspase inhibitor, Z-VAD-fmk. The maximum SD  
11 for the duplicated experiments was 5% (n=3). a, significantly different in apoptosis  
12 from each ( $\rho^+$  or  $\rho^0$ ) control (0  $\mu\text{M}$  BITC and z-VAD-fmk),  $P < 0.05$ ; b, significantly  
13 different in necrosis from each ( $\rho^+$  or  $\rho^0$ ) control (0  $\mu\text{M}$  BITC and z-VAD-fmk),  $P <$   
14 0.05; c, significantly different in total cell death (apoptosis + necrosis) from each ( $\rho^+$  or  
15  $\rho^0$ ) control (0  $\mu\text{M}$  BITC and z-VAD-fmk),  $P < 0.05$ .

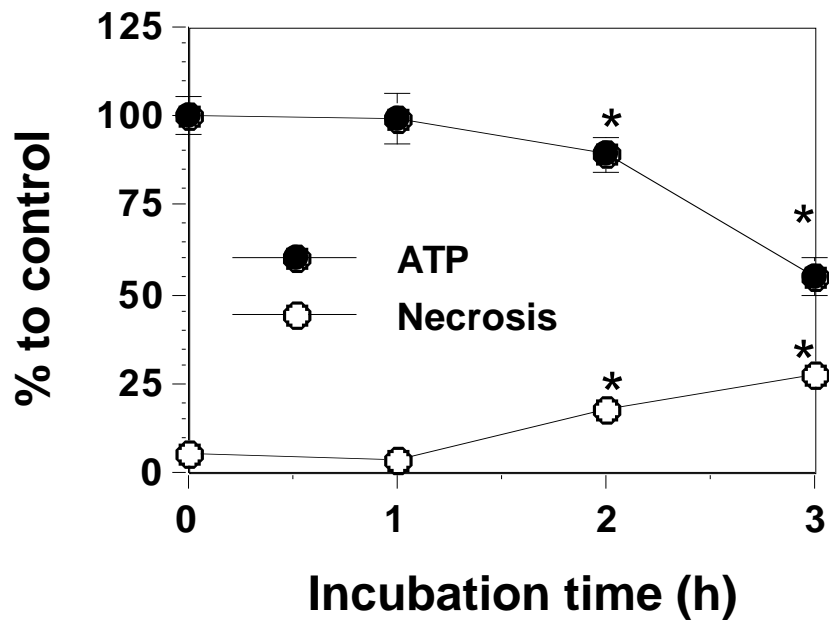
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Fig. 1, Miyoshi, *et al.*

