0026-895X/10/7703-378–387$20.00
MOLECULAR PHARMACOLOGY
Vol. 77, No. 3
60475/3564123
Mol Pharmacol 77:378–387, 2010
Printed in U.S.A.

Tirucallic Acids Are Novel Pleckstrin Homology Domain-Dependent Akt Inhibitors Inducing Apoptosis in Prostate Cancer Cells[S]

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Received August 20, 2009; accepted December 16, 2009

ABSTRACT

Activation of the serine/threonine kinase Akt is associated with aggressive clinical behavior of prostate cancer. We found that the human prostate cancer cell lines LNCaP and PC-3 express predominantly Akt1 and Akt2. Selective down-regulation of Akt1, but not Akt2, by short-hairpin RNA reduced the viability of prostate cancer cells. In addition, structurally different Akt inhibitors were cytotoxic for the prostate cancer cells, confirming that the Akt pathway is indispensable for their viability. We have purified the tetracyclic triterpenoids 3-oxo-tirucallic acid, 3-α-acetoxy-tirucallic acid, and 3-β-acetoxy-tirucallic acid from the oleogum resin of *Boswellia carterii* to chemical homogeneity. The acetoxy-derivatives in particular potently inhibited the activities of human recombinant Akt1 and Akt2 and of constitutively active Akt immunoprecipitated from PC-3 cells, whereas inhibitor of nuclear factor-κB kinases remained unaffected. Docking data indicated that these tetracyclic triterpenoids form hydrogen bonds within the phosphatidylinositol binding pocket of the Akt pleckstrin homology domain. Accordingly, 3-β-acetoxy-tirucallic acid did not inhibit the activity of Akt1 lacking the pleckstrin homology domain. In the prostate cancer cell lines investigated, these compounds inhibited the phosphorylation of cellular Akt and the Akt signaling pathways, including glycogen synthase kinase-3β and BAD phosphorylation, nuclear accumulation of p65, the androgen receptor, β-catenin, and c-Myc. These events culminated in the induction of apoptosis in prostate cancer, but not in nontumorigenic cells. The tirucallic acid derivatives inhibited proliferation and induced apoptosis in tumors xenografted onto chick chorioallantoic membranes and decreased the growth of pre-established prostate tumors in nude mice without overt systemic toxicity. Thus, tirucallic acid derivatives represent a new class of Akt inhibitors with antitumor properties.

Prostate cancer is the most frequently diagnosed malignancy in men in the United States and most Western countries. Upon therapy, androgen-dependent tumors may relapse, finally giving rise to advanced disease characterized by loss of androgen dependence and resistance to therapy (Syrovets et al., 2005b; Armstrong and Carducci, 2006; Morgan et al., 2009).

The phosphatidylinositol 3-kinase/Akt signaling pathway plays a key role in the regulation of cell division and survival in cancer cells (Engelman, 2009; Liu et al., 2009). Loss of phosphatase and tensin homolog deleted on chromosome ten (PTEN), a negative regulator of Akt activation, results in constitutive activation of Akt, which is frequent in prostate cancer (Stambolic and Woodgett, 2006) and correlates with poor prognosis (Armstrong and Carducci, 2006). This renders the Akt pathway a promising target for the development of novel therapeutic approaches (Liu et al., 2009).

The Akt family of serine/threonine kinases is composed of Akt1, Akt2, and Akt3. Upon activation of growth factor receptors, Akt family members become phosphorylated on two residues (e.g., Thr308 and Ser473) by the phosphoinositide-dependent kinase 1 (PDK1) and the mammalian target of rapamycin-rictor complex, respectively (Stambolic and Woodgett, 2006; Engelman, 2009). Disruption of the expression of Akt isoforms revealed that they have

This work was supported by the Deutsche Krebshilfe [Grant 102383] (to T.S. and T.S.).
A.C.E. and T.Sy. contributed equally to this work.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.109.060475.
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: PTEN, phosphatase and tensin homolog deleted on chromosome ten; PDK1, phosphoinositide-dependent kinase 1; GSK-3, glycogen synthase kinase-3; IKK, IκB kinase; IκB, inhibitor of nuclear factor-κB; CAM, chorioallantoic membrane; ERK, extracellular signal-regulated kinase; OTA, 3-oxo-tirucallic acid; αATA, 3-α-acetoxy-tirucallic acid; βATA, 3-β-acetoxy-tirucallic acid; PVP, polyvinylpyrrolidone K10; PBMC, peripheral blood mononuclear cell; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PCR, polymerase chain reaction; siRNA, small interfering RNA; shRNA, short-hairpin RNA; PH, pleckstrin homology; SPR, surface plasmon resonance.

overlapping but not identical functions. Thus, Akt kinases have isoform-specific functions in the regulation of cell migration, invasion, and metastasis (Stambolic and Woodgett, 2006).

Akt regulates at least four different but interacting pathways: cell survival, growth, metabolism, and progression through the cell cycle (Engelman, 2009). Glycogen synthase kinase-3 (GSK-3), which phosphorylates and inactivates crucial cell cycle regulators and transcription factors, including β-catenin, cyclin D1, and c-Myc, is a negatively regulated key target of Akt (Majumder and Sellers, 2005; Liu et al., 2009). Another target of Akt is the human double minute-2 (HDM2) E3 ubiquitin ligase. Activation of human double minute-2 represses p53 activity and targets it for degradation (Engelman, 2009). In addition, activated Akt targets several proteins that regulate the process of apoptosis, such as BAD, caspase 9, and Mcl-1 (Amaravadi and Thompson, 2005; Li et al., 2005).

Plants are a rich source of antitumor compounds (Newman and Cragg, 2007). Some plant-derived compounds, such as taxanes, etoposide, teniposide, and camptothecin derivatives, are of considerable clinical significance (Bailly, 2009). The plant-derived docetaxel was approved by the FDA for combination treatment of metastatic androgen-independent prostate cancer. Although, this was the first demonstration of survival benefit in advanced prostate cancer patients, the progress was rather limited (Armstrong and Carducci, 2006). Oleogum resins from various *Boswellia* species contain triterpenoids with antitumor properties (Akihisa et al., 2006). We have recently demonstrated that distinct pentacyclic triterpenoids from *Boswellia serrata* inhibit prostate cancer growth in vitro and in vivo, acting via inhibition of IκB kinases (IKK) (Syrovets et al., 2005a,b).

Here we identify tirucallic acids isolated from the oleogum resin of *Boswellia carterii* as potent Akt inhibitors that exert cytotoxic effects in human prostate cancer cell lines in vitro and in vivo when xenografted onto chick chorioallantoic membranes (CAM) and in nude mice.

Materials and Methods

Materials. Antibodies used were Akt2, c-Myc, IKKα, β-catenin, androgen receptor and phosphorylated forms of Akt (Ser473), ERK1/2, GSK-3β, BAD (Ser136) (Cell Signaling Technology, Danvers, MA); actin (Millipore Bioscience Research Reagents, Temecula, CA); p65, topoisomerase I (Santa Cruz Biotechnology, Santa Cruz, CA); Akt1, active recombinant kinases Akt1, Akt2, IKKα, IKKβ (Millipore, Billerica, MA); Akt inhibitors IV, VIII [Akt-I-1,2; 1,3-dihydro-1-(1-((4-(6-phenyl-1*H*-imidazo[4,5-*g*]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2*H*-benzimidazol-2-one], and X [10-(4′-(*N*-diethylamino) butyl)-2-chlorophenoxazine] (Calbiochem, San Diego, CA).

Tirucallic Acids. 3-Oxo–tirucallic acid (OTA), 3-α- and 3-β-acetoxy-tirucallic acids (ATAs) (Supplemental Fig. S1) were isolated from oleogum resin of *B. carterii* and purified to chemical homogeneity (>99.0% purity) by reversed-phase high-performance liquid chromatography (Büchele et al., 2003). The compounds were further characterized by UV-spectroscopy, mass spectrometry (Supplemental Fig. S1), and one- and two-dimensional nuclear magnetic resonance spectroscopy. Stock solutions of the tetracyclic triterpenoids and the Akt inhibitors were prepared in dimethyl sulfoxide and further diluted with media. All controls contained solvent, which had no effect on the parameters investigated.

