Down-regulation of Survivin enhances sensitivity to BPR0L075 in human cancer cells via caspase-independent mechanisms

Chun Hei Antonio Cheung[1], Ching-Chuan Kuo[1], Chi-Yen Chang[1], Mohane Selvaraj Coumar[2], Hsing-Pang Hsieh[2] and Jang-Yang Chang[1,*]

[1]*National Institute of Cancer Research, National Health Research Institutes, Tainan 704, Taiwan R.O.C.*

[2]*Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Zhunan, Miaoli County 350, Taiwan R.O.C.*

**Corresponding author:* National Institute of Cancer Research, National Health Research Institutes, 2F, No. 367 Sheng Li Road, Tainan 704, Taiwan R.O.C.

Tel: +886 6 7000123; fax: +886 6 2083427

E-mail: jychang@nhri.org.tw

ABSTRACT

Background: BPR0L075 [6-methoxy-3-(3',4',5'-trimethoxy-benzoyl)-1H-indole] is a novel anti-cancer compound. It inhibits tubulin polymerization and induces mitochondrial-dependent apoptosis in various human cancer cells with different multi-drug resistance (MDR) status. Over-expression of an anti-apoptotic molecule, survivin, causes drug-resistance in various cancers. Survivin inhibits apoptosis by interfering caspase-3 and promotes cell growth by stabilizing microtubule networks. Here, we determined the effects of down-regulation of survivin in BPR0L075 (L075) treatment. *Methods:* Western blot analysis was used to determine the expression level of survivin in L075-untreated/-treated human oral carcinoma KB and nasopharyngeal carcinoma HONE-1 cancer cells. siRNA was used to down-regulate endogenous survivin. MTT cell viability assay, real-time caspase-3 activity assay and immuno-fluorescence microscopy were used to analyze downstream effects. *Results:* Survivin expression was up-regulated in both KB and HONE-1 cells in response to L075 treatment. Down-regulation of survivin induced hyper-sensitivity to L075 in KB and re-stored sensitivity to L075 in KB-derived L075-resistant KB-*L30* cancer cells. At the molecular level, down-regulation of survivin induced changes in microtubule dynamics in both KB and KB-*L30* cells. Surprisingly, down-regulation of survivin did not enhance the activity of caspase-3 in L075 therapy. Instead, down-regulation of survivin induced translocation of the apoptosis-inducing factor (AIF) from cytoplasm to nucleus. *Conclusion:* Down-regulation of survivin improved drug sensitivity to L075 in both KB and L075-resistant KB-*L30* cancer cells, possibly through a tubulin-dependent and caspase-independent

mechanism. We suggest that combining BPR0L075 and survivin inhibitor may give better clinical outcome than the use of BPR0L075 monotherapy in future clinical trials.

Keywords:

Tubulin, survivin, caspase, p53, apoptosis, apoptosis inducing factor

INTRODUCTON

Microtubules are protein filaments of cytoskeleton composed of α-tubulin and β-tubulin molecules (Sawada & Cabral, 1989; Wade & Hyman, 1997). In cells, microtubule filaments rapidly alternate between phases of growth and shrinkage (dynamic instability) during cell cycle. Since microtubules play crucial roles in the regulation of the mitotic apparatus, disruption of microtubules can induce cell cycle arrest in M phase, the formation of abnormal mitotic spindles, and final triggering of signals for apoptosis. The discovery that the cytotoxic activity of various compounds is through interference with the mitotic spindle apparatus has attracted much attention within the past two decades, and microtubules have become an attractive pharmacologic target for anticancer drug discovery (Kiselyov et al., 2007; Shi et al., 1998). Anti-mitotic compounds such as vincristine, vinblastine (microtubule-destabilizing *Vinca* alkaloid) and paclitaxel (microtubule-stabilizing taxane) have been developed to target cancers recently (Arrieta et al., 2006; Raitanen et al., 2002; Tanaka et al., 2004). Although the taxanes and *Vinca* alkaloids are effective for the management of different malignancies, their potential is limited by the development of multidrug resistance (MDR) (Cornelissen et al., 1994; Dumontet & Sikic, 1999). MDR is multi-factorial, with one pathway leading to resistance mediated by over-expression of transmembrane efflux pumps, namely, the M_r 170,000 P-glycoprotein (P-gp170/MDR) and the multidrug resistance protein (MRP) (Deng et al., 2002). Therefore, there has been great interest in identifying novel microtubule inhibitors that can overcome various modes of resistance and have improved pharmacology profiles.

BPR0L075 [6-methoxy-3-(3',4',5'-trimethoxy-benzoyl)-1H-indole] is a novel synthetic compound discovered through research to identify new microtubule inhibitors. It is a heterocyclic Combretastatin A-4 (CA-4) analog, which is derived from the South African tree *Combretum caffrum*, and inhibits tubulin polymerization by binding to tubulin at the colchicine-binding site (Kuo et al., 2004a; Liou et al., 2004). Unlike traditional microtubule inhibitors such as vincristine and paclitaxel; BPR0L075 (L075) is effective in suppressing cell growth of both MDR-positive and -negative tumor cell lines (Kuo et al., 2004a). L075 also induces phosphorylation of Bcl-2 and activation of caspase-3 (Kuo et al., 2004a). *In-vivo*, L075 shows potent activity against the growth of xenograft tumors of the gastric carcinoma MKN-45, human cervical carcinoma KB, and KB-derived P-gp170/MDR-overexpressing KB-VIN10 cells in nude mice (Kuo et al., 2004a). Thus, L075 is a promising anticancer compound with anti-mitotic activity that can be applied to the management of various malignancies, particularly for patients with MDR-related drug resistance. Phase I clinical trial of L075 is planned and will be initiated in the near future.

Besides overexpression of MDR, inaccurate expression of survivin also induces drug resistance in various cancers (Zaffaroni & Daidone, 2002). Survivin belongs to the family of inhibitors of apoptosis (IAPs). Unlike other IAPs such as XIAP and IAP-1, survivin is not expressed in differentiated normal tissue (Reed, 2001; Satoh et al., 2001; Tanaka et al., 2003). In clinical situations, survivin seems to play an important role in the transition of adenoma with low dysplasia to high dysplasia during human colorectal

tumorigenesis (Kawasaki et al., 2001). The median survival period for patients with high survivin expression was less than that for patients with low levels of expression after chemotherapy treatment, suggesting that survivin expression correlates with a poor prognosis (Kato et al., 2001). Expression of survivin has been related to the causation of cancer-drug resistance in various studies (Pennati et al., 2007). Growing evidence indicates that transient upregulation of survivin by VEGF and bFGF in normal endothelial cells of blood vessel is partially responsible for tumor angiogenesis and tumor resistance against chemotherapeutic drugs (O' Connor et al., 2000; Tran et al., 2002). At the molecular level, survivin is a bifunctional protein that acts as a suppressor of apoptosis and plays a central role in cell division. It has been suggested that survivin, possibly the mitochondrial fraction instead of the cytosol fraction, inhibits apoptosis through interference with caspases (Dohi et al., 2004; Shin et al., 2001; Tamm et al., 1998). Survivin also promotes cell survival through interference with cell cycle-related kinases and microtubule networks. Complex formation between survivin, INCENP and Aurora-B kinase was found on the centromere, and over-expression of survivin has been shown to elevate Aurora-B activity *in-vitro* (Geddis & Kaushansky, 2004). Over-expression of survivin reduced centrosomal microtubule nucleation and suppressed both microtubule dynamics instability in mitotic spindles and bidirectional growth of microtubules in midbodies during cytokinesis (Rosa et al., 2006). It has been shown that intracellular loading of a polyclonal antibody to survivin induced microtubule defects and resulted in formation of multipolar mitotic spindles (Fortugno et al., 2002). In addition, gene knockout studies and tubulin staining revealed that the absence of normal

mitotic spindle structures and intercellular midbodies were related to the absence of survivin protein (Giodini et al., 2002; Li et al., 1998; Rosa et al., 2006).

