Title: Adenovirus-mediated transfer of siRNA against survivin enhances the radiosensitivity of human non-small cell lung cancer cells

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Running title: Survivin inhibition and radiation in NSCLC cells

ABSTRACT

Expression of survivin has been reported to be correlated with shorter survival in patients with non-small cell lung cancer (NSCLC), and overexpression of survivin may lead to radioresistance in various human cancers. In this study, we inhibited survivin expression by using an adenoviral vector (AdsiSurvivin)-mediated RNAi to elucidate the combined effect of survivin-targeting gene therapy and radiotherapy on the NSCLC cells. Our data revealed that AdsiSurvivin exerted survivin gene silencing, induced apoptosis, and significantly attenuated the growth potential in NSCLC cells within 72 hours after infection. The combined treatment modalities with AdsiSurvivin infection and radiation were significantly more potent on cell-growth inhibition than monotherapy. In H1650, H460, A549, and H1975 human NSCLC cells, the survival ratios of AdsiSurvivin-treated groups at m.o.i. of 25 and 50 were significantly lower than those of control groups at varying radiation dose (0~8 Gy; three-way ANOVA, $p < 0.05$). The cytotoxicity of combined AdsiSurvivin infection and irradiation increased in a dose-dependent manner in both the virus and the irradiation treatment. Knockdown of the survivin gene expression appears to be a promising treatment strategy for NSCLC. Our data warrants the need for further effort to develop survivin-targeted radiosensitizer for lung cancer treatment.

INTRODUCTION

Survivin is a member of the inhibitor of apoptosis family (IAP) that acts as a suppressor of apoptosis and plays a critical role in tumor initiation, progression, and drug resistance.[1] High levels of survivin have been found in most human cancers, including cancers of the lung, colon, pancreas, prostate, breast, and stomach.[2-5] Expression of survivin has been reported to be correlated with shorter survival in patients with non-small cell lung cancer (NSCLC).[3] Survivin is expressed in the G_2/M phase in a cell cycle-regulated manner, and its interaction with microtubules of mitotic spindle at the beginning of mitosis is essential for its antiapoptotic function. Disruption of the survivin-microtubules interaction results in the loss of survivin's anti-apoptotic function and increases the caspase-3 activity.[6, 7] Overexpression of survivin has oncogenic potential because it may overcome the G_2/M-phase checkpoint to compel the cell cycle to progress through mitosis.[6] In addition, survivin overexpression has been shown to result in accelerated S phase shift, resist G_1 arrest, activate cyclin-dependent kinase 2/cyclin E complex and thereby result in Rb phosphorylation.[8] Moreover, the expression of survivin could be significantly upregulated by a sublethal dose of irradiation, which might cause radioresistance.[9] Lu *et al.* reported that overexpression of survivin in human embryonic kidney cells (293 cells) prevented irradiation-induced apoptosis and increased cell viability after irradiation.[10] Increasing evidences indicated that the expression of survivin correlated with radioresistance in various cancers, thereby implying that survivin may be a potential target for radiosensitization during cancer treatment.[11-14]

Radiotherapy is commonly used in NSCLC treatment for either curative or palliative purposes. However, majority of patients are most likely to suffer regional failure as a part of their disease recurrence process.[15, 16] Recent researches to improve the clinical

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outcome in such patients include altered irradiation fraction schedule and the introduction of chemotherapy, biotherapy, immunotherapy, virotherapy, or gene therapy on a concurrent or adjuvant basis.[17-22] Survivin gene expression has been identified in a vast majority of NSCLC.[3] Additionally, Tamm *et al.* reported that among all the human tumor cells screened, lung cancer cells expressed the highest levels of survivin.[23] Since survivin overexpression may lead to resistance to radiotherapy by inhibiting apoptosis and enhancing cell viability, knockdown of the survivin gene expression by RNA interference (RNAi) should be a promising approach to ameliorate the efficacy of radiotherapy during NSCLC treatment. In the present study, we inhibited survivin expression by using RNAi to elucidate the combined effect of survivin-targeting gene therapy and radiotherapy on NSCLC cells. To achieve sufficiently high level of gene suppression in adequately large numbers of target cells, we constructed an adenoviral vector expressing short hairpin RNA (shRNA) with fold-back stem-loop structure.[24] We demonstrate here that the impact of adenovirus-mediated transfer of small interfering RNA (siRNA) targeting survivin on human NSCLC cells and the potential synergy with irradiation in cell killing.

MATERIALS AND METHODS

Cell Lines

The NSCLC cell lines A549 (ATCC CCL-185), H460 (ATCC HTB-177), H1650 (ATCC CRL-5883), and H1975 (ATCC CRL-5908) were purchased from American Type Culture Collection (ATCC; Manassas, Virginia, United States). All were cultured in DMEM complete media containing 10% fetal bovine serum (FBS).

Development of Stable 293 Cell Lines with High Survivin Expression

PcDNA3-Survivin plasmid was constructed by subcloning the fragment of the entire survivin encoding gene from plasmid pORF5-hSurvivin (Invivogene, San Diego, CA) into the pcDNA3-flag at *Bam*HI site. The vector of pcDNA3-flag was the same as previously described.[25] Human embryonic kidney cells (E1-transformed; 293 cells) were subsequently transfected with pcDNA3-Survivin; transfection was conducted by using a liposome-mediated transfection technique with lipofectamine 2000 reagent (Invitrogen, Grand Island, NY) and in accordance with the manufacturer's protocol. The transfected cells were replated at a 1:20 dilution in DMEM complete media containing 10% FBS and 1000 μg/ml neomycin (G418; Gibco-BRL Life Technologies Inc, Rockville, MD), and cultured for 14 days with replacement of the selective media every 3–4 days. The surviving transfectants that were localized in distinct "islands" were then maintained with the growth medium. Then, real-time PCR was used to screen the survivin expression of individual clones. The stable cell lines with high survivin expression, namely, 293S cells were used for further experiments.

Adenoviral Vectors

A 19nt survivin-targeting sequence of oligonucleotides was designed by Oligoengine, Inc. (Seattle, WA) and selected as the template: TTCGTCCGGTTGCGCTTTC. By BLAST analysis, it was confirmed that the template shared no homology with other coding sequences in human beings. A ring sequence of 6 base pairs (ATCGAT) existed between the sense and antisense strands. Plasmid pUC-U6, which was kindly gifted by Dr. Tang (College of Agriculture and Natural Resources, National Chung Hsing University, Taiwan), containing the U6 promoter was inserted between the *Xho*I and the *Kpn*I sites of pUC-18 (Fermentas, Burlington, Ontario, Canada). The siRNA-expressing cassette was subcloned into pUC-U6 between the *Kpn*I and the *Xba*I sites. Then, the *Xho*I-*Hin*dIII fragment containing the tandem arranged U6 promoter-driven siRNA expression cassette was double enzyme-cut by restriction endonuclease and ligated to the pAdTrack vector.[26] The resultant plasmid was linearized by digesting with restriction endonuclease, and subsequently cotransformed into *E. coli* BJ5183 cells with an adenoviral backbone plasmid, pAdEasy-1.[26] Recombinants were selected for kanamycin resistance, and recombination was confirmed by restriction endonuclease analysis. The linearized recombinant plasmid was then transduced into the 293 cells. A recombinant adenovirus expressing shRNA against survivin (AdsiSurvivin) was generated. The recombinant adenovirus AdCtrl, which carries a green fluorescence protein (GFP) gene regulated by the cytomegalovirus (CMV) promoter, was constructed with pAdTrack and pAdEasy-1, and used as the control in these experiments.[27, 28] These adenovirus vectors were amplified by using the 293S cells, and titered by using both Adeno-X™ Rapid Titer Kit (BD Biosciences, San Jose, CA) and 293S cells.

