Vacuolar-ATPase Inhibition Blocks Iron Metabolism to Mediate Therapeutic Effects in Breast Cancer

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Abstract

Generalized strategies to improve breast cancer treatment remain of interest to develop. In this study, we offer preclinical evidence of an important metabolic mechanism underlying the antitumor activity of inhibitors of the vacuolar-type ATPase (V-ATPase), a heteromultimeric proton pump. Specifically, our investigations in the 4T1 model of metastatic breast cancer of the V-ATPase inhibitor archazolid suggested that its ability to trigger metabolic stress and apoptosis associated with tumor growth inhibition related to an interference with hypoxia-inducible factor-1α signaling pathways and iron metabolism. As a consequence of disturbed iron metabolism, archazolid caused S-phase arrest, double-stranded DNA breaks, and p53 stabilization, leading to apoptosis. Our findings link V-ATPase to cell-cycle progression and DNA synthesis in cancer cells, and highlight the basis for the clinical exploration of V-ATPase as a potentially generalizable therapy for breast cancer. Cancer Res; 75(14); 2863–74. ©2015 AACR.

Introduction

Breast cancer is a major health issue, which worldwide causes almost 500,000 female fatalities each year, being the most lethal cancer for women (1). Therefore, it is of utmost importance to find new therapeutics to combat this disease. Nature is still one of the most essential sources for new chemotherapeutics, as approximately 60% of the new agents discovered in the last decades were classified as naturally derived or inspired (2). The myxobacterial macrolide archazolid, which was first isolated from Archangium gephyra (3), is a highly potent vacuolar-type ATPase (V-ATPase) inhibitor (4). It showed first promising cytotoxic effects on diverse cancer cell lines (5, 6) proposing pharmacologic V-ATPase inhibition as a new strategy to abrogate solid tumor growth. However, the precise mode of action is not defined yet.

V-ATPases are proton pumps located in the endomembrane system of eukaryotic cells as well as in the plasma membrane. They are heteromultimeric enzymes consisting of two functional domains: the cytosolic hydrolytic active V1 domain and the membrane integral V0 complex, which is responsible for proton translocation. V-ATPases actively transport protons from the cytoplasm into intracellular compartments or across the outer membrane. As a consequence of the acidification of endosomes and lysosomes, V-ATPases play a crucial role in the receptor-mediated endocytosis and the endosomal trafficking (7). Besides a variety of transporter and channel proteins, plasma membrane localized V-ATPase is reported to modulate the tumor microenvironment (7, 8). V-ATPase function can be inhibited by the myxobacterial compound archazolid, which binds to subunit c of the membrane integral V0 domain (4).

Our earlier studies revealed a strong cytostatic effect of archazolid on diverse cancer cell lines in vitro and showed an induction of cellular stress response involving the stabilization of the hypoxia-inducible factor 1α (HIF1α) protein (6). Yet, it stayed unclear how inhibition of V-ATPase generates HIF1α stabilization. The aim of this study was now to illuminate this matter and to extend the in vitro data for in vivo efficacy. Thereby, we uncover that V-ATPase inhibition impedes the iron metabolism of cancer cells, which opens up new therapeutic options for V-ATPase inhibitors.

This work unveils that the natural derived V-ATPase inhibitor archazolid disrupts endocytic transferrin receptor (TfR) recycling, leading to iron depletion in the cytosol followed by stabilization of the HIF1α protein and reduction of ribonucleotide reductase (RNR) activity. Finally, this leads to induction of apoptosis in vitro and reduction of tumor growth in vivo. These results suggest V-ATPase as a highly promising target for breast cancer treatment at the interplay of iron metabolism and apoptotic processes.

