ORIGINAL ARTICLE

Novel Survivin Inhibitor YM155 elicits Cytotoxicity in Glioblastoma Cell Lines with Normal or Deficiency DNA-Dependent Protein Kinase Activity

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Background: Pediatric glioblastoma is a malignant disease with an extremely poor clinical outcome. Patients usually suffer from resistance to radiation therapy, so targeted drug treatment may be a new possibility for glioblastoma therapy. Survivin is also overexpressed in glioblastoma. YM155, a novel small-molecule survivin inhibitor, has not been examined for its use in glioblastoma therapy.
Methods: The human glioblastoma cell line M059K, which expresses normal DNA-dependent protein kinase (DNA-PK) activity and is radiation-resistant, and M059J, which is deficient in DNA-PK activity and radiation-sensitive, were used in the study. Cell viability, DNA fragmentation, and the expression of survivin and securin following YM155 treatment were examined using MTT (methylthiazolyldiphenyl-tetrazolium) assay, ELISA assay, and Western blot analysis, respectively.
Results: YM155 caused a concentration-dependent cytotoxic effect, inhibiting the cell viability of both M059K and M059J cells by 70% after 48 hours of treatment with 50 nM YM155. The half-maximal inhibitory concentration (IC50) was around 30—35 nM for both cell lines. Apoptosis was determined to have occurred in both cell lines because immunoreactive signals from the DNA fragments in the cytoplasm were increased 24 hours after treatment with 30 nM

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YM155. The expression of survivin and securin in the M059K cells was greater than that measured in the M059J cells. Treatment with 30 nM YM155, for both 24 and 48 hours, significantly suppressed the expression of survivin and securin in both cell lines.

Conclusion: The novel survivin inhibitor YM155 elicits potent cytotoxicity in glioblastoma cells *in vitro* via DNA-PK-independent mechanisms. YM155 could be used as a new therapeutic agent for the treatment of human glioblastomas.

1. Introduction

Brain tumors are one of the most common solid neoplastic disorders that present in children.[1] The death of a pediatric patient with a brain tumor, especially a high-grade glioma, usually results from cancer.[2,3] Glioblastoma demonstrates the worse therapeutic response among high-grade gliomas, with a very low 5-year survival rate despite aggressive chemotherapy and/or radiation therapy.[4–6] Thus, new strategies for treating glioblastomas should be investigated.

Survivin, a member of the inhibitor of apoptosis protein family, is overexpressed in human glioblastoma tissues.[7] Studies have shown that the 3-year survival rate of glioblastoma tissues with positive nuclear survivin expression is zero; thus, survivin may be a useful biomarker for predicting clinical prognosis in patients with glioblastoma.[8] Treatments aimed at inhibiting survivin expression could be a new strategy for the treatment of glioblastomas.[9–13] However, specific survivin suppressants have not been examined for use in glioblastoma treatments.

A small molecule, YM155 {1-(2-Methoxyethyl)-2-methyl-4,9-dioxo-3- (pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho [2,3-d] imidazolium bromide}, has been identified by high-throughput screening as an inhibitor of the activity of the survivin gene promoter.[14] YM155, as a survivin suppressant, has been shown to inhibit the progression of some neoplasms *in vitro* and/or *in vivo* (e.g., prostate cancer[14,15] and lung cancer).[16,17] Until now, YM155 has not been studied for its use in glioblastoma therapy. Therefore, we conducted an *in vitro* study to evaluate the therapeutic effects of YM155 in two glioblastoma cell lines: one cell line that expresses normal DNA-dependent protein kinase (DNA-PK) activity, and another cell line that is deficient in DNA-PK activity. The former is resistant to radiation, whereas the latter is sensitive to radiation.[18] This study's aim was to demonstrate that YM155 exerts potent cytotoxicity through survivin inhibition and induces apoptosis in both of these glioblastoma cell lines.