For generation of tirucallic acid microsuspensions used for xenograft treatment, the respective tirucallic acids and polyvinylpyrrolidone K10 (PVP) (Sigma, St. Louis, MO) were separately dissolved in ethanol, mixed together in a molar ratio of 1:5, and stirred for 10 min at room temperature. Ethanol was removed under a stream of nitrogen at 60°C. The residue was lyophilized and reconstituted as required with 0.9% NaCl, for 2 min in an ultrasonic bath; the microsuspension remained stable for at least 3 days at room temperature.

Cytotoxicity and Apoptosis Parameters. Cell lines were from the American Type Culture Collection (Manassas, VA). PTEN-overexpressing PC-3 cells, PTEN-PC-3, were kindly provided by Dr. D. LeRoith (Zhao et al., 2004), and maintained in F12K supplemented with 10% fetal calf serum, penicillin/streptomycin, and 0.5 mg/ml G418 (Geneticin; Karl Roth, Karlsruhe, Germany). Peripheral blood mononuclear cells (PBMC) were isolated by autologous plasma-Percoll gradient centrifugation (Syrovets et al., 1997; Colognato et al., 2003). Cytotoxicity of the tetracyclic triterpenoids and the Akt inhibitors IV, VIII, and X was analyzed by monitoring the mitochondrial reduction of XTT (Roche Diagnostics, Mannheim, Germany). Where applicable, control samples were treated with solvent. For DNA laddering, the Suicide Track DNA Isolation Kit (Calbiochem) was used (Syrovets et al., 2005b).

Xenografts. LNCaP and PC-3 cells (0.75×10^6) were seeded in 20 μl of medium/Matrigel [1:1 (v/v); BD Biosciences, San Jose, CA] onto the CAM of chicken eggs 6 days after fertilization (Syrovets et al., 2005b). Starting from day 2 after seeding, cells were topically treated once daily either with tirucallic acid derivatives incorporated into PVP microemulsions or with unloaded microemulsions alone for 4 days. On day 12 after fertilization, the xenografts were histologically analyzed. Serial sections (5 μm) were stained for the human proliferation antigen Ki-67 (Dako Denmark A/S, Glostrup, Denmark) or other antigens (Syrovets et al., 2005b). The images were digitally recorded at a magnification of 200× with an Axiophot microscope (Carl Zeiss, Göttingen, Germany) and a Sony MC-3249 CCD camera using Visupac 22.1 software (Carl Zeiss). For the detection of apoptotic cells in the tissue sections, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was used (Roche Diagnostics); the sections were counterstained with hematoxylin.

For xenotransplantation, male NMRI/nu-nu mice (Janvier, Le Genest Saint Isle, France) were inoculated subcutaneously at both dorsal sides in the subscapular region with 10^6 PC-3 cells in 0.2 ml medium/injection. After 1 week, treatment commenced by single daily intraperitoneal injections of βATA-PVP at 10 μmol/kg or equivalent amounts of PVP vehicle for 2 weeks. The tumor volume was calculated according to the formula $0.5 \times \text{length} \times \text{width} \times \text{thickness}$ (Syrovets et al., 2005b).

Immunoprecipitation and Kinase Assays. The kinase assays were performed as described previously (Syrovets et al., 2001, 2005b). Akt and IKK were immunoprecipitated from lysates of 10^7 PC-3 cells using 4 μg of antibodies specific for Akt1 or IKK. Recombinant tagged fusion proteins corresponding to full-length IκBα (Santa Cruz Biotechnology) or GSK-3β (Cell Signaling Technology) served as substrates. The kinases were preincubated with the respective tirucallic acid derivatives for 20 min at 30°C before addition of the substrates and [γ-^{32}P]ATP. The samples were resolved by SDS-polyacrylamide gel electrophoresis, blotted on polyvinylidene difluoride membranes, and visualized with the use of a PhosphorImager (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). As loading controls, the blots were immunostained for Akt1 or IKKα. Alternatively, 100 nM active human recombinant His-Akt1, His-Akt2, His-ΔPH-Akt1, GST-IKKα, and His-IKKβ fusion proteins (Millipore) were treated with 0.1 to 10 μM concentrations of either triterpenoid, Akt inhibitor, or the solvent dimethyl sulfoxide and analyzed as indicated above.

Reverse Transcription-PCR. Total RNA (1.5 μg) was extracted with TRIzol (Invitrogen, Carlsbad, CA) and analyzed by reverse transcription-PCR. Primers for Akt were as described previously (Nakatani et al., 1999). To evaluate the expression of Akt1, Akt2, and

Akt3, all three Akt isoforms were amplified using 24 cycles. Conditions were such that the PCR reactions did not reach the saturation phase. Control experiments showed no DNA contaminations; normalization was carried out using GAPDH (Katoh et al., 2000). The amplification products had the appropriate size and their identity was confirmed by direct sequencing (ABI Prism 310; Applied Biosystems, Foster City, CA).

Cell Extracts and Western Immunoblots. Aliquots of whole-cell lysates or nuclear extracts (Syrovets et al., 2001) containing equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred, probed with specific antibodies, and detected as described previously (Burysek et al., 2002).

siRNA Knockdown Experiments. For in vitro knockdown of Akt1 or Akt2, we used the commercial small interfering RNA (siRNA) expression plasmids pKD-Akt1/PKBα-v2 and pKD-Akt2/PKBβ-v1 (Millipore), respectively. When expressed in cells, these plasmids encode short-hairpin RNA (shRNA), which are processed into the corresponding siRNAs. Control cells were transfected with pKD-NegCon-v1 (Millipore). The cells (10^6) were transfected in Cytomix buffer (van den Hoff et al., 1992) with 1 μg of plasmid and radiofrequency electroporation (Gene Pulser II; Bio-Rad Laboratories, Hercules, CA) (Chang et al., 1991). Cells were allowed to recover for 72 and 96 h for Western immunoblotting and analysis of cell viability by XTT, respectively.

Surface Plasmon Resonance Analysis. Measurements were performed on an IBIS optical sensor device (XanTec Bioanalytics, Münster, Germany). Human recombinant active Akt1 was bound to the surface of linear polycarboxylated hydrogel 80M sensor chips to a density of 600 m°. Serial dilutions of βATA in kinase buffer containing 0, 0.1, or 1 mM ATP were applied to the chip. The changes in refractive index in m° related to the amount of molecules bound to the sensor surface were recorded. The increase of the response after injection of the tirucallic acid derivative reflects binding to the immobilized Akt1. Relevant kinetic information was obtained with the IBIS kinetic evaluation program from the parameter k_s: $-k_s = (k_a C + k_d)$. A plot of $-k_s$ values versus concentration was used for linear regression to obtain the association rate constant from the slope and the dissociation rate constant from the y-intercept. Data from the association phase were used to determine the kinetic constants (Syrovets et al., 2000).

Molecular Modeling. Computational docking and scoring studies of the interaction of tirucallic acids with Akt were performed using AutoDock 3.0 (Morris et al., 1998; Hetényi and van der Spoel, 2002) and Molegro Virtual Docker 2008 (Thomsen and Christensen, 2006) with essentially the same results. The original parameters of blind docking were used in combination with an evaluation scheme based on Gibbs free energy change (ΔG) (Hetényi and van der Spoel, 2002). The protein structures were obtained from the Protein Data Bank using the following identification codes: 1unp (residues 1–121, for AKT1 PH domain), 3cqu (residues 144–478 for kinase and AGC kinase C-terminal domains), and 1gzn (residues 146–480 for kinase and AGC kinase C-terminal domains).

Statistical Analysis. Results are expressed as mean ± S.E.M. Significances were analyzed using the Newman-Keuls test for multigroup comparisons or the Mann-Whitney U test (tumor volumes).