Materials and Methods

Cell lines and reagents

The mammary cancer cell lines MDA-MB-231 cells were recently purchased from Cell Line Service Eppelheim, MCF7 from DSMZ, and 4T1-Luc (4T1) from PerkinElmer. MCF7 cells were grown in RPMI-1640 supplemented with 10% FCS, 1% pyruvate, 1% nonessential amino acids, and 1% sodium bicarbonate. The MDA-MB-231 and 4T1-Luc cell lines were grown in DMEM/F12 supplemented with 10% FCS, 1% pyruvate, and 1% nonessential amino acids for 1 week before they were further used for experiments. The cell lines MCF7 and MDA-MB-231 were grown in RPMI-1640 supplemented with 10% FCS, 1% pyruvate, and 1% sodium bicarbonate. The cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2 with a 95% air and monitored for cell proliferation and cell death. The cell lines were grown to 90% confluency, and cell lines were harvested by trypsinization at 72 h after treatment with archazolid. The cell lines were counted to analyze cell survival.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Measurement of intracellular iron levels

Iron levels were measured by atomic absorption spectrometry (PerkinElmer 2100). Treated cell pellets were digested with 60% citric acid and 10% nitric acid at 80°C for 3 hours. Spectra were recorded in continuous mode at 240 nm.

Measurement of cell death and cell cycle

Cell pellets were stained with Hoechst 33342 and propidium iodide (PI) and analyzed by flow cytometry. Cells with higher fluorescence intensities compared with control were considered apoptotic.

Clonogenic assay

Clonogenic assay was performed as described previously (6).

Measurement of dCTP levels

dCTP levels were measured as described previously (5). Therefore, cells were seeded on 10-cm culture dishes, allowed to grow overnight and treated for 24 hours.

qRT-PCR analysis

Total RNA was extracted using the RNeasy mini Kit (Qiagen GmbH) according to the manufacturer’s instructions. For cDNA synthesis, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. qRT-PCR was performed with the AB 7300 RealTime PCR system, the TaqMan Gene Expression Master Mix (Applied Biosystems) and the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. All designed primers were purchased from Metabion. ATP6V0C primers were purchased from Applied Biosystems.

Nuclei extraction

Stimulated MCF7 cells were trypsinized. Cell pellet was resuspended in nuclear extraction buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 1 mmol/L Complete EDTAfree; Roche) and incubated on ice for 15 minutes. Non-ident P-40 (0.625%) was added, probes were vortexed and centrifuged. Pellet was resuspended in nuclear extraction buffer B (20 mmol/L HEPES, pH 7.9, 0.4 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 25% glycerol, 1 mmol/L Complete EDTAfree). After 15-minute incubation on ice and centrifugation, supernatants were used for Western blot analysis.
Statistical analysis
GraphPad Prism Software was used for all statistical analysis. Error bars indicate SEM. Synergy was calculated with the Bliss formula: $v = \frac{v_{\text{combination}}}{(v_1 + v_2) - (v_1 \times v_2)}$, with $v > 1$ indicating synergism (13).

Results
Archazolid abrogates tumor growth in vivo and induces HIF1α stabilization in vitro
To start with, archazolid was confirmed to induce cell death in breast cancer cell lines MDA-MB-231 and MCF-7 as well as in mouse 4T1 cells (Supplementary Fig. S1). These results were extended by an in vivo model based on the 4T1 mouse mammary tumor cell line to assess therapeutic relevance of V-ATPase inhibition. In fact, archazolid reduced the tumor growth rate significantly with an average reduction of 24.4%, while the mean mouse weight did not differ (Fig. 1A). Tumor tissues of archazolid-treated mice showed induced caspase-3 activity (Fig. 1B).

To explore the molecular mechanism of archazolid-induced cell death, the HIF1α protein came into focus. We confirmed a strong HIF1α expression after 24 hours of archazolid treatment in MCF7 and MDA-MB-231 breast cancer cell lines (Fig. 1C). Importantly, silencing of ATP6V0C, the subunit c of V-ATPase, by siRNA resulted in stabilization of HIF1α in both cell lines after 72 hours (Fig. 1C), suggesting a V-ATPase–dependent effect. Silencing efficiency is shown in Supplementary Fig. S2 both on the mRNA and protein level. In addition to the expression status of the HIF1α protein, we analyzed the mRNA levels via qPCR analysis in MDA-MB-231 cells after 24 hours of treatment, which showed no significant changes (Supplementary Fig. S3), indicating an effect on stabilization of HIF1α by archazolid.