Western Blot

Cells (1×10^6) were plated onto 10-cm dishes and incubated for 16 hours at 37°C prior to infection. They were subsequently mock-infected or infected with either AdCtrl or AdsiSurvivin, and incubated for 24~96 hours. Total cell lysates from the infected cells were extracted with lysis buffer (M-PER Mammalian Protein Extraction Reagent; Thermo Fisher Scientific Inc, Rockford, IL). The primary antibodies used were mouse anti-survivin monoclonal antibody (2802; Cell Signaling Technology, Boston, MA), goat anti-γ tubulin polyclonal antibody (sc-7396; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti- poly-(ADP-ribose) polymerase (PARP) polyclonal antibody (9542; Cell Signaling Technology). Horseradish peroxidase conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat antibody was used as the secondary antibody (Santa Cruz Biotechnology). Chemiluminescence detection was carried out by using ECL Plus™ (GE Healthcare, Piscataway, NJ) and executed according to the manufacturer's instructions.

Cellular Proliferation

Cells (1×10^5/well) were plated in 12-well culture plates and incubated for 16 hours at 37°C prior to infection. They were then mock-infected or infected with either AdCtrl or AdsiSurvivin at varying doses (multiplicity of infection [m.o.i.] of 10, 25, 50, 100, and 200). On day 1, 2, 3, 4, or 5 after the infection, the remaining cells in the wells were collected by trypsinization and suspended in PBS. An equal volume of 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO) was added to the cell suspension. Viable cells were subsequently determined by direct microscopic counting with trypan blue exclusion. All counts were done on triplicate samples.

Cell Cycle Analysis

Cells (1×10^6) were plated onto 10-cm dishes, incubated for 16 hours at 37°C, then mock-infected or infected with either AdCtrl or AdsiSurvivin (m.o.i. of 50 each). Five days after the infection, the cells were collected by trypsinization, washed twice with 0.1% glucose in PBS without Mg^{++} or Ca^{++} (sample buffer), and then fixed in ice-cold 70% ethanol overnight at 4°C. Subsequently, the cells were centrifuged for 5 min at 1,500 rpm. After decanting the 70% ethanol without disturbing the pellet, the cells were stained with 50 µg/ml propidium iodide (PI), 100 U/ml RNase A in a sample buffer. The samples were subsequently analyzed by two-dimensional flow cytometry (Epics® Ultra™, Beckman Coulter, Fullerton, CA) to detect PI. The cell cycles were determined by EXPO32 ADC analysis software.

Irradiation

Cells (1×10^5/well) were plated onto 6-well culture plates and incubated for 16 hours at 37°C prior to infection. Subsequently, they were mock-infected or infected with either AdCtrl or AdsiSurvivin at m.o.i. of 10, 25, or 50. Forty-eight hours after the infection, the cells were irradiated (0, 2, 4, or 8 Gy in a single fraction) with a 6-MeV electron beam generated by a linear accelerator (Clinac 21EX; Varian, Palo Alto, CA) at a dose rate of 300 cGy/min. On day 3 after irradiation, triplet cultures for each combination treatment were counted for viable cells.

Statistical Analysis

The numeric variables are presented as mean and standard deviation (SD). All statistical analyses were performed by using Stata version 10.1 statistical software. (StataCorp. 2008. Stata Statistical Software: Release 10.1. College Station, TX: StataCorp LP). Statistical comparisons were made by using the two-sided *t-test*. Three-way analysis of variance (ANOVA) was used to compare the mean (survival ratios) and the SD at the three MOI levels (10, 25, and 50) and varying radiation doses with the treatment of AdsiSurvivin or AdCtrl. Two-way ANOVA was used to analyze the data acquired over time from both the treatment groups. The first factor was the treatment (AdsiSurvivin *vs.* AdCtrl groups), and the second factor was time (day 3–day 5). Tukey's HSD test was used for pair-wise comparisons. A *p* value of 0.05 or less indicated significant statistical difference.

RESULTS

Survivin expression after treatment with AdsiSurvivin

We generated stable 293S cell lines containing CMV promoter-controlled survivin expression. As illustrated in Fig. 1, the expression of survivin in 293S cells was strongly detected by using immunoblotting analysis; further, the expression was markedly higher than that observed in the 293 cells. We used the 293S cells to amplify and titer the adenoviral vector expressing shRNA against survivin. By using the AdEasy system, we generated an adenoviral vector, AdsiSurvivin, which carries both shRNA expression cassette for silencing of survivin gene and the GFP gene under control of the U6 promoter and CMV promoter, respectively. AdCtrl, which is similar to AdsiSurvivin except that it lacks the U6 promoter-driven shRNA expression cassette, was used as the control vector.[26] To evaluate the effect of AdsiSurvivin infection on survivin expression in NSCLC cells, we infected the H460, A549, H1650, and H1975 NSCLC cell lines with AdsiSurvivin or AdCtrl at m.o.i. of 100 or conducted mock-infection. After a 4-day period, survivin expression in the AdsiSurvivin-treated cells was markedly decreased than those in the AdCtrl-treated or mock-infected cells (Fig. 2).

Effects of AdsiSurvivin treatment on cell growth

In order to find an optimal dose for further studies, we treated the H460 and A549 cells with varying doses of AdsiSurvivin or AdCtrl (m.o.i. of 0, 10, 25, 50, 100 or 200). At 5 days, the numbers of viable cells in the AdsiSurvivin-infected group were significantly lower than those in the AdCtrl-infected groups even at m.o.i. of 10 in both the cell lines (1,025,000 ± 352,232 *vs.* 1,350,000 ± 178,841 and 575,000 ± 25,000 *vs.* 1,000,000 ± 25,000, for H460 and A549 cell lines, respectively; $p < 0.0001$ in both comparisons), and

the cytotoxicity of AdsiSurvivin increased in a dose-dependent manner (Fig. 3). The numbers of viable cells in the AdCtrl-infected group were lower than those of the mock-infected cells for both the cell lines, which indicates the cytotoxicity from the expressed viral proteins and the overexpression of GFP. The tested cell lines had different sensitivities to this virus-related toxicity. At m.o.i. of 50, the ratios of viable cell numbers in the AdCtrl-treated group to those in the mock-infected group (survival ratio) were not less than 50% (50.0% ± 3.09% and 64.2% ± 1.89%, respectively) and the ratios of viable cell numbers in the AdsiSurvivin-treated group to those in the AdCtrl-infected group were not more than 20% (12.9% ± 0.86% and 15.7% ± 1.09%, respectively) in H460 and A549 cells. Therefore, we chose the dose of m.o.i. as 50 to examine the effect of AdsiSurvivin infection on the cell growth in all the four NSCLC cell lines.

H460, A549, H1650, and H1975 NSCLC cells were subsequently treated with mock-infection or infection with AdCtrl or AdsiSurvivin at m.o.i. of 50. The numbers of viable cells were counted from day 1 to day 5 after the treatments. As shown in Fig. 4, the growth of AdsiSurvivin-infected cells was substantively suppressed by survivin gene silencing. Day 3 onwards, the numbers of viable cells in the AdsiSurvivin-infected groups were significantly lower than those in the AdCtrl-infected groups in the H460, A549, and H1650 cell lines ($p = 0.0022$, $p = 0.00005$, and $p = 0.00056$, respectively). The H1975 cells seemed more vulnerable to AdsiSurvivin treatment. The number of viable AdsiSurvivin-infected H1975 cells was significantly less than that infected with AdCtrl since day 2 ($p = 0.0257$, Fig. 4D). The cytotoxicity of AdsiSurvivin was significantly more potent than that of AdCtrl from day 3 to day 5 in all the four cell lines (two-way ANOVA, $p < 0.05$ in four comparisons).

Apoptosis induced by AdsiSurvivin treatment

In order to assess the apoptosis induced by survivin inhibition, the H460 cells were treated with mock-infection, or either AdsiSurvivin or AdCtrl at m.o.i. of 50. On day 5 following the infection, cell cycle analysis revealed marked increase in the subG1 population of the AdsiSurvivin-treated cells (Fig. 5A). To confirm the presence of the apoptotic process, the H460 cells were also treated with either AdsiSurvivin or AdCtrl at m.o.i. of 50; we then examined the cleavage of PARP by Western blot analysis. Day 3 onwards, degradation of PARP was detected only in the AdsiSurvivin-treated cells, but not in the AdCtrl-infected cells (Fig. 5B). The above findings indicated that survivin inhibition by siRNA induced programmed cell death, which was accompanied by the increased protein cleavage for PARP.

