Damnacanthal, an effective inhibitor of LIM-kinase, inhibits cell migration and invasion

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ABSTRACT **LIM-kinases (LIMKs) play crucial roles in various cell activities, including migration, division, and morphogenesis, by phosphorylating and inactivating cofilin. Using a bimolecular fluorescence complementation assay to detect the actin–cofilin interaction, we screened LIMK1 inhibitors and identified two effective inhibitors, damnacanthal (Dam) and MO-26 (a pyrazolopyrimidine derivative). These compounds have already been shown to inhibit Lck, a Src family tyrosine kinase. However, in vitro kinase assays revealed that Dam inhibited LIMK1 more effectively than Lck. Dam suppressed LIMK1-induced cofilin phosphorylation and deceleration of actin retrograde flow in lamellipodia in N1E-115 cells. Dam impaired CXCL12-induced chemotactic migration of Jurkat T lymphocytes and Jurkat-derived, Lck-deficient JCaM1.6 cells and also inhibited serum-induced migration and invasion of MDA-MB-231 breast carcinoma cells. These results suggest that Dam has the potential to suppress cell migration and invasion primarily through the inhibition of LIMK kinase activity. Topical application of Dam also suppressed hapten-induced migration of epidermal Langerhans cells in mouse ears. Dam provides a useful tool for investigating cellular and physiological functions of LIMKs and holds promise for the development of agents against LIMK-related diseases. The bimolecular fluorescence complementation assay system used in this study will provide a useful method to screen for inhibitors of various protein kinases.**

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INTRODUCTION

Actin cytoskeletal dynamics and remodeling are central to a variety of cell activities, including cell migration, division, morphogenesis, and gene expression. Among numerous actin-regulatory proteins, the actin-depolymerizing factor (ADF)/cofilin family proteins bind to G- and F-actin and play an essential role in regulating actin cytoskeletal dynamics and reorganization by severing and disassembling actin filaments (Bamburg and Wiggan, 2002; Pollard and Borisy, 2003; Ono, 2007). The actin-binding, -severing, and -disassembling activities of ADF/cofilin are inhibited by the phosphorylation of its serine residue at position 3 (Ser-3) near the N-terminus. In most cells, the level or turnover rate of Ser-3 phosphorylation of ADF/cofilin dramatically changes in response to extracellular and intracellular stimuli, crucially affecting actin dynamics and cell activities; hence, the protein kinases and phosphatases responsible for ADF/cofilin phosphorylation and dephosphorylation play essential roles in regulating actin cytoskeletal dynamics and actin-related cell activities (Meberg *et al.*, 1998; Bravo-Cordero *et al.*, 2013; Mizuno, 2013).

LIM-kinases (LIMKs), consisting of LIMK1 and LIMK2 in mammals, specifically phosphorylate ADF/cofilin at Ser-3, thereby inhibiting the actin-binding, -severing, and -disassembling activities (Arber *et al.*, 1998; Yang *et al.*, 1998). LIMKs have characteristic

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Abbreviations used: ADF, actin-depolymerizing factor; BiFC, bimolecular fluorescence complementation; CaMK, Ca^{2+}/calmodulin-dependent kinase; CFP, cyan fluorescent protein; Dam, damnacanthal; FCS, fetal calf serum; FRAP, fluorescence recovery after photobleaching; LIMK, LIM-kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PAK, p21-activated kinase; P-cofilin, Ser-3–phosphorylated cofilin; PKC, protein kinase C; ROCK, Rho-associated kinase; TNCB, 2,4,6-trinitrochlorobenzene; VC210, Venus C-terminal fragment (210–238); VN210, Venus N-terminal fragment (1–210); WT, wild type; YFP, yellow fluorescent protein.

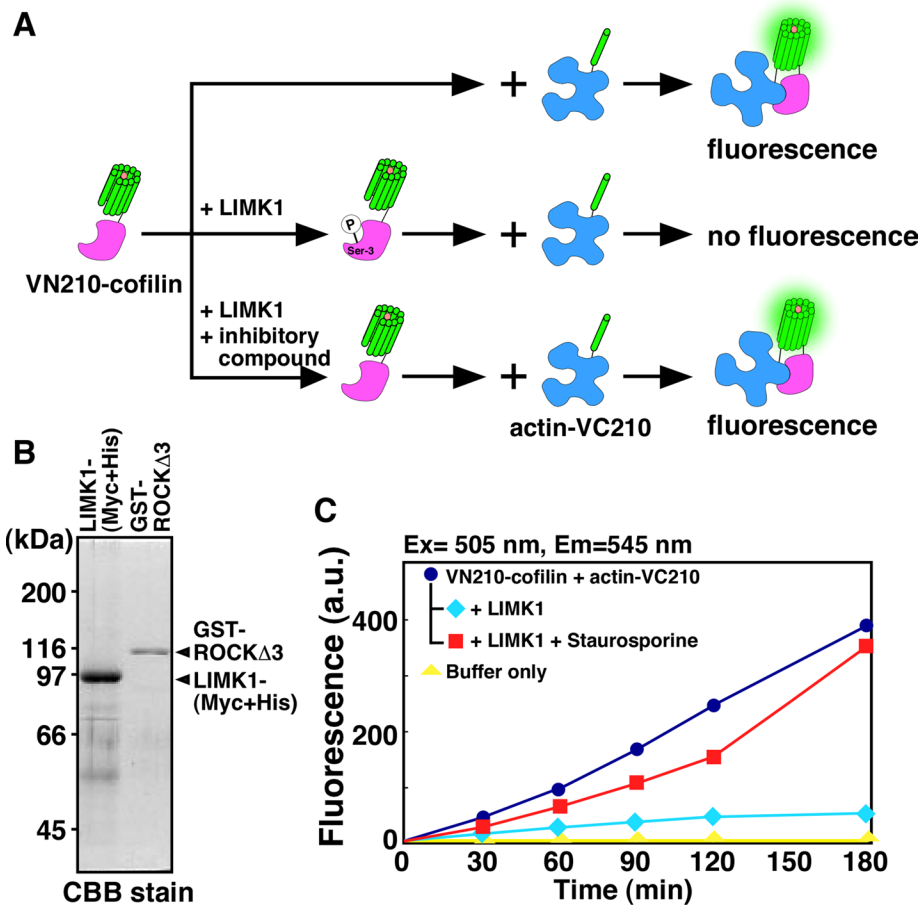

FIGURE 1: BiFC-based screening of LIMK1 inhibitors. (A) Schematic diagram of the BiFC assay used to screen for LIMK1 inhibitors. (B) Purification of LIMK1-(Myc+His) and GST-ROCKΔ3. Proteins expressed in Sf21 cells were purified, separated on SDS–PAGE, and analyzed by Coomassie brilliant blue (CBB) staining. (C) In vitro BiFC assays to detect the LIMK1-inhibiting activity of staurosporine. VN210-cofilin was preincubated with or without LIMK1, or with LIMK1 and staurosporine, and then mixed with actin-VC210. The time-dependent recovery in fluorescence of the BiFC probes is shown. a.u., arbitrary unit.

structural features, consisting of two N-terminal LIM domains, an internal PDZ-like domain, and a C-terminal protein kinase domain (Mizuno *et al.*, 1994; Okano *et al.*, 1995). The kinase domains of LIMKs contain the protein kinase consensus sequence but are unique in that they have an unusual motif (DLNSHN) in the kinase catalytic loop in subdomain VIB. Phylogenetic analysis of protein kinases places LIMKs within a tyrosine kinase-like family (Manning *et al.*, 2002). LIMKs are activated via a number of routes: phosphorylation of the conserved threonine residue in the kinase catalytic domain (Edwards *et al.*, 1999; Ohashi *et al.*, 2000; Amano *et al.*, 2001) by Rho-associated kinase (ROCK), p21-activated protein kinase (PAK), myotonic dystrophy kinase–related Cdc42-binding kinase-α (MRCKα), and Ca^{2+}/calmodulin-dependent kinase (CaMK)-II and -IV (Scott and Olson, 2007; Mizuno, 2013); phosphorylation at Ser-323 by mitogen-activated protein kinase (MAPK)–activated protein kinase-2 (MAPKAPK2); limited proteolysis at Asp-240 by caspase-3; and binding of bone morphogenic protein receptor II to the LIM domain of LIMK1 (Scott and Olson, 2007; Mizuno, 2013).