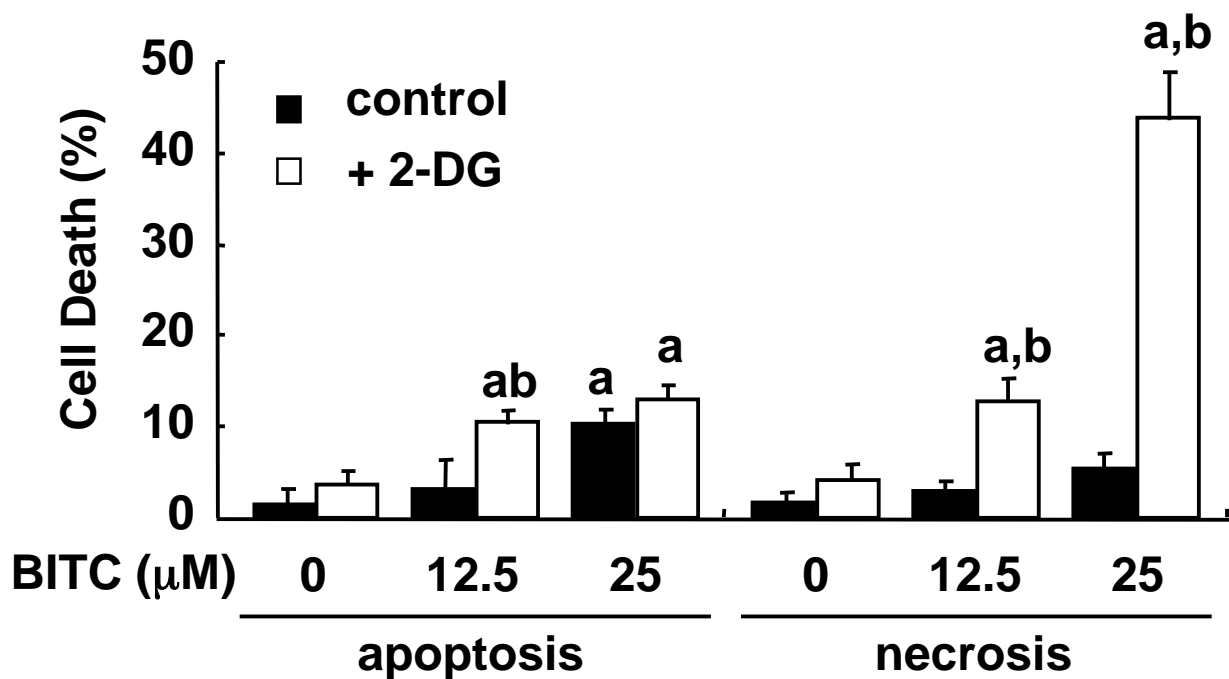


**Fig. 1, Miyoshi, *et al.***