Effects of concurrent treatments of the NSCLC cells with AdsiSurvivin and radiation

We then investigated the cytotoxicity due to the combined effects of radiation and survivin inhibition on the NSCLC cells. The H1650, H460, A549, and H1975 lung cancer cells were treated with AdsiSurvivin or AdCtrl at m.o.i. of 10, 25, or 50. The culture medium alone was used for mock infection. These cells were subsequently treated with radiation at 0, 2, 4, or 8 Gy 48 hours after infection. Triplet cultures for each combination treatment were counted for the detection of any viable cells on day 3 after the radiation, i.e., day 5 after infection. As shown in Fig. 6, increase in the radiation dose significantly decreased the survival ratio in a dose-dependent manner in the AdCtrl-infected groups at m.o.i. of 10, 25, and 50 in all the four cell lines (three-way ANOVA, $p < 0.05$ in all the 12 comparisons). In H460, A549, and H1975 cells, the survival ratios of AdsiSurvivin-

treated groups at m.o.i. of 10, 25, and 50 were significantly lower than those of the AdCtrl-treated groups at varying radiation doses (three-way ANOVA, $p < 0.05$ in all the nine comparisons; Figs. 6B, 6C, and 6D). At m.o.i. of 10, there were no significant differences in the survival ratios between AdsiSurvivin and AdCtrl-infected groups in the H1650 cells concurrently treated with radiation at varying doses (three-way ANOVA, $p = 0.3571$; Fig. 6A). While the viral dose increased to m.o.i. of 25 and 50, the survival ratios of the AdsiSurvivin-infected H1650 cells were significantly lower than those of the AdCtrl-infected cells concurrently treated with radiation at varying doses (three-way ANOVA, $p < 0.05$ in both comparisons; Fig. 6A). When combined with the treatment of AdsiSurvivin at high dose (m.o.i. of 50), irradiation-induced cell killing did not occur in a dose-dependent pattern in both the H1650 and A549 cells. The survival ratio was significantly higher in non-irradiated A549 cells than when treated with irradiation at 2 Gy (21.62% ± 4.35% *vs.* 16.22% ±1.35%, $p = 0.002$) in high dose AdsiSurvivin-infected groups; however, there were no significant differences between the 2-Gy, and 4-Gy, or 8-Gy radiation ($p > 0.05$ in both the comparisons, Fig. 6C). In H1650 cells treated with AdsiSurvivin at m.o.i. of 50, the survival ratio of the non-irradiated cells was not significantly different from those irradiated with 2 Gy or 4 Gy ($p > 0.05$ in both comparisons, Fig. 6A); however, at 8 Gy, there was a significant difference between the non-irradiated and irradiated H1650 cells (28.7% ± 0.86% *vs.* 13.27% ± 1.14%, $p = 0.000046$).

DISCUSSION

NSCLC is the leading cause of cancer-related deaths worldwide. Unfortunately, standard therapies remain inadequate for attaining disease control. The conventional chemotherapy regimens and radiotherapy are more of a hindrance due to their limited efficacy, significant toxicity, the development of resistance and high relapse rates; this warrants the need for newer therapeutic approaches. More recently, modest success has been achieved using novel agents to target signaling transduction pathways, growth factor receptors, oncogenes, and tumor-suppressor genes, which are known to become aberrant in lung cancer.[29] For example, the epidermal growth factor receptor tyrosine kinase inhibitors are a major advancement in lung cancer treatment.[30, 31] However, the eventual development of acquired resistance in the responders also hampers the clinical benefit of these agents during NSCLC treatment.[32] Therefore, further studies targeting multiple treatment modalities and/or the inhibition on multiple signaling pathways are required in our efforts to develop a novel therapeutic strategy to enhance anti-cancer efficacy and/or prevent drug resistances. Due to the high expression level in NSCLC and significant clinical association with survival outcome and treatment resistance, survivin has been widely evaluated as a potential therapeutic target for radiation sensitization in lung cancer. By using antisense oligonucleotides, several studies have shown the potential of survivin inhibition on sensitizing lung cancer cells to radiation.[10, 33] In order to achieve such a radiosensitization, we combined gene transfer by adenoviral vector and RNAi technology to improve the efficacies of both gene transduction and expression inhibition in the NSCLC cells. Uchida *et al.* used adenovirus-mediated transfer of siRNA against survivin to induce apoptosis and attenuate tumor cell growth in HeLa, U251, and MCF-7 cells.[34] However, our preliminary data revealed that replication of AdsiSurvivin resulted in

marked survivin gene silencing and cell death in both 293 and 293T cells (ATCC CRL-11268), which resulted in the failure of the virus yield (data not shown). Therefore, we generated 293S cells to overcome this toxicity and succeeded in constructing and amplifying AdsiSurvivin for further studies. However, the yield of AdsiSurvivin is only 10% of AdCtrl despite using 293S cells for viral amplification.

In the present study, our data revealed that AdsiSurvivin successfully exerted survivin gene silencing, induced apoptosis, and significantly attenuated the growth potential in NSCLC cells. These effects may set in early within 72 hours after infection. Furthermore, the effect of combined treatment modalities including AdsiSurvivin infection and radiation are significantly more potent on cell-growth inhibition than monotherapy involving either one of the above. This synergy was noted even at low virus dose (m.o.i. of 10) in most of the cells that were tested. The cytotoxicity of combined AdsiSurvivin infection and irradiation increased in a dose-dependent manner for both the treatment modalities. However, the increase of radiation dose during concurrent treatment with high dose (m.o.i. of 50) of AdsiSurvivin did not increase cell death in A549 and H1650 cells. It should be due to the superiority of AdsiSurvivin to irradiation in cytotoxicity at high viral dose.

We demonstrate that the adenovirus-mediated transfer of siRNA against survivin may enhance the radiosensitivity of human NSCLC cells. Due to this synergy of survivin gene knockdown and irradiation on cell death in NSCLC, this combined treatment modality has the potential to improve the anti-cancer efficacy and/or thus reduce radiation dose to attenuate collateral damage to normal tissue if applied clinically. Our data warrants the need for further effort to develop survivin-targeted radiosensitizer for NSCLC treatment.

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AUTHOR DISCLOSURE

All authors certify that there is no actual or potential conflict of interest in relation to this article.

