Pro-apoptosis and Anti-proliferation Effects of a Recombinant Dominant-negative Survivin-T34A in Human Cancer Cells

CHANGXIAN SHEN[1*], WEIJIN LIU[2], ANDREAS K. BUCK[1§] and SVEN N. RESKE[1]

[1]*Department of Nuclear Medicine, University of Ulm, Ulm, Germany;*
[2]*Department of Life Sciences, Huaihua University, Huaihua, China*

Abstract. *Background: Survivin is an attractive target for anti-cancer drug development; however targeting it by small molecules or antibodies is difficult, as survivin is neither a kinase nor a cell surface protein. Protein transduction domain (PTD)-mediated macromolecular therapeutics provides an alternative avenue for targeting survivin. Materials and Methods: A plasmid expressing a dominant-negative survivin-T34A fused with the immunodeficiency virus protein transduction domain TAT was constructed. The fusion protein was expressed and purified from E. coli. The inhibition of proliferation and induction of apoptosis was tested in human lung carcinoma cell line A549 by directly adding survivin-T34A to the cell culture medium. Results: Recombinant survivin-T34A was efficiently expressed and purified by affinity chromatography. It induced cell apoptosis as demonstrated by induction of caspase 3 activation and higher percentage of Annexin V staining, and inhibited cell proliferation as determined by cell number counting. Conclusion: This functional recombinant protein is promising for development of macromolecular therapeutics targeting survivin.*

The bottleneck of cancer therapy was thought to be the inability to find the chemicals that can kill cancer cells but spare normal cells (1). Exploring the cancer kinome for anti-cancer drug development is achievable, however the 95% attrition rate for small molecule and antibody-based oncology drugs in the clinical setting is unacceptably high. Targeting of protein-protein interactions, phosphatases and other important factors such as p53, Ras and Myc, is still being developed but is very promising (2). Cancer gene therapy is theoretically attractive but studies of gene transfer in human subjects have shown that an effective gene therapy of cancer remains elusive (3).

Protein transduction domain (PTD)-based macromolecular therapeutics provides an alternative avenue for anticancer drug development (4). Human immunodeficiency virus (HIV) TAT (5) is one of the best-studied PTDs. PTD vectored peptides, such as TAT-Smac (6), r8-BadBH3 and r8-BidBH3 (7), have been successfully exploited as laboratory tools and for pre-clinical drug development.

Survivin is a member of the inhibitor of apoptosis (IAP) family which antagonizes apoptosis by suppressing the processing and catalytic activity of caspases (8). Survivin is overexpressed in most types of human cancer, but is either undetectable or found at very low levels in normal adult cells (9). Genome-wide transcription profiling has shown that survivin is one of the most tumor-specific genes thus far identifiable (10). Therefore, survivin has been proposed as a promising target for anticancer drug development (11). Indeed, suppressing survivin by oligonucleotides such as RNAi (12), antisense (13), ribozyme (14) or triplex forming oligonucleotides (15), or interfering with its function by a dominant-negative mutant of survivin (16) result in apoptosis in a wide range of cancer cells.

In this study, the 11 amino acid PTD of TAT was fused to the dominant-negative survivin-T34A to form TAT-survivin-T34A. This fusion protein was expressed in *Escherichia coli* and purified by Ni-NTA affinity chromatography. Cell culture data demonstrate that this recombinant TAT-survivin-T34A inhibits growth and induces apoptosis in A549 cells.

[*]*Present address:* Department of Molecular Pharmacology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, U.S.A.

[§]*Present address:* Nuklearmedizinische Klinik und Poliklinik, Klinikum rechts der Isar der Technischen Universität München, Ismaninger Strasse 22, D-81675 München, Germany.

Correspondence to: Changxian Shen, Ph.D., Department of Molecular Pharmacology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, U.S.A. Tel: +1 901 595 2111, Fax: +1 901 595 4290, e-mail: shenchangxian@yahoo.com

Key Words: Apoptosis, macromolecular therapeutics, protein transduction domain, survivin.

Materials and Methods

Cell culture. Human lung carcinoma cell line A549 was from DSMZ (German Institute for Micro-organism and Cell Culture). Cells were grown in DMEM supplemented with 10% fetal calf serum, 100 μg/mL G418 and 100 μg/mL streptomycin, and cultured at 37˚C with 85% humidity and 5% CO_2.