2. Materials and Methods

2.1. Cell culture

Human glioblastoma cell lines M059J and M059K were obtained from Dr. Shu Jun Chiu (Department of Life Sciences, Tzu Chi University, Hualien, Taiwan). M059J and M059K exhibit deficient DNA-PK and normal DNA-PK activities, respectively. The cells were cultured in F-12/ Dulbecco's modified Eagle medium in combination with 10% fetal bovine serum (FBS) and 1% penicillin plus streptomycin.[19]

2.2. MTT cytotoxicity assay

For the cytotoxicity assay, 1.0×10^4 glioblastoma cells per well were seeded onto 96-well plates. After overnight incubation, the cells adhered to the plate. YM155 (Selleck, Houston, TX, USA) at a concentration of 0–50 nM was dissolved in dimethyl sulfoxide (DMSO; J.T. Baker, Phillipsburg, NJ, USA) and incubated for 48 hours. The MTT (methylthiazolyldiphenyl-tetrazolium bromide; Sigma, St. Louis, MO, USA) assay was then performed, using the protocol described in a previous study.[20]

2.3. DNA fragmentation assay

The Cell Death Detection ELISA[PLUS] (Roche, Mannheim, Germany) assay kit was used to differentiate apoptotic and necrotic glioblastoma cells after treatment with YM155. Twenty-four hours after drug treatment, both supernatants of the cultured medium and the cytoplasmic fraction from 2.0×10^4 cells were collected in 24-well dishes. The optical density (OD) value at 405 nm, which represents the extent of DNA fragmentation, was measured.

2.4. Western blot analysis

After YM155 treatment, the expression levels of survivin and securin in the glioblastoma cells were detected using Western blot analysis. The procedures we followed were described in our previous report.[20] Primary antibodies, including anti-survivin (#2808; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-securin (ab3305; Abcam, Cambridge, MA, USA), were used. The expression of α-tubulin (anti-α-tubulin, sc-8305; Santa Cruz, CA, USA) was used as the internal standard. The intensity of the immunoreactive proteins was calculated using NIH software ImageJ V.1.40. The intensity ratio, which was calculated by dividing the intensity of survivin or securin to that of α-tubulin, was used to compare the effects of YM155 treatment.

2.5. Statistical analysis

Data are presented as the mean ± standard error of mean (SEM) and were analyzed using the Student *t* test. In all
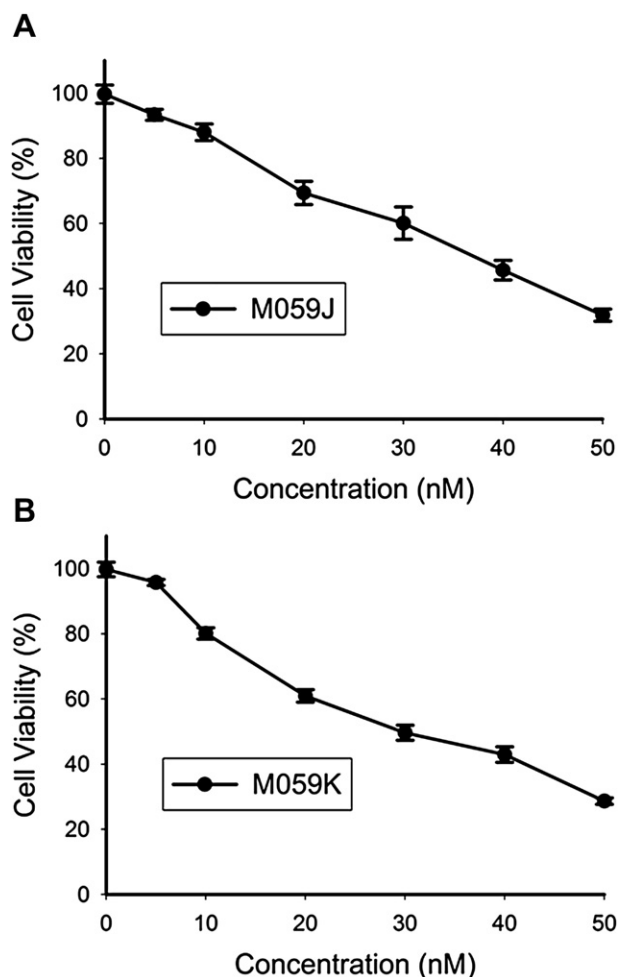
A