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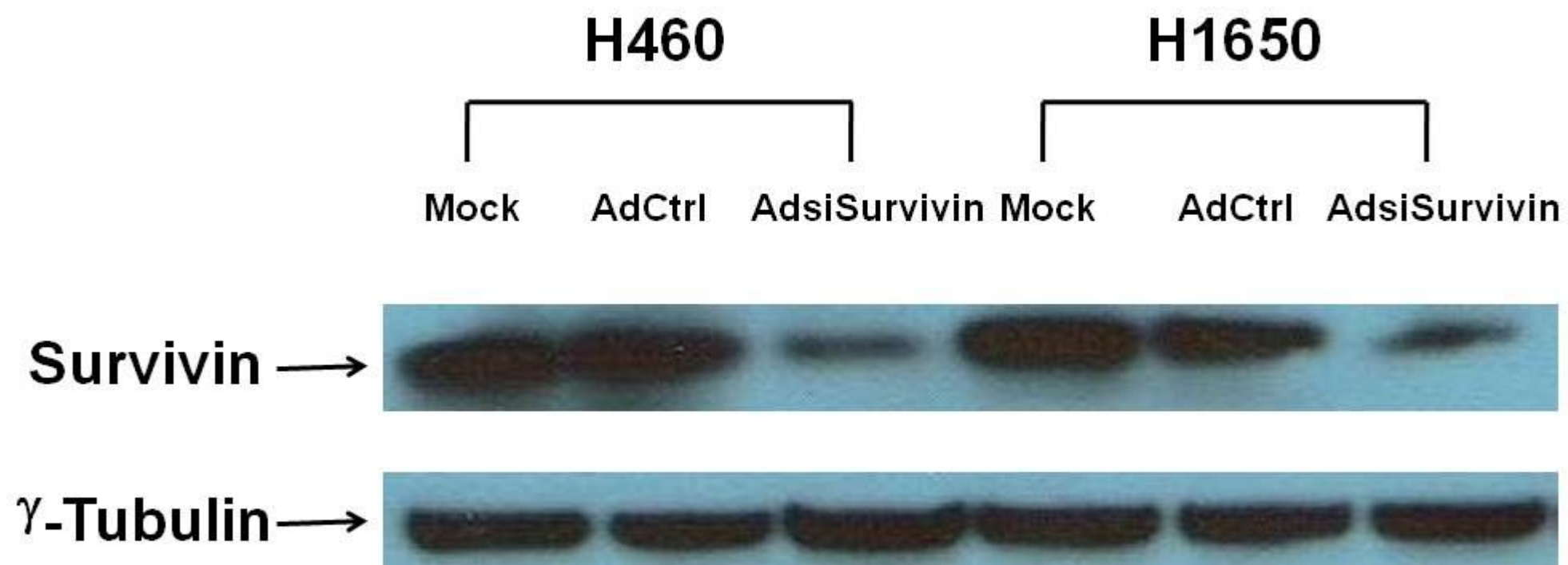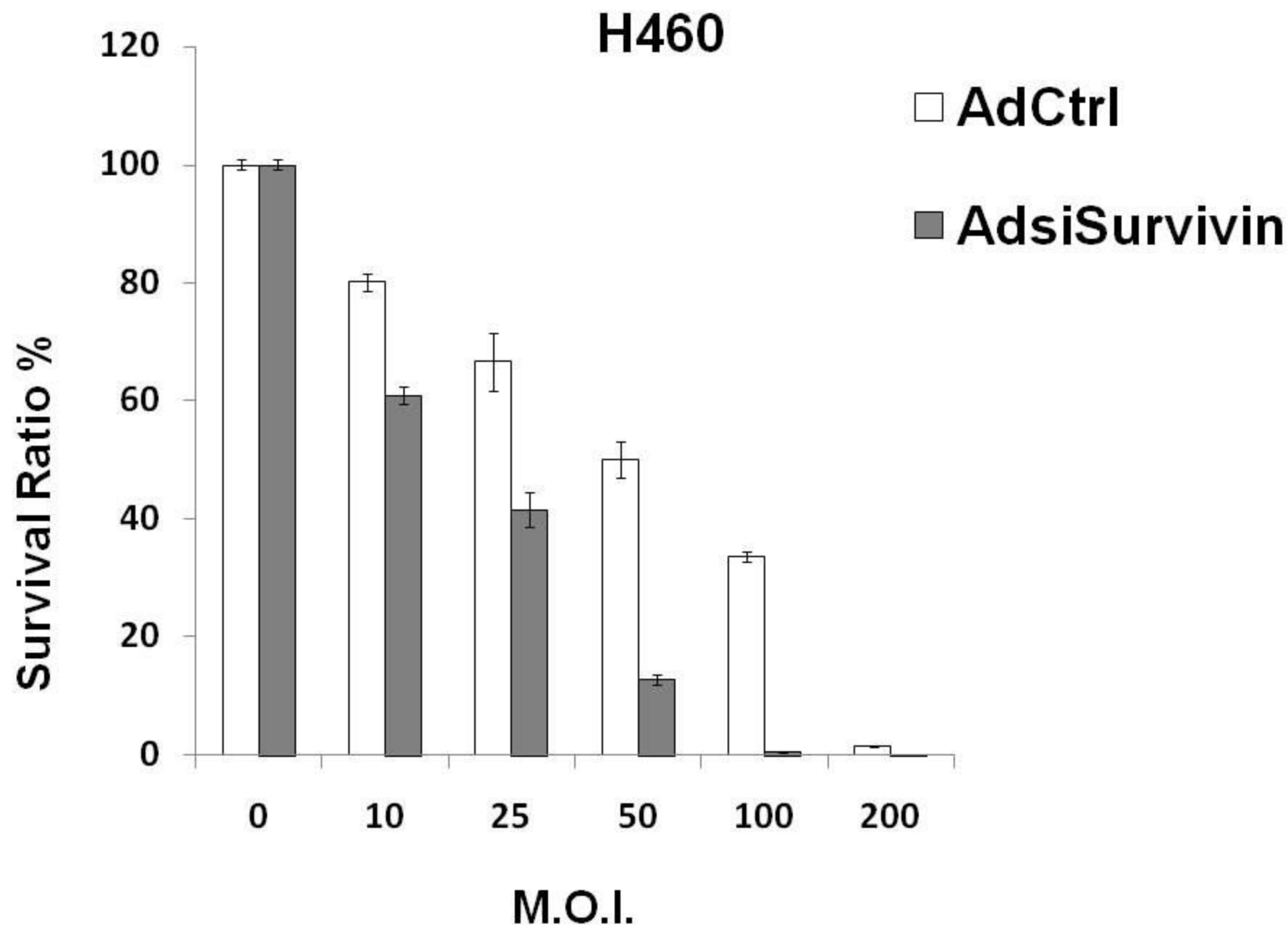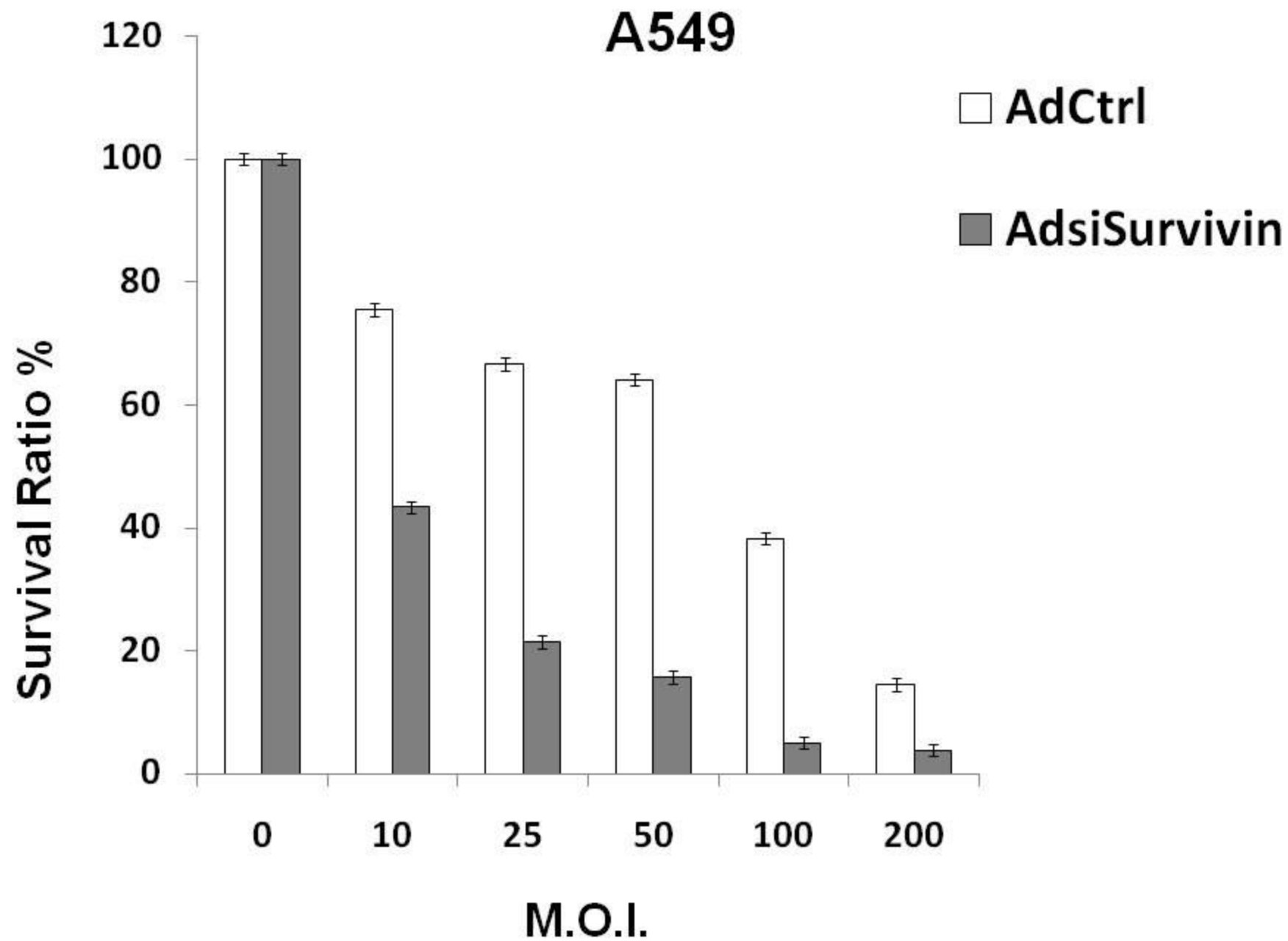
Fig. 1