Since survivin is associated with microtubules, apoptosis, and drug resistance, it is possible that this molecule can contribute to the cell response to our microtubule-destabilizing compound, BPR0L075. In this study, we describe the role of survivin in interfering sensitivity to BPR0L075 in human oral carcinoma cells.

MATERIALS AND METHODS

Drug

The compound BPR0L075 was synthesized at the Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Zhunan, Taiwan, ROC. BPR0L075 was obtained in 72% yield from 6-methoxyindole and 3,4,5-trimethoxybenzoyl chloride. The detailed synthetic method was previously published.(Liou et al., 2004)

Cell lines, antibodies and reagents

The human oral carcinoma cells (KB) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). KB and human nasopharyngeal carcinoma (HONE-1) cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 5% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL) and L-glutamine (0.29 mg/mL), at 37^{o}C. The antibodies used in this study included: a mouse anti-αTubulin antibody (Upstate Cell signaling, Lake Placid, NY), a mouse anti-βTubulin antibody (BD PharMingen, Franklin Lakes, NJ), a mouse anti-Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit anti-Survivin antibody (R&D Systems, Minneapolis, MN), a rabbit anti-AIF antibody (R&D Systems, Minneapolis, MN), a mouse anti-Bcl2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

RT-PCR

Total RNA was extracted with the use of TRIzol reagent (Invitrogen, Carlsbad, CA) and complementary DNA was synthesized from RNA with the SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Polymerase chain reaction was performed with target-specific primers. Survivin forward primer: 5' ATGGGTGCCCCGACGTT; Survivin reverse primer: 5' TCAATCCATGGCAGCCAG; GAPDH: 5' ACCACAGTCCATGCCATCAC and GAPDH reverse primer: 5' TCCACCACCCTGTTGCTGTA.

siRNA

Target-validated siRNA oligos (Santa Cruz Biotechnology, Santa Cruz, CA) were transfected into cells using the Lipofectamine-2000 reagent (Invitrogen, Carlsbad, CA). Briefly, cells were seeded onto 96-well plates or chamber-slides, and cultured overnight in 100 μl of antibiotic-free RPMI media. siRNA oligomers (8 pmol in 0.4 μl) were diluted in 25 μl of Opti-MEM® I medium (Invitrogen, Carlsbad, CA) without serum, and then mixed with 0.2 μl of Lipofectamine-2000 transfection reagent for 25 min at room temperature. Cells were overlaid with the transfection mixture, and incubated for various times.

SDS-PAGE and Western Blot Analysis

Cells were lysed with ice-cold lysis buffer (10 mM Tris, 1 mM EDTA, 1 mM DTT, 60 mM KCl, 0.5% NP-40 and protease inhibitors). Total cell lysates, fractions of supernatant

or pellet were resolved on 10% and 12% polyacrylamide SDS gels under reducing conditions. The resolved-proteins were electrophoretically transferred to PVDF membranes (Amersham Life Science, Amersham, U.K.) for Western blot analysis. The membranes were blocked with 5% non-fat milk powder at room temperature for two hours, washed twice with PBST (1% Tween) and then incubated with primary antibody for 90 minutes at room temperature. The membranes were washed twice with PBST then subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (dilution at 1:10000, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected by Enhanced Chemiluminescence (van Loo et al.) (Amersham International, Buckingham, U.K.) and autoradiography.

MTT cell viability assay

3×10^3 cells in 100 μL of drug-free culturing medium were seeded on 96-well plates for 24 hours before treatments. Cells were then treated with various concentration of BPR0L075 for 72 hours. 25 μL of MTT (5 mg/mL) was added into each sample and incubated for 4 hours, under 5% CO_2 and 37^{o}C. 100 μL of lysis buffer (20% SDS, 50% DMF) was subsequently added into each sample and further reacted for 16 hours. IC_{50} value resulting from 50% inhibition of cell growth was calculated graphically as a comparison with control growth.

Immunofluorescent microscopy

Cells were cultured in 8-well chamber slides, fixed with 4% paraformaldehyde, and permeabilized with TPBS. They were incubated with primary antibody for 60 min at

room temperature followed by FITC-conjugated secondary antibody. Slides were examined by microscopy using an Olympus BX50 microscope (Olympus optical co., LTD, Tokyo, Japan). Images were taken on the Olympus microscope with the use of software CoolSNAP (Roper Scientific, Inc.)

Real-time Caspase-3/-7 activity imaging and caspase-3/-7 activity assay

Caspase-3/-7 activity was analyzed with a MagicRed^{TM}-DEVD Caspase Detection Kit (Immunochemistry Technologies LLC, Bloomington, MN). Briefly, cells were cultured in chamber-slides and incubated with test agents. Cells were then incubated with caspase substrate MR-($DEVD_2$) in culture medium for 60 min, and then with Hoechst 33342 stain for 15 min. Cells were viewed with a UV-enabled inverted-microscope at an excitation wavelength of 540 nm – 560 nm and emission at 610 nm.

Quantitative analysis of caspase-3/-7 activity was performed on 96-well plates. Cells were cultured on 96-well plates and incubated with test agents. Treated-cells were then incubated with caspase substrate MR-($DEVD_2$) in culture medium for 90 min. Caspase-3/-7 activity was measured with a 96-well plate reader at an excitation wavelength of 590 nm and emission at 615 nm.

Visualization of apoptosis by the TUNEL assay

Cells were seeded and cultured in 8-well chamber-slides, and treated with various treatments. The cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min on ice, and permeabilized with TPBS at room temperature. Apoptotic cells were stained by the TUNEL agent using an In-Situ Apoptosis Detection TMR Kit (Roche Diagnostic,

Mannheim, Germany). Cells were counter-stained with DAPI to detect nucleus, and examined by fluorescence microscopy.

RESULTS

BPR0L075 induces up-regulation of survivin protein expression

Human p53-wildtype KB and p53-mutated HONE-1 cancer cell lines were used in this study. Cells were treated with 8 nM (IC_{50} value) of L075 for 24 h. Cell lysate were extracted and expressions of various intracellular proteins were analyzed by western blotting. A time-dependent phosphorylation of Bcl2 in both cell lines was shown and this result is consistent with our previously published data (Figure 1A) (Kuo et al., 2004a). Expression of p53 was also increased in L075-treated KB cells in a time-dependent manner where an increase of p53 in L075-treated HONE-1 cells was observed at 24 h post-treatment (Figure 1A). Both un-treated cell lines expressed survivin endogenously (Figure 1A). Surprisingly, treatment of L075 induced over-expression of survivin in a time-dependent manner (Figure 1A). KB cells were chosen for further *in-vitro* investigation; consider that the same cell line was chosen in our previously published report (Kuo et al., 2004a).

BPR0L075 induces survivin gene transcription

To determine whether L075-induced over-expression of survivin in KB cells was caused by changes in the rate of survivin gene transcription, RT-PCR was performed. As was observed for the protein, level of survivin mRNA transcripts was increased in KB cells after 24 h of L075 incubation (Figure 1B). Western blot analysis revealed that co-incubation with a gene transcription inhibitor, actinomycin-D, was able to reduce the amount of survivin protein in L075-treated cells (Figure 1C). Heat shock protein 90

(Hsp90) has been suggested to stabilize survivin *in-vitro* (Fortugno et al., 2003b; Suriawinata, 2004). Here, co-incubation of a Hsp90 inhibitor, geldanamycin, was unable to reduce the amount of intracellular survivin protein in L075-treated KB cells (Figure 1C). However, changes in cell morphology were shown by phase-contrast microscopy (Figure 1D).