B

Figure 1 Cytotoxic effects of YM155 on glioblastoma cells. The viability of (A) M059J ($n = 3$) and (B) M059K ($n = 3$) cells was measured using an MTT assay 48 hours after treatment with YM155. The viability ratio is the ratio of the optical density value obtained from the drug-treated sample divided by that of the control sample that was only treated with phosphate-buffered saline.

cases, $p < 0.05$ was considered statistically significant and is labeled using an asterisk (*) in the figures. Double and triple asterisks (** and ***) in the figures indicated $p < 0.01$ and $p < 0.005$, respectively.

Table 1 Cell viability of glioblastoma cells 48 hours after YM155 treatment.

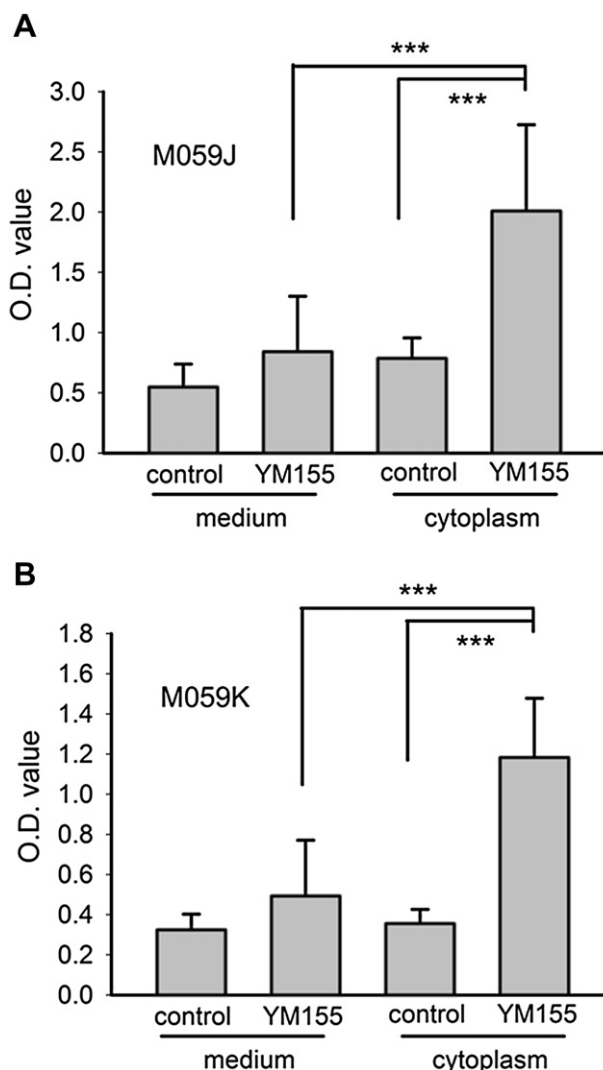| Concentration of YM155 (nM)	M059J ($n = 3$)	M059K ($n = 3$)
0*	99.7 ± 2.8	99.8 ± 2.2
5	93.4 ± 1.7	95.8 ± 0.9
10	88.0 ± 2.6	80.1 ± 1.7
20	69.4 ± 3.6	60.9 ± 1.9
30	60.1 ± 5.0	49.6 ± 2.3
40	45.7 ± 3.0	42.9 ± 2.4
50	31.9 ± 1.9	28.7 ± 1.0

* Only DMSO was administered.

