Department of Pharmacy[1], Pharmaceutical Biology, Ludwig-Maximilians-University, Munich; Kekulé-Institut für Organische Chemie und Biochemie der Universität Bonn[2], Germany

In vitro anti-cancer effects of the actin-binding natural compound rhizopodin

S. Zhang[1], D. Menche[2], S. Zahler[1], A. M. Vollmar[1], J. Liebl[1,*], F. Förster[1,*]

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Johanna Liebl, Ph.D., Ludwig-Maximilians-University, Department of Pharmacy, Pharmaceutical Biology, Butenandtstr 5-13, 81377 Munich, Germany
johanna.liebl@cup.uni-muenchen.de

[*]These authors contributed equally to this work.

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Several natural compound interfere with microtubules or the actin cytoskeleton. Compounds interfering with the microtubules like *Vinca*-alkaloids or taxanes, are extensively used for cancer therapy. In contrast, knowledge about pharmacological properties of actin binding drugs is poor and drugs interfering with actin are far from clinical use. Rhizopodin is a natural compound that strongly affects the actin cytoskeleton at nanomolar concentrations. Initial work revealed interesting anti-bacterial and cytotoxic effects, but the cellular effects and pharmacological properties of rhizopodin have not been characterized. We hypothesized that rhizopodin might exert anti-cancer activity. Therefore, the aim of this study was to characterize the cellular and pharmacological effects of rhizopodin in cancer. Effects of rhizopodin demonstrated prominent effects on the actin cytoskeleton as shown in the actin-pyrene assay and by immunostaining of cancer cells. To investigate cellular effects of rhizopodin, we analyzed cell proliferation, cell death induction by propidium iodide exclusion and western blot, as well as migration by impedance measurement using the xCELLligence device in MDA-MB-231 breast cancer and T24 bladder cancer cell lines. Rhizopodin inhibited proliferation and induced cell death of MDA-MB-231 and T24 cells at nanomolar concentrations. PARP cleavage by rhizopodin suggests caspase-dependent cell death induction. Importantly, rhizopodin potently inhibited MDA-MB-231 and T24 cancer cell migration at subtoxic doses where no actin aggregation was observed, indicating a specific underlying signaling of rhizopodin. In summary, our study elucidates rhizopodin as actin-binding natural compound that exerts potent anti-cancer effects. Therefore, our work provides the basis for further in depth characterization of rhizopodin as an antitumoral agent.

1. Introduction

Natural compounds represent an invaluable source for the development of powerful therapeutics. Because of their high structural diversity, substances from biologic origin are used as lead structures for drug discovery. Moreover, natural compounds show high effectiveness by addressing targets that exert central functions because nature has used targeting structures that are of utmost importance for most organisms (Koehn and Carter 2005; Newman and Cragg 2007; von Schwarzenberg and Vollmar 2013). Since the actin cytoskeleton is essential for central cellular functions, it is addressed by secondary metabolites and a large number of natural compounds interferes with the actin cytoskeleton (Allingham et al. 2006). The actin cytoskeleton essentially contributes to cytokinesis during cell division, cellular transport processes, cell contractility and motility (Hall 2009). Formation of stress fibres and cell protrusions by actin assembly and disassembly are prerequisites for cell migration and metastasis (Franklin-Tong and Gourlay 2008; Kunda and Baum 2009; Mitchison and Cramer 1996; Olson and Sahai 2009). Based on these pivotal roles of the actin cytoskeleton during cell growth and motility, actin-interfering drugs represent interesting compounds for cancer therapy (Giganti and Friederich 2003; Nurnberg et al. 2014; Rao and Li 2004). However, only few actin-interfering agents have been characterized

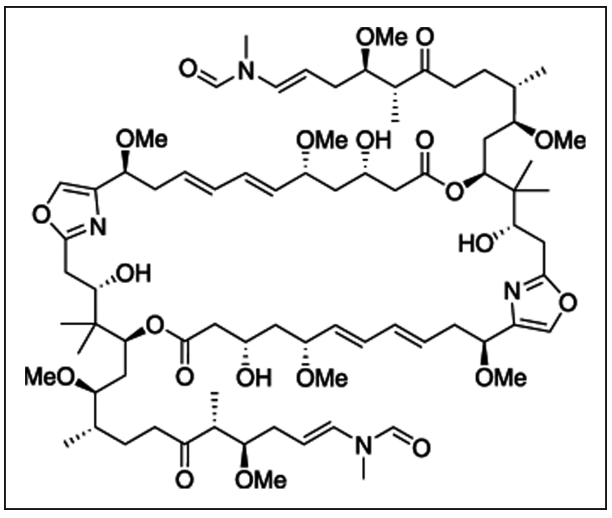

Fig. 1: Chemical structure of rhizopodin.

