**Proapoptotic Activity of Cell-Permeable Anti-Akt Single-Chain Antibodies**

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Abstract

We developed anti-Akt1 single-chain antibodies (scFv) by panning a mouse phage–displayed scFv recombinant antibody library. Recombinant scFv that bound glutathione S-transferase (GST)-Akt1 were screened for their ability to inhibit Akt activity in vitro in a kinase reaction containing human recombinant Akt1 and an Akt/serum glucocorticoid-inducible kinase (SGK) substrate. Michaelis-Menten analysis of kinase inhibition by a selected scFv was consistent with scFv-mediated competition with enzyme's substrate for the catalytic site of Akt. To generate a membrane-permeable version of the anti-Akt1 scFv, the scFv gene was subcloned into a GST expression vector carrying a membrane-translocating sequence (MTS) from Kaposi fibroblast growth factor. A purified GST–anti-Akt1–MTS fusion protein accumulated intracellularly in 293T, BT-474, and PyVmT cells in a dose- and sequence (MTS) from Kaposi fibroblast growth factor.

GST–anti-Akt1–MTS is a novel cell-permeable inhibitor of Akt, capable of suppressing Akt activity in intact cells both in vitro and in vivo. 

Introduction

Antibodies and antibody-based reagents have been used for the treatment of cancer (1, 2). For example, the humanized IgG1 trastuzumab (Herceptin) is an effective treatment for breast cancers that overexpress the HER2/neu (erbB2) proto-oncogene (3). The chimeric IgG2 cetuximab (Erbitux, C225) was recently approved for the treatment of epidermal growth factor (EGF) receptor–positive metastatic colorectal carcinoma (4). Genetic engineering of antibodies can be used to modify and enhance antibody efficacy. For example, mouse monoclonal antibodies can be chimerized by such approaches to prevent the production of human antimurine antibodies (HAMA) when administered to immune-competent humans (5). An alternative strategy is to replace the antibody gene present in mouse B cells with human antibody genes. These modified B cells can then be used to produce hybridoma cell lines that express fully humanized monoclonal antibodies that avoid cross-species immune response (i.e., HAMA) and, in addition, can trigger human host cell effector functions, such as complement fixation (6).

The two variable domains of an antibody binding site can be cloned and reconstituted in a variety of molecular forms and expressed in various hosts from bacteria to transgenic animals and plants (7). Over a decade ago, McCafferty et al. (8) first described that recombinant antibody fragments could be displayed on the tip of M13 bacteriophage, a bacterial virus (phage) than infects Escherichia coli. Some of the advantages of phage-displayed recombinant antibodies over the conventional polyclonal or monoclonal antibodies are quick generation time, cheap production cost, and, importantly, accessibility to the antibody DNA for further genetic manipulations (9).

The serine-threonine kinase protein kinase B or Akt, the cellular homologue of the AKT8 retrovirus oncogene, has been shown to play an important role in cell survival and proliferation (10, 11). The antiapoptotic functions of Akt are mediated, in part, by phosphorylation and functional inactivation of proapoptotic molecules, such as Bad (12), Forkhead transcription factors (13), and caspase-9 (14) among others. By promoting cell survival and proliferation, Akt signaling contributes to cancer progression (15). Akt1 gene amplification was first described in gastric adenocarcinomas (16). The Akt2 gene is amplified in some ovarian and breast carcinomas (17). High levels of Akt2 protein have been reported in pancreatic adenocarcinoma (18).

The potential involvement of Akt activity in cancer progression has suggested its role as a therapeutic target. Inhibition of Akt signaling has been accomplished with LY294002 (19) and Wortmannin (20), two small-molecule inhibitors of phosphatidylinositol-3 kinase (PI3K), a lipid kinase upstream of Akt (21). However, these small molecules are not suitable for use in humans. In addition, because of the many cellular targets of this lipid kinase, use of PI3K inhibitors may be associated with undesirable side effects (22). In this report, we describe the development of a novel recombinant Akt inhibitor that binds to and specifically blocks Akt activity in vitro and in intact cells. Recombinant single-chain antibodies (scFv) from a mouse phage–displayed antibody library
that recognized Akt1 were screened for inhibitory activity against the Akt kinase. To generate a fusion protein that can translocate into cells, the gene from the bacterial clone producing the anti-Akt scFv was subcloned into an expression vector containing a membrane-translocating sequence (MTS; refs. 23, 24). We describe, herein, a chimeric glutathione S-transferase (GST)–anti-Akt1–MTS protein that is efficiently imported into intact cells in an MTS-dependent manner, resulting in inhibition of Akt function and, in turn, tumor cell survival in vivo.