A

B

Figure 2 DNA fragments in the culture medium and cytoplasm of M059J and M059K cells detected using ELISA. After treatment with 30 nM YM155 for 24 hours, the supernatants of both the culture medium and the cytoplasmic fraction of 2×10^4 (A) M059J ($n = 6$) and (B) M059K ($n = 6$) glioblastoma cells were collected. Increased expression of DNA fragments in the medium indicates necrosis due to membrane rupture, while increased expression in the cytoplasm alone indicates apoptosis. *** indicates $p < 0.005$.

3. Results

3.1. Cytotoxic effects of YM155 on glioblastoma cells

After 48 hours of treatment, YM155 elicited potent cytotoxicity in a concentration-dependent manner in both DNA-PK-deficient M059J (Figure 1A) and DNA-PK-normal M059K (Figure 1B) human glioblastoma cells. The half-maximal inhibitory concentration (IC50) was about 30—35 nM (Table 1). YM155 exerted significant cytotoxicity on both cell lines when the concentrations were > 10 nM. The viabilities of the M059J and M059K cells were not
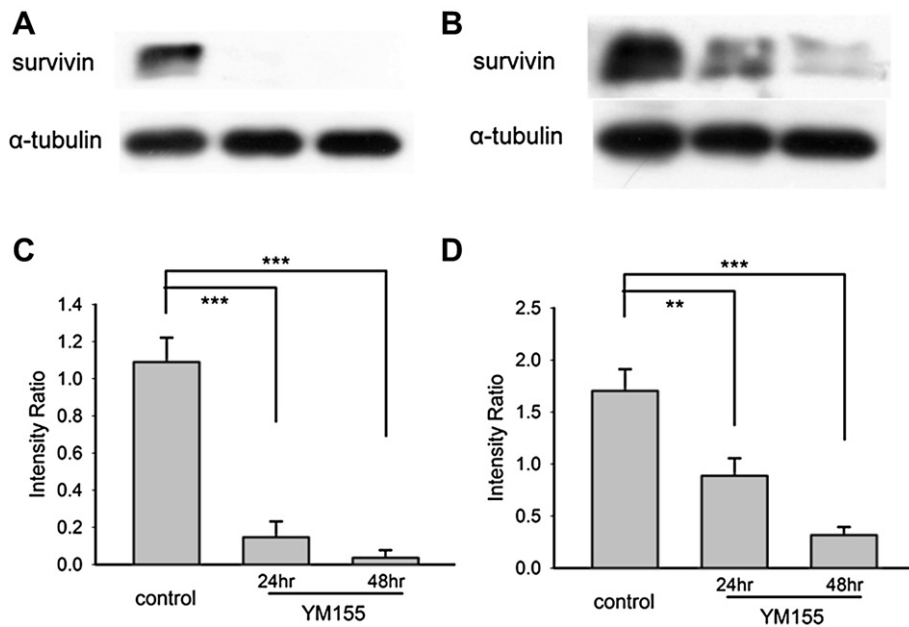
Figure 3 Expression of survivin after YM155 treatment. Figures A, B and C, D refer to M059J ($n = 3$) and M059K ($n = 3$) cells, respectively. (A, C) Representative Western blots of survivin expression that were examined 24 and 48 hours after treatment with 30 nM YM155. Expression of α-tubulin was used as the internal standard. (B, D) Quantitative comparisons of survivin expression. ** indicates $p < 0.01$; *** indicates $p < 0.005$.

significantly different at each concentration of YM155 (5–50 nM; Table 1).