As HIF1α has many target genes involved in glycolysis, we investigated the impact of archazolid on glycolysis and oxidative phosphorylation. Western blot analysis after 24 hours of archazolid treatment showed induced hexokinase II protein levels in breast cancer cell lines (Fig. 1D) and also other glycolytic gene products [6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase-3 (6PF2K3), aldolase C, Supplementary Fig. S4]. Moreover, measurement of oxygen consumption demonstrated a reduction in MCF7 cells after 4 hours of archazolid treatment compared with control cells (Fig. 1E). Oligomycin served as a positive control. Interestingly, the ratio of metabolic activity of MCF7 cells grown in medium with glucose to cells cultivated with galactose increases after 48 hours of archazolid treatment further implicating a shift to glycolysis (Fig. 1F). Galactose forces the cells to derive their energy by oxidative phosphorylation. Hence, an increased ratio of metabolic activity in glucose to galactose medium implicates a mitochondrial impairment.

Iron depletion leads to HIF1α stabilization
To unveil how V-ATPase is implicated in HIF1α stabilization, it is important to know that the hydroxylation of HIF1α by prolyl hydroxylases (PHD) requires oxygen, 2-oxoglutarate and iron. To examine whether a lack of iron is responsible for the stabilization of HIF1α after V-ATPase inhibition, cell culture medium was supplemented with iron citrate. Indeed, Western blot analysis of breast cancer cells after 24 hours of treatment showed a complete abolishment of archazolid-induced HIF1α stabilization in the presence of additional iron (Fig. 2A). Hence, expression of HIF1α is due to iron depletion in the cell.

Iron depletion leads to iron depletion
Next, we investigated whether iron depletion is responsible for archazolid-induced cell death. Interestingly, flow-cytometric analysis of MCF7 and MDA-MB-231 cells after 48 hours of archazolid treatment showed a reduction of PI-positive cells when iron citrate was added (Fig. 3A). In addition, 4T1 cells showed rescue of metabolic activity in medium with iron excess (Supplementary Fig. S6). PARP-1 cleavage was abolished in human breast cancer cell lines after 48 hours by adding iron (Fig. 3B). Along this line, we could see a synergistic cell death induction in MCF7 cells after 48 hours by combining archazolid with the iron chelators deferoxamine and 3-AP (Fig. 3C). In MDA-MB-231 cells, combination of archazolid and 3-AP led to reduced formation of colonies after 24 hours of treatment (Fig. 3D). We could verify these findings in three-dimensional growing MCF7 mammospheres after 48 hours of treatment by PI staining (Fig. 4A and B) and CTB assay (Fig. 4C).

Iron depletion leads to dysfunction of RNR
To more deeply analyze the consequences of archazolid-induced iron depletion, we took a closer look at the iron-dependent target RNR. The formation of deoxyribonucleotides (dNTP) in a cell is iron dependent and catalyzed by RNR (14). For analyzing RNR activity, dCTP levels were measured as described previously (12). Interestingly, treatment of MCF7 and MDA-MB-231 cells with archazolid for 24 hours resulted in reduced dCTP levels in both cells lines (Fig. 5A). As dNTPs are needed for DNA synthesis, cell-cycle analysis was examined in MDA-MB-231 cells 48 hours after archazolid treatment, showing S-phase block, which could be rescued by adding iron (Fig. 5B). Phosphorylation of the H2AX histone (γH2AX), indicating double-strand breaks (DSB), was seen after 48 hours of archazolid treatment in all three cell lines, further suggesting a reduced RNR activity after archazolid treatment. Again, these effects were abolished by additional iron (Fig. 5C and Supplementary Fig. S7). Combination of the DNA intercalating drug doxorubicin with archazolid showed reduced formation of colonies in MDA-MB-231 cells (Fig. 5D). These findings connect V-ATPase inhibition to fundamental cellular processes such as DNA synthesis and DNA repair.
p53 is implicated in archazolid-induced cell death in p53 wild-type MCF7 cells

