The V-ATPase-inhibitor Archazolid abrogates tumor metastasis via inhibition of endocytic activation of the Rho-GTPase Rac1

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ABSTRACT

The abundance of the multimeric vacuolar ATP dependent proton pump, V-ATPase, on the plasma membrane of tumor cells correlates with the invasiveness of the tumor cell, suggesting the involvement of V-ATPase in tumor metastasis. V-ATPase is hypothesized to create a proton efflux leading to an acidic pericellular microenvironment that promotes the activity of pro-invasive proteases. An alternative, not yet explored possibility is that V-ATPase regulates the signaling machinery responsible for tumor cell migration. Here, we show that pharmacological or genetic reduction of V-ATPase activity significantly reduces migration of invasive tumor cells in vitro. Importantly, the V-ATPase inhibitor archazolid, abrogates tumor dissemination in a syngeneic mouse 4T1 breast tumor metastasis model. Pretreatment of cancer cells with archazolid impairs directional motility by preventing spatially-restricted, leading edge localization of epidermal growth factor receptor (EGFR) as well as of phosphorylated Akt. Archazolid treatment or silencing of V-ATPase inhibited Rac1 activation, as well as Rac1-dependent dorsal and peripheral ruffles by inhibiting Rab5-mediated endocytotic/exocytotic trafficking of Rac1. The results indicate that archazolid effectively decreases metastatic dissemination of breast tumors by impairing the trafficking and spatially-restricted activation of EGFR and Rho-GTPase Rac1, which are pivotal for directed movement of cells. Thus, our data reveals a novel mechanism underlying the role of V-ATPase in tumor dissemination.
INTRODUCTION

Cancer is a major cause of mortality primarily due to tumor metastasis (1). The vacuolar (V)-ATPase is emerging as a prominent player in the invasiveness of cancer cells (2, 3). Consistently, pharmacological inhibition of V-ATPase has been proposed as a strategy to oppose metastatic processes (3-5).

V-ATPases are ATP-dependent proton pumps ubiquitously expressed and thought to regulate the pH in endomembrane systems. V-ATPase comprises two major domains: the cytoplasmic V₁ domain, which consists of 8 subunits (A-H), mediates ATP hydrolysis; the membrane-bound V₀ subunit is composed by subunits a, d, e and the proteolipids c, c' and c''. The c subunits form an H⁺-binding rotor ring which transports protons from the cytoplasm to the endosomal/lysosomal lumen or the extracellular space (6, 7).

Few specific small molecule inhibitors of V-ATPase have been developed and their binding properties have been extensively studied (8). The archazolids are a novel group of V-ATPase inhibitors, first isolated from cultivated myxobacteria Archangium gephyra (9). Like the known V-ATPase inhibitors bafilomycin A₁ or concanamycin, archazolids bind to the V₀ subunit c of V-ATPase. However, the molecular mechanism of their inhibitory action is different. Archazolids do not penetrate in between two c subunits, but most likely bind to the membrane facing side of a single subunit c (10). Archazolids potently inhibit V-ATPase activity (IC₅₀: nanomolar range) exerting little or no effects on Na+/K+-ATPases and mitochondrial F-ATPases (8). To date, archazolid A, B and F have been isolated from their natural producer and archazolid A and B have also been successfully chemically synthesized (9, 11-13).

It has been observed that the abundance of V-ATPase on the plasma membrane correlates with an invasive phenotype of tumor cells (3, 4). In addition, cell surface V-ATPase activity has been postulated to create an acidic extracellular microenvironment, which is required for the activation of proteases known to be important for tumor cell invasion (14).
Consistent with this, V-ATPase blockade reduces the activity of matrix metalloproteinase (MMP)-9, albeit it increases MMP-2 \textit{in vitro} (4). Besides plasma membrane-localized V-ATPase, also intracellular V-ATPase may be involved in proteolytic activation of proteases acting within the lysosome or facilitating the traffic of secretory vesicles containing MMPs or cathepsins to the cell surface (2, 4, 15). Most of the work on V-ATPase in tumor cell invasion has focused on the impact of this proton pump on matrix degradation, while little has been done to assess whether V-ATPase may also influence the signaling leading to tumor cell motility and migration.

