An α-Acetoxy-Tirucallic Acid Isomer Inhibits Akt/mTOR Signaling and Induces Oxidative Stress in Prostate Cancer Cells

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ABSTRACT

Here we provide evidence that αATA(8,24) (α-acetoxy-tir-8,24-dien-21-oic acid) inhibits Akt/mammalian target of rapamycin (mTOR) signaling. αATA(8,24) and other tirucallic acids were isolated from the acetylated extract of the oleo gum resin of Boswellia serrata to chemical homogeneity. Compared with related tirucallic acids, αATA(8,24) was the most potent inhibitor of the proliferation of androgen-insensitive prostate cancer cells in vitro and in vivo, in prostate cancer xenografted onto chick chorioallantoic membranes and in recurrent, late-stage, castration-resistant, and metastatic prostate cancer cells in vitro. At the molecular level, αATA(8,24) destabilized lysosomal and mitochondrial membranes and induced reactive oxygen species production via the inhibition of mTOR signaling and to induce simultaneously oxidative stress in cancer cells. The ability of αATA(8,24) to inhibit Akt/mTOR signaling and to induce simultaneously oxidative stress could be exploited for the development of novel antitumor therapeutics with a lower profile of toxic side effects.

Introduction

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death in males worldwide (Siegel et al., 2013). In fact, it represents a considerable health problem for men, accounting for 27,000 deaths in 2009 in the United States alone (Freedland, 2011). Death rates from prostate cancer continue to decline, reflecting improvements in early diagnosis and therapy (Siegel et al., 2013). Potentially curative options for patients with localized disease include radical prostatectomy, radiation, and cryotherapy (Freedland, 2011). About 35% of patients will experience disease recurrence within 10 years of primary therapy. Treatment of recurrent, late-stage, castration-resistant, and metastatic prostate cancer remains an unresolved therapeutic challenge. The small positive impact of docetaxel chemotherapy on survival (median survival gain of less than 3 months) (Freedland, 2011) has necessitated novel treatment strategies for advanced prostate cancer.

The evolutionarily conserved checkpoint protein kinase, mammalian target of rapamycin (mTOR), has emerged as a major effector of cell growth and proliferation via the regulation of protein and lipid synthesis (Zoncu et al., 2011). The activity of mTOR, a key regulator of protein synthesis, is exacerbated in diseases that are characterized by deregulated growth, such as cancer or type 2 diabetes (Zoncu et al., 2011). mTOR is positively regulated by Akt and both kinases are frequently constitutively activated in many high-grade human malignancies, including advanced prostate cancer, where their activity is correlated with poor prognosis (Brown et al., 2008; Graff et al., 2009; Antonarakis et al., 2010; Zoncu et al., 2011). Akt and mTOR pathways are tightly interconnected by a number of positive and negative feedback loops and are often considered as a single Akt/mTOR pathway (Efeyan and Sabatini, 2010; Porta et al., 2014). Thus, in clinical trials, the mTOR inhibitor rapamycin and its analogs showed limited efficacy as anticancer drugs because of activation of prosurvival and proliferative signals through Akt, as well as extracellular signal-regulated kinase and other cascades (Efeyan and Sabatini, 2010; Zoncu et al., 2011). New inhibitors of the Akt/mTOR pathway with different mechanisms of action are believed to overcome the shortcomings of conventional inhibitors (Zoncu et al., 2011).

Between 1981 and 2002, about two-thirds of the drugs approved for cancer treatment were natural products (Aggarwal et al., 2008).

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ABBREVIATIONS: αATA(7,24), 3α-acetoxy-tir-7,24-dien-21-oic acid; αATA(8,24), 3α-acetoxy-tir-8,24-dien-21-oic acid; CDK, cyclin-dependent kinase; FACS, fluorescence-activated cell sorting; H2DCFDA, 2’-7’-dichlorodihydrofluorescein diacetate; mTOR, mammalian target of rapamycin; OTA, 3oxo-tir-8,24-dien-21-oic acid (elemenonic acid); ROS, reactive oxygen species; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-[phenylaminocarbonyl]-2,3-dihydroxide; Z-DEVD-R110, rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide).
et al., 2004). The *Boswellia species* (*Burseraceae*), trees native to Ethiopia, Somalia, India, and the Arabic peninsula, produce an oleo gum resin known as olibanum or frankincense. This oleo gum resin has been traditionally used for the treatment of rheumatoid arthritis and other inflammatory diseases in many countries (Ammon, 2006). In clinical pilot studies, beneficial effects of frankincense extracts have been reported in a variety of inflammatory diseases, such as allergic asthma, inflammatory bowel diseases, arthritis, or peritumoral brain edema (Ammon, 2006; Abdel-Tawab et al., 2011). Some reports suggest that bioactive triterpenoids may represent the pharmacologically active principle of frankincense (Syrovets et al., 2000, 2005a; Poeckel and Werz, 2006; Cuaz-Peronil et al., 2008; Lu et al., 2008; Wang et al., 2009; Abdel-Tawab et al., 2011). Furthermore, many studies show antineoplastic activities of these triterpenoids, especially boswellic acids and their derivates, on different types of cancer (Syrovets et al., 2000, 2005a; Akihisa et al., 2006; Büchele et al., 2006; Lu et al., 2008; Bhushan et al., 2009; Park et al., 2011; Yadav et al., 2012; Morad et al., 2013).

