V-ATPase Inhibition Regulates Anoikis Resistance and Metastasis of Cancer Cells

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Abstract

Fighting metastasis is a major challenge in cancer therapy and novel therapeutic targets and drugs are highly appreciated. Resistance of invasive cells to anoikis, a particular type of apoptosis induced by loss of cell–matrix contact, is a major prerequisite for their metastatic spread. Inducing anoikis in metastatic cancer cells is therefore a promising therapeutic approach. The vacuolar-ATPase (V-ATPase), a proton pump located at the membrane of acidic organelles, has recently come to focus as an antimetastatic cancer target. As V-ATPase inhibitors have shown to prevent invasion of tumor cells and are able to induce apoptosis, we proposed that V-ATPase inhibition induces anoikis-related pathways in invasive cancer cells. We used the V-ATPase inhibitor archazolid to investigate the mechanism of anoikis induction in various metastatic cancer cells (T24, MDA-MB-231, 4T1, 5637) in vitro. Anoikis induction by archazolid was characterized by decreased c-FLIP expression and caspase-8 activation as well as reduction of active integrin-β1 and an early increase of the proapoptotic protein BIM. However, we observed that archazolid also induces mechanisms opposing anoikis such as degradation of BIM mediated by extracellular signal-regulated kinase (ERK), Akt and Src kinases at later time points and induction of reactive oxygen species. Still, intravenous injection of archazolid-treated 4T1-Luc2 mouse breast cancer cells resulted in reduced metastasis in mouse lungs. Thus, V-ATPase inhibition is not only an interesting option to reduce cancer metastasis, but also to better understand anoikis resistance and to find choices to fight against it. Mol Cancer Ther; 13(4); 926–37. ©2014 AACR.

Introduction

The cause of death by cancer is mainly not the primary tumor but the development of metastases in distinct organs (1). Metastasis is a highly complex process following sequential steps that comprise dissociation of cancer cells from the site of origin, their survival and travel in the circulation as well as migration and proliferation in distinct target organs (2–4). Because of the clinical importance, there is still the need to better understand the major determinants of metastasis and to identify therapeutic targets and pathways suitable for fighting the metastatic process.

We recently introduced the vacuolar-ATPase (V-ATPase) as a promising new antimetastatic target showing that the V-ATPase inhibitor archazolid inhibits cancer cell migration (5).

V-ATPases are ATP-dependent proton pumps ubiquitously expressed, regulating the pH in endomembrane systems thus affecting receptor-mediated endocytosis and intracellular trafficking (6). Archazolid is a myxobacterial second metabolite, first isolated from Archangium gephyra (7), which binds the subunit c in the V0 domain of the V-ATPase and thereby inhibits its activity (8).

Besides affecting motility of invasive cancer cells, archazolid also induces apoptotic cell death. We therefore hypothesized that archazolid might also affect the initial steps of metastatic dissemination, which is characterized by the ability of invasive tumor cells to survive in a state of detachment from the extracellular matrix (9).

Although nontumoral cells respond to loss of cell–matrix contact by a particular type of apoptosis, which has been termed anoikis (10, 11), metastatic cancer cells are insensitive to anoikis. This allows their survival after detachment from the primary tumor and their travel through the bloodstream to distant organs (3, 11). In other words, anoikis resistance is a precondition and a hallmark for the metastatic spread of tumor cells (12). Inducing anoikis by drugs is a promising option for the treatment of metastatic cancer but calls for a profound understanding of the mechanisms underlying anoikis resistance in invasive cancer.

Anoikis is initiated by disruption of integrin ligation of cells to the ECM. Integrin binding activates distinct
cell survival signaling cascades comprising downstream players such as focal adhesion kinase (FAK), Src kinase, PI3K/Akt, and extracellular signal-regulated kinase (ERK). Detachment of cells, that is, loss of integrin signaling not only inhibits survival signals, but also activates specific apoptotic processes (13–15).