In agreement with the central role of ADF/cofilin in actin filament remodeling, previous studies demonstrated that LIMK1 plays a crucial role in diverse cell activities, including cell shape change, migration, division, and gene expression, and in pathophysiological processes, including organogenesis, cancer invasion and metastasis, angiogenesis, axon guidance, and immune and inflammatory responses (Scott and Olson, 2007; Manetti, 2012a; Mizuno, 2013). In fact, LIMK1 is activated after cell stimulation with growth factors and chemokines, and the depletion of LIMK1 suppresses cell migration and tumor cell invasion and metastasis (Nishita *et al.*, 2002, 2005; Davila *et al.*, 2003; Yoshioka *et al.*, 2003; Wang *et al.*, 2007; Horita *et al.*, 2008; Mishima *et al.*, 2010; Scott *et al.*, 2010). To explore the functional roles of LIMKs in cellular and pathophysiological processes, it is useful to identify the chemical compounds that specifically inhibit the kinase activity of LIMKs. Several inhibitors of LIMKs have been reported (Ross-Macdonald *et al.*, 2008; Harrison *et al.*, 2009; Sleebs *et al.*, 2011; Prudent *et al.*, 2012), but more potent and selective LIMK inhibitors are needed for the development of effective therapeutic agents against LIMK-related diseases such as cancer metastasis, glaucoma, and neurological and cardiovascular disorders (Manetti, 2012a,b).

We recently developed a new bimolecular fluorescence complementation (BiFC) assay system to detect the interaction between G-actin and cofilin (Ohashi *et al.*, 2012). This assay is based on the reassembly of fluorescent Venus, a variant of yellow fluorescent protein (YFP; Nagai *et al.*, 2002), from its nonfluorescent fragments. The reassembly occurs in a manner dependent on the interaction between two proteins of interest fused to each fragment (Kerppola, 2009). In the cofilin–actin interaction BiFC assay, incubation of the actin probe with the wild-type (WT) cofilin probe resulted in fluorescence; however, incubation with the phosphomimic S3E-cofilin mutant probe (with a replacement of Ser-3 by Glu) did not (Ohashi *et al.*, 2012), indicating that the recovery of Venus fluorescence reflects the nonphosphorylated state of cofilin at Ser-3. Because LIMK-mediated cofilin phosphorylation blocks the cofilin–actin interaction, the BiFC assay is an ideal tool for screening LIMK inhibitors.

In this study, LIMK1 inhibitors were screened using the in vitro BiFC assay. One compound, damnacanthal (3-hydroxy-1-methoxy-9,10-dioxoanthraquinone-2-carbaldehyde [Dam]), was identified as an effective inhibitor of LIMK1. Using Dam, we explored the role of LIMK in cell migration and invasion, confirming its use as a powerful tool for analyzing cellular and physiological functions of LIMK.

RESULTS

A BiFC-based screen of LIMK1 inhibitors

The in vitro BiFC assay system was previously developed to detect the interaction between actin and cofilin. The assay uses a pair of probes composed of the C-terminal fragment (amino acids 210–238) of Venus fused to the C-terminus of actin (actin-VC210) and the N-terminal fragment (amino acids 1–210) of Venus fused to the N-terminus of cofilin (VN210-cofilin; Figure 1A; Ohashi *et al.*, 2012).

The assay is based on the complementary reassembly of the fluorescent protein Venus from its nonfluorescent fragments (VC210 and VN210), whose association depends on the interaction between actin and cofilin (Ohashi *et al.*, 2012). Incubation of actin-VC210 with VN210-cofilin(WT) results in Venus fluorescence; however, incubation of actin-VC210 with phospho-mimic VN210-cofilin(S3E) does not recover fluorescence, indicating that phosphorylation of cofilin at Ser-3 prevents fluorescence recovery of the BiFC probes (Ohashi *et al.*, 2012). LIMK1 phosphorylates cofilin at Ser-3 and inhibits the actin–cofilin interaction; hence, incubation of VN210-cofilin with LIMK1 inhibits recovery of BiFC probe fluorescence, whereas coincubation with LIMK1 and LIMK-inhibitory compound(s) recovers fluorescence by abolishing LIMK1-mediated cofilin phosphorylation (Figure 1A). To perform the BiFC assay, we individually expressed hexahistidine (His_6)-tagged actin-VC210 and VN210-cofilin in Sf9 cells and purified them as previously reported (Ohashi *et al.*, 2012). Similarly, we individually expressed (Myc+His)-tagged LIMK1 and glutathione *S*-transferase (GST)–tagged active ROCK (ROCKΔ3) in Sf21 cells and purified them (Figure 1B). LIMK1 was preincubated with GST-ROCKΔ3 to fully activate LIMK1 (Ohashi *et al.*, 2000). The in vitro BiFC assay was performed as follows: LIMK1 was first incubated with ROCKΔ3 and ATP for 10 min, with chemical compounds for 5 min, and then with VN210-cofilin for 60 min at 30°C. The actin-VC210 probe was then added and incubated for another 30–180 min at 30°C. As previously reported (Ohashi *et al.*, 2012), when VN210-cofilin was incubated with actin-VC210 without LIMK1 preincubation, fluorescence intensity gradually increased (Figure 1C). By contrast, when VN210-cofilin was preincubated with LIMK1 and then incubated with actin-VC210, fluorescence recovery was markedly suppressed (Figure 1C). The addition of the general protein kinase inhibitor staurosporine to the preincubation mixture blocked LIMK1-mediated inhibition of fluorescence recovery and resulted in increase in fluorescence intensity (Figure 1C), demonstrating that the in vitro BiFC assay can be used to screen for LIMK1 inhibitors.

Identification of Dam and MO-26 as LIMK1 inhibitors

We used a library of 958 small–molecular weight compounds that consists of 209 known inhibitors (including anticancer drugs), three natural compounds, and 746 chemically synthesized compounds. The chemically synthesized compounds were designed to consist of unbiased compounds (Kusumoto and Oikawa, 2001; Oikawa *et al.*, 1995, 2005; Oikawa, 2010). The library was screened for LIMK1 inhibition using the in vitro BiFC assay. Four compounds (daunorubicin, doxorubicin, MO-204, and MO-273) were excluded from the screen because of their high intrinsic fluorescence. Three compounds (staurosporine, Dam, and MO-26) clearly exhibited recovery of BiFC probe fluorescence in the presence of LIMK1 (Figure 2A). Dam is a natural product purified from the roots of a tropical plant native to Thailand, *Morinda citrifolia* (*Noni* or *Yor* in Thai; Figure 2B; Faltynek *et al.*, 1995). MO-26 is a chemically synthesized pyrazolo-[3,4-*d*]pyrimidine derivative, and its structure (Figure 2B) is closely related to that of a Lck inhibitor, A-420983 (Borhani *et al.*, 2004). Dam and MO-26 were reported to inhibit Lck, a member of the Src family tyrosine kinases (Faltynek *et al.*, 1995; Burchat *et al.*, 2002). Detailed in vitro BiFC assays were conducted (Figure 2B), which demonstrated that these compounds inhibited the kinase activity of LIMK1 in the micromolar (5–10 μM) range.