293   293S(1) 293S(2)

SURVIVIN ⟶

Fig. 2

Fig. 3A

Fig. 3B

Fig. 4A

H460
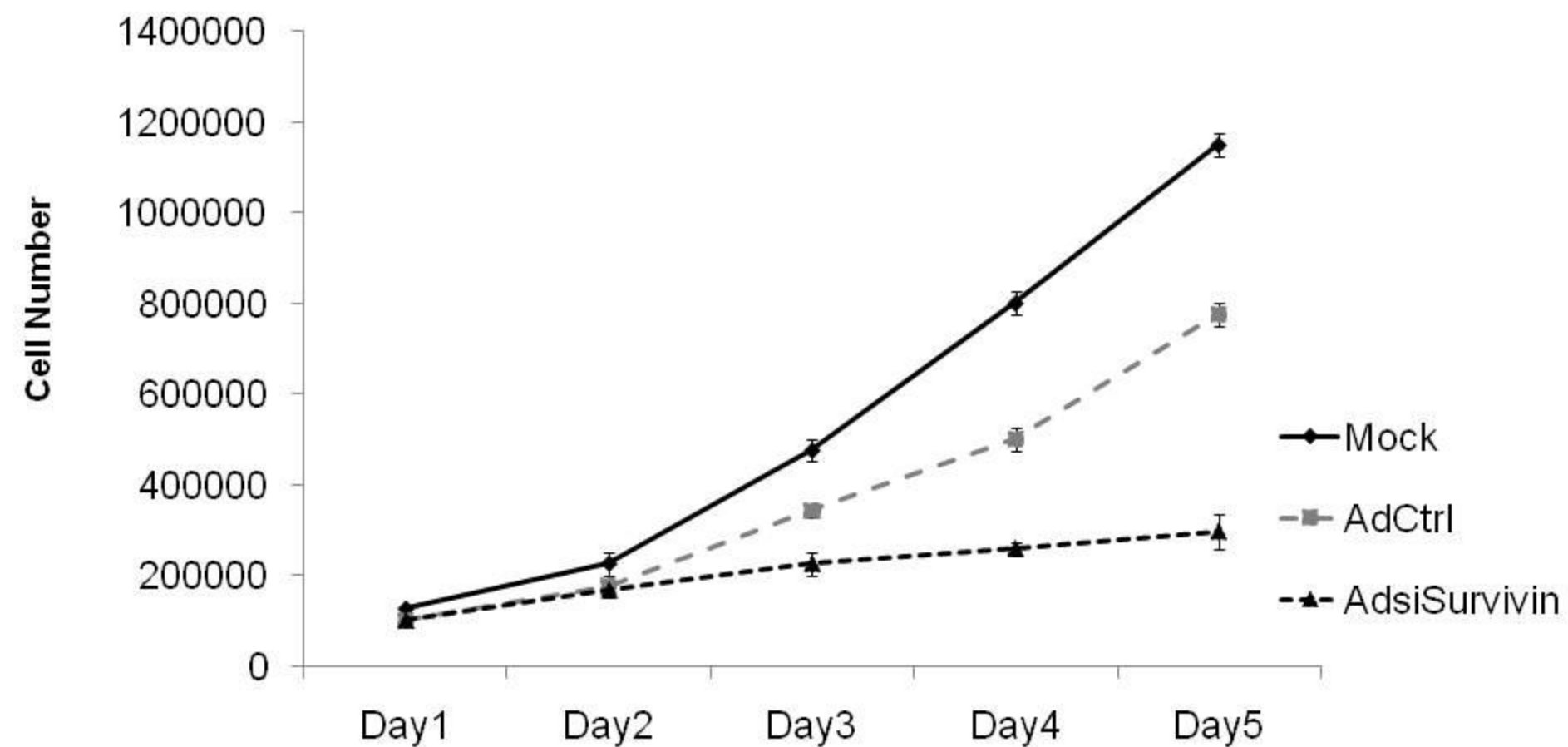
Fig. 4B

A549

Fig. 4C

H1650

Fig. 4D

H1975

Cell Number

300000
250000
200000
150000
100000
50000
0

Day1   Day2   Day3   Day4   Day5

Mock
AdCtrl
AdsiSurvivin

Fig. 5

A.

B.

