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Chelerythrine perturbs lamellar actomyosin filaments by selective inhibition of myotonic dystrophy kinase-related Cdc42-binding kinase

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ABSTRACT

Cell movement requires forces generated by non-muscle myosin II (NM II) for coordinated protrusion and retraction. The Cdc42/Rac effector MRCK regulates a specific actomyosin network in the lamella essential for cell protrusion and migration. Together with the Rho effector ROK required for cell rear retraction, they cooperatively regulate cell motility and tumour cell invasion. Despite the increasing importance of ROK inhibitors for both experimental and clinical purposes, there is a lack of specific inhibitors for other related kinases such as MRCK. Here, we report the identification of chelerythrine chloride as a specific MRCK inhibitor. Its ability to block cellular activity of MRCK resulted in the specific loss of NM II-associated MLC phosphorylation in the lamella, and the consequential suppression of cell migration.

Structured summary of protein interactions:
DMPK phosphorylates **MYPT** by protein kinase assay (View interaction)
PAK alpha phosphorylates **MLC** by protein kinase assay (View interaction)
MRCK alpha phosphorylates **MLC** by protein kinase assay (View interaction)
CRIK phosphorylates **MLC** by protein kinase assay (View interaction)
MRCK beta phosphorylates **MLC** by protein kinase assay (View interaction)
ROK alpha phosphorylates **MLC** by protein kinase assay (View Interaction 1, 2)
MRCK beta phosphorylates **MYPT** by protein kinase assay (View interaction)
MRCK alpha phosphorylates **MYPT** by protein kinase assay (View interaction)
ROK alpha phosphorylates **MYPT** by protein kinase assay (View interaction)
MLCK phosphorylates **MLC** by protein kinase assay (View interaction)

1. Introduction

Members of the Rho GTPase family are important regulators of cytoskeletal organization that underlies diverse cellular processes ranging from migration, differentiation to cell cycle progression [1,2]. Cell migration in particular requires polarization for the formation of the front-rear axis and generation of force for cell body translocation [3]. Non-muscle myosin II (NM II) molecules present in all non-muscle eukaryotic cells are responsible for most of the contractile forces required for cell body translocation and tail retraction in cell migration [4]. These bipolar actin-based motor proteins are comprised of a heavy chain and two light chains, namely the regulatory (MLC) and essential light chains (ELC). Activation of the catalytic ATPase domain residing in the N-terminus of the heavy chain relies on the reversible phosphorylation of the associated MLC on Ser19 (monophosphorylation), or in some cases on both Thr18 and Ser19 (diphosphorylation) [4]. Besides the first identified myosin light chain kinase (MLCK) [5], multiple Rho GTPase effector kinases including Rho kinase (ROK/ROCK/Rho-kinase) [6,7], citron kinase (CRIK) [8], myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) [9], p21-activated kinase (PAK) [10] have also been shown to activate NM II by phosphorylating MLC. A PAK-related Misshapen/NIKs-related kinase (MINK) has also been shown to regulate cytoskeletal organization and cell motility [11]. In addition, inhibition of myosin phosphatase by ROK, MRCK and myotonic dystrophy protein kinase (DMPK), through phosphorylation of its targeting subunit MYPT, is also important for myosin activation [6,12,13]. Importantly, collective

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findings from the studies of these MLC kinases reveal that rather than playing redundant roles, their separate regulation of NM II appears to be spatiotemporally distinct and coordinated. CRIK is primarily involved in the regulation of contractility at the cleavage furrow and cell cycle progression [8]. For interphase cells, MLCK has been shown to regulate focal complex formation during lamellipodial extension crucial for leading edge advancement [14,15]. Whereas ROK activity, known for inducing stress fiber and focal adhesion formation, is required for tail retraction and prevention of cell rear protrusion [16,17], the Cdc42/Rac effector kinase MRCK

