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# Intravenous siRNA silencing of survivin enhances activity of mitomycin C in human bladder RT4 xenografts

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## Abstract

**Purpose—**Survivin inhibits apoptosis and enables tumor cells to escape from therapy-induced senescence. High expression of survivin is associated with bladder cancer aggressiveness and recurrence. The present study evaluated if survivin expression is reduced by siRNA and if survivin silencing enhances the activity of mitomycin C (MMC), in human RT4 bladder transitional cell tumors *in vitro* and *in vivo*.

**Materials and Methods—**The effectiveness of siRNA therapy was evaluated using two newly developed pegylated cationic liposome carriers (PCat, PPCat). Both carries used a fusogenic lipid to destabilize the endosomal membrane. One carrier (PPCat) further contained paclitaxel to enhance the *in vivo* delivery and transfection of survivin siRNA (siSurvivin). *In vitro* antitumor activity was evaluated using short and long term cytotoxicity assays (microtetrazolium and clonogenicity). *In vivo* intravenous therapy was evaluated in mice bearing subcutaneous tumors.

**Results—**The nontarget-siRNA had no antitumor activity *in vitro* or *in vivo*. Treatment of cultured cells with MMC at 50% cytotoxic concentration enhanced survivin mRNA and protein levels; addition of PPCat or PCat containing siSurvivin reversed the survivin induction and enhanced the MMC activity ($p<0.05$). In tumor-bearing mice, single agent MMC delayed tumor growth and nearly tripled the survivin protein level in residual tumors, whereas addition of PPCat-siSurvivin, which by itself yielded a minor survivin reduction (<10%), completely reversed the MMC-induced survivin and enhanced the MMC activity ($p<0.05$).

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**Conclusions—**The results indicate effective *in vivo* survivin silencing and synergism between MMC and PPCat-siSurvivin. This combination represents a potentially useful chemo-gene therapy for bladder cancer.

### Keywords

Bladder cancer; survivin; siRNA silencing; mitomycin C; chemo-gene therapy

## INTRODUCTION

At presentation, more than 80% of bladder tumors are organ-confined, among which 70–80% are nonmuscle-invading that include Ta tumors located in the urothelium, T1 tumors in the lamina propria, and carcinoma *in situ*. This group is managed by transurethral tumor resection with or without fulguration, plus neoadjuvant or adjuvant intravesical immunotherapy or chemotherapy. Intravesical therapy involves instilling a drug solution into the bladder through a catheter, typically for 2 hr and repeated weekly for 6 weeks or longer. Recurrence occurs in 40 to 80% of patients, and between 10 to 20% of recurrences are accompanied by grade and/or stage progression (including the more fatal metastatic disease)[1].

Through a series of preclinical and clinical studies[2,3], our group has established that the efficacy of intravesical chemotherapeutics is limited by two factors: inadequate drug delivery to tumors and low chemosensitivity (especially the more aggressive tumors). We then identified a method that uses pharmacokinetic interventions to maximize the MMC delivery to nonmuscle-invading tumors. This method was tested in a multi-center, randomized phase III trial; the results confirm our hypothesis that improving drug delivery significantly improves the 5-yr recurrence-free rate of high risk patients (from 23.5% to 42.6%)[2].

As a part of our ongoing efforts to enhance the bladder tumor sensitivity to MMC, the present study evaluated the potential usefulness of silencing survivin, a chemotherapy-induced anti-apoptotic gene. Survivin is highly and selectively expressed in a majority of human cancers including bladder cancer[4,5], and is a marker/predictor of bladder cancer aggressiveness and recurrence[6–8]. In addition, high survivin expression is correlated with chemo/radio-resistance in multiple tumor types whereas low expression enhances cell death[9,10]. Inhibition of survivin by antisense, siRNA or shRNA enhances the sensitivity of tumor cells to chemotherapy including MMC[10–12].

siRNA produces post-transcriptional gene silencing and represents a promising gene therapy approach. We have shown that intraperitoneal administration of siRNA reverses chemotherapy-induced survivin expression in pancreatic xenograft tumors[13]. The potential utility of survivin silencing in bladder cancer was shown in a recent study where intratumoral injection of siRNA-containing nanoparticles reduced survivin expression and growth of subcutaneous xenograft tumors[14]. However, the feasibility of intravenous siRNA therapy is not known because these earlier studies used locoregional injections and because systemic siRNA therapy is impeded by inadequate delivery and transfection[15]. We recently reported that tumor priming using apoptosis-inducing chemotherapy enhances the *in vivo*

effectiveness of siRNA therapy[13]. This earlier study used paclitaxel as the tumor priming agent. In the current study, MMC was used as the chemotherapy and, due to its ability to induce apoptosis[16], also as the priming agent. For the siRNA carriers, we evaluated two pegylated cationic liposomes. One carrier was identical to the PCat carrier used in our earlier study[13], whereas the second carrier PPCat contained, in addition, paclitaxel. This study shows that both carriers yielded survivin silencing and enhanced MMC activity, *in vitro* and *in vivo*, in human bladder RT4 xenograft tumors.

## MATERIALS AND METHODS

### Chemicals and Supplies

MMC was purchased from A.G. Scientific (San Diego, CA), paclitaxel from Bioxel (Quebec, Canada), lipids (1,2-dioleoyl-3-trimethylammoniumpropane or DOTAP, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine or DOPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] or DSPE-PEG, cholesterol) from Avanti Polar Lipids (Alabaster, AL), and all cell culture supplies from Life Technologies (Grand Island, NY). Survivin siRNA (siSurvivin, human-specific, #6351), nontarget siRNA (NT-siRNA) and primer probe sets were purchased from IDT (San Diego, CA), rabbit monoclonal HLA-A antibody (EP1395Y) from Abcam (Cambridge, MA), survivin monoclonal antibody (71G4B7E) from Cell Signaling Technology (Danvers, MA), Ki67 antibody from Novocastra Lab (UK), and In Situ Cell Death Detection Kit (TUNEL) from Roche Diagnostics (Indianapolis, IN). All chemicals and reagents were used as received.