PARP

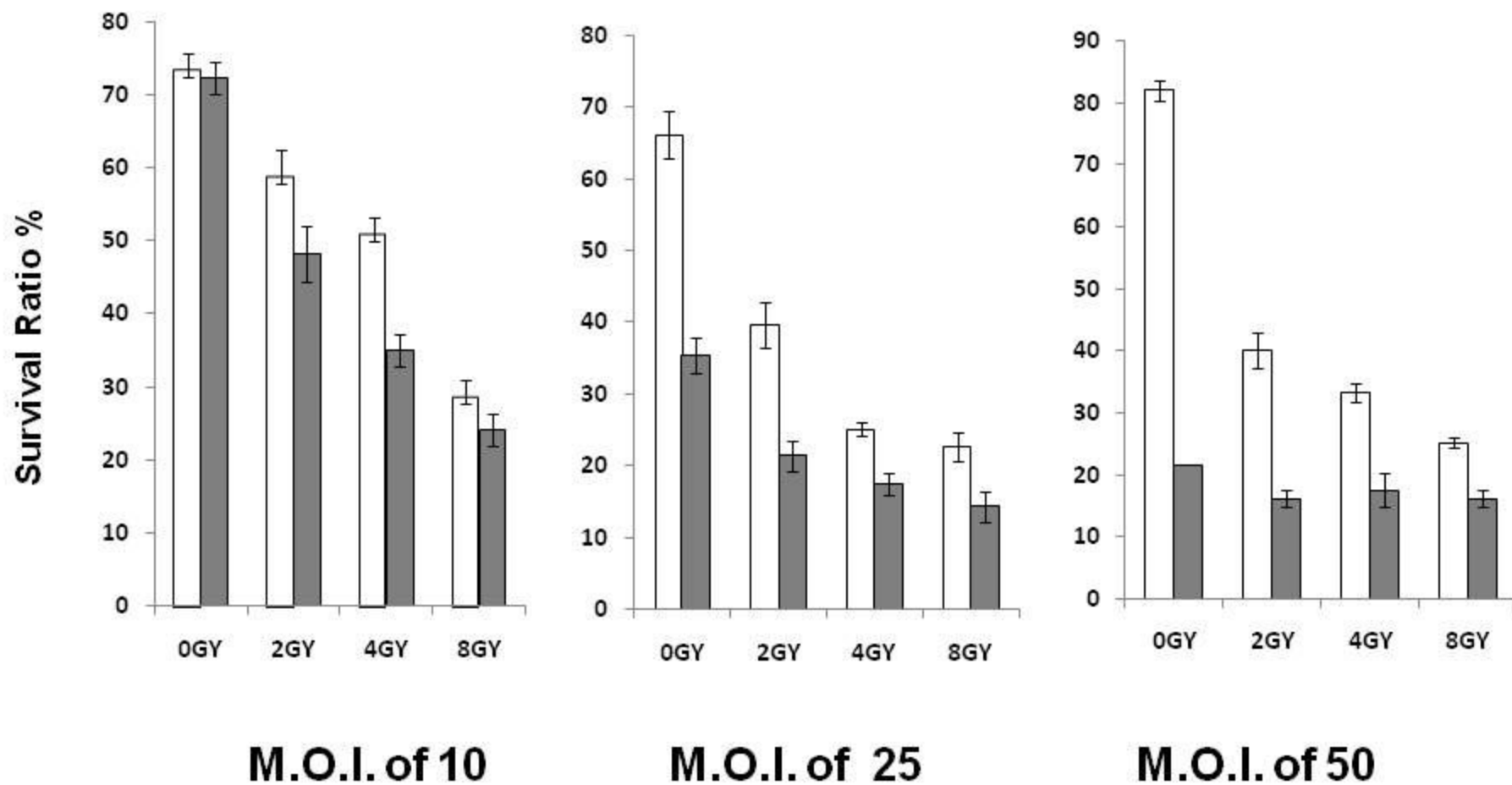# Fig. 6A

Fig. 6B

H460
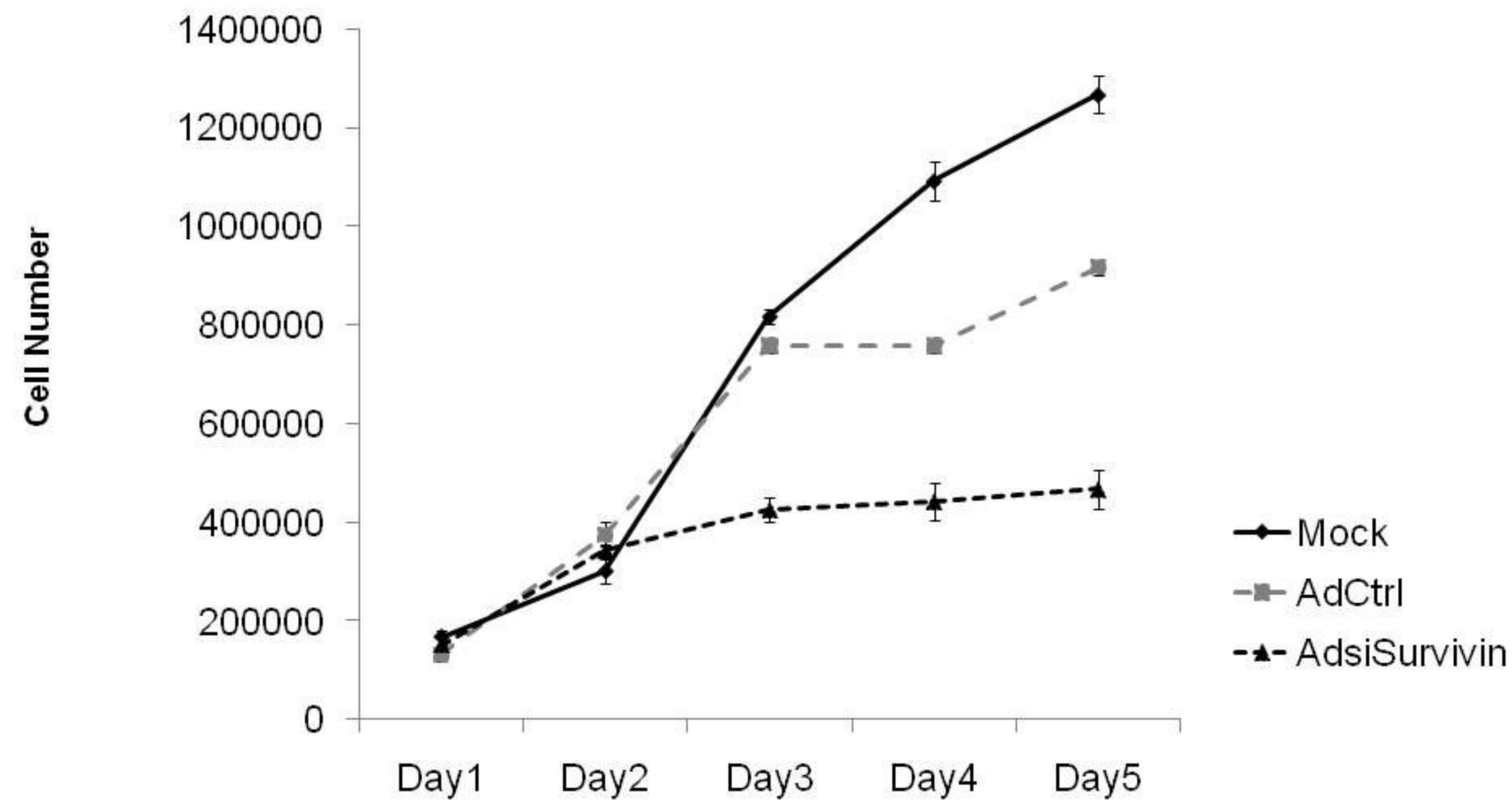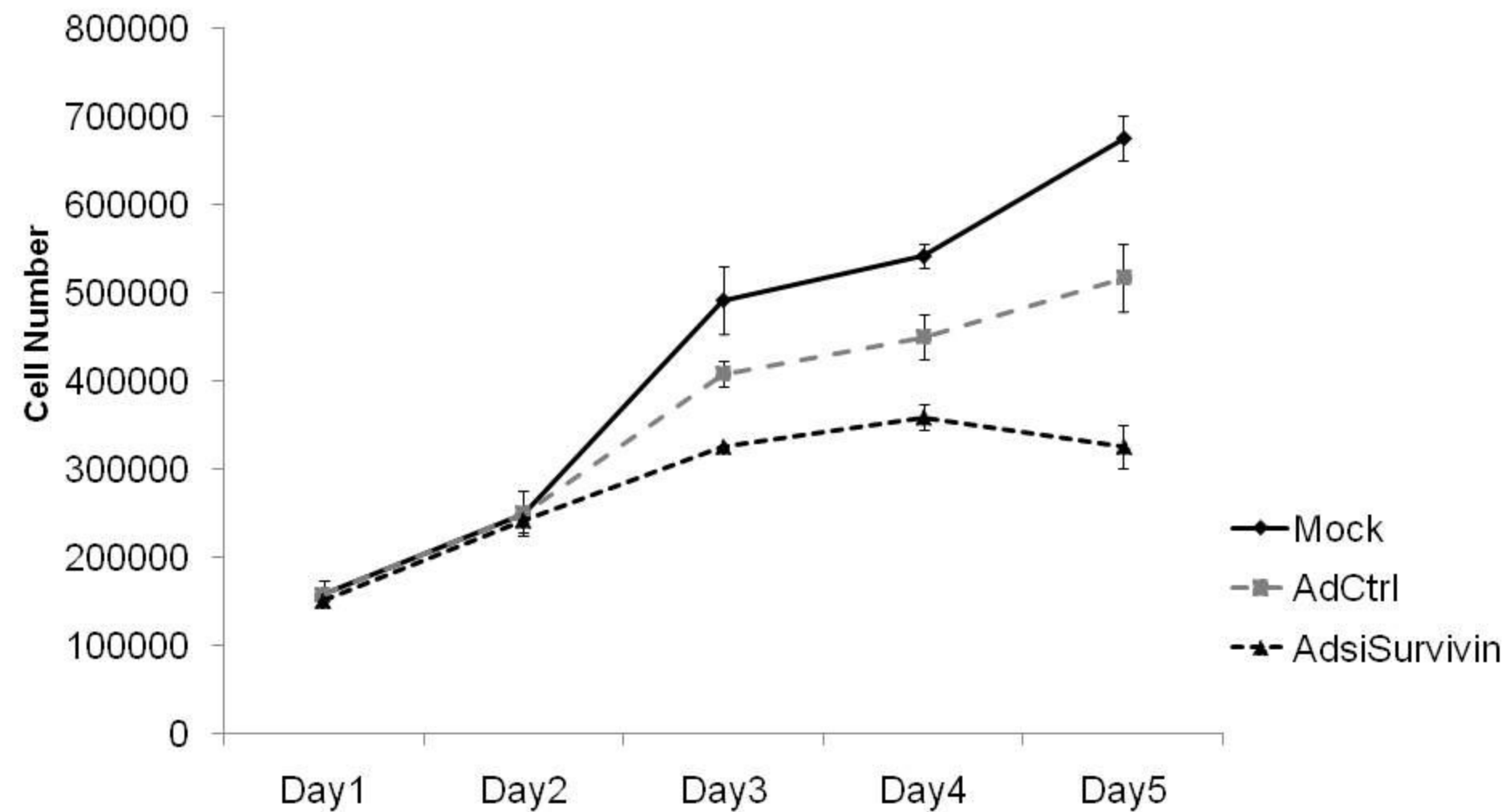