Materials and Methods

Expression of glutathione S-transferase–Akt1 fusion protein. The Akt1 plasmid was provided by Bakesh Kumar (University of Texas M. D. Anderson Cancer Center, Houston, TX). Akt1 was PCR-amplified and subcloned into the BamHI site of the GST expression vector pGEX-3X (Amersham, Piscataway, NJ) via TA cloning vector pGEM-T-Easy (Promega, Madison, WI). The primers used, each containing BamHI sites at their 5' end, were 5'CCGGATCCCTGGACACCTAGGGATCCGGT-3' and 5'-CCGGATCCCCGGCCGCGGTTCCAGCGGATCCGGATCTCCG-3'. DNA sequencing indicated that the Akt1 gene was inserted in the right orientation and in frame with GST. The resulting pGEX-3X-Akt1 was transformed into E. coli DH5α. Akt1–GST fusion protein expression and purification by reduced glutathione (GSH)–agarose affinity chromatography were done as previously described (23).

Panning of phage-displayed single-chain antibody library and antibody purification. One milligram of GST-Akt1 fusion protein was dialyzed against 5,000 volumes of 0.2 mol/L NaCl for 48 hours. The fusion protein was concentrated by ultrafiltration using a PM10 membrane (Amicon, Beverly, MA) and then used for phage selection and ELISA assays as described in Supplementary Methods.

Recombinant Akt1 kinase assay and single-chain antibody kinase inhibition analysis. Preactivated Akt1 [with mitogen-activated protein kinase (MAPK)–activated protein (MAPKAP) kinase 2, Upstate Biotechnology, Lake Placid, NY] was dissolved in assay dilution buffer [ADB; 20 mmol/L MOPS (pH 7.5), 25 mmol/L β-glycerophosphate, 5 mmol/L EGTA, 1 mmol/L Na3VO4, 1 mmol/L DTT]. E-tag ELISA-positive scFvs (0.15 μg each) were dissolved in 10 μL ADB and incubated with 0.15 μg recombinant Akt1 (rAkt1) for 1 hour at room temperature. An irrelevant scFv (0.15 μg) was used as control. The kinase reaction was started by the addition of 40 μmol/L ADB/sucrose/corticoid-inducible kinase (SGK) substrate peptide (Upstate Biotechnology) and 0.2 μCi [γ-32P]ATP (specific activity 3,000 Ci/mmol, DuPont-NEN, Boston, MA) diluted in 10 μL of 500 μmol/L cold ATP containing 75 mmol/L MgCl2. After a 10-minute incubation at 30°C with continuous shaking, the reaction was quenched with the addition of 20 μL of 40% trichloroacetic acid; 25 μL of the reaction supernatant were spotted onto phosphocellulose paper disks (Upstate Biotechnology). The disks were washed thrice with 0.75% phosphoric acid and once with acetone (5 minutes each). Disk-associated dpm values were measured in a liquid scintillation counter (Beckman, Fullerton, CA).

To determine the kinetics of the rAkt1 kinase reaction, Michaelis-Menten plots and corresponding double-reciprocal Lineweaver-Burk plots were generated. Kinase reactions were done in vitro with Akt/SGK substrate concentrations ranging from 10 to 480 μmol/L in the absence or presence of the anti-Akt scFv. rAkt1 (0.15 μg) was preincubated with 0.45 μg scFv (clone N5) for 1 hour before the kinase reaction. Dpm values incorporated onto the Akt/SGK substrate were converted to Ci by using the dpm values in standard samples. The activity in Ci was transformed to moles units using the specific activity of [γ-32P]ATP. Nonlinear Michaelis-Menten regression plots and Lineweaver-Burk plots were generated by GraphPad Prism software version 3.03 (GraphPad Software, San Diego, CA).

Generation of glutathione S-transferase–anti-Akt1–MTS fusion protein. The scFv gene in pCANTAB 5E phagemid clone N5 was PCR amplified. The sequences of degenerative primers used were 5'-CGGATCCATGCGCAAGTSMARCCTGACGASGTWCWG-3' and 5'-CGGATCCCTGGCCGCGGTTCCAGCGGATCTCCG-3'. The PCR product including the E-tag at the 5' end of VJ1 was first subcloned into pGEM-T-Easy, digested with BamHI, and ligated in BamHI-digested pGEX-3X-MTS2 (a gift from Mauricio Rojas, Emory University, Atlanta, GA; ref. 23). DNA sequence analyses confirmed that GST, scFv, and MTS translational frames were maintained. To test the specificity of GST–anti-Akt1–MTS, an irrelevant control scFv was also subcloned into pGEX-3X-MTS2. The expression and purification of the GST fusion proteins was done as described (23).

