**Natural Product Sciences**
23(1) : 35-39 (2017)
https://doi.org/10.20307/nps.2017.23.1.35

# Inhibitory Effect of D-chiro-inositol on Both Growth and Recurrence of Breast Tumor from MDA-MB-231 Cancer Cells

**Yoon-seob Kim, Ji-sung Park, Minji Kim, Bang Yeon Hwang, Chong-kil Lee, and Sukgil Song***

*College of Pharmacy, Chungbuk National University, Chungdae-ro 1, Cheongju-si Seowon-gu, Chungcheongbuk-do, Republic of Korea*

**Abstract** – D-chiro-inositol (DCI) is a secondary messenger in insulin signal transduction. It is produced *in vivo* from myo-inositol via action of epimerase. In this study, we evaluated antitumor activity of DCI against human breast cancer both *in vitro* and *in vivo*. In order to determine the inhibitory effects of DCI on growth of human breast cancer cells (MDA-MB-231), two different assessment methods were implemented: MTT assay and mouse xenograft assay. MTT assay demonstrated downturn in cell proliferation by DCI treatment (1, 5, 10, 20 and 40 mM) groups by 18.3% ($p < 0.05$), 17.2% ($p < 0.05$), 17.5% ($p < 0.05$), 18.4% ($p < 0.05$), and 24.9% ($p < 0.01$), respectively. Also, inhibition of tumor growth was investigated in mouse xenograft model. DCI was administered orally at the dose of 500 mg/kg and 1000 mg/kg body weight to treat nude mouse for 45 consecutive days. On the 45th day, tumor growth of DCI (500 mg/kg and 1000 mg/kg) groups was suppressed by 22.1% and 67.6% as mean tumor volumes were $9313.8 \pm 474.1$ mm$^3$ and $3879.1 \pm 1044.1$ mm$^3$, respectively. Furthermore, breast cancer stem cell (CSC) phenotype ($CD44^+/CD24^-$) was measured using flow cytometry. On the 46th day, CSC ratios of DCI (500 mg/kg) and co-treatment with doxorubicin (4 mg/kg) and DCI (500 mg/kg) group decreased by 24.7% and 53.9% ($p < 0.01$), respectively. Finally, from tumor recurrence assay, delay of 5 days in the co-treatment group compared to doxorubicin (4 mg/kg) alone group was observed. Based on these findings, we propose that DCI holds potential as an anti-cancer drug for treatment of breast cancer.
**Keywords** – D-chiro-inositol, Breast cancer, MDA-MB-231, Cancer stem cell, Recurrence

## Introduction

D-chiro-inositol (DCI) is a secondary messenger in insulin signal transduction. It is produced *in vivo* from myo-inositol via action of an epimerase.[1] Clinical test results have shown that DCI treatment lowers testosterone level and increases insulin sensitivity and frequency of ovulation in women.[2]

Breast cancer is a major health problem that is detrimental to the lives of millions of women. For the year 2015, it was estimated that 19,465 women in Republic of Korea will be diagnosed with breast cancer and that 2,367 women will succumb to it. With these numbers, breast cancer is the most commonly diagnosed cancer among Korean women and comes second in terms of total cancer incidence.[3] From 1999 to 2012, annual percentage change of breast cancer incidence rate increased by 6.1% in Republic of Korea.[4]

Recurrence is hardly rare in breast cancer, and is a major contributor to breast cancer-related deaths.[5] Identified causes of recurrence are cancer stem cell (CSC)[6,7], epithelial-mesenchymal transition (EMT)[8], $\beta$ 1-integrin[9], notch signaling[10], wnt signaling,[11,12] hedgehog signaling[13,14] and miRNA.[15,16] CSCs are group of cancer cells capable of self-renewing and producing heterogeneous lineages of cancer cells.[17] A number of cell surface markers such as $CD44^+/CD24^-$ are related to CSCs.[18]

In this study, we evaluated the antitumor activity of DCI against human breast cancer was both *in vitro* and *in vivo*.