Specificity of Dam and MO-26 inhibition of LIMK1 kinase activity

To determine the specificity of Dam and MO-26, we tested the compounds against the activity of various protein kinases, including LIMK1, LIMK2, Lck, and Src, using in vitro kinase assays. Protein kinases were expressed in COS-7 cells, purified by immunoprecipitation, and then subjected to in vitro kinase assays in the presence of [γ-^{32}P]ATP, substrate proteins (cofilin for LIMK1 and LIMK2; myelin basic protein [MBP] for other kinases), and various concentrations of inhibitors. Dam exhibited effective inhibition toward LIMK1, LIMK2, and Lck kinase activities, weak inhibition toward Src kinase activity (Figure 3A), and no inhibition of ROCK, PAK3, protein kinase C (PKC)-α, and CaMKIα kinase activities (Supplemental Figure S1A). The half-maximal inhibitory concentrations (IC_{50}) of Dam for LIMK1 (0.80 μM), LIMK2 (1.53 μM), and Lck (1.62 μM) were lower than those for Src, ROCK, PAK3, PKCα, and CaMKIα (>20 μM; Figure 3B and Table 1), indicating that Dam is an

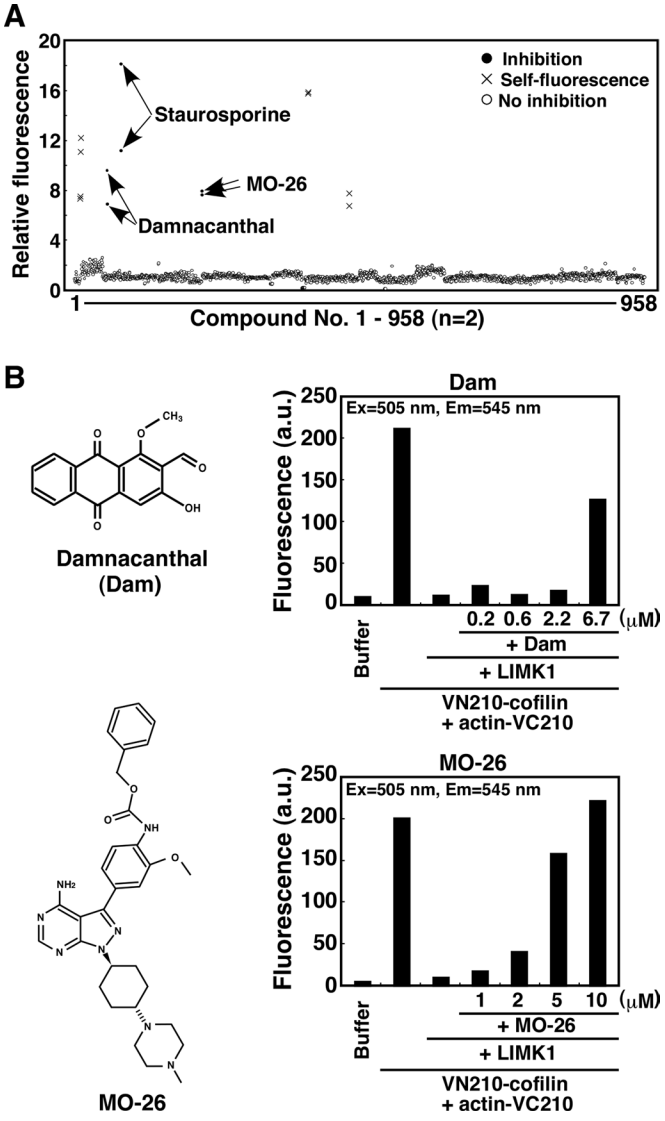

FIGURE 2: Identification of Dam and MO-26 as LIMK1 inhibitors. (A) Screening of LIMK1 inhibitors. The in vitro BiFC assay was carried out in duplicate for each chemical compound as described in *Materials and Methods*. Recovery of fluorescence intensity was calculated relative to the control. Four compounds (indicated as x) had intrinsic fluorescence. (B) LIMK1-inhibiting activity of Dam and MO-26. In vitro BiFC assays were conducted with a range of inhibitor concentrations as described in *Materials and Methods*. The chemical structures of Dam and MO-26 are shown on the left.

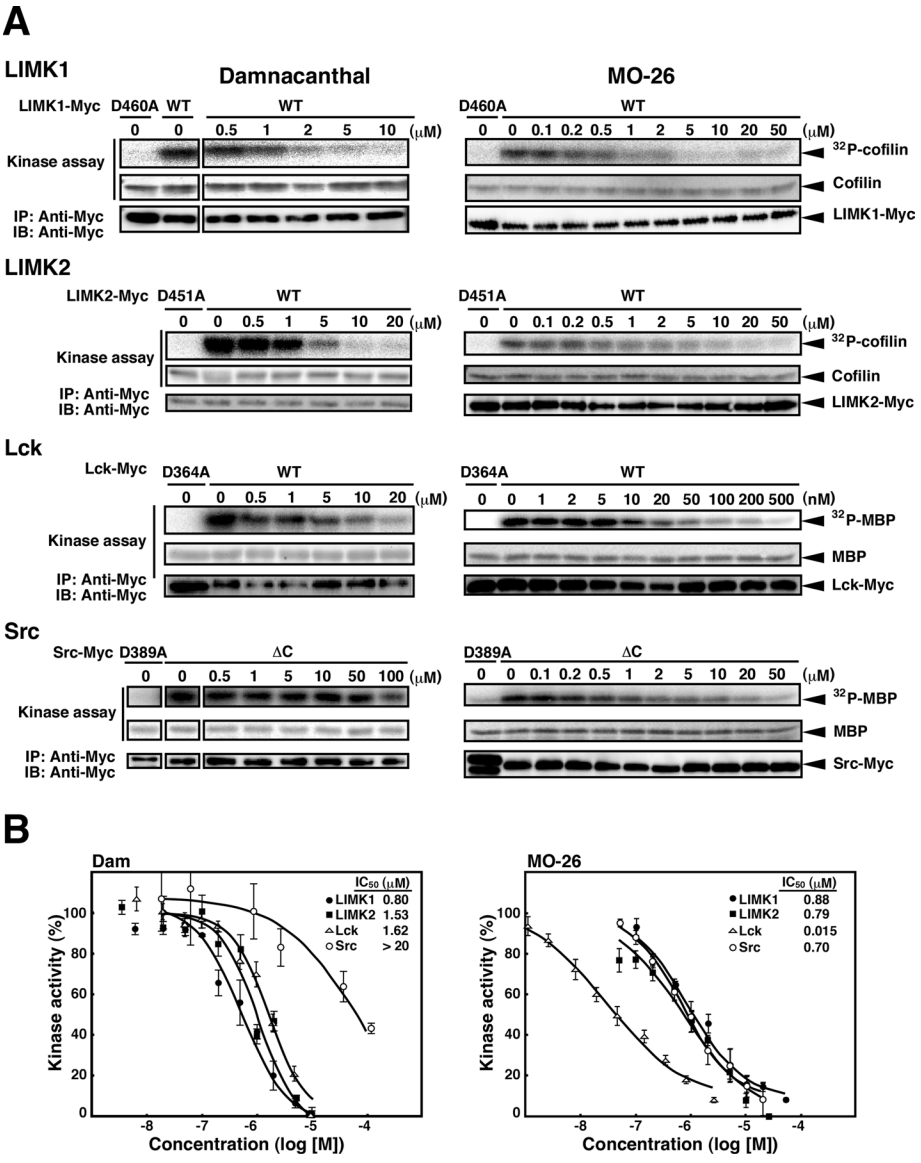

FIGURE 3: The effect of Dam and MO-26 on the kinase activity of LIMK1, LIMK2, Lck, and Src. (A) Dose-dependent effect of Dam and MO-26 on the kinase activity of LIMK1, LIMK2, Lck, and active Src (Src(ΔC)). Myc-tagged protein kinases were expressed, purified, and subjected to in vitro kinase assays, using cofilin or MBP as a substrate, in the presence of Dam or MO-26 as described in *Materials and Methods*. Kinase-inactive mutants, LIMK1(D460A), LIMK2(D451A), Lck(D364A), and Src(D389A), were used as controls. (B) Dose-dependent curves of the inhibition of kinase activity by Dam and MO-26. Data are mean values ± SD of three independent experiments.

effective inhibitor of LIMK1/2 and Lck and inhibits LIMK1 more effectively than its previously reported target, Lck. MO-26 inhibited LIMK1 and LIMK2 with an efficacy similar to that of Dam; however, it was more effective against Lck (Figure 3, A and B), with an IC_{50} value of 0.015 μM that was ~50 times lower than that for LIMK1 (0.88 μM), LIMK2 (0.79 μM), or Src (0.70 μM; Figure 3B and Table 1). MO-26 showed no inhibition of ROCK, PAK3, PKCα, and CaMKIα (IC_{50} > 20 μM; Table 1 and Supplemental Figure S1B), demonstrating that MO-26 has the potential to inhibit LIMK1, LIMK2, Src, and Lck, with particular efficacy toward Lck. Because Dam inhibited LIMK1 more effectively than Lck, the following experiments focused on the effects of Dam on the cellular functions of LIMK1.