Cell lines. 293T human embryonic fibroblasts, BT-474 human breast cancer, and U87-MG human glioma cells were purchased from American Type Culture Collection (Rockland, ME). The PyVMt mouse cell line derived from a mammalian tumor arising in a mouse mammary tumor virus/polyomavirus middle T antigen (MMTV/PyVMt) transgenic mouse and has been described previously (25). All cell lines, except BT-474 cells, were maintained in DME/10% FCS. BT-474 cells were kept in IMEM/10% FCS.

Immunofluorescence analysis and confocal microscopy. Subconfluent cell monolayers on cover slips placed in 12-well plates were treated with 10 μmol/L GST–anti-Akt1–MTS or GST-MTS for 6 hours. The cells were washed five times with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton in PBS, and then incubated with 3% milk in PBS for 30 minutes followed by an E-tag monoclonal antibody (0.5 μg/mL in 1% milk in PBS; Amersham) for 1 hour. After three washes with PBS, the samples were incubated with Oregon green-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) for 1 hour. The cover slips were mounted on glass slides with Poly/Mount (Polysciences, Warrington, PA) and subjected to Z-axis optical sectioning (1 μm/section) in a Zeiss laser scanning confocal microscope (LSM 510).

Immunoblot analysis. Subconfluent cell monolayers were treated with GST–anti-Akt1–MTS or GST–control scFv–MTS. The cells were washed five times with PBS and then lysed in a buffer containing 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP40, 10% glycerol, 20 mmol/L NaF, 1 mmol/L Na3VO4, and protease inhibitor cocktail (Roche, Nutley, NJ). Protein extracts (30 μg/lane) were separated by 10% SDS-PAGE and subjected to immunoblot analysis as described (26). Horseradish peroxidase–conjugated secondary antibodies were from Amersham; immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Pierce, Rockford, IL). Akt, p-ser473 Akt, p-Thr308 Akt, p-p38, GSK-3α, p-GSK-3α, cleaved caspase-3, phosphoinositide-dependent kinase 1 (PDK1), and p-ser341 PDK1 antibodies were from Cell Signaling (Beverly, MA). Antibodies to p-MAPK and total MAPK were from Promega. The monoclonal antibody to p38 was from Santa Cruz Biotechnology (Santa Cruz, CA). The GST and E-tag antibodies were from Amersham. The HER2 and p-Tyr antibodies were from Neomarkers (Freemont, CA) and Upstate Biotechnology, respectively. ZD1839 (Iressa, gefitinib) was provided by Alan Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom).

Expression of Akt isoforms. Hemagglutinin-tagged Akt1DD, Akt2DD, myr-Akt1, myr-Akt2, and myr-Akt3 were provided by Gordon Mills (University of Texas M. D. Anderson Cancer Center). 293T cells on 100-mm dishes were transfected with 5 μg of each plasmids using Fugene 6 (Roche). After 48 hours, the cells were lysed in NP40 buffer (described above). Cell lysates (500 μg) were precipitated with a hemagglutinin antibody (1 μg; Santa Cruz Biotechnology) and protein A-Sepharose (50% slurry) overnight at 4°C. The precipitated hemagglutinin-Akt was washed twice with lysis buffer and twice with (cold) kinase buffer (25 mmol/L Tris (pH 7.5), 5 mmol/L β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na3VO4, 10 mmol/L MgCl2). Pellets were suspended in 40 μL kinase buffer supplemented with 200 μmol/L ATP and 1 μg of a fusion protein containing a GSK-3β peptide (Cell Signaling) and incubated for 30 minutes at 30°C. The kinase reaction was terminated by addition of SDS sample buffer and boiling. Reaction products were subjected to SDS-PAGE and immunoblot analyses with total and phospho-GSK-3β/α antibodies.

Apoptosis assay. Cells (5 × 105/well) were seeded in six-well plates in triplicate in serum-containing medium. The following day, the medium was changed to serum-free medium with 10 μmol/L GST–control scFv–MTS or...
GST–anti-Akt1–MTS. Both floating cells and adherent cells were collected 72 hours later. Pooled cells were washed with PBS and then subjected to terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) analysis with the use of an Apo-bromodeoxyuridine (BrdUrd) assay kit (Phoenix Flow Systems, San Diego, CA) according to the manufacturer’s protocol. TUNEL+ cells were quantitated in a FACSCalibur Flow Cytometer (BD Biosciences, Mansfield, MA).