### Preparation of liposomes and lipoplexes

Cationic liposomes form lipoplexes with anionic siRNA, which improves the siRNA stability and internalization[15,17]. Both PCat and PPCat comprised cationic lipid (DOTAP), neutral lipids (cholesterol, DOPE) and pegylated lipid (DSPE-PEG2000), at a ratio of 50:30:19:1. The fusogenic lipid DOPE destabilizes the endosomal membrane. PCat was prepared as previously described[13], whereas the PPCat preparation used an extra step of adding paclitaxel dissolved in methanol at 0.0406 weight % of total lipids. PCat or PPCat were gently mixed with an aqueous siRNA solution (10 μM) at room temperature, in a 1:4 siRNA-to-DOTAP charge ratio. Particle size distribution and zeta potential were measured using Zetasizer Nano ZS90.

### Measurement of survivin mRNA and protein levels

mRNA and protein levels were respectively measured using real time (RT)-PCR and Western blotting as previously described[13]. Briefly, total RNA was extracted using the Rneasy Plus Mini Kit (Qiagen, Valencia, CA), and reversed transcribed to cDNA using qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). RT-PCR (triplicate samples, 5 μL cDNA per reaction) was performed with PerfeCTa® MultiPlex qPCR SuperMix (Quanta) in CFX96™ RT-PCR Detection Systems (BioRad, Hercules, CA). The prime probe sets were: survivin, forward: 5’- CAACCGGACGAATGCTTTT-3’; reverse: 5’-AAGAACTGGCCCTTCTTGGA-3’; probe: 5’-/5HEX/CCAGATGAC/ZEN/GACCCCATAGAGGAA/3IABkFQ/-3’; GAPDH, forward: 5’-

AATCCCATCACCATCTTCCAG-3'; reverse: 5'-AAATGAGCCCCAGCCTTC-3'; probe: 5'-/5Cy5/CCAGCATCGCCCCACTTGATTTT/3IAbRQSp/-3'. The multiplex thermal reaction program was: 3 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 61°C. Survivin mRNA expression relative to GAPDH expression was calculated using the ΔΔCt-method[18]. For protein analysis, cells were lysed and analyzed for protein concentrations using BCA kit (ThermoFisher, Rockford, IL). Samples (1.5 μg protein/μl) were run on Bolt™ 10% Bis-TrisPlus Gel (Life technologies, Carlsbad, CA). The gels were transferred to a nitrocellulose membrane using Trans-blot Turbo Transfer System (BioRad). Blots were probed with survivin and β-actin primary antibodies (Cell Signaling). Chemi-luminescence was detected and the band intensity analyzed using BioRad Molecular Imager. Protein levels were normalized to loading control (β-actin for cultured cell lysates and HLA-A for animal tumor lysates). HLA-A is the major histocompatibility complex specific to humans; it was used to eliminate ambiguity due to the presence of mouse tissues.

### *In vitro* studies

Human bladder transitional RT4 cancer cells (ATCC, Manassaas, VA) were maintained in complete medium comprising Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 100 μg/ml penicillin and 100 μg/ml streptomycin, in 5% $CO_2$ at 37°C.

Antitumor activity was measured using the short term microtetrazolium (MTT) cytotoxicity assay and the long term clonogenic assay. All cytotoxicity studies used 9 groups, including two control groups (untreated or treated with nontarget siRNA or siNT), and groups treated with siSurvivin, two concentrations of MMC (10% and 50% cytotoxicity, or $IC_{10}$ and $IC_{50}$), and combinations of MMC and siNT or siSurvivin (i.e., $IC_{10}$+siNT, $IC_{10}$+siSurvivin, $IC_{50}$+siNT, $IC_{50}$+siSurvivin). The transfection medium was the complete medium minus FBS and antibiotics. The siRNA concentration was 100 nM. The MMT assay used PPCat carrier, whereas the clonogenic assay used PCat in order to avoid potential complications due to the delayed cytotoxicity of paclitaxel embedded in PPCat[19]. For both assays, cells were treated with MMC (2 hr), PPCat-siSurvivin (4 hr), or their combinations (in the sequence of siSurvivin followed by MMC). For the MTT assay, cells were seeded in 96-well plates (5,000–15,000 cells/well), allowed to attach to the growth surface for 24 hr, treated, and the numbers of metabolically active cells remaining at 48 hr post-treatment were measured. For the clonogenic assay, cells were seeded in culture dishes (100 $mm^2$) for 24 hr, treated, harvested by trypsinization, re-plated (1,000 cells per dish), incubated with medium change every 4 days, and stained with the violet crystal dye on day 23. Pictures were analyzed using ImageJ (NIH, Bethesda, MD); the numbers of colonies with a size >0.1 $mm^2$ (>100 cells) were counted.

The effect of MMC and siRNA treatments on survivin expression was studied using two concentrations of MMC (0.1 and 3 μM) and one concentration of siRNA (100 nM in PPCat). Cells were collected 48 hr after treatment, and analyzed for survivin mRNA and protein levels.

### *In vivo* studies

Female athymic nude mice (NCI, Frederick, MD), 5 to 6 weeks old, were cared for according to Institutional Animal Care and Use Committee-approved protocols. Subconfluent RT4 cells were harvested and implanted subcutaneously into the left and right flanks (3 million cells per side). Treatments were initiated after 28 days when tumors reached at least 3 mm in width. MMC solution (1 mg/ml physiological saline) was prepared immediately before treatment. Mice were randomized according to initial tumor size and body weight, to six treatment groups to receive intravenous injections of physiological saline, single agents (4mg/kg MMC per dose, 1 nmole PPCat-siNT or PPCat-siSurvivin per dose), or their combinations (MMC plus PPCat-siNT, MMC plus PPCat-siSurvivin). MMC was given every 4 days for a total of 3 doses and siRNA was given 2 days after each MMC treatment.