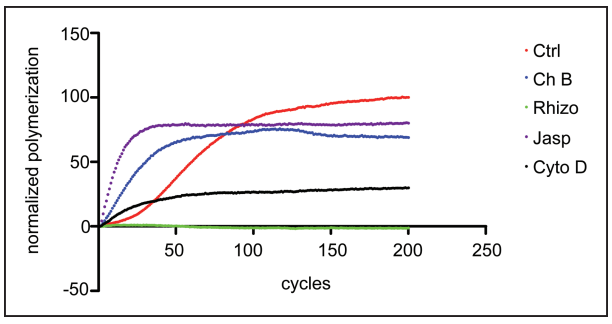

Fig. 2: Rhizopodin inhibits actin polymerization *in vitro*. Polymerization assay of purified pyrenylated actin. Indicated compounds or DMSO (solvent control) were incubated at a concentration of 10 μM with the actin polymerization cocktail as described in materials and methods. Following compounds were used: Chondramide B (Ch B), jasplakinolide (Jasp) as overpolymerizing compounds; cytochalasin D (Cyto D) as polymerization inhibitor and rhizopodin (Rhizo). The assay was performed for each compound in duplicates (n = 1).

in detail and their cellular effects and mode of action are still largely elusive.

Rhizopodin is a natural compound that has been isolated from the myxobacterium *Myxococcus stipitatus* in 1993 (Sasse et al. 1993). Rhizopodin is a polyketide that binds to monomeric G-actin and inhibits actin polymerization by induction of G-actin dimerization by addressing the gelsolin binding site (Gronewold et al. 1999; Hagelueken et al. 2009; Sasse et al. 1993). Other natural compounds targeting the actin cytoskeleton include polyketides like latrunculin, trisoxazoles, and bistramides, and cyclic depsipeptides like phalloidin, jasplakinolide, chondramides and doliculide (Allingham et al. 2006; Herkommer et al. 2015). Importantly, rhizopodin is available by chemical synthesis (Dalby et al. 2013; Dieckmann et al. 2012; Kretschmer et al. 2013) and served as basis for synthesis of simplified rhizopodin-hybrids (Herkommer et al. 2015; Nicolaou et al. 2011). Although rhizopodin strongly affects the actin cytoskeleton at nanomolar concentrations (Nicolaou et al. 2011) and initial work provided evidence for anti-bacterial and cytotoxic effects (Sasse et al. 1993), potential anti-cancer effects have not been characterized.

Along this line, we hypothesized that rhizopodin might exert anti-cancer properties. Therefore, the aim of this study was to elucidate cellular effects and mode of action of rhizopodin in highly proliferating and migratory cancer cells.

2. Investigations and results

2.1. Rhizopodin inhibits polymerization of purified actin in vitro

The chemical structure of the polyketide rhizopodin is shown in Fig. 1 By using pyren-labeled actin, effects of rhizopodin on purified actin were proven. Our setup used several actin-binding compounds at a concentration of 10 μM (Fig. 2. Next to the compounds that induce a polymerization of actin named jasplakinolide and chondramide B, also cytochalasin D was included as an example for an inhibitor of polymerization. As expected, the two overpolymerizing compounds jasplakinolide and chondramide B lead to a left shift of the graph, thus indicating an increased polymerization. The polymerization inhibitor cytochalasin D leads to a diminished polymerization according to its binding mechanism, that inhibits the elongation of f-actin filaments (Allingham et al. 2006). A different graph is obtained for rhizopodin, which completely inhibits any nucleation or polymerization events, thus, pointing to the outstanding binding mechanism of rhizopodin to g-actin monomers. In line with previous studies (Nicolaou et al. 2011), rhizopodin strongly inhibited actin polymerization.