Tumor window chamber model. This model has been described previously (27). Briefly, a small metal frame was surgically implanted in a dorsal skin flap in FVB mice. An 8-mm-diameter hole was dissected in the surface of the skin flap, which was next retracted away from the s.c. dermis. The underlying fascia was dissected away until a facial plane with associated vasculature was exposed. An 0.5 mm3 fragment from an MMTV/PyVmT transgenic mammary tumor (25) was implanted onto the facial plane of the window. Sterile saline solution (100 mL) was added and the window was sealed with glass cover slips to protect it from the outside environment. The resulting tissue plane within the window is 200 mm thick and is semitransparent. Mice bearing the dorsal chambers were housed individually in pathogen-free units in compliance with Vanderbilt University Institutional Animal Care and Use Committee regulations. Upon the formation of vascularized tumors in the window chambers after 7 days, mice were randomly assigned to receive anti-Akt scFv or control antibody, respectively. The first treatment was injected locally into the window chamber (120 g/mouse in a 100 mL volume), followed by two similar doses delivered i.p. every other day. Tumor neovascularization was scored using an Olympus PROVIS AX70 microscope (Olympus, New York, NY) on days 3 and 7. The tumor vascular length density as a functional index of tumor neovascularization was calculated from the tumor images within the window chambers using previously described methods (27). After 7 days of treatment, tumor tissues were harvested and subjected to H&E staining and TUNEL analysis as described (25). To obtain tumor size, H&E-stained sections representing the largest cross-sectional areas were photographed and both tumor thickness and diameter were used to calculate tumor volume in mm3 as described (27).

Statistical analysis. Results were reported as mean ± SD for both tumor volume and vascular density for each group. A two-tailed Student’s t test was used to analyze statistical differences between them. Differences were considered to be statistically significant at \( P < 0.05 \).

Results

Anti-Akt1 single-chain antibodies competitively inhibit Akt kinase in vitro. A GST-Akt1 fusion protein was utilized to pan a large (\( \sim 2.9 \times 10^9 \) members) mouse phage–displayed scFv library. The first treatment was injected locally into the window chamber (120 g/mouse in a 100 mL volume), followed by two similar doses delivered i.p. every other day. Tumor neovascularization was scored using an Olympus PROVIS AX70 microscope (Olympus, New York, NY) on days 3 and 7. The tumor vascular length density as a functional index of tumor neovascularization was calculated from the tumor images within the window chambers using previously described methods (27). After 7 days of treatment, tumor tissues were harvested and subjected to H&E staining and TUNEL analysis as described (25). To obtain tumor size, H&E-stained sections representing the largest cross-sectional areas were photographed and both tumor thickness and diameter were used to calculate tumor volume in mm3 as described (27).
More than 50 scFv clones interacted with GST-Akt1 as determined by E-Tag ELISA. To screen for scFv-mediated inhibition of Akt activity, we developed an in vitro kinase assay utilizing rAkt1 and a synthetic Akt/SGK peptide. In dose-dependent experiments, 0.15 μg rAkt1 showed kinase activity within a linear range (Fig. IA). Using this concentration of rAkt1, maximal incorporation of \(^{32}P\)ATP onto the SGK substrate occurred after 40 minutes with 50% incorporation being achieved after only 10 minutes of reaction time (Fig. 1B). Anti-Akt1 scFv phage, which bound to GST-Akt1, was screened for its inhibitory activity against Akt in the kinase reaction. Of >50 scFv tested, two clones (H7 and N5) exhibited a dose-dependent inhibitory effect against Akt in vitro (Fig. 1C).