Antitumor activities were monitored using two sets of pharmacodynamic endpoints. The macroscopic endpoint was change in tumor size (calculated as $0.5 \times (\text{width})^2 \times (\text{length})$). The microscopic cellular and molecular endpoints were survivin protein level, apoptosis and antiproliferation, as described previously[13]. Briefly, two days after the last treatment, tumors were excised from anesthesized mice and divided into two halves. One half was stored frozen at −80°C, and later lysed with M-PER and the lysates analyzed for survivin protein level. The second-half was fixed with 10% formalin, embedded in paraffin, cut into 10 μm histologic sections, and processed for immunostaining. Micrographs were taken with a Leica SP8 confocal microscope and images were analyzed for survivin-, Ki67-, and TUNEL-positive cells. We determined the number of labeled and unlabeled tumor cells in randomly selected microscopic fields at 200× magnification, and counted, on average per animal, >500 cells per data point.

### Statistical Analysis

For comparison of *in vivo* antitumor activity, differences between treatment groups were analyzed using analysis of variances (ANOVA with repeated measures). p values of less than 5% were considered statistically significant. The concentration-response curves were analyzed using GraphPad Prism (La Jolla, CA).

## RESULTS

### Characterization of siRNA carriers

Blank PCat and PPCat liposomes (i.e., no siRNA cargo) were stable at 4°C, with no significant changes in particle size and zeta potential after 18 months. Upon mixing with siRNA, the respective particle size increased from 110±4 to 170±3 nm and from 110±1 to 164±2 nm, and the respective zeta potential reduced from 62±1 to 34±1 mV and from 59±1 to 33.5±1mV (mean±SD, n=3 experiments).

### Effect of MMC and siSurvivin on survivin expression in cultured RT4 cells

Pilot studies showed that MMC transiently elevated the mRNA and protein levels of survivin, which reached maximal levels on day 2 and returned to baseline levels on day 4, whereas PPCat-siSurvivin (100 nM) showed the opposite profiles (maximum knockdown on

day 2 and full recovery on day 4). Subsequent studies on survivin expression used the day 2 time point; the results are summarized in Figure 1 and Table 1A.

MMC induced survivin expression in a dose-dependent manner; MMC treatment at 0.1 and 3 μM (equivalent to the $IC_{10}$ and $IC_{50}$ in MTT assay) yielded, respectively, about 30% and 70% increases in mRNA levels and about 20% and 50% increases in protein levels, compared to untreated controls ($p<0.05$). Single agent PPCat-siSurvivin (100 nM) significantly reduced the survivin mRNA and protein levels. PPCat-siSurvivin also reversed the survivin induction by MMC, with complete/partial reversal of mRNA induction and more-than-complete reversal of protein induction (i.e., below the level in untreated control) ($p<0.05$ compared to MMC alone).

### Activity of MMC and siSurvivin in cultured cells

Figures 2A and 2B show the results of the short term cytotoxicity MTT assay (measured at 48 hr post-treatment) and the long term clonogenic assay (measured on day 23 post-treatment), respectively.

PCat- and PPCat-siRNA lipoplexes loaded with siNT (i.e., no siSurvivin), at up to 1,000 nM siRNA, were nontoxic, as shown by the lack of cytotoxicity in the two assays. MMC showed dose-dependent cytotoxicity in MTT and clonogenic assays whereas siSurvivin produced different outcomes in the two assays. In MTT assay, PPCat-siSurvivin had no activity and did not alter the activity of MMC. In contrast, in the clonogenic assay, PCat-siSurvivin treatment yielded minor but significant growth inhibition (7±2%, $p<0.05$ compared to untreated control and siNT) as well as significantly enhanced the MMC activity (from 13±3% to 30±2% inhibition at 0.05 μM MMC and from 48±2% to 62±2% inhibition at 0.2 μM MMC, $p<0.05$ in both cases). Furthermore, the average sizes of colonies in groups treated with PCat-siSurvivin (with or without MMC) were slightly but significantly smaller compared to without PCat-siSurvivin treatment (0.18 vs. 0.16 $mm^2$ and 0.12 vs. 0.11 $mm^2$, respectively, $p<0.05$). Collectively, these results indicate siSurvivin had long term cytotoxicity but no short term cytotoxicity.

### *In vivo* antitumor activity

Figure 3 shows the tumor size changes over time. The untreated control showed continuous tumor growth, reaching ~700% of the initial size on day 58 (day 0 is day of first treatment). Tumor growth was not affected by PPCat-siSurvivin ($p>0.8$ compared to untreated control), indicating the lack of activity of PPCat carrier and siSurvivin siRNA. In contrast, single agent MMC (3 mg/kg per dose×3 doses) caused tumor regression (maximal 16% size reduction on day 12) and reduced tumor growth (to <430% of initial size on day 58) ($p<0.05$ compared to untreated control and the two single agent siRNA groups). Addition of PPCat-siSurvivin (1 nmole per dose × 3 doses) to MMC further improved the MMC activity (i.e., maximal 32% tumor regression and <300% tumor growth, $p<0.05$ compared to single agent MMC). The times for tumor size to increase by 50% were similarly extended by MMC and PPCat-siSurvivin (Table 1B).

Figure 4 and Table 1B show the results on several cellular and molecular pharmacodynamic endpoints in tumors (i.e., survivin expression, proliferating and apoptotic cell fractions).

