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Evidence for a role of MRCK in mediating HeLa cell elongation induced by the C1 domain ligand HMI-1a3

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ABSTRACT

Diacylglycerol (DAG) is a central mediator of signaling pathways that regulate cell proliferation, survival and apoptosis. Therefore, C1 domain, the DAG binding site within protein kinase C (PKC) and other DAG effector proteins, is considered a potential cancer drug target. Derivatives of 5-(hydroxymethyl)isophthalic acid are a novel group of C1 domain ligands with antiproliferative and differentiation-inducing effects. Our previous work showed that these isophthalate derivatives exhibit antiproliferative and elongation-inducing effects in HeLa human cervical cancer cells. In this study we further characterized the effects of bis(3-trifluoromethylbenzyl) 5-(hydroxymethyl)isophthalate (HMI-1a3) on HeLa cell proliferation and morphology. HMI-1a3-induced cell elongation was accompanied with loss of focal adhesions and actin stress fibers, and exposure to HMI-1a3 induced a prominent relocation of cofilin-1 into the nucleus regardless of cell phenotype. The antiproliferative and morphological responses to HMI-1a3 were not modified by coexposure to pharmacological inhibition or activation of PKC, or by RNAi knock-down of specific PKC isoforms, suggesting that the effects of HMI-1a3 were not mediated by PKC. Genome-wide gene expression microarray and gene set enrichment analysis suggested that, among others, HMI-1a3 induces changes in small GTPase-mediated signaling pathways. Our experiments revealed that the isophthalates bind also to the C1 domains of β2-chimaerin, protein kinase D (PKD) and MRCK, which are potential mediators of small GTPase signaling and cytoskeletal reorganization. Pharmacological inhibition of MRCK, but not that of PKD attenuated HMI-1a3-induced cell elongation, suggesting that MRCK participates in mediating the effects of HMI-1a3 on HeLa cell morphology.

Keywords: protein kinase C, C1 domain, isophthalate, cell proliferation, cell elongation, myotonic dystrophy kinase-related Cdc42-binding kinase

Chemical compounds studied in this article

chelerythrine (PubChem CID: 72311); CID755673 (PubChem CID: 755673); Gö6983 (PubChem CID: 3499); phorbol 12,13-dibutyrate (PubChem CID: 37783); phorbol 12-myristate 13-acetate (PubChem CID: CID 27924); U0126 (PubChem CID: 3006531)

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1. INTRODUCTION

Diacylglycerol (DAG) is one of the key second messengers mediating signals initiated by G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). Agonist binding to various GPCRs or RTKs induces activation of phospholipase C (PLC), which then hydrolyses cell membrane phospholipids. Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generates inositol 1,4,5-trisphosphate (IP_3) and DAG, both of which play roles in activating intracellular signaling cascades. IP_3 diffuses throughout the cytosol and acts to release calcium from the endoplasmic reticulum (ER), while DAG remains at the inner layer of the phospholipid membrane and serves as a ligand for proteins that contain a specialized DAG recognition motif, the C1 domain.

The cysteine-rich C1 domain was first found in protein kinase C (PKC), a family of serine/threonine kinases that were also the first known effectors for DAG (Nishizuka, 1992). The C1 domains are approximately 50 amino acids long conserved cysteine-rich protein structures that form a globular domain with a polar binding cleft for DAG (Zhang et al., 1995). In addition to binding DAG they serve as receptors for phorbol esters, which are plant-derived PKC activators with tumor-promoting properties. There are two types of C1 domains: those that bind DAG and phorbol esters and those that do not: the so-called typical and atypical C1 domains, respectively (Hurley et al., 1997). The C1 domains of conventional (cPKCs; α, βI, βII and γ) and novel (nPKCs; δ, ε, θ and η) PKC isoforms serve as paradigms for DAG-responsive C1 domains, while the C1 domains of atypical PKC isoforms (aPKCs; ζ and λ/ι) are unresponsive to DAG or phorbol esters (Newton, 2001).

Besides PKC, there are six other classes of proteins with DAG-responsive C1 domains. Protein kinase D (PKD) isoforms are serine/threonine kinases that are classified as a subgroup of calcium/calmodulin-dependent protein kinase (CAMK) family (Fu and Rubin, 2011; Manning et al., 2002). Diacylglycerol kinases (DGKs) are a family of lipid kinases that modulate intracellular levels of the lipid signaling molecules DAG and phosphatidic acid (PA) by phosphorylating DAG to yield PA (Merida et al., 2008). The DGK isoforms β and γ contain a DAG and phorbol-responsive C1 domain (Shindo et al., 2003). The chimaerins are a family of four Rac-GTPase-activating proteins (Rac-GAPs) (Yang and Kazanietz, 2007). Ras guanylyl-releasing proteins (RasGRPs) are guanine nucleotide exchange factors that activate Ras and related small GTPases (Stone, 2006). The myotonic dystrophy kinase-related Cdc42-binding kinases (MRCKs) are a family of three serine/threonine kinases that act as effectors for the small GTPase Cdc42 (Leung et al., 1998). The four Munc13 scaffolding proteins are important regulators of exocytosis (Brose et al., 2000; Rhee et al., 2002). Binding of DAG to the C1 domain of these proteins may regulate either their activity, intracellular localization or binding to other protein partners (Colón-Gónzalez and Kazanietz, 2006).

As the first recognized receptor for the tumor-promoting phorbol esters, PKC became a popular target in cancer research already in 1980s, and vast evidence substantiates an important role for PKC in controlling processes linked to cell proliferation and apoptosis as well as tumor invasion and angiogenesis (Griner and Kazanietz, 2007; Hofmann, 2004). Several of the other DAG effectors are also implicated in modulating cellular processes related to cancer, such as cell proliferation and apoptosis (Griner and Kazanietz, 2007). The actin cytoskeleton participates in the regulation of various cellular processes that are important in cancer development and progression including adhesion, morphology and migration (Hall, 2009). Several of the DAG effectors also participate in regulating these processes through their effects on actin cytoskeleton. For example, PKC phosphorylates a number of proteins directly linked to cytoskeleton (e.g. integrins), and it is known to play a role in cell spreading, cell migration, and neurite outgrowth (Larsson, 2006). PKD has been implicated in cytoskeleton reorganization during directed movement (Eiseler et al., 2009; Olayioye et al., 2013), and chimaerins exert their effects on cytoskeleton by inhibiting activity of the small GTPase Rac1 and thereby control e.g. membrane protrusions (Bosco et al., 2009). Furthermore, MRCKs, the effectors of another small GTPase Cdc42, play a crucial role in cytoskeleton organization by regulating myosin phosphorylation, lamellar actomyosin retrograde flow and reorientation of the microtubule-organizing center (Gomes et al., 2005; Tan et al., 2008; Wilkinson et al., 2005).

Targeting the C1 domain thus provides an attractive way to influence multiple signaling pathways mediating carcinogenesis and tumor progression (Blumberg et al., 2008). Furthermore, compounds that are selective for distinct PKC isoforms or DAG effectors would be valuable tools in discriminating their roles in controlling processes that lead to cancer. Several families of C1 domain ligands have been discovered, and most of them induce antiproliferative or proapoptotic responses in cancer cell lines (Boije af Gennäs et al., 2011). Various PKC- or C1 domain-targeted compounds have been and are currently being studied in clinical trials against cancer. The first PKC-targeting drug, the plant-derived C1 domain ligand ingenol-3-angelate (ingenol mebutate) (Kedei et al., 2004) has recently been approved for clinical use against actinic skin

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keratosis, a preliminary stage of squamous cell carcinoma (EMA, 2012; FDA, 2012). However, most of the currently known C1 domain ligands are complex in their chemical structure and thus difficult to synthesize. Therefore, simpler C1 domain-binding compounds are needed as lead molecules for drug development.

Our group has previously described a series of dialkyl 5-(hydroxymethyl)isophthalates as novel synthetic C1 domain ligands (Boije af Gennäs et al., 2009). Several isophthalates were shown to possess antiproliferative activity, and bis(3-trifluoromethylbenzyl) 5-(hydroxymethyl)isophthalate (HMI-1a3) was recognized as a promising antiproliferative compound for further studies (Talman et al., 2011). Furthermore, our previous work showed that HMI-1a3-induced inhibition of HeLa cell proliferation was accompanied with a distinct morphological change, cell elongation. The aim of this work was to further characterize HMI-1a3-induced effects on HeLa cell proliferation and phenotype.