3.2. YM155 induces apoptosis in glioblastoma cells

To determine the characteristics of the cytotoxicity that is mediated by YM155 in glioblastoma cells, a DNA fragmentation ELISA assay was carried out. Low basal levels of DNA fragments bound to anti-histone plus anti-DNA antibodies were detected in the culture medium of the M059J and M059K cells 24 hours after treatment with 30 nM YM155. A significantly large amount of cytoplasmic DNA fragments was found after treating the M059J and M059K cells with 30 nM YM155 in comparison with the control
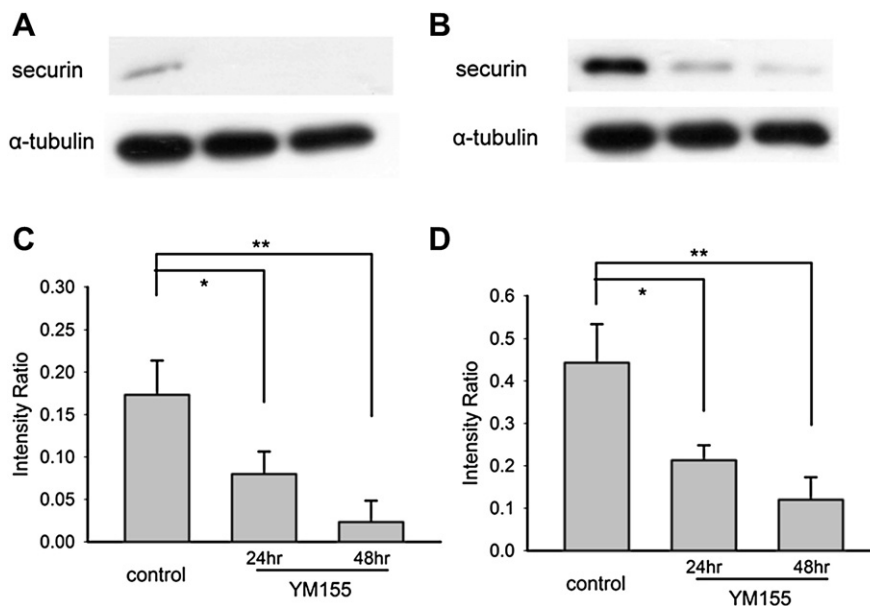

Figure 4 Expression of securin after YM155 treatment. Figures A, B and C, D refer to M059J ($n = 3$) and M059K ($n = 3$) cells, respectively. (A, C) Representative Western blots of securin expression that were examined 24 and 48 hours after treatment with 30 nM YM155. Expression of α-tubulin was used as the internal standard. (B, D) Quantitative comparisons of securin expression. * indicates $p < 0.05$; ** indicates $p < 0.01$.

(Figure 2A and 2B). These results indicated that YM155 induces apoptosis in M059J and M059K glioblastoma cells.

3.3. Expression of survivin in glioblastoma cells after YM155 treatment

As shown in Figure 3A and 3B, survivin expression in the M059J cells was inhibited by about 80% at 24 hours after treatment with 30 nM YM155, and almost the complete inhibition (96.6%) of survivin expression was noted 48 hours after treatment. Figure 3C shows that the expression of immunoreactive survivin in M059K cells was more abundant (1.56-fold higher, $p = 0.01$) than that measured in M059J cells. Survivin expression in M059K cells was also significantly inhibited 24 hours after treatment with 30 nM YM155, and $> 80\%$ inhibition was noted 48 hours later (Figure 3C and 3D).

3.4. Expression of securin in glioblastoma cells after YM155 treatment

Comparisons of the immunoreactive signals between the two glioblastoma cell lines shows that securin was much more strongly expressed in M059K cells (2.56-fold higher, $p = 0.01$) than M059J cells (Figure 4A and 4C). Securin expression in the M059J cells (Figure 4A and 4B) and M059J cells (Figure 4C and 4D) was significantly inhibited at 24 and 48 hours after treatment with 30 nM YM155.

4. Discussion

DNA-PK is a nuclear protein kinase that consists of a catalytic subunit (DNA-PKcs) and a heterodimeric DNA targeting subunit (Ku).[21] DNA-PK is directly activated by double-stranded DNA after exposure to ionizing radiation (IR),[22] and it plays an important role in the repair of double-stranded breaks via nonhomologous end-joining (NHEJ) and V(D)J recombination.[23] Cells that are deficient in DNA-PK activity are extremely sensitive to IR.[24] Conversely, increased expression or activity of DNA-PK promotes cancer cell resistance to chemotherapeutic agents or IR.[25,26] Thus, the M059K human glioblastoma cell line with normal DNA-PK may be a good cell model for investigating new treatment strategies against radiation-resistant glioblastoma.