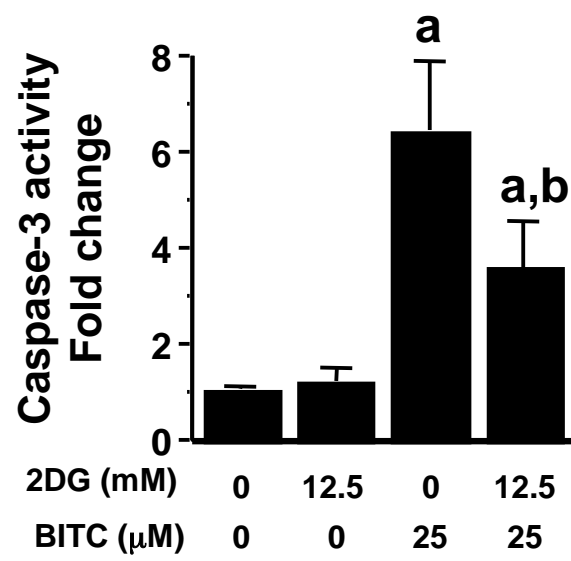
A



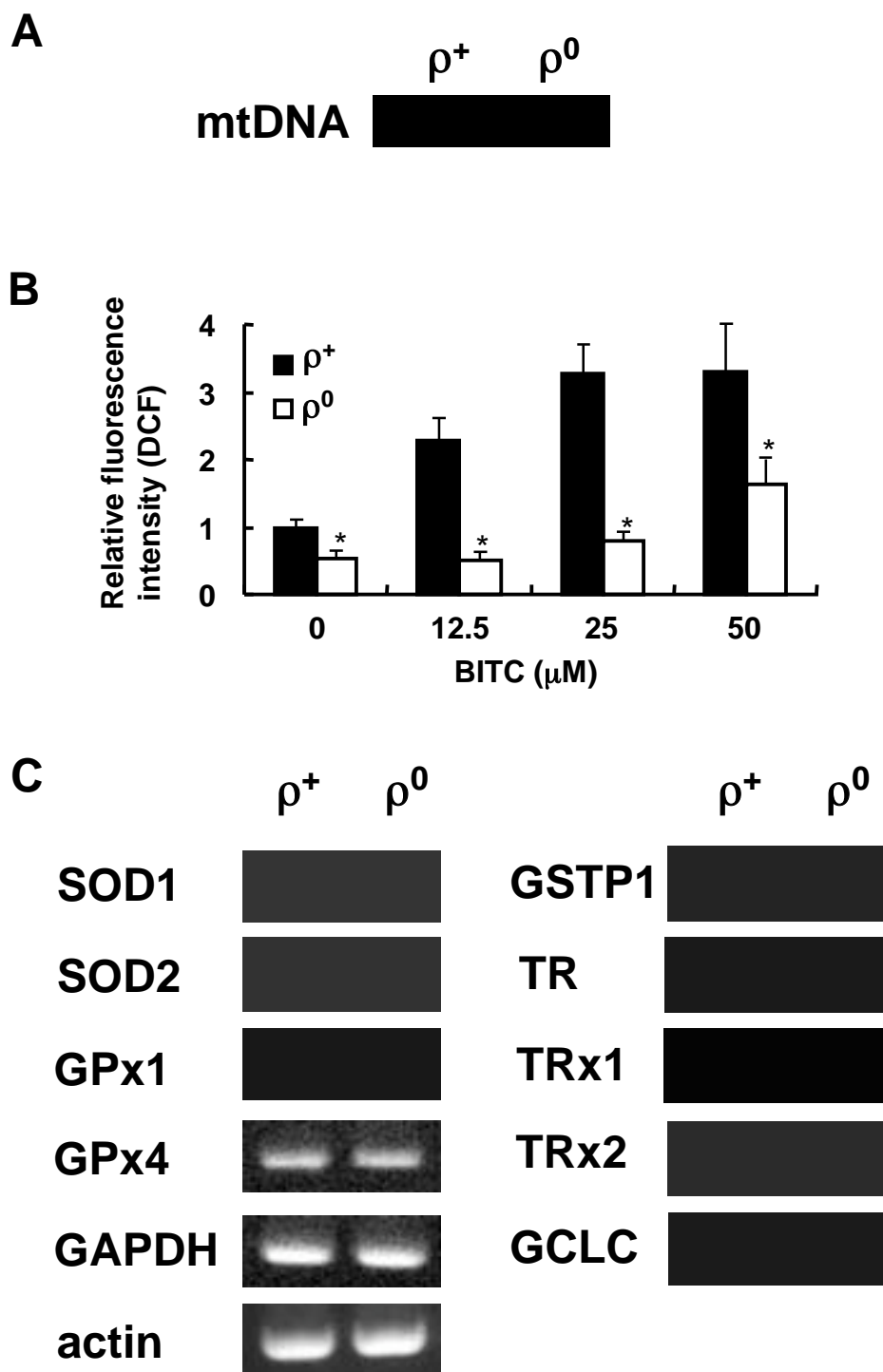
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