2. MATERIALS & METHODS

2.1 Materials

All chemicals and reagents were commercially available except for isophthalic acid derivatives bis(3-trifluoromethylbenzyl) 5-(hydroxymethyl)isophthalate (HMI-1a3) and bis(3-trifluoromethylbenzyl) 5-nitroisophthalate (NI-15e) (see Fig. 1 for structures), which were synthesized at the Division of Pharmaceutical Chemistry, University of Helsinki as described (Boije af Gennäs et al., 2009). [20-^{3}H]Phorbol-12,13-dibutyrate ([^{3}H]PDBu) was custom labeled by Amersham Radiolabeling Service (GE Healthcare, Little Chalfont, UK). Phorbol 12-myristate-13-acetate (PMA), phosphatidyl-l-serine (PS), bovine immunoglobulin G (IgG), reduced L-glutathione, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Triton X-100, chelerythrine chloride and U0126 were purchased from Sigma-Aldrich (Steinheim, Germany). Gö6983 was purchased from Calbiochem (EMD Biosciences Inc., La Jolla, CA). Bicinchoninic acid (BCA) protein assay reagents (BCA Protein Assay Kit) and enhanced chemiluminescence (ECL) reagents (SuperSignal-West-Pico-Chemiluminescent-Substrate-Kit) were purchased from Pierce (Thermo Fisher Scientific Inc., Rockford, IL, USA). Cell culture solutions and reagents were from Invitrogen (Carlsbad, CA, USA), except for Dulbecco's modified Eagle's medium (DMEM), which was purchased from Sigma-Aldrich. Recombinant purified PKCμ (PKD1; #14-508) and the PKD inhibitor CID755673 were bought from Millipore (Merck Millipore, Dundee, UK). Protease inhibitors (Complete Protease Inhibitor Cocktail Tablets) and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail Tablets) as well as X-tremeGENE siRNA transfection reagent and FugeneHD transfection reagent were purchased from Roche Diagnostics (Mannheim, Germany). PKC isozyme-specific small interfering RNA (siRNA) molecules (ON-TARGETplus SMARTpool PRKCA and ON-TARGETplus SMARTpool PRKCD) and the scrambled siRNA (ON-TARGETplus siCONTROL non-targeting Pool) were purchased from Dharmacon RNA Technologies (Lafayette, CO). Primary antibodies used were as follows: anti-phospho-cofilin (Ser3) was from Cell Signaling Technology (Danvers, MA, USA); anti-vinculin was purchased from Sigma-Aldrich; and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). For anti-cofilin-1, see (Hotulainen et al., 2005). The secondary antibodies used in immunofluorescence stainings were: Alexa Fluor® 488 Goat Anti-Mouse IgG (Invitrogen); Alexa Fluor® 488 Goat Anti-Rabbit IgG (Invitrogen); Alexa Fluor® 594 Goat Anti-Mouse IgG1 (Invitrogen) and Alexa Fluor® 647 Goat Anti-Rabbit IgG (Invitrogen). Horseradish peroxidase (HRP)-linked secondary antibodies anti-mouse IgG and anti-rabbit IgG were from Cell Signaling Technology (Danvers, MA, USA). Phalloidins (Alexa Fluor® 488 and 568) were acquired from Invitrogen and 4',6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich.

2.2 Cell culture

HeLa human cervical adenocarcinoma cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cell culture medium for both maintenance and all drug exposures consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

2.3 Immunofluorescence stainings

HeLa cells were seeded onto 13 mm cover slips in 6-well plates, grown overnight and exposed to test compounds. After 24-h exposures to HMI-1a3 or NI-15e (at 10 μM and 20 μM) or vehicle (DMSO 0.1% and

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0.2%) the coverslips were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature (RT). Coverslips were then washed 2 times with Dulbecco + 0.2% BSA (DB) and the cells were permeabilized with 0.1% Triton X-100 for 7 min. Coverslips were then washed with DB, incubated with primary antibodies (1:50 in DB) for 60 min, washed again with DB and incubated with secondary antibodies (1:200 in DB) for 45 min. If phalloidin (1:250) and/or DAPI (1:500) were used, they were added to DB with the secondary antibodies. After several washes with DB the coverslips were mounted onto Mowiol containing DABCO. Images were acquired through a microscope (Axio Imager.M2; Zeiss) equipped with charge-coupled device camera (AxioCam HRm; Zeiss) and PlanApo 63x/1.40 (oil) objective (Zeiss), using AxioVision Rel. 4.8 software (Zeiss). Confocal imaging was performed on a Zeiss LSM 700 microscope equipped with ZEN Software and LCI Plan-Neofluar 63x/1.30 Imm Corr (glycerol) objective.

2.4 Immunoblotting

HeLa cells were seeded onto 6-well plates (4×10^5 cells/well) and allowed to attach overnight. Cells were exposed to 20 μM HMI-1a3 or 0.2% DMSO for various durations (5 min – 24 h), washed with PBS and lysed with 1% SDS in 1 mM of Tris-HCl (pH 7.0). The lysates were passed several times through a 25 gauge needle to shear genomic DNA and centrifuged at 16000 g for 20 min at 4 °C. Protein concentration of the supernatant was measured using a BCA protein assay kit according to manufacturer's instructions. Samples were diluted in Laemmli sample buffer and stored at −20 °C until use. Equal amounts of protein per lane were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. Membranes were washed for 5 min with 0.1% Tween 20 in Tris-buffered saline (TTBS) and then blocked for 1 h with 5% nonfat milk powder in TTBS (milk-TTBS) or 5% BSA in TTBS (BSA-TTBS) at RT depending on the primary antibody (according to manufacturer's instructions, if available). Membranes were incubated at 4 °C overnight with the primary antibodies (1:1000) in milk-TTBS or BSA-TTBS, washed with TTBS and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) in milk-TTBS or BSA-TTBS for 1 h at RT. The membranes were washed again and the immunoreactive bands were visualized with ECL. GAPDH bands were used as controls to verify equal protein loading. Western blots were quantified by measuring the optical densities (OD) of the immunoreactive bands with ImageJ software.

2.5 Cell viability assay and live-cell imaging with Cell-IQ

Effects of HMI-1a3 (0.1-20 μM) on HeLa cell viability were studied using a standard MTT assay as described earlier (Talman et al., 2011). When PMA (10 nM or 100 nM), Gö6983 (1 μM) or U0126 (10 μM) were used, they were added to the cells 15 min prior to the isophthalate. For live cell imaging and analysis of cell proliferation and morphology, HeLa cells were seeded onto 48-well plates and left to attach overnight. Cells were exposed to HMI-1a3 (0.1; 1; 4; 10 and 20 μM) alone or in combination with various inhibitors (Gö6983 at 0.01-10 μM; chelerythrine at 0.5-10 μM; CID755673 at 1 μM or 10 μM), which were added to the cells 15 min prior to HMI-1a3, and grown for 72 h on a continuous cell culturing platform with integrated optics and informatics (Cell-IQ®, CM Technologies Ltd, Tampere, Finland) in a humidified, 5%-CO_2 atmosphere at 37 °C. Images were captured automatically from 4 positions per well at 1 h intervals. The cells in each image were counted and classified into healthy, elongated, dividing or dead cells with Cell-IQ Analyzer® employing computer vision technology and a protocol specifically created for HeLa cells as previously described (Talman et al., 2011).

2.6 RNA interference with siRNA

HeLa cells were seeded onto 12-well plates (2×105 cells/well) and transfected the next day with siRNA constructs using X-tremeGENE transfection reagent according to manufacturer's instructions. Cells were detached after 4 h, seeded onto 48-well plates (2500 cells/well) and grown for 3 days preceding drug treatments to allow for optimal silencing of target genes. Cells were then exposed to various concentrations of HMI-1a3 (at 0.1-20 μM) and imaged with Cell-IQ® as described above. Downregulation of PKCα and PKCδ expression after siRNA transfections was verified with immunoblotting from cells harvested in 1% SDS at post-transfection days 1, 2, 3 and 6.