MMC treatment tripled the protein level whereas PPCat-siSurvivin significantly reduced the protein level, compared to untreated control. Adding PPCat-siSurvivin to MMC more than reversed the survivin induction by MMC (i.e., below the level in untreated control). These findings were confirmed by the immunohistochemical results; MMC significantly increased the survivin-positive cell fraction whereas PPCat-siSurvivin significantly reduced the fraction. Consistent with the *in vitro* data in cultured cells, PPCat-siSurvivin yielded more-than-complete reversal of the MMC induction in animal tumors. MMC significantly reduced the Ki67-positive proliferating cell fraction and increased the TUNEL-positive apoptotic cells ($p<0.05$). Although PPCat-siSurvivin had no effects as single agent, its addition enhanced the MMC effects, i.e., significantly lower proliferating fraction and greater apoptotic fraction compared to MMC alone ($p<0.05$).

In contrast to siSurvivin which produced significant changes, the nontarget siRNA had no effects *in vivo*, i.e., it did not alter survivin expression or the proliferating/apoptotic cell fractions in tumors, and did not slow tumor growth or enhance the MMC activity.

## DISCUSSION

The present study shows several interesting findings. First, MMC induced survivin expression, in a dose-dependent manner, in cultured RT4 cells and RT4 xenograft tumor-bearing animals; this is consistent with the literature results[20]. Second, PCat and PPCat were efficient siSurvivin carriers and produced survivin mRNA and protein knockdown *in vitro*. Third, survivin silencing produced different outcomes in short term MTT assay and long term clonogenic assay. Fourth, PCat- and PPCat-siSurvivin reversed the survivin induction by MMC, and enhanced the MMC cytotoxicity in cultured cells and animal tumors. In contrast, the nontarget siRNA had no activities, confirming the role of survivin silencing of MMC chemosensitization. Fifth, because single agent PPCat-siSurvivin was inactive *in vivo*, the enhanced activity for the combination of MMC and PPCat-siSurvivin indicates synergy. The potential clinical implications of these findings are as follows.

The expression and prognostic value of several anti-apoptotic genes, including survivin, XIAP, p53, bcl2 and bcl-xL, in bladder cancer have been compared[6–8,21,22].The expression of survivin is the most frequently upregulated in tumors (65–100%) and the most highly correlated with poor prognosis, whereas the expression of the other genes has no or inferior correlations, indicating the more important role of survivin among the known anti-apoptotic genes. This is further supported by the finding in monolayer EJ28 and J82 bladder tumor cells that siRNA-mediated survivin silencing yielded greater antitumor effect compared to silencing of XIAP, bcl2 and bcl-xL[10]. Our finding that PCat-siSurvivin produced a minor but significant growth reduction only in the clonogenic assay and not in the MTT assay indicates slow onset cytotoxicity such as cytostasis instead of cell kill by survivin knockdown in RT4 cells.

The successful *in vivo* siRNA transfection using the PPCat carrier is noteworthy as the current study used intravenous administration, which subjects siRNA to multiple transport and elimination barriers that have limited the efficacy and development of siRNA therapeutics[15]. There have been very few successful gene silencing by intravenous RNAi.

The first demonstration of RNAi in humans was in 2010 and was accomplished using pegylated cyclodextrin, transferrin-targeted nanoparticles (target gene was ribonucleotide reductase)[23]. A 2013 study used pegylated cationic liposomes in a phase I trial and detected VEGF and/or KSP siRNA in tumor biopsies of all 12 patients plus partial gene knockdown in 3 of 12 patients[24]. To our knowledge, the present study is the first to demonstrate survivin silencing in bladder tumors by intravenous RNAi. For example, the earlier study demonstrating the *in vivo* activity of survivin siRNA was accomplished using the intratumoral injection route[14].

Survivin is universally upregulated by cytotoxic treatments, i.e., radiation and drugs used for intravesical or intravenous therapy of organ-confined or metastatic bladder cancer (e.g., doxorubicin, cisplatin, gemcitabine, methotrexate, vincristine, paclitaxel, docetaxel)[9,25–30]. Hence, the *in vivo* synergy between intravenous MMC and PPCat-siSurvivin observed in the current study, plus the *in vivo* synergy between intraperitoneal paclitaxel and PCat-siSurvivin observed in our earlier study[13], indicate siSurvivin gene therapy can be used to overcome drug resistance in bladder cancer, in either the intravesical or the systemic therapy setting.

At present, there is limited information on the disposition of siRNA therapeutics, e.g., the dose fraction that is delivered intact to tumors, residence time, and duration of gene silencing. These data, however, are needed to determine the dose intensity and frequency. Another consideration for intravesical therapy is the ability of the siRNA carrier to reach the target site, as the urothelium is a well-known barrier to the absorption of intravesical therapeutics (e.g., <3% of the intravesical MMC dose reaches the locations of Ta and T1 tumors[3]). Additional pharmacokinetic and pharmacodynamic studies are needed to facilitate the successful translation of PCat/PPCat-siSurvivin to clinical application.

## CONCLUSIONS

The synergy between intravenous MMC and PPCat-siSurvivin *in vivo* supports further development of chemo-survivin gene therapy for organ-confined and metastatic bladder cancer.

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## Abbreviations used

| | |
|---|---|
| **DOPE** | 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine |
| **DOTAP** | 1,2-dioleoyl-3-trimethylammoniumpropane |
| **DSPE-PEG** | 1,2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[methoxy(polyethylene glycol)-2000] |
| **FBS** | fetal bovine serum |
| **MMC** | mitomycin C |
| **MTT** | microtetrazolium |
| **PCat** | liposomes comprising DOTAP:cholesterol:DOPE:DSPE-PEG in 50:30:19:1 ratio |
| **PPCat** | paclitaxel-containing PCat |
| **siNT** | non-target siRNA |
| **siRNA** | small interfering RNA |
| **siSurvivin** | survivin siRNA |