Plasmids and bacterial strains. Plasmids containing the complete ORF of wild-type survivin and a phosphorylation-deficient Thr34 to Ala mutant survivin-T34A were provided by Dario C. Altieri (Yale University School of Medicine, USA). Vector pTAT-HA was provided by Steven F. Dowdy (Howard Hughes Medical Institute, UCSD School of Medicine, USA). *E. coli* DH5α and BL21 (DE3) pLys were from Novagen, Germany.

Construction of plasmids pTAT-HA-survivin and pTAT-HA-survivin-T34A. The complete ORFs of survivin and survivin-T34A were amplified by PCR with the forward primer 5' CTAGCT CGAGATGGGTGCCCCGACGTTG 3' and reverse primer 5' GATCGAATTCGGCCTCAATCCATGGCAGCC 3' from plasmids containing survivin and survivin-T34A. The purified PCR products were cleaved by Xho I and EcoR I, and then cloned into the vector pTAT-HA cut with the same enzymes to get plasmids pTAT-HA-survivin and pTAT-HA-survivin-T34A. The cloned DNA fragments were confirmed by sequencing. A diagram of the structure of pTAT-HA-survivin-T34A is shown in Figure 1.

Plamid transformation and confirmation of gene expression. Plasmids pTAT-survivin and pTAT-survivin-T34A were transformed into BL21 (DE3) pLys. Five clones from the plate of each plasmid were picked and inoculated into 2 mL LB supplemented with 100 μg/mL ampicillin and 0.5 mM IPTG. The cultures were incubated at 37˚C overnight. The bacterial mass was pelleted, washed with PBS and resuspended in 100 μL 2 × SDS sample buffer. The protein sample was heated at 95˚C for 5 min and analyzed by 15% SDS-PAGE, and stained with Coomassie blue. Glycerol stocks of 2-3 BL21 (DE3) pLys isolates with highest expression were prepared in 15% glycerol and stored at –80˚C. A single isolate was streaked on an LB plate for large-scale expression and purification.

Affinity chromatography. A single clone (plasmids in BL21 (DE3) pLys S) from an LB plate was inoculated into 100 mL LB with 100 μg/mL ampicillin and incubated at 37˚C overnight. The overnight culture was inoculated into 1 liter LB with ampicillin and 0.5 mM IPTG and incubated at 37˚C for 7 h. The cell pellet was collected, washed once with 20 mL 1×PBS in a 50 mL tube and resuspended in 20 mL buffer Z [8 M urea, 100 mM NaCl, 20 mM Hepes, pH 8.0 (or 20 mM Tris-Cl, pH 8.0)]. The bacterial suspension was sonicated on ice for 10×30 seconds (to optimize) and clarified by centrifugation out 16,000 x*g* at 4˚C for 10 min. The supernatant was saved and equilibrated in 20 mM imidazole. Ni-NTA agarose (1.5 mL Ni-NTA agarose plus 1.5 mL storage buffer) (Qiagen, Germany) was mixed well, packed into a column, washed 2 times with double-distilled water and 3 times with buffer Z supplemented with 20 mM imidazole. The 20 mL protein sample was loaded onto the column, washed 3-6 times with buffer Z plus 20 mM imidazole (optional: washed for an additional 3 times with buffer Z plus 40 mM imidazole). The TAT-fusion proteins were eluted by stepwise addition of 5×1 mL buffer Z containing 250 mM imidazole. The proteins in the start (St) flow through (FT) and each column fraction were analyzed by SDS-PAGE. Fractions with the desired amount were pooled.

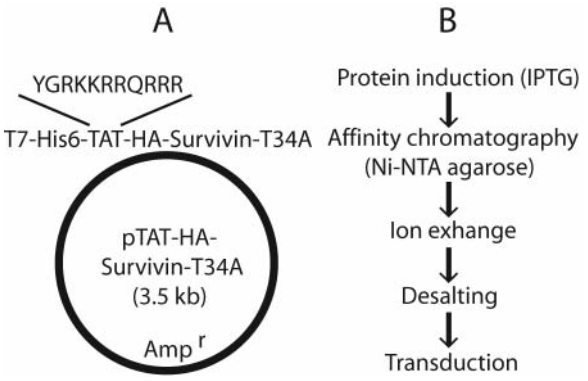

Figure 1. *A, Schematic representation of the construction of pTAT-HA-Survivin-T34A. B, Flowchart of the experiments.*