Anoikis in line with classical apoptosis can follow both the extrinsic pathway triggered by cell surface death receptors and the intrinsic pathway driven by mitochondria (12, 16). Activation of caspase-8 is considered as a major player of the extrinsic pathway, and the activation of proapoptotic members of the Bcl-2 family, in particular, the protein BIM and mitochondrial cytochrome C release as features of the intrinsic pathway of anoikis. However, a cross-talk between intrinsic and extrinsic pathways frequently occurs (3, 17, 18).

Detachment can also induce autophagy as a survival mechanism preventing anoikis. It has been shown that inhibition of autophagy increased caspase-3 activity in detached cells (19). BIM was also suggested to play a role in autophagy by interacting with beclin-1 and inhibiting autophagosome formation (20). V-ATPase inhibition on the other hand is known to block late-stage autophagy due to alkalization of lysosomes and therefore inhibition of protein degradation in autolysosomes. V-ATPase inhibition might also affect integrin signaling as integrin activities are dependent on fast endocytosis rates and V-ATPase regulates receptor recycling via acidification of endosomes and lysosomes (21–23). That is why it has been hypothesized that V-ATPase inhibition affects anoikis induction.

In fact, we report here, that archazolid induces anoikis in invasive urinary and breast cancer cells. Anoikis is provoked by a reduction of active integrin-β1, an activation of caspase-8, an early translocation of BIM to the mitochondria followed by release of cytochrome C. Of note, archazolid-treated breast tumor cells injected (intravenously) in mice showed a reduced formation of lung metastasis. Our study further identified counter mechanisms induced by archazolid treatment like a strong proapoptomal downregulation of BIM provoked by generation of reactive oxygen species (ROS) and activation of prosurvival kinase Akt.

Materials and Methods

Compounds

Archazolid (Supplementary Fig. S1) was purified and isolated as described previously (7). The proteasome-inhibitors MG-132 and bortezomib as well as the Akt-inhibitor LY 294002, ERK-inhibitor PD 98059, and c-Src-inhibitor saracatinib, all dissolved in dimethyl sulf-oxide (Sigma-Aldrich) were purchased from Selleckchem. N-Acetyl-L-Cystein (NAC; Sigma-Aldrich) dye, photographed, and analyzed by ImageJ software performed according to the guidelines of the German law for protection of animal life and approved by the local ethics committee.

Cell culture

The human urinary carcinoma cell line T24 was provided by Dr. B. Mayer (Surgical Clinic, LMU, Munich, Germany) in 2009 and authenticated in April 2013 by the DSMZ by DNA profiling of eight highly polymorph short tandem repeats (STR) regions. Cells were cultured in McCoy's medium containing 10% heat-inactivated fetal calf serum (FCS, Biochrom AG), 1% glutamine (1.5 mmol/L), and 1% penicillin-streptomycin at 37°C and 5% CO2. MDA-MB-231 and 5637 cells were purchased from CLS cell lines service GmbH in May 2011 and April 2013. CLS authenticates all cell lines by DNA profiling via STR analysis. The mouse breast cancer cell line 4T1-Luc2 was purchased from Caliper Life Science in January 2012. Caliper analyzed the cells by IMPACT 1 PCR profiling. 4T1-Luc2 and 5637 cells were maintained in RPMI-1640 medium with 10% FCS. MDA-MB-231 cells were cultivated in Dulbecco’s Modified Eagle Medium (High Glucose) with 10% FCS. All media were purchased from PAA laboratories.