2.2. Depolymerizing effect of rhizopodin on the cellular actin cytoskeleton

F-actin stainings of the breast cancer cell line MDA-MB-231 and the urinary bladder cancer cell line T24, showed depolymerizing effects of rhizopodin on the actin cytoskeleton (Fig. 3. The f-actin cytoskeleton was visualized with f-actin binding compound phalloidin-rhodamin, that has a similar binding mode as jasplakinolide and only binds to polymers/oligomers of f-actin (Allingham et al. 2006). At the very low concentration of 7.5 nM of rhizopodin a decrease in stress fibre formation can be observed. Higher concentrations of 50 nM rhizopodin show an intracellular loss of f-actin elements and some kind of actin aggregates at the border of the cells. Thus, our results reveal an influence of rhizopodin on the actin cytoskeleton also in a cellular setup even in low concentrations compared to the 10 μM of *in vitro* pyrene assay.

2.3. Rhizopodin inhibits cancer cell growth

Functional effects of rhizopodin on the proliferation of cancer cells were tested by using MDA-MB-231 and T24 cancer cells. In line with the cellular actin depolymerization, proliferation of MDA-MB-231 and T24 cancer cell lines was concentration-dependently decreased by rhizopodin treatment at nanomolar concentrations (EC 50 for MDA: 41.75 nM; EC 50 for T24: 19.27 nM) (Fig. 4).

2.4. Rhizopodin induces cell death of cancer cell lines

Cell death was quantified by propidium iodide exclusion assay in MDA-MB-231 breast cancer and T24 bladder cancer cell lines. Rhizopodin induced cell death of both MDA-MB-231 as well as T24 cells at nanomolar concentrations (Fig. 5a,b). PARP was analyzed as central apoptotic marker. Western blot analysis shows that PARP-cleavage was induced by rhizopodin (Fig. 5c), suggesting that cell death induced by rhizopodin was caspase dependent.

2.5. Rhizopodin inhibits cancer cell migration

Potential effects of rhizopodin on migration of MDA-MB-231 and T24 cells, both highly metastatic cell lines, were tested by impedance measurement using the xCELLigence device. Migration of both MDA-MB-231 as well as T24 cancer cell lines was potently inhibited by subtoxic doses of rhizopodin (Fig. 6). Our results show that rhizopodin inhibited cancer cell migration at concentrations that only affect the actin cytoskeleton slightly, suggesting interesting underlying signaling of rhizopodin.

3. Discussion

Since the cytoskeleton is essential for cell division and motility, processes highly activated in proliferating and metastasizing tumor cells, it represents an interesting target for cancer therapy. Drugs targeting the microtubule cytoskeleton, like taxanes or Vinca-alkaloids, are well established anti-cancer therapeutics that are extensively clinically used. Their mode of action and signaling mechanism is very well understood (Wertz et al. 2011). In contrast, actin-targeting compounds are far beyond clinical use. Although potent actin-binding natural compounds have been identified, in contrast to microtubule binding drugs,