Ion exchange. Source Q and S resin (1 mL) (GE Healthcare Europe GmbH, Germany) were mixed in a 15 mL Falcon tube and washed 2 times with 10 mL buffer A (50 mM NaCl, 20 mM Tris-HCl pH8.0). The above Ni-NTA eluate was then added directly onto the washed Source Q and S resin, mixed well, placed on a rotator at room temperature (RT) for 30 min followed by centrifugation at 2,000-4,000 x*g* for 5 min. The resin was then washed for an additional 3 times with 10 mL of buffer A. Buffer B (2.5 mL) (2MNaCl, 20 mM Tris-HCl pH 8.0) was then added, mixed well and placed on a rotator at RT for 10 min. The elution steps were repeated 2 more times then analyzed by SDS-PAGE. Eluates with acceptable amount of protein were pooled.

Desalting. A disposable PD-10 column filled with Sephadex-25 (GE Healthcare Europe GmbH) was held on a rack and washed with 25 mL of DMEM without serum. Sample (2.5 mL) purified by Ni-NTA and ion-exchange was added onto the column. When the fusion protein entered the gel bed, 3.5 mL of DMEM was added to top out the column. One mL of each fraction was collected immediately after addition of DMEM to the column. Proteins began eluting in fraction 1. Fractions were pooled and diluted with 15% glycerol. Finally, the protein was flash-frozen with liquid nitrogen and stored at –80˚C.

SDS-PAGE and Western-blot. For caspase 3 analysis, total proteins (25 μg per lane) were resolved in 10% SDS-PAGE and transferred onto a nylon membrane. An anti-activated caspase 3 antibody (Cell Signaling) was used to check the activation of caspase 3 during apoptosis and an anti-actin monoclonal antibody (Sigma) was used to control for total protein loading.

Apoptosis staining and cell proliferation assay. At different time points following treatment, cells were trypsinized, washed with PBS and resuspended in 200 μL 1 × binding buffer according to the manufacturer's instructions (Clontech). Five μL of EGFP-conjugated Annexin V were added to each sample and incubated at RT for 10 min in the dark. The cells were then washed with PBS, resuspended in 500 μL 1 × binding buffer and subjected to flow-

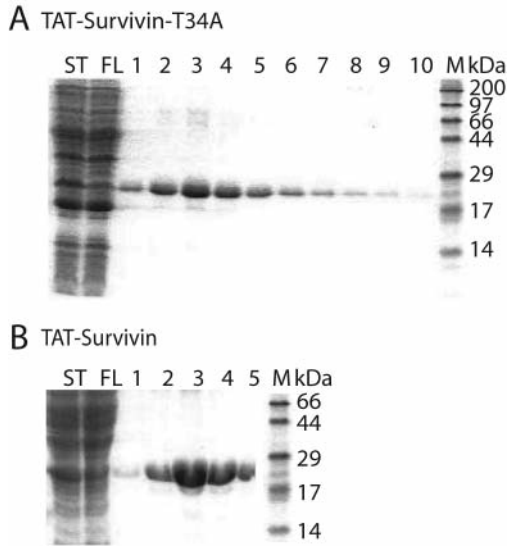

Figure 2. *Analysis of expressed and purified TAT-HA-Survivin-T34A (A) and TAT-HA-Survivin (B) by 15% SDS-PAGE. ST, total proteins of induced cells; FL, flowthrough of total proteins; lanes 1-5 indicate the elution fractions of purified proteins by Ni-NTA affinity chromatography; M, protein standards with molecular weights shown on the right lane.*

