

# RC-RNase-induced cell death in estrogen receptor positive breast tumors through down-regulation of Bcl-2 and estrogen receptor

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**Abstract.** RC-RNase exerts anti-cancer effects on many tumors. However, the mechanisms by which RC-RNase induces cytotoxicity in different tumor cells are unclear. Currently, estrogen receptor (ER)-positive and negative breast tumors are treated with RC-RNase. Our data demonstrate that RC-RNase induces cell death on ER-positive but not on ER-negative breast tumors. This study also shows that down-regulation of ER and Bcl-2 is found on RC-RNase-treated ER-positive breast tumors. Additionally, Bcl-2 overexpression can prevent ER-positive breast tumors from cell death treated with RC-RNase. In summary, this study demonstrates that

RC-RNase-induced cell death of ER-positive breast tumors is through regulation of ER and Bcl-2.

## Introduction

The anti-cancer effects of RNases have been demonstrated in several studies (1-3). Onconase, derived from *Rana pipiens* and RC-RNase from *Rana catesbeiana* both belong to the RNase family exerting anti-cancer activities (4-7). Presently, onconase has been used in the treatment of tumors in some clinical trails done in the USA and Europe (8-10). RC-RNase, with about 50% of its amino acid sequences homologous to that of onconase, exerts similar anti-cancer activities (5,7,11). Many studies have demonstrated that onconase and RC-RNase can induce cell death on many tumor cells (5,7,11-13). However, the mechanisms of RNase-induced cytotoxicity and therapeutic target have remained unclear.

Our previous study has demonstrated that RC-RNases can induce different cytotoxicity on different tumor cells in humans and showed that RC-RNase induces cell death on breast tumors, hepatic tumors and leukemia through different caspase pathways (11). Additionally, many studies have also demonstrated that the degree of cytotoxicity induced by RC-RNase correlates with the stages of differentiation of tumor cells (6,14,15). These studies indicated that RC-RNase strongly exerts its anti-cancer activity on poorly-differentiated tumors, however, exerts a much lower activity on those tumors that are well-differentiated. However, the site where the RC-RNase will target the tumor cells to exert its cytotoxic effect is still unclear.

In this study, our primary data showed that RC-RNase can induce cytotoxicity on MCF-7 and ZR-75-1 breast

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*Abbreviations:* XTT, sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate; RC-RNase, *Rana catesbeiana* ribonuclease; ER, estrogen receptor

*Key words:* estrogen receptor, Bcl-2, breast tumor

tumors but cytotoxicity was not induced on MDA-MB-231 and ZR-75-30 containing tumors. This result indicated that RC-RNase can induce different cytotoxicity on breast tumors depending on its receptors. Therefore, MCF-7, MDA-MB-231, ZR-75-1 and ZR-75-30 containing breast tumors are widely used to study the target sites of RC-RNase activity on breast tumors.

Many reports have shown that ER-positive breast tumors have MCF-7 and ZR-75-1 (16-19) while ER-negative tumors have MDA-MB-231 and ZR-75-30 (20-22). Primarily, data presented in this report show that RC-RNase induces cell death on MCF-7 and ZR-75-1 tumors, but not on MDA-MB-231 and ZR-75-30 tumors. That is, RC-RNase only induces cell death on ER-positive breast tumors. We further study estrogen receptor level on RC-RNase-treated ER-positive breast tumors. Our data showed that RC-RNase can induce down-regulation of ER. Therefore, ER is an important target of RC-RNase-induced cytotoxicity on ER-positive breast tumors. In addition, our data showed that down-regulation of Bcl-2 was found on RC-RNase-treated ER-positive breast tumors. Previous studies indicated that Bcl-2 and Bcl-XL belong to Bcl-2 family and demonstrated that overexpression of Bcl-2 or Bcl-XL has anti-apoptosis effects (23-26). However, our previous study demonstrated that overexpression of Bcl-XL can not inhibit RC-RNase-induced cytotoxicity on ER-positive breast tumors (5). We investigated whether Bcl-2 can inhibit RC-RNase-induced cytotoxicity. Our study showed that overexpression of Bcl-2 can inhibit RC-RNase-induced cytotoxicity on ER-positive breast tumors. Overall, we firstly demonstrated that RC-RNase induces cytotoxicity on ER-positive breast tumors, but not on ER-negative breast tumors through ER and Bcl-2 down-regulation.