Results

Akt Is Constitutively Active in Prostate Cancer Cells. At the mRNA level, the prostate cancer cells express mainly Akt1 and less Akt2. Androgen-dependent LNCaP cells, compared with androgen-independent PC-3 cells, expressed higher amounts of Akt2 mRNA (Fig. 1A). The expression of Akt3 mRNA is low in PC-3 cells, whereas LNCaP cells do not express Akt3, confirming cell-type-specific variations. Western immunoblotting confirmed the differential expression of the various Akt isoforms in the prostate cancer cell

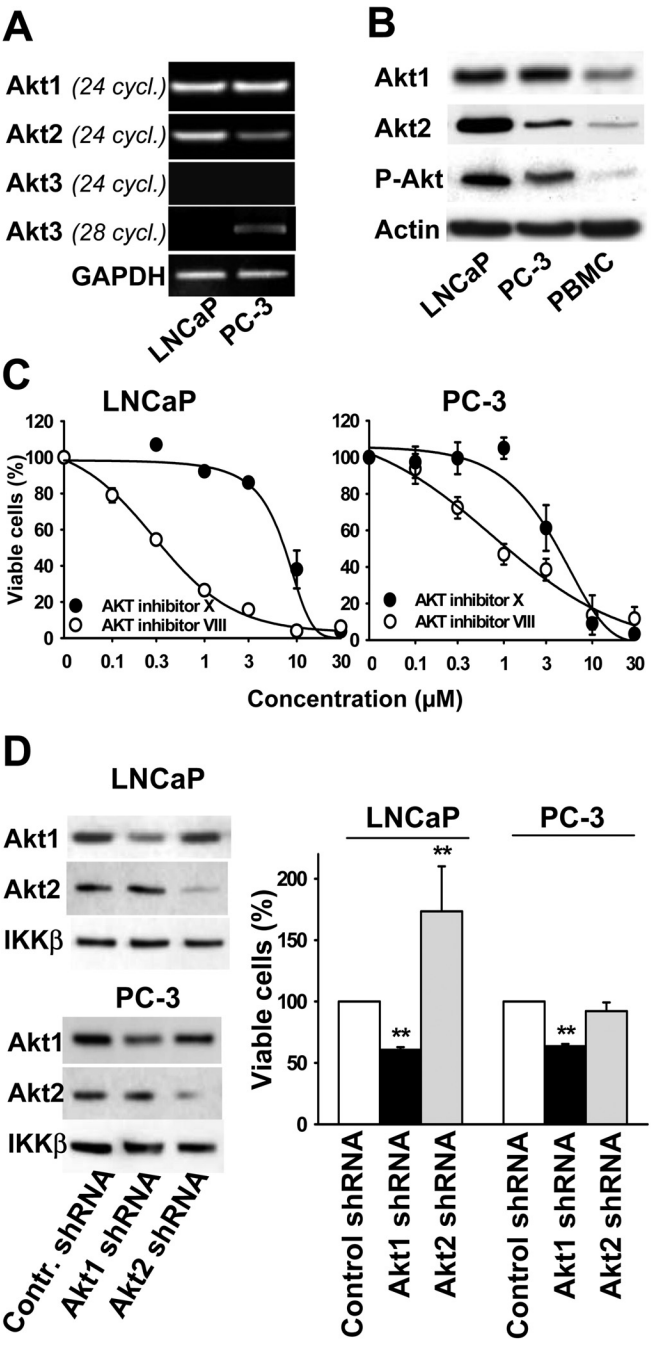


Fig. 1. Inhibition of Akt reduces the viability of prostate cancer cells. A, mRNA expression of Akt isoforms in prostate cancer cells. Equal amounts of total RNA were subjected to reverse transcription-PCR to enable comparison between the cell lines. GAPDH served as internal standard. Data shown are representative of three experiments. B, Western immunoblots of Akt protein expression and activation in whole-cell lysates from PC-3 and LNCaP cells; PBMCs served as controls. C, treatment of PC-3 and LNCaP cells for 48 h with the structurally different Akt inhibitors VIII and X reduces the cell viability as measured by the XTT assay (percentage of solvent controls); solvent had no effect on cell viability. Results are mean ± S.E.M. of three different experiments (each performed in triplicate). D, cells were transfected using radiofrequency electroporation with plasmids encoding shRNA, which are processed intracellularly either in siAkt1, siAkt2, or control siRNA. Cell lysates prepared 72 h after transfection were analyzed for Akt1 and Akt2 by immunoblotting. Immunoblots show specific downregulation of either Akt1 or Akt2 isoform. IKKβ is an additional control of the specificity of Akt down-regulation. Cell viability was analyzed by XTT 96 h after transfection (percentage of controls). Data are mean ± S.E.M. of three independent transfections each performed in triplicate. **, $p < 0.01$ versus control.

lines (Fig. 1B). Akt is constitutively active in the PTEN-deficient LNCaP and PC-3 cells as revealed by Akt phosphorylation (Fig. 1B). Next, we analyzed whether Akt inhibition by structurally different inhibitors would affect prostate cancer cell viability. The Akt inhibitor VIII potently inhibits Akt1, Akt2, and, to a much lesser extent, Akt3 (Lindsley et al., 2005). The Akt inhibitor X is pleckstrin homology (PH) domain-independent (Thimmaiah et al., 2005), and the Akt pathway inhibitor IV may target kinases upstream of Akt (Kau et al., 2003). All these structurally and functionally different Akt inhibitors induced cell death in both LNCaP and PC-3 cancer cells (Fig. 1C and Supplemental Fig. S2). To define the roles of individual Akt isoforms in prostate cancer cell growth, we have transiently down-regulated the expression of Akt1 and Akt2 by shRNA. Akt protein was already down-regulated 48 h after transfection and remained that way for the next several days. Down-regulation of Akt1 protein was accompanied by a significantly decreased viability of both LNCaP ($60.7 \pm 2.1\%$, $p < 0.01$) and PC-3 cells ($63.7 \pm 1.6\%$, $p < 0.01$) (Fig. 1D). Considering the extent of protein down-regulation, these data indicate that the expression of Akt1 is indispensable for prostate cancer cell viability. By contrast, down-regulation of Akt2 in PC-3 cells did not affect cell viability. Proliferation of the LNCaP cells, which constitutively express higher amounts of Akt2 than PC-3 (see Fig. 1A and B), was significantly enhanced. Similar observations have been made in differentiating myoblasts, where knockout of Akt2 prevented cells from exiting the cell cycle (Héron-Milhavet et al., 2006).

Tirucallic Acids Inhibit Akt. We purified the tetracyclic triterpenoids OTA, αATA, and βATA from oleogum resin of *B. carterii* to chemical homogeneity (Fig. 2A and Supplemental Fig. S1) and analyzed their effect on the activity of human recombinant Akt1, Akt2, IKKα, and IKKβ. In contrast to the previously isolated pentacyclic triterpenoids from the boswellic acid family (Syrovets et al., 2005a,b), the tirucallic acid derivatives inhibited the activity of neither human recombinant IKKα nor human recombinant IKKβ (Fig. 2B). However, αATA and βATA were potent inhibitors of the activity of human recombinant Akt1 and Akt2 (Fig. 2B). OTA was a weaker inhibitor of Akt, whereas αATA and βATA showed comparable potency and blocked Akt1 activity at concentrations as low as 1 μM. We further tested whether tirucallic acids also exert their inhibitory effects on Akt immunoprecipitated from PC-3 cells. The antibody used binds Akt1 but also partially recognizes Akt2. The acetoxy-tirucallic acids αATA and βATA potently inhibited endogenous Akt activity from PC-3 cells but had no effect on immunoprecipitated IKKα from the very same cells (Fig. 2C).