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2.7 Genome-wide gene expression microarray

HeLa cells were seeded onto 6-well plates ($5x10^5$ cells/well) and let to attach overnight, whereafter the cells were exposed to 0.1% DMSO or 10 µM HMI-1a3 in duplicate for 12 h. Cells were then washed with PBS, detached with Trypsin-EDTA, washed again with PBS, snap-frozen in liquid nitrogen and stored at -70 °C. RNA extraction and purification as well as genome-wide gene expression analysis were performed in the Biomedicum Functional Genomics Unit (FuGU), University of Helsinki (Helsinki, Finland). RNA was extracted and purified with RNeasy kit (Qiagen) according to manufacturer´s instructions. Total RNA integrity was verified using an Agilent Technologies 2100 Bioanalyzer with an RNA Integrity Number (RIN) value greater than or equal to 8. Amplified total RNA was labelled with Illumina® TotalPrep RNA Labeling Kit, and purified biotinylated cRNA was hybridized to Illumina® HumanHT-12 v4 Expression BeadChips for 18 h at 58 °C. BeadChips were then washed, blocked and stained with streptavidin-Cy3 and scanned with Illumina® iScan. The data was exported using Illumina® BeadStudio and analyzed with R/bioconductor (packages Limma/IBMT/ RankProd), BeadStudio and biomaRt. Microarray quality was evaluated using principal component analysis, hierarchical clustering and control probe analyses. The relative reliability of each array was then estimated with the arrayWeights function of Limma. Gene expression levels between sample sets were compared with moderated paired t-test (package IBMT, $P<0.05$) and a Benjamini-Hochberg multiple test correction was used to minimize selection of false positives. Gene set enrichment analysis was performed using GO*rilla* (http://cbl-gorilla.cs.technion.ac.il) (Eden et al., 2009).

2.8 Production of recombinant β2-chimaerin in Sf9 insect cells

The plasmid encoding human β2-chimaerin (pAcG2T; Pharmingen, San Diego, USA) was a generous gift from prof. Marcelo G Kazanietz (University of Pennsylvania, Philadelphia, PA, USA) (Caloca et al., 1997). Recombinant human β2-chimaerin was produced in *Spodoptera frugiperda* Sf9 cells. Recombinant baculoviruses were generated using Bac-to-bac® baculovirus expression system (Invitrogen) according to manufacturer's instructions. Sf9 cells were infected with an optimized amount of the recombinant baculovirus, harvested 2 days postinfection, washed with PBS, and frozen. Crude cell lysates were prepared by suspending the cell pellets in lysis buffer (25 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.1% Triton-X 100, and protease inhibitors). After a 30-min incubation on ice the lysates were centrifuged at 16000 g for 15 min at 4 °C and the supernatant was collected. The protein content of the supernatant was determined according to Bradford (Bradford, 1976) and the supernatant was used immediately for [^{3}H]PDBu binding experiments.

2.9 Production and purification of the C1 domain of MRCKα in *E. coli*

The pGEX-plasmid encoding the GST-fused C1 domain of human MRCKα (Choi et al., 2008) was a kind gift from prof. Peter M. Blumberg (Center for Cancer Research, NCI, USA). *E. coli* cells (strain BL21) transfected with the plasmid were cultured in Luria Bertani (LB) medium containing 100 µg/ml ampicillin. Expression of recombinant protein was induced by adding isopropyl-O-d-thiogalactopyranoside (IPTG, from Sigma-Aldrich) to a final concentration of 0.5 mM when the OD of the culture reached 0.5-0.6. The bacteria were harvested 4 h after induction at 37 ˚C, washed twice with PBS, frozen and stored at -70 ˚C until purification. The frozen pellets of *E. coli* were thawed and resuspended into lysis buffer (Tris-HCl 20 mM pH 7.8; 50 mM NaCl; 1% Triton X-100; 0.5% Igepal; 2 mM EDTA; 2 mM EGTA; 1 mM DTT, 2 mM PMSF). Cell suspensions were incubated on ice for 30 min and homogenized using French press. After centrifugation (11 200 *g* for 30 min at 4 ˚C) the supernatant was incubated with glutathione beads (Sigma-Aldrich) for 2 h at 4 ˚C. Beads were then washed and bound proteins were eluted with lysis buffer containing 10 mM reduced L-glutathione (Sigma-Aldrich).

2.10 Binding assays

Binding of HMI-1a3 to recombinant C1 domain of MRCK and recombinant β2-chimaerin was tested on 96-well plates using a **filtration method** as described earlier (Boije af Gennäs et al., 2009). Briefly, 20 µg of protein/well from lysates of Sf9 cells over-expressing β2-chimaerin or 40 ng/well of purified C1 domain of MRCKα was incubated with graded concentrations of HMI-1a3 (0.03-10 µM for β2chimaerin and 0.1-30 µM for MRCKα) and 25 nM [^{3}H]PDBu for 10 min at RT in a 96-well Durapore filter plate (Millipore, Bedford, MA, USA) in a total volume of 125 µL. The final concentrations in the assay were as follows: 20 mM Tris-HCl (pH 7.5), 40 µM $CaCl_2$, 10 mM $MgCl_2$, 400 µg/mL bovine IgG, 25 nM [^{3}H]PDBu, and 0.1 mg/mL phosphatidyl-L-

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serine. Proteins were precipitated by the addition of 125 µL of cold 20% poly(ethylene glycol) 6000, the filters were washed and the plates were dried before the addition of liquid scintillant. Radioactivity was measured using Wallac Microbeta Trilux microplate scintillation counter (PerkinElmer, Waltham, MA). Binding of HMI-1a3 to commercial recombinant PKD was investigated using the **centrifugation method** (Lewin and Blumberg, 2003). Briefly, 20 ng/tube of purified recombinant human PKD was incubated with graded concentrations of HMI-1a3 (0.1-100 µM) for 10 min at 37 °C in a reaction mixture consisting of 50 mM Tris-HCl (pH 7.4), 0.1 mg/mL phosphatidyl-L-serine, 1.8 mg/mL bovine IgG, and 10 nM [^{3}H]PDBu. After chilling the samples on ice for 10 min, 200 µL of 35% poly(ethylene glycol) 6000 was added, samples were mixed, and after a 15-min incubation on ice the samples were centrifuged at 15000g for 15 min at 4° C. Radioactivity was determined from 100 µL aliquots of supernatants and from dried pellets.

2.11 Data analysis

Data are expressed as the mean ± S.E.M. from at least three independent experiments unless otherwise stated. Cell-IQ® quantifications were analyzed using PASW Statistics 18 software (SPSS Inc., Chicago, IL, USA) using one-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test. Competition binding data was analyzed with GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, CA, USA) and one-site competition equation.

3. RESULTS

3.1 Effects of HMI-1a3 on actin stress fibers and focal adhesions

According to our previous studies (Talman et al., 2011), the antiproliferative isophthalate HMI-1a3 induces elongation of HeLa cells (Fig. 1A), while the inactive derivative NI-15e has no effect on cell morphology or proliferation (Fig 1B). The response is most prominent after 24 h of exposure, at which time point approximately 30% of cells are elongated. Since the morphological change implies major reorganization of actin cytoskeleton, we studied the effects of HMI-1a3 and NI-15e on actin stress fibers and focal adhesions using immunocytochemistry. After a 24-h exposure to HMI-1a3 (at 10 µM or 20 µM), elongated cells exhibited a substantially smaller number of stress fibers and focal adhesions (20 µM shown in Fig. 2). Neither stress fibers nor focal adhesions were changed in cells exposed to NI-15e or in cells with normal morphology despite HMI-1a3 exposure (data not shown).

3.2 Effects of HMI-1a3 on the localization and phosphorylation of cofilin-1

Reorganization of actin cytoskeleton, such as that observed in HMI-1a3-induced cell elongation, requires disassembly of actin filaments. Among the central proteins promoting actin filament disassembly is cofilin-1, a member of ADF/cofilin family that is expressed in non-muscle tissues and is required for the reorganization of the actin cytoskeleton during diverse processes (Bernstein and Bamburg, 2010; Paavilainen et al., 2004). To investigate the potential role of cofilin-1 in mediating the cytoskeletal effects of HMI-1a3, we studied its subcellular localization in response to HMI-1a3 exposure. In untreated or DMSO-treated cells cofilin-1 was localized mainly in cytosol or diffusely throughout the whole cell (Fig. 3). However, a 24-h exposure to HMI-1a3 (at 10 µM or 20 µM) induced a strong relocation of cofilin-1 into nucleus in both elongated and non-elongated cells, while NI-15e had no effect (Fig. 3 and Fig. 4A). To investigate possible changes in the phosphorylation state of cofilin-1 in response to 20 µM HMI-1a3 exposure, we determined the levels of total and phosphorylated cofilin-1 protein from total cell homogenates at various time points with Western blotting. HMI-1a3-exposure had no effect on the amount of phospho-cofilin-1 (Fig. 4B). However, it induced a transient downregulation of total cofilin-1 protein, which was most pronounced (45%, $p=0.06$) at 30 min, whereafter it partially recovered (Fig. 4C).