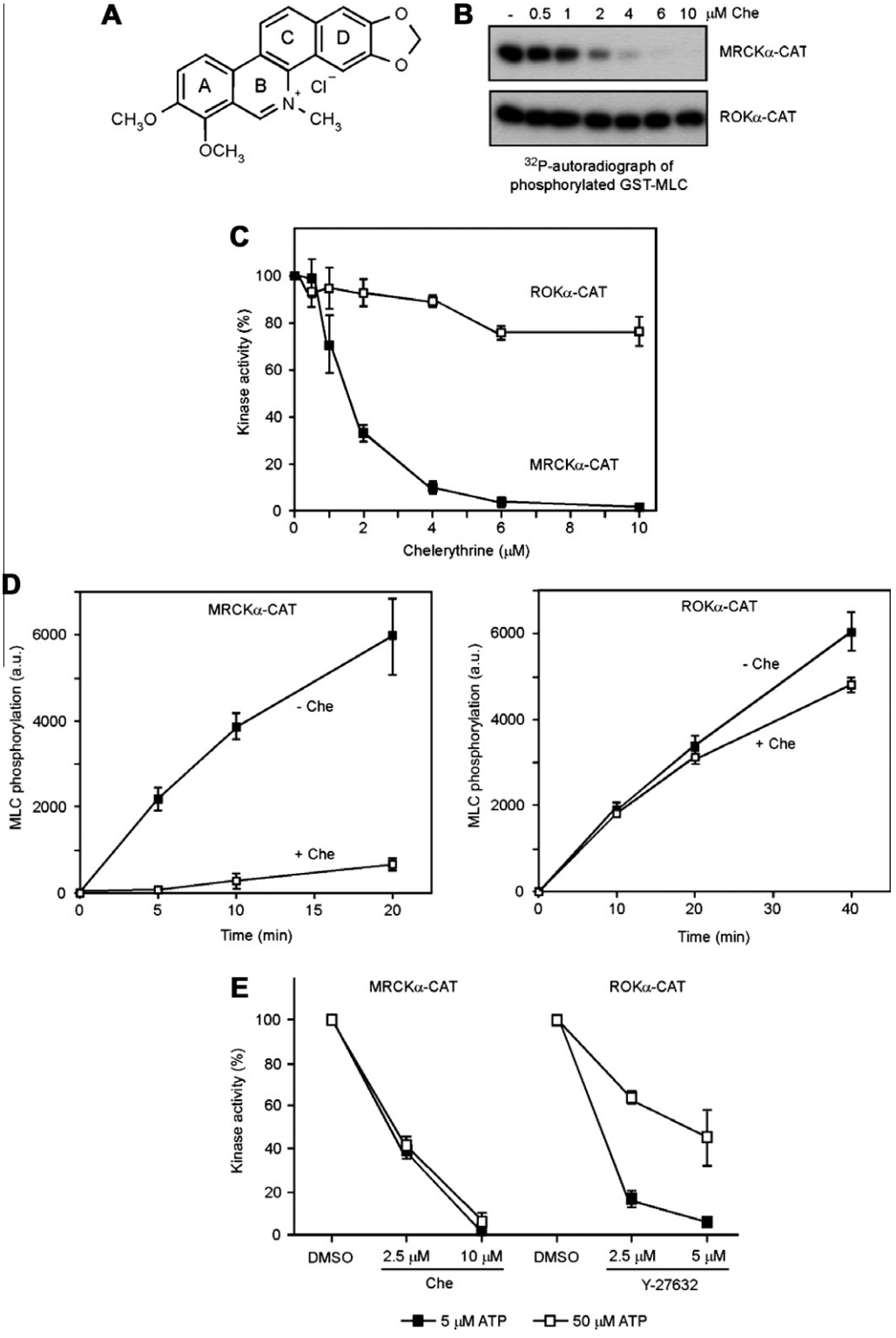

Fig. 1. Inhibition of MRCKa activity by chelerythrine. (A) Structure of chelerythrine chloride. (B) Kinase activity of recombinant catalytic domains of MRCKα and ROKα were assayed using GST-MLC as substrate, in the presence of increasing concentrations of chelerythrine. Samples were analyzed on SDS–PAGE and the ^{32}P autoradiograph is shown. (C) A graphical representation of the results in (B). Means and standard errors of data are shown. (D) Time course of MLC phosphorylation by MRCKα and ROKα in the presence or absence of 5 μM chelerythrine. Values represent means and standard errors of data from three experiments. (E) Kinase activity of MRCKα and ROKα were measured at 5 μM or 50 μM ATP, in the presence of increasing concentrations of chelerythrine and Y-27632, respectively. Means and standard errors of data are shown.

that colocalizes with and regulates the dynamics of lamellar actomyosin network has recently been shown to be essential for lamellar protrusion in a Cdc42-dependent manner, independent of MLCK and ROK [18]. The ability of these kinases to promote protrusion, traction and retraction in different parts of the cell is likely the result of their unique localization [19]. It is conceivable that their precise coordination is important for efficient cell movement.

In view of the complex regulation of actomyosin contractility by multiple protein kinases, it would be useful to develop specific inhibitors for each of the kinases involved, in order to gain further insight into their distinct roles. The widely used ROK inhibitor Y-27632 is crucial to our understanding of ROK functions. In addition, it has been found to be potentially useful for clinical conditions involving smooth muscle contraction dysfunction [20,21]. As Rho-ROK and Cdc42-MRCK signaling have been shown to cooperatively regulate the invasion of colorectal carcinoma cells into three-dimensional matrices [22], and that Cdc42-MRCK is important for collective invasion of squamous cell carcinoma cells [23], it is likely that MRCK may also serve as a potential drug target for blocking tumor cell invasion or metastasis. Here we describe the identification and characterization of chelerythrine chloride as a specific inhibitor of MRCK, and its specific disruptive effects on MRCK localization, lamellar actomyosin filaments and cell migration.