Interactions of Tirucallic Acids with Akt as Analyzed by Surface Plasmon Resonance and Molecular Docking. Surface plasmon resonance (SPR) analysis revealed that βATA binds to active Akt1 (Fig. 3A, 100 μM ATP) with a K_D of 8.0 ± 3.1 μM ($n = 3$). Addition of up to 1 mM ATP did not affect the binding affinity ($K_D = 12.0 \pm 2.1$ μM, $n = 3$), indicating that the tirucallic acids do not compete with ATP for the binding to Akt.

In addition, we have used a molecular docking approach to identify the best matching interaction between Akt1 and the tirucallic acid molecules. Results of docking simulation of the interaction of αATA, βATA, or OTA with the kinase and C-terminal domains of Akt1 (residues 144–478)

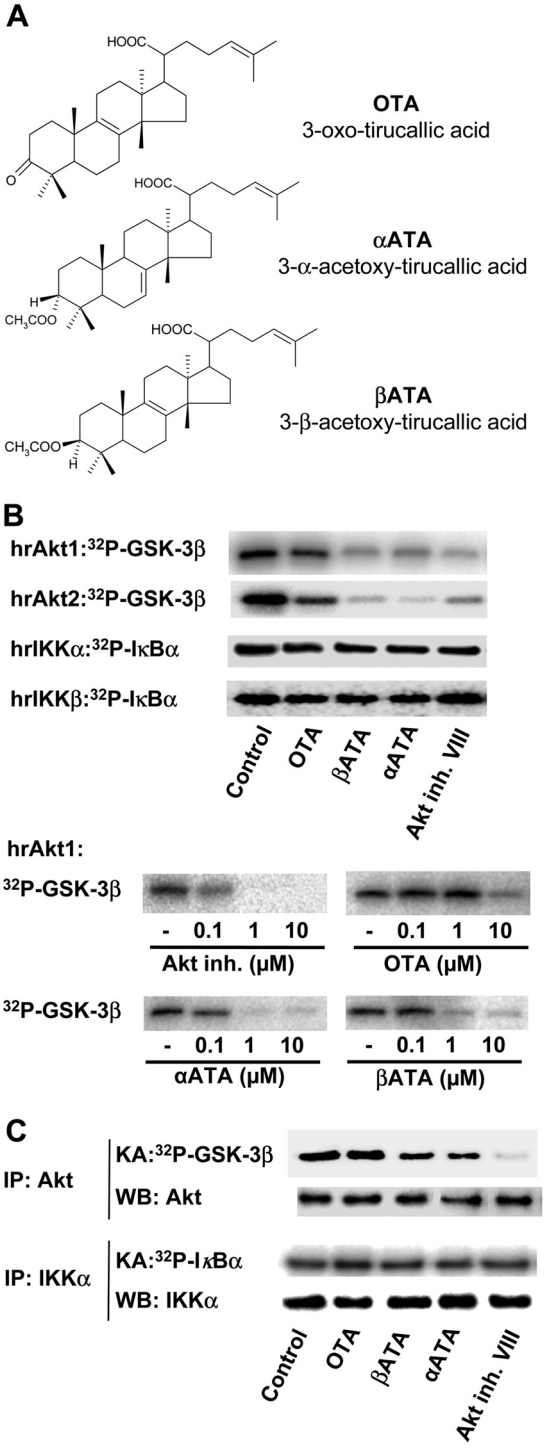
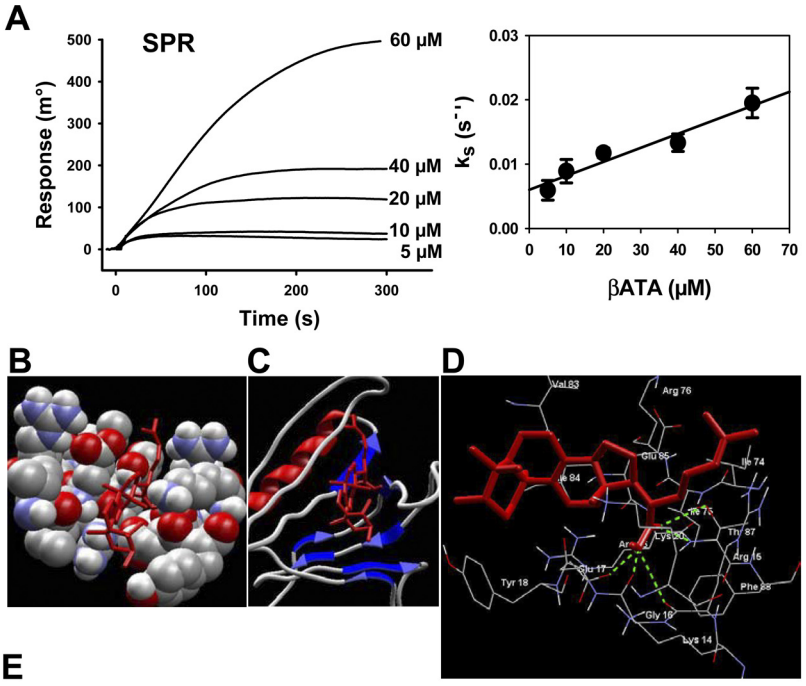


Fig. 2. Tetracyclic triterpenoids from the tirucallic acid family inhibit Akt. A, structures of the tirucallic acids. B, OTA, αATA, or βATA inhibit the activity of Akt in vitro. Active human recombinant (hr) Akt1, Akt2, IKKα, and IKKβ (100 nM) were pretreated for 20 min at 30°C with the tirucallic acids or the Akt inhibitor VIII (each at 10 μM) and analyzed in a kinase assay using GSK-3β or IκBα as kinase substrates, respectively. Alternatively, hrAkt1 was pretreated with different concentrations of the tirucallic acids or the Akt inhibitor VIII (0.1–10.0 μM each) and analyzed as above. C, tirucallic acids inhibit the activity of Akt but not of IKK immunoprecipitated from prostate cancer cells. Constitutively active Akt and IKK were immunoprecipitated from PC-3 cells, preincubated for 20 min at 30°C in the presence or absence of tirucallic acids or the Akt inhibitor VIII (10 μM each) and analyzed in a kinase assay using GSK-3β or IκBα as substrates. Control samples were treated with the solvent. Results of the kinase assays (KA) and immunoblots are representative of three experiments. WB, Western immunoblot; IP, immunoprecipitation.

showed no binding affinity between these molecules (high energy and low scoring function, not shown). In contrast, however, the computational approach indicated that tirucallic acids exhibit affinity binding to the PH domain of Akt1 (Fig. 3, B–E); a low (negative) energy of the interaction indicates a stable system and thus a favorable binding interaction (Thomsen and Christensen, 2006). Predicted hydrogen bonding interactions between βATA and residues Gly16, Glu17, Lys20, and Ile75 of Akt1 are highlighted with green dashed lines (Fig. 3D). Binding affinity data calculated by computational molecular docking were similar to those analyzed in experimental settings using SPR (Fig. 3E). The total hydrogen bonding energies correlated with the experimentally defined relative inhibitory efficacies of the tirucallic acids in the kinase assay (i.e., βATA ≥ αATA > OTA) (Fig. 2, B, C, and E).

It is noteworthy that active Akt1 lacking the PH domain (ΔPH-Akt1) proved to be resistant to inhibition with βATA (Fig. 3F). These data further support the notion that tirucallic acids inhibit Akt activity via binding to the PH domain.