Our results show that the novel survivin inhibitor YM155 elicits cytotoxicity in the glioblastoma cell lines M059K and M059J, indicating that DNA-PK is not involved in survivin inhibition-induced apoptosis of glioblastoma cells. Capalbo et al described the interaction between nuclear survivin and DNA-PKs following exposure to irradiation, demonstrating that survivin knockdown by RNAi impairs the repair of double-stranded DNA breaks.[27] Survivin-depleted cells also present with decreased DNA-PK activities.[27] This evidence explains why survivin expression in radiation-resistant M059K cells was greater than that measured in radiation-sensitive M059J cells in this study. This is consistent with the observation that survivin mediates radio-resistance in cancer cells.[28,29] Survivin is undetectable in normally differentiated human adult tissues.[30] Thus, the targeting of survivin inhibition may not influence the functioning of normal cells. Knockdown of survivin by shRNA delays the growth of glioblastoma xenografts in nude mice.[31] However, the clinical applications of genetic knockdowns may not be feasible in the near future. A few phase I/II clinical trials on the use of YM155 in cancer therapies have already been reported, and these treatments were well tolerated by patients with minimal adverse effects such as fatigue, nausea, and pyrexia.[32–34] Thus, YM155 may offer a new hope for glioblastoma patients, including those with radiation resistance.

Human securin is identical to the product of *PTTG* (pituitary tumor-derived transforming gene), and the overexpression of securin has been observed in several human cancers other than pituitary tumors.[35–37] The securin that is expressed in the nuclei was originally found to be an inhibitor of premature sister chromatid separation and a potential activator of transcription, but recently it has also been found to mediate tumor invasiveness and recurrence.[38] Until now, there have been no studies that have investigated the expression of securin in human glioblastoma cells and tissues, and our results provide the first results. In our previous report, concomitantly decreased expression of survivin and securin in bladder cancer cells was noted after TrkB antibody treatment.[39] Chao and Liu have also demonstrated that the transfection of survivin siRNA significantly induces apoptosis and decreases the levels of survivin and securin proteins in A549 cells.[40] Thus, the decreased expression of securin and elevated apoptosis following YM155 treatment observed in our study is consistent with previous studies. Furthermore, Chen et al reported that securin mediates radiosensitivity.[41] Thus, the higher expression level of securin that was observed in M059K cells in comparison with M059J cells may at least partly explain why M059K cells exhibit radiation resistance.

In conclusion, survivin inhibition by YM155 elicits potent cytotoxicity in human glioblastoma cells via DNA-PK-independent mechanisms. YM155 could be used as a new therapeutic drug for treating glioblastomas, including those that are resistant to radiation.

Acknowledgments

References

1. Grondin RT, Scott RM, Smith ER. Pediatric brain tumors. *Adv Pediatr* 2009;**56**:249–69.
2. Cohen KJ, Broniscer A, Glod J. Pediatric glial tumors. *Curr Treat Options Oncol* 2001;**2**:529–36.
3. Tamber MS, Rutka JT. Pediatric supratentorial high-grade gliomas. *Neurosurg Focus* 2003;**14**:e1.
4. Wrensch M, Minn Y, Chew T, Bondy M, Berger MS. Epidemiology of primary brain tumors: current concepts and review of the literature. *Neuro Oncol* 2002;**4**:278–99.
5. Davis FG, Freels S, Grutsch J, Barlas S, Brem S. Survival rates in patients with primary malignant brain tumors stratified by patient age and tumor histological type: an analysis based on Surveillance, Epidemiology, and End Results (SEER) data, 1973–1991. *J Neurosurg* 1998;**88**:1–10.