Akt activity can be regulated by site-specific phosphorylation. Thr\(^{308}\) and Ser\(^{473}\) phosphorylation have been shown to be required for full activation of Akt1 (10). Therefore, if the scFvs inhibit Akt by binding to either of these sites and blocking their phosphorylation, we proposed that the antibodies will act as allosteric inhibitors and the kinetics of enzyme inhibition will be noncompetitive with the kinase substrate. An alternative inhibitory mechanism would be binding of the scFv to the catalytic site of Akt1. In this scenario, the inhibitor should compete with the enzyme substrate for the active site of Akt1, thus showing kinetics of competitive inhibition. To elucidate the mechanism of scFv-mediated inhibition of Akt, Michaelis-Menten enzyme kinetics and double-reciprocal Lineweaver-Burk plots were generated using varying concentrations of enzyme substrate (Fig. 1D). The anti-Akt1 scFv inhibited rAkt1 activity at SGK substrate concentrations ranging from 10 to 480 μmol/L, suggesting that the scFv can compete with the enzyme’s substrate for binding to Akt1. Lineweaver-Burk plots revealed a \(V_{\text{max}}\) for the kinase reaction of 30.03 and 32.88 fmol \(^{32}P\)/min in the presence and absence of the anti-Akt1 scFv, respectively. Consistent with the competitive inhibition of rAkt1, the \(K_m\) (the concentration of substrate showing half-maximal velocity) of the reaction was 24.81 versus 75.84 μmol/L in the absence and presence of the anti-Akt1 scFv, respectively.

**Anti-Akt1 single-chain antibodies localize intracellularly and bind to and inhibit Akt.** To deliver the anti-Akt1 scFv intracellularly, we generated a fusion protein that also contained a MTS derived from the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor (24). This modified 12 amino acid sequence (AAVLLPVLLAAP) functions as a cellular import signal. Therefore, we subcloned the anti-Akt1 scFv gene from clone N5 phagemid DNA (Fig. 2A) into pGEX-3X-MTS2 by PCR with degenerative primers. A PCR product of ~670 bp containing both antibody DNA heavy chain (≈340 bp) and light chain (≈325 bp) chains and linker DNA (9) was identified on agarose gels (Fig. 2B). The resulting 60 kDa fusion recombinant scFv containing GST on its amino terminus and MTS on its carboxyl terminus was expressed in E. coli and purified by GSH-agarose affinity chromatography (Fig. 2C).

Human embryonic 293T fibroblasts, BT-474 human breast cancer cells, and mouse cancer cells expressing the PyVmT (middle T antigen) oncogene were used to determine if the recombinant fusion protein could penetrate the membranes of living cells. After treatment with purified GST–anti-Akt1–MTS or GST-MTS, the cells were assayed by immunofluorescence microscopy using a monoclonal antibody specific for the E-tag present in the scFv. Whereas GST-MTS–treated cells did not show any E-tag fluorescent signal, GST–anti-Akt1–MTS–treated cells exhibited a punctate staining pattern (Fig. 3A). In 293T and BT-474 cells, GST–anti-Akt1–MTS was found mainly in the cytosol whereas in PyVmT-expressing cells the fluorescent signal was more diffuse. Staining with a GST antibody showed fluorescent signals in both GST-MTS– and GST–anti-Akt1–MTS–treated cells (data not shown). To confirm that GST–anti-Akt1–MTS was localized intracellularly, we used confocal laser scanning microscopy. Z-axis optical sectioning from bottom (0 μmol/L) to top (11 μmol/L) of cells indicated that the GST–anti-Akt1–MTS fusion protein localized in the midsections of the cells, supporting that intracellular translocation had occurred (Fig. 3B and C).

**Figure 2.** PCR amplification of scFv gene from pCANTAB SE. **A,** schematic map of the pCANTAB SE plasmid containing the scFv gene library. **B,** agarose gel showing the PCR-amplified scFv gene from the N5 clone. **C,** pGEX-3X-MTS2 containing the scFv gene was transformed in E. coli (BL21). Expression of the GST fusion protein was induced by 0.1 mM IPTG. The bacterial lysates were resolved on SDS-PAGE and stained with Coomassie blue. Lanes 1 to 4, empty vector encoding GST-MTS; lanes 5 and 6, clones transformed with GST–anti-Akt1–MTS.
We next determined whether the recombinant scFv inhibited Akt signaling in intact cells. 293T cells were treated with GST–anti-Akt1–MTS or control (GST-MTS) for 24 hours. Cells were then permeabilized, blocked with 5% bovine serum albumin, and stained with an E-tag monoclonal antibody (1:2,000) for 1 hour. After washing thrice with PBS, cells were incubated with anti-mouse IgG-Oregon green (1:1,000) for 1 hour. The samples were washed with PBS, mounted on glass slides, and observed with a confocal microscope. Figures 3A, 3B, and 3C, the same samples were subjected to serial Z-coordinate optical sections by using the Z-sectioning function of a Zeiss laser-scanning confocal microscope: 293T (B) and BT-474 (C).