As the HIF1α protein and the occurrence of DSB are known inducers of the p53 protein, we analyzed its implication in archazolid-induced cell death in p53 wild-type MCF7 cells. Figure 6A shows elevated p53 mRNA levels after 24 hours of V-ATPase inhibition, which was compensated by additional iron. Along this line, archazolid-treated MCF7 cells indicate higher p53 protein levels after 48 hours within the nucleus (Fig. 6B). Silencing HIF1α in MCF7 cells reduced the induction of p53 protein levels (Fig. 6C) revealing an impact of HIF1α on p53 expression in this context. The ataxia telangiectasia mutated (ATM) kinase mediates the induction of p53 upon DSB. To unveil whether in addition the presence of DSB elevates p53 protein levels, we used the ATM inhibitor KU55933. Western blot analysis after 48 hours of archazolid treatment in MCF7 cells indicates that this is the case (Fig. 6D). Finally, to prove that p53 plays a role in archazolid-induced cell death, we used the p53 inhibitor pifithrin-α. Flow-cytometric analysis of MCF7 cells after 48 hours shows a significant reduction of cell death in the presence of pifithrin-α (Fig. 6E) providing evidence that p53 is involved in V-ATPase–dependent cell death.

To sum up in a cartoon, Fig. 7 illustrates that V-ATPase inhibition by archazolid leads to cell death induction, which is mediated by iron depletion, resulting in the stabilization of the HIF1α protein and the inhibition of RNR together leading to p53 induction.

Discussion

Our data suggest V-ATPase as a highly interesting and druggable target to abrogate solid breast tumors by interfering with the iron homeostasis of cancer cells. Since the first advent of V-ATPase inhibitors, evidence is increasing that V-ATPases are implicated in cancer. Reports show that...
V-ATPase is overexpressed in invasive pancreatic tumors (15) and that the mass of V-ATPases located at the plasma membrane of breast cancer cells correlates with their invasiveness (16). Among various other transporter proteins such as monocarboxylate transporter (MTC), Na⁺/H⁺ exchanger (NHE1), carbonic anhydrase IX (CAIX) plasma membrane located V-ATPase contribute to dysregulated pH in the tumor microenvironment favoring tumor progression and metastasis (8, 17). Moreover, overexpression of

Figure 2.
Archazolid affects Ti/TIR internalization and induces iron depletion. A, HIF1α expression was detected by Western blot analysis upon archazolid and iron citrate treatment (24 hours). B, archazolid-treated MDA-MB-231 cells (24 h) were incubated with calcein-AM for 30 minutes and analyzed by confocal microscopy. C, calcein-AM–stained MCF7 and MDA-MB-231 cells were analyzed by flow cytometry after 24 hours of archazolid and iron citrate treatment. Bars are the SEM of three independent experiments performed in triplicates; *, P < 0.05 (one-way ANOVA, Newman–Keuls multiple comparison test). D, MCF7 and MDA-MB-231 cells were incubated with Tf-rhodamine conjugate after 24 hours of archazolid treatment. Cells were analyzed by confocal microscopy. A, B, and D, representative experiments out of three independent experiments are shown.
Figure 3.
Induced iron deprivation leads to archazolid-induced cell death. A and C, cell death induction after archazolid, iron citrate, deferoxamine (DFO), and 3-AP treatment (48 hours in MCF7 cells and 72 hours in MDA-MB-231 cells) was assessed by flow cytometry. Bars are the SEM of three independent experiments performed in triplicates, \( P < 0.05 \) (one-way ANOVA, Newman-Keuls multiple comparison test). C, synergism was calculated with the Bliss formula (value for deferoxamine, 3; value for 3-AP, 1.93). B, PARP-1 cleavage was detected by Western blot analysis upon archazolid and iron citrate treatment (MCF7 cells, 48 hours; MDA-MB-231, 72 hours). One representative blot out of three independent experiments is shown. D, clonogenic survival after 20 hours of archazolid and 3-AP treatment (4 hours archazolid pretreatment) was analyzed by crystal violet staining. Absorption of resolved crystal violet was measured with SpectraFluor Plus reader. Bars are the SEM of three independent experiments performed in triplicates. Synergism was calculated with the Bliss formula (value: 2.05)
Figure 4.
Combination of archazolid and 3-AP induces synergistic cell death in mammospheres. A, MCF7 mammospheres were treated with archazolid for 48 hours, stained with PI and Hoechst, and analyzed by confocal microscopy. B, MCF7 mammospheres seeded in Poly-HEMA plates were incubated with archazolid and 3-AP for 48 hours. A and B, representative experiments out of three independent experiments are shown. C, metabolic activity of MCF7 mammospheres after archazolid and 3-AP treatment was assessed by CTB assay. Bars are the SEM of three independent experiments performed in triplicates. *, *P* < 0.05 (one-way ANOVA, Newman-Keuls multiple comparison test).
subunit c of V-ATPase in Drosophila induces a tumor-like tissue transformation (18). Nontumor cells showed less cytotoxic sensitivity toward V-ATPase inhibition compared with breast carcinoma cells and hepatoblastoma cells (6, 19). It has been shown that V-ATPase inhibition abrogates tumor cell migration and dissemination through disturbed endocytotic activation of Rac1 (20). Furthermore, cell death induction was observed in various cancer cell lines by bafilomycin, a V-ATPase inhibitor from the first family (21–23). Consistently, we did find induced apoptosis in breast cancer cells through archazolid treatment. Here, we could show for the first time that V-ATPase inhibition by archazolid reduced the growth rate of 4T1 mammary tumors significantly in vivo, indicating therapeutic relevance of V-ATPase inhibition.