Among the pharmacologically active triterpenoids present in the oleo gum resin of *Boswellia* species are terpenoids with tirucallane skeletons. Thus, α-ATA(7,24) (3α-acetoxy-tir-7,24-dien-21-oic acid), 3α-ATA(7,24), and OTA (3oxo-tir-8,24-dien-21-oic acid) have been shown to inhibit the 12-O-tetradecanoyl phorbol-13-acetate–induced inflammation in mice (Banno et al., 2006). We have previously demonstrated that tirucallic acids, including OTA and α-ATA(7,24), induce prostate cancer cell death via apoptosis and that they decreased the growth of pre-established prostate tumors in nude mice without overt systemic toxicity (Estrada et al., 2010). It has also been reported that tirucallic acids might inhibit cell-free 5-lipoxygenase activity but induce 5-lipoxygenase product formation in intact polymorphonuclear cells. OTA was also shown to increase phosphorylation of mitogen-activated protein kinase 1/2 (Boden et al., 2001).

In light of the pharmacologic potential of natural compounds derived from *Boswellia* oleo gum resins, we have investigated its constituents regarding their potential anticancer efficacy. In the present work, we have isolated the tirucallic acid derivative α-ATA(8,24) (3α-acetoxy-tir-8,24-dien-21-oic acid) from *Boswellia serrata* Roxb. and show that it effectively inhibits Akt/mTOR signaling and induces reactive oxygen species (ROS) formation, as well as apoptosis in androgen-insensitive human prostate cancer cells in vitro in and in chick chorioallantoic membrane xenografts in vivo.

**Materials and Methods**

The tirucallic acid isomers, ATA(8,24) and OTA, were isolated from an oleo gum resin extract of Indian *B. serrata* Roxb. (85%; Biomex, Heidelberg, Germany) and purified to chemical homogeneity (≥99.0% purity; Supplemental Figs. 1 and 2; see Supplemental Methods for details). Rapamycin was from Calbiochem (Merck, Darmstadt, Germany). Docetaxel, propidium iodide, acridine orange, and DNase-free RNase A were purchased from Sigma-Aldrich (St. Louis, MO). XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)–2H-tetrazolium–5-carboxanilide–2H-tetrazolium] and labeled UTP were from Roche Diagnostics (Filderstadt, Germany). Matrigel and Annexin V fluorescent isothiocyanate were from BD Biosciences (Heidelberg, Germany). Z-DEVD-R110 (rhodamine 110 bis-(N-CBZ-t-aspartyl-t-glutamyl-t-valyl-t-aspartic acid amide), H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate), MitoSOX, and JC-1 were purchased from Molecular Probes (San Diego, CA). Antibody against the human proliferation antigen Ki-67 was from DakoCytomation (Glostrup, Denmark). The following antibodies were used: antibodies against cyclin D1, cyclin E, cyclin-dependent kinases CDK4 and CDK2, S6K1 (Santa Cruz Biotechnology, Dallas, TX), p-S6K1 (Ser240/244), p-Rb (Ser807/811), cyclin B1 (Cell Signaling Technology, Danvers, MA), and actin (Chemicon/EMD Millipore, Billerica, MA). Stock solutions of the studied compounds were prepared in dimethylsulfoxide and further diluted with media (F12K; Life Technologies, Carlsbad, CA) supplemented with 1% heat-inactivated fetal calf serum just before the biologic experiments.

**Cell Lines and Xenografts.** The treatment-resistant PC-3 human prostate adenocarcinoma cell line was from the American Type Culture Collection (Rockville, MD). Peripheral blood mononuclear cells were isolated by Ficoll density gradient separation (Syrovets et al., 1997; Colognato et al., 2003). For the in vivo experiments, PC-3 xenografts (0.75 × 10^6 cells) were xenotransplanted in medium Matrigel (1:1, v/v) onto the chick chorioallantoic membrane 8 days after fertilization. Commencing the day after, the xenografts were topically treated for 3 consecutive days with 20 μl of each compound. On day 12 after fertilization, the xenografts were collected, fixed, paraffin-embedded, and analyzed histologically; serial sections (5 μm) were stained for the human proliferation antigen Ki-67.