Down-regulation of survivin enhances sensitivity to BPR0L075 in KB cells

To address whether survivin plays a role in sensitivity to L075 in KB cells, siRNA was applied to down-regulate survivin. Cells were transfected with validated-siRNA oligomers, siR-C (scramble control) or siR-S (survivin specific) by liposomal reagent. Successful down-regulation of survivin by siR-S after 48 h of transfection was shown by immunofluorescence microscopy (Figure 2A). Down-regulation of survivin mRNA transcripts by siRNA after 36 h of transfection was further confirmed by RT-PCR (Fig. 2B).

Changes in cell morphology were observed in survivin down-regulated KB cells and revealed by phase-contrast microscopy. Survivin-targeted cells became round and slightly enlarged in size (Figure 3A). Although inhibition of survivin by siR-S induced morphological changes in KB cells under L075-free conditions (Figure 3A), the same treatment did not reduce cell viability (Figure 3B). Interestingly, down-regulation of survivin by siR-S enhanced sensitivity to L075 in KB cells *in-vitro*. Treatment with siR-S reduced cell viability of KB by 21-31% among various concentrations of L075 compared to L075 monotherapy (Figure 3B).

Inhibition of survivin enhances sensitivity to BPR0L075 via a caspase-independent mechanism

To determine whether down-regulation of survivin enhances sensitivity to L075 in KB cells via a caspase-dependent mechanism, a real-time caspase-3/-7 activity imaging was performed. KB cells were seeded on 8-well chamber slides. Cells were transfected with siR-C and siR-S for 24 h and co-incubated with L075 for various times. Real-time caspase-3/-7 activity assay was used and cells were viewed under microscope. Incubation of L075 induced caspase-3/-7 activity as expected (Figure 4A). Surprisingly, inhibition of survivin by siR-S did not induce caspase-3/-7 activity in KB cells under L075-free condition (Figure 4A and 4B). In addition, co-treatment of siR-S and L075 did not enhance caspase-3/-7 activity, compared to the L075 monotherapy at various time points (Figure 4B).

Nucleus degradation was observed with KB cells treated with 16 nM (twice of IC_{50} value) of L075 after 72 h (data not shown) (Kuo et al., 2004a). Here, combination of siR-S and 4 nM of L075 induced massive degradation of nucleus in KB cells. In contrast, cells treated with 4 nM of L075 did not induce similar molecular phenotype (Figure 4A). DNA fragmentation was further analyzed by TUNEL assay and immunofluorescence microscopy. In TUNEL analysis, nucleus of cells co-treated siR-S and L075 were stained red, suggesting that DNA fragmentation occurred in the nucleus (Figure 4C). In contrast, nucleus of KB cells treated with siR-C, siR-S or L075 were not stained red by the TUNEL reagent (Figure 4C).

Down-regulation of survivin induces translocation of apoptosis-inducing factor in KB cells

Translocation of apoptosis-inducing factor (AIF) occurs during caspase-independent apoptosis. The possibility that siRNA-mediated down-regulation of survivin leads to the translocation of AIF was explored. KB cells were treated with siR-C, siR-S, or drug L075 alone, and AIF expression was examined by immunofluorescence microscopy using an anti-AIF antibody. The cytoplasm of cells treated with siR-C for 48 h was stained green with a ring-like pattern around the nucleus by the anti-AIF antibody, as was the cytoplasm of cells treated with L075 (Figure 4D). In marked contrast, it was the nucleus that was stained green by the anti-AIF antibody in cells treated with the siR-S against survivin, suggesting that AIF translocated from cytoplasm to the nucleus when survivin was targeted with siRNA (Figure 4D). Cells treated with a combination of siR-S and L075 induced similar phenotype to siR-S mono-treatment (data not shown).

Survivin interferes with the microtubule dynamics

To determine whether sensitivity to L075 in KB cells is also related to survivin-interfered microtubule dynamics, insoluble fraction of cell lysate were analyzed by western blotting. Application of siR-S reduced the amount of insoluble α-tubulin in KB cells after 72 h of transfection (Figure 5A). In contrast, down-regulation of survivin did not induce any change in general expression of α-tubulin (Figure 5A). Immuno-staining of α-tubulin in KB cells was performed to determine the integrity of microtubule networks. Normal KB cells showed intact microtubule networks (Figure 5B). In contrast, down-regulation

of survivin by siR-S induced disorganization of the microtubule networks (Figure 5B). Furthermore, combination of siR-S and 4 nM of L075 induced synergistic microtubule depolarization and changes in cell morphology (Figure 5B).

A KB-derived L075-resistant cancer cell line, KB-*L30*, was recently generated in our laboratory. This drug-resistant cell line was shown to withstand 30 nM of L075 under culturing condition (data not shown). In comparison to KB cells, western blot analysis revealed that expression of survivin was increased by approximately 2-fold in L075-resistant KB-*L30* (Figure 5C). In correlation, amount of insoluble α-tubulin and β-tubulin were also increased in the cells (Figure 5D). siRNA was used to down-regulate survivin in KB-*L30* cells. Levels of survivin mRNA transcript were successfully down-regulated by siR-S after 36 h of transfection (Figure 5E). Application of siR-S reduced the amount of insoluble α-tubulin in KB-*L30* cells after 72 h of transfection (Figure 5F).

Down-regulation of survivin enhances sensitivity to BPR0L075 in KB-*L30* cells

Since down-regulation of survivin induced changes in the microtubule dynamics in drug-resistant KB-*L30* cells, siRNA was used to determine whether down-regulation of survivin also restores L075 sensitivity in the same cell line. Result from phase contrast microscopy and cell viability showed that down-regulation of survivin by siR-S induced massive cell death in KB-*L30* cancer cells under normal culture conditions (30 nM of L075) (Figure 6A and 6B). In contrast, down-regulation of survivin did not induce KB-*L30* cells cell death under L075-free conditions (Figure 6A and 6B). Co-treatment of siR-

S and L075 reduced cell viability of KB-*L30* by 20-52% among various concentrations of L075 *in-vitro* (Figure 6B).

DISCUSSION

Over-expression of survivin has been shown to induce drug resistance in cancers. In an example, over-expression of wildtype survivin by gene transfection mediated resistance to anti-androgen therapy in prostate cancer cells (Zhang et al., 2005a). In addition, adenovirus-mediated inhibition of survivin expression sensitized human prostate cancer cells to paclitaxel *in-vitro* and *in-vivo* (Zhang et al., 2005b). Given such evidences implicating survivin's role in drug resistance, survivin may also play a role in the sensitivity and resistancy to our newly developed tubulin de-polymerizing compound BPR0L075 in cancers.