Tirucallic Acids Inhibit Akt Activity and Down-Regulate Pathways Activated by Akt. Within 30 min, tirucallic acids induced rapid inhibition of Akt phosphorylation in the prostate cancer cells that persisted over several hours, whereas the ERK1/2 activity was increased (Fig. 4A). Accordingly, the phosphorylation of GSK-3β, the main target of Akt, was inhibited (Fig. 4B). It has been demonstrated that Akt stabilizes β-catenin involved in TCF-mediated gene transcription and the transcription factor c-Myc (Dang et al., 2006). Indeed, inhibition of Akt by the tirucallic acids reduced the nuclear levels of β-catenin and c-Myc (Fig. 4B). Whereas treatment of PC-3 cells for 6 h with the Akt inhibitor VIII did not decrease the nuclear accumulation of c-Myc, this was observed later (Supplemental Fig. S3). In addition, in LNCaP cells, the nuclear translocation of the androgen



E

Ligand	Hydrogen bonding energy kcal/mol	Interaction energy between ligand and protein kcal/mol	Calculated Kd μM
αATA	-7.41	-28.70	14.64
βATA	-10.47	-28.29	17.16
OTA	-5.23	-22.29	149.80

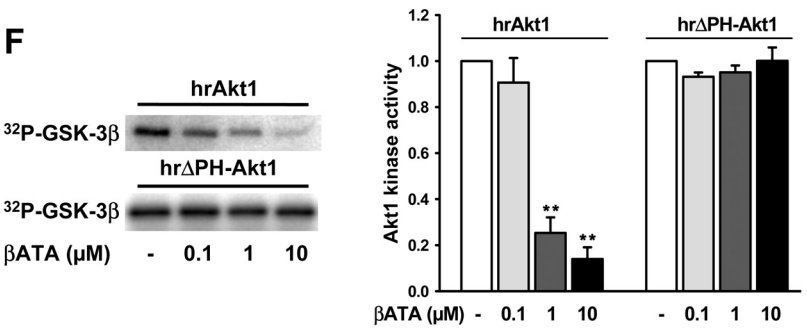


Fig. 3. The tetracyclic tirucallic acids interact with Akt1. Identification of the binding site. A, surface plasmon resonance analysis of the interaction of βATA with Akt1. Active human recombinant Akt1 was immobilized on the surface of linear polycarboxylated hydrogel 80M SPR sensor chips. The SPR responses indicate binding of the increasing concentrations of βATA to Akt1 (overlay plot, left). The rates were calculated from the entire association phase using an integrated rate method resulting in determination of the equilibrium dissociation constant K_D (n = 3). B–D, predicted binding mode of βATA to the PH domain of Akt1 (Protein Data Bank identification code 1unp). Molecular modeling was performed with Molegro Virtual Docker 2008. B, βATA is shown as red carbons. Large gray spheres, carbons of the Akt1 PH domain; small gray spheres, hydrogen; red, oxygen; violet, nitrogen. C, βATA is shown in red. Red helix, α-helix of the Akt1 PH domain; blue arrows, β-sheets; gray, random coils. D, predicted hydrogen bonding interactions between βATA (red) and residues Gly16, Glu17, Lys20, and Ile75 of Akt1 of the phosphatidylinositol binding pocket are highlighted with green dashed lines. E, calculated binding energy and calculated K_D of the interaction between Akt1 and tirucallic acids. F, βATA does not inhibit active Akt1 lacking the PH domain. Active Akt1 and ΔPH-Akt1 (100 nM) were pretreated with βATA (0.1–10.0 μM) or solvent (control) and analyzed in a kinase assay using GSK-3β as a substrate. The graph shows quantification of the kinase assays (n = 3, Newman-Keuls test; **, $p < 0.01$ versus control).

receptor was inhibited (Fig. 4B). Nuclear factor κB is another important target of Akt, which activates nuclear factor κB and induces nuclear translocation of the p65/RelA subunit after phosphorylation by IKK (Gustin et al., 2004). Inhibition of Akt by the tirucallic acids consistently resulted in decreased nuclear accumulation of p65 (Fig. 4B). In addition, inactivation of Akt resulted in hypophosphorylation and activation of proapoptotic BAD (Fig. 4C), creating a direct link between Akt inhibition and apoptosis (Liu et al., 2009). It is noteworthy that hypophosphorylation of BAD was also observed, when Akt1, but not Akt2, was down-regulated in PC-3 cells by shRNA (Fig. 4D). Thus, the hypophosphorylation/activation of BAD correlated with the induction of apoptosis in PC-3 cells treated with Akt1-specific shRNA (Fig. 1D). Phosphorylation of PDK1, another kinase containing a PH domain, was only negligibly affected by the tirucallic acids, indicating selectivity toward Akt (Supplemental Data, Fig. S4).

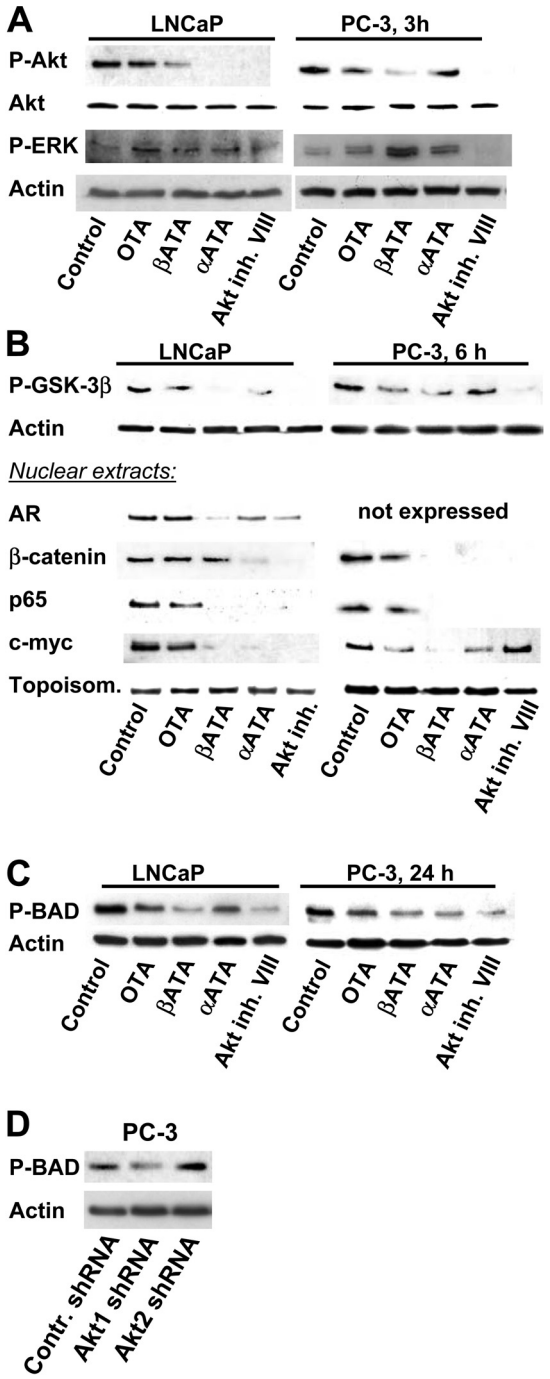


Fig. 4. Inhibition of Akt activity by tirucallic acids leads to time-dependent inhibition of Akt-mediated signaling in prostate cancer cells. A, LNCaP and PC-3 cells were treated for 3 h with OTA, αATA, βATA, or Akt inhibitor VIII (each at 10 μM) and analyzed by immunoblotting. B, cells were treated as in A but for 6 h. Proteins were analyzed in whole-cell lysates and in nuclear extracts by Western immunoblotting. Staining for the nuclear protein topoisomerase I (topoisom.) ensured equal extraction and loading of nuclear proteins. C, the cells were treated as in A, but for 24 h. All controls were treated with solvent. Data shown are representative of three experiments. D, cells were transfected using radiofrequency electroporation with plasmids encoding shRNA, which are processed intracellularly either into siAkt1, siAkt2, or control siRNA. Cell lysates prepared 72 h after transfection were analyzed for phosphorylated BAD by immunoblotting. Actin, loading control.