In vivo experiments

Twenty, 4- to 6-week old female BALB/cByJRj mice (Janvier) were housed in individually ventilated cages with a 12-hour day/night cycle and food and water ad libitum. Mice were injected with archazolid pretreated (10 nmol/L, 24 hours) or untreated 4T1-Luc2 cells (1 × 10⁶) via the tail vein. Bioluminescence of metastasized cells was monitored at day 8 after cell injection under anesthesia (2% isoflurane in oxygen) using the IVIS Lumina system with Living Image software 3.2 (Caliper Life Sciences) 15 minutes after intraperitoneal injection of 6 mg Na-lucif-erin (Promega). Thereafter, mice were sacrificed by cervical dislocation, their lungs harvested, imaged, and weighted. The total flux/area of the defined region of interest was calculated as photon/second/cm². All in vivo experiments were performed according to the guidelines of the German law for protection of animal life and approved by the local ethics committee.

Colony formation assay

Archazolid-treated T24 cells (5 × 10⁵, 24 hours) were suspended in a 0.4% agarose-medium mix (low-melting temperature agarose, LONZA), seeded on 6-well plates precoated with 1% agarose, and incubated for 9 days at 37°C. Evolved colonies were stained with MTT (Sigma-Aldrich) dye, photographed, and analyzed by ImageJ 1.46r software.

Detachment-induced anoikis assay

T24, MDA-MB-231, 4T1, or 5637 cells (7 × 10⁴) were kept in suspension by using poly-HEMA [poly(2-hydroxyethyl methacrylate); Sigma-Aldrich] coated culture dishes to prevent adhesion (24, 25). The culture medium was supplemented with 1% methicellulose (Sigma-Aldrich) to
increase viscosity to prevent cell clumping. Apoptotic death was analyzed as described (26): permeabilized cells stained with propidium iodide (50 μg/mL) were analyzed for their subdiploid DNA content by flow cytometry (FACSCanto II, BD Biosciences). Cell death was further analyzed by the propidium iodide exclusion assay counting propidium iodide (5 μg/mL) positive, nonpermeabilized cells by flow cytometry.

**Western blot analysis and cytosol-mitochondria fractionation**

Floating cells were treated with archazolid and the listed inhibitors for the indicated timeframes and subsequently lysed. Equal amounts of the proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes by tank blotting. For detection of specific proteins, the ECL detection system (Amersham Pharmaacia Biotech) or the Odyssey Infrared Imaging system version 2.1 (LI-COR Biosciences) was used.

For cytosol-mitochondria fractionation, cells were harvested, incubated with a permeabilization buffer (mannitol 210 mmol/L, sucrose 200 mmol/L, HEPES, pH 7.2, 10 mmol/L, Na2EGTA 0.2 mmol/L, succinate 5 mmol/L, bovine serum albumin 0.15%, digitonin 80 μg/mL; 20 minutes, on ice), and centrifuged (10 minutes, 1,300 rpm, 4°C). The supernatant was collected (cytosolic fraction) and the pellet permeabilized with 0.1% TritonX-100 (15 minutes, on ice; mitochondrial fraction).

**Flow cytometry analysis of cell surface integrin**

Active integrin-β1 on the cell surface was examined using the conformation specific integrin-β1 active form
12G10 antibody (Abcam). Floating and adherent cells were treated with archazolid (10 nmol/L, 24 hours), harvested on ice (adherent cells were trypsinized with trypsin/EDTA first), washed once with PBS, and incubated with the active integrin-β1 antibody (45 minutes, 4°C). After washing with PBS, cells were incubated with a fluorescent secondary antibody (45 minutes, 4°C) and analyzed by flow cytometry.

**Caspase activity**

After treating cells with archazolid (1 nmol/L, 10 nmol/L, 48 hours), the activity of caspase-8 was measured using a commercial caspase-8 activity assay (Calbiochem) based on the cleavage of a caspase-8–specific 7-amino-4-trifluoromethyl coumarin labeled peptide sequence. The fluorometric shift over 5 hours was monitored by a fluorescent plate reader (SpectraFluorPlus, Tecan) calculating the relative enzyme activity displayed as the relative fluorescence signal (RFU).