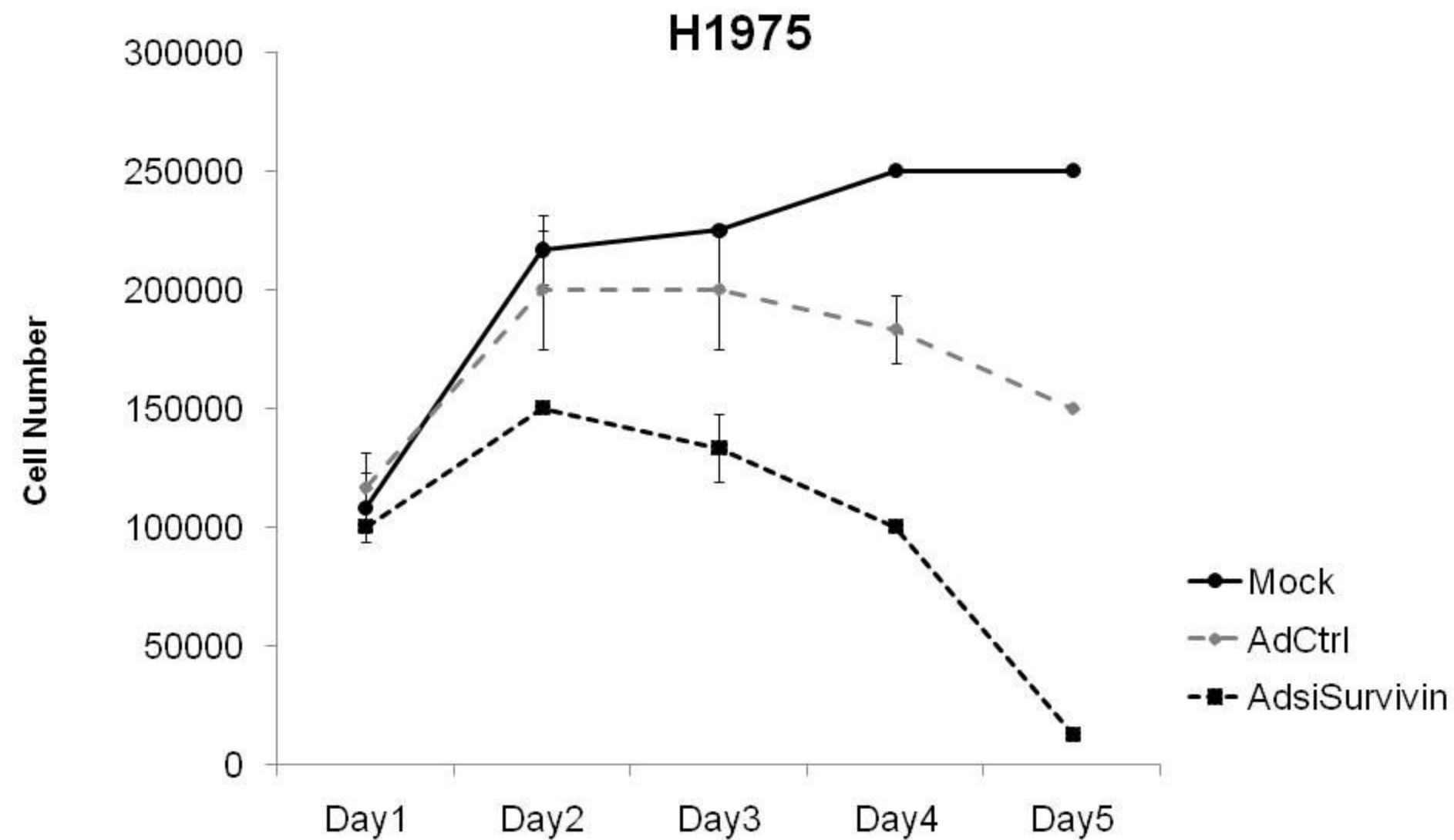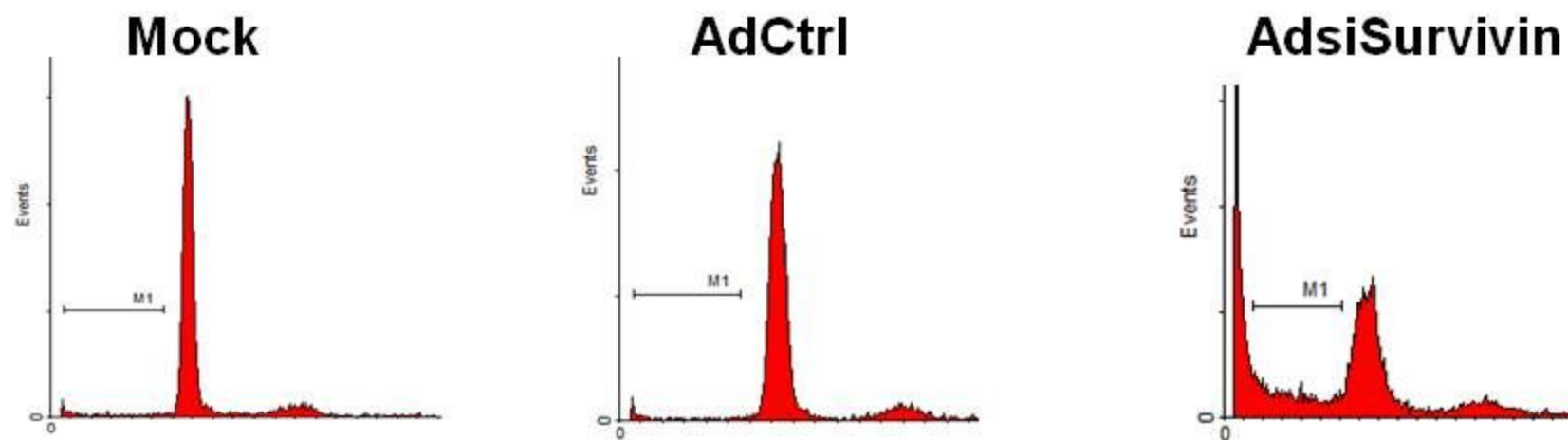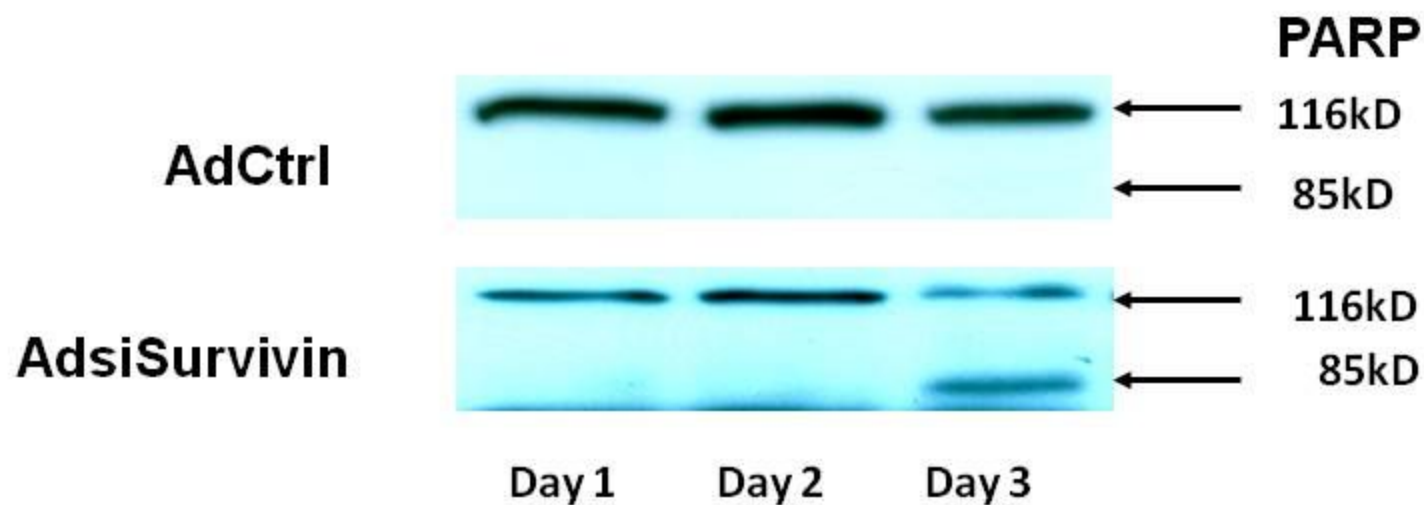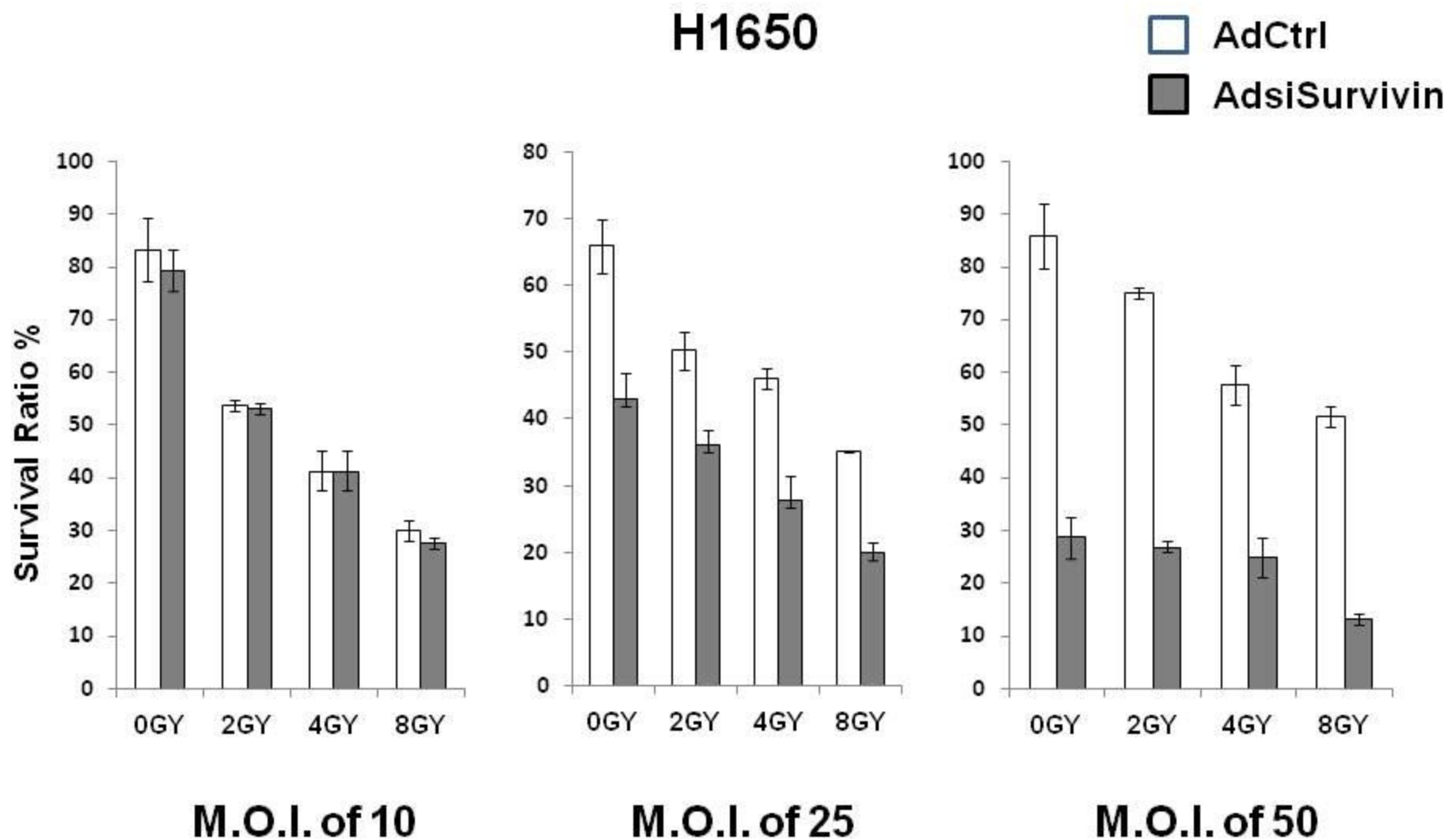