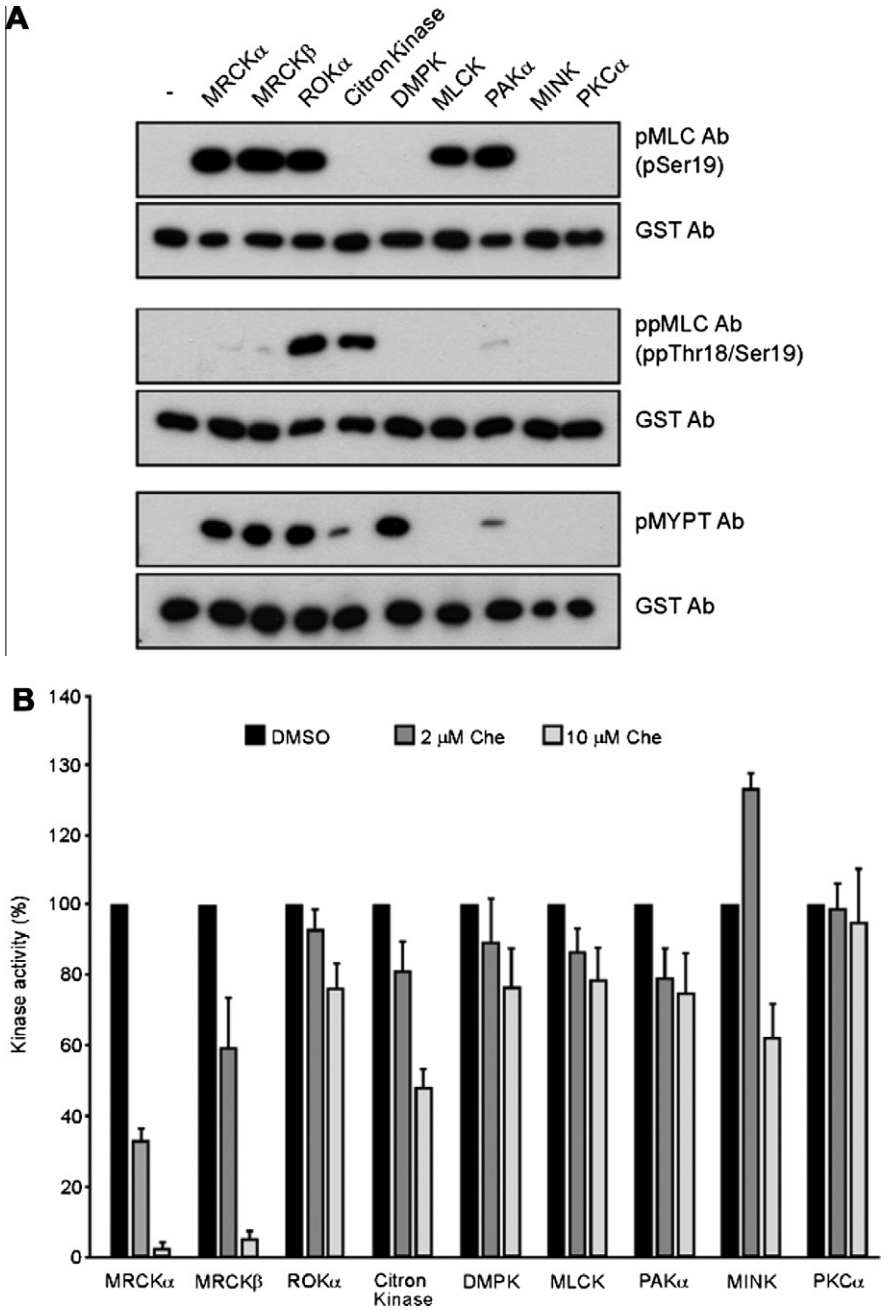

Fig. 2. Inhibition profile of chelerythrine on MRCK-related kinases. (A) *In vitro* phosphorylation of GST-MLC and GST-PIM by various kinases as indicated. Monophosphorylation on Ser19 (pMLC) and diphosphorylation on Thr18/Ser19 (ppMLC) of MLC were detected using specific phospho-antibodies. Phosphorylation of MYPT-PIM was detected by anti-pMYPT antibody. (B) Activity of various kinases was assayed using appropriate substrates (MLC as substrates for MRCKα/β ROKα, CRIK, MLCK and PAKα; MBP for PKCα; MYPT-PIM for DMPK and Histone H1 for MINK), in the presence of 2 μM or 10 μM chelerythrine. Incorporation of ^{32}P into substrate was measured by phosphorimager for determining kinase activity. Means and standard errors of data from three experiments are shown.

2. Materials and methods

2.1. cDNA constructs and purification of recombinant proteins

Catalytic domains of MRCKα, MRCKβ, CRIK, DMPK and MINK1 were cloned in bacterial expression vector pQE60 and expressed as C-terminal hexa-His-tagged fusion proteins. Full-length PAKα, MLC and the phosphorylation inhibitory motif (PIM) of MYPT were cloned in bacterial expression vector pGEX4T1 and purified as N-terminal-tagged GST-fusion proteins. The catalytic domains of PKCα and ROKα were cloned in mammalian expression vector pXJ40 and expressed as N-terminal GST-fusion proteins.