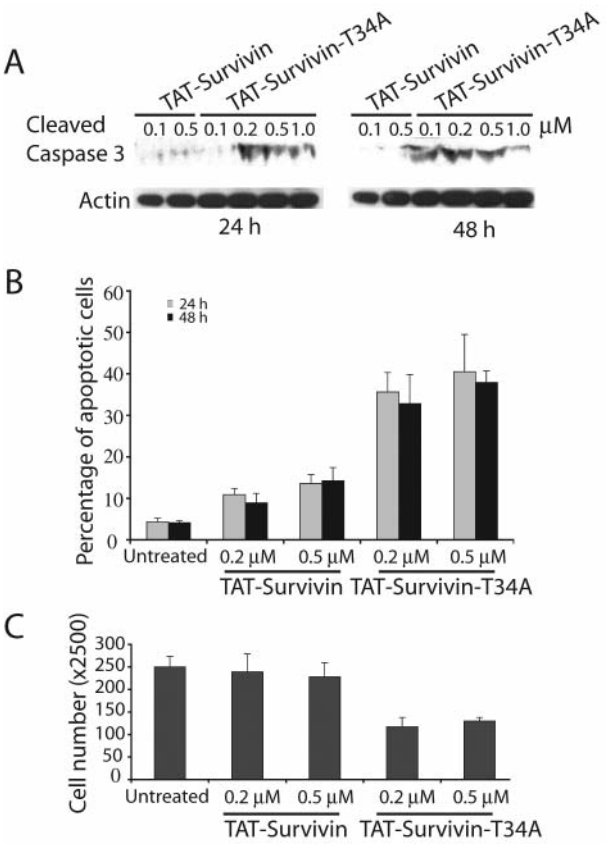

Figure 3. *TAT-Survivin-T34A induced apoptosis and inhibited proliferation of A549 cells. A, The induction of apoptosis by TAT-Survivin-T34A was demonstrated by its ability to induce the cleavage and hence activation of caspase 3. In these Western blots, a cleaved caspase 3-specific antibody was used to detect the activation of caspase 3. Actin staining served as a control for total protein loading. B, Staining A549 cells with EGFP-conjugated Annexin followed by FACS to test TAT-Survivin-T34A induced apoptosis. Grey bars, 24 h of treatment; dark bars, 48 h of treatment. C, TAT-Survivin-T34A inhibited cell proliferation. Cell number was determined with a hemocytometer 48 h following treatment, after detaching the cells by trypsinization. All experiments were repeated three times and data are presented as mean±SD.*

cytometry analysis (FACS). For the proliferation assay, at different time points following treatment, cell number was determined with a hemocytometer after detaching the cells in a solution of 1 mg/mL trypsin and 1 mM EDTA.

Results

Construction of plasmids pTAT-HA-survivin-T34A and pTAT-HA-survivin. The vector pTAT-HA is driven by a T7 promoter, which can be induced by IPTG. Nine bp following the ATG start codon is an 18 bp fragment encoding 6 histidines. This is followed by a sequence encoding 11 amino acids of the protein transduction domain of TAT (transactivator of transcription). An HA encoding sequence was placed after the TAT. The DNA sequence of interest can be cloned into the MCS (multiple cloning site). PCR was used to amplify the complete ORF of either wildtype survivin or survivin-T34A from plasmids. The purified PCR products were cloned into the Xho I and EcoR sites of pTAT-HA to get plasmid pTAT-HA-survivin and pTAT-HA-survivin-T34A. The cloned DNA fragments were confirmed by DNA sequencing. The structure of pTAT-HA-survivin-T34A is shown in Figure 1A. Plasmids were transformed into *E. coli* BL21 (DE3) pLys S) for expression of the fusion proteins. Figure 1B shows the experimental flowchart.

Expression and purification of TAT-survivin-T34A and TAT-survivin proteins. The expression of proteins was induced with 0.5 M IPTG and incubated at 37˚C for 7 h with high speed shaking (225 rpm). The culture was sonicated and the 6xHis tagged fusion proteins were purified by Ni-NTA affinity chromatography. The expression level of TAT-survivin-T34A (Figure 2A) was lower than that of TAT-survivin (Figure 2B), but both proteins migrated to the predicted molecular weight of 25 kDa. From one liter of culture, 20 mg of purified TAT-Surivin-T34A or 49 mg of TAT-survivin were typically retrieved. The high concentration of imidazole was removed by ion exchange. The proteins were then eluted into 50 mM NaCl, 20 mM Tris-HCl pH8.0. Finally, the proteins were in DMEM without serum by desalting with

disposable PD-10 column filled with Sephadex-25. After determination of protein concentration, the proteins were flash-frozen and stored at –80˚C.