Cell migration is the result of a complex interplay of signaling machineries. Among these signaling axes, those emanating from growth factor receptors, such as EGF-R, are important for directed migration, while Rho-GTPases, a subgroup of the Ras superfamily of GTPases, regulate cell morphology and actin cytoskeleton, and are therefore indispensable for controlling the machinery of cell migration (16-18).

Here, we report that the V-ATPase inhibitor archazolid hinders the migration of invasive cancer cells \textit{in vitro} and \textit{in vivo}. We suggest that V-ATPase contributes to spatially restricted activation of migratory transducers such as EGF-R and Rac1, which is required for the acquisition of polarized and directed tumor cell migration.
MATERIAL AND METHODS

Compounds

Archazolid A was purified and isolated as described previously (9), archazolid B was synthesized chemically as described (12) (Fig.S1A). Both compounds were dissolved in DMSO and show similar bioactivity (Fig.S1B). The following antibodies were used: Ductin-antibody (ATP6L, Millipore), actin, cortactin and Rab5A (Santa Cruz), anti-pAkt/Akt, pEGFR/EGFR (cell signaling technologies), anti-Rac1 (Millipore), goat-anti rabbit-488, goat-anti mouse-488 (AlexaFluor®, Life technologies), goat-anti rabbit (Dianova), goat-anti mouse IgG2b (Biozol). Phalloidin-rhodamine was purchased from Sigma.

Cell culture

The human breast cancer cell line SKBR3 was provided in 2009 by Dr. B. Mayer (University of Munich, Germany). Cells were tested every 6 month for their ER, PgR, Her2 and EGF-R status. Hela cells were from ATCC and routinely tested for Mycoplasma contamination with MycoAlert™ Mycoplasma Detection Kit (LONZA) and cell cross-contamination with StemElite™ ID System (Promega). SKBR3 were maintained in McCoy’s (PAA) medium supplemented with 10 % heat-inactivated fetal calf serum (FCS, Biochrom AG) and 1% glutamine. Hela cells were grown in DMEM medium supplemented with 10 % South American serum (EuroClone) and 1 % L-glutamine. 4T1-Luc cells were recently (< 6 month) purchased from Caliper (Alamenda, CA, USA) and cultivated in RPMI with 10% FCS.

Immunocytochemistry/ flow cytometry

SKBR3 cells were seeded on IBIDI-µ-slides (IBIDI,Martinsried, Germany), fixed with 4% formaldehyde (15 min, RT) and permeabilized with 0.2% Triton X-100/PBS. Antibodies were diluted in 1% BSA/PBS containing 0.1% Triton. For uptake of EGF-rhodamine (Invitrogen,) archazolid treated as well as control cells were starved for 2 h and incubated for 5 to 15 min.
with EGF-rhodamine and analyzed by confocal microscopy (LSM 510 Meta; Zeiss, Oberkochen, Germany).

For analysis of EGF-R and Her2 on the plasma membrane SKBR3 cells were treated with archazolid (10nM, 24h), trypsinized, washed with ice cold HEPES buffered saline, labeled anti EGF-R antibody and AlexaFluor488-trastuzumab, respectively (Fig.S3) and analyzed with FACScan flow cytometer.

**Rac1 activation assay**

Rac1 was induced in cells treated with archazolid (24 h, DMEM media) by adding epidermal growth factor (EGF 100ng/ml) for 2 min followed by a pulldown assay according to the manufacturer’s instructions (Thermo Fisher Scientific, Bonn, Germany).

**Western blot**

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using tank blotting. For the detection of protein levels the ECL™ detection system (Amershan Pharmacia Biotech) or the Odyssey Infrared Imaging system version 2.1 (LI-COR Biosciences, Lincoln, NE, USA) was used.

**Quantitative real-time PCR**

The ABI 7300 RealTime PCR system with the TaqMan Universal PCR Mastermix (Life Technologies Corporation, Carlsbad, CA, USA) was used. Probe and primers for the V-ATPase subunit c (ATP6L) and GAPDH (housekeeping gene) were supplied as mix (Life Technologies Corporation). Fluorescence increase was analyzed using the ABI 7300 system software. Calculation of relative mRNA was done according to (19).