## Experimental

**Materials** – The materials used and its vendors are as follows: d-chiro-inositol, doxorubicin hydrochloride, metformin, bovine serum albumin (BSA), 2,2,2-tribromoethanol, 2-methyl-2-buranol from Sigma (St. Louis, MO, USA); Rosewell Park Memorial Institute medium 1640 (RPMI 1640), fetal bovine serum (FBS), trypsin-

*Author for correspondence
Dr. Sukgil Song, College of Pharmacy, Chungbuk National University, Chungdae-ro 1, Cheongju-si Seowon-gu, Chungcheongbuk-do, Republic of Korea.
Tel: +82-43-261-2817; E-mail: songs@chungbuk.ac.kr

EDTA solution (10X), penicillin-streptomycin and phosphate buffered saline (PBS) from GIBCO-BRL (Grand Island, NY, USA); FITC-mouse anti human CD44, PE-mouse anti human CD24, 1 ml syringe, 1 ml insulin syringe and cell strainer from Becton Dickinson (San Diego, CA, USA); thiazolyl blue tetrazolium bromide (MTT) from Amresco (Solon, OHIO, USA); triton X-100 from USB (Cleveland, OHIO, USA); and hydrochloric acid and isopropyl alcohol from Deajung (Siheung-si, Gyeonggi-do, Korea).

**Cell culture** – Human breast adenocarcinoma MDA-MB-231 Cells were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin. Cells were maintained at 37 ºC in humidified atmosphere with 5% $CO_2$.

**MTT assay for cell viability** – MDA-MB-231 ($5 \times 10^4$ cells/200 μl) cells were incubated in 96-well plate for 24 hr. Incubated cells were treated with doxorubicin hydrochloride (final concentration: 1 μM), metformin (final concentration: 5 mM) and DCI (final concentration: 1, 5, 10 mM) for 48 hr. After 48 hr, cells in each well were treated with MTT solution (5 mg/ml) and incubated for 2 hr. The culture medium was aspirated from each well. Then, 100 μl MTT solvent (HCl 50 μl, isopropyl alcohol 15 ml, 10% triton X-100) was added to each well to dissolve formazan crystals for 20 min. The absorbance was measured at 590 nm by microplate reader using Molecular device spectramax M2.

**Animal** – Four-week-old anthymic nude female mice were purchased from Orient bio (Seongnam-si, Gyeonggi-do, Korea). Mouse xenograft experiment (CBNUA-586-13-01) was performed from semi-SPF area of Chungbuk National University Laboratory Animal Research Center (Cheongju-si, Chungcheongbuk-do, Korea). Nude mice were maintained under a 12-hr light/12-hr dark cycle and 23 ± 1 ºC with 50% humidity.

**Nude mouse xenograft assay for tumor growth** – Nude mice were stabilized for one week in semi-SPF area. On day 0, MDA-MB-231 cells ($9 \times 10^6$ cell/mouse) in 100 μl of PBS were injected subcutaneously into nude mice. When the tumor sizes reached 50 $mm^3$, mice were randomly distributed into equal groups (7 mice per group). DCI at a dose of 500 mg/kg and 1000 mg/kg body weight was administered orally to treat nude mouse for 45 consecutive days. Metformin was administered at a dose of 500 mg/kg by the same method as DCI group. Doxorubicin hydrochloride was injected intratumorally once every five days (day 0, 5, 10, 15) at a dose of 4 mg/kg. Tumor volume was measured once every other day. Tumor volumes were estimated by the following formula: length (mm) × width (mm) × height (mm) / 2. To determine the toxicity of DCI, body weight changes of the mice were measured once every other day. On day 25 and 45, tumor was separated from mouse.

**Nude mouse xenograft assay for tumor recurrence** – In tumor recurrence assay, DCI at a dose of 500 mg/kg body weight was administered orally to treat nude mouse for 63 consecutive days. Doxorubicin hydrochloride was injected intratumorally once every five days (day 0, 5, 10, 15) at a dose of 4 mg/kg. Other conditions were the same as mouse xenograft assay for tumor growth.

**Cancer stem cell ratio analysis** – Single-cell suspensions were obtained from tumor tissue, and lysis red blood cell using ACK solution. Single cells ($1 \times 10^6$ cell) were washed with 1% BSA/PBS. After single cells were suspended in 50 μl of 1% BSA/PBS, they were stained with CD44 antibody and CD24 antibody for 20 min on ice. Breast cancer stem cell phenotype ($CD44^+/CD42^-$) was measured using BD canto II.

**Statistical analysis** – Statistical analysis of data were performed using IBM SPSS statistics 18 software (IBM corporation, Armonk, NY, USA). Statistical significance ($p < 0.05$) was assessed by one-way analysis of variance (ANOVA) coupled with scheffe test.