**Cell Viability and Analysis of Apoptosis.** Cells treated with the respective compounds were analyzed using an XTT assay that relies on the formation of water-soluble formazan salt by viable cells. Apoptotic and necrotic cells were quantified by flow cytometric analysis of annexin V/propidium iodide double-stained cells. The percentage of the subdiploid G1 cells undergoing DNA fragmentation was determined by propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis (Nicolletti et al., 1991). Caspase-3 activity was analyzed using fluorometric detection of the cleaved caspase-3 substrate Z-DEVD-R110 using flow cytometry. For the detection of apoptotic cells in prostate cancer xenografts, DNA strand breaks were visualized by the terminal deoxynucleotidyl transferase dUTP nick-end labeling method (Syrovets et al., 2005b). The sections were counterstained with H&E and images were digitally recorded with an Axioskop microscope (Carl Zeiss, Göttingen, Germany) and a Sony MC-3249 CCD camera using Visupac 22.1 software (Carl Zeiss), and quantified using ImageJ (NIH, Bethesda, MD).

**Determination of ROS Production.** Cellular production of ROS was determined by the cell-permeant dye H_2DCFDA. Upon cleavage of the acetate groups by intracellular esterases and by oxidation, the nonfluorescent H_2DCFDA is converted to the highly fluorescent 2′,7′-dichlorofluorescein (Loos et al., 2014). PC-3 cells were treated with either α-ATA(8,24) (10 μM), OTA (10 μM), docetaxel (100 nM), or rapamycin (10 nM) for 24 hours. Alternatively, cells were treated with H_2O_2 (200 μM) for 48 hours as positive control. At the end of the treatment, cells were incubated with 10 μM H_2DCFDA for 30 minutes at 37°C, and analyzed by flow cytometry. Superoxide anions were determined by MitoSOX Red, a fluorogenic dye targeting specifically mitochondria in live cells. Oxidation of MitoSOX Red by superoxide produces a fluorescent product with absorption/emission maxima at ~510/580 nm. After treatment, cells were loaded with 5 μM MitoSOX for 30 minutes and analyzed by flow cytometry using BD FACSVerse flow cytometer and BD FACSuite software (BD Biosciences).

**Determination of the Mitochondrial Membrane Potential.** PC-3 cells were treated with either α-ATA(8,24) (10 μM), OTA (10 μM), docetaxel (100 nM), or rapamycin (10 nM) for 24 and 48 hours. Changes in the mitochondrial membrane potential were determined using JC-1 sensor, a lipophilic cationic dye concentrated selectively within intact mitochondria, where it forms red-fluorescent multimeric aggregates. A monomeric form of the dye found in the cytoplasm emits green fluorescence. Treated cells were loaded with 5 μM JC-1 dye for 20 minutes followed by flow cytometric analysis.

**Analysis of Acidic Vesicular Organelles.** The number and integrity of acidic vesicular organelles were analyzed using acridine orange showing red (640 nm) fluorescence of precipitated dye within intact acidic vesicular organelles (Loos et al., 2014). PC-3 cells treated with
for 24 and 48 hours were stained with 2.5 μg/ml acridine orange for 15 minutes at 37°C and analyzed by flow cytometry.

**Cell Cycle Analysis.** Following a 24-hour treatment, PC-3 cells were stained by propidium iodide and analyzed by flow cytometry (Nicolletti et al., 1991) using FlowJo software (TreeStar Inc., Ashland, OR).

**Western Immunoblotting.** PC-3 cells were starved in serum-free medium overnight, pretreated with the compounds for 30 minutes, and stimulated with 1% fetal calf serum for a total of 6 or 8 hours. Collected cells were lysed in cold radioimmunoprecipitation assay buffer in the presence of protease and phosphatase inhibitors. Cell debris was removed by high-speed centrifugation and protein concentration of the samples was determined by BCA assay. Equal amounts of protein (60 μg) were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. Proteins were visualized with specific antibodies and detected using corresponding horseradish peroxidase–coupled secondary antibodies as described (Syrovets et al., 2001).

**Kinase Assay.** The kinase assays were performed as described (Syrovets et al., 2005a,b; Estrada et al., 2010; Morad et al., 2013). Active human recombinant His-Akt1 (100 nM) fusion protein (Biaffin GmbH, Kassel, Germany) was treated either with 3–30 μM tirucallic acid, Akt inhibitor VIII (Akt-1/2, 10 μM; Calbiochem), or the solvent dimethylsulfoxide for 15 minutes at 30°C before addition of the substrate and [γ-32P]ATP. Recombinant tagged fusion glycogen synthase kinase 3β (Cell Signaling Technologies) served as substrate. The samples were resolved by SDS-PAGE and visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Statistical Analysis.** All values are expressed as mean ± S.E.M. Statistical analysis was performed using the Newman-Keuls test and Statistica software (StatSoft, Hamburg, Germany).