To further examine the specificity of Dam, we analyzed the effect of Dam on the profile of phosphorylated proteins in cultured cells. We analyzed the changes in the profile of ^{32}P-labeled proteins between cell extracts from untreated and Dam-treated cells by two-dimensional (2D) gel. Autoradiography and immunoblot analysis of 2D gels showed that the amount of Ser-3–phosphorylated cofilin (P-cofilin, indicated by arrows) decreased in Dam-treated cells compared with that in untreated cells, whereas no appreciable difference in the profile of ^{32}P-labeled proteins other than P-cofilin was observed, as a whole (Supplemental Figure S2). This suggests that the pathway of cofilin phosphorylation is at least one of the major targets of Dam.

Structural requirements for the LIMK1-inhibiting activity of Dam

Dam is an anthraquinone derivative with a structure related to that of other anthraquinone derivatives, emodin and mitoxantrone (Supplemental Figure S3, A and B). Emodin inhibits Lck with an IC_{50} of 18.5 μM (Jayasuriya *et al.*, 1992), and mitoxantrone is used as an anticancer agent (Parker *et al.*, 2010); hence these compounds were examined for efficacy against LIMK1 kinase activity. However, in vitro kinase assays demonstrated that at concentrations of 1–30 μM, emodin and mitoxantrone had no appreciable effect on the kinase activity of LIMK1 (Supplemental Figure S3, A and B), indicating that the side-chain structure of Dam plays an important role in its inhibition of LIMK1.

Dam has an aldehyde group on an anthraquinone ring. To examine the function of this group, we analyzed the effect of a nucleophilic agent, hydroxylamine, on the LIMK1-inhibiting activity of Dam. Preincubation of Dam with hydroxylamine almost completely blocked the inhibitory activity of Dam (Supplemental Figure S4A). Hydroxylamine alone had no effect on LIMK1 activity. These results suggest that the aldehyde group of Dam is involved in the LIMK1-inhibiting activity of Dam.

To examine whether Dam reversibly or irreversibly inhibits LIMK1, we analyzed the effect of repeated washout on the inhibitory activity of Dam. Cyan fluorescent protein (CFP)–tagged LIMK1 was immunoprecipitated and treated with Dam or untreated for 10 min and then the precipitates were washed three times with the kinase reaction buffer or left unwashed. The LIMK1-inhibiting activity of Dam was not affected by repeated washing (Supplemental Figure S4B). Thus it is presumed that Dam may irreversibly inhibit LIMK1 by forming a stable adduct with LIMK1.

Kinase	Dam (μM)	MO-26 (μM)
LIMK1	0.80	0.88
LIMK2	1.53	0.79
Lck	1.62	0.015
Src	>20	0.70
ROCK	>20	>20
PAK3	>20	>20
PKCα	>20	>20
CaMKIα	>20	>20

IC_{50} values were determined by in vitro kinase assays using cofilin (for LIMK1 and LIMK2) and MBP (for other kinases) as substrates. See Figure 3 and Supplemental Figure S1.

TABLE 1: IC_{50} values of Dam and MO-26 toward the kinase activities of LIMKs and other protein kinases.

Dam inhibits LIMK1-mediated cofilin phosphorylation and deceleration of actin retrograde flow in cultured N1E-115 cells

Previous studies showed that overexpression of LIMK1 slows the rate of actin retrograde flow in the lamellipodia by phosphorylating and inactivating cofilin (Ohashi *et al.*, 2011). To examine whether Dam can inhibit LIMK1 activity within living cells, we analyzed the effect of Dam on LIMK1 overexpression–induced deceleration of actin retrograde flow in the lamellipodia of active RacV12-expressing N1E-115 neuroblastoma cells by fluorescence recovery after photobleaching (FRAP) time-lapse analysis of YFP-actin. After cotransfection of the cells with YFP-actin and RacV12, YFP-actin fluorescence in a rectangular region of lamellipodium was photobleached, and signal recovery was monitored every second for 40 s (Figure 4A). Kymograph analysis showed that the tip of the lamellipodium did not move substantially during time-lapse observations and that YFP-actin fluorescence was recovered gradually from the tip of the lamellipodium and moved inward at a near-constant rate via actin retrograde flow (Figure 4A, control). The rate of actin retrograde flow was measured as the rate at which the boundary between the bright and dark areas of the YFP-actin signal migrated inward from the initial cell margin. In lamellipodia of control RacV12-expressing cells, the average rate of actin retrograde flow was 5.7 μm/min (Figure 4B, control). As reported (Ohashi *et al.*, 2011), the rate of actin retrograde flow was markedly decreased in LIMK1-CFP–overexpressing cells (2.0 μm/min) compared with the rate in control cells (Figure 4, A and B). Immunoblot analysis of cell lysates showed that the amount of LIMK1-CFP was ~10 times higher than that of endogenous LIMK1 (Figure 4C). Considering the transfection efficiency of LIMK1-CFP into N1E-115 cells (~40%), the amount of LIMK1-CFP was estimated to be 25 times higher than that of endogenous LIMK1 in LIMK1-CFP–overexpressing cells. When LIMK1-overexpressing cells were pretreated with Dam at concentrations of 0.3–10 μM for 30 min and then subjected to FRAP time-lapse analysis, the decelerating effect of LIMK1 overexpression on the actin retrograde flow was significantly inhibited by Dam in the 3–10 μM range (Figure 4, A and B). To examine whether the effect of Dam on the actin retrograde flow is the result of LIMK1 inhibition, we analyzed the effect of Dam on the level of cofilin phosphorylation. The level of P-cofilin was drastically increased in LIMK1-CFP–overexpressing cells but significantly decreased in the cells after exposure to 5–10 μM Dam (Figure 4D). No apparent change in the level of total cofilin was observed. These results suggest that Dam is cell permeable and has the potential to inhibit LIMK1 kinase activity and cofilin phosphorylation in N1E-115 cells.

Dam inhibits chemotactic migration of Jurkat cells and Lck-deficient JCaM1.6 cells

It was previously reported that Dam inhibits CXCL12 (SDF-1)-induced chemotactic migration of Jurkat T-cells by inhibiting the kinase activity of Lck (Inngjerdingen *et al.*, 2002); however, LIMK1 is also required for CXCL12-induced chemotaxis in Jurkat cells (Nishita *et al.*, 2002, 2005). The results given here demonstrate that Dam inhibits the kinase activity of LIMK1 more effectively than that of Lck. Thus, to determine whether Dam suppresses the chemotactic response of Jurkat cells through Lck inhibition or through LIMK1 inhibition, we examined the effect of Dam on CXCL12-induced chemotaxis of both Jurkat and Jurkat-derived, Lck-deficient JCaM1.6 cells. Immunoblot analyses revealed that Lck was expressed in Jurkat cells but not in JCaM1.6 cells, whereas LIMK1 expression was similar in both cells (Figure 5A). Chemotactic migration was analyzed using Transwell chambers in which CXCL12 was added to the lower chamber. When Jurkat and JCaM1.6 cells were pretreated with Dam for 30 min and then subjected to chemotaxis assays, Dam inhibited CXCL12-induced chemotaxis in the 3–10 μM range in both Jurkat and JCaM1.6 cells (Figure 5, B and C). Because Lck is not expressed in JCaM1.6 cells, these results suggest that Dam inhibited CXCL12-induced chemotaxis not through the inhibition of Lck, but probably through the inhibition of LIMK1, at least in JCaM1.6 cells. To further address this issue, we next examined the effect of Dam on CXCL12-induced cofilin phosphorylation. As previously reported (Nishita *et al.*, 2002, 2005), the level of P-cofilin in Jurkat cells increased 5 min after CXCL12 stimulation, but this increase was suppressed after exposure of cells to 3–10 μM Dam (Figure 5D). By contrast, the increase in phosphorylated MAPK (P-MAPK) after CXCL12 stimulation was unaffected by Dam treatment (Figure 5D). These results suggest that Dam has the potential to inhibit CXCL12-induced LIMK1 activation and cofilin phosphorylation in Jurkat cells.