Figure 3. Intracellular localization of GST–anti-Akt1–MTS. A, 293T, BT-474, and PyVmT cells on cover slips were treated with 10 μmol/L GST–anti-Akt1–MTS or control (GST-MTS) for 24 hours. Cells were then permeabilized, blocked with 5% bovine serum albumin, and stained with an E-tag monoclonal antibody (1:2,000) for 1 hour. After washing thrice with PBS, cells were incubated with anti-mouse IgG-Oregon green (1:1,000) for 1 hour. The samples were washed with PBS, mounted on glass slides, and observed with a confocal microscope. B and C, the same samples were subjected to serial Z-coordinate optical sections by using the Z-sectioning function of a Zeiss laser-scanning confocal microscope: 293T (B) and BT-474 (C).

Antitumor Effect of Intracellular Akt Antibodies
as measured by immunoblot analyses using phosphospecific antibodies, were not modified by the anti-Akt1 scFv, suggesting that its effects were Akt specific. This inhibition was dose dependent with partial reduction of p-Ser\(^{\text{Tyr}}\) Akt and p-GSK at a concentration of 1 \(\mu\text{mol/L}\). GST-anti-Akt1-MTS (Fig. 4B). The GST-scFv-MTS control fusion also transferred efficiently into 293T cells (10 \(\mu\text{mol/L}, 6\) hours) as indicated by GST immunoblot. However, it did not inhibit p-Ser\(^{\text{Tyr}}\) Akt and GSK-3\(\beta\) phosphorylation (Fig. 4B, lane 5). To confirm binding of the GST-anti-Akt1-MTS to its target (Akt) intracellularly, we precipitated Akt from lysates of cells treated with anti-Akt or control scFv. The anti-Akt scFv, as measured by E-tag immunoblot, but not the control antibody, was present in the Akt precipitates (Fig. 4C), thus providing direct evidence of association of GST-anti-Akt-MTS with its molecular target in intact cells.

Similar findings were observed in BT-474- and PyVmT-expressing cells. Both cells exhibit constitutive activation of Akt. In BT-474 cells, it has been suggested that this is the result of HER2 (erbB2) overexpression and heterodimerization with erbB3 (26). In PyVmT transgenic cells, middle T antigen binds to and potently activates PI3K and Akt (28). In all three cell lines, GST-anti-Akt1-MTS effectively blocked Ser\(^{\text{Tyr}}\) Akt and GSK-3\(\beta\) phosphorylation (Fig. 4D). However, phosphorylation of Akt on Thr\(^{308}\), the PDK1 phosphorylation site (10), and autophosphorylation of PDK1 on Ser\(^{341}\) were not affected. Again, active MAPK and active p38 were not inhibited by the anti-Akt1 fusion protein. Because activation of HER2-containing receptor heterodimers activates PI3K/Akt signaling in HER2 overexpressing cells, such as BT-474 (26), we controlled for the effect of GST-anti-Akt1-MTS on HER2 phosphorylation. GST-anti-Akt1-MTS did not inhibit HER2 levels or its phosphorylation as measured by p-Tyr immunoblot of HER2 precipitates from treated BT-474 cells. The small-molecule EGF receptor tyrosine kinase inhibitor ZD1839, which has been shown to inhibit HER2 phosphorylation in BT-474 cells (30), was used as a positive control (Fig. 4E).

**Anti-Akt1 single-chain antibodies inhibit Akt2 and Akt3 and induce apoptosis in vivo.** To determine the effect of GST-anti-Akt1-MTS on other Akt isoforms, hemagglutinin-tagged, constitutively active versions of Akt2 and Akt3 were expressed in 293T cells (24). A phosphomimetic mutant of Akt1, where Thr\(^{306}\) and Ser\(^{373}\) are replaced with Asp (Akt1DD), has been shown to exhibit constitutive Akt catalytic activity (31). In this study, we used an Akt2\(^{D\text{D}}\) mutant, where Asp has replaced Thr\(^{309}\) and Ser\(^{474}\), corresponding to Thr\(^{308}\) and Ser\(^{473}\) in Akt1.
and Ser473 in Akt1 (32). Addition of a c-Src–derived myristoylation (myr) signal (MGSSKSKPK) to the amino terminus of Akt renders its kinase constitutively active by tethering Akt to the plasma membrane (33). Expression of Akt1DD, Akt2DD, myr-Akt1, myr-Akt2, and myr-Akt3 in transfected 293T cells was confirmed by hemagglutinin immunoblot analysis (Fig. 5A).