## Result and Discussion

To determine the effects of DCI on the growth of human breast cancer cells, growth inhibitory effect was evaluated by MTT assay and mouse xenograft assay. MDA-MB-231 cells were treated with doxorubicin hydrochloride (final concentration: 1 μM), metformin (final concentration: 5 mM) and DCI (final concentration: 1, 5, 10, 20 and 40 mM) for 48 hr. Cell proliferation of doxorubicin hydrochloride group decreased by 21.7% ($p < 0.01$), compared to V.C group; metformin group decreased by 12.7%; and DCI (1, 5, 10, 20 and 40 mM) groups decreased by 18.3% ($p < 0.05$), 17.2% ($p < 0.05$), 17.5% ($p < 0.05$), 18.4% ($p < 0.05$), and 24.9% ($p < 0.01$), respectively (Fig. 1).

Also, suppression of tumor growth was investigated in mouse xenograft model. DCI at a dose of 500 mg/kg and 1000 mg/kg body weight was administered orally to treat nude mouse for 45 consecutive days. Metformin at a dose of 500 mg/kg was administered by the same method as DCI group. Doxorubicin hydrochloride was injected intratumorally once every five days (day 0, 5, 10, 15) at a dose of 4 mg/kg. On day 0, MDA-MB-231 cells ($9 \times 10^6$ cell/mouse) in 100 μl of PBS were injected subcutaneously into nude mice. When the tumor size reached 50 $mm^3$,

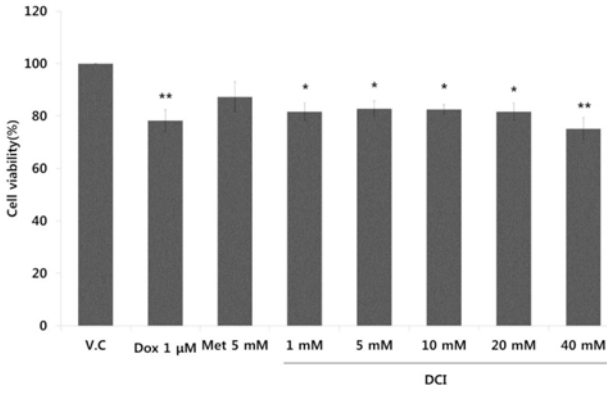


**Fig. 1.** Growth inhibition of DCI on breast cancer cell lines. MDA-MB-231 cell lines were treated with 1 - 40 mM DCI for 48 hours. Doxorubicin (1 μM) and metformin (5 mM) were used as comparisons. Cell viability of MDA-MB-231 cell lines was estimated by MTT assay ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

mice were randomly distributed into equal groups (7 mice per group). The body weights of tumor-bearing nude mice were measured to evaluate overall toxicity of DCI. After drug administration, body weights had been reduced insignificantly, disproving the toxicity of DCI (Fig. 2.B). On day 25, tumor growth of doxorubicin (4 mg/kg) group was inhibited by 94.1%; and mean tumor volume was $190.8 \pm 105.2$ mm$^3$ ($p < 0.05$), compared to V.C group. Tumor growth of metformin (500 mg/kg) group was inhibited by 77.3%; and mean tumor volume was $736.3 \pm 409.2$ mm$^3$. Tumor growth of DCI (500 mg/kg and 1000 mg/kg) groups were inhibited by 22.8% and 66%; and mean tumor volumes were $2503.1 \pm 714.5$ mm$^3$ and $1101.4 \pm 564.1$ mm$^3$, respectively. Tumor growth of co-treatment with doxorubicin (4 mg/kg) and metformin (500 mg/kg) was inhibited by 96.4%; and mean tumor volume