**Intracellular ROS level**

ROS was measured by using the 2',7'-dichlorofluorescin diacetate dye (DCFDA; Sigma-Aldrich). Cells were harvested after incubation with the indicated substances and stained with 20 μmol/L DCFDA for 30 minutes at 37°C, washed once with PBS, and measured by flow cytometry (excitation 488 nm, emission, excitation 535 nm, FACSCanto II, BD Biosciences).
Statistical analysis

All experiments were performed at least three times in triplicate. All statistic analyses were performed using GraphPad Prism 5.0 software. One-way ANOVA with Tukey posttest and for two column comparison unpaired Students t-test was performed as significance analysis. Error bars indicate SEM.

Results

Archazolid impairs anchorage-independent growth and induces anoikis in invasive cancer cells

To start with, anoikis resistance of the invasive cancer cell lines T24, 5637 (urinary), MDA-MB-231 (mammary), and 4T1-Luc2 (mouse mammary) was shown by their ability to grow and proliferate in an anchorage-independent state (Fig. 1A and B).

Interestingly, pretreatment of T24 cells with archazolid (Supplementary Fig. S1A) for 24 hours impaired anchorage-independent growth as shown by a reduction of viable colonies in a soft agar colony formation assay (Fig. 1A). To confirm that archazolid in fact inhibits V-ATPase, endolysosomal pH was monitored by staining cells with Lysotracker dye (Supplementary Fig. S1B). To exclude apoptosis responsible for the shown results cells were stained with propidium iodide before seeded to the soft agar (Supplementary Fig. S2A). Inhibition of colony formation could also be observed by the V-ATPase inhibitor concanamycin (Supplementary Fig. S2B), suggesting a V-ATPase–dependent effect.

To further analyze effects on anoikis, cells were kept in a detached status using poly-HEMA–coated culture dishes. Treatment with archazolid (10 nmol/L) induced anoikis in detached cancer cell lines T24, 5637, and 4T1 after 48 hours and MDA-MB-231 after 72 hours, respectively (Fig. 1B). In comparison, adherent T24 cells showed a higher sensitivity toward archazolid after 48 hours (Supplementary Fig. S3) pointing to death resistance mechanisms of detached cells.

Archazolid-treated tumor cells lose their metastatic potential in vivo

The anoikis inducing effect of archazolid was hypothesized to affect the invasive potential of tumor
cells in vivo. To confirm this hypothesis, an in vivo model based on the 4T1-Luc2 mouse mammary tumor cell line has been used. These cells are highly metastatic and disseminate quickly to the lungs when injected intravenously and are therefore resistant to anoikis. 4T1-Luc2 cells are further engineered to express a luciferase reporter to enable real-time monitoring of developing tumors by live imaging. As shown in Fig. 2A, 4T1-Luc2–injected animals formed easily detectable lung metastases; however, 4T1-Luc2 cells pretreated with 10 nmol/L archazolid for 24 hours showed a significant reduction of lung metastases (Fig. 2B). Archazolid-treated cells did not show signs of apoptosis at the time of the intravenous injection (Supplementary Fig. S4).

Underlying mechanisms

Archazolid treatment reduces active β1-integrin and FAK activity in detached cells. Integrin-β1 is a major player responsible for cell adhesion to the ECM. Activated by attachment integrin-β1 signaling inhibits anoikis and promotes cell survival (27).

Figure 3A shows that the level of active integrin-β1 (analyzed by an integrin-β1 conformation specific antibody) is similar between adherent and detached (24 hours) T24 cells. However, archazolid-treated detached cells (24 hours) display a reduction of active integrin-β1 compared with detached control cells, whereas surface level of total integrin-β1 was not affected by archazolid treatment.

The FAK is recruited and activated by integrin binding to their ECM ligands, leading to the activation of several downstream survival signals via the PI3K/Akt and the Raf/MEK/ERK pathway (14). As shown in Fig. 3B archazolid-treated floating cells showed a reduced phosphorylation of FAK in the total cell lysate after 24 and 48 hours of treatment.