Here, we report that BPR0L075 induced co-expression of survivin and p53 in both p53-wildtype KB and p53-mutated HONE-1 cancer cells in a time-dependent manner. The amount of survivin within cells seems to be controlled by two different mechanisms, namely gene transcription and protein stabilization (Fortugno et al., 2003a; Li & Altieri, 1999; Zhu et al., 2004). Mutations in the CDE/CHR repressor element within survivin promoter region were shown to increase survivin gene transcription (Xu et al., 2004). At post-translational level, molecular chaperones such as heat shock protein 90 (Hsp90) were shown to stabilize survivin protein *in-vitro* (Fortugno et al., 2003b; Suriawinata, 2004). In our study, the Hsp90 inhibitor, geldanamycin, did not alter the over-expression of survivin protein in response to BPR0L075 while the gene transcription inhibitor, actinomycin-D, completely abrogated BPR0L075-induced changes in survivin protein expression. Together with the result from RT-PCR, decreases in survivin protein in

response to BPR0L075 in the presence of actinomycin-D may reflect increased gene transcription. Expression of survivin is thought to be negatively regulated by p53 (Ikeda et al., 2007; Mirza et al., 2002; Raj et al., 2008; Yonesaka et al., 2006; Zhou et al., 2002; Zhu et al., 2006). However, whether p53 plays a definite role in survivin regulation under various chemo-stresses remains agurable. In fact, a new class of anticancer drug, Geranylgeranyltransferase I inhibitors (GGTIs), induce cancer cells cell death by down-regulation of survivin through a p53-independent pathway (Dan et al., 2004). Furthermore, co-expression of survivin and p53 in response to a flavonoid, Quercetin, has been demonstrated previously (Kuo et al., 2004b). Interestingly, co-incubation of a p53-inhibitor, pifithrin-α, was unable to increase the level of survivin expression in BPR0L075-treated KB cells (supplemental data). The present study suggests that BPR0L075 induces overexpression of survivin through a p53-independent pathway. Indeed, further investigation should be undertaken to determine the pathway of BPR0L075-induced survivin expression.

As mentioned previously, survivin plays important roles in the dynamic polymerization of microtubule and induction of drug resistance. Here, survivin clearly plays a role in the sensitivity to BPR0L075 in human oral carcinoma cells. Survivin also plays a role in the polymerization of microtubule in our tested cell lines. It has been reported that VEGF-induced expression of survivin preserved the microtubule integrity of both CDDP and paclitaxel-treated HUVEC cells. Stabilization of microtubule networks subsequently induced drug-resistance in cells (Tran et al., 2002). Furthermore, a role for altered microtubule polymer levels in vincristine resistance of acute lymphoblastic leukemia has

been demonstrated *in-vivo* (Ong et al., 2008). In Ong et al's study, a vincristine-resistant xenograft with high levels of polymerized tubulin was also relatively sensitive to the microtubule-polymerizing drug paclitaxel (Ong et al., 2008). Interestingly, the BPR0L075-resistant KB-*L30* with high levels of polymerized tubulin was relatively sensitive to paclitaxel (data not shown). In addition, KB-*L30* cells were relatively resistant to the microtubule-depolymerizing agent BPR0L075, suggesting a positive correlations between the amounts of intracellular survivin, tubulin polymers and levels of drug sensitivity in KB and KB-*L30* cells. In our study, survivin stabilizes tubulin polymers in both KB and KB-*L30* cells. In contrast, anti-mitotic compound BPR0L075 induces de-polymerization of the microtubule networks. Taken together, over-expression of survivin in response to the BPR0L075 reflects one of the possible cell survival mechanisms by stabilizing microtubule.

In addition to dynamic formation of the microtubule networks, survivin is also involved in the process of both caspase-dependent and -independent apoptosis (Liu et al., 2004; Shankar et al., 2001). It has been demonstrated that survivin enhances radiation resistance in primary human glioblastoma cells via a caspase-independent mechanism (Chakravarti et al., 2004). Our previous study revealed that BPR0L075 induced activation of caspase-3 and DNA fragmentation in KB cells (Kuo et al., 2004a). Our current study shows that down-regulation of survivin did not induce caspase-3 activity in the same cell line. However, increased DNA fragmentation was observed in the combination treatments when compared to BPR0L075 monotherapy. During one form of caspase-independent apoptosis, apoptosis-inducing factor (AIF) translocates from the mitochondria to the

nucleus (Bajt et al., 2006; Hisatomi et al., 2001; Ye et al., 2002). AIF is a flavoprotein that is normally confined to the mitochondrial intermembrane space, but induces chromatin condensation and fragmentation of DNA into high molecular weight forms of >50 kb when it translocates to nuclei (Lu et al., 2003; Wang et al., 2002). Structure-based mutagenesis of AIF revealed that replacing positively charged residues at the protein surface with alanines caused AIF to be defective in DNA-binding (Cande et al., 2002). It has been suggested that survivin interferes with the translocation of AIF. In fact, translocation of AIF from the mitochondria to the nucleus was shown in YUSAC2 melanoma cells treated with a cell-permeable dominant-negative survivin protein (Yan et al., 2006). Translocation of AIF from the cytoplasm to the nucleus following the down-regulation of survivin indicates that survivin inhibits caspase-independent apoptosis in KB cells. Thus, both caspase-dependent and caspase-independent DNA fragmentation could be initiated during survivin-targeted BPR0L075 combination therapy.

Drug resistance is a common problem in the management of neoplastic diseases. The effectiveness of a single anti-cancer agent is limited by the fact that “anti-drug” mechanisms can be introduced by cancer cells during cell evolution or re-arrangement of tumor micro-environment. In clinical situations, rather than treating patients with a single anti-cancer agent, combination therapies are preferred. BPR0L075 is a tubulin-targeting compound capable of inducing cytotoxic effect among various MDR-positive/-negative cancers both *in-vitro* and *in-vivo*. Here, we propose a novel survivin-involved microtubule-dependent and caspase-independent mechanism which interferes with the BPR0L075-drug sensitivity in human oral carcinoma cells. Since survivin plays an

important role in BPR0L075 sensitivity, the use of survivin-targeted agents such as oxaliplatin and SPC3042 (Santaris Pharma) in BPR0L075-combinational therapy maybe of clinical benefit (Fujie et al., 2005; Hansen et al., 2008). Also, unlike other potential therapeutic targets, survivin is expressed in few adult tissues. Thus, survivin-specific BPR0L075 combination therapy is likely to produce few adverse effects.

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Figure Legends

Fig. 1 – BPR0L075 induces over-expression of survivin in human KB and HONE-1 cancer cells. (**A**) Cells were treated with 8 nM of L075 for 6 h, 12 h and 24 h. Cell lysate were extracted and proteins were resolved by SDS-PAGE. Western blot analysis was performed as described in Materials and Methods. (**B**) KB cells were treated with 8 nM of L075 for 24 h and total RNA was extracted. Level of survivin mRNA was determined by RT-PCR. GAPDH was included for the use of semi-quantization. (**C**) KB cells were pre-treated with either 1 μg/ml of actinomycin-D or 56 ng/ml of geldanamycin for 6 h, and follwed by incubation with 8 nM of L075 for 24 h. Cell lysate were extracted and protein were resolved by SDS-PAGE. Western blot analysis was performed as described in Materials and Methods. (**D**) KB cells were cultured on chamber-slides, treated with various compounds for 24 h and analyzed by phase-contrast microscopy.

Fig. 2 – Down-regulation of survivin in KB cells by siRNA. (**A**) Cells were seeded on 8-well chamber slides overnight and transfected with siR-C (scramble control) or siR-S (survivin specific) for 48 h. Cells were labeled with FITC-coupled anti-human survivin antibody and counter-stained with DAPI nucleus stain. Slides were analyzed with fluorescent microscopy. (**B**) Cells were seeded on 24-well plates overnight and transfected with siR-C or siR-S for 36 h. Total RNA were extracted by Trizol reagent. RT-PCR was performed and PCR products were resolved by DNA electrophoresis.

Fig. 3 – Down-regulation of survivin increases sensitivity to BPR0L075 in KB cells. KB cells were seeded on 96-well plates overnight. Cells were pre-transfected with siR-C (scramble control) or siR-S (survivin specific) for 24 h and incubated with various concentrations of BPR0L075 for 48 h. Cells were analyzed by (**A**) phase-contrast microscopy and (**B**) MTT cell viability assay. Mean difference in cell viability between treatments at same dose of BPR0L075 was labeled on the graph.