**Results**

**αATA(8,24) Inhibits Proliferation of Prostate Cancer Cells In Vitro and In Vivo.** αATA(8,24), with the chemical structure shown in Fig. 1, A and B, as well as other known tirucallic acids, OTA and αATA(7,24), decreased the viability of androgen-independent chemoresistant prostate cancer cells PC-3 in a concentration- and time-dependent manner (Fig. 1C). With an IC50 of 6.2 ± 0.4 μM, αATA(8,24) was the most effective among the tirucallic acid derivatives tested. The antimitotic compound docetaxel (Taxotere; Sanofi-Aventis, Bridgewater, NJ) used to treat advanced prostate cancer (Freedland, 2011) partially reduced the viability of the prostate cancer cells. However, about one-third of the cells exhibited resistance to docetaxel, even when rather high concentrations were applied (Fig. 1C). Similarly, the mTOR inhibitor rapamycin exhibited cytostatic activity on PC-3 cells with about 50% remaining viable irrespective of the applied rapamycin concentration (Fig. 1C). The cytotoxic effect of αATA(8,24) on the cancer cells appeared to be specific, because the tirucallic acid was not toxic for peripheral blood mononuclear cells (Fig. 1D). Similarly, αATA(8,24) and OTA inhibited the proliferation of PC-3 xenografts on chorioallantoic membranes of fertilized chicken eggs as assessed by the expression of the proliferation antigen Ki-67 (Fig. 1E); αATA(8,24) (3 μM) was as active as docetaxel (100 nM) and more active than rapamycin (10 nM).

**αATA(8,24) Triggers Apoptosis in Prostate Cancer Cells In Vitro and In Vivo.** Caspase-3 is an effector caspase that when activated cleaves many important cellular substrates, which causes membrane blebbing, disassembly of the cell structure, and DNA fragmentation leading finally and inevitably to cell death. Caspase-3 is activated in apoptotic cells, both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways (Lawen, 2003). About 17% of the cells treated with αATA(8,24) for 24 hours exhibited active caspase-3 (Fig. 2A); after 48 hours, the number of cells with active caspase-3 increased up to 50%. Activation of caspase-3 was observed in cells treated with docetaxel, but not in those treated with rapamycin. In fact, the latter is known to be rather cytostatic than cytotoxic (Zoncu et al., 2011; Morad et al., 2013) (Fig. 2A).

Analysis of the cell membrane asymmetry revealed that treatment of PC-3 cells with αATA(8,24) for 48 hours resulted in a significant increase in the percentage of annexin V–positive cells, whereas the increase caused by OTA was much lower and not statistically significant compared with control (Fig. 2B). αATA(8,24) was as active as docetaxel in inducing the phosphatidylserine expression on the cells surface, whereas rapamycin had no effect on this parameter, indicating no induction of apoptosis in PC-3 cells (Fig. 2B). These data are consistent with the observation that rapamycin did not induce caspase-3 activation and DNA fragmentation in prostate cancer cells (Fig. 2, A and C). In contrast, the majority of cells treated with αATA(8,24) for 72 hours were subdiploid, and in the group treated with OTA, about 30% of the cells were apoptotic. In agreement with the data demonstrating that about the half of PC-3 cells remain viable after treatment with docetaxel (Fig. 1B), DNA fragmentation was observed only in about 40% of the prostate cancer cells treated with docetaxel (Fig. 2C).

The in vitro data were further validated by experiments performed in an in vivo xenotransplantation model using fertilized chicken eggs. Thus, αATA(8,24) potently induced DNA strand breaks, which indicates induction of apoptosis in the prostate cancer xenografts (Fig. 2D). The amount of apoptotic cells was also increased in xenografts treated with OTA and docetaxel, although to a lesser extent compared with αATA(8,24). Rapamycin, on the other hand, did not induce apoptosis.