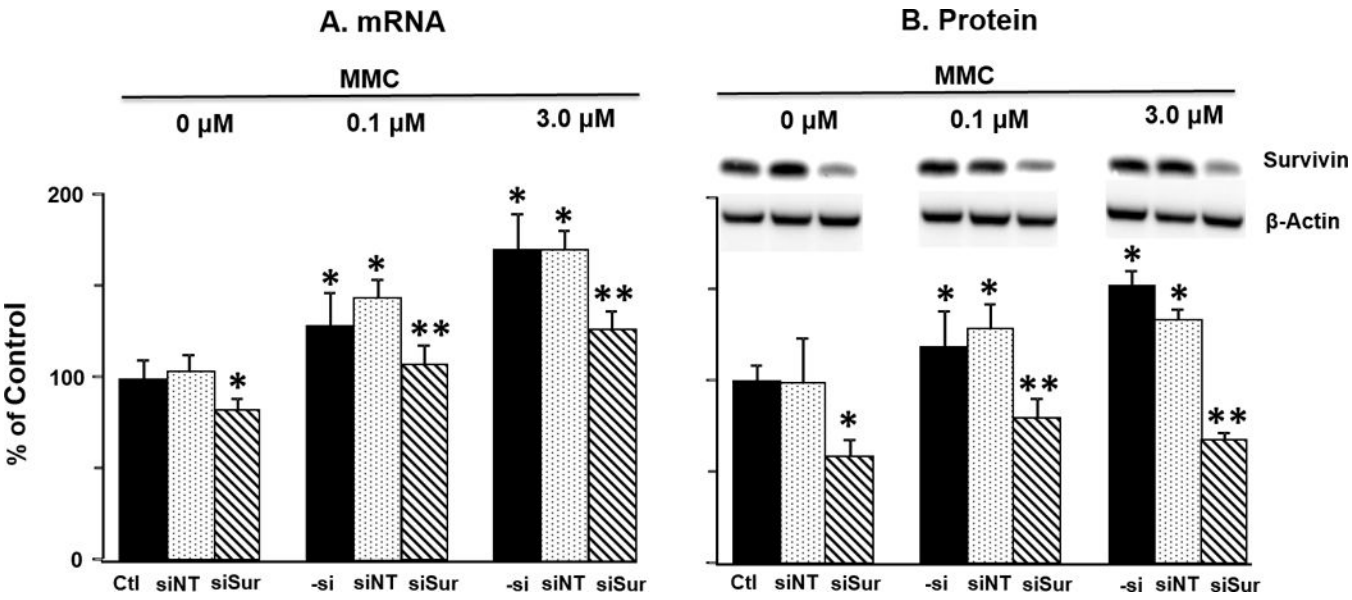



**Figure 1. Effect of MMC and siRNA on survivin expression *in vitro***

RT4 cells were incubated for 4 hr in transfection medium (control), with PPCat-siNT (100 nM) or PPCat-siSurvivin (100 nM), followed by 2 hr treatment with complete medium or MMC (0.1 and 3 μM). At 48 hr post-treatment, cells were collected and analyzed for survivin mRNA and protein levels. Ctl: control. –si: no siRNA, siNT: nontarget siRNA, siSur: survivin siRNA. Data for mRNA levels are mean+1 SD (n=3 experiments, with triplicate samples per experiment). Data for protein levels are mean+1 SD (n=3 experiments, single sample per experiment). *p<0.05 compared to control. **p<0.05 compared to MMC and MMC+PPCat-siNT.