## Materials and methods

**Reagents and cell culture.** RC-RNase was purified with the modified methods described in previous studies (5,6,11). Ac-DEVD-pNA (Acetyl-Asp-Glu-Val-Asp-p-nitroanilide) was purchased from Anaspec (San Jose, CA). XTT assay kit was procured from Roche (Mannheim, Germany). Bcl-2 antibody was purchased from Upstate. ER and actin antibodies were commissioned from Pharmingen Laboratories (San Diego, CA), and Chemicon Laboratories (Temecula, CA), respectively. Dr Jiang (Tzu Chi General Hospital) provided the human breast carcinoma cells with MCF-7, MDA-MB-231, ZR-75-1 and ZR-75-30 and cultures were made using Dulbecco's Modified Eagle Medium (Gibco BRL) supplemented with 10%-heat-inactivated-fetal-bovine serum (Hyclone® Laboratories, Inc., Logan, UT), 2 mM L-glutamine (Gibco BRL), 100 IU/ml penicillin G sodium (Gibco BRL), 100 µg/ml streptomycin sulfate (Gibco BRL), 1 mM sodium pyruvate (Sigma Chemical Co., St. Louis, MO) and 0.1 mM non-essential amino acids (Gibco BRL).

**Survival rate assay.** Cell survival rate was determined using XTT {sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate} kit which analyzes the activity of mitochondrial dehydrogenase. Briefly,  $2 \times 10^3$ -cells were grown in each well of 96-well-

containing cell culture plates overnight. The following day, these cells were treated with RC-RNase. XTT assays were carefully performed every 24 h following instructions from the manufacturer. Absorbance was determined at 492 nm using a multi-well ELISA reader (Molecular Devices, Sunnyvale, CA).

**Caspase activity assay.** Cells were treated with the lytic buffer (50 mM Tris-HCl, 120 mM NaCl, 1 mM EDTA, 1% NP-40, pH 7.5) supplemented with protease inhibitors. Cell pellets were removed via centrifugation at 15000 x g for 20 min at 4°C. The caspase activity assay was determined in a reaction solution containing 40 µl cell lysates (80 µg total protein), 158 µl of reaction buffer (20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol, 100 mM HEPES, pH 7.5) and 2 µl of fluorogenic Ac-DEVD-pNA and was incubated at 37°C for 6 h. The fluorogenic substrate cleavage readout was the p-nitroanilide release as detected at 405 nm in an ultra-microplate reader (Bio-Tek instruments).

**Western blot analysis.** Cells were collected using cell scrapers and lysed in RIPA buffer (10 mM Tris-base, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Calbiochem, La Jolla, CA). Equal amounts of the total proteins were loaded into a 13.3% SDS-polyacrylamide gel and underwent electrophoresis. They were then transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). The membranes were blocked with 5% skim milk and 1% NP-40 in TBS-T (0.8% NaCl, 0.02% KCl, 25 mM Tris-HCl, 0.05% Tween-20, at pH 7.4) for 1 h, incubated with the primary antibody (1:500 dilution in the blocking buffer) at 4°C overnight, and subsequent incubation was done with biotinylated anti-mouse or anti-rabbit IgG (1:1000 or 1:10000 dilution in the blocking buffer) and streptavidin-horseradish peroxidase conjugates (1:2000 dilution in PBS). The membranes were developed using the Super Signal™ chemiluminescent-HRP substrate system (Pierce, Rockford, IL).

**Establishment of transfectants overexpressing Bcl-2 and Bcl-XL.** Human Bcl-2/PCR 3.1 and Bcl-XL/PCR 3.1 plasmids were constructed employing methods as mentioned previously (5). MCF-7 cells were transfected with Bcl-2/PCR 3.1 or Bcl-XL/PCR 3.1 plasmids using lipofectamine (Gibco) and selected by 400 µg/ml geneticin (Gibco). These transfectants were cultured in the 96-well plates to make a single cell per well. After a single cell became confluent, they were transferred to 25-T flasks and cultured with complete medium using 400 µg/ml geneticin. Transfectants overexpressing Bcl-2 and Bcl-XL were determined using the Western blot method.

