Docetaxel Followed by Castration Improves Outcomes in LNCaP Prostate Cancer—Bearing Severe Combined Immunodeficient Mice

Yao Tang,1,3 Mohammad A. Khan,3 Olga Goloubeva,2 Dong I. Lee,3 Danijela Jelovac,4 Angela M. Brodie,3,4 and Arif Hussain1,3,5

Abstract Purpose: Androgen ablation is the standard initial treatment for advanced prostate cancer; however, tumors eventually develop androgen independence and become incurable. Chemotherapy is commonly used after hormone treatment fails but has not shown significant survival benefit. Studies suggest that androgen ablation can select for a population of hormone-independent cells that are also relatively chemotherapy resistant. Thus, it may be therapeutically advantageous to target prostate cancer with chemotherapy before hormone ablation. This study was undertaken to determine the relative efficacy of such an approach in a preclinical model of prostate cancer.

Experimental Design: Severe combined immunodeficient mice bearing human LNCaP prostate tumors were treated with docetaxel and/or surgical castration applied singly, concurrently, or in different sequences. Treatment efficacy was determined by tumor volume and growth delay measurements. The extent of apoptosis in tumors in response to treatments was assessed via terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assays. In addition, Western blots were done to study the relative expression of Bcl-2 and Bax in the tumors.

Results: Docetaxel followed by castration showed the most potent antitumor effects. In contrast, with the exception of castration alone, castration followed by docetaxel produced the least antitumor activity. TUNEL assays confirmed that the density of apoptotic tumor cells was significantly greater for docetaxel followed by castration than for any other treatment. In tumors of mice treated with single modality therapies, Bax to Bcl-2 ratios decreased significantly after castration, whereas this ratio remained high after docetaxel treatment.

Conclusion: A treatment sequence of docetaxel followed by hormone ablation may be more effective in treating prostate cancer than concurrent docetaxel/hormone therapy or hormone ablation followed by docetaxel.

Hormone ablation represents one of the most effective therapies for advanced prostate cancer, particularly hormone-sensitive prostate cancer, and has remained the mainstay of systemic treatment for this disease for >60 years (1). Although hormone ablation is initially highly effective in a majority of patients with advanced prostate cancer, men eventually develop hormone-resistant prostate cancer (HRPC), which is generally fatal. Chemotherapy has not been shown to significantly alter survival rates in advanced prostate cancer (2–4). However, in recent years, clinical trials have shown that chemotherapy can play an important palliative role in HRPC, and docetaxel has become a standard chemotherapeutic agent for treating metastatic HRPC (3, 4).

Preclinical and clinical studies suggest that androgen ablation can select for a population of androgen-independent prostate cancer cells that may also be relatively resistant to chemotherapy (5, 6). This might account, in part, for the apparent ineffective- ness of chemotherapy in HRPC. It is likely that cytotoxic agents, such as docetaxel, that have shown activity in HRPC may also be active, and possibly more so, in hormone-sensitive prostate cancer. Thus, one potential way to improve the outcome of the traditional treatment modalities, like hormone ablation and chemotherapy, might be to use chemotherapy relatively early in the disease process rather than in the HRPC stage.

Here, we present our recent studies on severe combined immunodeficient mice bearing human LNCaP prostate cancer xenografts; the results show that of the numerous treatment methods tested, sequential therapy with docetaxel followed by castration provided the best antitumor effects.

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Materials and Methods

**Animals and tumor inoculation.** Male severe combined immunodeficient mice (National Cancer Institute, Frederick, MD), 4 to 6 weeks old, were inoculated with human LNCaP prostate cancer cells (American Type Culture Collection, Rockville, MD) at 2 × 10^5 per flank, resulting in two tumors per mouse. Three to 4 weeks after inoculation, mice were assigned to different treatment groups such that the average tumor volume in each group was ~300 mm³. There was no difference in mean tumor volumes across all groups \((P = 0.78)\). At this time point (designated week 0), treatments were initiated. The average body weight of the mice at week 0 was 19.36 ± 2.46 g; there was no significant difference in mean body weight across all groups \((P = 0.10)\). All animal studies were done under a protocol approved by the Institutional Animal Care and Use Committee, University of Maryland, Baltimore, MD.