Tirucallic Acids Induce Apoptosis in Prostate Cancer Cell Lines. Consistent with the described molecular mechanisms, the tetracyclic tirucallic acids exerted potent cytotoxic effects on the prostate cancer cell lines LNCaP and PC-3 (Fig. 5A). The efficacy of the triterpenoids in this cell-based assay correlated well with their relative potencies to inhibit Akt activity in the kinase assays; i.e., βATA and αATA proved to be more active than OTA. LNCaP cells, which show the highest level of Akt activation (Fig. 1B) were more sensitive, compared with PC-3 cells, to Akt inhibition either by the commercially available inhibitors (Fig. 1C) or by the tirucallic acids (Fig. 5A). It has previously been shown that overexpression of PTEN in PC-3 cells is associated with down-regulation of Akt signaling (Zhao et al., 2004). Consistent with these findings, we observed that this PTEN-overexpressing PC-3 cell line was largely resistant to tirucallic acids, indicating specific targeting of Akt by these triterpenoids (Fig. 5A). When the nontumorigenic human prostate epithelial cell line *RWPE-1* or peripheral blood mononuclear cells (PBMC) were exposed to the same concentrations of tirucallic acids, we could detect no significant reduction in cell viability (Fig. 5A), suggesting specificity for tumor cells.

Prolonged exposure of the prostate cancer cells to tirucallic acids led to formation of DNA laddering, the terminal hallmark of apoptosis (Fig. 5B). Transfection of a constitutively active Akt1 mutant significantly increased the viability of βATA-treated LNCaP cells (Fig. 5C); their viability was increased from 37.2 ± 5.4% (pCMV5) to 69.0 ± 9.5% (Akt1-DD), $p < 0.01$. This functional antagonism supports the notion that the proapoptotic effects of tirucallic acids are due to their inhibition of Akt signaling.

Tirucallic Acids Inhibit Growth of Prostate Cancer Xenografts. To verify the proapoptotic and antitumor activity of the tirucallic acids in vivo, we first xenotransplanted LNCaP and PC-3 cells on CAM of fertilized chicken eggs (Syrovets et al., 2005b). For in vivo application of the highly lipophilic triterpenoids, we generated PVP microemulsions. PVP vehicle alone had no effect on the parameters analyzed. Immunohistochemical analysis of the tumor xenografts confirmed that in LNCaP and PC-3 cells, Akt is constitutively activated as demonstrated by intense phospho-Akt staining in control xenografts (Fig. 6, top rows). The Akt phosphorylation was inhibited by treatment of the xenografts with the tirucallic acids. c-Myc, a downstream target of Akt, was predominantly localized within the nuclei in control xenografts (Fig. 6, bottom row), whereas treatment with the tirucallic acids resulted in predominantly cytosolic localization of

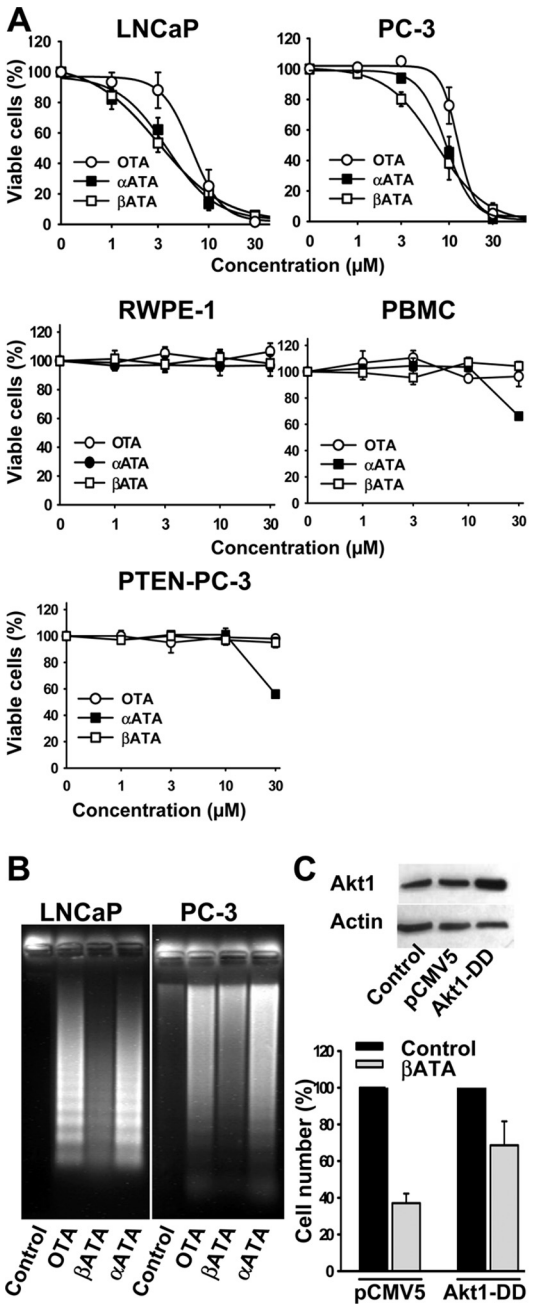


Fig. 5. Tirucallic acids induce apoptosis in prostate cancer cells, but not in nontumorigenic prostate epithelial or blood cells. A, treatment with OTA, αATA, or βATA for 48 h concentration-dependently reduced the viability of LNCaP and PC-3 prostate cancer cells, whereas nontumorigenic RWPE-1 cells, PBMCs, and PC-3 cells overexpressing PTEN (PTEN-PC-3) were resistant to treatment with tirucallic acids as measured by XTT (percentage of solvent-treated control cells). Results represent the mean ± S.E.M. of three experiments each performed in triplicate. B, tirucallic acids induce DNA laddering when cells were treated for 72 h (LNCaP) or 96 h (PC-3) with 10 μM OTA, αATA, or βATA. Low-molecular-weight DNA was extracted, separated on agarose gels, and stained with ethidium bromide. Controls were treated with solvent. C, expression of constitutively active Akt1 mutant (Akt1-DD) increased the viability of LNCaP cells treated with βATA. Cells were transfected with plasmid encoding Akt1-DD or empty vector (pCMV5) by radiofrequency electroporation. Seventy-two hours after transfection, cells were either analyzed by Western blotting using antibody against Akt1 (top) or treated for 48 h with βATA (10 μM), and cell viability was analyzed by XTT (percentage of controls; bottom). Data are mean ± S.E.M. of three independent experiments.

c-Myc. Thus, the tirucallic acids inhibited Akt phosphorylation in LNCaP and PC-3 cells and down-regulated the nuclear localization of the Akt-dependent nuclear phosphoprotein c-Myc that is essential for cell growth and proliferation.

Analysis of the proliferation antigen Ki-67 revealed that the xenografts treated with the tetracyclic compounds exhibited significantly lower proliferation compared with the control groups (Fig. 7A, top rows); the reduced expression of the of the proliferation antigen after treatment with tirucallic acids was additionally quantitated by histomorphometry (Fig. 7B). With the TUNEL technique, we confirmed the induction of apoptosis in LNCaP and PC-3 prostate cancer cells in vivo (Fig. 7A, bottom rows).

We further xenotransplanted PC-3 tumors subcutaneously at both dorsal sides in the subscapular region of nude mice. After the tumors were grown for 1 week, the mice were randomly distributed into two groups and treated with either vehicle or βATA. Treatment of mice bearing the pre-established tumors for 2 weeks with 10 μmol/kg βATA resulted in a significant reduction of the tumor growth compared with mice treated with PVP vehicle alone (Fig, 7C); vehicle had no detectable effect on the tumor growth (not shown).