## Results

**RC-RNase induces cytotoxicity and caspase-3-like activity on MCF-7 and ZR-75-1 breast tumors.** The following observations were made and recorded in the course of this study. The survival rate of MCF-7 and ZR-75-1 breast tumor cells was below 50%, noted on day 3 after treatment with RC-RNase while >80% of MDA-MB-231 and ZR-75-30 breast

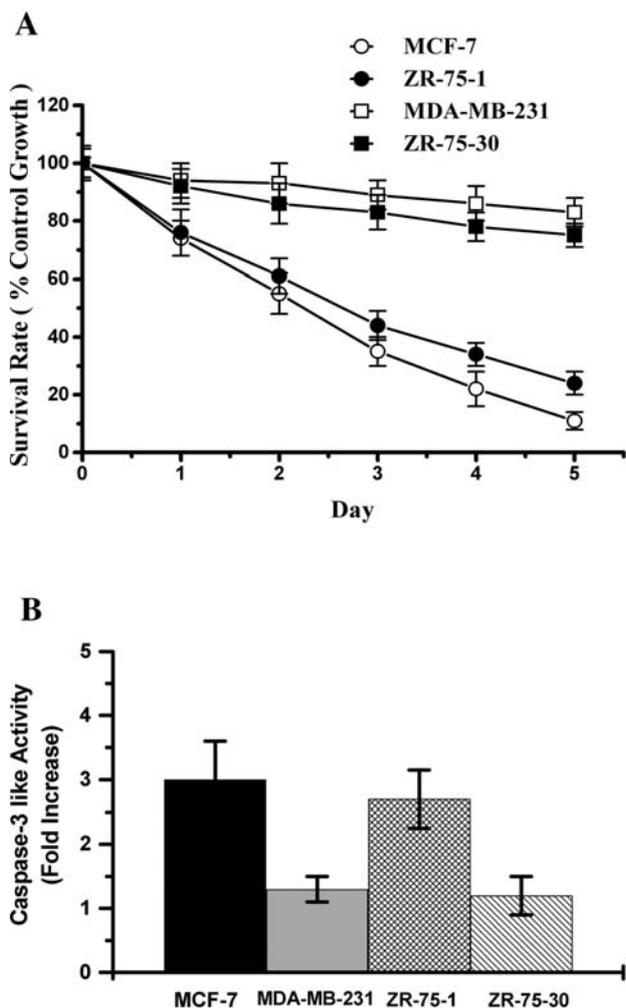


Figure 1. Survival rate and caspase-3-like activity. The survival rates of the 4 indicated breast tumor cells were treated with 20 µg/ml for 4 days (A). The caspase-3 like activities of the indicated cells were treated with 20 µg/ml at day 3 (B). Data were obtained from three independent triplicate experiments and presented as the mean ± SD.

tumor cells survived after treatment with RC-RNase (Fig. 1A). The data indicated that RC-RNase induces cytotoxicity on MCF-7 and ZR-75-1 breast tumor cells. However, RC-RNase induces lesser cytotoxicity on MDA-MB-231 and ZR-75-30 tumor cells. Caspase-3 like activity was also tested in this study. The result showed that RC-RNase induces caspase-3-like activity on MCF-7 and ZR-75-1 breast tumor cells with no activity observed on MDA-MB-231 and ZR-75-30 tumor cells (Fig. 1B). These observations are highly suggestive of RC-RNase ability to induce cytotoxicity on MCF-7 and ZR-75-1 breast tumor cells through the caspase pathway.

*Down-regulation of estrogen receptor and Bcl-2 on ER-positive breast tumors with RC-RNase treatment.* MCF-7 and ZR-75-1 cells are ER-positive breast tumor cells. They cannot survive without estrogen. However, MDA-MB-231 and ZR-75-30 cells are ER-negative breast tumor cells and can survive without estrogen. As shown in Fig. 1A, RC-RNase induce cytotoxicity and caspase-3 like activity on MCF-7 and ZR-75-1 breast tumor cells but not on MDA-MB-231 and

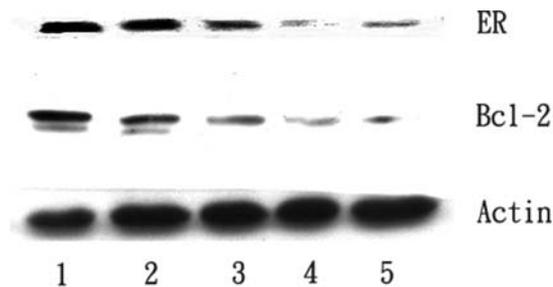


Figure 2. Down-regulation of estrogen receptor (ER) and Bcl-2 was analyzed by Western blot analysis on MCF-7 breast tumor cells. Cells were treated with 0 µg/ml (lane 1), 5 µg/ml (lane 2), 10 µg/ml (lane 3), 20 µg/ml (lane 4) and 40 µg/ml RC-RNase (lane 5) for 3 days.