**αATA(8,24) Induces Loss of Mitochondrial Membrane Potential and Oxidative Stress in Prostate Cancer Cells.** The observation that αATA(8,24) induces activation of caspase-3 in prostate cancer cells suggests that it might activate the mitochondrial apoptotic pathway. Indeed, some cytotoxic drugs induce early loss of mitochondrial membrane potential, opening of the mitochondrial permeability transition pore, release of cytochrome c and other proapoptotic molecules, formation of the apoptosome, and caspase-3 activation (Lawen, 2003). The mitochondrial integrity was analyzed using JC-1 dye, which accumulates within mitochondria in a potential-dependent manner characterized by a fluorescence emission shift from green to red. Consequently, mitochondrial depolarization is characterized by a decrease in the red/green fluorescence intensity ratio. Treatment of cells with αATA(8,24) for 24 hours led to an increase in the red/green fluorescence intensity ratio indicating hyperpolarization, which was followed by a dramatic loss of the mitochondrial membrane potential after 48 hours (Fig. 3A). Likewise, a loss of the mitochondrial membrane potential was observed in cells treated with docetaxel, whereas OTA and rapamycin induced mitochondrial membrane hyperpolarization but no loss of the membrane potential (Fig. 3A). These data are consistent with those demonstrating that OTA is a weak inducer of apoptosis and rapamycin is cytostatic rather than proapoptotic (Figs. 1, B and D, and 2).

Most cancer cells exhibit increased aerobic glycolysis and oxidative stress compared with their normal counterparts (Trachootham et al., 2009). An increase in the production of
Free oxygen radicals (ROS) above a certain level may enhance the susceptibility of cancer cells to a chemotherapeutic drug. Dissipation of the mitochondrial membrane potential in PC-3 cells treated with oATA(8,24) might amplify ROS formation and increased oxidative stress. Indeed, following 24-hour treatment of PC-3 cells with oATA(8,24), the cells exhibited

Fig. 1. The tirucallic acid isomer oATA(8,24) inhibits proliferation of prostate cancer cells in vitro and in vivo. (A) Structures of oATA(8,24), oATA(7,24), and OTA. (B) Overlay of oATA(8,24) (gray carbon sticks) with oATA(7,24) (green carbon sticks), and oATA(8,24) with oATA(7,24) (violet carbon sticks) (oxygen, red; hydrogen atoms omitted for clarity. Superimpositions of three-dimensional structures were performed using the Discovery Studio Software Suite (v 3.5) from Accelrys Software Inc. (San Diego, CA) (C) oATA(8,24) is more potent than OTA and oATA(7,24). PC-3 cells were treated with different concentrations of oATA(8,24) and known tirucallic acid derivatives, OTA and oATA(7,24), for 48 or 72 hours, and cell proliferation was analyzed by XTT assay. Docetaxel and rapamycin were used as positive controls. (D) Peripheral blood mononuclear cells (PBMC) are relatively resistant to oATA(8,24). Cells were analyzed after 48 hours as in (B). (E) oATA(8,24) inhibits proliferation of pre-established PC-3 xenografts on the chick chorioallantoic membranes (CAM). Cells were grafted onto CAM 8 days after fertilization. The next day, PC-3 xenografts were topically treated with 20 μl of either oATA(8,24) (3 μM), OTA (3 μM), docetaxel (100 nM), or rapamycin (10 nM) for 3 consecutive days. Paraffin-embedded PC-3 xenografts were immunohistochemically analyzed for the proliferation antigen Ki-67 (red stain). Representative pictures are shown. Original magnification, 400×. Graph shows the fraction of proliferating, Ki-67–positive cells, in high-power field (HPF). Data are mean ± S.E.M. (n = 6); **P < 0.01; ***P < 0.001 versus control.
an increased production of ROS and superoxide, as analyzed by H$_2$DCFDA and MitoSOX fluorescent dyes, respectively (Fig. 3, B and C). OTA and docetaxel also induced oxidative stress in prostate cancer cells, although to a lesser extent compared with αATA(8,24). No significant oxidative stress was detected in cells treated with rapamycin (Fig. 3, B and C).

**αATA(8,24) Increases the Activity of Acidic Vesicular Organelles in Prostate Cancer Cells.** There is increasing evidence of cross-talk between mitochondria and lysosomes or other acidic vesicular organelles in the execution of apoptosis (Aits and Jaattela, 2013). Thus, lysosomes contain cysteine cathepsins, which, when released through limited permeabilization of the...
lysosomal membrane, might promote apoptotic cell death. On the other hand, mitochondrial ROS could destabilize the lysosomal membrane by lipid peroxidation and damage of membrane proteins (Aits and Jaattela, 2013). Staining with the acidophilic dye acridine orange, which is protonated and emits orange light at low pH, allows analysis of the activity of the proton pump of acidic vesicular organelles (Moriyama et al., 1982). αATA(8,24) and OTA, as well as docetaxel and, to a lesser extent, rapamycin induced activation of the proton pump in PC-3 cells, as manifested by a fluorescence shift to