To further elucidate the mechanism by which Dam suppresses chemotactic migration of Jurkat cells, we analyzed changes in cell morphology and actin cytoskeleton by time-lapse fluorescence analysis. Jurkat cells expressing YFP-actin were treated with 3 μM Dam or control vehicle for 30 min and then stimulated with CXCL12. Before CXCL12 stimulation, the untreated control Jurkat cells exhibited a round cell morphology, but within 1–5 min of CXCL12 stimulation, there were multiple F-actin–rich lamellipodial protrusions around the circumference of the cell that were converted into a single lamellipodium on one side of the cell within 20 min (Figure 6A and Supplemental Movie S1). By contrast, Dam-treated cells formed only faint and immature lamellipodial protrusions before and after CXCL12 stimulation (Figure 6A and Supplemental Movie S2). Changes in cell morphology and actin cytoskeleton were also assessed using rhodamine–phalloidin staining before and 20 min after CXCL12 stimulation. Quantitative analysis confirmed that after CXCL12 stimulation, Dam-treated cells had fewer cells with large lamellipodial protrusions and more cells with small or no lamellipodial protrusions than the control cells (Figure 6B). The phenotypes of Dam-treated cells are similar to those of LIMK1-knockdown cells (Nishita *et al.*, 2005). Taken together, these findings suggest that Dam suppresses Jurkat cell migration by suppressing stimulus-induced F-actin assembly and the formation and maintenance of lamellipodial membrane protrusions, presumably by inhibiting the F-actin–stabilizing activity of LIMK1. Dam did not affect the cell morphology of non-stimulated cells, which suggests that LIMK1 is primarily involved in

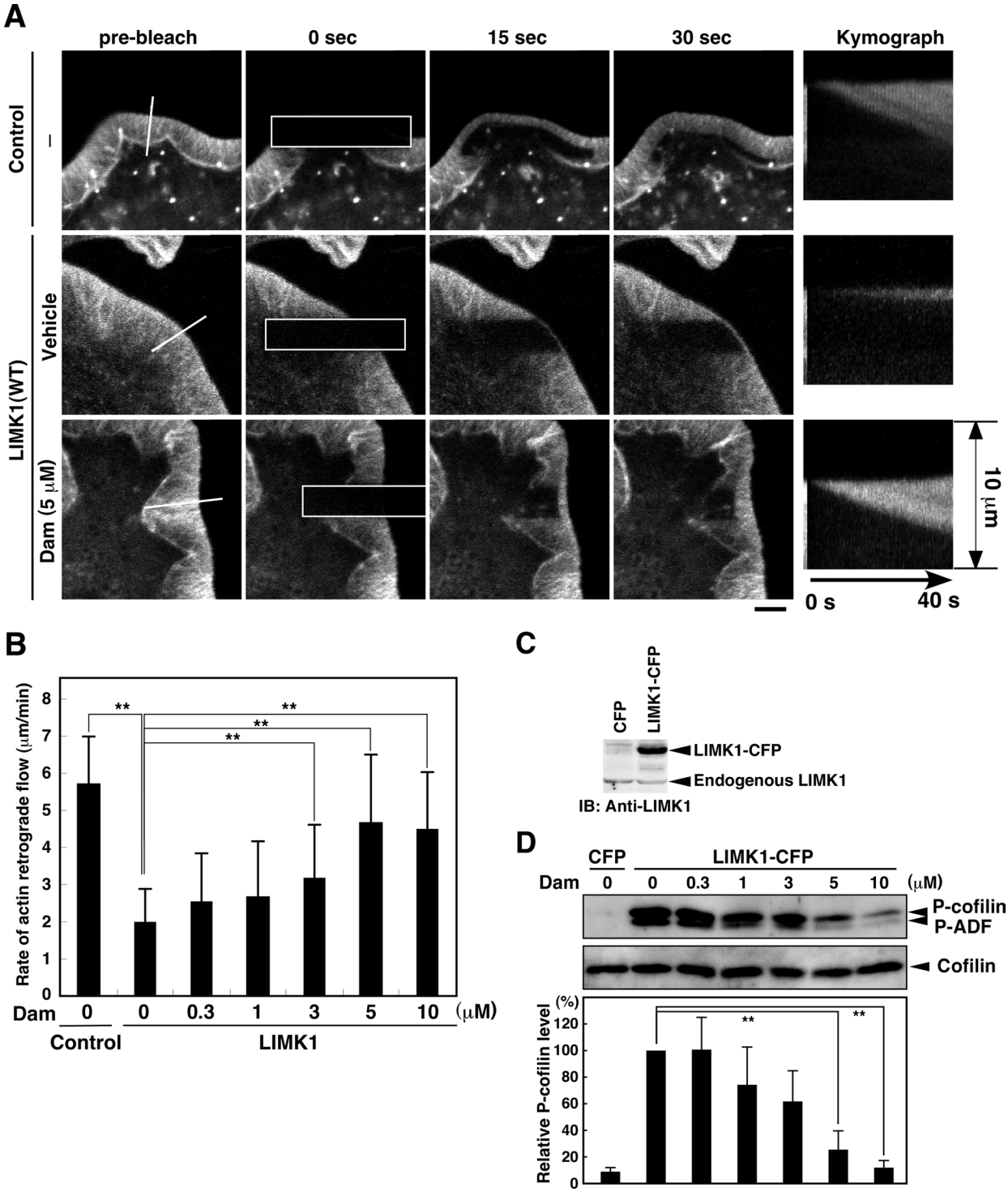

FIGURE 4: Dam inhibits LIMK1-induced deceleration of actin retrograde flow and cofilin phosphorylation. (A) FRAP time-lapse imaging of YFP-actin in RacV12-expressing N1E-115 cells. Cells were cotransfected with CFP (control) or CFP-LIMK1. Cells were pretreated with Dam or control vehicle (1% DMSO) for 30 min. After photobleaching of a rectangular region (white box), fluorescence images were acquired every 1 s for 40 s. Scale bar, 10 μm. Far right, kymographs of the white lined region (perpendicular to the cell margin) depicted at the far left. (B) Quantitative analysis of the effect of Dam on LIMK1-induced deceleration of the rate of actin retrograde flow in lamellipodia, measured by kymograph analysis. Data are mean values ± SD of three independent experiments. $^{**}p < 0.01$ by one-way ANOVA followed by Dunnett's test. (C) Level of LIMK1-CFP overexpression. N1E-115 cells were cotransfected with CFP (control) or LIMK1-CFP, and cell lysates were analyzed by immunoblotting with anti-LIMK1 antibody. (D) The effect of Dam on the level of cofilin phosphorylation. N1E-115 cells were cotransfected as before and treated with indicated concentrations of Dam for 30 min. Cell lysates were analyzed by immunoblotting with anti–P-cofilin and anti-cofilin antibodies. Bottom, relative P-cofilin levels, with the value in Dam-untreated, LIMK1-overexpressing cells taken as 100%. Data are mean values ± SD of three independent experiments. $^{**}p < 0.01$ by one-way ANOVA followed by Dunnett's test.