**Measurement of apoptosis**

Apoptotic cell death was quantified as described (20) permeabilizing cells, staining with propidiumiodide (50 µg/µl) and analysis by flow cytometry (FACSCalibur, Becton Dickinson).
siRNA transfection

1 x 10^6 SKBR3 cells were transfected with control siRNA or siRNA targeting ATP6L (Thermo Scientific Dharmacon) using the Amaxa system. Downregulation of ATP6L was proved by real-time PCR.

Liposome-based cell transfection (Fugene)

The Fugene transfection reagent (Roche) was used to transfect plasmids: pCMV-BamHI, pCMV-Rab5, pGFP-Rac1-C1,1014, pRaichu-CRIB (CFP-CRIB-YFP/PAK-CRIB), pCDNA3-Rac1-HA (RacWT) and pCDNA3-RacQ61L-HA (RacQL).

Boyden chamber assay

Cells were treated with archazolid at a confluence of 70% for 24/16h. 1x10^5 cells were suspended in media without FCS and added on top of the Boyden chamber membrane (Corning). Culture medium with 10% FCS was added on the bottom of the membrane. Chambers were incubated at 37°C, 5% CO_2 for 4 h. Migrated cells were fixed and stained with crystal violet/methanol. Cells not migrated were removed with a q-tip. Pictures (10 x magnifications) were taken and migrated cells were counted and normalized to control cells (100% migration). For control purpose cells were not exposed to FCS gradient (-Co, 0% migration).

Chemotaxis

SKBR3 (5 x 10^6) cells containing 2 % FCS were seeded on IBIDI chemotaxis µ-slides and allowed to attach for 16 h. FCS gradient and chemotaxis analysis was performed as described before (21).
**Cell adhesion/xCelligence**

4 x 10^4 SKBR3 cells/well were allowed to adhere on fibronectin coated IBIDI µ-slides (30 min, 37°C) and fixed with 4% paraformaldehyde. Pictures were taken using the Axiovert, Zeiss and number of adhered cells was counted.

For impedance/adherence time measurement 5x10^3 cells/well were seeded in the respective electrode plate (xCelligence, Roche, E-Plate 16). Cell impedance was monitored and adhesion time analyzed using the manufacturer's software (Software RTCA 1.2.1).

**In vivo experiments**

20 female BALB/cByJRj mice (Janvier, Le Genest-St-Isle, France) were housed in individually ventilated cages with a 12 h day/night cycle and food and water ad libitum. Mice were inoculated with 1 x 10^5 4T1-Luc cells via the tail vein. 24 and 4 h before tumor cell injection the treatment group (n=10) was medicated with 1mg/kg archazolid (5% DMSO/PBS) intravenously. On day eight after tumor cell inoculation bioluminescence imaging of the mice was performed under anesthesia (2% isoflurane in oxygen) using the IVIS Lumina system with Living Image software 3.2 (Caliper Life Sciences, Hopkinton, USA) 15 min after intraperitoneal injection of 6mg Na-luciferin (Promega). Mice were sacrificed through cervical dislocation, lungs were harvested, weighed and imaged. Regions of Interest (ROIs) were defined and the total signal per ROI was calculated as photons/second/cm^2 (total flux/area).

All animal experiments were performed according to the guidelines of the German law for protection of animal life and approved by the local ethics committee.

**Statistical analysis**

All experiments were performed at least three times in duplicates/triplicates. Results are expressed as mean value ±SEM. One-way ANOVA/Dunnett and individual students t-tests were performed using GraphPadPrism™. P-values < 0.05 were considered as significant.
RESULTS

Archazolid inhibits cell migration

Treatment with archazolid (Fig. S1A inhibits migration of the breast cancer cell lines SKBR3 and MDA-MB 231 as well of the pancreatic cancer cell line L3.6pl using either Boyden chamber (Fig.1A, Fig.S2A) or wound healing-type of assays (Fig.S2B). Importantly, archazolid, at all the concentrations and timepoints tested, had no effects on cell survival (Fig.1A, S2A lower right panel). Archazolid affects chemotaxis of SKBR3 cells exposed to a diffusive gradient of FCS (0 to 10%) (Fig.1B). Whereas control cells move toward the highest FCS concentration, cells treated with archazolid clearly display reduced directional migration (expressed as y-forward migration index). Only slight alterations in the cumulative distance and in the velocity of cell movement are observed suggesting that directed migration rather than cell mobility is mainly affected by archazolid.