Archazolid induces activation of caspase-8 and downregulation of c-FLIP

As caspase-8 induction is a known hallmark of anoikis, its activity was measured in cell lysates using a fluorophore generating substrate for caspase-8. Floating T24 and 4T1 cells were stimulated for 48 hours with different

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**Figure 3.** (Continued.) C, treated (48 hours) T24 and 4T1 cells were harvested and the total cell lysate was analysed for caspase-8 activity. TRAIL, as a positive control, was applied 1 hour before activity measurement. Top, representative graph of caspase-8 activity measurement over 6 hours (T24 cells) displayed as RFU. Bottom, evaluation of three independent experiments for T24 and 4T1 cells presented as relative RFU minute. ***P < 0.001; ***P < 0.05; n = 3. D, left, the level of caspase inhibitor c-FLIP was investigated by Western blot analysis in T24 cells after archazolid treatment (48 hours). Right, quantification of three independent experiments. Bars always represent the mean ± SEM of three independent experiments conducted in triplicate. All Western blot experiments show a representative blot out of three independent experiments.

1, P < 0.01.
archazolid concentrations, displaying a significant increase in caspase-8 activity compared with control cells (Fig. 3C). TRAIL was used as a positive control.

Also, the FLICE-like inhibitory protein (c-FLIP), a well-described inhibitor of caspase-8 (28), which contributes to anoikis resistance by overexpression in malignant cells (3), is reduced after 48 hours of treatment in floating T24 cells (Fig. 3D).

Archazolid treatment rapidly induced BIM translocation to the mitochondria leading to cytochrome C release

The Bcl-2 protein BIM is considered to be the major player of the intrinsic-mediated anoikis pathway (29). Figure 4A indicates that cellular BIM expression is not affected by archazolid treatment for 5 hours; however, BIM is translocated and enriched to mitochondria at early time points after archazolid exposure (i.e., 3 and 5 hours). BIM localized at the mitochondrial outer membrane is responsible for Bax and Bak activation leading to the release of cytochrome C in the cytosol and subsequently to apoptosis (30). A decrease of cytochrome C in the mitochondrial fraction was observed after 24-hour treatment (Fig. 4B). Obviously, both the intrinsic and the extrinsic pathways are activated by archazolid.

Archazolid triggers mechanisms opposing anoikis

Sustained exposure of detached cells to archazolid results in a strong reduction of BIM (Fig. 5A and Supplementary Fig. S5), which is due to proteasomal degradation shown by the use of two proteasome inhibitors (MG-132 and bortezomib; Fig. 5B and Supplementary Fig. S6). Interestingly, archazolid treatment increases ROS level significantly after 16 to 48 hours (Fig. 6C and Supplementary Fig. S7A) compared with floating control cells and especially after 24 hours (Fig. 6D). BIM localized at the mitochondrial outer membrane is responsible for Bax and Bak activation leading to the release of cytochrome C in the cytosol and subsequently to apoptosis (30). A decrease of cytochrome C in the mitochondrial fraction was observed after 24-hour treatment (Fig. 4B). Obviously, both the intrinsic and the extrinsic pathways are activated by archazolid.

In addition, kinases involved in anoikis resistance (Akt, ERK, Src) were shown to be affected by detachment of T24 cells shown by an early increase of their phosphorylation (Supplementary Fig. S8A and S8B). Treatment with archazolid even further increased the phosphorylation of Akt and did not downregulate phosphorylation of ERK and Src (Fig. 5D and Supplementary Fig. S8C), suggesting archazolid induced counter mechanisms. Of note, using specific inhibitors, that is, LY 294002 (Akt), PD 98059 (ERK), and Saracatinib (c-Src) leads to apoptosis (Supplementary Fig. S9) and prevents the decrease of BIM in archazolid-treated cells (Fig. 5D).