Fig. 4 – Down-regulation of survivin with co-incubation of BPR0L075 cannot enhance activity of caspase-3 in KB cells. (**A**) Cells were seeded on 8-well chamber slides

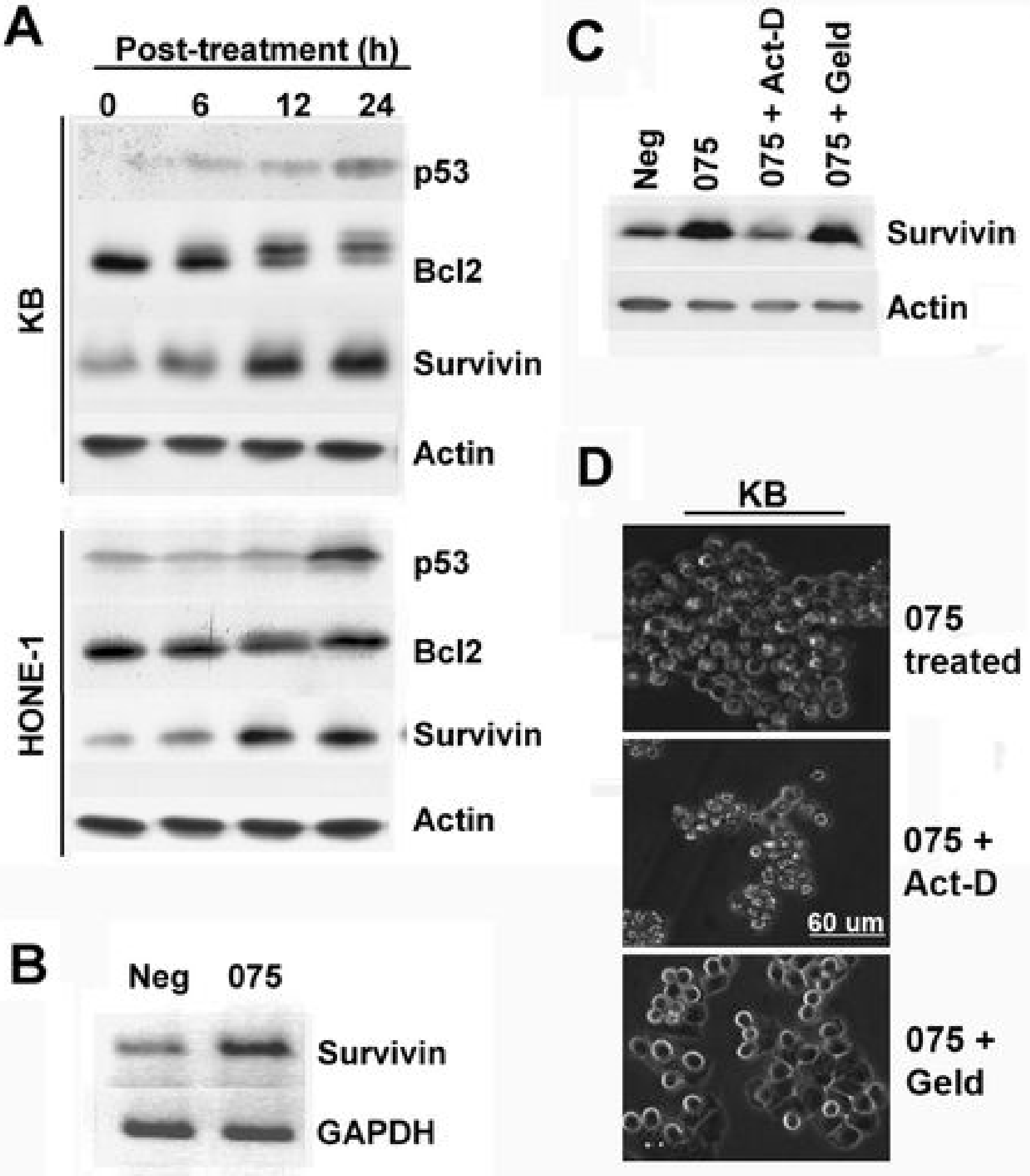
overnight. Cells were pre-transfected with siR-C (scramble control) or siR-S (survivin specific) for 24 h and co-incubated with various concentrations of BPR0L075. MagicRed™-DEVD real-time caspase-3/-7 activity kit (Immunochemistry Technologies LLC) was used. Activated-caspase-3/-7 was stained red and nucleus was counter-stained blue by Hoechst 33342. White arrows indicate cells with nucleus degradation. (**B**) Quantitative measurement of caspase-3/-7 activity. Cells were seeded on 96-well plate and transfected with siR-C or siR-S for 24 h. siRNA-treated cells were incubated with 4 nM of L075 for various times. Caspase-3/-7 activity was analyzed by the use of MagicRed™-DEVD real-time caspase-3/-7 activity kit with a 96-well plate-reader. (**C**) Analysis of DNA fragmentation by TUNEL assay. Cells were pre-transfected with siR-C or siR-S for 24 h and co-incubated with 4 nM of L075 for 48 h. DNA fragmentations were analyzed by the use of *In-Situ* Cell Death Detection kit. Nucleus with DNA fragmentation was stained red. (**D**) Immunofluorescence staining of the AIF molecule. Cells were treated with siR-C, siR-S and L075 for 72 h. AIF was detected by the use of anti-AIF antibody and FITC-conjugated anti-rabbit IgG antibody.

Fig. 5 – Donw-regulation of survivin changes the dynamic of microtubule networks (**A**) KB cells were transfected with siR-C (scramble control) and siR-S (survivin specific) for 48 h. Supernatant and pellet fractions of total cell lysate of un-treated cells were extracted. Proteins were resolved by SDS-PAGE and analyzed by western blotting. Anti-actin was used as internal control. (**B**) KB cells were seeded on 8-well chamber slides. Cells were pre-transfected with siR-C (control) or siR-S for 24 h and co-incubated with 4 nM of BPR0L075 for 24 h. Cells were fixed and labeled with mouse anti-human α-tubulin antibody and FITC-coupled anti-mouse secondary antibody. Slides were analyzed by fluorescent microscopy. (**C**) Total cell lysate of both KB and KB-*L30* were extracted. Proteins were resolved by SDS-PAGE and analyzed by western blotting. Anti-actin was used as internal control. Relative band intensities were labeled (**D**) Both KB and KB-*L30* cells were seeded and cultured in BPR0L075-free RPMI for 72 h. Supernatant and pellet fractions of total cell lysate of un-treated cells were extracted. Proteins were resolved by SDS-PAGE and analyzed by western blotting. Anti-actin was used as internal control. Relative band intensities were labeled. (**E**) KB-*L30* cells were transfected with siR-C or

siR-S for 36 h. Total RNA was extracted by Trizol reagent. RT-PCR was performed and PCR products were resolved by DNA electrophoresis. (**F**) KB-*L30* cells were transfected with siR-C and siR-S for 48 h. Supernatant and pellet fractions of total cell lysate of un-treated cells were extracted. Proteins were resolved by SDS-PAGE and analyzed by western blotting. Anti-actin was used as internal control.

Fig. 6 - Down-regulation of survivin increases sensitivity to BPR0L075 in KB-*L30* cells. Cells were seeded on 96-well plates overnight. Cells were pre-transfected with siR-C (scramble control) or siR-S (survivin specific) for 24 h and incubated with various concentrations of BPR0L075 for 48 h. Cells were analyzed by (**A**) phase-contrast microscopy and (**B**) MTT cell viability assay. Mean difference in cell viability between treatments at same dose of BPR0L075 was labeled on the graph.