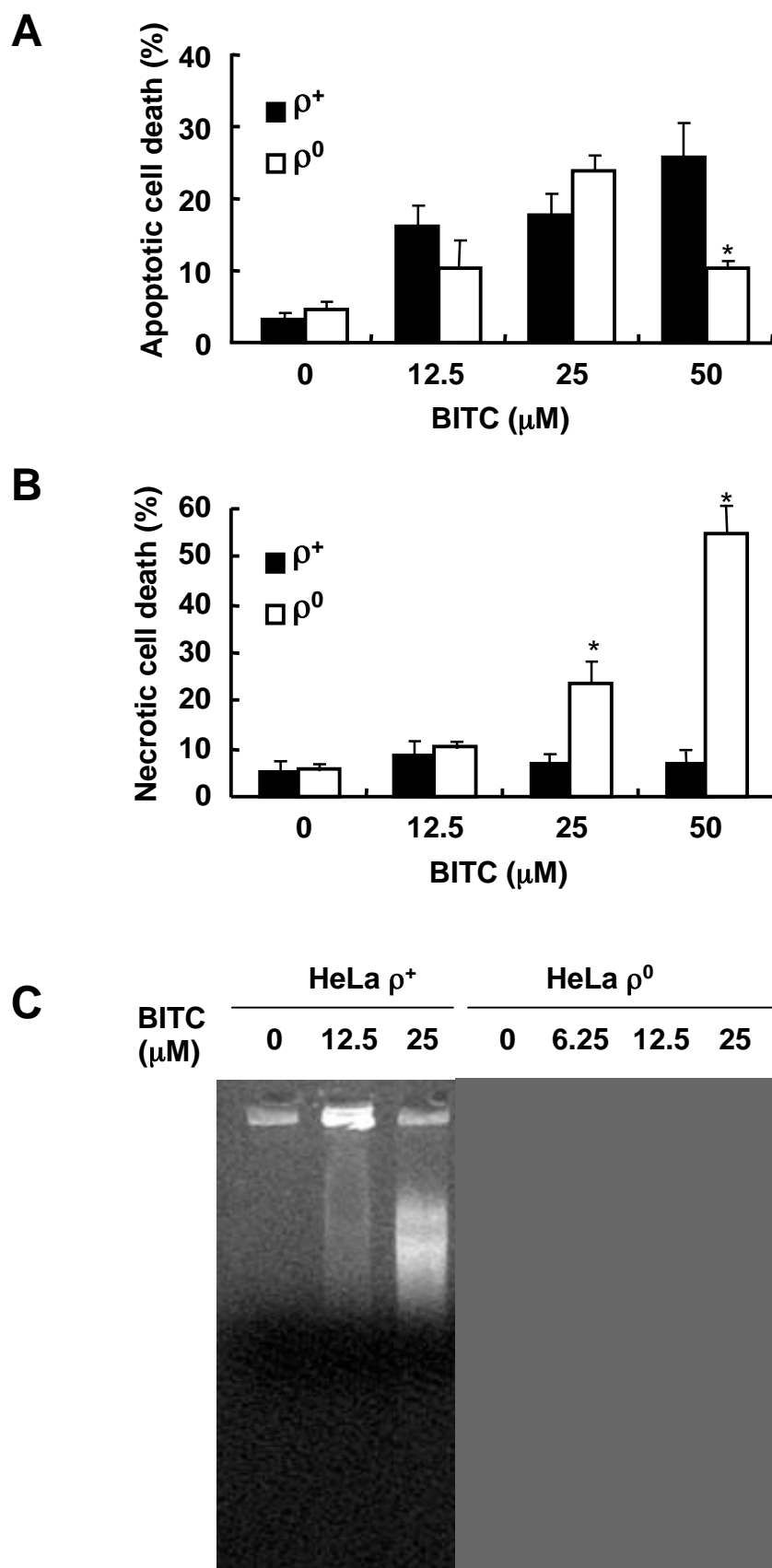
Fig. 2, Miyoshi, *et al.*