Hemagglutinin precipitates from 293T cells transfected with the Akt isoform vectors were preincubated with GST–anti-Akt1–MTS or its control and then tested in the Akt in vitro kinase reaction using 200 μmol/L cold ATP and 1 μg GSK-3β fusion protein. Before addition to the kinase reaction, the hemagglutinin precipitates were incubated for 1 hour at room temperature with 1 μg GST–anti-Akt1–MTS or GST–control scFv–MTS. Kinase reaction products were resolved by SDS-PAGE followed by immunoblot analyses with p-GSK-3β and total GSK-3β antibodies.

Akt function is causally associated with cell survival (10, 11). Thus, we next examined if GST–anti-Akt1–MTS exhibited proapoptotic effects.

Figure 6. GST–anti-Akt1–MTS induces apoptosis of cells in culture. A, 293T, BT-474, PyVmT, and U87-MG cells were treated with 10 μmol/L GST–anti-Akt1–MTS or GST–control scFv–MTS in serum-free medium for 72 hours. Controls were maintained in 10% fetal bovine serum. Cell lysates were subjected to an immunoblot procedure using an antibody specific to cleaved caspase-3. B, cells were treated as in A and fixed in 1% paraformaldehyde. The proportion of apoptotic cells was quantitated by Apo-BrdUrd analysis and flow cytometry as described in Materials and Methods. The proportion of FITC+ (TUNEL+) cells ± SD (n = 3 wells) is indicated in the gated area in each panel.
activity in cell lines with constitutive Akt activity. U87-MG human glioma cells contain a homozygous deletion of the phosphatase PTEN resulting in high PI3K and Akt activities (34). Subconfluent 293T, BT-474, PyVmT, and U87-MG cells were treated with GST–anti-Akt1–MTS or GST–control scFv–MTS. All four cell lines exhibited markedly enhanced cleavage of caspase-3, a hallmark of apoptosis (35), when treated for 72 hours with the anti-Akt1 scFv but not with the control fusion protein (Fig. 6A). Furthermore, quantification of cell death by TUNEL analysis of all four cell lines showed a 2-fold to >4-fold increase in the proportion of TUNEL+ cells as a function of treatment with the anti-Akt fusion protein (Fig. 6B). Furthermore, quantification of cell death by TUNEL analysis of all four cell lines showed a 2-fold to >4-fold increase in the proportion of TUNEL+ cells as a function of treatment with the anti-Akt1 fusion protein (Fig. 6B).

Finally, we determined the inhibitory activity of GST–anti-Akt1–MTS against MMTV/PyVmT transgenic tumor transplants in vivo. Tumorigenicity and metastases in this transgenic mouse model of mammary cancer depend on the ability of middle T to activate PI3K, the major kinase upstream Akt (28). In addition, inhibition of PI3K and Akt in these cells has been shown to reduce cell motility, survival, and metastases (25, 36). Mice bearing established and vascularized MMTV/PyVmT transgenic transplants in dorsal mouse chambers were treated systemically with GST–anti-Akt1–MTS or the control scFv. Both tumor volume and tumor neovascularization, as inferred from the vascular length density, were markedly reduced in tumors present in mice treated with the anti-Akt scFv treated for 7 days compared with controls (Fig. 7A). In addition, there was an 8-fold increase in the average proportion of TUNEL+ cells in the former tumors compared with those treated with the control scFv (Fig. 7B).

**Discussion**

The serine-threonine kinase Akt promotes tumor cell proliferation and survival both in vitro and in vivo. Several components of the PI3K/Akt pathway are dysregulated in a variety of human cancers. These include inactivation and mutation of the tumor suppressor phosphatase PTEN (34, 37, 38), PI3K gene amplification and/or mutation (39–42), and Akt gene amplification or Akt protein overexpression (16, 17). Studying the specific role of Akt in cancer biology has been somewhat limited by the lack of specific inhibitors of Akt function. We report herein that phage-displayed scFvs screened for inhibitory activity in vitro against the Akt kinase can be genetically engineered to acquire cell membrane permeability and inhibit Akt function in intact cells. GST–anti-Akt1–MTS specifically inhibited Akt but had no detectable activity against...
PKD-1, MAPK, p38, and HER2 kinases (Fig. 4). This effect cannot be subscribed to GST action in that a similar fusion protein containing an irrelevant scFv had no effect on Akt or cell survival (Figs. 6 and 7) both in cultured cells and in a P3K/Akt-dependent tumor model in vivo. GST–anti-Akt1–MTS also inhibited constitutive active versions of Akt2 and Akt3 in vitro (Fig. 5). Because the anti-Akt1 scFv acts as a competitive substrate inhibitor for rAkt1 (Fig. 1D), the anti-pan Akt activity of GST–anti-Akt1–MTS (Fig. 5B) suggests that the fusion protein binds the catalytic domain of the enzyme, which is highly conserved in all three Akt isoforms (10).

