

Rapid Assay for Cell Death

Correlation of Caspase Activity and Chemo-Response in Epithelial Ovarian Cancer Cell Lines

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Abstract

The immediate assessment of response to therapy is most beneficial to ovarian cancer patients. This study shows the correlation of drug-induced caspase activation determined by Western blot analysis and by the Caspase-Glo™ Assay. Our initial research demonstrates that the Caspase-Glo™ Assay provides a simple, fast and sensitive alternative for the evaluation of in vitro response to cytotoxic agents. The clinical correlation of caspase activity and response to treatment is under investigation.

Our results show that the response of EOC cell lines to treatment can be evaluated in a couple of hours using the Caspase-Glo™ Assay.

Introduction

Epithelial ovarian cancer (EOC) is the fourth leading cause of cancer-related deaths in women and is the most lethal of the gynecological malignancies (1). One of the major limitations in EOC treatment is the development of resistance to commonly used chemotherapeutic agents. This is confounded by the lack of practical means to predict clinical response.

It is now well-documented that apoptosis or programmed cell death is the key mechanism by which chemotherapeutic agents exert their cytotoxicity (2). Apoptosis is mediated by a cascade of intracellular factors known as caspases, which are highly specific proteases synthesized as zymogens and activated by cleavage (3). Caspases can be divided into “initiators” or “effectors” of apoptosis. Initiator caspases, such as caspase-8 and -9, mediate their oligomerization and autoactivation in response to upstream signals, while the effector caspases, which include caspase-3, -6, and -7, cleave cellular substrates and precipitate apoptotic death.

Recently, we demonstrated a correlation between caspase-3 activity in EOC cells isolated from malignant ascites and the in vivo response to chemotherapy in patients with recurrent ovarian cancer (4). That study was performed using Western blot analysis to detect the expression of the active forms of caspase-3. Western blot analysis, however, has some limitations. It is time-consuming, has low sensitivity and requires a large amount of material.

The objective of this study was to evaluate the sensitivity and specificity of the Caspase-Glo™ Assay in EOC cells treated with Docetaxel and Triapine®. Here we present the correlation between the expression of the active forms of caspases determined by Western blot analysis and caspase activity measured by the Caspase-Glo™ Assays.

Methods

Caspase-Glo™ Assays

Caspase-3/7, -8, and -9 activities were measured using the Caspase-Glo™ 3/7, 8, and 9 Assays^(a,b), respectively. We evaluated caspase activity in three different systems: 1) cell lysates, single assay, 2) protein extracts from tumor samples, and 3) whole cell cultures, 96-well plate assay.

Cell lysates. EOC cells ($5\text{--}10 \times 10^5$) were plated in 100 × 20mm dishes (BD Discovery Labware, Bedford, MA) and grown to 70–80% confluency. At this point, medium was changed to Opti-MEM® I reduced-serum medium (GIBCO, Carlsbad, CA) for 4 hours, and cells were then treated with either 100ng/ml Docetaxel (Aventis) or 10mM Triapine® (Vion) for 0, 24, 48, and 72 hours. After treatment, cells were scraped from the culture vessel, pelleted at 1,500rpm at 4°C and lysed in phosphate-buffered saline with 1% NP40 and 0.1% sodium dodecyl sulfate. After 20 minutes on ice, debris was removed by centrifugation at $14,000 \times g$ at 4°C. Ten micrograms of protein in a 50µl total volume was mixed with 50µl of equilibrated Caspase-Glo™ 3/7, Caspase-Glo™ 8, or Caspase-Glo™ 9 Reagent and incubated for 1 hour at room temperature. Afterwards, luminescence was measured using a TD-20/20 Luminometer.

Protein extracts from tumor samples. Tumor samples obtained from an ovarian cancer xenograft model were homogenized, and protein was extracted as previously described (5). Five to ten micrograms of protein in a 50µl total volume was mixed with 50µl of equilibrated Caspase-Glo™ 3/7, Caspase-Glo™ 8, or Caspase-Glo™ 9 Reagent and incubated for 1 hour at room temperature. Afterwards, luminescence was measured using a TD-20/20 Luminometer.

96-well plate assay. EOC cells ($2\text{--}5 \times 10^3$ cells/well) were plated in a 96-well plate and incubated for 24 hours prior to treatment. Cells were then incubated for 4 hours in Opti-MEM® medium followed by treatment with docetaxel for another 24 hours. At the end of the treatment, 50µl of Caspase-Glo™ 3/7 Reagent was added to each well and incubated for 1 hour. Luminescence was measured using a Reporter® microplate luminometer from Turner Designs.