TAT-survivin-T34A induces apoptosis and inhibits proliferation of A549 cells. Previous studies demonstrated that a survivin-T34A mutant interfered with the functions of overexpressed wild-type survivin and hence induced cell apoptosis (16-19). The functions of the purified TAT-survivin-T34A were tested in human lung carcinoma A549 cells. A549 cells were allowed to grow to 30-50% confluence in 6-well plates and treated with wild-type TAT-survivin and mutant TAT-survivin-T34A at the indicated concentrations as shown in Figure 3A. Twenty-four and 48 hours after treatment, total proteins were extracted from each well. Since caspase 3 is one of the major effector caspases in cell apoptosis pathways and its cleavage and activation is a marker of apoptosis, the anti-cleaved caspase 3-specific antibody was used to check the effects of TAT-survivin-T34A on cell apoptosis. As shown in Figure 3A, at 24 h, 0.2 μM TAT-survivin-T34A induced caspase 3 cleavage with no enhanced activation of caspase 3 under higher concentration of TAT-survivin-T34A. At 48 h, activation of caspase 3 was induced by 0.1 μM of TAT-survivin-T34A. Similarly, there was no apparent increase in the cleavage of caspase 3 under higher concentration of TAT-survivin-T34A at 48 h. At both time points, wild-type TAT-survivin did not display effects on the activation of caspase 3 at concentrations of either 0.1 μM or 0.5 μM. Thus, 0.2 μM and 0.5 μM of both proteins were applied in the following experiments. These Western blots demonstrated that recombinant TAT-survivin-T34A is a functional dominant-negative mutant of survivin.

To further test this conclusion, apoptotic cells were stained with EGFP-conjugated Annexin V and were analyzed by flow cytometry. TAT-survivin-T34A 0.2 or 0.5 μM, was directly added to the medium of 30-50% confluent A549 cells in 6-well plates. Twenty-four and 48 h later, cells were stained and subjected to flow cytometry. Compared to TAT-survivin, TAT-survivin-T34A treated cells showed an increased induction of apoptosis (Figure 3B), which was consistent with enhanced activation of caspase 3 by TAT-survivin-T34A (Figure 3A). Surprisingly, wild-type TAT-survivin also induced a certain level of apoptosis (8-15%) compared with untreated controls (3-5%), as TAT-survivin theoretically should inhibit apoptosis. Whether this is due to the non-specific cytotoxicity of TAT fused protein is to be determined. Moreover, there was no increase or even some decrease in apoptosis of cells treated for 48 h compared to those at 24 h. This may be due to the degradation or escape of TAT-survivin-T34A from endosomes.

The effects of TAT-survivin-T34A on cell proliferation were further tested. A549 cells were treated as in Figure 3B, cell number was determined with a hemocytometer after 48 h. In contrast to untreated controls, TAT-survivin-T34A inhibited cell proliferation, while TAT-survivin displayed no apparent effect on cell proliferation.

Discussion

With the commercially available protein purification techniques, a functional cell-permeable dominant-negative survivin-T34A was expressed and purified, and demonstrated pro-apoptotic and anti-proliferative activities in A549 cells, suggesting that targeting survivin by cell permeable peptides is a promising strategy for anticancer drug development.

Cell apoptosis plays a pivotal role in the maintenance of homeostasis of multicellular organisms. Deregulated cell apoptosis results in clinically important malignancies such as cancer and autoimmune diseases (20). One hallmark of cancer is the evasion of apoptosis (21). Among the regulators of apoptotic pathways, members of the IAP family antagonize apoptosis by suppressing the processing and catalytic activity of capases (8). Survivin is a structurally and functionally unique protein of this family (11). Suppression of survivin by oligonucleotides or interference with survivin function by dominant-negative mutant of survivin (13-19) results in polyploidy, aneuploidy and apoptosis, which support the proposal that targeting survivin is a promising strategy for anticancer drug development (11, 22). For instance, the Altieri group has targeted survivin pathway with replication deficient adenovirus encoding a survivin Thr^{34}-Ala mutant, which abolishes a phosphorylation site for $p34^{cdc2}$-cyclin B1. They showed that the survivin mutant virus caused initiation of the mitochondrial apoptotic pathway in various tumor cell types and suppressed tumor growth in three different xenograft breast cancer models *in vivo* (23).