Targeting regulators of BIM degradation increases archazolid-induced anoikis

Combination of archazolid with the proteasome inhibitors MG-132 (100 nmol/L) and bortezomib (5 ng/mL) induced significant higher apoptosis rates (synergistic) in T24 cells and rescued BIM from degradation (Fig. 6A). NAC as well was able to increase cell death in combination with archazolid (Fig. 6B) and rescued BIM (Fig. 5C), suggesting a role for ROS in anoikis induction. The mechanisms leading to archazolid induced anoikis and counter mechanisms are further depicted in a cartoon (Supplementary Fig. S10).

Discussion

This work disclosed that pharmacologic inhibition of V-ATPase by archazolid induces anoikis in invasive cancer cells, which contributes to the distinct antimetastatic action of archazolid in vivo. Thus we could add important new information about the role of V-ATPase in cancer dissemination.

Up to now, it has been reported that the abundance of V-ATPase on the plasma membrane of tumor cells correlates with their invasiveness. Several well-known V-ATPase inhibitors like concanamycin and bafilomycin were shown to lead to growth arrest and cell death induction in a variety of tumor cells (6, 31, 32). Also, the newly developed V-ATPase inhibitors salicylihalamide (33) and NIK-12192 (34) have demonstrated antitumor activity although the exact molecular mechanisms of V-ATPase inhibitors leading to inhibition of tumor cell invasion remain to be elucidated. Cell surface located V-ATPase is hypothesized to create a proton efflux leading to an acidic pericellular microenvironment that promotes the activity of proinvasive proteases (35). However, recently there is increasing evidence that the endolysosomal V-ATPase is important as antitumor-al/antimetastatic target. Recent own work showed that V-ATPase inhibition by archazolid impairs endocytotic traffic of migratory signaling molecules such as Rac1 and EGF receptor (EGFR, which is pivotal for directed growth. In other words, most cell types need proper cell–matrix interactions to survive. Cell to ECM interactions occur mainly through specific integrins and especially anoikis resistance in cancer.

From a physiologic point of view, anoikis is an important mechanism to remove cells that are currently not in their correct location lacking a correct adhesion and thus to guarantee tissue homeostasis and prevent dysplastic growth. In other words, most cell types need proper cell–cell and cell–matrix interactions to survive. Cell to ECM interactions occur mainly through specific integrins and trigger a cascade of prosurvival and proliferative signals (12, 14).

Cancer cells in contrast, do not require adhesion to the ECM to survive and proliferate, they are mostly insensitive to anoikis and in fact resistance to anoikis is a key
regulator for tumor cell invasion and metastasis (12). Tumor cells use various strategies to acquire anoikis resistance such as the constitutively activation of survival pathways (PI3K/Akt, MEK/ERK, and Src family kinases), alteration of the integrin expression pattern or generating oxidative stress, and inhibition of apoptotic pathways (3, 12, 38, 39).

In experimental anoikis models, cells are forced to grow anchorage independent, which triggers the above-mentioned stress and survival responses leading to a selection of the “fittest,” which are often resistant to chemotherapeutics. This is supported by our observation that adherent cancer cells are significantly more sensitive to archazolid than detached cells (Supplementary Fig. S3). The clear effect of archazolid on anchorage-independent growth of cancer cells as well as the induction of cell death in cells forced to stay detached is thus remarkable. Even more so having learned that archazolid treatment itself potently induces anoikis resistance via ROS generation and activation of Akt, which together with constitutive active ERK and c-Src leads to the degradation of BIM.

Obviously it is important to find out how a compound like archazolid finally achieves anoikis induction, meaning which pathways are involved.

Anoikis is characterized as an apoptotic cell death executed by features of the intrinsic and extrinsic apoptotic pathway and the loss of survival signals by unligated anchorage proteins like integrins (4). The extrinsic pathway is initiated by caspase-8 activating effector caspases or promoting mitochondrial cytochrome C release (4).