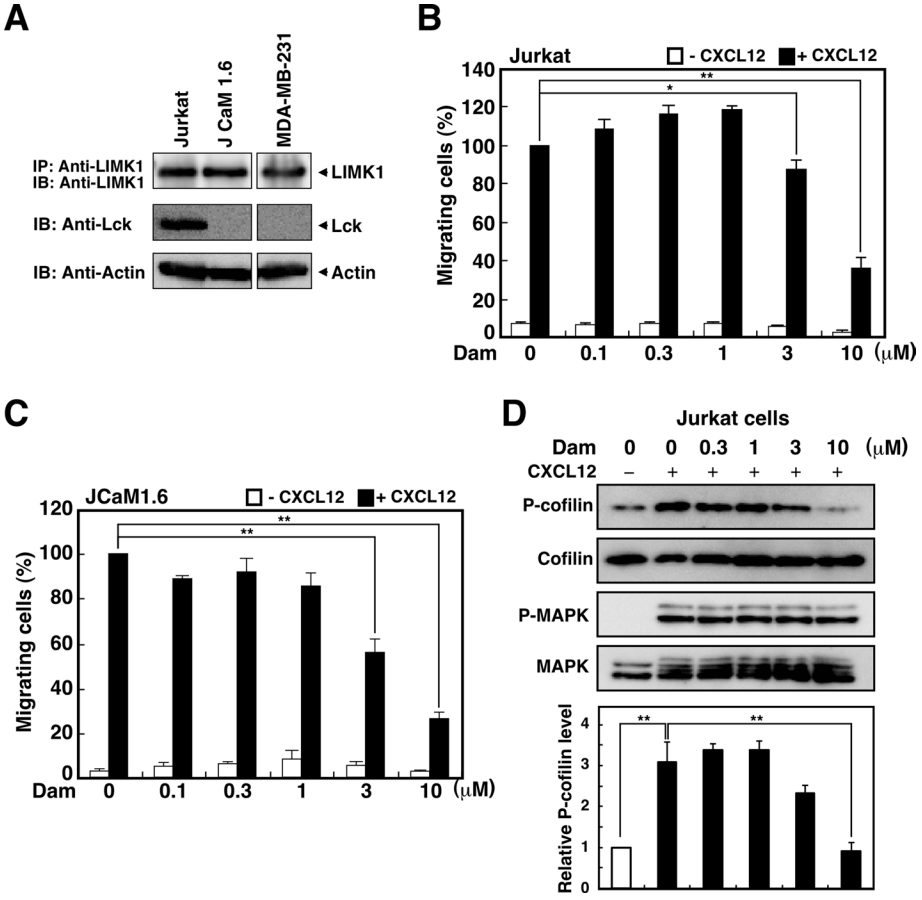

FIGURE 5: Dam inhibits chemotactic migration of Jurkat T-cells and Jurkat-derived, Lck-deficient JCaM1.6 cells. (A) Immunoblot analysis of the expression of LIMK1 and Lck in Jurkat, JCaM1.6, and MDA-MB-231 cells. Expression of Lck and control actin was analyzed by immunoblotting of cell lysates with anti-Lck and anti-actin antibodies. Expression of LIMK1 was analyzed by immunoprecipitation of cell lysates, followed by immunoblotting with anti-LIMK1 antibody. (B, C) Effect of Dam on CXCL12-induced chemotaxis of Jurkat (B) or JCaM1.6 (C) cells. Cells were pretreated with Dam and then loaded into the upper well of the Transwell chambers in the presence or absence of CXCL12 in the lower well. After incubation for 3 h, migrating cells in the lower well were counted. Data are mean values ± SD of four independent experiments. *$p < 0.05$, **$p < 0.01$, by one-way ANOVA followed by Dunnett's test. (D) Effect of Dam on CXCL12-induced cofilin phosphorylation in Jurkat cells. Cells were stimulated with 5 nM CXCL12 for 5 min and cell lysates analyzed by immunoblotting using antibodies to P-cofilin, cofilin, P-MAPK, and MAPK. Bottom, relative P-cofilin levels, with the value in control cells taken as 1.0. Data are mean values ± SD of three independent experiments. **$p < 0.01$ by one-way ANOVA followed by Dunnett's test.

stimulus-induced actin cytoskeletal remodeling and changes in cell morphology.

To further elucidate the pharmacological efficacy of Dam under various conditions, we analyzed the effect of washout on the inhibitory activity of Dam. Jurkat cells were treated with 5 μM Dam or control vehicle for 30 min and then washed three times with culture medium. Immediately or 30–180 min after washing, cells were stimulated with CXCL12 for 20 min and then fixed and stained with rhodamine–phalloidin, and the lamellipodium formation was analyzed as in Figure 6B. In accord with the result shown in Supplemental Figure S4B, washout of Dam before CXCL12 stimulation did not substantially affect the inhibitory activity of Dam on CXCL12-induced lamellipodium formation (Supplemental Figure S5, a–d), which suggests that Dam has the long-lasting inhibitory effect, presumably by forming a stable adduct with the target kinase. In addition, application of Dam immediately after CXCL12 stimulation inhibited CXCL12-induced lamellipodium formation, but application of Dam 10 min after CXCL12 stimulation reduced the inhibitory effect of Dam on CXCL12-induced large lamellipodium formation (Supplemental Figure S5, a, e, and g), which suggests that Dam inhibits CXCL12-induced lamellipodium formation by predominantly inhibiting the early step (occurring within 10 min after cell stimulation) of lamellipodium formation.

Dam inhibits migration and invasion of MDA-MB-231 breast carcinoma cells

Several lines of evidence show that LIMK1 is required for tumor cell migration and invasion (Yoshioka *et al.*, 2003; Horita *et al.*, 2008; Mishima *et al.*, 2010). Because MDA-MB-231 human breast carcinoma cells are known to express LIMK1 and require it for cell migration and invasion (Scott *et al.*, 2010), we investigated the effect of Dam on serum-induced chemotactic migration and invasion of MDA-MB-231 cells. Immunoblot analysis revealed that LIMK1, but not Lck, is expressed in MDA-MB-231 cells (Figure 5A). Serum-induced chemotactic migration of MDA-MB-231 cells was significantly suppressed by exposure of cells to 1–10 μM Dam (Figure 7A). To examine the effect of Dam on the invasive activity of MDA-MB-231 cells, we loaded the cells on Matrigel in the upper chamber of Transwell chambers and added serum to the lower chamber. After incubation for 15 h, the cells that had invaded in the lower chamber were counted. Serum-induced invasion of MDA-MB-231 cells was significantly suppressed by exposing the cells to 3–10 μM Dam (Figure 7B). Thus Dam is a potent inhibitor of migration and invasion of MDA-MB-231 breast carcinoma cells.

Topical application of Dam inhibits hapten-stimulated migration of epidermal Langerhans cells in mouse ears

The epidermal Langerhans cell is a member of the dendritic cell family, and it initiates cutaneous immune responses by transporting foreign antigen for presentation to T lymphocytes in the skin-draining lymph nodes. To do this, the cells migrate from the epidermis to draining lymph nodes in response to chemical allergens on the skin surface (Cumberbatch *et al.*, 2000). To examine whether Dam affects cell migration in vivo, we analyzed the effect of topical administration of Dam on hapten-stimulated migration of epidermal Langerhans cells in mouse ears. Mouse ears were painted with 3% (wt/vol) 2,4,6-trinitrochlorobenzene (TNCB) in acetone/olive oil (1:4) or vehicle (acetone/olive oil) alone as a control. After 24 h of TNCB exposure, the epidermal sheets were prepared from the ear skins and stained with fluorescein isothiocyanate (FITC)–labeled anti-mouse