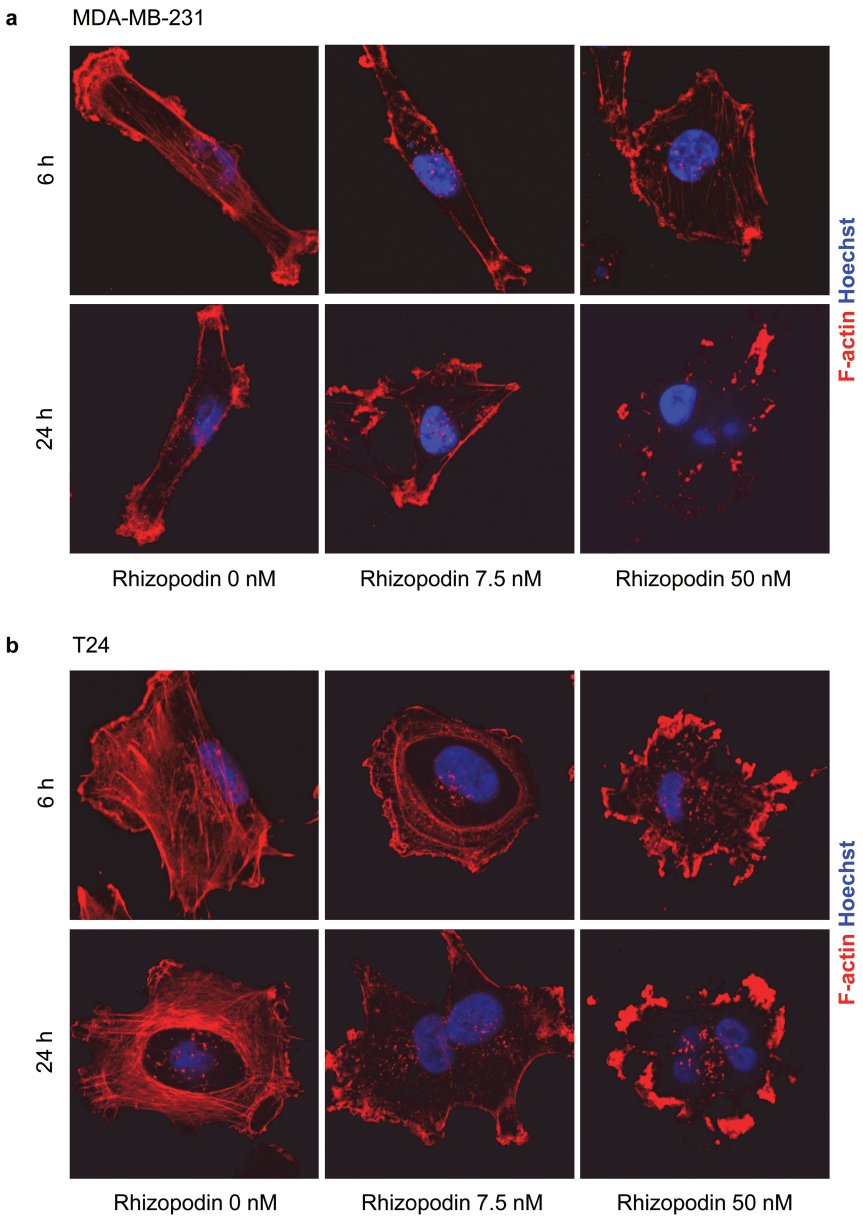

Fig. 3: Rhizopodin exerts a depolymerization effect on the actin cytoskeleton. f-Actin Staining of MDA-MB-231 (A) and T24 (B) cell lines treated with rhizopodin at indicated concentrations for 6 h and 24 h are shown.

knowledge about their anti-tumor effects and pharmacological properties of actin-targeting compounds is poor. Actin binding agents include compounds that inhibit actin filament assembly or lead to actin filament depolymerization like latrunculin B and cytochalasin D or compounds that stabilize or overpolymerize the actin cytoskeleton like phalloidin, chondramide and jasplakinolide. Based on the central functions of the actin cytoskeleton, potential severe side effects of agents interfering with the actin cytoskeleton, like an increased cardiotoxiticy, are suspected. Interestingly, recent studies demonstrate *in vivo* efficacy against solid tumor growth as well as metastasizing tumor cells and tolerability of actin-interfering agents (Foerster et al. 2014b; Menhofer et al. 2014a; Menhofer et al. 2014b). Moreover, influence of tumor-specific factors by interference with the actin cytoskeleton suggests tumor-specificity of actin-binding compounds (Foerster et al. 2014b). Therefore, actin-binding compounds are suggested as potential new class of anti-cancer agents.

Along this line, the aim of our present study was to characterize cellular effects of the actin binding compound rhizopodin. Our study shows that rhizopodin exerts prominent anti-cancer effects. In line with a study evaluating pharmacologic effects of the actin binding compound doliculide (Foerster et al. 2014a), rhizopodin induced PARP cleavage, one central player in caspase-dependent cell death. Moreover, besides inhibiting cell growth and inducing cell death, migration was potently inhibited by rhizopodin. Interestingly, rhizopodin diminished cell motility at subtoxic doses that did not induce actin aggregation, pointing to a specific signaling mechanism of rhizopodin. Actin turnover, i.e. assembly, elongation and dissociation, represents a highly regulated mechanism that involves a variety of actin-related proteins (Pollard and Cooper 2009). In line with previous studies from our group that showed that the actin-binding natural compound chondramide exerts prominent anti-cancer effects by influencing actin-related proteins (Foerster et al. 2014b; Menhofer et al. 2014a; Menhofer et al. 2014b), rhizopodin might influence (an) actin-related protein(s), leading to its prominent effect in inhibiting tumor cell migration.