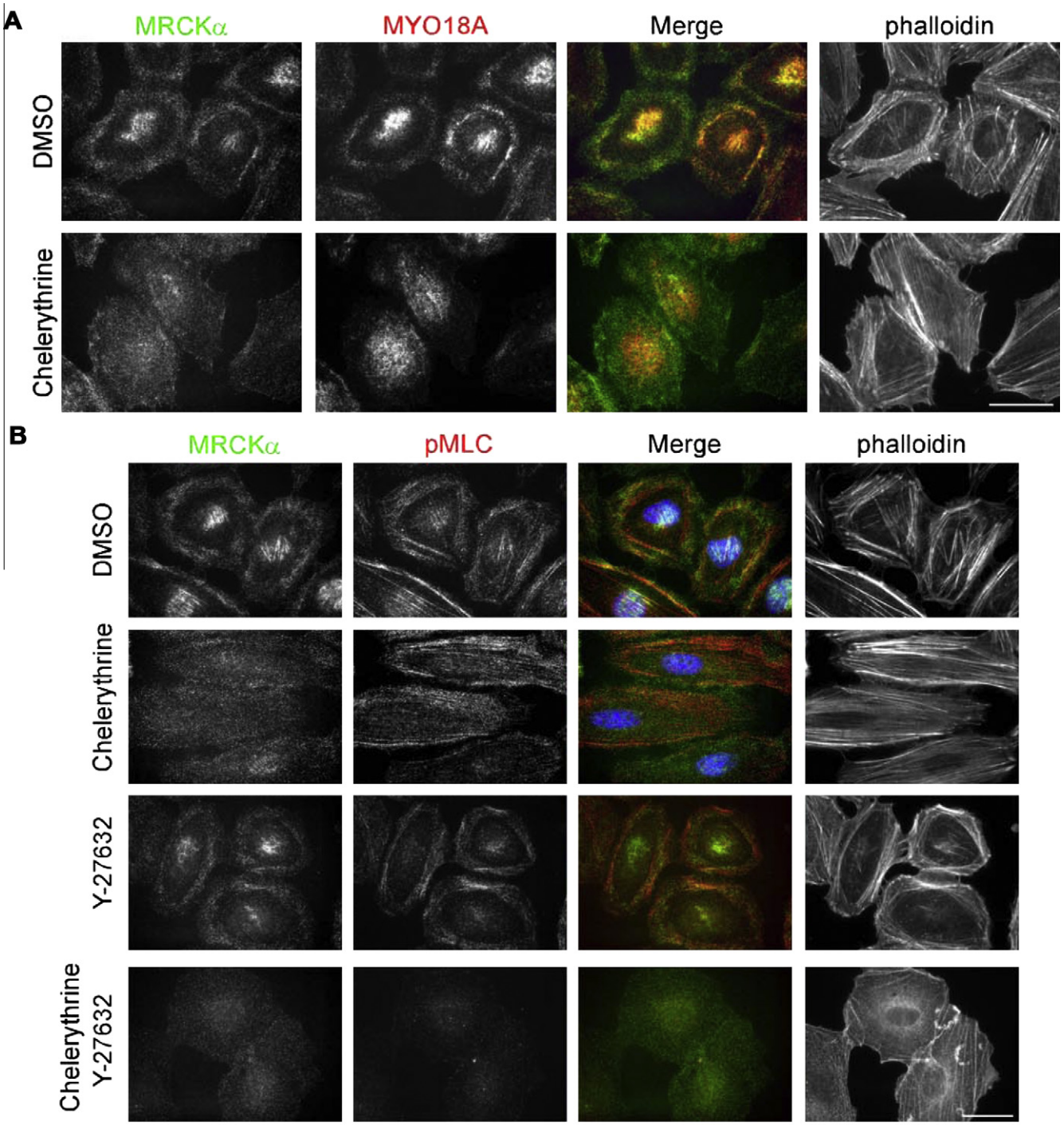

Fig. 3. Chelerythrine disrupts localization of endogenous MRCK. (A) HeLa cells on coverslips were treated with either DMSO or chelerythrine (7.5 μM for 45 min). Cells were fixed and stained for endogenous MRCKa, MYO18A and F-actin (phalloidin). Scale bar: 20 μm. (B) HeLa cells were treated with either DMSO, chelerythrine (7.5 μM for 45 min), Y-27632 (10 μM for 30 min) or a combination of chelerythrine and Y-27632 for 30 min. Cells were fixed and stained for endogenous MRCKα, pMLC, F-actin and nucleus (DAPI, in selected images). Scale bar: 20 μm. (C) The degrees of colocalization between MRCKa and pMLC in HeLa cells treated with DMSO, chelerythrine or Y-27632 were determined using Metamorph software. Means and standard errors were derived from measurements of 30–40 cells in each category. (D) HeLa cells treated similarly as (B) were stained for endogenous MRCKα, ppMLC and F-actin. (E) HeLa cells treated with either DMSO or chelerythrine (7.5 μM for 30 min) with or without wash-out for the indicated times were stained for endogenous MRCKα and F-actin. Scale bar: 20 μm.