Today, cancer is still being predominately treated by traditional surgery, radiotherapy and chemotherapy, the first generation of cancer drugs based on the theory that cancer cells proliferate and metabolize faster than normal cells (24). With the elucidation of the molecular circuitry of cancer cells and the advance of biotechnology, targeted molecular therapy is the current wave of anticancer drug development (25). This is exemplified by the success of imatinib to target Bcr-Abl (26) and Herceptin™ to target Her2 (27). A favorite druggable target for small molecular inhibitors is believed to be a kinase with a defined catalytic cleft. Current cancer drug development is focusing on targeting the cancer kinome. However, with the advance and wide use of structural biology to provide the exact domains of protein interactions, even to a peptide of several amino acids, targeting of protein-protein interactions is very promising (2).

The research in protein transduction domains (PTD) of naturally occurring or synthetic peptides has advanced very fast and provides an alternative avenue for anticancer drug

development (4). With the progress of macromolecular therapeutics, several protein transduction domains have been identified. Among them, the human immunodeficiency virus (HIV) TAT protein (5), the *Drosophila antennapedia* penetratin (28) and human herpes simplex virus (HSV) tegument VP22 (29) have been well studied. The short 11 amino acid peptide of human immunodeficiency virus PTD of TAT or full-length TAT protein have been efficiently used to deliver peptides, proteins and nucleic acids into many kinds of mammalian cells and helped to address a number of biological questions, such as cell cycle progression, apoptosis and cellular architecture, suggesting TAT is a promising vehicle for post genome macromolecular cancer therapy (5, 30).

With protein transduction domains and advanced techniques for recombinant protein purification, the development of a cancer specific macromolecular therapy to interfere with survivin was attempted by expressing and purifying a cell permeable dominant-negative survivin mutant protein. In order to increase targeting efficiency, an 11 amino acid of human immunodeficiency virus (HIV) TAT protein was fused to the dominant-negative survivin mutant protein. This fusion gene was cloned into the pTAT vector, expressed in *E. coli* and purified by Ni-NTA affinity chromatography. This fusion protein was added directly to cell culture medium at different concentrations to test its anti-proliferation and anti-apoptosis effects on cancer cell lines. Data showed that this TAT-survivin-T34A protein inhibited cell growth and induced cell apoptosis, suggesting that it is promising for further development of this protein as a cancer specific protein therapy.

Acknowledgements

We thank Stephen Meier for technical assistance, Dario C. Altieri (Yale University School of Medicine) for kindly providing plasmids containing the complete ORFs of wild-type survivin and mutant survivin-T34A, and Steven F. Dowdy (Howard Hughes Medical Institute, UCSD School of Medicine) for kindly providing the vector pTAT-HA. This work is partially supported by a German BMBF grant to Sven N. Reske.