Archazolid affects EGF-R cellular distribution and cell polarization

Growth factor receptors, including the epidermal growth factor receptor (EGF-R), play a crucial role in directed cell migration as confirmed by employing an EGF-R inhibitor (Fig.S4). Whereas the amount of EGF-R protein on the cell surface increased upon archazolid treatment (24h) (Fig.2A left panel, Fig.S3), the total amount of EGF-R was unchanged (Fig.2A right panel) and was still able to be activated as indicated by phosphorylation at Tyr 1068, although the extent and kinetic of activation were slightly decreased (Fig.2A lower panel). Conversely, we observed dramatic changes of EGF-R surface distribution after archazolid treatment by confocal microscopy analysis of not-permeabilized cells. In control cells, EGF-R localizes and is enriched at the leading edge of migrating cells. In archazolid-treated cells, in contrast, EGF-R was distributed all over the cell surface and failed to accumulate along the leading edge extending in the direction of migration. This altered cellular distribution of EGF-R likely reflects archazolid inhibition of lamellipodia extension as revealed by anti-actin staining (Fig.2C left panel). Furthermore the localization of the
microtubule organizing center (MTOC) was affected. Whereas in control cells the MTOC is localized at the very front of the nucleus in the direction of migration, the MTOC of archazolid treated cells was randomly positioned with respect to the nucleus and the front of migration (Fig.2C right panel).

We tested if archazolid affects EGF-R internalization and trafficking, which may in turn control EGF-R signaling to actin remodeling. EGF-R is rapidly internalized upon ligand binding. However, in archazolid treated cells EGF-R internalization was significantly delayed (Fig.3A) paralleled by a delay in maximal activation and a significant reduction in the duration of Akt signaling in archazolid treated cells (Fig.3B). Likewise for EGF-R, the polarized distribution of activated Akt in migrating cells was significantly impaired (Fig.3C).

**Archazolid inhibits ruffle formation by preventing Rac1 activation**

Cells that attach and spread on ECM substrates are known to activate signaling pathways and undergo morphological changes in their shape and remodelling of the actin cytoskeleton that recapitulate events typical of migratory cells (22). Thus, we tested the effect of archazolid on cell spreading onto fibronectin coated plates. Archazolid treatment did not significantly altered the number of adhered cells, suggesting that adhesion receptors responsible for fibronectin interaction are functional (Fig.4A left panel). Conversely, archazolid-treated, freshly adherent SKBR3 cells underwent prominent morphological changes that were monitored in real time both by changes in impedance, which is directly proportional to the area of cells in contact with the substrate (xCelligence, Roche, Fig.4A right panel) and by determination of ruffle formation (Fig.4B left panel). In contrast to control, archazolid-treated cells were significantly less efficient in spreading and failed to form prominent actin rich peripheral lamellipodia and ruffles.

Lamellipodia extension and ruffle formation are primarily controlled by the Rho GTPase Rac1, which localizes to leading edges to promote these migratory structures. Thus,
we tested whether archazolid may affect either the localization or activation (or both) of Rac1. Confocal analysis of freshly adherent control cells showed a distinct localization of Rac1, which, as expected, accumulated at the cell periphery where actin-rich lamellipodial sheets are localized. Archazolid completely abrogated the distribution of Rac1 to the plasma membrane most likely because the peripheral enrichment of filamentous actin (ruffles) also disappears upon archazolid treatment (Fig.4B, right panel). A similar picture was observed in migrating cells in a scratch assay. Under these conditions, archazolid inhibited the formation of wound–directed, Rac1-, cortactin-, and F-actin-positive polarized lamellipodia (Fig.4D). All these morphological phenotypes can be easily accounted by a direct inhibitory effect of archazolid on Rac1 activation. Consistent with this hypothesis, EGF–induced Rac-GTP levels were significantly reduced by archazolid treatment (Fig.4C), suggesting that pathways leading to the activation of this GTPase are a primary target of archazolid action. This is further supported by overexpression of either wild type Rac1 (RacWT) or constitutive active Rac1 (RacQL), which could both rescue the inhibition of migration caused by Archazolid (Fig.4E).