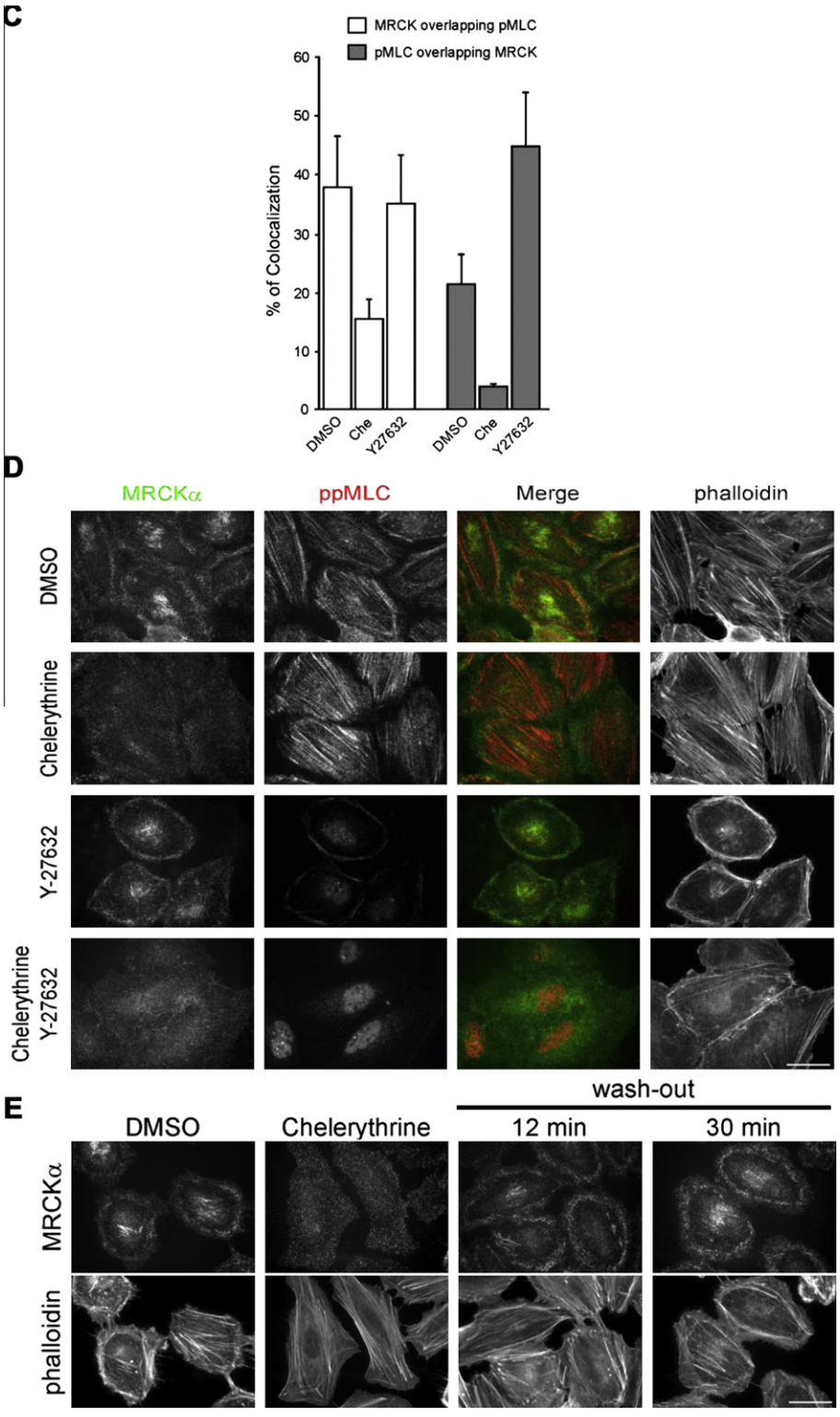

Fig. 3 (*continued*)

Each bacterially expressed recombinant protein was purified from 800 ml IPTG-induced culture. Briefly, bacterial cells were lysed in a buffer solution containing 25 mM Hepes (pH 7.3), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, the protease inhibitor PMSF and 0.25 mg/ml lysozyme, followed by sonication. The buffer for purification of His-tagged proteins contained 25 mM Imidazole. Post-centrifuged lysates were incubated with corresponding affinity beads (Ni-NTA agarose or Glutathione-coated sepharose beads) for 3 h at 4 °C. After an extensive wash with the same lysis buffer, proteins were eluted with the appropriate elution buffer containing 250 mM Imidazole for His-fusion proteins or 10 mM Glutathione for GST-fusion proteins. Each of the kinase domains cloned in the mammalian pXJ40 vector was expressed in 10 dishes (10 cm diameter) of COS7 cells for 24 h and purified essentially as the above-mentioned steps for GST-fusion proteins (in a 5 times smaller scale).

2.2. Chemicals, antibodies and immunoblot analysis

Chelerythrine chloride and Y-27632 were obtained from Calbiochem. Anti-MRCKα monoclonal antibody (clone 2A3) was obtained as described [16]. Both anti-pMLC (pSer19) and anti-ppMLC (ppThr18/Ser19) antibodies were obtained from Cell Signaling Technology. Anti-pMYPT and anti-GST antibodies were obtained from Santa Cruz Biotech. and Sigma-Aldrich, respectively.

For immunoblot analysis, 10-30 ng of phosphorylated protein samples were resolved by SDS–PAGE and transferred onto PVDF membrane for probing with specific antibodies as indicated.

2.3. Kinase activity measurements

Kinase assays were carried out at 30 °C using appropriate substrates in a buffer containing 10 μM ATP (unless otherwise indicated), 0.2 μl [γ-^{32}P]ATP (3000 Ci/mmol; Perkin Elmer), 25 mM HEPES (pH 7.3), 25 mM KCl, 5 mM β-glycerolphosphate, 2.5 mM NaF, 5 mM $MgCl_2$, and 0.025% Triton X-100. Radioactive ATP was excluded from reactions intended for immunoblot analysis. For the experiment in Fig. 2B, reaction time was 20 min for all kinases except for MRCKα-CAT at 10 min. MLC was used as substrate for MRCKα/β, ROKα, CRIK, MLCK and PAKα, myelin basic protein (MBP) was used as PKCα substrate, while GST-MYPT-PIM and Histone H1 were for DMPK and MINK1 respectively.

In the initial MRCKα inhibitor screening, a panel of 159 known chemical compounds (Sigma-Aldrich) at 10 μM each was screened using Kinase-Glo system (Promega). Reactions in duplicates were performed in a multi-well plate with 5 μM ATP, 120 ng His-tagged MRCKα-CAT and 3.5 μg GST-PIM as substrates in a final volume of 20 μl. Luminescent signals reflecting MRCKα-CAT kinase activity were measured using Tecan Safire2 microplate reader.

2.4. Cell culture, immunofluorescence and image processing

HeLa and COS7 cells were cultured as described [18]. U2OS cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS). For immunofluorescence studies, cells on cover slips were fixed in 4% paraformaldehyde for 20 min. For immunostaining of endogenous proteins, the incubation with primary antibody was carried out for 16 h at 4 °C. Alexa 488- and Alexa 546-conjugated secondary antibodies against mouse and

