

# 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 Shun-Yuan 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<sup>®</sup> Laboratories, Inc., Logan, UT), 2 mM L-glutamine (Gibco BRL), 100 IU/ml penicillin G sodium (Gibco BRL), 100  $\mu$ g/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  $\mu$ l cell lysates (80  $\mu$ g total protein), 158  $\mu$ l of reaction buffer (20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol, 100 mM HEPES, pH 7.5) and 2  $\mu$ l 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<sup>™</sup> 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  $\mu$ g/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  $\mu$ g/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

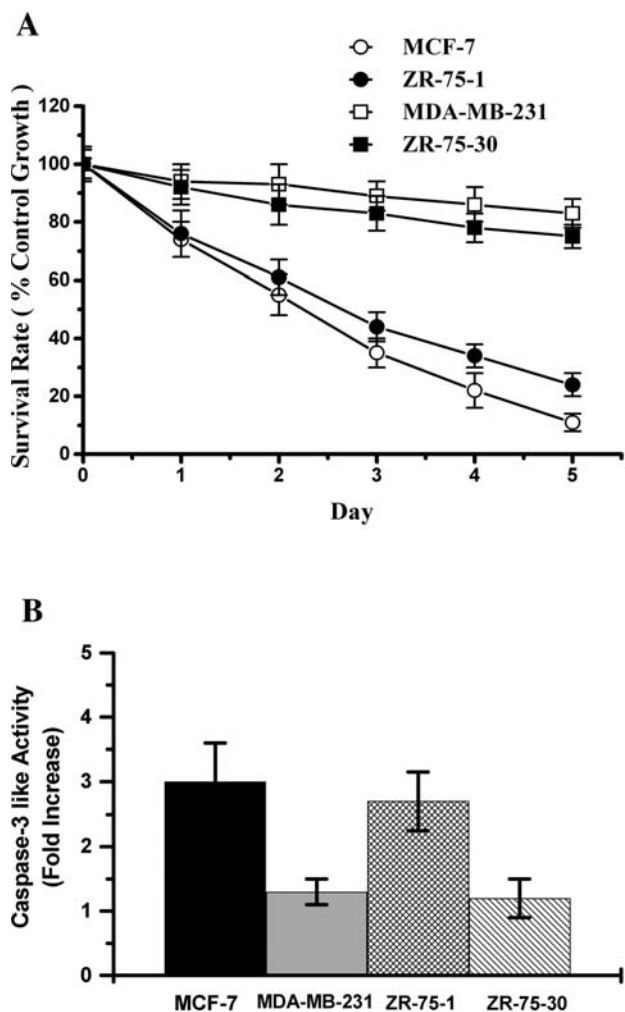


Figure 1. Survival rate and caspase-3-like activity. The survival rates of the 4 indicated breast tumor cells were treated with 20  $\mu\text{g/ml}$  for 4 days (A). The caspase-3 like activities of the indicated cells were treated with 20  $\mu\text{g/ml}$  at day 3 (B). Data were obtained from three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

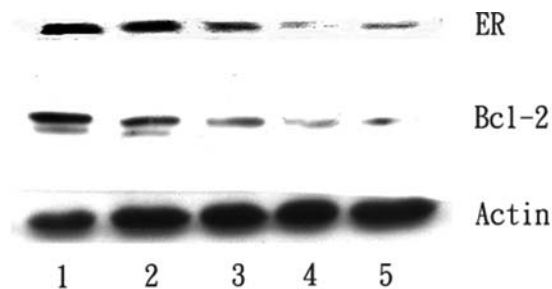


Figure 2. Down-regulation of estrogen receptor (ER) and Bcl-2 was analyzed by Western blot analysis on MCF-7 breast tumor cells. Cells were treated with 0  $\mu\text{g/ml}$  (lane 1), 5  $\mu\text{g/ml}$  (lane 2), 10  $\mu\text{g/ml}$  (lane 3), 20  $\mu\text{g/ml}$  (lane 4) and 40  $\mu\text{g/ml}$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

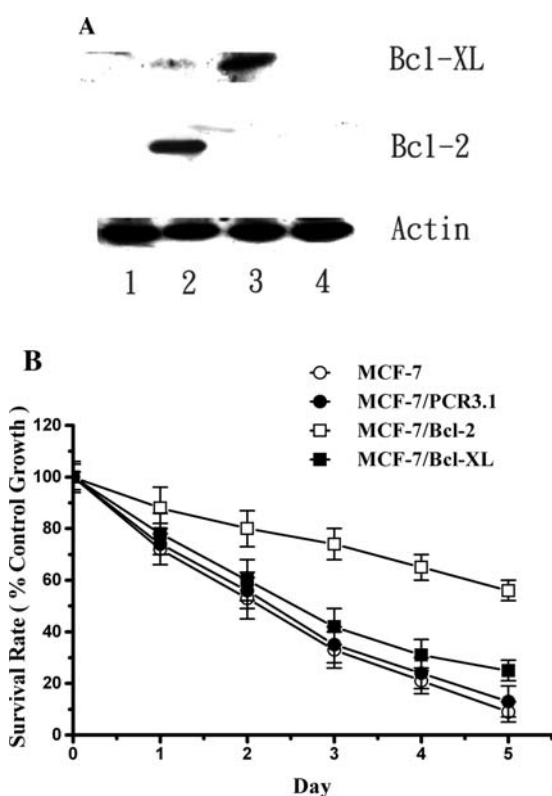


Figure 3. Transfectants and the survival rates. Transfectants (MCF-7, lane 1; MCF-7/Bcl-2, lane 2; MCF-7/Bcl-XL, lane 3; MCF-7/PCR 3.1, lane 4) with related-overexpression proteins were checked by Western blot analysis (A). The survival rates of these cells with RC-RNase treatments for 5 days (B). Data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

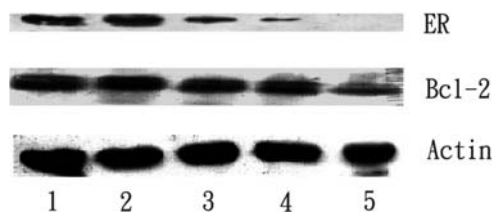


Figure 4. Down-regulation of ER and Bcl-2 was analyzed by Western blotting on MCF-7/Bcl-2 transfectants. Cells are treated for 3 days with RC-RNase 0  $\mu\text{g/ml}$  (lane 1), 5  $\mu\text{g/ml}$  (lane 2), 10  $\mu\text{g/ml}$  (lane 3), 20  $\mu\text{g/ml}$  (lane 4) and 40  $\mu\text{g/ml}$  (lane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