Supplemental data – BPR0L075 induces overexpression of survivin via p53-independent mechanism. KB cells were pre-incubated with 30 μM of a p53-inhibitor, pifithrin-α, for 12 h. Cells were subsequently co-incubated with 8nM of BPR0L075 for 24 h. Cell lysate were extracted and proteins were resolved with SDS-PAGE. Expressions of various proteins were analyzed by western blotting. Relative band intensities were labeled.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Post-treatment (h)
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6
12
24
KB
p53
Bcl2
Survivin
Actin
HONE-1
p53
Bcl2
Survivin
Actin
B
Neg
075
Survivin
GAPDH
C
Neg
075
075 + Act-D
075 + Geld
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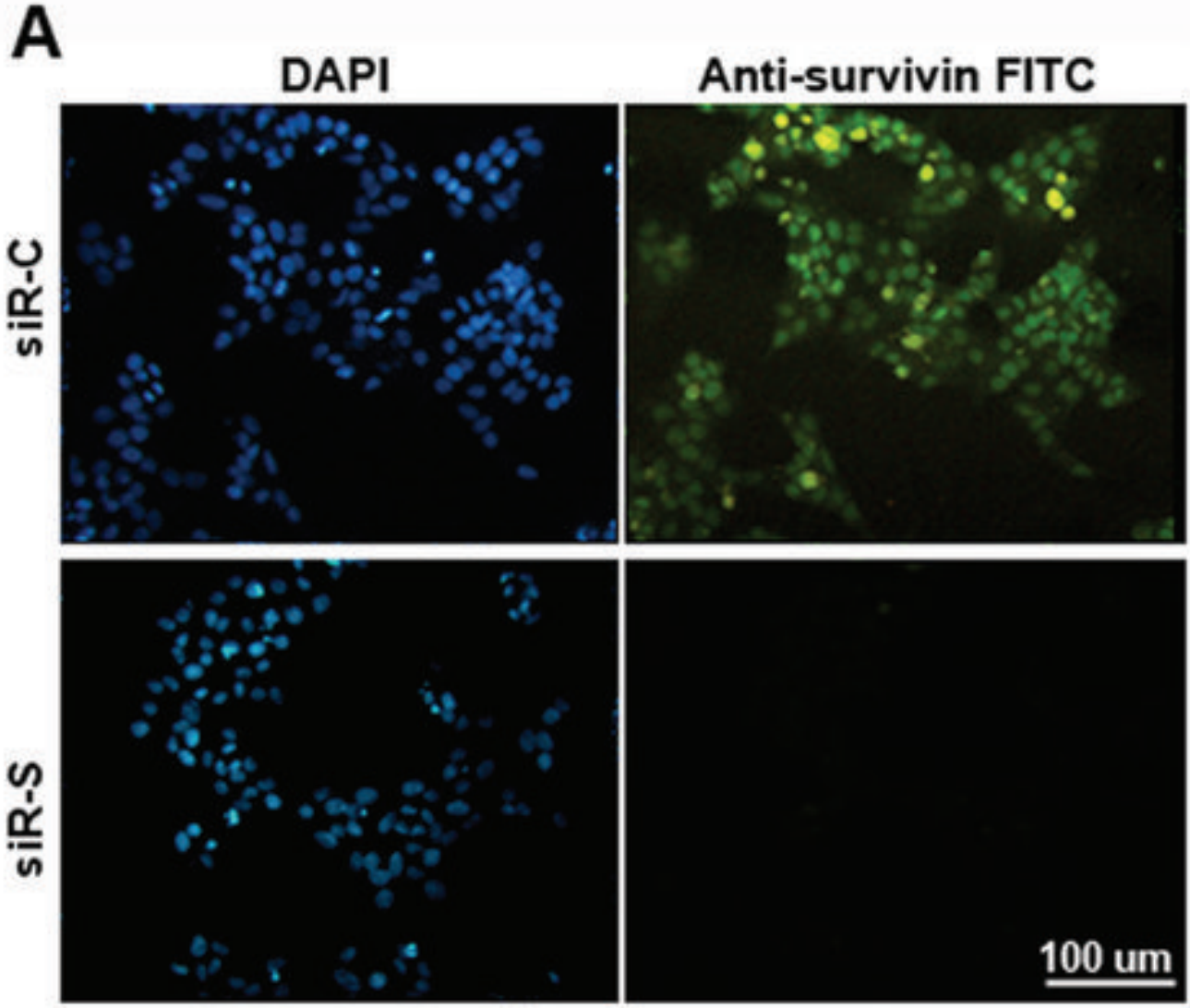
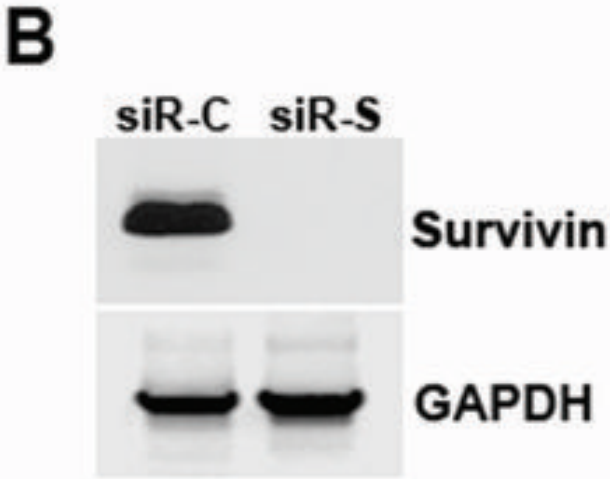
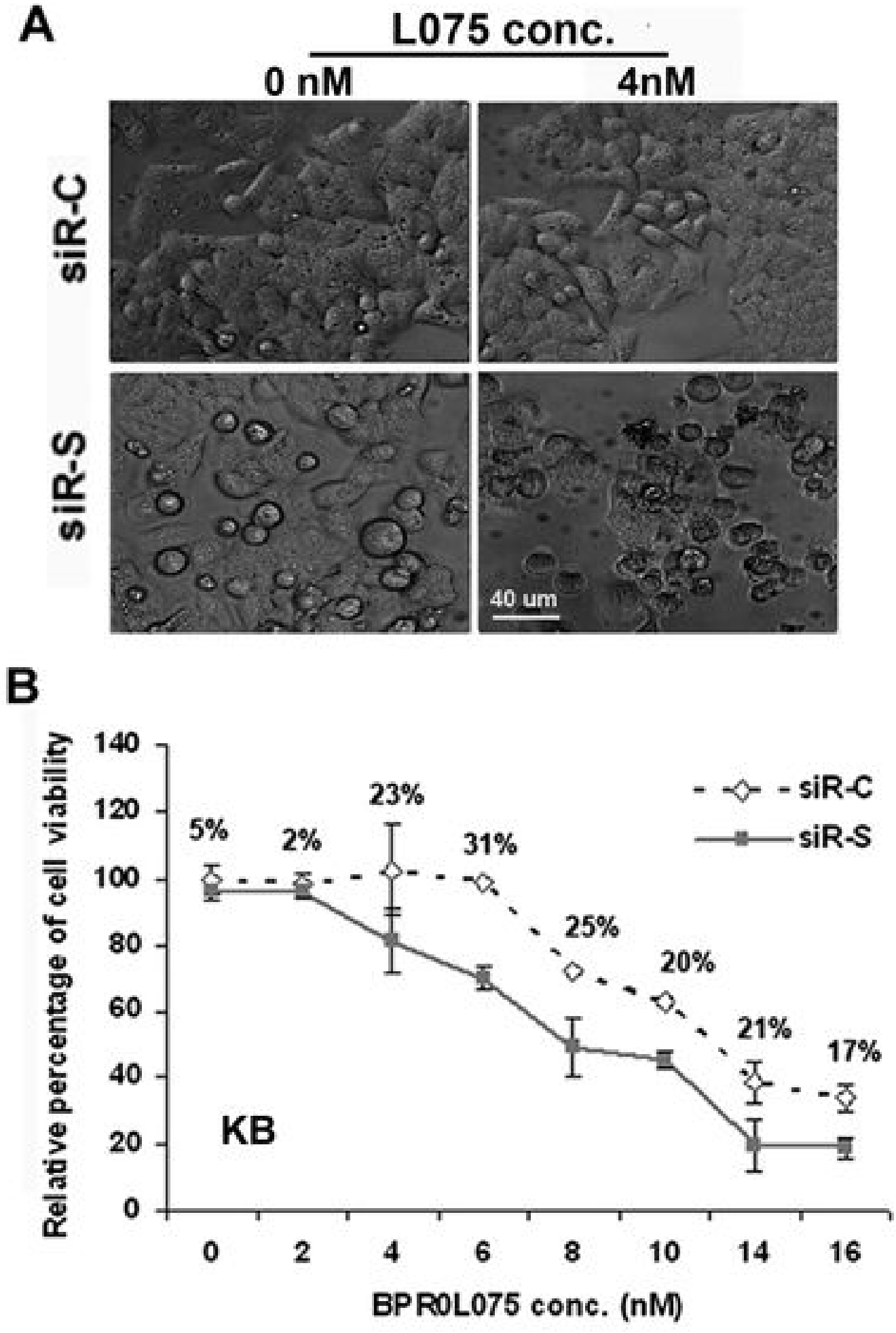
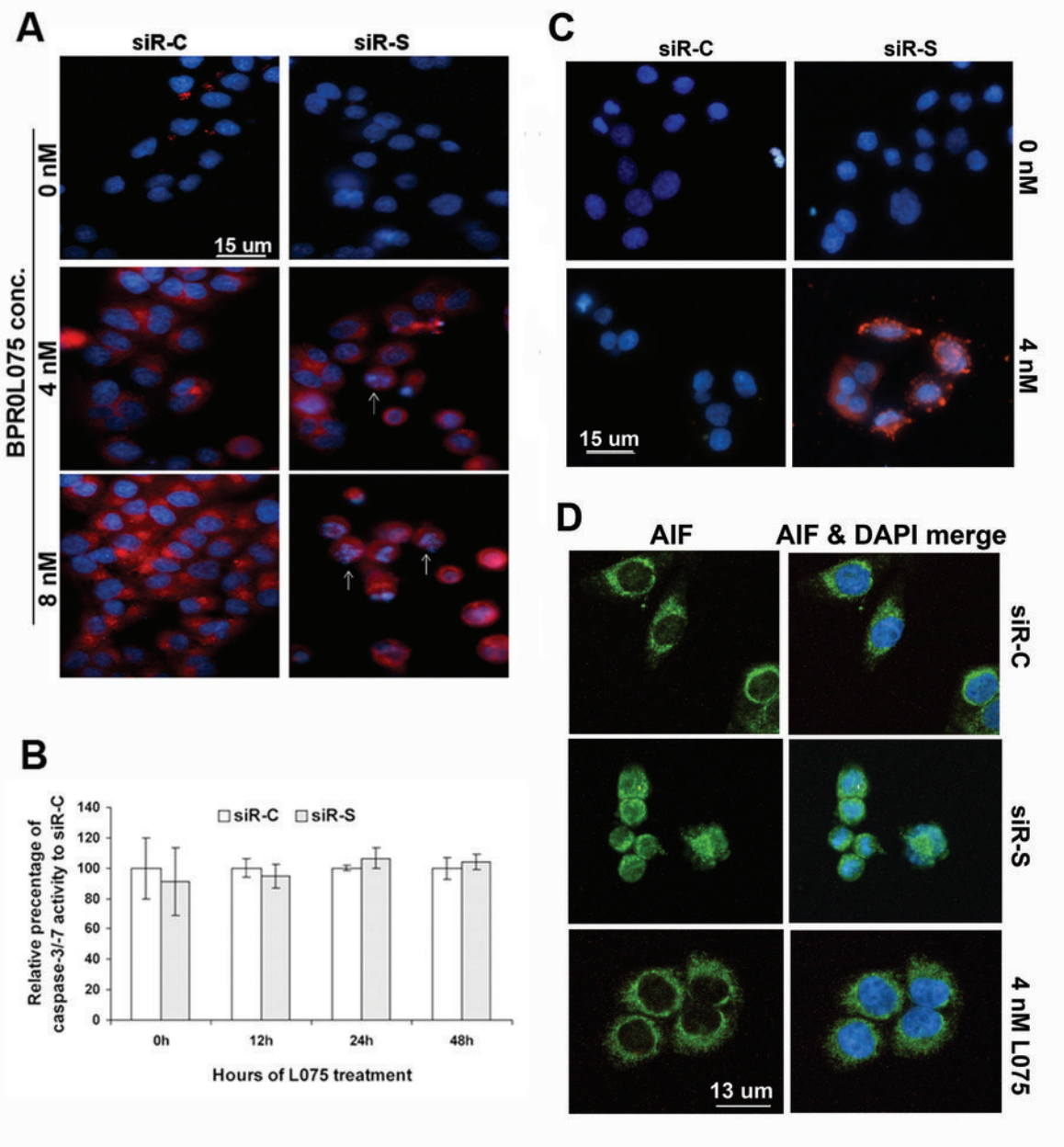
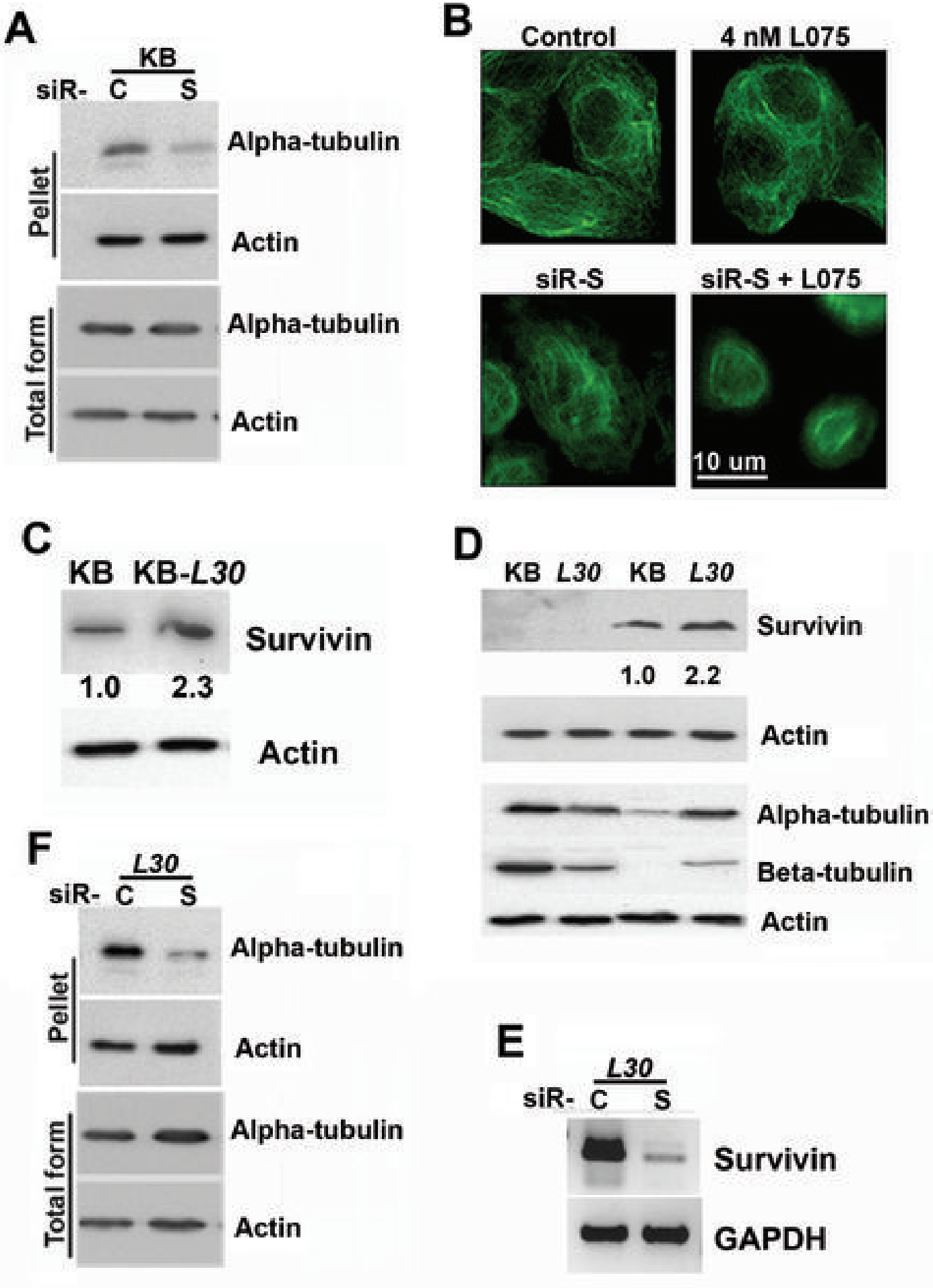
Actin
D
KB
075 treated
075 + Act-D
60 um
075 + Geld