3.3 Effects of pharmacological modulation of PKC or the ERK1/2 pathway on HeLa cell response to HMI-1a3

Since our previous data suggested that HMI-1a3 induces PKC-dependent phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in HeLa and SH-SY5Y cells (Gonelli et al., 2009; Talman et al., 2013), we investigated the roles of PKC and ERK1/2 pathways in HMI-1a3-induced cytotoxicity, cell elongation and

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antiproliferative effects by using pharmacological tools. Inhibition of PKC by the pan-PKC inhibitor Gö6983, activation of PKC by PMA, or MEK inhibition by U0126 were unable to protect HeLa cells from HMI-1a3-induced toxicity (Fig. 5A). PMA treatment alone induced cytotoxicity at concentrations 10 nM and 100 nM (32% and 19% cytotoxicity, respectively; not shown), while treatments with Gö6983 (10 μM) or U0126 (1 μM) alone did not affect cell viability (4.5% and 5.2% cytotoxicity, respectively; not shown). According to Cell-IQ® imaging, Gö6983 (at 10 nM - 10 μM) had no effect of its own, nor did it modify the antiproliferative effect of HMI-1a3 (Fig 5B and 5C). Furthermore, it had no influence on HMI-1a3-induced cell elongation (data not shown).

3.4 Effects of PKCα or PKCδ knock-down on HeLa cell response to HMI-1a3

To further determine whether PKC plays a role in mediating the effects of HMI-1a3, we investigated the effects of silencing separately the expression of two PKC isoforms (PKCα and PKCδ) that are highly expressed in HeLa cells. Inhibition of PKCα or PKCδ expression by siRNA transfection inhibited HeLa cell proliferation, whereas transfection with the non-targeting control siRNA had no effect (Fig. 5D). Despite the slower proliferation rate of PKC siRNA-transfected cells, downregulation of PKCα or PKCδ expression had no influence on the concentration-response to HMI-1a3 (Fig. 5D) or on morphological changes evoked by the isophthalate (data not shown). Transfection of isozyme-specific siRNAs induced specific downregulation of PKCα or PKCδ that was clearly visible at 48 h and more pronounced at 72 h post-transfection as determined by Western blotting (Fig. 5E-F). Downregulation lasted for at least until 6 days post-transfection, and the non-targeting control siRNA transfections had no effect on PKC protein levels (Fig. 5E-F).

3.5 Effects of HMI-1a3 on genome-wide gene expression

To characterize the effects of HMI-1a3 and NI-15e on gene expression in HeLa cells, genome-wide gene expression microarray and subsequent gene set enrichment analysis (GSEA) were carried out. When compared to DMSO-exposed cells, a 12-h exposure to 10 μM HMI-1a3 induced statistically significant changes in the expression of 1411 genes, while exposure to NI-15e (10 μM for 12 h) had a significant effect on 9 genes only (data not shown). Furthermore, there was a statistically significant difference in the expression of 1277 genes in HMI-1a3-exposed versus NI-15e-exposed cells (data not shown). Significantly altered biological processes in response to HMI-1a3 exposure included those linked to cell proliferation, cell cycle progression, cytoskeleton organization and cell death (Table 1), as expected based on the cellular response. Additionally, gene expression changes linked to processes such as lipid and amino acid metabolism, intracellular transport and macromolecular complex assembly were significantly enriched according to GSEA (Table 1). Furthermore, gene expression changes were significantly enriched in gene ontologies of molecular functions related to small GTPase signaling (Table 1) emphasizing potential changes in small GTPase-related signal transduction.

3.6 Binding of HMI-1a3 to the C1 domains of β2-chimaerin, MRCKα and PKD

Based on the HMI-1a3-evoked morphological changes and genome-wide gene expression microarray we considered chimaerins, PKD and MRCK among the C1 domain-containing proteins as potential mediators of HMI-1a3-induced effects in HeLa cells. We therefore examined the binding of HMI-1a3 *in vitro* to these DAG effectors. HMI-1a3 displaced [^{3}H]PDBu from β2-chimaerin, PKD1 and the C1 domain of MRCKα within a similar range of low micromolar concentrations (Fig. 6).

3.7 Effects of pharmacological inhibition of MRCK or PKD on HeLa cell response to HMI-1a3

We then investigated which of the DAG effectors are expressed in HeLa cells. Expression of PKD1, PKD2 and MRCKα was detected in HeLa cells, while β2-chimaerin protein levels were undetectable with Western blotting (data not shown). The PKD inhibitor CID755673 (at 1 μM or 10 μM) alone had no effect on HeLa cell proliferation (data not shown) or morphology (Fig. 7), and it did not modify the HMI-1a3-evoked cellular responses (Fig. 7). The MRCK inhibitor chelerythrine (Tan et al., 2011) induced rapid cell death at 4 μM and 10 μM preventing analysis with these higher concentrations (data not shown). At lower concentrations of 0.5-2 μM it had no effect on cell proliferation or morphology when administered alone (data not shown and Fig 7). However, at concentrations of 1 and 2 μM chelerythrine attenuated HMI-1a3-induced cell elongation (Fig. 7). The effect was most prominent at 24 h, when the percentage of elongated cells in HMI-1a3-exposed wells

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was at its highest: at that time point, chelerythrine (1 μM and 2 μM) reduced HMI-1a3-induced cell elongation by 38% and 42%, respectively ($p<0.05$, ANOVA and Tukey's HSD) (Fig. 7). There was, however, no significant difference in the percentage of healthy, dead or dividing cells after co-exposure to chelerythrine and HMI-1a3 in comparison to HMI-1a3 exposure alone (Fig. 7).

4. DISCUSSION

The seven families of C1 domain-containing DAG effectors regulate cellular functions that are linked with cell growth, proliferation, survival and morphology and several of them are considered potential cancer drug targets. The derivatives of 5-(hydroxymethyl) isophthalic acid were initially designed to target the C1 domain of PKC (Boije af Gennäs et al., 2009). Targeting the C1 domain enables the development of PKC activators and inhibitors (reviewed in Boije af Gennäs et al., 2011). Both PKC activators and inhibitors bear the potential of becoming cancer therapeutics, since the role of PKC isoforms in cancer depends on the original tissue and on the cell type (Griner and Kazanietz, 2007; Hofmann, 2004). In our previous studies in HeLa and SH-SY5Y cells the isophthalate HMI-1a3 induced phosphorylation of ERK1/2, and the effect was inhibited by the pan-PKC inhibitor Gö6983, showing that HMI-1a3 functions as a PKC-activator (Talman et al., 2011; Talman et al., 2013). HMI-1a3 was able to inhibit HeLa cell proliferation and to induce a distinct morphological change, cell elongation, in HeLa cells (Talman et al., 2011). The present study aimed to further characterize the effects of HMI-1a3 in HeLa cells and to determine, whether activation of PKC plays a role in the cellular response.

The morphological changes in HeLa cells as a response to HMI-1a3 exposure were most obviously due to a marked loss of actin stress fibers and focal adhesions. Actin stress fibers are contractile actomyosin structures that play an important role in regulating cell morphology, motility and adhesion (Tojkander et al., 2012). Focal adhesions consist of protein complexes, which connect the cytoskeleton to the extracellular matrix (Wolfenson et al., 2009). We propose that compromised focal adhesion and stress fiber formation in HMI-1a3-treated cells leads to decreased cell adhesion and contractility, which are manifested by cell elongation. Alternatively, disruption of the contractile stress fiber network may lead to an increase in the assembly of protrusive actin filament arrays, which would be expected to enhance cell migration and/or elongation. Because of the cytotoxic properties of HMI-1a3, reliable analysis of its effect on cell migration is unfeasible and was not carried out.

The actin-depolymerizing protein cofilin-1 plays a fundamental role not only in regulating reorganization of actin cytoskeleton (Paavilainen et al., 2004), but also in the regulation of apoptosis, phospholipid metabolism and nuclear import of actin (Bernstein and Bamburg, 2010). The cofilin sequence contains a nuclear localization signal (Iida et al., 1992), and nuclear cofilin-1 has been shown to regulate transcription elongation by controlling dynamics of gene-associated actin (Obrdlik and Percipalle, 2011). Thus, HMI-1a3-induced nuclear translocation of cofilin observed in this study may contribute to the prominent HMI-1a3-induced changes in gene expression. The actin-severing activity of cofilin is inhibited by phosphorylation on Ser3 (see Bernstein and Bamburg, 2010; Paavilainen et al., 2004). Among the C1 domain-containing proteins, MRCK and PKD are known to induce cofilin-1 phosphorylation (Bernstein and Bamburg, 2010; Eiseler et al., 2009; Spratley et al., 2011; Sumi et al., 2001). Furthermore, since PKD activity is largely controlled by PKC (Steinberg, 2012), PKC activation could also induce cofilin-1 phosphorylation. To our surprise, HMI-1a3 had no effect on the levels of phosphorylated cofilin-1. Since cofilin-1 phosphorylation can be regulated in a highly localized manner (van Rheenen et al., 2009), the present results do not however rule out the possibility that HMI-1a3 induces cofilin-1 phosphorylation: Small but locally significant changes in cofilin-1 phosphorylation may not be detectable with the methods employed. Furthermore, it can be speculated that the increase in the proportion of phosphorylated cofilin-1 (relative to total cofilin-1) affects cofilin-1 function and thereby plays a role in mediating the cellular response.