6. Song KS, Phi JH, Cho BK, et al. Long-term outcomes in children with glioblastoma. *J Neurosurg Pediatr* 2010;**6**:145—9.

7. Xie D, Zeng YX, Wang HJ, et al. Expression of cytoplasmic and nuclear Survivin in primary and secondary human glioblastoma. *Br J Cancer* 2006;**94**:108—14.

8. Shirai K, Suzuki Y, Oka K, et al. Nuclear survivin expression predicts poorer prognosis in glioblastoma. *J Neurooncol* 2009;**91**:353—8.

9. George J, Banik NL, Ray SK. Survivin knockdown and concurrent 4-HPR treatment controlled human glioblastoma in vitro and in vivo. *Neuro Oncol* 2010;**12**:1088—101.

10. Kang DW, Choi CH, Park JY, Kang SK, Kim YK. Ciglitazone induces caspase-independent apoptosis through down-regulation of XIAP and survivin in human glioma cells. *Neurochem Res* 2008;**33**:551—61.

11. Kardosh A, Soriano N, Pyrko P, et al. Reduced survivin expression and tumor cell survival during chronic hypoxia and further cytotoxic enhancement by the cyclooxygenase-2 inhibitor celecoxib. *J Biomed Sci* 2007;**14**:647—62.

12. Blum R, Jacob-Hirsch J, Rechavi G, Kloog Y. Suppression of survivin expression in glioblastoma cells by the Ras inhibitor farnesylthiosalicylic acid promotes caspase-dependent apoptosis. *Mol Cancer Ther* 2006;**5**:2337—47.

13. Schultze K, Bock B, Eckert A, et al. Troglitazone sensitizes tumor cells to TRAIL-induced apoptosis via down-regulation of FLIP and Survivin. *Apoptosis* 2006;**11**:1503—12.

14. Nakahara T, Takeuchi M, Kinoyama I, et al. YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. *Cancer Res* 2007;**67**:8014—21.

15. Wang Q, Chen Z, Diao X, Huang S. Induction of autophagy-dependent apoptosis by the survivin suppressant YM155 in prostate cancer cells. *Cancer Lett* 2011;**302**:29—36.

16. Nakahara T, Yamanaka K, Hatakeyama S, et al. YM155, a novel survivin suppressant, enhances taxane-induced apoptosis and tumor regression in a human Calu 6 lung cancer xenograft model. *Anticancer Drugs* 2011;**22**:454—62.

17. Iwasa T, Okamoto I, Suzuki M, et al. Radiosensitizing effect of YM155, a novel small-molecule survivin suppressant, in non-small cell lung cancer cell lines. *Clin Cancer Res* 2008;**14**:6496—504.

18. Allalunis-Turner MJ, Barron GM, Day 3rd RS, Dobler KD, Mirzayans R. Isolation of two cell lines from a human malignant glioma specimen differing in sensitivity to radiation and chemotherapeutic drugs. *Radiat Res* 1993;**134**:349—54.

19. Hisatomi T, Sueoka-Aragane N, Sato A, et al. NK314 potentiates antitumor activity with adult T-cell leukemia-lymphoma cells by inhibition of dual targets on topoisomerase IIα and DNA-dependent protein kinase. *Blood* 2011;**117**:3575—84.

20. Huang YT, Lai PC, Wu CC. BDNF mediated TrkB activation is a survival signal for transitional cell carcinoma cells. *Int J Oncol* 2010;**36**:1469—76.

21. Wang J, Satoh M, Pierani A, et al. Assembly and DNA binding of recombinant Ku (p70/p80) autoantigen defined by a novel monoclonal antibody specific for p70/p80 heterodimers. *J Cell Sci* 1994;**107**:3223—33.

22. Jackson SP. DNA-dependent protein kinase. *Int J Biochem Cell Biol* 1997;**29**:935—8.

23. Weaver DT. What to do at an end: DNA double-strand-break repair. *Trends Genet* 1995;**11**:388—92.

24. Muller C, Calsou P, Frit P, Cayrol C, Carter T, Salles B. UV sensitivity and impaired nucleotide excision repair in DNA-dependent protein kinase mutant cells. *Nucleic Acids Res* 1998;**26**:1382—9.