A
DAPI
Anti-survivin FITC
siR-C
siR-S
100 um


B
siR-C
siR-S
Survivin
GAPDH


A
L075 conc.
0 nM
4nM
siR-C
siR-S
40 um
B
Relative percentage of cell viability
140
120
100
80
60
40
20
0
siR-C
siR-S
5%
2%
23%
31%
25%
20%
21%
17%
KB
0
2
4
6
8
10
14
16
BPR0L075 conc. (nM)


A
siR-C
siR-S
0 nM
4 nM
8 nM
BPR0L075 conc.
15 um
C
siR-C
siR-S
0 nM
4 nM
15 um
B
siR-C
siR-S
Relative precentage of caspase-3/-7 activity to siR-C
140
120
100
80
60
40
20
0
0h
12h
24h
48h
Hours of L075 treatment
D
AIF
AIF & DAPI merge
siR-C
siR-S
4 nM L075
13 um


A
KB
siR- C S
Pellet
Alpha-tubulin
Actin
Total form
Alpha-tubulin
Actin
B
Control
4 nM L075
siR-S
siR-S + L075
10 um
C
KB KB-L30
Survivin
1.0 2.3
Actin
D
KB L30 KB L30
Survivin
1.0 2.2
Actin
Alpha-tubulin
Beta-tubulin
Actin
F
L30
siR- C S
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Actin
Total form
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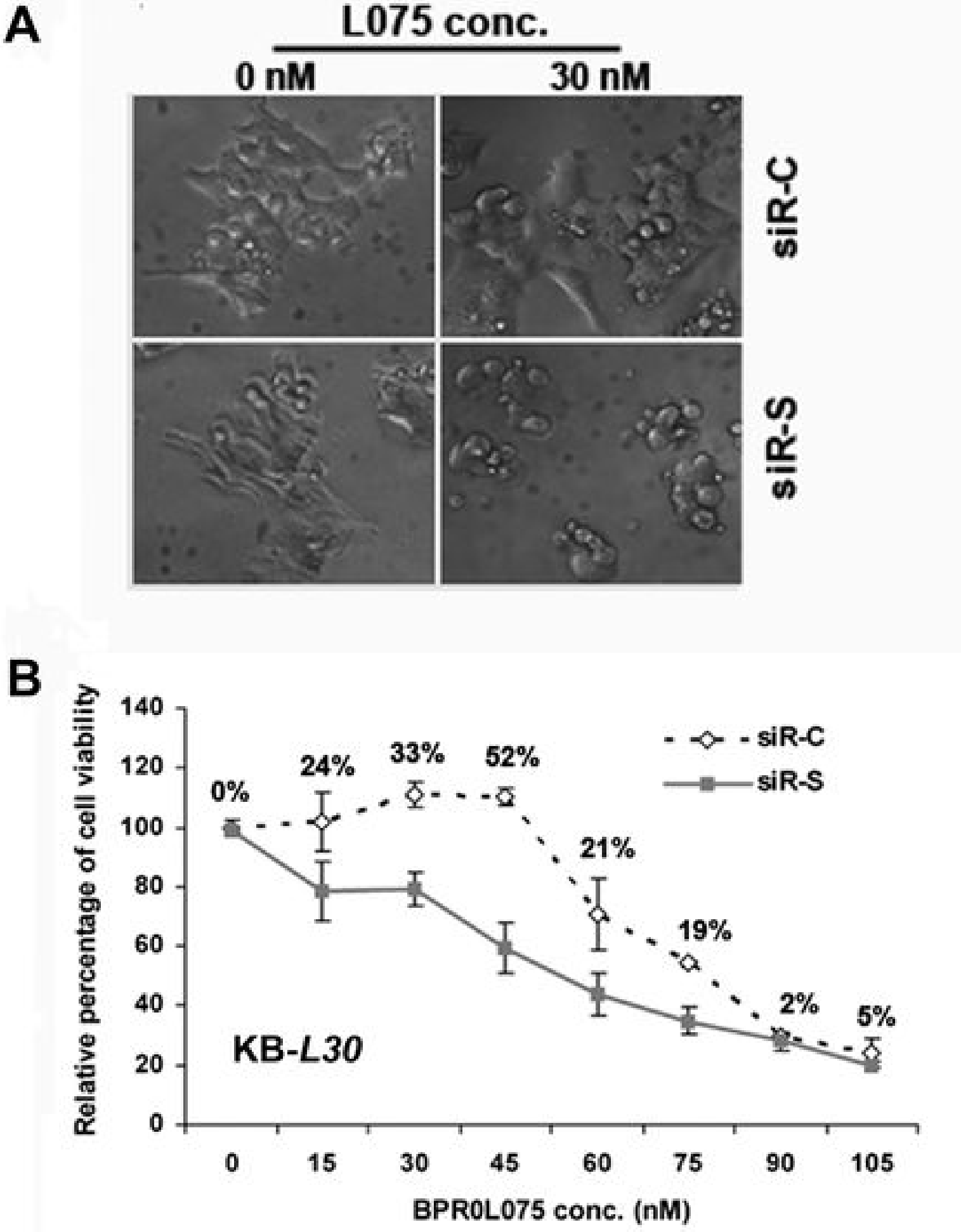
Actin
E
L30
siR- C S
Survivin
GAPDH


A
L075 conc.
0 nM
30 nM
siR-C
siR-S
B
Relative percentage of cell viability
140
120
100
80
60
40
20
0
siR-C
siR-S
0%
24%
33%
52%
21%
19%
2%
5%
KB-L30
0
15
30
45
60
75
90
105
BPR0L075 conc. (nM)