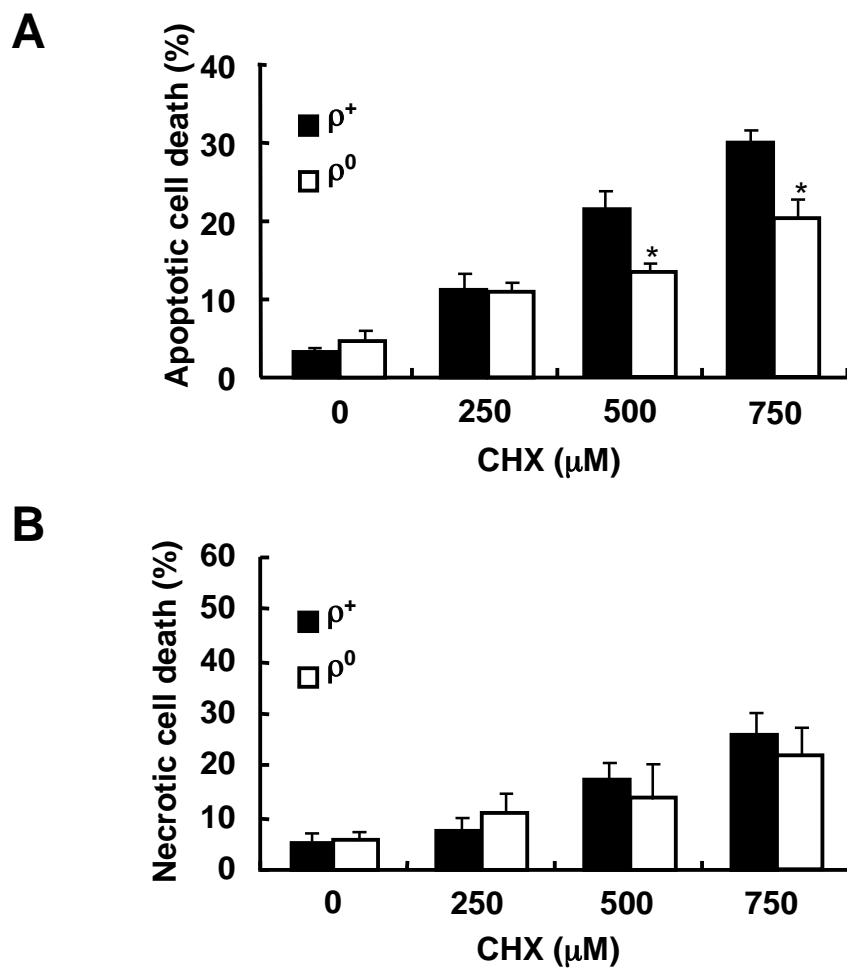
C

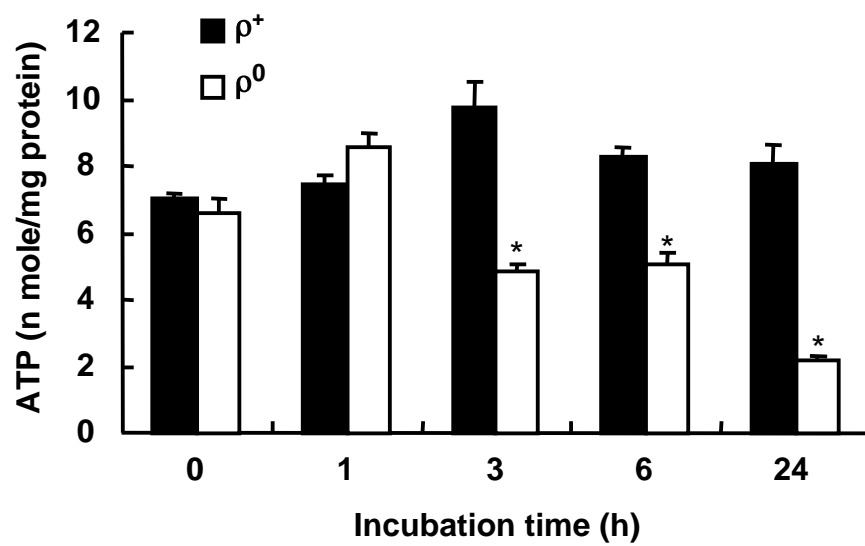
Fig. 2, Miyoshi, *et al.*



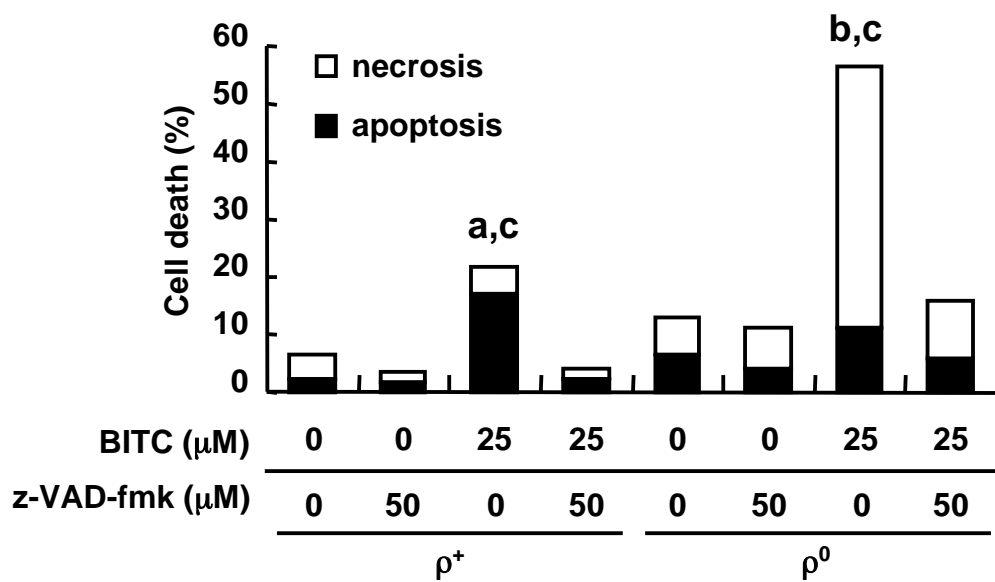
Fig. 3, Miyoshi, *et al.*

Fig. 4, Miyoshi, *et al.*

Fig. 5, Miyoshi, *et al.*



**Fig. 6, Miyoshi, *et al.***

Fig. 7, Miyoshi, *et al.*