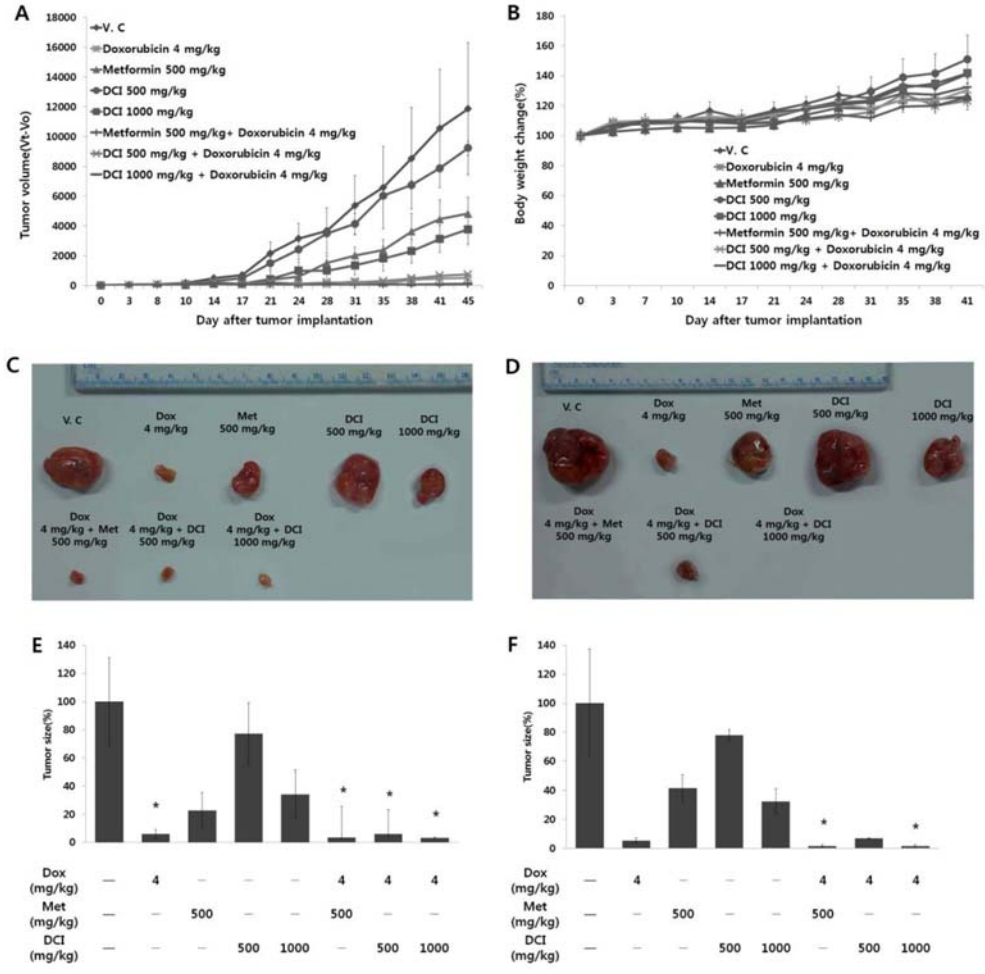


**Fig. 2.** Inhibition of breast tumor growth by DCI in nude mouse xenograft assay. On day 0, nine million MDA-MB-231 cells were implanted subcutaneously into nude mice. DCI at a dose of 500 mg/kg or 1000 mg/kg body weight was administered orally for 45 consecutive days. (A) The body weights of the tumor-bearing nude mice were measured to evaluate overall toxicity of DCI. (B) Tumor volumes were estimated by the formula: length (mm) × width (mm) × height (mm)/2. Photographs of representative MDA-MB-231 tumor mass on (C) day 25 and (D) day 45 were shown. On (E) day 25 and (F) day 45, relative tumor size was measured ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

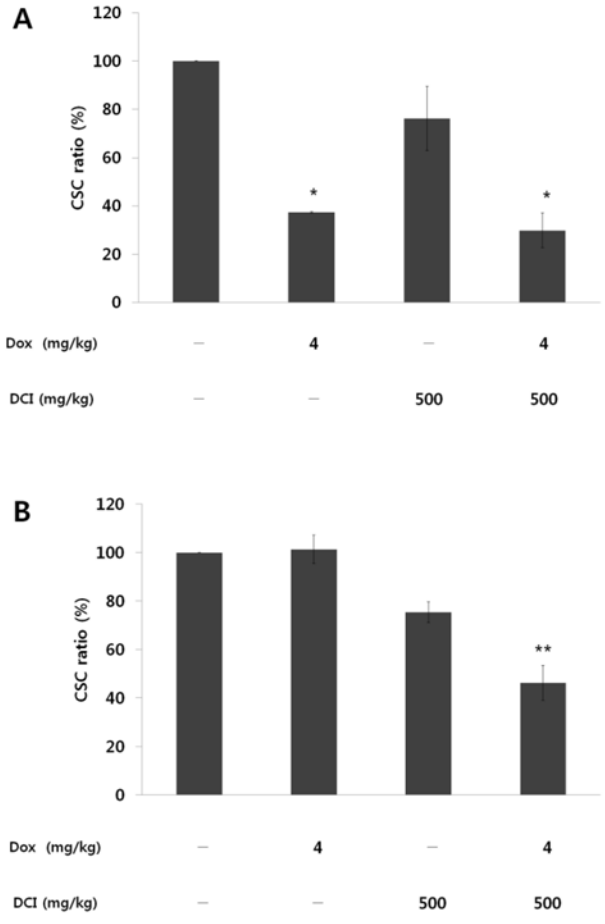


**Fig. 3.** Growth inhibition of CSCs within tumor mass by DCI. On (A) day 25 and (B) day 46, the mice were sacrificed and population of CSCs of the breast tumors was measured by flow cytometry analysis after double staining on anti-CD44 and anti-CD24 ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