Discussion

The LNCaP and PC-3 prostate cancer cell lines harbor mutations in PTEN, rendering them PTEN-null. As a result, Akt is constitutively active in these cells (Majumder and Sellers, 2005). PTEN loss and Akt activation correlate with

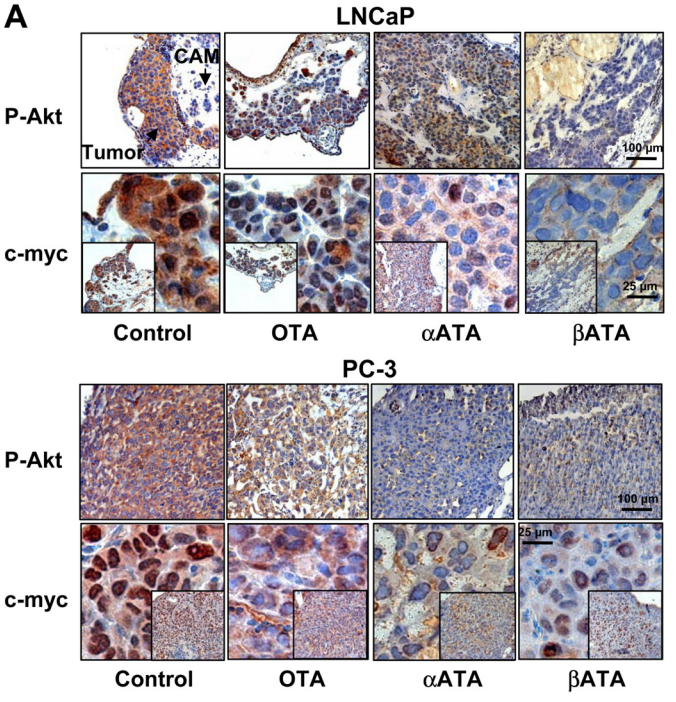


Fig. 6. Tirucallic acids inhibit Akt activation and nuclear localization of c-Myc in LNCaP and PC-3 cells grafted onto the chick chorioallantoic membrane of fertilized chicken eggs. After 2 days, the cells were topically treated with 20 μl of PVP microsuspensions (control), or OTA-, αATA-, or βATA-loaded PVP microsuspensions (each at 30 μM) for 4 days. The tumors were analyzed by immunohistochemistry. Microsections of the tumor specimens were immunostained: top row, phosphorylated/activated Akt (Ser473); bottom row, c-Myc (brown stain) and photomicrographed at 200× magnification. The sections were counterstained with hematoxylin (blue). Data shown are representative center tissue sections of the xenografts of four eggs each.

high Gleason grade and advanced stage of prostate cancer. In addition, the phospho-Akt level is considered to be an independent negative prognostic indicator for the survival of patients with advanced prostate cancer (Li et al., 2005). These clinical observations point to an extraordinary role of Akt activation in the progression of prostate cancer.

A number of inhibitors targeting Akt signaling have been described. Several of them, such as rapamycin and everolimus, inhibiting mammalian target of rapamycin, or GSK690693, an ATP-dependent Akt inhibitor, are in clinical phase I-II studies for prostate and hematologic malignancies (Garcia-Echeverria and Sellers, 2008; Morgan et al., 2009). Perifosine, a synthetic alkyl-lysophospholipid and Akt inhibitor is currently in phase II trials against several cancers. Although perifosine yielded some benefit in patients with refractory solid tumors, treatment was complicated by fatigue and gastrointestinal toxicity (Amaravadi and Thompson, 2005). Unfortunately, no significant clinical activity against androgen-independent prostate cancer was observed (Posadas et al., 2005; Morgan et al., 2009). It is noteworthy that in multiple myeloma, perifosine induced recruitment of Fas/CD95 death receptor and procaspase-8 into lipid rafts, leading to death-inducing signaling complex formation and subsequently to the induction of apoptosis rather than to inhibition of Akt activity (Gajate and Mollinedo, 2007). The potent Akt inhibitor VIII used in our study was unsuitable for in vivo studies because of poor solubility and pharmacokinetics (Cheng et al., 2005).

PH domains are about 100 amino acid motifs with low sequence homology, but a remarkable homology in their three-dimensional structures, which specifically bind phosphoinositides (Toker, 2002). Recent studies, however, have shown that the PH domains might be less conserved than it has previously been assumed and that they can bind ligands other than phosphoinositides (Lemmon, 2004). The PH domain of Akt specifically binds phosphoinositides, which induce conformational changes, expose the critical Thr308 residue, and facilitate Akt activation (Toker, 2002). Our data indicate that tirucallic acids inhibit Akt through binding to the Akt PH domain. However, the activity of PDK1, another

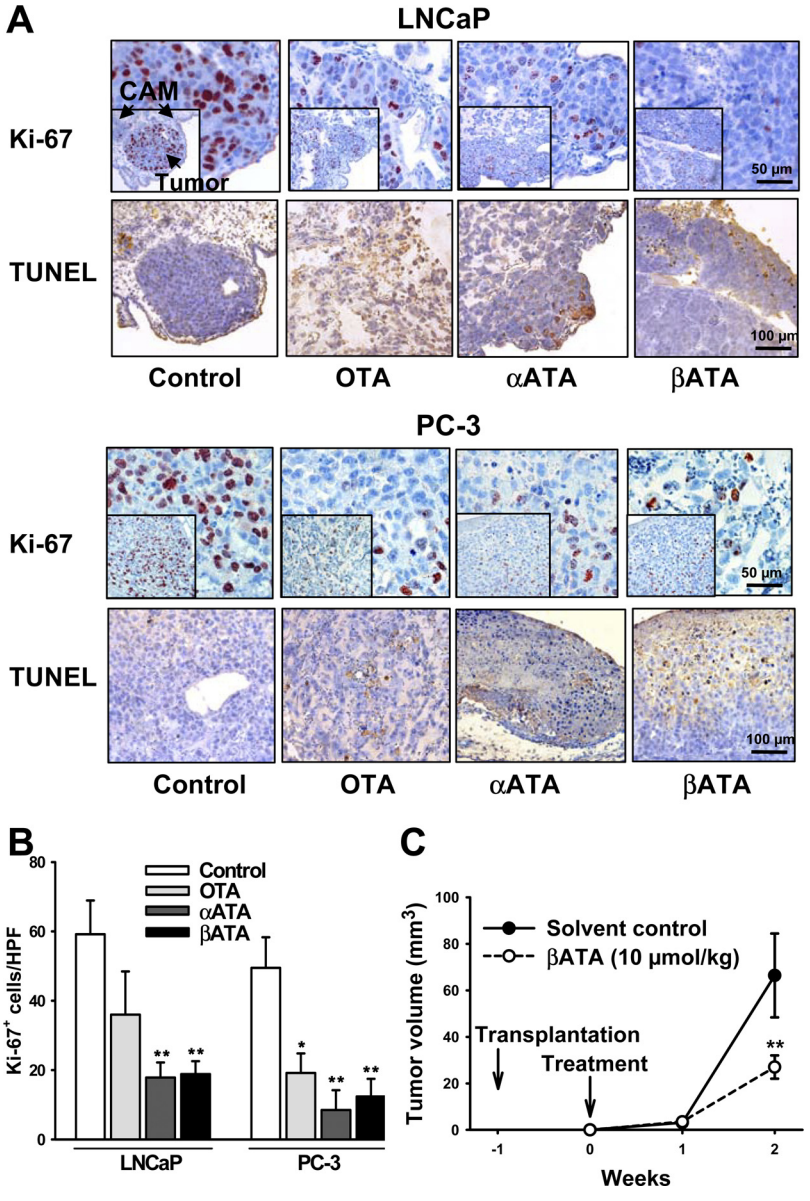


Fig. 7. Tirucallic acids inhibit cell proliferation and induce apoptosis in prostate cancer cells in vivo in chick chorioallantoic membrane xenografts and reduce prostate tumor growth in nude mice. A, prostate cancer cells were grafted onto the chorioallantoic membrane of fertilized chicken eggs and after 2 days topically treated with 20 μl of PVP microsuspensions (control), or OTA-, αATA- or βATA-loaded PVP microsuspensions (each at 30 μM) for 4 days. The tumors were analyzed by immunohistochemistry. A, the tissue sections were immunostained: top row, proliferation antigen Ki-67 (brown-stained nuclei, magnification 200×); bottom row (TUNEL; brown-stained cells) and counterstained with hematoxylin. Data shown are representative of 4 eggs each. B, the treatment groups expressed significantly less Ki-67 antigen than the control group. Histomorphometric analysis of the proliferation antigen Ki-67. Serial 5 μm microsections 100 μm apart from each other (n = 4 eggs in each group). Results are mean ± S.E.M., *, $p < 0.05$; **, $p < 0.01$ as compared with controls. C, βATA inhibits growth of PC-3 xenografts in nude mice. PC-3 cells (10^6) were inoculated subcutaneously into the subscapular region of both sides. Starting from day 8, the animals were treated intraperitoneally either with βATA-PVP (10 μmol/kg) or PVP vehicle alone (control) once daily for 2 weeks (n = 6 mice each). **, $p = 0.028$ versus control.