**Treatments.** The different treatments are listed in Table 1. Most of the treatments were tested in three to four separate groups of mice (four to five mice per group) in independent experiments.

To determine the optimum dose of docetaxel, initial experiments were conducted in tumor-bearing mice in which the drug was injected i.p. twice a week for up to 4 weeks at doses of 2, 4, 6, 8, or 10 mg/kg body weight. After 4 weeks, antitumor effects and relative toxicity (weight loss and urine stain) were assessed, and the 8 mg/kg dose was selected for all subsequent studies. Mice received docetaxel twice a week for either 2 or 4 weeks, depending on the group. Hormone ablation was achieved via surgical castration; orchidectomy was done after mice were anesthetized with ketamin-xylazine (Ben Venue Laboratories, Bedford, OH), according to instructions provided by the manufacturer. For the concurrent therapies, mice were allowed to recover from surgery for 1 to 2 days before docetaxel treatment was initiated. The control group received vehicle (PBS) only. Mice were sacrificed at the end of the experiments, following procedures initiated. The control group received vehicle (PBS) only. Mice were sacrificed at the end of the experiments, following procedures established by the Animal Facility Research Department, University of Maryland School of Medicine.

Tumors from 30 additional treated mice were used for terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assays and Western blot analysis. Tumors were harvested at different time points posttreatment (two to three mice for each time point). For the TUNEL assays, the time points were 1 or 2 weeks after castration (C-1 or C-2), 1 or 2 weeks of docetaxel (D-1 or D-2), or 2 weeks of treatment consisting of D-1 followed by C-1 (D-1/C-1) or castration first followed by D-1 (C/D-1). For the Western blots, the time points were 2 or 4 weeks after castration (C-2 and C-4) or 2 or 4 weeks of docetaxel treatment (D-2 and D-4). Tumors from mice treated with vehicle only served as controls.

**Tumor measurements.** Tumors were measured weekly during the first 4 weeks after treatment started and twice a week thereafter. Tumor volumes were calculated using the following formula: \(V = \frac{4}{3} \pi a b^2\), where \(a = 0.5 \text{ width}; \ b = 0.5 \text{ length} (a < b)\). Efficacy of the different treatment methods was assessed using two metrics: \(A\) average tumor volumes per group after 4 weeks of treatment (i.e., week 4 time point) and \(b\) growth delay, measured as the time from the start of treatment for either tumor to a mouse to reach a volume of 800 mm³.

**TUNEL assay.** Tumor tissues were fixed in 10% formaldehyde for routine paraffin sections and H&E staining. TUNEL assays for apoptosis were done using the ApopTag kit (Intergen, Norcross, GA) as per the manufacturer’s instructions. After counterstaining with hematoxylin (Sigma, St. Louis, MO), slides were observed under a Nikon TE2000 microscope. Based on the morphology of H&E stains, areas of tumors free of necrosis and with similar cellular densities were selected. Within these areas, 10 random fields \((>400)\) per slide were captured with Nikon Digital Still Camera DXM1200 and saved as image files with Act-1 software (Nikon Corp., Tokyo, Japan). The apoptotic index in each field was calculated using the formula: apoptotic index \(= A \times 100 / (A + C)\), where \(A = \text{apoptotic cells}, \ C = \text{unlabeled cells}\). For each tumor, the mean of the apoptotic index and the SE were calculated using SigmaPlot.

**Western blot analysis.** Tumor tissues were homogenized in Tissue Protein Extraction Reagent (Pierce, Rockford, IL) with brief ultrasonication. Equal amounts of protein were loaded onto 12% Tris-glycine polyacrylamide gels (Bio-Rad, Hercules, CA), separated, and then transferred onto Hybond-P polyvinylidene difluoride membrane (Amersham, Piscataway, NJ). Nonspecific signals were blocked by incubating the membranes in 10% nonfat dry milk in TBST (TBS plus 0.05% Tween 20) solution for 1 hour. The membranes were briefly rinsed with 1% bovine serum albumin in TBST and incubated overnight in 1:200 diluted anti-human Bcl-2 monoclonal antibody (Oncogene, San Diego, CA), anti-human Bax monoclonal antibody (Upstate, Lake Placid, NY), or anti-human p-Bcl-2 polyclonal antibodies (which recognize phosphorylated serine residues 70 and 87; Santa Cruz Biotechnology, Santa Cruz, CA). Anti-human β-actin antibody (Sigma), diluted 1:1,000, was used to assess housekeeping protein expression. Signals were detected with an enhanced chemiluminescence–linked detection kit (Amersham) after incubating the membranes with horseradish peroxidase–coupled secondary antibodies. Signal intensity was measured via a Molecular Dynamics Image Quant Densitometer and was normalized to β-actin expression.