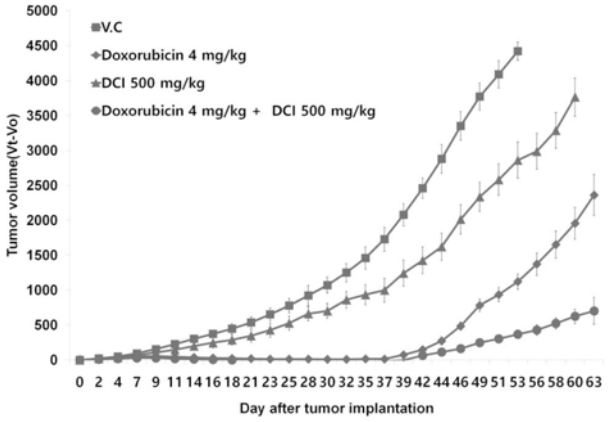


**Fig. 4.** Delay of breast tumor recurrence in mouse xenograft assay by DCI. DCI at a dose of 500 mg/kg body weight was administered orally for 63 consecutive days. Doxorubicin at a dose of 4 mg/kg was injected within breast tumor four times a day for 15 days.

was $117 \pm 17$ mm$^3$ ($p < 0.05$). Tumor growth of co-treatment doxorubicin (4 mg/kg) and DCI (500 mg/kg or 1000 mg/kg) were inhibited by 22.8% and 66%; and mean tumor volumes were $190.6 \pm 49.7$ mm$^3$ ($p < 0.05$) and $99.3 \pm 8$ mm$^3$ ($p < 0.05$), respectively (Fig. 2 A, C, E). On day 45, tumor growth of doxorubicin (4 mg/kg) group was inhibited by 94.6%; and mean tumor volume was $648.1 \pm 194.5$ mm$^3$, compared to V.C group. Tumor growth of metformin (500 mg/kg) group was inhibited by 58.5%; and mean tumor volume was $4967 \pm 1124.8$ mm$^3$. Tumor growth of DCI (500 mg/kg and 1000 mg/kg) groups were inhibited by 22.1% and 67.6%; and mean tumor volumes were $9313.8 \pm 474.1$ mm$^3$ and $3879.1 \pm 1044.1$ mm$^3$, respectively. Tumor growth of co-treatment with doxorubicin (4 mg/kg) and metformin (500 mg/kg) group was inhibited by 98.5%; and mean tumor volume was $181.3 \pm 96.3$ mm$^3$ ($p < 0.05$). Tumor growth of co-treatment with doxorubicin (4 mg/kg) and DCI (500 mg/kg or 1000 mg/kg) were inhibited by 93.1% and 98.5%; and mean tumor volumes were $821.1 \pm 27.9$ mm$^3$ and $184.9 \pm 110.2$ mm$^3$ ($p < 0.05$), respectively (Fig. 2 A, D, F).

Furthermore, the population of the CSCs was investigated using flow cytometry. Single-cell suspensions were obtained from tumor tissue, and lysis red blood cell using ACK solution. Single cells ($1 \times 10^6$ cell) were stained with CD44 antibody and CD24 antibody for 20 min on ice. Breast cancer stem cell phenotype ($CD44^+/CD24^-$) was measured using BD canto II. On day 25, CSCs ratio of doxorubicin (4 mg/kg) group decreased by 62.7% ($p < 0.05$), compared to V. C group; and DCI (500 mg/kg) and co-treatment with doxorubicin (4 mg/kg) and DCI (500 mg/kg) decreased by 23.8% and 70.2% in CSCs population ($p < 0.05$). (Fig. 3A) On day 46, CSCs ratio of doxorubicin (4 mg/kg) group increased by 1.3%, compared to V.C group; and DCI (500 mg/kg) and co-treatment with doxorubicin (4 mg/kg) and DCI (500 mg/kg) decreased by 24.7% and 53.9% ($p < 0.01$), respectively (Fig. 3B).