PH domain-containing kinase upstream of Akt, was only marginally affected by the tirucallic acids, indicating specific inhibition of Akt. Specific inhibition of the Akt pathway by these tetracyclic triterpenoids was also implied by the fact that selective inactivation of the Akt pathway by PTEN overexpression drastically reduced the sensitivity of PC-3 cells to tirucallic acids.

In our study, the tetracyclic tirucallic acids, specifically αATA and βATA, efficiently inhibited the Akt activity. At the same time, increased ERK1/2 phosphorylation was observed. Activation of ERK1/2 as a result of Akt inhibition is not surprising. Knockdown of Akt1 in breast epithelial cells resulted in activation of ERK1/2 and a functional ERK1/2-dependent increase in cell migration (Irie et al., 2005). The exact mechanisms of the Akt1-mediated inhibitory effects on ERK1/2 are currently unknown. It has been demonstrated that Akt might negatively regulate the Raf/ERK pathway through phosphorylation of B-Raf (Guan et al., 2000). It is noteworthy that the commercial Akt inhibitor VIII (Akti 1,2) (Lindsley et al., 2005) inhibited both Akt and ERK1/2 activation, suggesting that this compound might also target ERK1/2 or a kinase upstream of ERK.

The oncogenic effects of Akt1, Akt2, and Akt3 seemed to be indistinguishable when analyzed in vitro and in animal models (Mende et al., 2001). However, in these experiments, myristoylated forms of Akt isoforms have been expressed that might not mimic the endogenous nuclear localization of Akt (Martelli et al., 2006). Other studies point to differential roles of Akt isoforms. Whereas Akt1 was shown to be directly involved in motility and invasion of fibrosarcoma cells, possibly via expression of matrix metalloproteinase 9, Akt2, but not Akt1 or Akt3, has been reported to promote invasion of human breast and ovarial cancer cells, probably as a result of integrin β1 expression (Stambolic and Woodgett, 2006). In addition, Akt1 is important for the organization of the actin cytoskeleton and the assembly of integrin adhesion complexes. Accordingly, expression of a dominant-negative Akt mutant in mammalian fibroblasts decreased their motility (Higuchi et al., 2001). On the other hand, down-regulation of Akt1 in breast epithelial cells enhanced cell migration (Irie et al., 2005).

To analyze the functions of Akt isoforms in LNCaP and PC-3 cells, we have used a vector-based shRNA expression system, which allows gene silencing for a longer time compared with synthetic siRNA. Down-regulation of Akt1 in prostate cancer cells was sufficient to significantly reduce cell proliferation. Taking into account the extent of the protein down-regulation, one may conclude that Akt1 expression is indispensable for prostate cancer cell viability. This is in line with the acknowledged role of Akt in cell cycle progression and the suppression of apoptosis (Héron-Milhavet et al., 2006; Liu et al., 2009). In contrast, down-regulation of Akt2 in slowly dividing LNCaP cells drastically increased cell proliferation, indicating that Akt2, different from Akt1, inhibits the cell cycle progression, probably via p21 binding (Héron-Milhavet et al., 2006). Down-regulation of Akt2 did not affect proliferation of PC-3 cells. It is noteworthy that the level of expression of Akt2 and p21 are low in PC-3 compared with LNCaP. Accordingly, PC-3 cells proliferate more quickly. Recent data using an siRNA approach also pointed to differential roles of Akt1 and Akt2 in the cell cycle regulation. Thus, knockdown of Akt2 in differentiating C2 myoblasts reduced the ability of these cells to stop proliferation before undergoing differentiation. The authors concluded that Akt1 is indispensable for proliferation, whereas Akt2 promotes the cell cycle exit using p21 (Héron-Milhavet et al., 2006).

In addition to cell proliferation, Akt isoforms are essential for cell invasion and metastasis (Stambolic and Woodgett, 2006; Engelman, 2009). Akt1 and Akt2 down-regulation in breast epithelial cells resulted in different phenotypes. Down-regulation of Akt1 led to an ERK-dependent increase in cell migration, which was abolished when Akt2 was concomitantly down-regulated (Irie et al., 2005). These data suggest that inhibition of all Akt isoforms might be necessary for the efficient targeting of Akt signaling in antitumor therapy. Three structurally different Akt inhibitors exerted cytotoxic effects on the prostate cell lines. The effect was observed at concentrations sufficient to inhibit all three Akt isoforms (Lindsley et al., 2005; Thimmaiah et al., 2005). Likewise, a membrane-permeable single-chain antibody that recognizes all three Akt isoforms induced apoptosis and exerted antitumor activity in human cell lines and in mice bearing mammary tumors (Cheng et al., 2005).

The molecular modeling data with βATA predicted that the tetracyclic tirucallic acids bind to the PH domain residues Gly16, Glu17, Lys20, and Ile75 of Akt. These residues form a phosphatidylinositol-phosphate binding pocket; in particular, residue Glu17 was shown to be directly involved in the phosphatidylinositol (3,4,5)-trisphosphate binding to Akt1 (Thomas et al., 2002). The clinical importance of this Akt region was recently highlighted when it was discovered that a substitution of Glu17 to Lys17 in Akt1 is a frequent mutation in human cancers. It alters the electrostatic interaction in the pocket, leading to stronger binding of a phosphatidylinositol ligand, targeting Akt1 to the cellular membrane, and finally resulting in increased Akt1 activation; in mice, this mutation induced leukemia (Carpten et al., 2007; Engelman, 2009).

The total hydrogen-bonding energies of OTA are equivalent, whereas those of αATA and βATA are higher than those reported for phosphatidylinositol phosphates (Rong et al., 2001). Thus, occupation of the PH domain especially by αATA and βATA might hinder binding of phosphatidylinositols and the subsequent activation of Akt. On the other hand, PH-domain-dependent oligomerization of Akt molecules, which induce conformational changes and contribute to Akt activation (Datta et al., 1995), might be also be obstructed in the presence of the tetracyclic tirucallic acids. Likewise, the Akt kinase activity in in vitro kinase assays and in cells as well as the leukemia cell viability were inhibited by a peptide mimicking the Akt-binding domain of TCL1 and binding to the PH domain of Akt (Hiromura et al., 2004).

Inhibition of Akt represents a promising therapeutic approach. Considerable efforts are currently being made to develop selective small molecule inhibitors of Akt, yet none made it to clinic so far (Majumder and Sellers, 2005; Garcia-Echeverria and Sellers, 2008; Morgan et al., 2009). Obstacles might be a complex network of mutual regulations between different signaling pathways, so that inhibition of a single pathway will lead only to a limited response (Faivre et al., 2006). Therefore, it has been suggested that simultaneous inhibition of a number of key kinases might improve the clinical outcome. Definition of the combination of the kinase inhibitors, which are likely to be effective in tumors with

specific genetic background is an ongoing challenge. Discovery of specific kinase pathway inhibitors is a step toward this goal. In such settings, tetracyclic triterpenoids from the tirucallic acid family or derivatives thereof might possibly, in combination with MAPK inhibitors, provide an opportunity to counteract activation of Akt signaling to combat advanced prostate cancer.

Acknowledgments

The plasmid encoding constitutively active Akt1 (T308D/S473D, Akt1-DD) was a kind gift from Prof. Dario R. Alessi (University of Dundee, Dundee, UK). The PC-3 cell line overexpressing PTEN (PTEN-PC-3) was a generous gift from Dr. Derek LeRoith (Mount Sinai School of Medicine, New York, NY).

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