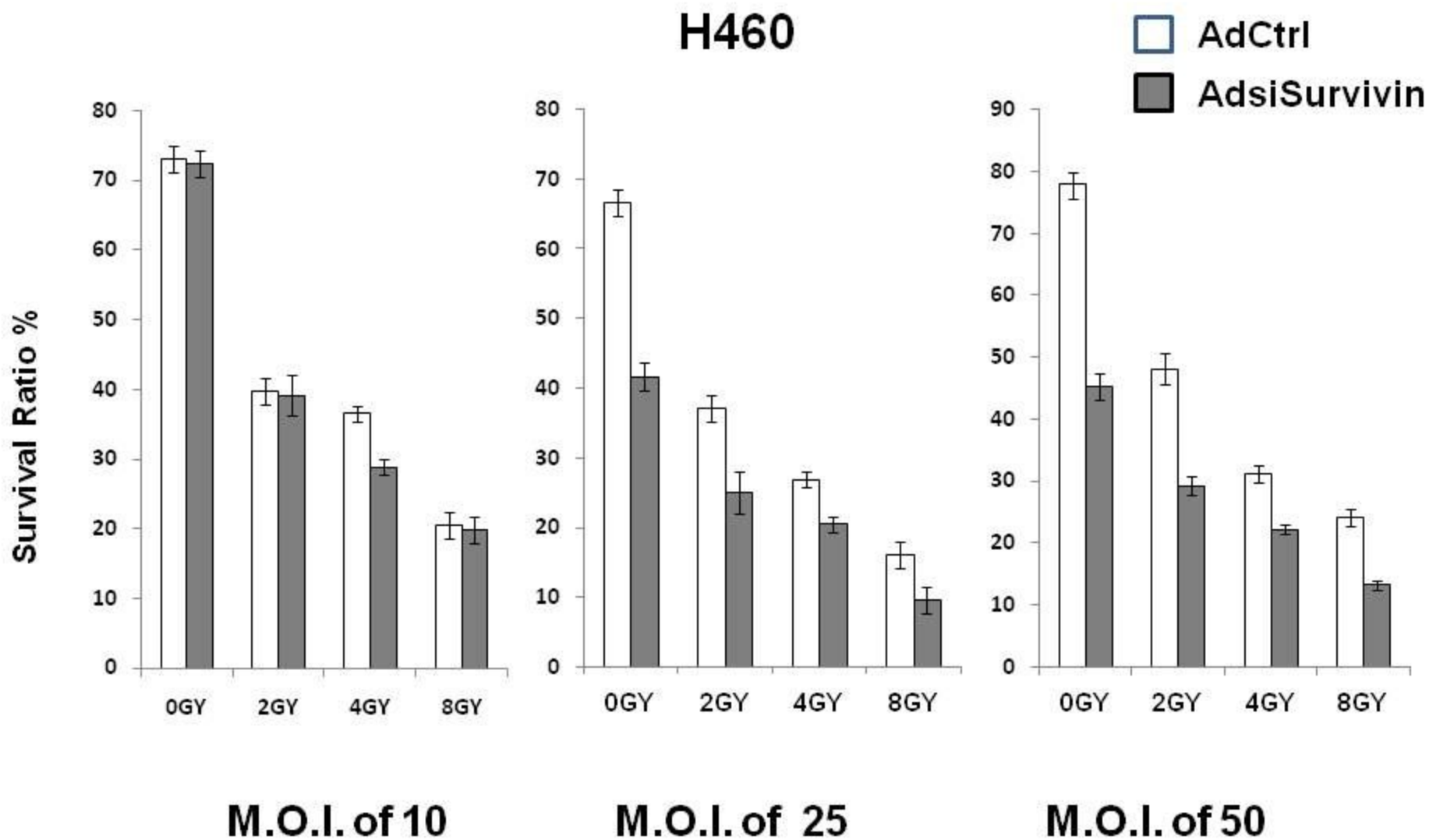
Fig. 6C

A549

AdCtrl
AdsiSurvivin

M.O.I. of 10   M.O.I. of 25   M.O.I. of 50

Fig. 6D