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**Table 1. Mean tumor volumes in the different treatment groups at week 4**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Therapy</th>
<th>Sample size* (no. mice)</th>
<th>Mean (SE) tumor volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle only</td>
<td>17</td>
<td>819 (60)</td>
</tr>
<tr>
<td>C-only</td>
<td>Castration only</td>
<td>18</td>
<td>602 (67)</td>
</tr>
<tr>
<td>D-2</td>
<td>Docetaxel only, 2 wks</td>
<td>18</td>
<td>177 (42)</td>
</tr>
<tr>
<td>D-2+C</td>
<td>Concurrent: castration plus docetaxel 2 wks</td>
<td>17</td>
<td>148 (42)</td>
</tr>
<tr>
<td>D-2/C</td>
<td>Sequential: docetaxel for 2 wks followed by castration</td>
<td>14</td>
<td>93 (46)</td>
</tr>
<tr>
<td>C/D-2</td>
<td>Sequential: castration followed 2 wks later by docetaxel for 2 wks</td>
<td>18</td>
<td>283 (41)</td>
</tr>
<tr>
<td>D-4</td>
<td>Docetaxel only, 4 wks</td>
<td>14</td>
<td>191 (40)</td>
</tr>
<tr>
<td>D-4+C</td>
<td>Concurrent: castration plus docetaxel, 4 wks</td>
<td>14</td>
<td>163 (42)</td>
</tr>
<tr>
<td>D-4/C</td>
<td>Sequential: docetaxel for 4 wks followed by castration</td>
<td>10</td>
<td>122 (48)</td>
</tr>
<tr>
<td>C/D-4</td>
<td>Sequential: castration followed 2 wks later by docetaxel for 4 wks</td>
<td>5</td>
<td>228 (102)</td>
</tr>
</tbody>
</table>

*The sample size is pooled from three to four independent experiments (except for the last two groups). In each experiment, four to five mice were assigned to each specified treatment.
Statistical analyses. The experiment/cage effect was tested in a statistical model (ANOVA). No sufficient evidence was found that experiment/cage effect was present \( (P = 0.28-0.51 \text{ for the treatment groups}) \). Therefore, groups that were identical with respect to the treatments given were pooled for the statistical modeling.

Linear mixed-effects models were used to estimate average animal body weight, tumor volume, and growth rate across the treatment groups \( (7) \). The treatment groups were compared with one another at a 0.05 level of statistical significance. All statistical tests were two sided. No adjustments for multiple comparisons were made due to the exploratory nature of the analysis. The results of the comparisons across treatment groups are presented as the difference in group means, along with the corresponding SE or 95% confidence interval. Tumor growth delay was modeled using the time-to-event approach \( (\text{i.e., the time required for a tumor to grow to the prespecified volume of 800 mm}^3) \). The Kaplan-Meier estimates of growth delay function were compared using the log-rank test for right-censored data.

The morphologic data were compared between the different treatment groups using standard or Welch modified two-sample \( t \) test.