**Downregulation of V-ATPase subunit c inhibits migration and Rac1 activation**

Archazolid binds to the V-ATPase subunit c. To unequivocally establish that the effects of archazolid are due to the inhibition of V-ATPase subunit c, we genetically silenced the V₀ subunit c (ATP6L) using siRNA. Downregulation of ATP6L impaired both cell migration and polarized lamellipodia and ruffle formation observed in a scratch wound healing assay (Fig.5A/B). This effect is not cell type specific, since pancreatic cancer cells L3.6pl were equally inhibited by silencing of ATP6L (Fig. S5). Notably and similar to archazolid, silencing of ATP6L inhibited both the activation of Rac1 (Fig.5C) and its accumulation at the leading edge of migrating SKBR3 cells due to loss of ruffles (Fig.5B, right panel). These results support the hypothesis that the V-ATPase subunit c plays a crucial role during the migration process by critically participating in the activation of Rac1.
V-ATPase-inhibition affects Rab5-induced Rac1 activation

Recently, the recycling molecule Rab5 has been reported to play an important role in the activation of the Rac1-mediated actin remodeling and cell migration (23). In keeping with these observations, we show that Rab5 is sufficient to induce the formation of Rac1-dependent actin-rich migratory structures, including circular dorsal ruffles and peripheral ruffles (Fig.6A). Thus we tested whether archazolid interfered also with Rab5-evoked morphological changes. Both, peripheral and circular dorsal ruffle-formation was significantly reduced in Rab5-expressing Hela cells after 24h of archazolid treatment (Fig.6A). Similarly, Rab5-mediated activation of Rac1 (Fig.6B left panel) was significantly inhibited by archazolid treatment (Fig.6B right panel).

In Rab5-expressing, untreated cells, Rac1 can, as previously reported (23), localize to enlarged early endosomes [revealed by the presence of the early endosome antigen-1 (EEA1)] (Fig.6C left panel). Treatment with archazolid, however, prevented the accumulation of Rac1 on Rab5 vesicles, as revealed both by directly monitoring Rac1 cellular distribution (Fig. S6) or using a Rac1 biosensor (PAK-CRIB) that specifically binds to activated Rac1 (Fig.6C right panel).

Archazolid inhibits metastasis in vivo

To extend the relevance of the inhibitory effects of archazolid on chemotactic migration and cell invasion, we next tested whether this drug was effective in impairing cancer dissemination in xenograft mouse models. To this end we employed the 4T1-Luc syngeneic metastatic mouse breast cancer model (24-27). This model is based on the 4T1-Luc mouse mammary tumor cell line, which when injected intravenously disseminates to distant organs, homing to lungs, liver, bone and brain over a short period of time. These cells are engineered to express a luciferase reporter to enable real time monitoring of developing tumor by live imaging. Under these conditions, mock treated, 4T1-injected animals formed easily detectable lung metastases, which were drastically inhibited by intravenous injection of...
archazolid, which at the concentrations tested (1mg/kg i.v.) did not cause any obvious signs of toxicity (Fig.7A/B and Fig.S7A-B).
DISCUSSION

This study gives evidence for a role of V-ATPase in tumor metastasis and reveals novel molecular mechanisms of its action (Fig.S8).

It has been reported for breast and pancreatic cancer cells that the abundance of V-ATPase on the plasma membrane correlates with an invasive phenotype (3, 28-31). Our observation that V-ATPase is localized on the plasma membrane of the invasive cancer cell line SKBR3, but not in the non-tumor mammary epithelial cells MCF-10A (Fig.S9) is consistent with this notion. Genetic knock down of ATP6L leads both to the disappearance of V-ATPase at the leading edge of breast cancer cells as well as to a significant decrease of their migratory activity extending and corroborating data by Lu et al. using hepatocellular carcinoma cells (32).

With respect to the mode of action of V-ATPase in cancer metastasis, one currently accepted model invokes that V-ATPase, highly concentrated on the cell surface, may provide a localized proton efflux, thus generating an acidic extracellular microenvironment, which is, in turn, favorable for the activation of a variety of pro-invasive proteases (4, 33).

Our work unveils an alternative mode of action for V-ATPase. We propose that V-ATPase regulates the signaling machinery responsible for tumor cell migration. More specifically, we showed that V-ATPase inhibition by archazolid impairs spatial restriction of migratory signaling molecules such as Rac1 and EGF-R, which is pivotal for directed and polarized cell movement.