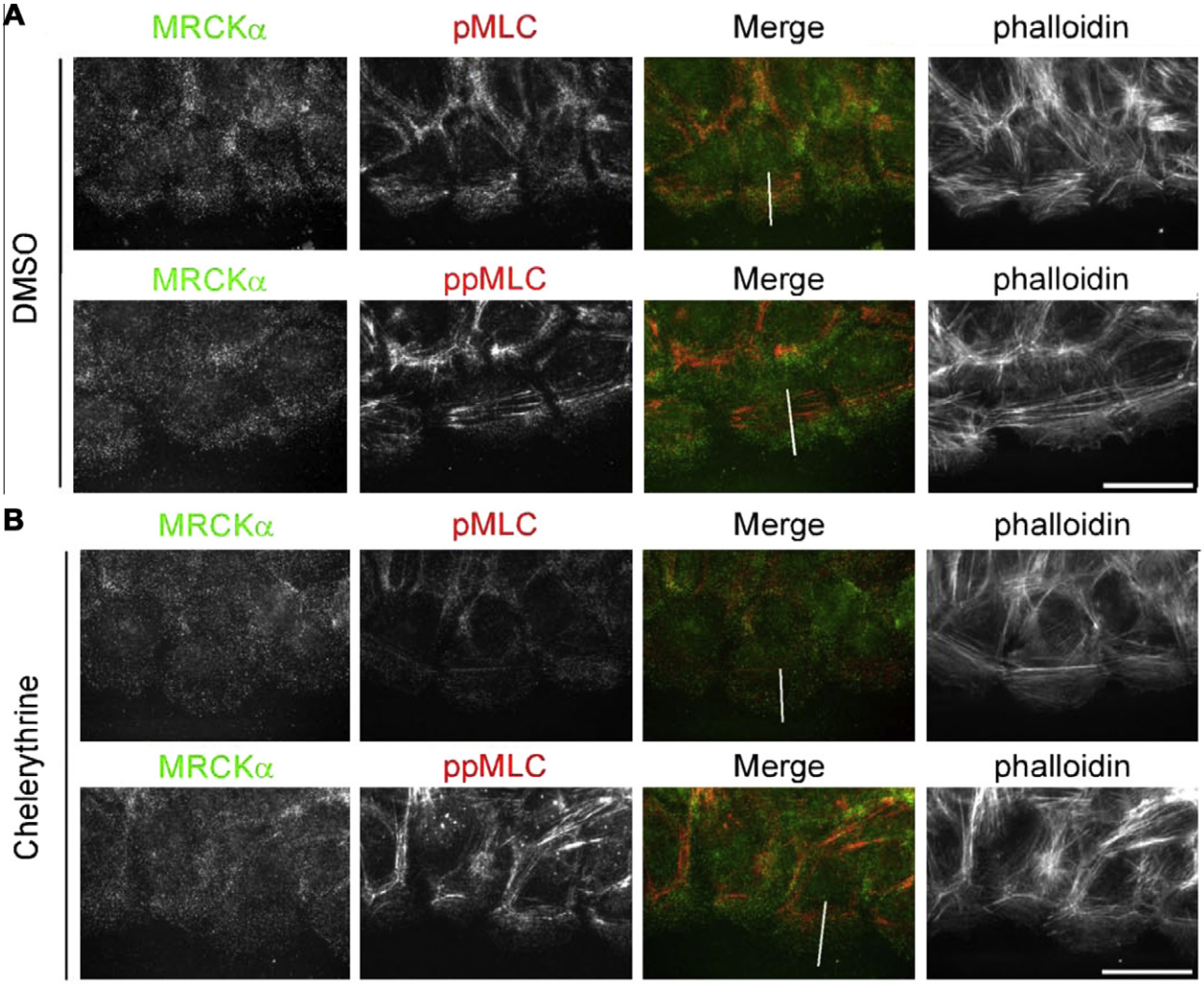

Fig. 4. Chelerythrine affects polarized distribution of MRCK, MLC phosphorylation and cell motility. (A) U2OS cells at wound edges treated with DMSO for 30 min were stained for F-actin, endogenous MRCKα, and pMLC (top) or ppMLC (bottom). White lines correspond to regions for line scan analysis in (C). Scale bar: 20 μm. (B) U2OS cells at wound edges treated with 6 μM chelerythrine for 30 min were stained similarly as (A). White lines correspond to regions for line scan analysis in (C). Scale bar: 20 μm. (C) Line scan profiles of co-stained MRCKα/βMLC or MRCKα/ppMLC derived from cells treated with DMSO or chelerythrine. (D) U2OS cells were pretreated with either DMSO or 6 μM of chelerythrine for 15 min, followed by wounding and subjected to live-cell imaging to follow their migration. Images taken from time 0 h and 10 h are shown. Scale bar: 50 μm. (E) Average velocity of migrating cells treated with either DMSO or chelerythrine. Values represent means and standard errors of data from three experiments. (F) U2OS cells were exposed to chelerythrine at various concentrations for 12 h. Cell lysates prepared were probed with anti-cleaved caspase-3 antibody.