Results

Tumor size at week 4. The mean tumor volumes at week 4 of treatment are shown in Table 1. As expected, the average tumor volume in the castration-only group \( (\text{C-only}; 602 \text{ mm}^3) \) was significantly less than in the control group \( (819 \text{ mm}^3) \), with \( P = 0.009 \) (Table 1). However, compared with the C-only group, the mean tumor volumes were at least 50% smaller for all groups whose treatment included docetaxel. The group with the smallest tumor size at week 4 was the one that received docetaxel for 2 weeks followed by castration \( \text{(D-2//C)} \). The opposite treatment sequence, \( \text{C//D-2 (castration followed 2 weeks later by 2 weeks of docetaxol)} \), resulted in tumors about thrice as large. No significant difference was found between the groups treated with concurrent docetaxel and castration \( \text{(D-2+C) versus docetaxel only (D-2).} \)

Growth delay in tumors after treatment. The median time for tumors to reach 800 mm\(^3\) in the control group was about 3.5 weeks; castration delayed tumor growth by only 7 to 10 days relative to the controls (data not shown). Growth delay results for other treatment groups are shown in Fig. 1A and B. Among the four groups that received docetaxel for 2 weeks (Fig. 1A), \( \text{D-2//C was again the most effective. It produced the longest growth delay, whereas C//D-2 had the shortest. Among the groups treated with docetaxel for 4 weeks (Fig. 1B), chemotherapy followed by castration (D-4//C) also resulted in the best outcome.} \)

Table 2 compares the median growth delay times for different treatment groups \( (\text{except C-only}) \), along with the associated confidence intervals and \( P \) values. Comparing the groups that included docetaxel for 2 weeks, D-2 was significantly better than C//D-2 in delaying tumor growth \( (P = 0.009) \). Concurrent chemotherapy and hormone therapy \( (\text{D-2+C}) \) had antitumor effects similar to chemotherapy alone \( (\text{D-2}; P = 0.64); \) it also produced a significant delay in tumor growth compared with C//D-2 \( (P = 0.003) \). D-2//C was more effective than D-2+C \( (P = 0.01) \) or D-2 \( (P = 0.03) \) and resulted in the most significant delays in tumor growth compared with the reverse treatment sequence, C//D-2 \( (P = 0.0003) \). When docetaxel treatment was extended to 4 weeks, the comparative results changed somewhat, which may in part reflect the small number of animals in groups C//D-4 and D-4//C \( (\text{Table 1}) \). However, docetaxel followed by castration \( \text{(D-4//C) continued to provide a more effective outcome when compared with C//D-4} \ (P = 0.054), \text{or to D-4+C and D-4} \ (P = 0.04). As expected, extending the docetaxel treatment to 4 weeks had a greater growth delay benefit compared with 2 weeks of treatment \( (\text{Table 2}) \). Apoptosis in treated tumors. To assess the effects of the various treatments at the tissue level, we determined the relative degree of apoptosis using TUNEL assay. Typical examples of apoptosis at different time points posttreatment are shown in Fig. 2A, and the apoptotic index in different tumors is summarized in Fig. 2B. As shown in these figures, docetaxel induced more apoptosis than did castration, and 2 weeks of docetaxel treatment \( \text{(D-2) resulted in more apoptotic cells than did 1 week (D-1). However, if docetaxel was given after castration (C//D-1), its effectiveness in causing apoptosis was reduced relative to both D-1} \ (P = 0.067) \text{ and D-2} \ (P < 0.0001) \text{. In contrast, fewer apoptotic cells were observed at the 2-week time point after castration (C-2) relative to the amount measured after 1 week (C-1). If castration followed docetaxel treatment (D-1/C-1), the apoptotic cell count was significantly increased relative to C-1 and C-2} \ (P < 0.0001 \text{ for both}). \) In addition, twice as many apoptotic cells were observed in the docetaxel followed by castration group \( \text{(D-1/C-1) compared with castration first and then docetaxel (C//D-1; Fig. 2B). Thus, chemotherapy followed by castration resulted in the highest density of apoptotic cells among the tumor tissues tested.} \)