Results

Caspase-3 Activity in Cell Lysates

The presence of the active forms of caspase-3 was first evaluated by Western blot analysis. Figure 1, Panel A, shows the expression of the active forms of caspase-3 (p19, p17) in the sensitive cells (CP70) but not in the resistant cells (R182) after treatment with Docetaxel. We then evaluated caspase-3 activity in the same sample preparations using Caspase-Glo™ 3/7 Assay. As shown in Figure 1, Panel B, caspase-3 activity corresponds to the presence of p19/p17 determined by the Western blot. Thus, high levels of activity were found in CP70 cells treated with Docetaxel for 24 hours. Caspase-3 activity further increased after 48 hours of treatment and declined after 72 hours. No appreciable caspase-3 activity was observed in the samples from R182 cells treated with Docetaxel.

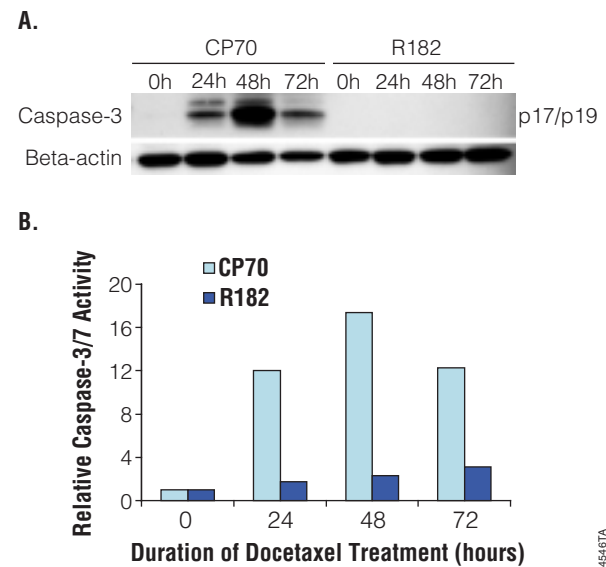


Figure 1. Measuring caspase-3 activity in cell lysates. CP70 and R182 cells were treated with 100ng/ml of Docetaxel for 0, 24, 48, and 72 hours. Caspase-3 activity was determined using cell lysates by Western blot analysis (Panel A) and Caspase-Glo™ 3/7 Assay (Panel B) (p17 and p19 are the active forms of caspase-3).

Caspase Activity in Tumor Samples

The objective of this experiment was to determine whether the activities of caspase-3, -8 and -9 could be measured in protein extracts obtained from tumor samples of animals treated with chemotherapy. We used as little as 5µg protein for this assay and were able to obtain excellent signals. As shown in Figure 2, the Caspase-Glo™ assay was sensitive enough to detect caspase activity in tumor samples that responded to treatment.

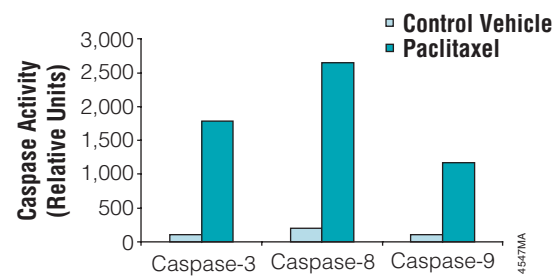


Figure 2. Caspase-3,-8 and-9 activities were determined in tumor samples obtained from animals treated with paclitaxel for 10 days or control vehicle.

Caspase Activity in Whole Cells Plated in a 96-Well Plate

To determine the correlation between the expression of the active forms of caspase-3 in cell lysates determined by Western blot and caspase-3 activity measured in whole cells by Caspase-Glo™ Assay, CP70 and R182 cells were seeded in a 96-well plate and then treated with tenfold dilutions of Docetaxel (0 to 500ng/ml) for 24 hours. As shown in Figure 3, the 96-well plate assay showed similar results to those obtained with Western blot using cell lysates.

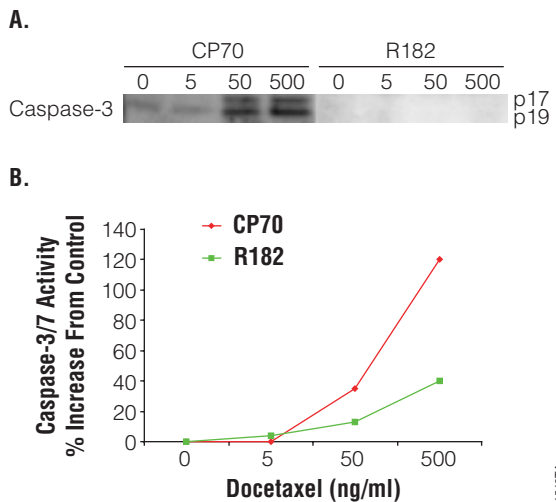


Figure 3. Caspase activities in whole cells in 96-well plates. Panel A. CP70 and R182 cells were treated with increasing concentrations of Docetaxel for 24 hours. Caspase-3 activity was determined by Western blot analysis using cell lysates. Panel B. CP70 and R182 cells were plated on a 96-well plate and then treated with Docetaxel as in Panel A. Caspase-3 activity was measured from whole cells using the Caspase-Glo™ 3/7 Assay (p17 and p19 are the active forms of caspase-3).