Spatial restriction of signaling, achieved by redistributing signaling molecules to confined areas of the plasma membrane (e.g. leading edge) upon chemotactic stimuli, has emerged as essential for directed cell migration (17). Within this context, endocytosis has been recently proposed to serve as an integrated trafficking network to direct where signaling molecules are activated and is thus important for virtually all polarized functions, first and foremost cell migration (34). Consistently endo/exocytic cycles of receptor tyrosine kinases,
such as EGF-R, are essential for guided motility (35, 36). EGF-R is also a potent guidance receptor, which induces actin remodeling leading to the formation of various migratory protrusions such lamellipodia (peripheral ruffles) or dorsal surface circular ruffles, an obligatory step for two-dimensional cell motility (16). Own experiments employing an EGF-R inhibitor support this notion (Fig. S4).

V-ATPase inhibition by archazolid in migrating tumor cells alters EGF-R localization, reduces its internalization, and impairs EGF-R downstream signaling by ablating Akt activation and preventing its accumulation at the leading edge. These combined effects, ultimately, impact on the ability of cells to polarize. Not surprisingly, the PI3K/Akt pathway plays a major role in the migratory programs of cells (17).

Notably, a general influence of V-ATPase inhibition on internalization as well as recycling processes has been reported before (37, 38). This finding was also confirmed for archazolid, which inhibits uptake of dextran, but not of transferrin/transferrin-R after short time of treatment, however abrogates transferrin/transferrin-R uptake after prolonged exposure (Fig. S10A/B).

Regarding mechanisms underlying V-ATPase action on internalization/recycling, it is important to know that endosomes and lysosomes, where recycling and degradation takes place, are acidified by V-ATPases (39). The acidic pH is pivotal for the dissociation of ligands from its receptors and degradation/recycling processes (35). Increase of pH induced by bafilomycin, for example, has been shown to affect the delivery of endocytosed molecules to late endosomes or lysosomes in some, but not all cell lines tested (37, 40, 41). We analyzed SKBR3 cells by transmission-electron-microscopy (Fig. S10C) and found considerably more and enlarged multivesicular-bodies (MVBs) in cells after archazolid treatment. MVBs are degradative endosomal organelles, mediating trafficking between early and late endosomes (40, 42). Interestingly, this phenomenon was also described in a study focusing on the involvement of V-ATPase activity in Notch-Signaling (42). Since an even more acidic pH is
necessary for lysosomal degradation process, V-ATPase inhibition by archazolid might inhibit the final fusion with lysosomes, halting the degradative, and probably also recycling routes, leading to enlargement of MVBs. Likely these “high pH” MVB are inefficient degradative factory that may trap cargos and endosomal associated molecules that would otherwise be routed to recycling pathways for subsequent round of signaling. Ultimately, this may result in an impaired delivery of internalized molecules, such as EGF-R, to the proper location on the plasma membrane preventing their proper and sustained activation. Similar alteration in trafficking of endosomal membrane associated molecules recycling back to the plasma membrane may also occur for impaired activation of Rac1, which after archazolid treatment, is no longer able to enter the Rab5-mediated endosomal routes for subsequent delivery back to the plasma membrane. How changes in endosomal pH affect Rac1 recruitment to these organelles is unclear at this stage. One possibility is that local changes of the pH at the plasma membrane, where Rac1 or its GEFs are primarily localized and activated, are required for Rac1 (or its activators) to subsequently enter endocytic organelles. Indeed similar reduction of Rac1 in endosomes could be observed after interference with clathrin (23) suggesting that plasma membrane localized Rac may follow a canonical endocytic routes that may require local changes of pH to be effective. Whether this is indeed the case remains, however, to be tested in future investigations.

Whatever the case, impaired endocytic traffic induced by V-ATPase is hypothesized to affect the spatially restricted activation of Rac1, a central regulator of actin remodeling and cell migration (17, 43). Consistently, archazolid treatment or silencing the V_0 subunit c of the V-ATPase, the binding site of archazolid, abrogate Rac1-dependent F-actin remodeling, F-actin-mediated formation of ruffles or lamellipodia as well as the recruitment of active Rac1 to these migratory structures. These results also demonstrate that archazolid exerts its effect by specifically targeting V-ATPase, or more precisely of the V_0 subunit c.