Bax and Bcl-2 expression in tumors after treatment. An example of Western blots for Bax and Bcl-2 expression in tumor specimens is shown in Fig. 3, and the expression relative to that in untreated controls is summarized in Table 3. Compared with
untreated tumors, increased Bcl-2 expression is observed in tumors 2 weeks after castration (2.9-fold increase), and even higher levels are seen at the week 4 time point (7.9-fold increase; Table 3). In contrast, in tumors treated with docetaxel for 4 weeks, Bcl-2 levels are reduced by more than half relative to that in the untreated controls, although an ~1.8-fold increase occurs after 2 weeks of docetaxel therapy (Table 3). A well-documented mechanism by which docetaxel can diminish the antiapoptotic effects of Bcl-2 is by phosphorylating the protein (8, 9); however, enhanced Bcl-2 phosphorylation was not observed in either the 2-week or 4-week docetaxel-treated tumors (data not shown). Interestingly, Bax levels are only minimally modulated by either castration or docetaxel treatment (Table 3). Bax levels relative to Bcl-2 within tumors from different groups are shown in Table 4; each ratio is the mean of two or three independent Western blots. In the untreated tumors, Bax is present in 12-fold excess over Bcl-2. With hormone ablation, the Bax to Bcl-2 ratio decreases, being lower at the week 4 time point (1.0-fold) than at week 2 (1.8-fold) after castration (Table 4). After docetaxel treatment, the Bax to Bcl-2 ratio at the week 2 time point is 5.6, and this ratio increases to ~22.7 at the week 4 time point (Table 4).

**Discussion**

The different treatment modalities were tested several times in independent experiments. Statistical analysis revealed that individual experiment per cage effect did not occur among the identically treated groups in the repeat experiments. This allowed results from the independent experiments to be pooled.

The LNCaP cell line (10) has been widely used for both in vitro and in vivo prostate cancer studies due primarily to its androgen-sensitive characteristics. Surgical castration in mice bearing LNCaP tumors arrests tumor growth (11). However, this antitumor effect of androgen ablation is temporary. In our experiments, tumors resumed growth between 2 and 3 weeks after castration, suggesting that they had evolved to be hormone independent by this time.

All groups that received docetaxel for 2 weeks showed significantly longer growth delays (over 6 weeks) than did the castration-only group (7-10 days). Extending docetaxel treatment provided an even greater antitumor effect; the median growth delay time in D-2 mice was 9 weeks, whereas in the D-4 group it was 17 weeks (Table 2). However, significant loss of body weight was also associated with the extended chemotherapy (data not shown).

It is noteworthy that the antitumor effects of docetaxel were reduced if the docetaxel treatments were begun after the mice had been castrated compared with being given alone. This is evidenced by the significant difference in growth delay between the D-2 and C/D-2 treatment groups (P = 0.009; Fig. 1A; Table 2). A similar difference is also seen between the D-4 and C/D-4 groups (Fig. 1B), although the statistical significance is reduced (P = 0.12); this might be influenced by the smaller sample size of the C/D-4 group (n = 5). TUNEL assays confirmed that apoptosis in tumors was significantly reduced if docetaxel followed castration rather than being used alone (Fig. 2). Taken together, these observations suggest that after castration therapy, LNCaP tumors become less sensitive to chemotherapy.

The sequential treatment consisting of docetaxel followed by castration (D-2/C and D-4/C) provided the most potent antitumor effects among all the treatments tested, as shown by both metrics (i.e., tumor volumes at week 4 and growth delay; Tables 1 and 2; Fig. 1). The TUNEL assay also showed...
that castration after docetaxel treatment results in a further increase in apoptotic cells over either docetaxel or castration alone.

An intricate network of cellular mediators modulates apoptosis. Mechanistically, altered expression in some of these mediators could contribute, at least in part, to the observed outcomes in the above xenograft model. As shown in Fig. 3 and Table 3, enhanced Bcl-2 expression occurs in the LNCaP tumors after hormone ablation, consistent with several prior preclinical studies (12–14). Other reports have also shown that with the development of clinical hormone resistance, Bcl-2 expression in men with prostate cancer tends to increase (15–18). In our studies, we noted that the tumors start to regrow around 2 to 3 weeks after castration (data not shown); this growth reflects resistance to the hormone ablation. In parallel, higher Bcl-2 levels are found at both weeks 2 and 4 after castration compared with control and docetaxel-treated tumors (Fig. 3; Table 3).