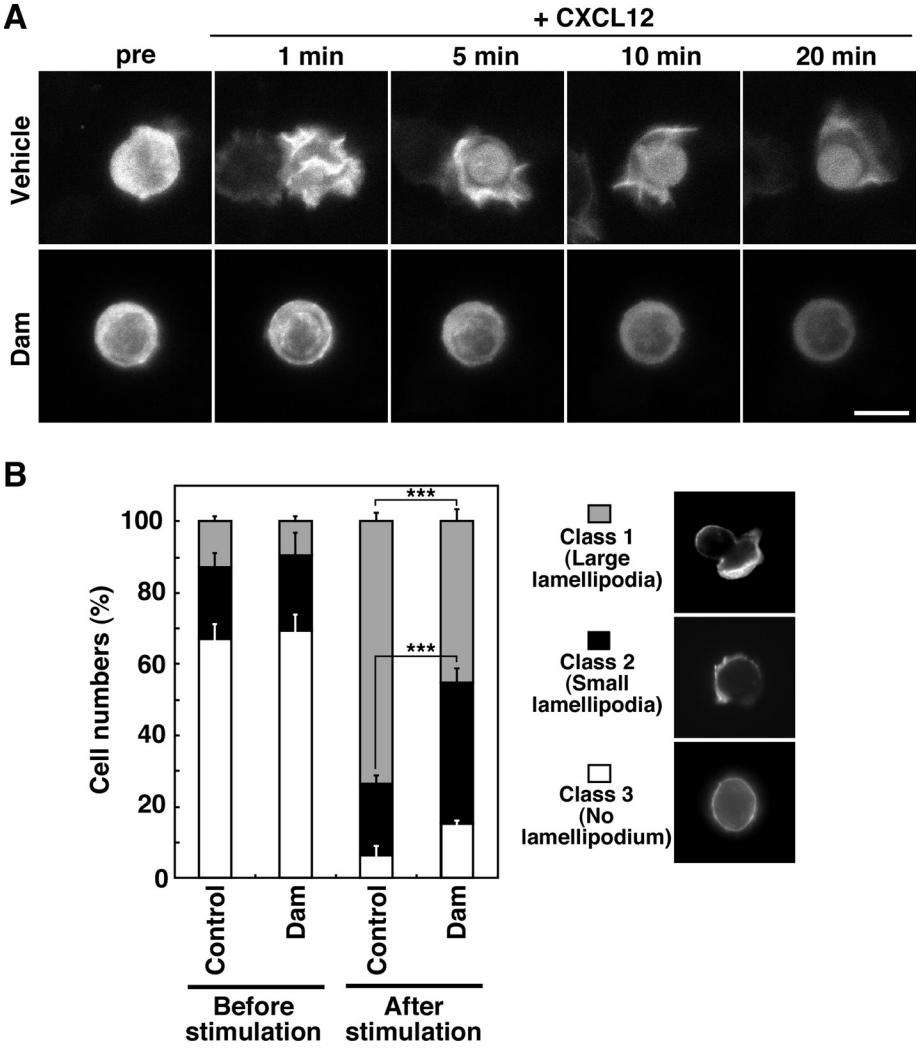

FIGURE 6: Effect of Dam on CXCL12-induced F-actin assembly and membrane protrusion formation in Jurkat cells. (A) Time-lapse fluorescence analysis. Jurkat cells transfected with YFP-actin were treated with 5 μM Dam or control vehicle (0.1% DMSO) for 30 min and then stimulated with 5 nM CXCL12 for 20 min. The levels of F-actin assembly and membrane protrusion were analyzed by time-lapse analysis of YFP fluorescence. Scale bar, 10 μm. See also Supplemental Movies S1 and S2. (B) The effect of Dam on the lamellipodium formation of Jurkat cells before and after CXCL12 stimulation. Cells were treated with 5 μM Dam or control vehicle for 30 min and then stimulated with 5 nM CXCL12 for 20 min. Cells were fixed and stained with rhodamine–phalloidin to detect F-actin and categorized into three classes: 1, cells with large lamellipodia; 2, cells with small lamellipodia; 3, round cells without a lamellipodium. Percentages of cells in each class are shown as mean values ± SD of three independent experiments (200–300 cells were counted in each experiment). ***$p < 0.005$ by Student's t test.

I-Ad monoclonal antibody to visualize class II MHC in epidermal Langerhans cells on the ear epidermis (Aiba and Katz, 1990). Treatment with TNCB significantly decreased the density of Langerhans cells on the ear epidermis (Figure 8, A–C and F), indicating that TNCB administration induced migration of epidermal Langerhans cells to the lymph nodes. To examine the effect of Dam on in vivo cell migration, Dam (20 μM) or vehicle solution was topically applied on the ear skin 30 min before, immediately after, and 12 h after TNCB painting. The density of Langerhans cells was analyzed as before, and in the TNCB-administrated ear epidermis, it was found to be significantly higher in Dam-treated mouse ears compared with vehicle treatment (Figure 8, C, E, and F), whereas Dam treatment of control ears had no effect on Langerhans cell density (Figure 8, D and F). These results indicate that Dam has the potential to suppress hapten-induced migration of epidermal Langerhans cells. Further studies are required to determine whether Dam suppressed Langerhans cell migration via LIMK1 inhibition. On hapten stimulation, Langerhans cells appeared to increase cell size, as reported (Aiba and Katz, 1990; Kubo *et al.*, 2009), but Dam treatment had no apparent effect on the cell size changes (Figure 8, C and E).

DISCUSSION

In this study, two compounds, Dam and MO-26, were identified as effective inhibitors of LIMKs. Both were previously reported to be effective inhibitors of Lck tyrosine kinase (Faltynek *et al.*, 1995; Burchat *et al.*, 2002). Despite the different backbone structures (Dam is an anthraquinone derivative, whereas MO-26 is a pyrrazolopyrimidine derivative), both exhibited effective inhibition of LIMKs and Lck, with no apparent effect on ROCK, PAK3, PKCα, and CaMKIα. However, Dam is a better inhibitor of LIMK1, whereas MO-26 is a better inhibitor of Lck. Although LIMKs are known to phosphorylate the Ser-3 residue of cofilin/ADF family proteins, they share sequence similarities with tyrosine kinases and possess unique sequence motifs, such as DLNSHN in the VIB subdomain of the kinase catalytic domain (Okano *et al.*, 1995; Manning *et al.*, 2002). In addition, unpublished results from this lab indicate that LIMK1 has weak tyrosine kinase activity. These results suggest that the active-site tertiary structures of LIMKs and Lck are sufficiently similar to allow binding and inhibition by Dam and MO-26. Although several inhibitors of Lck (Meyn and Smithgall, 2008) and LIMKs (Ross-Macdonald *et al.*, 2008; Harrison *et al.*, 2009; Prudent *et al.*, 2012; Manetti, 2012a) have been reported, in light of the results presented here, it is clear that there is potential for cross-specificity of inhibition; hence it is important to reexamine whether these compounds are indeed specific for each of these kinases. Moreover, it is important to determine the target specificity of Dam and MO-26 more precisely by using comprehensive kinase profiling. Research from this lab is focused on the development of more-selective LIMK inhibitors, using Dam and MO-26 as lead compounds.

Based on the results that Dam inhibits Lck and suppresses CXCL12-induced migration of Jurkat cells, it was postulated that Lck plays a crucial role in T-cell chemotaxis (Inngjerdingen *et al.*, 2002). In this study, Dam suppressed CXCL12-induced chemotactic migration in both Jurkat and Jurkat-derived, Lck-deficient JCaM1.6 cells. Dam also caused inhibition of CXCL12-induced cofilin phosphorylation and chemotactic migration of Jurkat cells in a similar dose-dependent manner. Moreover, we previously showed that LIMK1 knockdown suppresses CXCL12-induced chemotaxis of Jurkat cells (Nishita *et al.*, 2002, 2005). Together these results suggest that Dam suppresses T-cell chemotaxis primarily by inhibiting LIMK1, at