Fig. 3. αATA(8,24) affects mitochondrial membrane polarization and induces production of ROS in prostate cancer cells. PC-3 cells were treated with either αATA(8,24) (10 μM), OTA (10 μM), docetaxel (100 nM), or rapamycin (10 nM) for 24 and 48 hours. (A) Mitochondrial membrane potential was analyzed using JC-1 dye followed by flow cytometry. Mitochondrial membrane potential (ΔΨm) was measured as red/green fluorescence intensity ratio. Dot plot shows representative analysis of cells treated for 48 hours. (B) Staining with H2DCFDA was used to analyze ROS production by flow cytometry. Cells were treated as in (A). (C) After staining with MitoSOX, superoxide production was analyzed by flow cytometry. Cells were treated as in (A). H2O2 (200 μM) was used as positive control. All data are mean ± S.E.M., n = 3; *P < 0.05; **P < 0.01; ***P < 0.001 versus control.
the right. The activation was already evident after 24 hours of treatment, and was more pronounced after 48 hours (Fig. 4). Similarly, within this time, the number of cells with decreased fluorescence exhibiting lysosomal rupture was increased (shown by a fluorescence shift to the left). Docetaxel was the strongest inducer of lysosomal membrane permeability, followed by αATA(8,24) and OTA (Fig. 4). No fluorescence decrease and, thus, no damage of the lysosomal membrane was observed in cells treated with rapamycin.

**αATA(8,24) Inhibits Akt/mTOR Signaling and Induces Cell Cycle Arrest in Prostate Cancer Cells.** Membranes of functional lysosomes are the location for the activation of mTOR, which promotes cell growth and proliferation under conditions of abundant nutrient supply. On the other hand, inhibition of mTOR triggers activation of autophagy, which is characterized by the increased formation of acidic vesicular organelles (Zoncu et al., 2011). Indeed, treatment of cells with αATA(8,24) induced inhibition of the Akt/mTOR pathway as analyzed by the reduction of phosphorylation of its downstream target, S6K1, as soon as 6 hours after treatment, and thus, far ahead of any other effects observed in prostate cancer cells, such as mitochondrial membrane disipation and increase in the acidic vesicular organelle formation (Fig. 5A). Similar effects were observed after addition of the mTOR inhibitor rapamycin but not after treatment with the anti-mitotic drug docetaxel.

S6K1 is a downstream target of mTORC1, an mTOR complex that preferentially drives the translation of mRNAs for protumorigenic genes, including cell cycle regulators, thus promoting tumorigenesis (Zoncu et al., 2011). mTORC1 is, in turn, regulated by tuberous sclerosis complex 2, an Akt substrate (Fig. 5D). We have previously shown that tirucallic acids inhibit the Akt kinase activity (Estrada et al., 2010). Indeed, αATA(8,24) is a potent Akt inhibitor, which inhibited Akt1 activity in kinase assay more efficiently than known tirucallic acids (Fig. 5B).

As expected, treatment of PC-3 cells with αATA(8,24) for 8 hours resulted in decreased protein levels of cyclin D1 and its corresponding partner CDK4, cyclin E and its corresponding partner CDK2, as well as cyclin B1. The expression of the phosphorylated form of retinoblastoma protein was also significantly reduced by treatment with αATA(8,24) and rapamycin, and to a lesser extent by OTA. Interestingly, the antimitotic agent docetaxel inhibited slightly the expression of only cyclin E and CDK4, and significantly increased the expression of the cyclin B1 (Fig. 5C) owing to inhibition of the cyclin B1 proteolysis by the proteasome (Clute and Pines, 1999). Not unexpectedly, inhibition of the cell cycle regulators by αATA(8,24) and OTA resulted in dysregulation of the cell cycle of the prostate cancer cells within 24 hours. Docetaxel induced a strong accumulation of PC-3 cells in the G2 phase of the cell cycle, which might be a result of its binding and stabilization of microtubules, followed by mitotic catastrophe (Yvon et al., 1999). By contrast, rapamycin did not affect the cell distribution throughout the cell cycle within 24 hours after treatment (Fig. 5E).

**Discussion**

The present study provides evidence that the tirucallic acid isomer αATA(8,24) is more potent than tirucallic acid derivatives investigated so far with respect to induction of apoptotic cell death in prostate cancer cells in vitro and in vivo. Furthermore, the differential selectivity of the tirucallic acid derivatives in destroying cancer cells without overt cytotoxicity to normal cells encouraged us to analyze the mechanisms of their toxicity.

Cell death occurs mostly dichotomously as either apoptosis or necrosis. Apoptosis is an active, programmed process of autonomous cellular dismantling that does not elicit inflammation known to be produced by necrosis (Fink and Cookson, 2005). Therefore, drugs leading to apoptotic cell death are of great interest in biomedical and pharmaceutical research. On the basis of several markers of apoptosis, such as loss of membrane phospholipid asymmetry resulting in phosphatidylserine externalization, caspase-3 activation, and the late-occurring DNA fragmentation, we concluded that, following treatment with αATA(8,24), PC-3 cells succumb through apoptosis. The presence of an acetyl group instead of a keto group at the position 3 renders αATA(8,24) more lipophilic compared with OTA, as evidenced by the retention times upon reversed-phase high-performance liquid chromatography (Supplemental Figs. 2 and 3). This phenomenon might explain the higher cytotoxic and apoptosis-promoting capability of αATA(8,24) compared with OTA.