Perhaps a more critical event in regulating apoptosis is the Bax to Bcl-2 ratio (19). An excess of Bax relative to Bcl-2 favors formation of proapoptotic Bax/Bax homodimers. As this ratio decreases, enhanced Bax/Bcl-2 heterodimerization and Bcl-2/Bcl-2 homodimerization occur; these events have antiapoptotic effects on cells (19). In the LNCaP model, untreated tumors are relatively sensitive, at least initially, to either hormone ablation or docetaxel, in keeping with the significant excess of Bax over Bcl-2 at baseline (Table 4). The decrease in the Bax to Bcl-2 ratio after castration favors formation of Bax/Bcl-2 heterodimers and Bcl-2/Bcl-2 homodimers, resulting in a relatively apoptosis-resistant state. In contrast, after 2 weeks of docetaxel treatment, >5-fold excess of Bax over Bcl-2 is observed in the tumors, and this ratio increases further after 4 weeks of chemotherapy (Table 4).

Thus, initial hormone treatment can lead to the emergence of tumors that are relatively resistant to subsequent chemotherapy. With the use of docetaxel first, on the other hand, tumors retain the ability to respond to additional apoptotic stimuli, such as castration. That is, LNCaP tumors retain hormone sensitivity after chemotherapy. In addition to producing the smallest tumors at week 4, the D-2/C treatment resulted in tumors that were nearly half the size of those treated with D-2 only (Table 1). Furthermore, both D-2/C and D-4/C provided the longest growth delays among all 2-week and 4-week treatment groups, respectively (Table 2). These data show that castration after chemotherapy contributes to a further reduction in tumor size and a longer delay in tumor growth. A recent clinical trial evaluating the sequential use of docetaxel followed by hormone therapy in men with prostate cancer experiencing prostate-specific antigen failure after their definitive primary local treatments also showed that patients responded to hormone therapy after the initial docetaxel treatment (20).

In addition to the possible selection of cells with altered Bax/Bcl-2 ratios during hormone ablation, a subpopulation of hormone-sensitive prostate cancer cells could also potentially enter the G0 phase of the cell cycle (which is a relatively chemotherapy-resistant state) rather than undergo apoptosis. Thus, theoretically, concurrent docetaxel plus hormone therapy may also be less effective than sequential docetaxel hormone therapy. Consistent with this notion, tumor volumes at week 4 (Table 1) as well as growth delay measurements (Table 2) reveal the greater antitumor activity of D-2/C versus D-2+C and D-4/C versus D-4+C.

In summary, the above studies suggest that the earlier use of docetaxel (i.e., before the development of hormone resistance) is therapeutically advantageous. The relative insensitivity of prostate cancer to chemotherapy in the clinical setting could be due, in part, to the fact that patients generally receive chemotherapy after androgen ablation; the latter may be instrumental in desensitizing tumors to chemotherapy. More work is needed to fully understand the molecular mechanisms underlying the therapeutic outcomes of our studies, and to verify that these results can be translated to clinical practice. Nevertheless, our data suggest that a treatment sequence of docetaxel before hormone ablation might provide significant therapeutic benefits in androgen-sensitive prostate cancer.

**Table 3. Summary of relative Bax and Bcl-2 expression in tumors following different treatments**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>C-2</th>
<th>C-4</th>
<th>D-2</th>
<th>D-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>0.4 ± 0.11</td>
<td>0.8 ± 0.17</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.29</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>2.9 ± 1.2</td>
<td>7.9 ± 1.73</td>
<td>1.8 ± 0.42</td>
<td>0.4 ± 0.21</td>
</tr>
</tbody>
</table>

*Changes in Bax and Bcl-2 expression relative to respective control, after normalizing to β-actin. Data reflect mean ± SD of two or three experiments.

**Table 4. Bax/Bcl-2 ratio in tumors following different treatments**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Bax/Bcl-2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.0</td>
</tr>
<tr>
<td>C-2</td>
<td>1.8</td>
</tr>
<tr>
<td>C-4</td>
<td>1.0</td>
</tr>
<tr>
<td>D-2</td>
<td>5.6</td>
</tr>
<tr>
<td>D-4</td>
<td>22.7</td>
</tr>
</tbody>
</table>

*Bax/Bcl-2 ratio within each tumor is calculated after normalizing Bax and Bcl-2 to β-actin, respectively. The numbers represent the mean of two or three independent Western blots.
References

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