In summary, our study suggests the natural compound rhizopodin as new actin-interfering agent with promising anti-

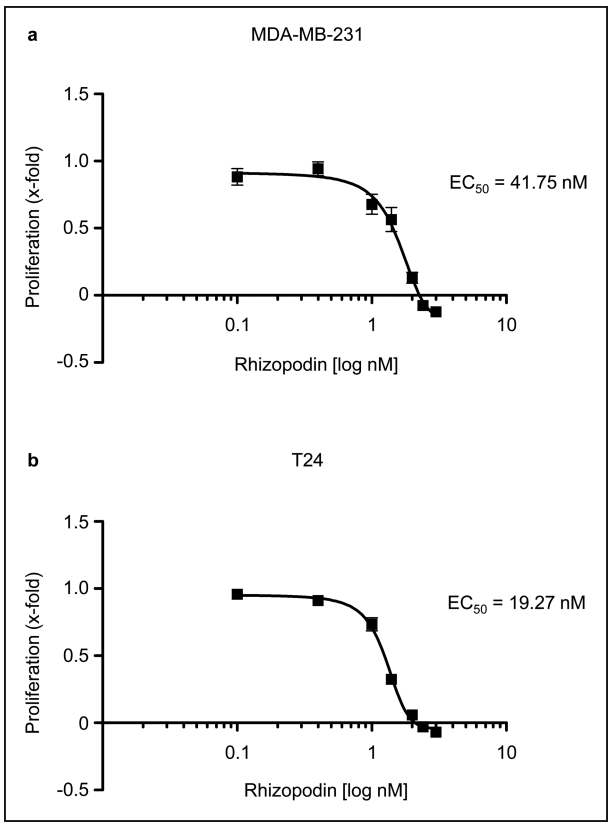

Fig. 4: Rhizopodin inhibits cancer cell growth. (A) Inhibition of proliferation of MDA-MB-231 cells after treatment with rhizopodin at indicated concentrations are shown. EC50 is indicated. n = 3. (B) Inhibition of proliferation of T24 cells after treatment with rhizopodin at indicated concentrations are shown. EC50 is indicated. n = 3.

cancer effects and implies for setting up to analyze the *in vivo* effectiveness of rhizopodin and its signaling mechanism in tumor growth and metastasis.

4. Experimental

4.1. Pyrene-actin polymerization assay

Actin polymerization was measured by using the f-actin buffer kit and purified pyrene actin 10% (Hypermol, Bielefeld, Germany) according to manufacturer's instructions. Fluorescence intensity was measured using a TECAN fluorescence reader (TECAN; Maennedorf, Switzerland) at an excitation of 360 nm and an emission of 407 nm in circles of 20 s/well for 200 circles. Blank measurement was subtracted and values were normalized to modified polymerization buffer as positive control. Actin polymerization was quantified by fitting a nonlinear dose response sigmoidal fit and calculating the Hill slope for all 200 circles followed by normalization to the positive control. Nucleation was quantified by fitting a linear regression curve into the first 10 circles to calculate the slope.

4.2. Cells

All cell lines were cultured under constant humidity at 37 °C and with 5% CO_2 in an incubator (Heraeus, Hanau, Germany). MDA-MB-231 breast cancer cells were purchased from Cell Line Services (Eppelheim, Germany) and cultivated in Dulbecco's modified eagle's medium (DMEM, PAN Biotech, Aidenbach, Germany) supplemented with 10% FCS. The human urinary bladder carcinoma cell line T24 was kindly provided by Barbara Mayer (Department of Surgery, University of Munich, Germany) and recently authenticated by the DSMZ (Braunschweig, Germany). T24 were maintained in McCoy's medium (PAA, Coelbe, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biochrom AG, Berlin, Germany), 1% glutamine and 0.1% penicillin/streptomycin (PAA).

4.3. Proliferation

Cell Titer Blue assay was performed according to manufacturer's instructions. Briefly, cells were seeded (96-well plates, 3×10^3 cells/well) for 24 h, treated with rhizopodin at indicated concentrations and incubated for 70 h. CTB solution (20 µl) was added and cells were incubated for 2 h. Fluorescence was measured with a Tecan reader (Maennedorf, Austria).