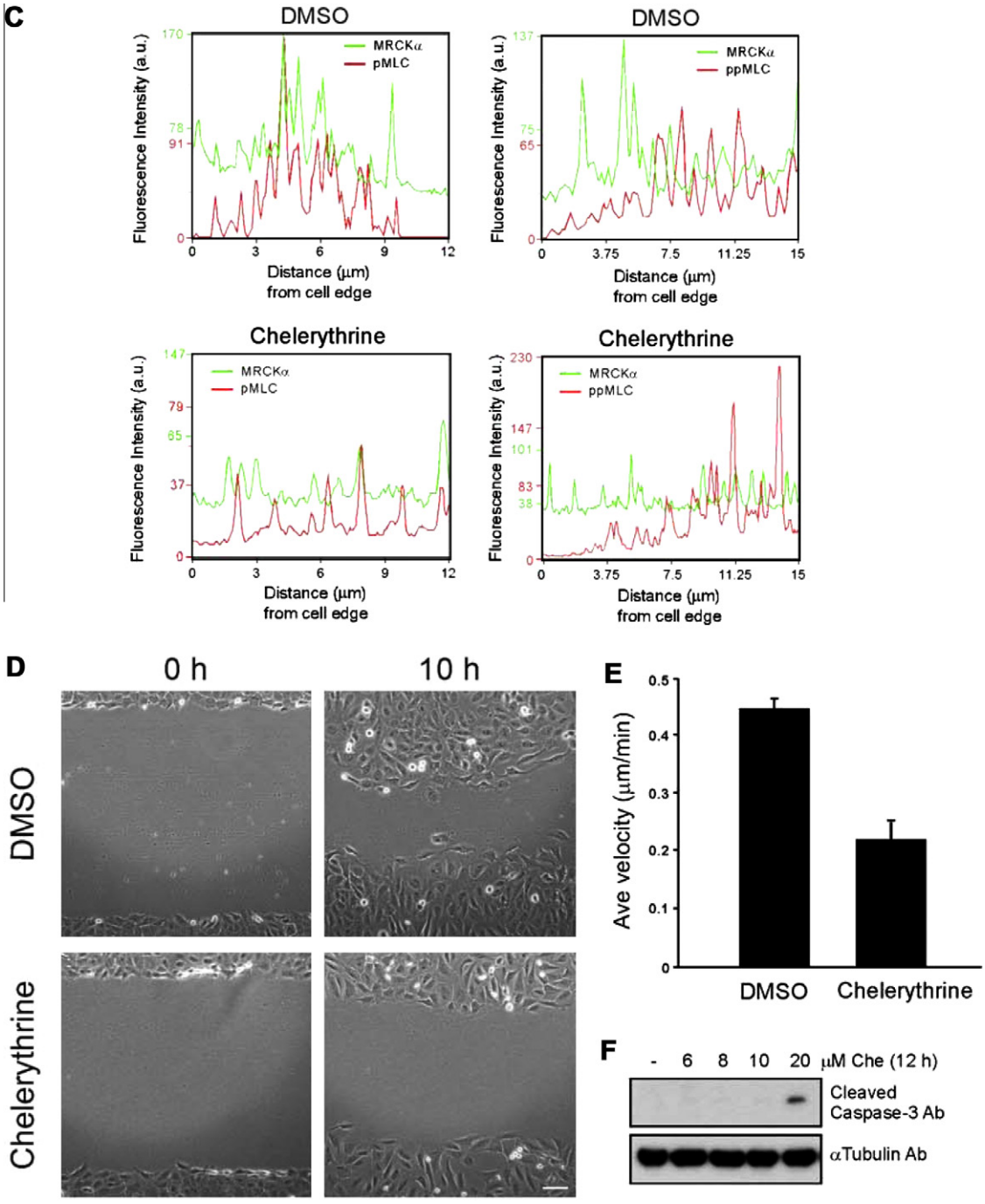

Fig. 4 (*continued*)

rabbit immunoglobulin were obtained from Molecular Probes. Cy3-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch Laboratory. Alexa 647-conjugated phalloidin were obtained from Molecular Probes. All images were captured using a Coolsnap HQ (Roper Scientific) adapted to a Zeiss Axioplan wide-field fluorescent microscope. Image processing, line scan and colocalization analyses were carried out using Metamorph software.

2.5. Wound healing assay

U2OS cells at about 90% confluence were starved overnight in Dulbecco's modified Eagle's medium containing 1% FBS. The resulting confluent cells were scratched with a pipette tip and replaced in 10% FBS-containing medium to induce cell migration in the presence of either DMSO or 6 μM Chelerythrine chloride. The migration events were followed for 10 to12 h by live recording. The average migration speed was derived from measurements of distance traveled using ImagePro software.

3. Results and discussion

3.1. Identification of chelerythrine as a potential MRCK inhibitor

In a search for chemical compounds that inhibit MRCK catalytic activity, chelerythrine chloride was identified from an *in vitro*

screen involving 159 compounds, of which most are known kinase inhibitors. As shown in Fig. 1, chelerythrine effectively inhibited the catalytic domain of MRCKα from phosphorylating MLC on Ser19 in a concentration-dependent manner (IC_{50} 1.77 μM). A similar inhibitory profile was also observed for a closely related compound sanguinarine (IC_{50} 1.25 μM), but no obvious inhibition was detectable with other related compounds that have fused ABC ring structures [24] (data not shown). The inhibition was specific as no significant inhibition was observed for the closely related ROKα kinase (80% homology) under similar conditions (Fig. 1B, C and D). Unlike ROK inhibition by its inhibitor Y-27632 which became ineffective in the presence of high ATP concentration, chelerythrine inhibition on MRCK was not affected by higher ATP concentration (Fig. 1E), indicating that it does not act through ATP competition.

3.2. Chelerythrine chloride selectively inhibits MRCK

We next examined the specificity of chelerythrine more vigorously against a panel of kinases, some of which have been shown to regulate NM II activity besides ROKα. They include kinases that share significant sequence homology to MRCK such as CRIK and DMPK, as well as those from different kinase subfamilies like MLCK, protein kinase C alpha (PKCα), p21-activated kinase alpha (PAKα) and its related MINK. We first determined the substrate specificity of each of these kinases in phosphorylating MLC and the MYPT-PIM using specific phospho-antibodies (Fig. 2A). We detected Ser19 of MLC as the common phosphorylation site for the catalytic domains of MRCKα/β, ROKα, MLCK and PAKα, but only ROKα and CRIK are able to phosphorylate both Thr18 and Ser19 residues causing diphosphorylation. These *in vitro* data correlate well with the cellular functions of ROKα and CRIK as the main regulators of highly contractile processes, such as the actomyosin contraction required for cell rear detachment during migration [25] and the contractile ring formation in cytokinesis crucial for daughter cells separation [8], as diphosphorylation of MLC has been shown to further increase myosin's actin-activated ATPase activity leading to a higher contractility and filament assembly [19,26,27]. In contrast, the monophosphorylation of MLC by MLCK, PAK and MRCK supports their roles in cell edge advancement and lamellar protrusion [10,14,18]. Likewise, the PIM of MYPT was effectively phosphorylated by MRCKα/β, ROKα and DMPK (Fig. 2A) [6,12,13]. These findings suggest that these kinases have the potential to cooperatively regulate myosin phosphatase activity and to maintain the MLC phosphorylation state. The inability of DMPK to phosphorylate MLC further suggests that its regulation on myosin activity may be solely dependent on the inactivation of myosin phosphatase. Both MINK and PKCα were found incapable of phosphorylating any of the three sites on MLC and MYPT probed.