Although cell death induction through V-ATPase inhibition was extensively studied, the precise causal mechanisms are still not well understood. Wu and coworkers attributed the induction of apoptosis in colon cancer by bafilomycin to the inhibition of macroautophagy (21). Others suggested a mechanism in which proteases are released after lysosomal dysfunction, leading to caspase-3 activation (22). Our group postulated a mechanism that involves the induction of a cellular stress response, including...
autophagy and the stabilization of HIF1α (6). This study here ties in with our earlier work and reveals a role for V-ATPase at the interplay of iron metabolism and apoptotic processes. Our findings illuminate that the cytotoxicity of V-ATPase inhibitors is mainly due to disturbed TfR recycling, which impairs the iron supply of cancer cells. As a consequence, the activity of iron-dependent enzymes is reduced, leading to DSB, S-phase arrest, and induction of p53.

In the last decades, it became more and more evident that iron is implicated in cancer development. Induction of malignant tumors by injecting iron dextran into rat was first reported in 1959 (24). This was confirmed later by the observation that patients injected with iron preparations developed sarcomas (25). Consistently, epidemiologic reports from the 1980s associated high body iron with the risk of cancer (26). The iron supply of cancer cells is mainly mediated by the TfR. The clearly defined function of the TfR is the interaction with the iron loaded plasma glycoprotein Tf initiating its endocytosis uptake (27). Since it has been reported that TfR is overexpressed in cancer cells (28, 29), targeted therapy became an attractive strategy for cancer treatment. In different studies, antibodies against TfR have successfully been used to abrogate tumor progression (30, 31). Others used toxic moieties conjugated to Tf to selectively target cancer cells (32). After internalization of Tf/TfR, iron dissociates from the receptor-ligand complex and enters the cytosol forming the labile iron pool where the complex gets recycled to the cell surface. Essential for the dissociation is the acid pH in the endosomes (33), which is maintained by V-ATPases. It is known that an inadequate acidification of endosomes inhibits trafficking out of them although precise mechanisms are not defined yet (34). Consistently, a role for V-ATPase in the internalization or processing of receptors has been shown such as for Notch (35, 36). Our present study in breast cancer cells shows impaired TfR recycling after archazolid treatment and connects this with disturbed iron metabolic pathways. The generated iron deprivation eventually induces apoptotic processes, leading to cell death. Therefore, we suggest inhibition of V-ATPase as a

![Figure 6](cancerres.aacrjournals.org)
new and effective way to target and interfere with the iron metabolism in cancer cells. Interestingly, there are other approaches to abrogate tumor growth by targeting iron homeostasis. Iron chelators such as deferoxamine and 3-AP showed antitumor effects in different reports (37). Combination of these iron chelators with archazolid showed synergistic cytotoxicity in breast cancer cells, which allowed for the reduction of both chelator and inhibitor concentrations. Up to now there are further chelators under evaluation as anticancer agents such as ciclopirox, tachpyridine, and deferasirox (38).