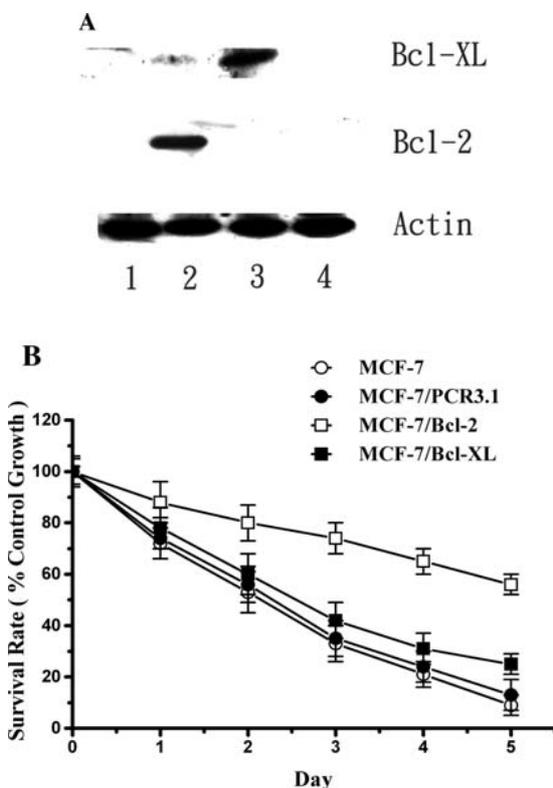


Figure 3. Transfectants and the survival rates. Transfectants (MCF-7, lane 1; MCF-7/Bcl-2, lane 2; MCF-7/Bcl-XL, lane 3; MCF-7/PCR 3.1, lane 4) with related-overexpression proteins were checked by Western blot analysis (A). The survival rates of these cells with RC-RNase treatments for 5 days (B). Data were obtained from three independent triplicate experiments and presented as the mean ± SD.

ZR-75-30 breast tumor cells. That is, RC-RNase has a stronger cytotoxicity on ER-positive breast tumor cells than ER-negative breast tumor cells. This result indicates that ER may be one of targets on RC-RNase-treated ER-positive breast tumors. To demonstrate this idea, ER was determined by Western blotting. Our result showed that degradation of ER can be found on RC-RNase-treated ER-positive breast tumor cells in a dose-dependent manner (Fig. 2). Additionally, degradation of Bcl-2 was found on RC-RNase-treated ER-positive breast tumor cells (Fig. 2). Based on the observations and results in this study, it is highly suggestive that RC-

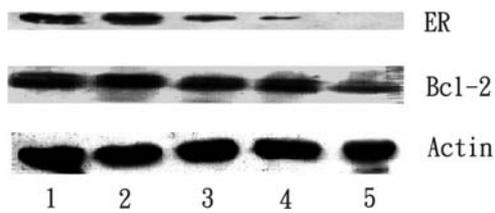


Figure 4. Down-regulation of ER and Bcl-2 was analyzed by Western blotting on MCF-7/Bcl-2 transfectants. Cells are treated for 3 days with RC-RNase 0  $\mu\text{g/ml}$  (lane 1), 5  $\mu\text{g/ml}$  (lane 2), 10  $\mu\text{g/ml}$  (lane 3), 20  $\mu\text{g/ml}$  (lane 4) and 40  $\mu\text{g/ml}$  (lane 5).

RNase induces cytotoxicity on ER-positive breast tumor cells through down-regulation of ER and Bcl-2.