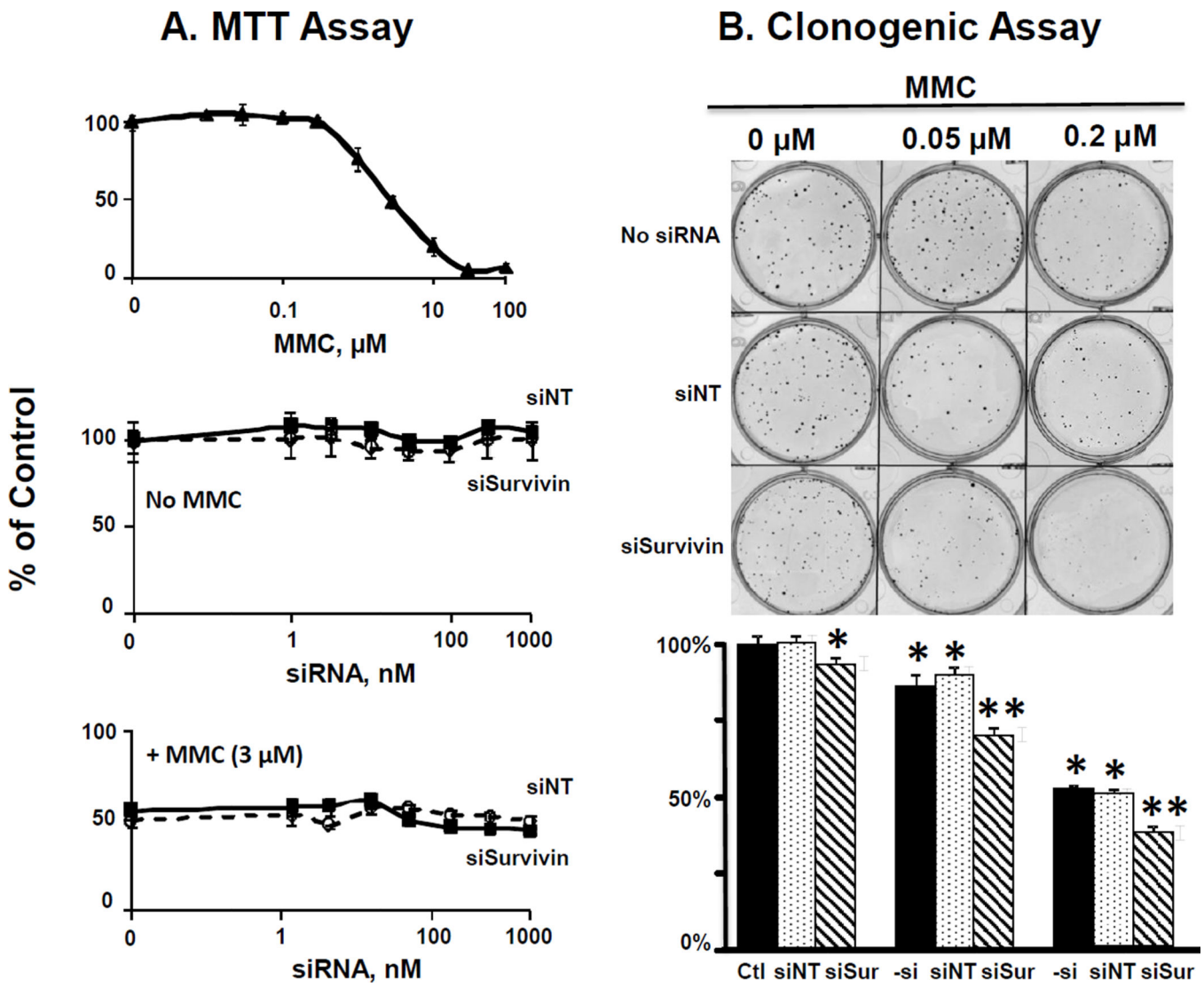



**Figure 2. Survivin silencing enhances antitumor activity of MMC *in vitro***

RT4 cells were incubated for 4 hr in transfection medium (control), with PPCat-siNT (100 nM) or PPCat-siSurvivin (100 nM), followed by 2 hr treatment without or with MMC in complete medium, and then processed for short term MTT assay (cytotoxicity measured at 48 hr post-treatment, Panel A), or the long term clonogenic assay (cytotoxicity measured at 23 days post treatment, Panel B). For Panel A, data are mean+1 