Archazolid evidently uses the extrinsic apoptotic pathway as shown by a downregulation of c-FLIP and a distinct caspase-8 activation. Recruitment of caspase-8 and its activation have been shown to occur by loss of
anchorage to ECM and unligated integrins (17, 40). Integrins with the β1-subunit in common are the major receptors for ECM components responsible for cell–ECM interactions (14). Interestingly cell surface β1-integrin in 24-hour floating cancer cells was still as active as in adherent cells, suggesting an inside-out integrin

Figure 5. Counter mechanism induced by archazolid (Arch.). A, BIM degradation. BIM protein level in the whole-cell lysate (left) and the mitochondrial fraction (right) after prolonged treatment with archazolid (24, 48 hours) were analyzed by Western blot analysis. B, BIM rescue. A combination of archazolid (10 nmol/L) with proteasome inhibitor MG-132 (24 hours; left) or bortezomib (BOR, 48 hours; right) was used to investigate changes in BIM protein level by Western blot analysis. The combination was applied simultaneously to the cells at time of detachment. C, ROS generation. Archazolid induces generation of ROS, which can be inhibited by the antioxidant NAC after 48 hours of treatment (left). ROS generation was measured as indicated in Materials and Methods. Bars, the mean ± SEM of three independent experiments conducted in triplicate. *** P < 0.001. Right, ROS generation is involved in BIM degradation by archazolid. Western blot analysis showed that ROS scavenger in combination with archazolid (24 hours) rescued the degradation of BIM. D, activation of survival kinases. Left, activation of Akt, ERK, and Src kinases was investigated after 48 hours for Akt and ERK and 24 hours for Src of archazolid treatment by Western blot analysis of their phosphorylated sites. Right, application of inhibitors, i.e., LY 294002 (Akt), PD 98059 (ERK), or saracatinib (c-Src), were used to analyze changes in BIM protein level. All Western blot experiments show a representative blot out of three independent experiments. All experiments were performed with T24 cells.
was measured by propidium iodide staining and combination with NAC (10 mmol/L) for 48 hours. Induction of apoptosis detached T24 cells were treated with archazolid (5 nmol/L) alone or in combination with moderate concentrations of kinase inhibitors rescued BIM decrease, indicating that cells actively block BIM activation by various pathways in response to archazolid treatment (Fig. 5D). This further underlines the highly efficient survival and anoikis resistance mechanisms of invasive tumor cells and highlights compounds such as archazolid still inducing cell death.

Unexpectedly, we did not observe inhibitory effects of archazolid on either Akt, ERK, or c-Src but an activation of Akt, which is considered to be part of the counter pro-survival mechanism induced by archazolid.

Anoikis due to the intrinsic pathway is mainly initiated by BIM (42). BIM, a member of the Bcl-2 family activates Bax and Bak, which leads to the permeabilization of the outer mitochondrial membrane and the release of cytochrome C to the cytosol thereby activating a caspase cascade inducing cell death (30, 43). In fact, BIM has been reviewed as a potential target for tumor therapy as BIM promotes anoikis in many tumor cell types and BIM suppression supports metastasis and chemoresistance (29). BIM regulation is dependent on cell surface molecules like integrins and the EGFR (18).

We found that BIM was rapidly translocated to the mitochondria (Fig. 4A) by archazolid treatment followed by cytochrome C release (Fig. 4B) pointing to an involvement of the intrinsic pathway. Of note, BIM inhibition seems to be also the major factor used by T24 cells to induce resistance to anoikis as BIM gets strongly degraded at later time points of archazolid exposure (Fig. 5A). The fact that rescue of BIM degradation using proteasome inhibitors MG-132 and bortezomib synergistically increase archazolid-induced cell death underscores the important role of this BH-3 only protein in the regulation of anoikis and its failure (Figs. 5B and 6A).