As shown in Fig. 2B, the kinase inhibition profile reveals that besides CRIK and MINK that were partially inhibited by 10 μM chelerythrine (~50% and ~40% inhibition respectively), no significant inhibition was detected for all other kinases examined. In line with previous reports, the lack of inhibition on PKCα observed confirms that chelerythrine does not effectively inhibit PKCα activity [28,29]. Together with a separate profiling study (involving 102 individual protein kinases) on chelerythrine specificity that also showed no significant inhibition of kinases known to directly phosphorylate cytoskeletal components (P. Cohen, personal communication), our findings indicate that chelerythrine has a high selectivity towards MRCK.

3.3. Chelerythrine treatment perturbs endogenous MRCK localization in HeLa cells

We previously showed that MRCK interacts with LRAP35a and MYO18A, forming a functional complex that colocalizes with and regulates specific actomyosin networks in the lamella and cell centre area beneath the nucleus [16]. Therefore, we tested if its localization and the cytoskeletal networks it regulates are sensitive to chelerythrine treatment. As shown in Fig. 3A, HeLa cells treated with 7.5 μM of chelerythrine for 45 min showed significantly perturbed MRCK and MYO18A localizations. Specific losses of actomyosin networks were also observed in the lamella and subnuclear region, as revealed by F-actin and monophosphorylated MLC staining (Fig. 3B and C). Importantly, RhoA/ROK-regulated stress fibers that contain diphosphorylated MLC were largely unaffected (Fig. 3D). By contrast, treatment of ROK inhibitor Y-27632 mainly resulted in the losses of stress fibers and diphosphorylated MLC, with little effect on MRCK-regulated actomyosin network (Fig. 3B and D). These observations correlate well with the *in vitro* findings on the mono- and diphosphorylaton of MLC by MRCK and ROK, as well as the specificity of chelerythrine and Y-27632 (Fig. 2A and B). Expectedly, co-treatment of chelerythrine and Y-27632 led to marked losses of both populations of phosphorylated MLC, resulting in an almost complete abolishment of the intracellular actomyosin filaments (Fig. 3B and D). These results strongly suggest that ROK and MRCK are the two major regulators of cellular actomyosin structures. The inhibitory effects of chelerythrine therefore mirror the reported effects of MRCK siRNAs [18], providing further support that it can inhibit MRCK function *in vivo* with high specificity. Furthermore, chelerythrine inhibition of MRCK is reversible as shown by the rapid reappearance of specific MRCK localization after its removal (Fig. 3E).

3.4. Chelerythrine treatment affects migration of U2OS cells

To further demonstrate the specificity of MRCK inhibition by chelerythrine in cell migration, a wound-healing assay on U2OS cells was performed. Endogenous MRCK in the lamella of migrating U2OS cells colocalizes significantly with monophosphorylated MLC (Fig. 4A and C) [18]. Together with the underlying F-actin, they form a specific network arranged perpendicular to the direction of wound edge advancement. This is slightly different from its non-polarized localization in HeLa cells that exhibit multiple lamellae around most of their periphery [18]. In contrast, the diphosphorylated MLC filaments showed little overlap with MRCK, they are more enriched in an inner region immediately behind the lamella (Fig. 4A and C). Remarkably, chelerythrine treatment almost completely disrupted the accumulation of MRCK in the lamella, with a marked drop in the level of monophosphorylated MLC in the same area (Fig. 4B and C). The level of diphosphorylated MLC was either not affected or increased moderately upon chelerythrine treatment (Fig. 4B and C). Furthermore, wound edge cells treated with 6 μM chelerythrine migrated significantly slower, with an average speed of about half of the control cells (Fig. 4D and E). The lack of detectable cleaved caspase-3 in cells exposed to chelerythrine at up to 10 μM rules out the possible influence of cell death on the migration defect (Fig. 4F). Taken together, our results show that chelerythrine treatment specifically affected MRCK-mediated MLC monophosphorylation in the lamella, leading to defective cell motility. This lends further support to the importance of MRCK in lamellar actomyosin function.

Here we have found chelerythrine to specifically perturb lamellar and subnuclear actomyosin filaments by selectively inhibiting MRCK. This is different from ROK inhibition by Y-27632 which mainly affects stress fibers. Although chelerythrine was initially reported as a selective PKC inhibitor [30], two later studies showed that this is not the case [28,29]. Our work further demonstrates that it selectively inhibits MRCK over other related kinases. While chelerythrine has also been shown to be an apoptosis-inducing agent through its ability to block BclXL-Bak interaction [31], this effect is only obvious upon a prolonged exposure of more than

16 h, or to high concentrations. The application of chelerythrine under our experimental conditions was deemed unlikely to induce significant cell death (Fig. 4). Our initial work on chelerythrine analogs shows that structurally related compounds with fused ABC rings of chelerythrine are ineffective in inhibiting MRCK while retaining the ability to induce apoptosis (data not shown), suggesting that the active structural component that directly inhibits MRCK may lie in the BCD rings portion. Thus, it should be possible to generate specific MRCK inhibitors with eliminated cellular toxicity. With the recent recognition of MRCK as the important counterpart of ROK in actomyosin regulation [18,22], and the involvement of these kinases in tumor metastasis [22,23], the identification of chelerythrine as a chemical inhibitor of MRCK should complement the use of ROK inhibitor Y-27632 in understanding their distinct but coordinated roles in cellular functions.

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