Spatial restriction of Rac1 signaling has been shown to depend on Rab5 endo/exocytic cycles (23). Rab5, in addition to be a master regulator of endocytosis (44) by
controlling the biogenesis and fate of early endosomes, was also shown to be sufficient to promote, through Rac1, the formation of a diverse set of migratory protrusions, including lamellipodia and circular dorsal ruffles (23). Notably, archazolid robustly inhibits all these structures. These effects are accompanied by loss of Rac1 from endosomal membrane, which results in reduced delivery of this GTPase to cell protrusion, and impaired directional migration. Thus, archazolid appears to interfere both with endocytic trafficking of Rac1 as well as EGF-R. Whether EGF-R needs to be internalized and recycled to trigger Rac1 internalization and activation or the two events are occurring at the same time independently of each other remains to be addressed in future work.

Finally, as in vivo data on V-ATPase inhibitors are very limited, we like to point to the fact that effects of archazolid in cell culture are recapitulated in a mouse model of tumor dissemination. This indicates that pharmacological interference with V-ATPase represents a viable strategy both to decipher functions of this enzyme in cancer progression as well as to prevent the acquisition of migratory and invasive properties, ultimately leading to metastasis.

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REFERENCES


FIGURE LEGENDS

Fig.1: Archazolid inhibits cancer cell migration. (A) SKBR3 cells treated with archazolid (24 h) were allowed to migrate in Boyden chambers (4 h). Bars (left panel) indicate percent of migration (cells stimulated with FCS (+Co) are set as 100%; -Co: no FCS gradient applied) and are the mean±S.E.M. of three independent experiments performed in duplicates, *p<0.05. Right panel shows that inhibition of migration by archazolid is not due to apoptosis induction. Bars represent the mean±S.E.M. of three independent experiments performed in triplicates, *p<0.05. (B) Movement of SKBR3 cells treated with archazolid (16 h) along a FCS gradient was monitored (24 h) by live cell imaging and analyzed (Image-J (NIH); IBIDI software). Bars represent the y-forward migration index (control cells are set as 1) as well as velocity and accumulative distance and are the mean±S.E.M. of three independent experiments, *p<0.05 (t-test).

Fig. 2: Archazolid affects EGF-R localization. (A) EGF-R on the plasma membrane was analyzed by FACS (left panel) and total EGF-R by Western blot after 24 h of archazolid treatment (right panel). EGF-R phosphorylation (Tyr1068) upon EGF stimulation was reduced in archazolid treated cells (lower panel). Representative experiments out of three independent experiments are shown. (B) Cells treated (archazolid, 24h) or untreated (control) were scratched, fixed and stained for EGF-R without permeabilization (controlled by negative staining for actin) after 5 h migration time. (C) Archazolid inhibits lamellipodia extension as observed by actin staining in permeabilized cells (left panel). The localization of the centrosome (MTOC) (stained for β-tubulin) is altered by archazolid (right panel, arrows). Representative images out of three independent experiments are shown.

Fig.3: Archazolid affects EGF-internalization and Akt (A) SKBR3 cells treated or untreated, starved for 2 h were exposed to EGF-rhodamine for 5 and 15 min and analyzed
by confocal microscopy (B) Phosphorylated Akt (Ser473) was detected by western blot upon exposure to EGF (100ng/ml) (C) Localization of pAkt was analyzed by confocal microscopy in control and archazolid treated cells. (A-C) Representative experiments out of three independent experiments are shown.

Fig.4: Archazolid affects localization and activation of Rac1. (A) Archazolid treated SKBR3 cells (24 h) were seeded on fibronectin (30 min), fixed and counted. Bars represent percent of adhered cells (control cells: 100%) (left panel). Kinetics of cell spreading (control cells: 100%) was analyzed by impedance measurement (xCELLigence RTCA, Roche) as shown by a representative graph (right panel). (B) The formation of ruffles (left panel) and cellular localization of Rac1 (right panel) in freshly adhered cells untreated or treated with archazolid was analyzed by confocal microscopy. (C) A Rac1-pulldown assay was performed in control and archazolid treated cells upon a 2 min EGF-stimulation. A representative experiment out of three independent experiments is shown. (D) SKBR3 treated with archazolid (24 h) were engaged in a scratch assay and stained for Rac1, cortactin and F-actin (left panel). Representative images out of three independent experiments are shown. (E) Overexpression of cells with a wild type Rac1 (RacWT) or a constitutive active Rac1 mutant (RacQL) was able to abrogate inhibition of migration by archazolid (boyden chamber setting). Representative images out of three independent experiments are shown. (A,B,E) Bars always represent the mean±S.E.M. of three independent experiments performed in triplicates. *p<0.05 (t-test).