As kinases such as ERK, Akt, and c-Src are known to regulate BIM degradation and expression, it was tempting to use specific kinase inhibitors to gain further insight in the archazolid triggered BIM removal and thus chemoresistance (29). The relevance of these kinases in survival was shown by the fact that kinase inhibitors alone at high concentration induce anoikis (Supplementary Fig. S9). Costimulation of archazolid with moderate concentrations of kinase inhibitors rescued BIM decrease, indicating that cells actively block BIM activation by various pathways in response to archazolid treatment (Fig. 5D). This further underlines the highly efficient survival and anoikis resistance mechanisms of invasive tumor cells and highlights compounds such as archazolid still inducing cell death.

In addition, ROS are considered critical players in anoikis resistance. Integrin-mediated adhesion induces a transient burst of high ROS levels transducing survival signals by activation of Src kinases. Activated Src kinases trans-phosphorylate EGFR ligands, activating the ERK and PI3K/Akt pathways. Scavenging of ROS in adherent cells leads to BIM induction and cell death (38, 44). ROS are also recognized as second messenger in cell growth, proliferation, adhesion, and cell spreading in transformed cells (45). Induction of ROS in tumor cells is correlated to tumor initiation and progression as well as with tumor invasiveness (46). Anoikis-sensitive cells show decreased ROS levels after detachment correlating with cell death induction (38). Now, we found that archazolid treatment of floating cells resulted in elevated ROS

activation supporting anoikis resistance of these cells (41). Of note, archazolid reduced the amount of active integrin-β1 on the cell surface leaving the total integrin level constant.

In consequence downstream prosurvival signals like the phosphorylation of one key player in anoikis protection, the FAK (27) was affected by archazolid treatment. FAK together with c-Src then interact with numerous molecules recruiting and activating other prosurvival proteins like PI3K/Akt or ERK (16). We observed that T24 cells shortly after detachment (5 hours) induce anoikis resistance by activating Akt and ERK, which decreases when cells were detached for 24 hours, suggesting a restored balance between apoptotic and survival players (Supplementary Fig. S8A and S8B). C-Src kinase phosphorylation is not changed despite detachment over 24 hours (Supplementary Fig. S8B).

![Figure 6](image-url)

Figure 6. Inhibition of resistance pathways triggers archazolid (Arch.)-induced anoikis. A, floating T24 cells were either treated with archazolid (10 nmol/L) alone or in combination with the proteasome inhibitor MG-132 (100 nmol/L) for 48 hours. Subsequently apoptosis induction was determined as described in Materials and Methods. ***, P < 0.001. B, detached T24 cells were treated with archazolid (5 nmol/L) alone or in combination with NAC (10 mmol/L) for 48 hours. Induction of apoptosis was measured by propidium iodide staining and flow cytometry. ***, P < 0.001. Bars always represent the mean ± SEM of three independent experiments conducted in triplicate.
levels with untreated cells (Fig. 5C and Supplementary Fig. S7A), but did not exceed the steady state level of ROS in untreated adherent cells (Supplementary Fig. S7B). Still, cell death was induced by archazolid. This elevated ROS could be a further explanation for the prominent removal of BIM protein. We showed in accordance to Giannoni and colleagues that cotreatment of archazolid with an ROS scavenger lead to increased cell death and increased BIM levels (38). Therefore, ROS must play a critical role in resistance to archazolid-induced anoikis.

In sum, we demonstrate that archazolid induces anoikis in highly invasive tumor cells (Supplementary Fig. S10). Anoikis induction is accompanied by initiation of highly productive resistant mechanisms especially degradation of BIM and induction of ROS. Understanding the mode of actions leading to cell death by archazolid treatment and the challenges of counter reactions can help gaining deeper insight in anoikis resistance mechanisms, chemoresistance and the metastatic transition of detached tumor cells.

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

References


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Conception and design: C.M. Schempp, K. von Schwarzenberg, R. Müller, A.M. Vollmar

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