Despite being able to activate PKC and ERK1/2 signaling in HeLa cells (Talman et al., 2011), the present results suggest that the effects of HMI-1a3 on HeLa cell proliferation and morphology are not a consequence of PKC activation or inhibition, or activation of the Raf-MEK-ERK1/2 cascade. However, PKC is also known to exhibit functions that are independent of its catalytic activity. For example, the pro-proliferative and pro-survival role of PKCα in several glioma cell lines was reported to be independent on kinase activity, but rather dependent on the protein itself as a non-catalytic scaffold protein (Cameron et al., 2008). In the same study, knock-down of PKCα or exposure to calphostin C, a C1 domain-targeting PKC inhibitor, inhibited glioma cell proliferation and reduced cell viability, while ATP-competitive inhibitors, such as Gö6983, had no effect. Furthermore, a conserved sequence N-terminal of the C1b domain of PKCε has been shown to mediate induction of neurites in neuroblastoma cells (Ling et al., 2005; Zeidman et al., 1999). Based

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on these findings, PKCs have been proposed to function as allosteric regulators with GTPase switch-like properties (Cameron and Parker, 2009). To investigate the possible non-catalytic role of PKC in mediating HMI-1a3-induced responses, we used a genetic approach to knock down PKCα and PKCδ one by one. The antiproliferative responses to PKC siRNA transfections suggest a pro-proliferative role for both PKCα and PKCδ in HeLa cells. Since the PKC inhibitor Gö6983 had no effect on cell proliferation, the responses to PKCα or PKCδ knock-down may illustrate non-catalytic functions of these PKC isoforms. However, since silencing PKCα or PKCδ expression had no effect on the response to HMI-1a3, such an activation-independent PKC-mediated mechanism of action for HMI-1a3 seems unlikely - or at least it is not mediated by PKC isoforms α or δ.

As shown previously (Talman et al., 2011; Talman et al., 2013) and in this study, the cellular responses to isophthalates correlate closely with the binding affinity of the isophthalate to the C1 domain. However, since the antiproliferative and morphological responses in HeLa cells seem to be independent of PKC, it is probable that they are mediated by some other DAG/phorbol ester-responsive protein(s). Small GTPase signaling, which was one of the biological processes highlighted in GSEA after HMI-1a3 exposure, plays a central role in regulating cytoskeleton reorganization (Hall, 1998; Nobes and Hall, 1995). Therefore, signaling pathways either up- or downstream of small GTPases may be responsible for mediating the cytoskeletal changes induced by HMI-1a3. C1 domain-containing proteins that are directly linked to small GTPase signaling include RasGRPs, chimaerins and MRCKs. Among these, especially chimaerins and MRCKs are recognized as central mediators of the cytoskeleton (Leung et al., 1998; Tan et al., 2008; Toker, 2005; Yang and Kazanietz, 2007). Furthermore, the PKD family also plays an important role in controlling cytoskeleton reorganization (reviewed in Olayioye et al., 2013).

Based on high similarity among the DAG-responsive C1 domains, it was expected that the isophthalates would bind to "non-PKC" DAG effectors as well. We selected β2-chimaerin, PKD1 and MRCKα for the binding experiments based on their potential role in mediating the morphological response to HMI-1a3. As expected, HMI-1a3 was able to displace [3H]PDBu from the C1 domains of all DAG effectors studied in a similar concentration range than from PKC in our previous studies (Boije af Gennäs et al., 2009). Even though the affinities are not directly comparable, since K_i values were not determined, the similar concentration range and the IC_{50} values indicate that there are no major differences in the affinities. Furthermore, based on studies with 19 isophthalate derivatives, the inactive isophthalate derivatives (i.e. those that are unable to displace [^{3}H]PDBu from PKC) are also unable to bind to the C1 domain of β2-chimaerin, and those that bind to PKC also bind to β2-chimaerin (Talman et al., unpublished data). The binding results thus confirm the isophthalate structure-activity relationship model created with PKC (Boije af Gennäs et al., 2009) and suggest that it can be extended to other proteins containing a DAG-responsive C1 domain.

We considered MRCK and PKD as the most potential DAG effectors mediating HMI-1a3-induced responses, since they are expressed in HeLa cells at detectable levels and since they have also been linked to regulation of cofilin activity. To characterize the role of PKD and MRCK in mediating the effects of HMI-1a3 in HeLa cells, we used pharmacological inhibitors of these kinases. CID755673 has been reported to be a selective PKD inhibitor (IC_{50} for PKD2: 280 nM) with antiproliferative effects in prostate cancer cells (Lavalle et al., 2010; Sharlow et al., 2008). Chelerythrine is marketed as a PKC inhibitor, but several reports demonstrate that it in fact does not inhibit PKC isoforms (Davies et al., 2000; Lee et al., 1998; Tan et al., 2011). Instead, it has been shown to be a non-ATP-competitive inhibitor of MRCK isoforms (IC_{50} for MRCKα: 1.77 μM) (Tan et al., 2011). In a screen of 70 protein kinases (not including MRCKs) chelerythrine exhibited no significant inhibition to any kinase tested at a concentration of 10 μM (Davies et al., 2000), and can thus be considered highly selective for MRCK. The present results with the PKD inhibitor indicate that (1) PKD activity is not crucial for HeLa cell proliferation or survival and (2) the antiproliferative or elongation-inducing effects of HMI-1a3 do not result from HMI-1a3-induced PKD activation. However, chelerythrine inhibited HMI-1a3-induced cell elongation suggesting that activation of MRCK may mediate the HMI-1a3-induced morphological change. The incomplete rescue of elongated phenotype by chelerythrine can be attributable to the low concentrations used because of significant cytotoxicity with the higher concentrations. The concentrations used (1 μM and 2 μM) are close to the reported IC_{50} value for MRCK kinase activity (1.77 μM) (Tan et al., 2011), indicating that the level of MRCK inhibition attained was only partial and thus insufficient for fully preventing HMI-1a3-induced cell elongation. MRCK is known to regulate cytoskeleton reorganization and actomyosin dynamics (Leung et al., 1998; Tan et al., 2008), and therefore the suggested role for MRCK in mediating HMI-1a3-induced effects in HeLa cells is logical. Indeed, it has been shown that Cdc42-MRCK signaling cooperates with Rho-ROCK signaling, and that either MRCK or ROCK signaling is required for elongated morphology of tumor cells (Wilkinson et al., 2005).

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Regarding cell proliferation and survival, MRCKα has been identified as a survival kinase in HeLa cells (MacKeigan et al., 2005), consistent with the toxicity observed in this study with higher concentrations of chelerythrine. However, the antiproliferative effects of HMI-1a3 do not seem to be attributable to modulation of MRCK activity, since chelerythrine at the lower concentrations did not significantly alter its antiproliferative or cytotoxic effects. Since none of the pharmacological inhibitors or genetic manipulations used in this study affected the inhibition of cell proliferation induced by HMI-1a3, its antiproliferative action is probably not mediated by a single C1 domain-containing protein but through modulation of several DAG effectors. Although specificity is often the aim in drug development, the feature that C1 domain-binding compounds can modulate various mitogenic signaling routes by targeting several DAG effectors may actually be advantageous in the treatment of cancer, since cancer cells often become resistant to treatments that target only one signaling cascade. Furthermore, the cross-talk between DAG effector-mediated signaling pathways also provides an opportunity to modify a specific signal transduction cascade at several stages. Although these aspects may be beneficial considering the anti-cancer efficacy of C1 domain ligands, the complicated signaling networks may impede determination of the exact mechanism of action for C1 domain-targeting compounds.