25. Shen H, Schultz M, Kruh GD, Tew KD. Increased expression of DNA-dependent protein kinase confers resistance to adriamycin. *Biochim Biophys Acta* 1998;**1381**:131—8.

26. Frit P, Canitrot Y, Muller C, et al. Cross-resistance to ionizing radiation in a murine leukemic cell line resistant to cis-dichlorodiammineplatinum, II: role of Ku autoantigen. *Mol Pharmacol* 1999;**56**:141—6.

27. Capalbo G, Dittmann K, Weiss C, et al. Radiation-induced survivin nuclear accumulation is linked to DNA damage repair. *Int J Radiat Oncol Biol Phys* 2010;**77**:226—34.

28. Khan Z, Khan N, Tiwari RP, Patro IK, Prasad GB, Bisen PS. Down-regulation of survivin by oxaliplatin diminishes radioresistance of head and neck squamous carcinoma cells. *Radiother Oncol* 2010;**96**:267—73.

29. Lei Y, Geng Z, Guo-Jun W, He W, Jian-Lin Y. Prognostic significance of survivin expression in renal cell cancer and its correlation with radioresistance. *Mol Cell Biochem* 2010;**344**:23—31.

30. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997;**3**:917—21.

31. Zhen HN, Zhang X, Shi CH, et al. Inhibition of growth and angiogenesis of U251 cell xenograft in vivo by short hairpin RNA targeting survivin gene. *Zhonghua Wai Ke Za Zhi* 2006;**44**:1270—4 [In Chinese].

32. Lewis KD, Samlowski W, Ward J, et al. A multi-center phase II evaluation of the small molecule survivin suppressor YM155 in patients with unresectable stage III or IV melanoma. *Invest New Drugs* 2011;**29**:161—6.

33. Satoh T, Okamoto I, Miyazaki M, et al. Phase I study of YM155, a novel survivin suppressant, in patients with advanced solid tumors. *Clin Cancer Res* 2009;**15**:3872—80.

34. Tolcher AW, Mita A, Lewis LD, et al. Phase I and pharmacokinetic study of YM155, a small-molecule inhibitor of survivin. *J Clin Oncol* 2008;**26**:5198—203.

35. Zou H, McGarry TJ, Bernal T, Kirschner MW. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* 1999;**285**:418—22.

36. Ogbagabriel S, Fernando M, Waldman FM, Bose S, Heaney AP. Securin is overexpressed in breast cancer. *Mod Pathol* 2005;**18**:985—90.

37. Shibata Y, Haruki N, Kuwabara Y, et al. Expression of PTTG (pituitary tumor transforming gene) in esophageal cancer. *Jpn J Clin Oncol* 2002;**32**:233—7.

38. Salehi F, Kovacs K, Scheithauer BW, Lloyd RV, Cusimano M. Pituitary tumor-transforming gene in endocrine and other neoplasms: a review and update. *Endocr Relat Cancer* 2008;**15**:721—43.

39. Huang YT, Lai PC, Wu CC, Cheng CC, Chiu TH. TrkB antibody elicits cytotoxicity and suppresses migration/invasion of transitional cell carcinoma cells. *Int J Oncol* 2010;**37**:943—9.

40. Chao JI, Liu HF. The blockage of survivin and securin expression increases the cytochalasin B-induced cell death and growth inhibition in human cancer cells. *Mol Pharmacol* 2006;**69**:154—64.

41. Chen WS, Yu YC, Lee YJ, Chen JH, Hsu HY, Chiu SJ. Depletion of securin induces senescence after irradiation and enhances radiosensitivity in human cancer cells regardless of functional p53 expression. *Int J Radiat Oncol Biol Phys* 2010;**77**:566—74.