Activation of caspase-3 was already observed 24 hours after treatment with αATA(8,24), ahead of any other biochemical or morphologic change associated with apoptosis. This suggests that caspase-3 is the initiator of apoptosis in cells treated with αATA(8,24). It is known that mitochondrial proteins can

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**Fig. 4.** αATA(8,24) affects acidic vesicular organelles in prostate cancer cells. PC-3 cells were treated with either αATA(8,24) (10 μM), OTA (10 μM), docetaxel (100 nM), or rapamycin (10 nM) for 24 and 48 hours, stained with acridine orange, and analyzed by flow cytometry. Graph shows the fraction of cells with an increased number of acidic vesicular organelles (A1). Data are mean ± S.E.M.; **P < 0.01; ***P < 0.001 versus control.
trigger initiation of apoptosis and, in particular, activation of caspase-3 (Green and Reed, 1998). The release of caspase activators (such as cytochrome c, second mitochondria-derived activator of caspases/DIABLO, Omi/HtrA2), alteration of cellular redox state (oxidative stress), disruption of electron transport and energy metabolism, and loss of mitochondrial transmembrane potential are the events responsible for the critical role of mitochondria in apoptotic cell death (Green and Reed, 1998; Fischer and Schulze-Osthoff, 2005). These events are the consequence of the mitochondrial membrane dysfunction that

Fig. 5. αATA(8,24) inhibits the Akt/mTOR signaling and induces cell cycle arrest in prostate cancer cells. (A) PC-3 cells were treated with either αATA (8,24) (10 μM), OTA (10 μM), docetaxel (100 nM), or rapamycin (10 nM) for 6 hours. Inhibition of the mTOR pathway was analyzed by Western immunoblotting. Actin served as a loading control. (B) Active human recombinant Akt1 (100 nM) was pretreated for 15 minutes at 30°C with different concentrations of the tirucallic acids or the Akt inhibitor VIII (Akti-1/2, 10 μM) and analyzed in a kinase assay using glycogen synthase kinase 3α/β as substrate. Control samples were treated with solvent. Results of the kinase assays and immunoblots are representative out of three. (C) Inhibition of the expression of cell cycle regulators was analyzed by Western immunoblotting in cells treated as in (A) for 8 hours. (D) Scheme of mTOR signaling. (E) Cell-cycle analysis was performed in cells treated as in (A) for 24 hours. DNA was stained with propidium iodide and cells were analyzed by flow cytometry. Representative pictures are shown. All data mean ± S.E.M., n = 3; *P < 0.05; **P < 0.01; ***P < 0.001. PI3K, phosphoinositide 3-kinase; TSC, tuberous sclerosis complex.
manifests itself in a collapse of transmembrane potential, uncoupling of respiration from oxidative phosphorylation, high ROS production, followed by activation of caspase-3 and cell death. Here, we provide evidence that tirucallic acids induce changes in mitochondrial membranes leading to an early hyperpolarization, followed by depolarization and collapse of the mitochondrial membrane potential. The hyperpolarization can be a sign of an increased number of mitochondria per cell or an increase in matrix volume. In turn, mitochondrial swelling could lead to the rupture of its outer membrane, followed by dissipation of the mitochondrial membrane potential, cytochrome c release, and caspase-3 activation (Green and Reed, 1998; Fischer and Schulze-Osthoff, 2005), events that take place in cells treated with \( \alpha \text{ATA}(8,24) \).

These processes were accompanied by an increase in ROS and superoxide in the cytosol, which can likewise act as mediators of apoptotic pathways, in particular through hydrogen peroxide, which reacts in lysosomes with ferruginous material delivered there by autophagy. Highly reactive ROS produced in lysosomes as a result of Fenton-type reactions can damage the lysosomal membrane and induce release of lysosomal cargo into the cytosol, thereby promoting apoptosis (Repnik et al., 2013). Overall, this leads to a point of no return during the commitment stage of apoptosis and suggests that apoptosis induced by tirucallic acids proceeds mainly through the mitochondrial pathway. Owing to the different redox states of normal and malignant cells and the higher susceptibility of cancer cells toward ROS-mediated apoptosis (Trachootham et al., 2009), the oxidative burst might be central to the tirucallic acid–induced apoptosis and explain the selectivity of those compounds toward cancer cells compared with normal, nonmalignant cells, such as peripheral blood mononuclear cells or, as observed previously, normal prostate epithelial cells (Estrada et al., 2010).