5. CONCLUSIONS

The present work shows that the isophthalate derivative HMI-1a3, which was originally designed to target the C1 domain of PKC, also binds to the C1 domains of β2-chimaerin, PKD and MRCKα, further confirming the structure-activity model created with PKC. Moreover, HMI-1a3-induced HeLa cell elongation is associated with loss of actin stress fibers and focal adhesions as well as prominent nuclear accumulation of cofilin-1 with no major changes in cofilin-1 phosphorylation. Although HMI-1a3 activates PKC to induce ERK1/2 signaling, its antiproliferative and elongation-inducing effects seem to be independent of PKC. The antiproliferative effect is hypothesized to be mediated by several DAG effectors, whose converging signaling pathways may render the determination of the mechanism of action unfeasible. However, the results suggest that the cell elongation evoked by HMI-1a3 exposure may be, at least partially, mediated by MRCK. The current results further support the potential of HMI-1a3 as a lead molecule for cancer drug development. Further studies using other cancer cell lines and, in particular, *in vivo* cancer models are however needed to assess its anti-cancer potential in more detail.

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FIGURES AND FIGURE LEGENDS

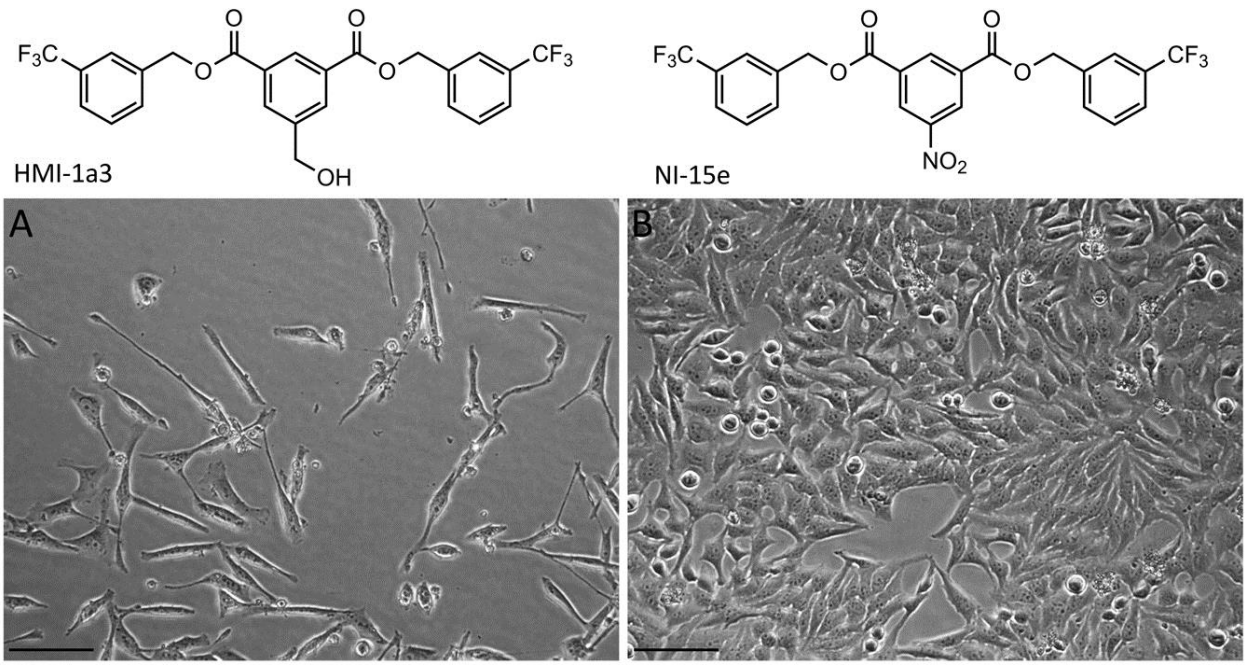

Figure 1. Structures of isophthalate derivatives and cell elongation induced by HMI-1a3. *A, HeLa cells exposed to 10 µM HMI-1a3 for 72 h. B, HeLa cells exposed to 10 µM NI-15e for 72 h. Scale bar, 100 µm.*

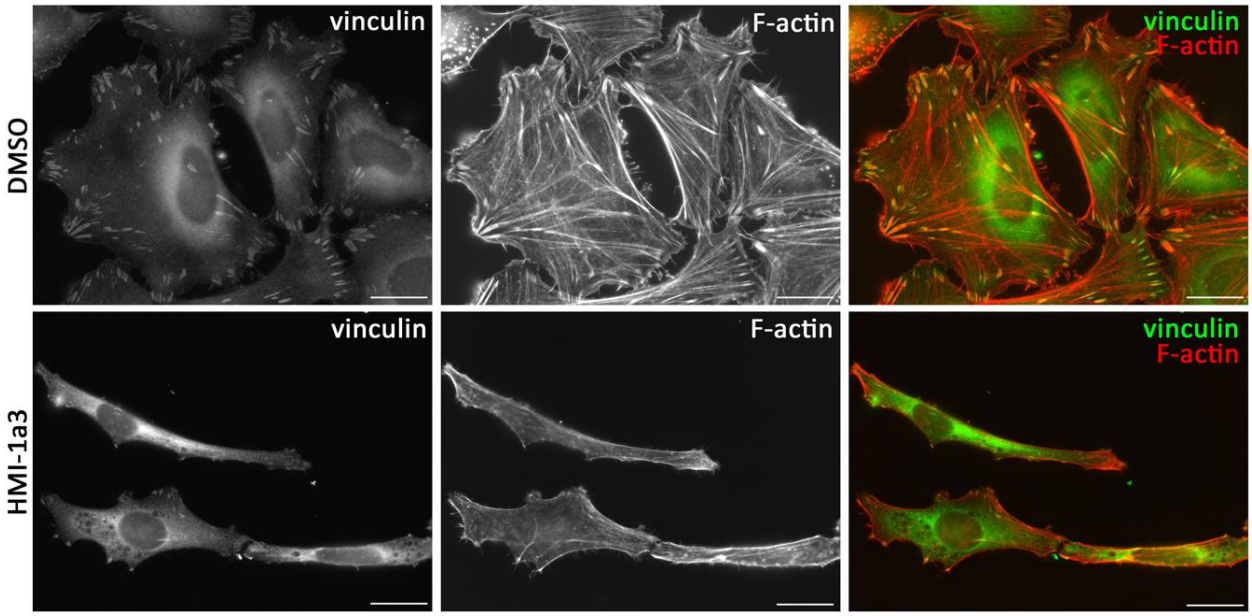

Figure 2. Effects of HMI-1a3 on focal adhesions and actin stress fibers. *HeLa cells were exposed to 0.2% DMSO or 20 µM HMI-1a3 for 24 h, fixed and stained. Focal adhesions were visualized with anti-vinculin antibody and actin filaments with phalloidin. Representative epifluorescence microscopic images are presented. Experiments were repeated 2 times for focal adhesions and 8 times for F-actin with similar results. Scale bar, 20 µm.*