*Overexpression of Bcl-2 inhibits RC-RNase-induced cytotoxicity on ER-positive breast tumors.* Transfectants overexpressing Bcl-2 and Bcl-XL was selected successfully in this study. As shown in Fig. 3A, MCF-7/Bcl-2 transfectants can express Bcl-2 and MCF-7/Bcl-XL transfectants can express Bcl-XL. MCF-7 cells and MCF-7/PCR 3.1 transfectants were used as a negative control. The survival rates of MCF-7, MCF-7/Bcl-2, MCF-7/Bcl-XL and MCF-7/PCR 3.1 with RC-RNase treatment showed that only MCF-7/Bcl-2 transfectants inhibited RC-RNase-induced cytotoxicity (Fig. 3B) while MCF-7/Bcl-XL transfectants did not (Fig. 3B). We can deduce from our study that only Bcl-2 can inhibit RC-RNase-induced cytotoxicity on MCF-7 cells despite Bcl-2 and Bcl-XL both belonging to the anti-apoptosis protein family.

*Overexpression of Bcl-2 inhibits down-regulation of Bcl-2.* We further determined the expression of ER and Bcl-2 on RC-RNase-treated MCF-7/Bcl-2 transfectants. The result is shown in Fig. 4. Comparing with Fig. 2, down-regulation of Bcl-2 is clearer on RC-RNase-treated MCF-7 cells than RC-RNase-treated MCF-7/Bcl-2 transfectants. Furthermore, our data indicate that down-regulation of ER was observed clearly on RC-RNase-treated MCF-7/Bcl-2 transfectants at day 3. This result is similar to that of ER down-regulation on RC-RNase-treated MCF-7 cells (Fig. 2).

## Discussion

Data from previous literature demonstrated that RC-RNase can induce cytotoxicity on breast tumors (MCF-7 cells) through caspase-7 activation (5,11). However, the target sites where RC-RNase will induce cytotoxicity on breast tumors are still unclear. In this study, our data showed that RC-RNase induces cytotoxicity on ER-positive breast tumors (MCF-7 and ZR-75-1) but fails to induce cytotoxicity on ER-negative breast tumors (MDA-MB-231 and ZR-75-30). In other words, RC-RNase has an anti-cancer effect only on ER-positive breast tumors. Additionally, our study demonstrates that RC-RNase can induce down-regulation of ER on ER-positive breast tumors. ER-positive breast tumor treatments have demonstrated that cell proliferation can be inhibited on ER-positive breast tumors by blocking the ER signal pathway (27-30). Based on these studies and our

results, we suggest that ER is an important target of RC-RNase-induced cytotoxicity on ER-positive breast tumors.

Bcl-2 with anti-apoptotic functions and survival effects have also been demonstrated in some studies (31-33). These studies indicated that various cell types cannot survive when Bcl-2 level decreased. Our study shows that down-regulation of Bcl-2 is expressed on ER-positive breast tumors treated with RC-RNase. Many studies have demonstrated that down-regulation of Bcl-2 can induce cell death on ER-positive breast tumors (34-36). These results are similar to our study. We therefore consider that Bcl-2 is also a target site of action for RC-RNase to induce cytotoxicity on ER-positive breast tumors. Our study demonstrates that RC-RNase can down-regulate ER and Bcl-2 levels resulting in cell death on ER-positive breast tumors.

Various reports have indicated that Bcl-2 and Bcl-XL are anti-apoptotic factors (23-26). These reports suggested that overexpression of Bcl-2 and Bcl-XL can inhibit cell death and the down-regulation of Bcl-2 and Bcl-XL can induce cell death. However, previous studies demonstrated that Bcl-2 overexpression cannot prevent hyperoxia-induced cell death on epithelial cells (37) and Bcl-XL overexpression cannot inhibit apoptosis on hepatocytes (38). Additionally, it has been reported that Bcl-2 and Bcl-XL inhibit cell death in a different manner (39). These studies indicated that anti-apoptotic effects between Bcl-2 and Bcl-XL on different cells vary depending on the target site of action and cellular function (37-40). In this study, our results demonstrate that Bcl-XL overexpression can not inhibit RC-RNase-induced cytotoxicity on MCF-7 cells. This result is similar with our previous study (5). Furthermore, our study shows that Bcl-2 overexpression can inhibit RC-RNase-induced cytotoxicity on ER-positive breast tumors indicating that the anti-apoptotic effect on RC-RNase-treated ER-positive breast tumors is dependent on Bcl-2 functions but not on Bcl-XL functions.

In summary, this study is able to demonstrate that RC-RNase induces cell death on ER-positive breast tumors but not on ER-negative breast tumors through down-regulation of ER and Bcl-2. In addition, the anti-cancer effect on RC-RNase-treated ER-positive breast tumors is related to Bcl-2 overexpression, but not to Bcl-XL overexpression.

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