Sufficient amount of labile iron is essential for fundamental cellular processes such as DNA synthesis and repair (39). The enzyme that connects these processes to iron homeostasis is the RNR. It catalyzes the formation of dNTPs by reducing the corresponding ribonucleotides (14). In mammalians, class I enzymes of RNR are present using an iron center for the catalytic reaction (40). Not surprisingly, depleted labile iron in the cytosol of cells affects the activity of RNR (41), thereby reducing the dNTP pool. Consistently, our observations show decreased dCTP levels after V-ATPase inhibition. In consequence, dNTP levels are decreased in the cytosol, generating S-phase block and DSB, finally leading to p53-dependent and p53-independent apoptosis.

Figure 7. Proposed mode of action for archazolid induced apoptosis. A, Tf-bound iron is taken up by endocytosis. The low pH of the lysosomes releases the iron from Tf, reaching the cytosol through divalent metal transporters 1 (DMT1) where it forms the labile iron pool. Iron is an essential cofactor for the hydroxylation of HIF1α via PHDs, initiating its degradation. Furthermore, catalytic formation of dNTPs by RNR is iron dependent. 2-OG, 2-oxoglutarate. B, archazolid inhibits acidification of endosomes, resulting in disrupted TfR internalization. Induced iron deprivation stabilizes HIF1α and reduces RNR activity. In consequence, dNTP levels are decreased in the cytosol, generating S-phase block and DSB, finally leading to p53-dependent and p53-independent apoptosis.
tumor cells and shifts the cellular metabolism toward glycolysis (45). For the proteasomal degradation of HIF1α via the von Hippel–Lindau tumor suppressor, HIF1α has to be hydroxylated by PDHs (46). This catalytic reaction is dependent on the presence of oxygen, 2-oxoguantar, and iron (47). Consistently, arachidol-induced iron depletion stabilizes HIF1α modifying the cellular glucose metabolism.

DNA damage as well as HIF1α can evoke the stabilization of the tumor suppressor p53 (48, 49). After activation of p53, it is able to induce either cell-cycle arrest or apoptosis, thereby reducing tumor progression. Cell-type origin, strength of p53-activating stimulus, and others can influence the outcome of p53 activation (50). p53 is widely mutated in tumors but at least 50% of all cancers are p53 wild-type tumors. Therefore, a lot of effort was put into the development of p53 activators. Up to now, the most advanced p53 activators are RG7112, MI-773, and DS-3032b being in phase I clinical trials (51). Nevertheless, it has been reported that DNA damage can trigger apoptosis independent of p53 in p53-deficient cells (52). Our work in p53 wild-type tumor cells (MCf7 cells) showed a clear involvement of p53 in V-ATPase–dependent cell death. Furthermore, arachidol successfully induced cell death in p53-mutated breast cancer cells (MDA-MB-231) and p53-null breast cancer cells (4T1). Hence this indicates that targeting the iron metabolism of cancer cells by V-ATPase inhibition can hit p53 wild-type tumors as well as mutated. Nonetheless one might speculate that a xenograft mouse model using a cell line expressing wild-type p53 should be more sensitive to tumor mass reduction than our p53-null 4T1 model.

Our observations that V-ATPase inhibition generates iron deprivation in the cell, thereby influencing activity of the iron-dependent enzymes PHDs and RNR evokes new options for interfering with the iron metabolism in cancer therapy. The depicted mechanism of action connects V-ATPase inhibition to fundamental cellular processes such as DNA synthesis and repair. We like to point to the fact that the growth-inhibiting effects of arachidol seen in vitro are recapitulated in a solid tumor growth model in vivo, suggesting inhibition of V-ATPase as highly promising and viable strategy for breast cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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