SD (n=3 experiments, with triplicate samples per experiment). Some SD values are smaller than the symbols. For Panel B, data are mean+1 SD (n=3 experiments, triplicate samples per experiment). Ctl: control. –si: no siRNA, siNT: nontarget siRNA, siSur: survivin siRNA. *p<0.05 compared to untreated control (i.e., no MMC, no siRNA). **p<0.05 compared to untreated control as well as single agent MMC at same MMC concentration (i.e., no siRNA or MMC+siNT).

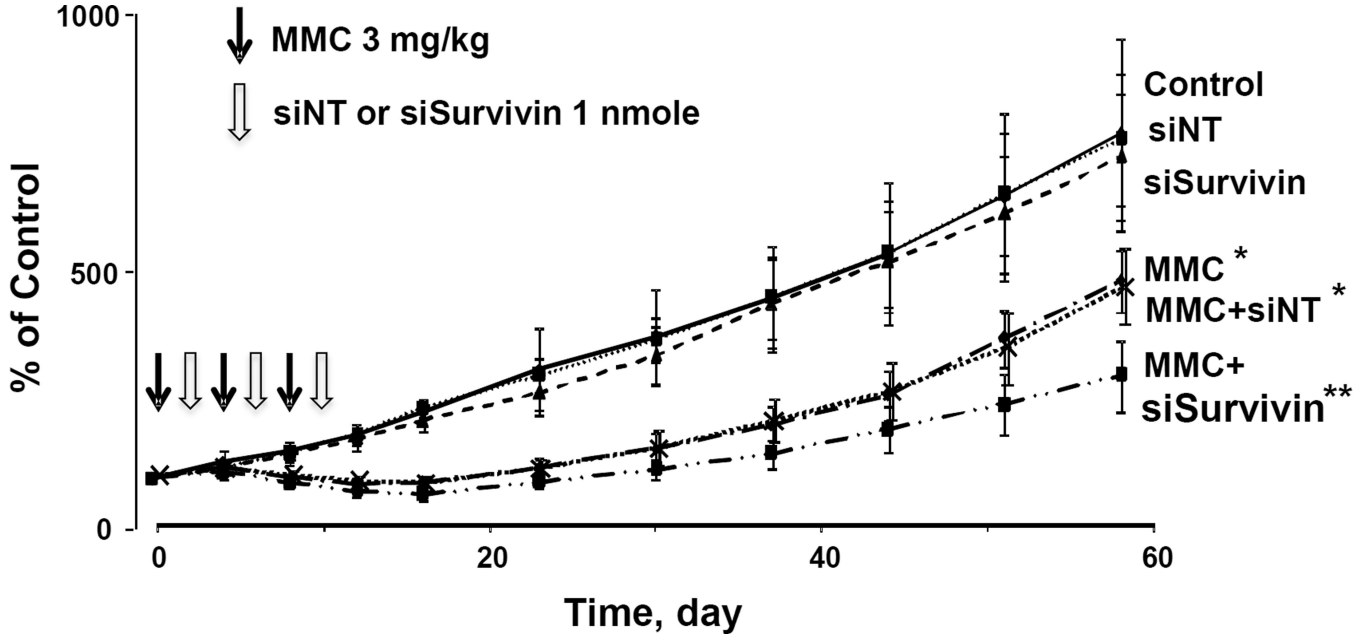
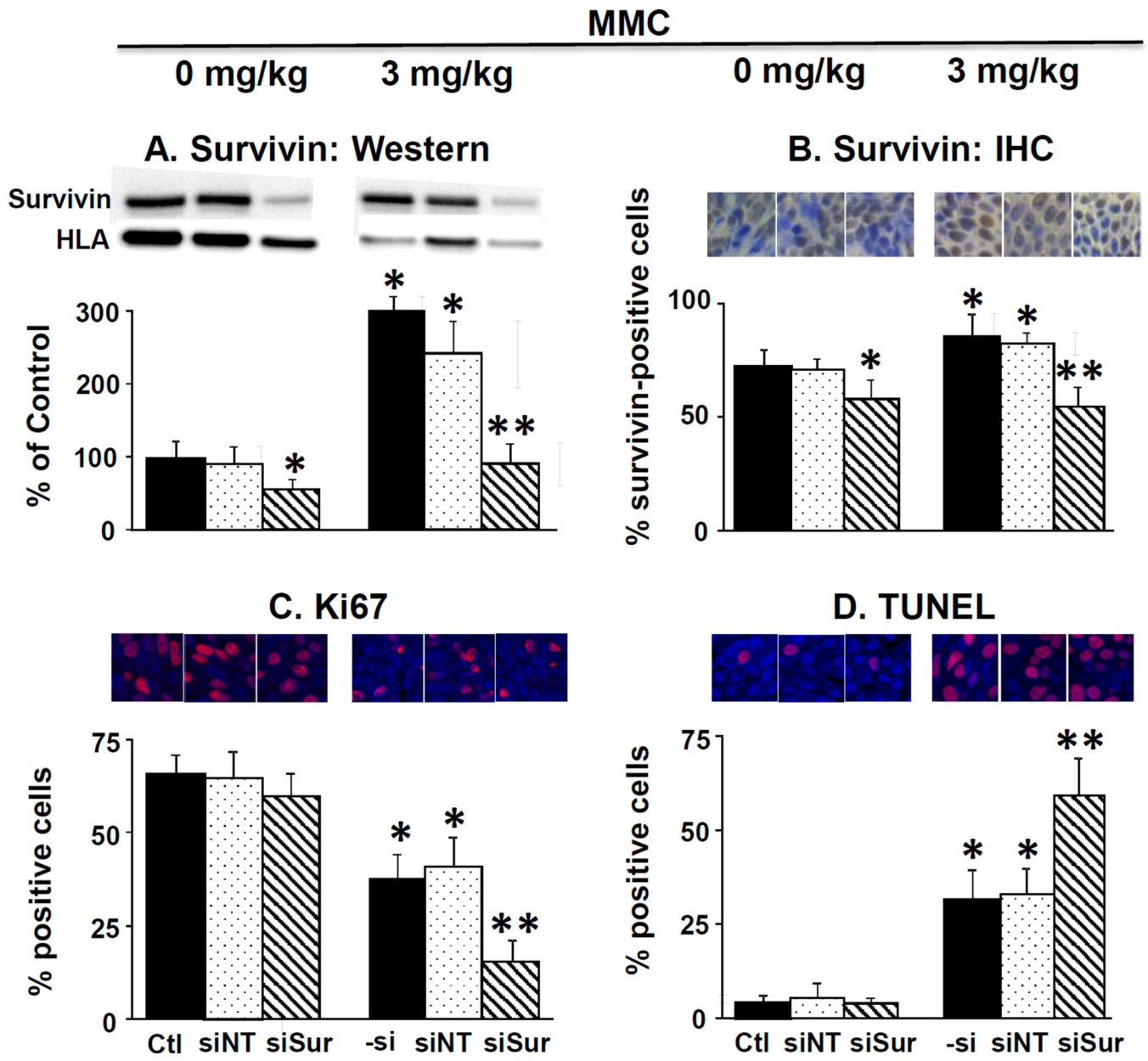