Moreover, mouse xenograft assay was implemented to determine the effect of DCI on recurrence of breast cancer. Tumor recurrence assay, DCI at a dose of 500 mg/kg body weight was administered orally to treat nude mouse for 63 consecutive days. On day 37, tumor recurred among doxorubicin (4 mg/kg) group; and, on day 42, co-treatment with doxorubicin (4 mg/kg) and DCI (500 mg/kg) also experienced tumor recurrence. Evidently, recurrence was delayed for 5 days in co-treatment with doxorubicin (4 mg/kg) and DCI (500 mg/kg) group compared to doxorubicin (4 mg/kg) group (Fig. 4).

In conclusion, inhibitory effect of DCI was confirmed

by MTT assay in MDA-MB-231 cells. Also, compared to the untreated control group of mice, tumor growth was hindered and CSCs ratio diminished in DCI administered nude mice. Additionally, tumor recurrence lagged in co-treatment group with DCI and doxorubicin compared to doxorubicin administration group. Thus, we propose DCI as a potential anti-cancer drug for the treatment of breast cancer.

## Acknowledgments

This work was supported by the research grant of Chungbuk National University in 2014.

## References

(1) Larner, J. *Int. J. Exp. Diabetes Res.* **2002**, *3*, 47-60.
(2) Nestler, J. E.; Jakubowicz, D. J.; Reamer, P.; Gunn, R. D.; Allan, G. *N. Engl. J. Med.* **1999**, *340*, 1314-1320.
(3) Jung, K. W.; Won, Y. J.; Oh, C. M.; Kong, H. J.; Cho, H.; Lee, D. H.; Lee, K. H. *Cancer Res. Treat.* **2015**, *47*, 142-148.
(4) Jung, K. W.; Won, Y. J.; Kong, H. J.; Oh, C. M.; Cho, H.; Lee D. H.; Lee K. H. *Cancer Res. Treat.* **2015**, *47*, 127-141.
(5) Moody, S. E.; Perez, D.; Pan, T. C.; Sarkisian, C. J.; Portocarrero, C. P.; Sterner, C. J.; Notorfrancesco, K. L.; Cardiff, R. D.; Chodosh, L. A. *Cancer Cell* **2005**, *8*, 197-209.
(6) Lacerda, L.; Pusztai, L.; Woodward, W. A. *Drug Resist. Updat.* **2010**, *13*, 99-108.
(7) McDermott, S. P.; Wicha, M. S. *Mol. Oncol.* **2010**, *4*, 404-419.
(8) Dave, B.; Mittal, V.; Tan, N. M.; Chang, J. C. *Breast Cancer Res.* **2012**, *14*, 202.
(9) Barkan D.; Chambers A. F. *Clin. Cancer Res.* **2011,** *17*, 7219-7223.
(10) Reedijk, M.; Pinnaduwage, D.; Dickson, B. C.; Mulligan, A. M.; Zhang, H.; Bull, S. B.; O'Malley, F. P.; Egan, S. E.; Andrulis, I. L. *Breast Cancer Res. Treat.* **2008**, *111*, 439-448.
(11) Gangopadhyay, S.; Nandy, A.; Hor, P.; Mukhopadhyay, A. *Clin. Breast Cancer* **2013**, *13*, 7-15.
(12) Karamboulas, C.; Ailles, L. *Biochim. Biophys. Acta.* **2013**, *1830*, 2481-2495.
(13) Izrailit J.; Reedijk M. *Cancer Lett.* **2012,** *317*, 115-126.
(14) Hassounah N. B.; Bunch T. A.; McDermott K. M. *Clin. Cancer Res.* **2012**, *18*, 2429-2435.
(15) Tang J.; Ahmad A.; Sarkar F. H. *Int. J. Mol. Sci.* **2012**, *13*, 13414-13437.
(16) Li L.; Xie X.; Luo J.; Liu M.; Xi S.; Guo J.; Kong Y.; Wu M.; Gao J.; Xie Z.; Tang J.; Wang X.; Wei W.; Yang M.; Hung MC.; Xie X. *Mol. Ther.* **2012**, *20*, 2326-2334.
(17) Sanguinetti A.; Bistoni G.; Avenia N. *G. Chir.* **2011**, 32, 438-446.
(18) Velasco-Velázquez M. A; Homsi N.; De La Fuente M.; Pestell R. *G. Int. J. Biochem. Cell Biol.* **2012**, *44*, 573-577.

Received June 14, 2016
Revised November 8, 2016
Accepted November 18, 2016