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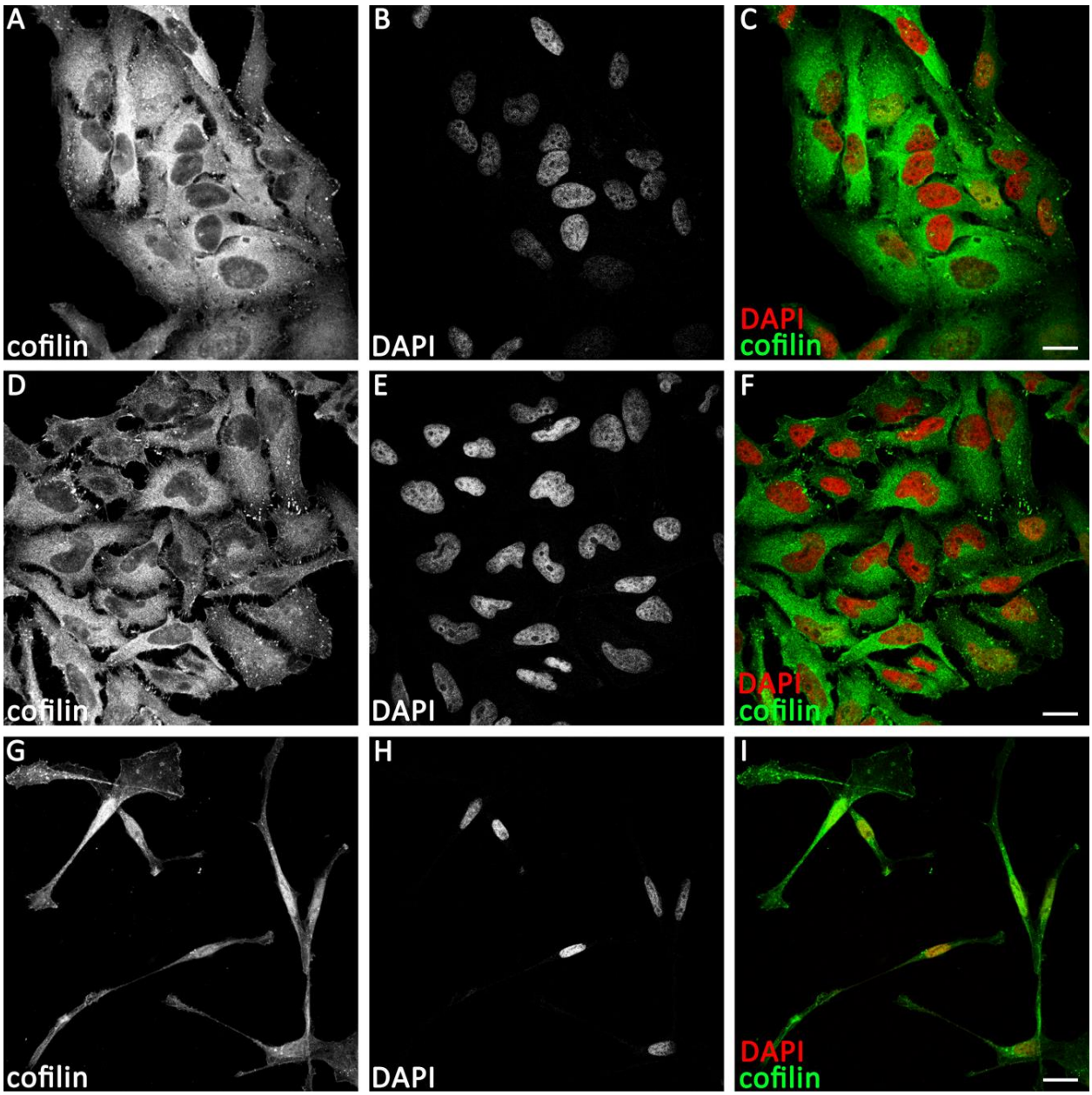

Figure 3. Effects of isophthalates NI-15e and HMI-1a3 on localization of cofilin-1. *HeLa cells were exposed to 0.2% DMSO (A-C); 20 μM NI-15e (D-F) or 20 μM HMI-1a3 (G-I) for 24 h, fixed and stained for cofilin-1 (A, D, G). The nuclei were visualized with DAPI (B, E, H). Merged images (C, F, I) show cofilin-1 in green and DAPI in red. Representative confocal microscopic images from 3 independent experiments are presented. Scale bar, 20 μm.*

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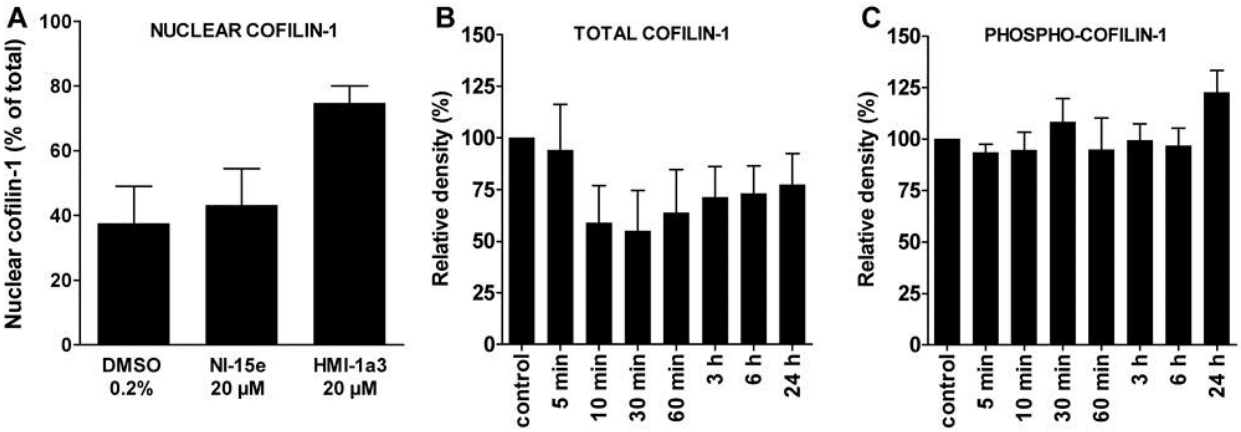

Figure 4. Effects of HMI-1a3 on cofilin-1 localization, phosphorylation and expression. *A, HeLa cells were exposed to DMSO, NI-15e or HMI-1a3 for 24 h, fixed and stained for cofilin-1. Samples were at Zeiss LSM 700 confocal microscope and the data was analyzed with ImageJ software. Fluorescence intensity in the nucleus is presented as percentage of total fluorescence within the same cell. 10 cells were analyzed from each sample. The experiment was repeated 3 times with similar results. B-C, Levels of phosphorylated (B) and total (C) cofilin were analyzed with Western blotting from total cell homogenates of HeLa cells exposed to 20 µM HMI-1a3 for the indicated times. Results are expressed as mean + S.E.M. from 3-4 independent experiments.*

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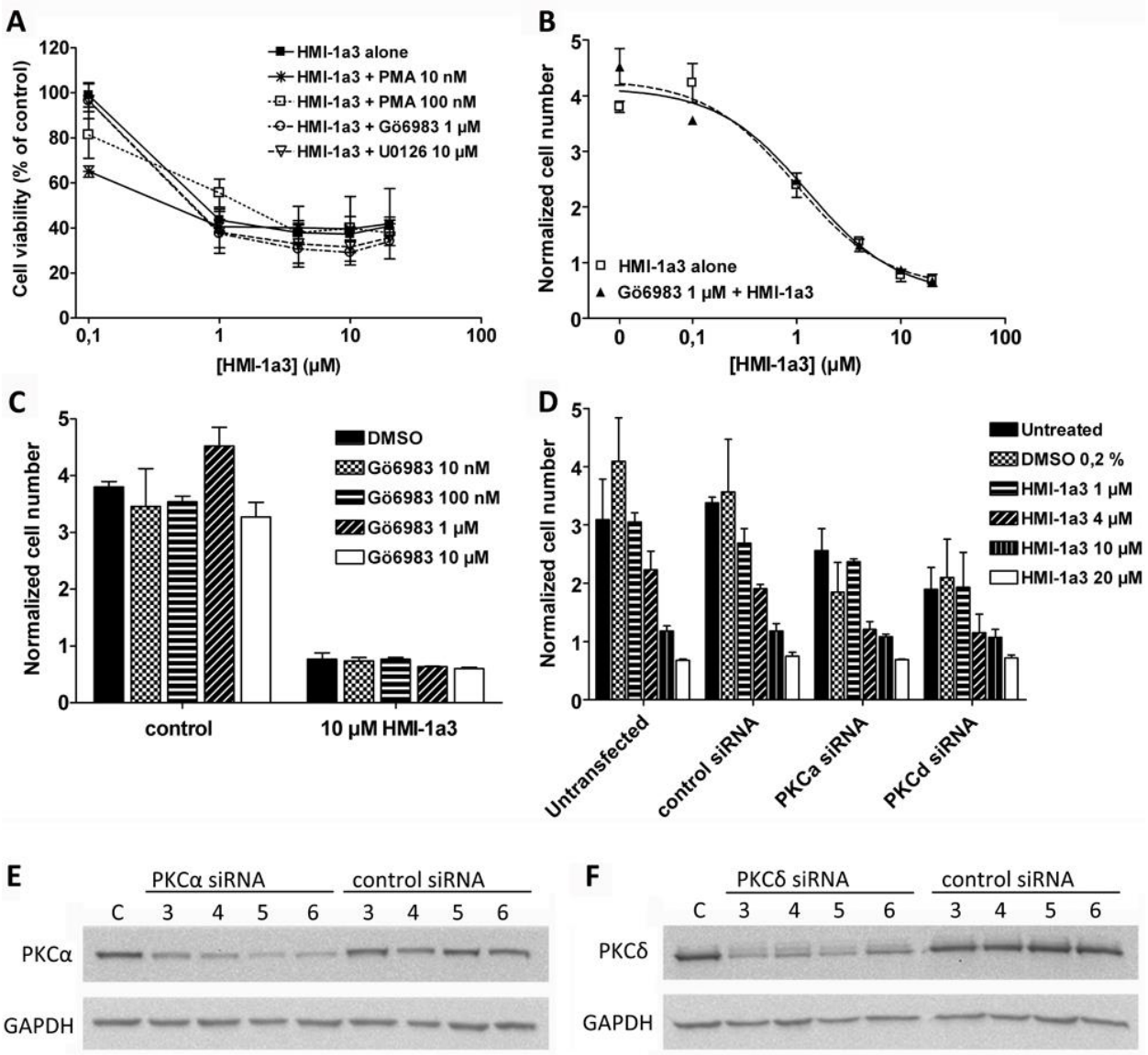