Treatment with GST–anti-Akt1–MTS resulted in inhibition of Ser473 but not Thr308 phosphorylation in Akt1 (Fig. 4). Although PKD1 has been well characterized as the kinase that phosphorylates Thr308 of Akt1 (11), the kinase responsible for Ser473 phosphorylation is a matter of debate (reviewed in ref. 43). In the presence of PKD1-interacting fragment from protein kinase C–related kinase-2, PKD1 phosphorylates both Thr308 and Ser473 of Akt1 in a phosphatidylinositol 3,4,5-triphosphate (PIP3)-dependent manner (44). Integrin-linked kinase has also been shown to regulate phosphorylation of Ser473 in Akt1 (45). For example, knocking-out integrin-linked kinase by double-stranded RNA interference inhibits Ser473 phosphorylation of Akt without affecting Thr308 phosphorylation (46). However, another report suggested that kinase-dead integrin-linked kinase may contribute to Ser473 phosphorylation by providing an adaptor function and, thus, recruiting other kinases to Akt (47). Akt Ser473 kinase activity was also found in detergent-insoluble plasma membrane rafts, which did not contain integrin-linked kinase (48). Protein kinase C βII has also been shown to phosphorylate Akt on Ser473 in a cell-type-specific fashion (49). Another study with kinase-dead and heat-inactivated Akt suggested that Ser473 is autophosphorylated by Akt itself (50). Our results support this last possibility in that the recombinant scFv exhibited kinetics consistent with competition with the kinase substrate, whereas blocking phosphorylation of Akt at Ser473 and downstream GSK-3β phosphorylation. However, we could not rule out the possibility that binding of GST–anti-Akt1–MTS to Akt may sterically hinder access of other kinase(s) capable of phosphorylating Ser473.

Recent reports have claimed the development of Akt inhibitors. Derivatives of dichlorotriazine and dichloropyrimidine were reported as Akt3 inhibitors with an IC50 of <1 μmol/L. However, these compounds were also effective against EGFR and insulin-like growth factor receptor tyrosine kinases (51). A series of phosphatidylinositol phosphate lipid analogues with modified 3-OH group have been developed. These analogues inhibit Akt indirectly via the suppression of PI3K-mediated formation of PIP3 (52). One of these derivatives was shown to act directly on Akt by binding to the pleckstrin homology domain of the serine-threonine kinase, thus inhibiting membrane translocation and/or dimerization of Akt (53). The alkyl-lysophospholipid perifosine has also been reported to perturb membrane translocation of the pleckstrin homology domain of Akt and, in turn, inhibit its activation by PKD1 (54). Because these compounds can, in principle, affect translocation of all pleckstrin homology–containing signal transducers, their Akt specificity is unclear. Finally, a small-molecule Akt pathway inhibitor (API-2), from the National Cancer Institute Diversity Set, was recently discovered (55). In this study, API-2, previously known as tricyclic nucleoside, exhibited predominant activity against tumor cells with aberrant Akt signaling. However, the ability of this compound to also inhibit DNA synthesis and viral activity clearly suggests that API-2 may have a broad spectrum of molecular targets in addition to Akt.

The penetration of MTS-containing fusion peptides into the cell membrane is thought to occur through interactions between the amino acid residues in the MTS and membrane phospholipids (56). Cell-penetrating peptides derived from either cell-permeable proteins or their signal sequence have been used for the delivery of macromolecules into cells and may have important therapeutic applications (reviewed in ref. 57). Dendritic cells loaded with a tyrosinase-related protein 2 peptide covalently linked to MTS showed prolonged presentation of class I MHC and protected immunized mice from B16 tumor formation and lung metastases (58). A fusion protein containing a growth factor receptor binding protein 2 (Grb2) SH2 domain and MTS entered cells and, in dominant-negative fashion, inhibited the association of the EGF receptor with Grb2 (23). An MTS peptide that was chemically attached to antibodies was shown to enter living cells in culture (59). To our knowledge, this is the first report of an scFv that is genetically engineered to have inherent cell membrane-translocating activity, whereas retaining biochemical inhibitory function against its molecular target as well as cellular activity in vivo. These results have important clinical implications as they suggest that this approach can be applied to the generation of compounds that target tumor cells dependent on aberrant Akt signaling for their survival.

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