4.4. Propidium iodide (PI) exclusion

Cells were seeded (12 well plates, 5×10^4 cells/well) for 24 h and treated with rhizopodin at indicated concentrations for 48 h before incubation with PI (5 µg/ml, 5 min) and analysis by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany).

4.5. Migration

Migration of cells was determined using the xCELLigence System (Roche Diagnostics). The lower compartment of CIM plates was filled with DMEM containing FCS (160 µl). After assembling lower and upper compartments, DMEM without FCS (27 µl) was added to the upper compartment and CIM plates were equilibrated at 37 °C for 1 h. 40 000 cells per Well (in 100 µl DMEM without FCS) were seeded. Cells were incubated for 30 min and migration was analyzed for 18 h in the xCELLigence device.

4.6. Immunoblotting

Immunoblotting was performed as described previously (Foerster et al. 2014b). The following primary antibodies were used: β-tubulin (2164 Cell Signaling Technology), PARP (9542, Cell Signaling Technology).

4.7. Confocal microscopy

Cells were plated in ibidi-µ-slides 8-well (ibidi, Martinsried, Germany), treated as indicated, with rhizopodin at indicated concentrations and times. Cells were fixed (4% PFA, 10 min), permablized (0.2% Triton X 100 in PBS, 5 min), blocked (0.2% BSA in PBS, 1 h) and incubated with rhodamin-phalloidin. f-Actin was stained with rhodamin-phalloidin (1:400, R-415, Molecular Pobes/Invitrogen) in 0.2% BSA/PBS (1 h). Subsequently, Hoechst33342 staining (5 µg/ml, 5 min) was performed and cells were mounted (Fluorsave, Millipore). Confocal microscopy was performed with a Zeiss LSM 510 META confocal microscope.

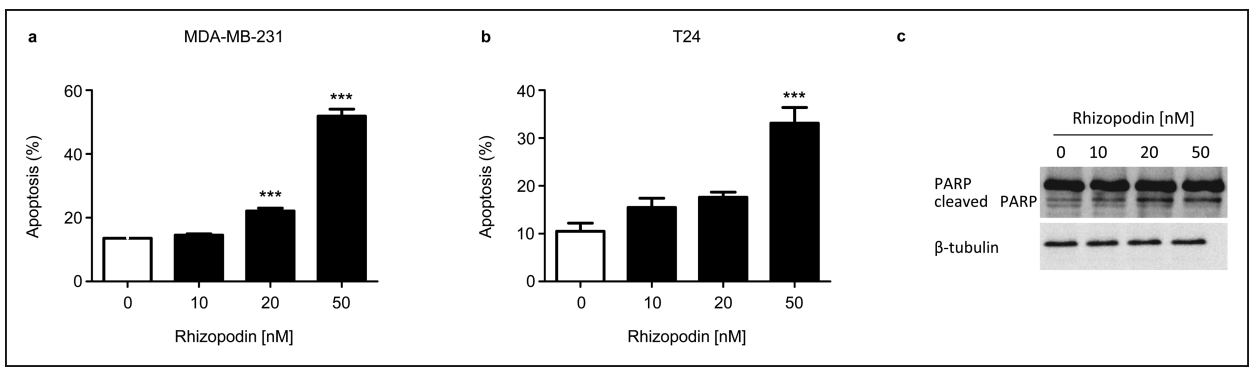

Fig. 5: Rhizopodin induces apoptosis of cancer cell lines. (A, B) Histograms of the PI-exclusion assay of MDA-MB-231 (A) and T24 (B) cells after treatment with rhizopodin at indicated concentrations. $^{***}p \leq 0.001$, One-Way ANOVA, n = 3. (C) Rhizopodin induces PARP cleavage. Immunoblots from T24 cells treated with rhizopodin at indicated concentrations developed with anti-PARP and anti-ß-tubulin antibodies are shown. Tubulin indicate equal loading. One representative blot out of three is shown.