Parallel to the mitochondrial dysfunction, tirucallic acids increased the activity of acidic vesicular organelles, which may be a result of an increased size of lysosomes as well as an increased lysosome number. Such increased staining of acidic vesicular organelles is also consistent with the induction of acidic autolysosomes, a late event in autophagy induction (Klionsky et al., 2012). The lysosomal membranes contain highly specialized glycosylated proteins that form a tight barrier for the luminal milieu with its acidic pH of <5 and about 50 highly destructive hydrolases. During lysosomal membrane permeabilization, commonly named LMP, detrimental lysosomal proteins may leak into the cytosol and damage membranes of other organelles. Thus, the mitochondrial membrane permeabilization could be a result of the increased permeabilization of the lysosomal membrane. ROS, in turn, may contribute to lysosomal membrane permeabilization by causing lipid peroxidation and damaging lysosomal membrane proteins as has been discussed above (Aits and Jaattela, 2013).

\( \text{mTOR} \) as well as Akt strongly suppress autophagy under nutrient-rich conditions, and \( \text{mTOR} \) inhibitors, such as rapamycin, for example, are strong inducers of autophagy (Zoncu et al., 2011). Therefore, accumulation of the acidic vesicular organelles in cells treated with \( \alpha \text{ATA}(8,24) \) might be a result of Akt/\( \text{mTOR} \) inhibition; a similar effect was observed with rapamycin. Additionally, activation of the Akt/\( \text{mTOR} \) pathway positively regulates cell proliferation by the Akt and \( \text{mTORC1} \)-mediated translation of mRNAs for protumorigenic genes, including cell cycle regulators contributing to the growth of many sporadic cancers (Zoncu et al., 2011; Porta et al., 2014). \( \alpha \text{ATA}(8,24) \) with similarity to rapamycin and in contrast to docetaxel, strongly inhibited theAkt/\( \text{mTORC1} \) signaling and the expression of different cell cycle regulators. Consistently, \( \alpha \text{ATA}(8,24) \) significantly disturbed the cell cycle of prostate cancer cells. The inhibition of the Akt/\( \text{mTOR} \) pathway by \( \alpha \text{ATA}(8,24) \) was already evident 6 hours after stimulation, before any mitochondrial or lysosomal dysfunction could be determined.

Inhibition of \( \text{mTORC1} \) by rapamycin was, however, not sufficient to trigger apoptosis in prostate cancer cells, as we show here, and as it has previously been shown by us and others (Zoncu et al., 2011; Morad et al., 2013). Rapamycin, which promotes autophagy, even protects cells against a range of proapoptotic insults. It is believed that autophagy, which enhances clearance of mitochondria, reduces cytotoxic cytochrome c release and downstream caspase activation thereby promoting survival (Repnik et al., 2013). All forms of autophagy, in turn, rely on lysosomes for the subsequent degradation of their cargo. Under conditions of oxidative stress, excess ROS diffusing into lysosomes and autolysosomes fully loaded with autophagic cargo can easily destabilize membranes and cause lysosomal membrane permeabilization (Aits and Jaattela, 2013). Furthermore, lysosomal membrane dysfunction not only leads to the release of lysosomal proteases into the cytosol but also reduces the degradation rate of the endocytotic system, impairing the cytoprotective role of autophagy and lowering the apoptotic threshold (Repnik et al., 2013). Indeed, in cells treated with \( \alpha \text{ATA}(8,24) \) the increased ROS and superoxide production might be amplified by the positive feedback loop between mitochondria and acidic vesicular organelles, whereas in cells treated with rapamycin, the ROS production and the feedback loop are absent.

Thus, the novel tirucallic acid derivative \( \alpha \text{ATA}(8,24) \) is a potent inhibitor of the Akt/\( \text{mTOR} \) pathway leading to prolonged autophagy, ROS production, permeabilization of the lysosomal membrane, dissipation of the mitochondrial membrane potential, and apoptosis in prostate cancer cells. Owing to the increased susceptibility of cancer cells to tirucallic acids, they may present a new class of antitumor lead compound exhibiting the desired selectivity for cancer cells. These properties of tirucallic acids warrant further therapeutic exploitation.

**Authorship Contributions**

**Participated in research design:** El Gaafary, Büchele, Syrovets, Simmet.

**Conducted experiments:** El Gaafary, Büchele, Agnolet, Schneider, Schmidt.

**Contributed new reagents or analytic tools:** Büchele, Agnolet, Schneider, Schmidt.

**Performed data analysis:** El Gaafary, Büchele, Syrovets, Agnolet, Schneider, Simmet.

**Wrote or contributed to the writing of the manuscript:** El Gaafary, Syrovets, Simmet.

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