Expression and Activity of Caspase-8 and -9

We also evaluated the correlation between the expression of the active forms of caspase-8 and -9 seen in Western blot and their in vitro activity using the Caspase-Glo™ 8 and 9 Assays. For this, we used CP70 and R179 cells treated with 10mM Triapine® for 0, 24, and 48 hours. Figure 4, Panel A, shows the expression of the active forms of caspase-8 (p45) and -9 (p36) in both cell lines with peak activity after 48 hours of treatment. Results from the Caspase-Glo™ 8 and 9 Assays showed the same time-dependent increase in caspase-8 and -9 activities in both cell lines (Figure 4, Panel B).

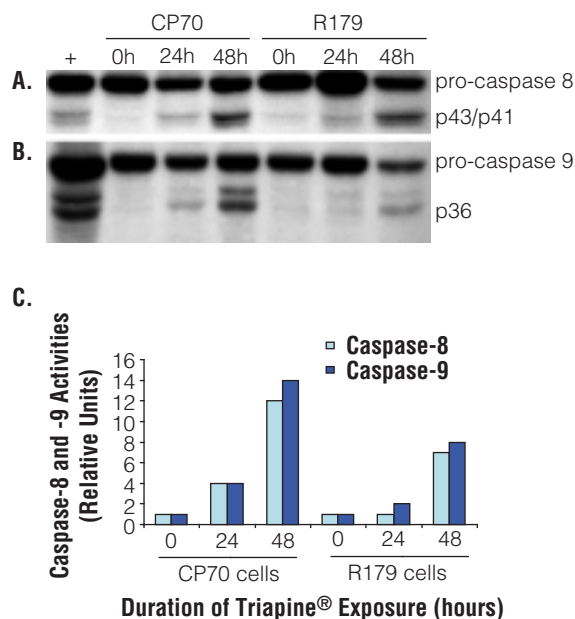


Figure 4. Correlation of expression of caspase-8 and -9 with activity. CP70 and R179 cells were treated with 10μM Triapine® for 0, 24, and 48 hours. Caspase-8 and -9 activities were determined using cell lysates by Western blot analysis (Panel A) and Caspase-Glo™ 8 and 9 assays (Panel B) (p43/41 is the active form of caspase-8; p36 is the active form of caspase-9).

Conclusion

The availability of a sensitive and specific assay, which can provide immediate assessment of treatment response, will be most beneficial to EOC patients. Our results show that the response of EOC cell lines to treatment can be evaluated in a couple of hours using the Caspase-Glo™ Assays. This is in contrast to performing Western blot analysis, which can take up to 3 days before results can be obtained. The assays can be performed with whole cells in a 96-well plate or with cell and tissue protein extracts.

The clinical correlation of caspase activity and response to treatment is under investigation.

References

- Schwartz, P.E. (2002) *Cancer Treat. Res.* **107**, 99–118.
- Kaufmann, S.H. and Earnshaw, W.C. (2000) *Exp. Cell Res.* **256**, 42–9.
- Cohen, G.M. (1997) *Biochem. J.* **326** (Pt 1), 1–16.
- Flick, M.B. et al. (2004) *Journal of the Society for Gynecologic Investigation*, in press.
- Kamsteeg, M. et al. (2003) *Oncogene* **22**, 2611–20.

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Protocols

- ◆ *Caspase-Glo™ 3/7 Assay Technical Bulletin* #TB323, Promega Corporation. (www.promega.com/tbs/tb323/tb323.html)
- ◆ *Caspase-Glo™ 8 Assay Technical Bulletin* #TB332, Promega Corporation. (www.promega.com/tbs/tb332/tb332.html)
- ◆ *Caspase-Glo™ 9 Assay Technical Bulletin* #TB333, Promega Corporation. (www.promega.com/tbs/tb333/tb333.html)



Ordering Information

Product	Size	Cat. #
Caspase-Glo™ 3/7 Assay ^(a,b)	2.5ml	G8090
	10ml	G8091
	100ml	G8092
Caspase-Glo™ 8 Assay ^(a,b)	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo™ 9 Assay ^(a,b)	2.5ml	G8210
	10ml	G8211
	100ml	G8212

For Laboratory Use.

^(a)U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents are pending.

^(b)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

Caspase-Glo is a trademark of Promega Corporation.

Opti-MEM is a registered trademark of Invitrogen Corporation. Reporter is a registered trademark of Turner Designs. Triapine is a registered trademark of Vion Pharmaceuticals, Inc.