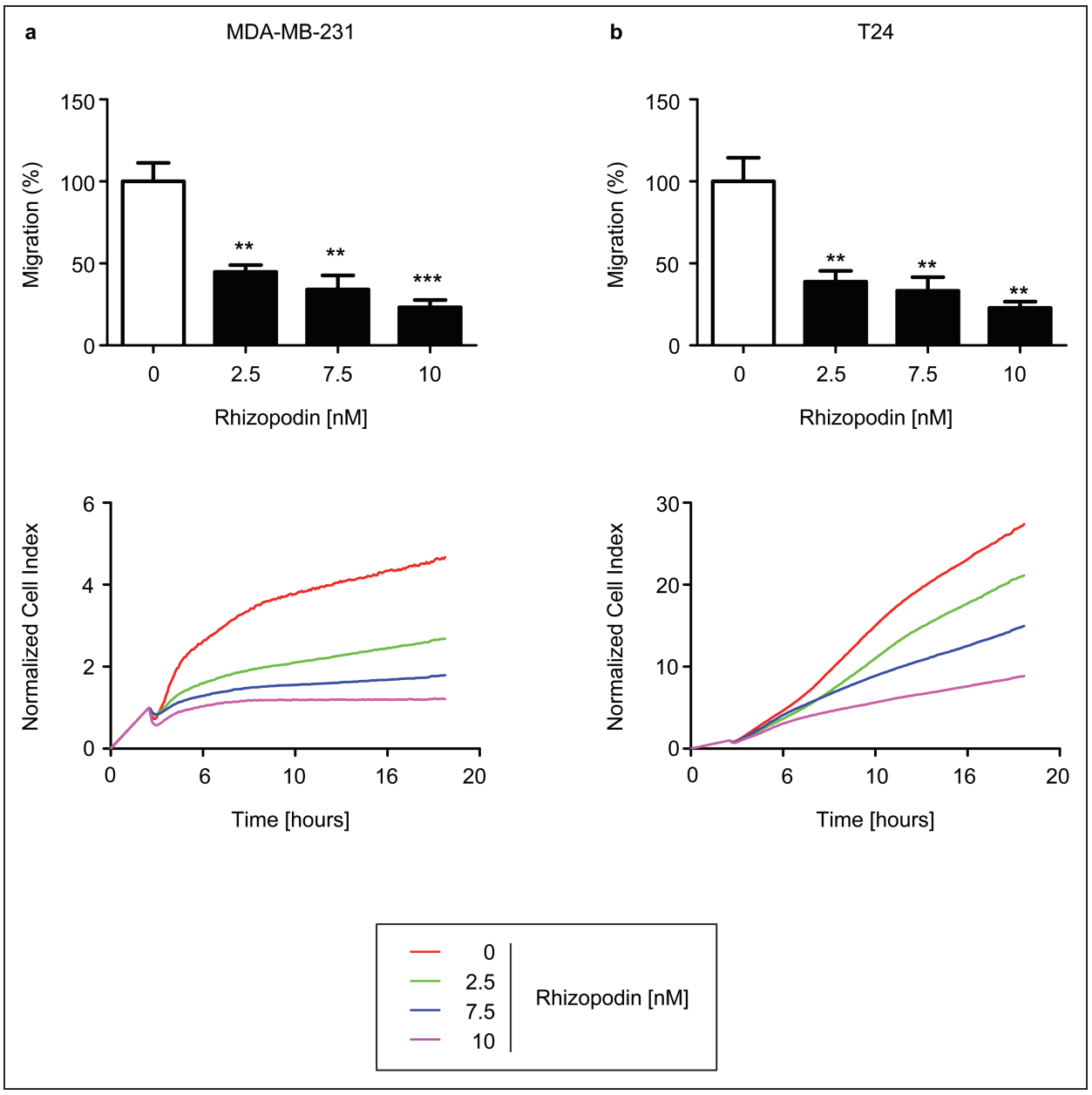

Fig. 6: Rhizopodin inhibits cancer cell migration. (A, B) Migration of MDA-MB-231 (A) and T24 (B) cells treated with rhizopodin at indicated concentrations determined by the xCELLigence device is shown (upper panel). The bar graphs show quantitative evaluation of migrated cells after treatment with rhizopodin at indicated concentrations. (A, B) $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, One-Way ANOVA, n = 3. In the lower panel a representative graph of one xCELLigence measurement is shown.

4.8. Statistic evaluation

All experiments were performed at least three times in duplicates/triplicates. Results are expressed as mean value +/- SEM. One-way ANOVA/Turkey and individual students t tests were conducted using GraphPad Prism (version 5.04, GraphPad Software, Inc.). P values less than 0.05 were considered as significant.

Conflict of interest: The authors declare no conflict of interest.

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