Fig.5: Downregulation of the V-ATPase subunit c abrogates cell migration (A) Cells were transfected with siRNA either non targeted (NT) or targeted against the subunit c (ATP6L) for 24 h, followed by a Boyden chamber assay and quantification of migrated cells (left panel). Downregulation of ATP6L was confirmed by real-time PCR (middle panel) and
no apoptotic cell death is induced by siRNA transfection (right panel). **(B)** Cells were transfected with siRNA-ATP6L and freshly seeded. Cells with ruffles were counted and expressed as percentage of total adhered cells (left panel). siRNA-ATP6L treated cells were employed in a scratch assay and stained for ATP6L, Rac1 and F-actin (phalloidin). Representative images out of three independent experiments are shown (right panel). **(C)** Rac1 pull-down assay was performed in ATP6L-silenced cells. One representative experiment out of three independent experiments is shown (left panel). Downregulation of ATP6L was proved for each single experiment by real-time PCR (right panel). **(A-C)** Bars represent always the mean±S.E.M. of three independent experiments, *p<0.05 (t-test).

**Fig.6: Rab5-dependent Rac activation is inhibited by archazolid** **(A)** Hela cells were transfected with Rab5WT and treated with archazolid (10 nM, 24 h). Ruffle formation was induced with 10% FCS and 10 ng/ml HGF for 5 min. Samples were stained for actin using phalloidin-FITC. Number of ruffles was counted (PR=peripheral ruffles; CDR=circular dorsal ruffles). Bars represent ruffling index and are the mean ± S.E.M. of three independent experiments performed in triplicates. *p < 0.05 (t-test). **(B)** Rab5 expression induces Rac1 activation (left panel) similar to EGF. Rab5-dependent Rac activation is reduced after archazolid treatment (right panel). SKBR3 cells were transfected with pcMV-Rab5 followed by a Rac1-Pulldown assay. Representative experiments out of three independent experiments are shown. **(C)** Rab5 expression leads to enlarged early endosomes containing Rac1 (arrows, left panel). Rab5 and Rac-GFP were expressed in Hela cells being stained for early endosome antigen-1 (EEA1). Localization of active Rac1 was analyzed in archazolid treated (24h) and untreated cells expressing Rab5, Rac1-HA and CRIB-YFP. Arrows show active PAK-CRIB at the enlarged endosomes (right panel). Representative images out of three independent experiments are shown.
Fig. 6: Metastasis of 4T1 Luc cells is decreased by archazolid in vivo.

Archazolid reduces the amount of metastasis in the lung of BALB/cByJ mice. $1 \times 10^5$ 4T1-Luc cells were injected intravenously into pretreated (1mg/kg archazolid) and untreated mice. (A) On day eight after cell inoculation bioluminescence signals were recorded by imaging the mice dorsoventral and ventrodorsal. Color bar scales were equalized. (B) Day eight, mice were sacrificed, lungs were harvested and imaged separately (Fig.S7). Regions of Interest (ROIs) were defined and the total signal per ROI was calculated as photons/second/cm$^2$ (total flux/area). Bars represent the mean ± S.E.M. *p < 0.05 (t-test).
Figure 1
Figure 2

A

B

C
Figure 3
Figure 4
Figure 5

A

siRNA-NT

siRNA-ATP6L

B

n

A

n

siRNA-NT

siRNA-ATP6L

C

Rac1-GTP

Rac1-total

siRNA control

siRNA ATP6L

(EGF)
Figure 6
A

control

1  2  3  4  5  6  7  8  9  10

dorsoventral

ventrodorsal

Archazolid treated

1  2  3  4  5  6  7  8  9  10

dorsoventral

ventrodorsal

B

Figure 7
The V-ATPase-inhibitor Archazolid abrogates tumor metastasis via inhibition of endocytic activation of the Rho-GTPase Rac1

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