**Figure 3. Survivin silencing enhances antitumor activity of MMC *in vivo*: Tumor growth**

Antitumor activity was evaluated in immunodeficient mice bearing subcutaneous RT4 human bladder xenograft tumors. All treatments (indicated by arrows) were administered by intravenous injections. Day 0 represents the day of treatment initiation, which corresponded to 28 days post-tumor implantation. The MMC dose was 3 mg/kg, given every 4 days for a total of 3 doses. The PPCat-siSurvivin dose was 1 nmole, given 2 days after each MMC treatment. Animals were maintained for 58 days post treatment, with tumor size measurements taken every 4 days for the first 16 days and once a week afterwards. From top to bottom: control (filled diamonds, n=5), siNT (open squares, n=5), siSurvivin (filled triangles, n=5), MMC (open diamonds, n=10), MMC+siNT (crosses, n=5), MMC +siSurvivin (filled squares, n=10). *$p<0.05$ *vs.* control and single agent siNT and siSurvivin. **$p<0.05$ *vs.* all other groups.



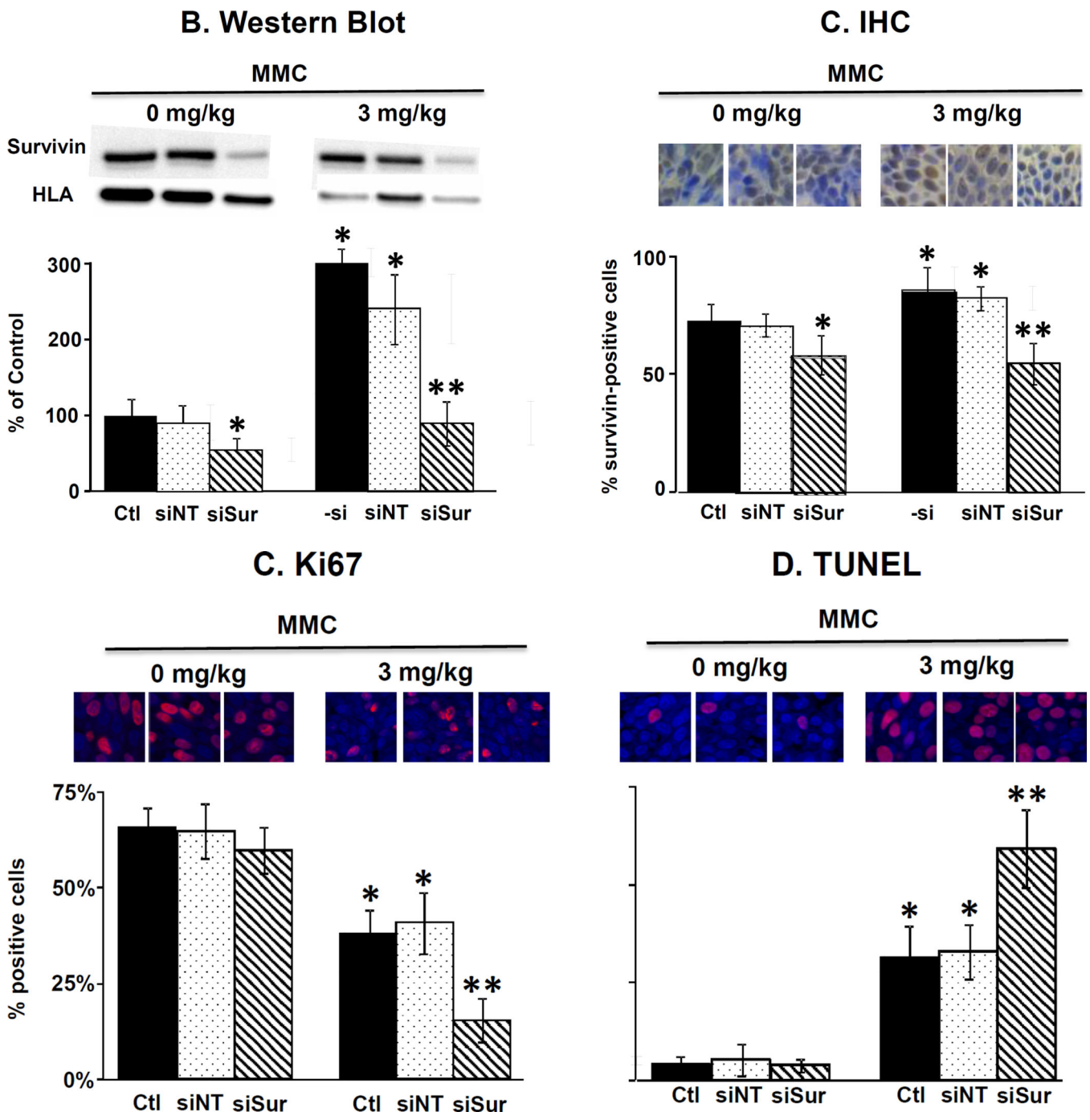



**Figure 4. Survivin silencing enhances antitumor activity of MMC *in vivo:* Cellular and molecular pharmacodynamic endpoints**

Animals were treated as described in the legend of Figure 3, with the exception that animals were anesthesized 2 days after the last treatment and their tumors excised and processed for Western blotting and for immunohistochemical staining and quantitative imaging analysis. (A) Survivin protein levels in tumors detected by Western blotting. Results were normalized by HLA. (B) Survivin expression detected by immunohistochemical staining (IHC, stained brown). Blue: nucleus dye DAPI. (C) Immunohistochemical staining of Ki67 (red). Blue:

nucleus dye DAPI. Note the Ki67 stain overlapped with the nucleus stain (i.e., stained cells showed only red stain). (D) Immunohistochemical staining of apoptotic cells (TUNEL, red).Blue: nucleus dye DAPI. Note the TUNEL stain overlapped with the nucleus stain (i.e., stained cells showed only red stain). Ctl: control. –si: no siRNA, siNT: nontarget siRNA, siSur: survivin siRNA. Data are mean+1 SD (3–5 tumors). *$p<0.05$ vs. control groups.**$p<0.05$ vs. MMC and MMC+PPCat-siNT.

**Table 1**

**Survivin silencing enhances MMC activity *in vitro* and *in vivo***

Cultured RT4 cells and mice bearing RT4 xenograft tumors were treated with MMC, nontarget siRNA and survivin siRNA (siRNA were delivered in PPCat), as described in the legends of Figures 1 and 3. For the *in vitro* studies, the MMC concentration was 3 μM and the siRNA concentration was 100 nM. For the *in vivo* studies, the MMC dose was 3 mg/kg given every 4 days for a total of 3 doses and the siRNA dose was 1 nmole given 2 days after each MMC treatment.

| Group | A. *In Vitro* results | | B. *In Vivo* results | | | |
|---|---|---|---|---|---|---|
| | Survivin expression mean±SD (3 experiments, 3 samples each) | | Time for 50% tumor size increase, median (range), days | Survivin protein level, % of control (n=3–5) | % Ki67-positive cells (n=3–5) | % TUNEL-positive cells (n=3–5) |
| | mRNA | Protein | | | | |
| Control | 1.00 ± 0.10 | 1.00 ± 0.09 | 8 (8-12, n=5) | 100 ± 21 | 66 ± 5 | 4 ± 1 |
| PPCat-siNT | 1.08 ± 0.05 | 0.99 ± 0.25 | 12 (8-12, n=5) | 90 ± 23 | 65 ± 7 | 5 ± 4 |
| PPCat-siSurvivin | 0.82 ± 0.02* | 0.58 ± 0.10* | 12 (8-12, n=5) | 55 ± 15* | 60 ± 6 | 4 ± 2 |
| MMC | 1.47 ±0.34* | 1.52 ± 0.08* | 37 (23-44, n=10)* | 302 ± 18* | 38 ± 6* | 31 ± 4* |
| MMC + PPCat-siNT | 1.53 ± 0.24* | 1.33 ± 0.06* | 37 (23-44, n=5)* | 240 ± 45* | 41 ± 8* | 33 ± 7* |
| MMC + PPCat-siSurvivin | 1.13 ± 0.17** | 0.90 ± 0.04** | 44 (37-58, n=10)*** | 90 ± 28** | 16 ± 5*** | 59 ± 6*** |

* p<0.05 *vs*. the untreated control and PPCat-siNT control.

** p<0.05 *vs*. MMC and MMC+PPCat-siNT, but not different from untreated control and PPCat-siNT control.

*** p<0.05 *vs*. all other groups.