Figure 5. Effects of HMI-1a3 were not altered by MEK-inhibition or by pharmacological or translational means of PKC modulation. *A, HeLa cells were exposed to different concentrations of HMI-1a3 in the presence or absence of PMA, U0126 or Gö6983 for 24 h and cell viability was measured with an MTT assay. Error bars represent the S.E.M. from 3 independent experiments. B and C, HeLa cells treated with graded concentrations of HMI-1a3 and/or Gö6983 were imaged automatically for 72 h with Cell-IQ and analyzed using Cell-IQ analyzer® software. Bars represent mean + S.E.M. of normalized cell numbers at 72 h from 3 independent experiments. D, PKCα and PKCδ expression was silenced with isozyme specific siRNA molecules after which cells were exposed to different concentrations of HMI-1a3 and imaged automatically for 72 h with Cell-IQ®. Non-transfected HeLa cells and cells transfected with scrambled siRNA sequence served as controls. Results are expressed as mean + S.E.M. of normalized cell number at 72 h from 3 independent experiments. E–F, Representative Western blots showing knock-down efficiency of siRNA against PKCα (E) and PKCδ (F) at 3-6 days post-transfection. Cells transfected with non-targeting siRNA (control siRNA) were used as controls. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.*

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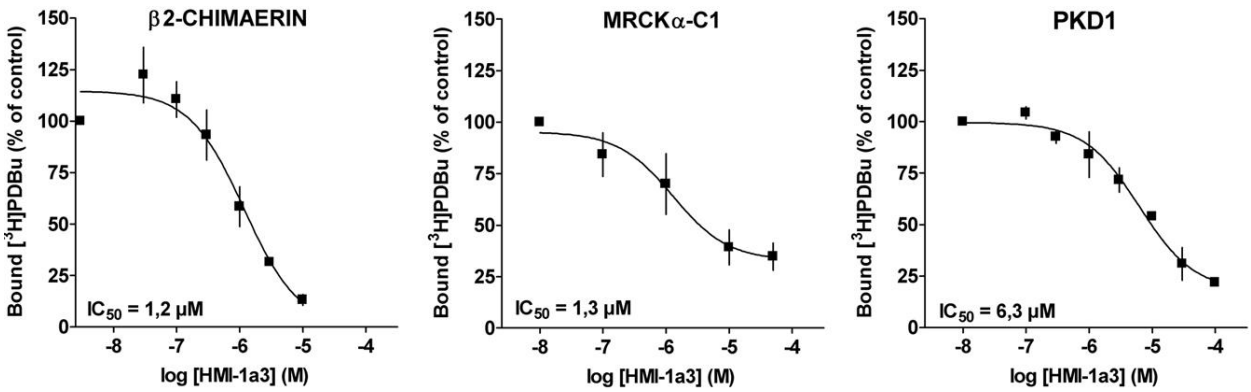

Figure 6. Binding of HMI-1a3 to C1 domains of β2-chimaerin, PKD and MRCKα. *The ability of HMI-1a3 to displace [³H]PDBu from recombinant full-length β2-chimaerin produced in Sf9 cells (left) and from the C1 domain of MRCKα produced in E.coli cells (middle) was determined with the filtration assay, and from purified recombinant full-length PKD1 (right) with the centrifugation assay. Results are expressed as mean ± S.E.M. from 3 independent experiments.*

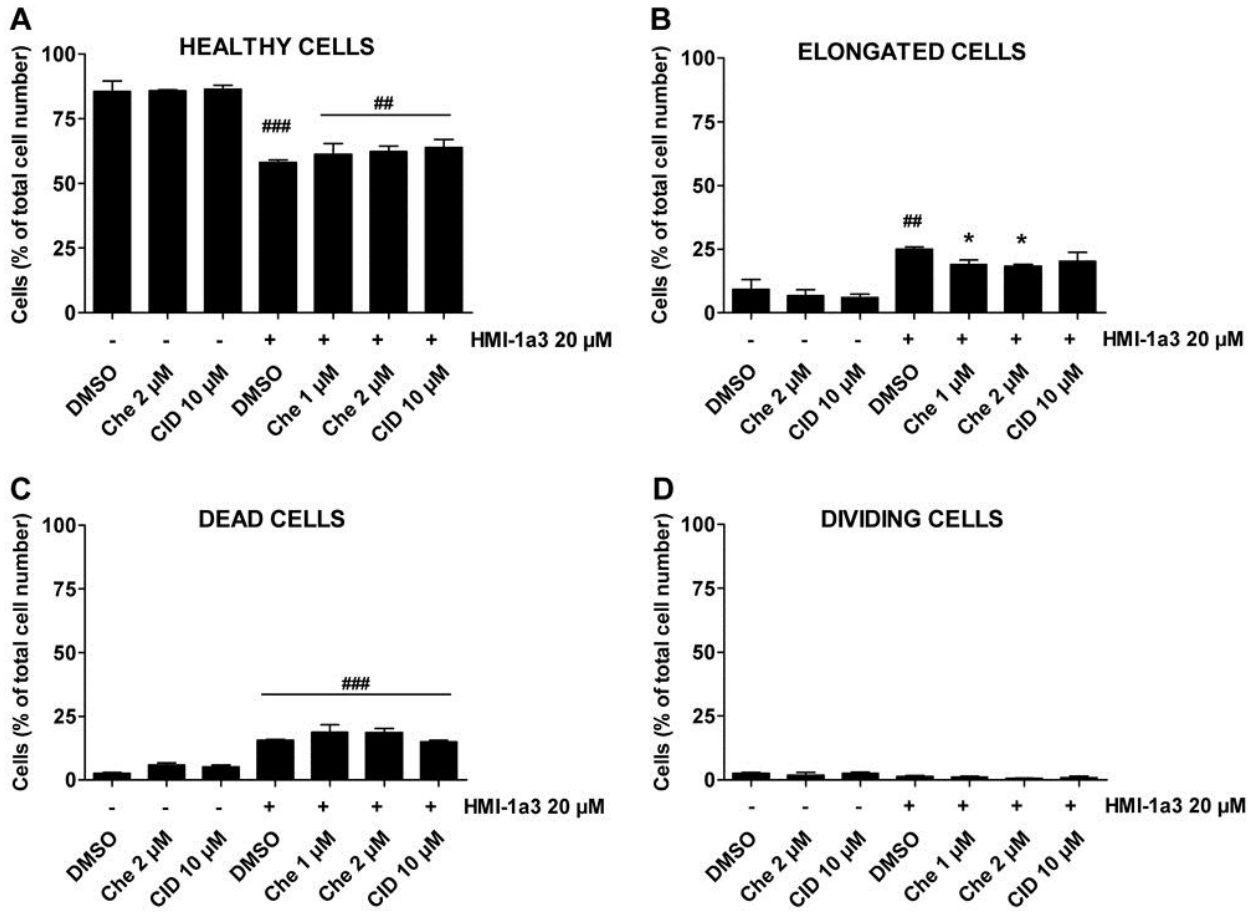

Figure 7. Effects of PKD and MRCK inhibitors on HMI-1a3-induced morphological changes. *HeLa cells were exposed to test compounds and imaged with Cell-IQ® at 1 h intervals for 72 h. Representative photomicrographs of HeLa cells exposed for 24 h to 0.2% DMSO (A), 20 μM HMI-1a3 (B), 2 μM chelerythrine (C) and 2 μM chelerythrine and 20 μM HMI-1a3 (D) are presented. Cells from each image were classified into healthy (E), elongated (F), dead (G) or dividing (H) cells with Cell-IQ Analyzer® software. Quantifications at time point 24 h are shown as mean + S.E.M. from 3 independent experiments. ###, p<0.001 vs. DMSO; ##, p<0.01 vs. DMSO; *, p<0.05 vs. DMSO + HMI-1a3 (One-way ANOVA and Tukey's HSD). Che, chelerythrine (MRCK inhibitor); CID, CID755673 (PKD inhibitor).*

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