References

1 Kaelin WG Jr: The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer *5*: 689-698, 2005.
2 Collins I and Workman P: New approaches to molecular cancer therapeutics. Nat Chem Biol *2*: 689-700, 2006.
3 Fichou Y and Ferec C: The potential of oligonucleotides for therapeutic applications. Trends Biotechnol *24*: 563-570, 2006.
4 Deshayes S, Morris MC, Divita G and Heitz F: Cell-penetrating peptides: tools for intracellular delivery of therapeutics. Cell Mol Life Sci *62*: 1839-1849, 2005.
5 Schwarze SR, Ho A, Vocero-Akbani A and Dowdy SF: *In vivo* protein transduction: delivery of a biologically active protein into the mouse. Science *285*: 1569-1572, 1999.
6 Fulda S, Wick W, Weller M and Debatin KM: Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma *in vivo*. Nat Med *8*: 808-815, 2002.
7 Goldsmith KC, Liu X, Dam V, Morgan BT, Shabbout M, Cnaan A, Letai A, Korsmeyer SJ and Hogarty MD: BH3 peptidomimetics potently activate apoptosis and demonstrate single agent efficacy in neuroblastoma. Oncogene *25*: 4525-4533, 2006.
8 Deveraux QL and Reed JC: IAP family proteins – suppressors of apoptosis. Genes Dev *13*: 239-252, 1999.
9 Ambrosini G, Adida C and Altieri DC: A novel anti-apoptosis gene, *survivin*, expressed in cancer and lymphoma. Nat Med *3*: 917-921, 1997.
10 Velculescu VE, Madden SL, Zhang L, Lash AE, Yu J, Rago C, Lal A, Wang CJ, Beaudry GA, Ciriello KM, Cook BP, Dufault MR, Ferguson AT, Gao Y, He TC, Hermeking H, Hiraldo SK, Hwang PM, Lopez MA, Luderer HF, Mathews B, Petroziello JM, Polyak K, Zawel L, Zhang W, Zhang X, Zhou W, Haluska FG, Jen J, Sukumar S, Landes GM, Riggins GJ, Vogelstein B and Kinzler KM: Analysis of human transcriptomes. Nat Genet *23*: 387-388, 1999.
11 Altieri DC: Survivin, cancer networks and pathway-directed drug discovery. Nat Rev Cancer *8*: 61-70, 2008.
12 Ling X and Li F: Silencing of antiapoptotic survivin gene by multiple approaches of RNA interference technology. Biotechniques *36*: 450-454, 456-460, 2004.
13 Grossman D, McNiff JM, Li F and Altieri DC: Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. J Invest Dermatol *113*: 1076-1081, 1999.
14 Pennati M, Colella G, Folini M, Citti L, Daidone MG and Zaffaroni N: Ribozyme-mediated attenuation of survivin expression sensitizes human melanoma cells to cisplatin-induced apoptosis. J Clin Invest *109*: 285-286, 2002.
15 Shen C, Buck A, Polat B, Schmid-Kotsas A, Matuschek C, Gross HJ, Bachem M and Reske SN: Triplex-forming oligodeoxynucleotides targeting survivin inhibit proliferation and induce apoptosis of human lung carcinoma cells. Cancer Gene Ther *10*: 403-410, 2003.
16 Grossman D, Kim PJ, Schechner JS and Altieri DC: Inhibition of melanoma tumor growth *in vivo* by survivin targeting. Proc Natl Acad Sci USA *98*: 635-640, 2001.
17 Li F, Ackermann EJ, Bennett CF, Rothermel AL, Plescia J, Tognin S, Villa A, Marchisio PC and Altieri DC: Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. Nat Cell Biol *1*: 461-466, 1999.
18 Kanwar JR, Shen WP, Kanwar RK, Berg RW and Krissansen GW: Effects of survivin antagonists on growth of established tumors and B7-1 immunogene therapy. J Natl Cancer Inst *93*: 1541-1552, 2001.
19 Yan H, Thomas J, Liu T, Raj D, London N, Tandeski T, Leachman SA, Lee RM and Grossman D: Induction of melanoma cell apoptosis and inhibition of tumor growth using a cell-permeable survivin antagonist. Oncogene *25*: 6968-6974, 2006.
20 Igney FH and Krammer PH: Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer *2*: 277-288, 2002.
21 Hanahan D and Weinberg RA: The hallmarks of cancer. Cell *100*: 57-70, 2000.
22 Altieri DC: Validating survivin as a cancer therapeutic target. Nat Rev Cancer 3: 46-54, 2003.

23 Mesri M, Wall NR, Li J, Kim RW and Altieri DC: Cancer gene therapy using a survivin mutant adenovirus. J Clin Invest *108*: 981-990, 2001.
24 Varmus H: The new era in cancer research. Science *312*: 1162-1165, 2006.
25 Sawyers C: Targeted cancer therapy. Nature *432*: 294-297, 2004.
26 Druker BJ: Imatinib as a paradigm of targeted therapies. Adv Cancer Res *91*: 1-30, 2004.
27 Shepard HM, Jin P, Slamon DJ, Pirot Z and Maneval DC: Herceptin™ . Handb Exp Pharmacol *181*: 183-219, 2008.
28 Gratton JP, Yu J, Griffith JW, Babbitt RW, Scotland RS, Hickey R, Giordano FJ and Sessa WC: Cell-permeable peptides improve cellular uptake and therapeutic gene delivery of replication-deficient viruses in cells and *in vivo*. Nat Med *9*: 357-362, 2003.
29 Elliott G and O'Hare P: Intercellular trafficking and protein delivery by a herpesvirus structural protein. Cell *88*: 223-233, 1997.
30 Becker-Hapak M, McAllister SS and Dowdy SF: TAT-mediated protein transduction into mammalian cells. Methods *24*: 247-256, 2001.\

Received October 2, 2008
Revised January 7, 2009
Accepted January 19, 2009