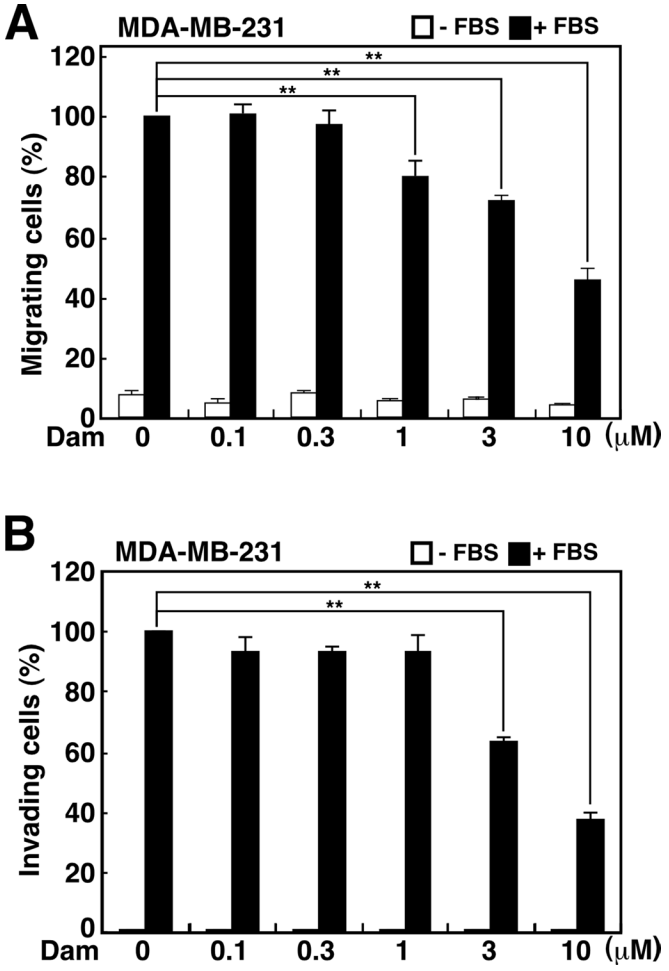

FIGURE 7: Dam inhibits migration and invasion of MDA-MB-231 breast carcinoma cells. (A) Migration assay. Cells were incubated with indicated concentrations of Dam or vehicle for 30 min and loaded into the upper well of the Transwell chambers in the presence or absence of 10% FCS in the lower well. After a 3-h incubation, the migrating cells on the lower face of the membrane were counted. (B) Invasion assay. Matrigel was placed onto the upper well of the Transwell chambers, cells were loaded onto Matrigel and cultured for 15 h, and the invading cells on the lower face of the membrane were counted. Data are mean values ± SD of four (A) or six (B) independent experiments. $^{**}p < 0.01$ by one-way ANOVA followed by Dunnett's test.

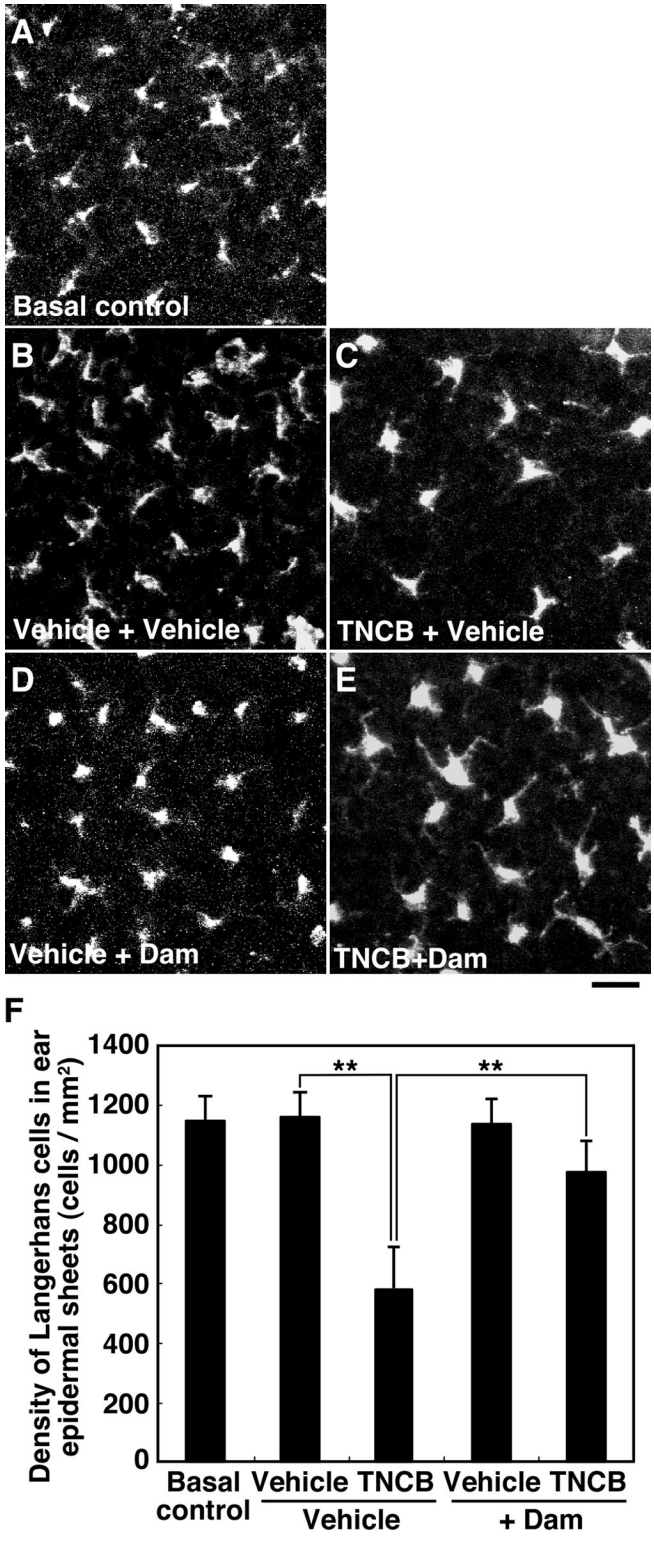

FIGURE 8: Topical application of Dam inhibits hapten-induced migration of epidermal Langerhans cells in mouse ears. (A) Direct immunofluorescence analysis of epidermal sheets, using FITC-labeled anti-mouse I-Ad monoclonal antibody. Epidermal sheets were obtained from normal skin (A) or skin painted with vehicle (B), TNCB (C), Dam (D), or TNCB and Dam (E). Scale bar, 20 μm. (F) Quantitative analysis of density of Langerhans cells in ear epidermal sheets. Data are mean values ± SD of five independent experiments (cell density was analyzed in 15 areas/mouse ear in each experiment). $^{**}p < 0.01$ by one-way ANOVA followed by Dunnett's test.

least in Lck-deficient JCaM1.6 cells. As for Jurkat cells, the possibility cannot be excluded that Dam may suppress migration through inhibition of both LIMK1 and Lck. Treatment with Dam suppressed CXCL12-induced lamellipodium formation in Jurkat cells, with a similar phenotype to that of LIMK1 knockdown cells (Nishita *et al.*, 2005), which indicates that LIMK1 plays a critical role in Jurkat cell migration by promoting stimulus-induced lamellipodium formation.

Dam also suppressed migration and invasion of MDA-MB-231 breast carcinoma cells, which have no detectable expression of Lck, further supporting the hypothesis that Dam suppresses MDA-MB-231 cell migration and invasion primarily through inhibition of LIMKs. Previous studies demonstrated the crucial role of LIMKs in tumor cell invasion and metastasis (Davila *et al.*, 2003; Yoshioka *et al.*, 2003; Wang *et al.*, 2007; Horita *et al.*, 2008; Mishima *et al.*, 2010; Scott *et al.*, 2010). Thus Dam could be used to develop a therapeutic agent against LIMK-mediated tumor cell invasion and metastasis.

In this study, topical application of Dam impaired hapten-induced migration of epidermal Langerhans cells in the mouse ear. Langerhans cells in the epidermis play a central role in the initiation and elicitation of cutaneous immune responses and the pathogenesis of allergic contact dermatitis. Langerhans cells recognize, internalize, and process the antigens and carry them from the epidermis to draining lymph nodes, where they are presented to antigen-responsive T-lymphocytes that drive primary immune responses (Cumberbatch *et al.*, 2000; Kimber *et al.*, 2000). Inhibition of Langerhans cell migration could be a promising strategy to alleviate allergic skin diseases, such as contact hypersensitivity and atopic dermatitis. Topical treatment with Dam suppressed this migration; hence Dam could be a suitable starting point for the development of agents against allergic inflammatory diseases.

LIMKs are key regulators of actin cytoskeletal reorganization through the phosphorylation and inactivation of cofilin. They play essential roles in diverse cell functions, including cell migration, morphogenesis, division, polarity formation, gene expression, neurite outgrowth, and cancer cell invasion (Scott and Olson, 2007; Mizuno, 2013). Identification of Dam and MO-26 as effective LIMK inhibitors provides a useful tool for investigating the functional roles of LIMKs in cellular and pathophysiological processes. Aberrant regulation of LIMK activity has been reported to be involved in the pathogenesis of cancer metastasis and cardiovascular and neurological disorders (Scott and Olson, 2007; Manetti, 2012a; Mizuno, 2013), and LIMK2 inhibitors have been used to treat ocular hypertension and associated glaucoma in model animals (Harrison *et al.*, 2009). Thus LIMKs are promising therapeutic targets, and specific inhibitors of LIMKs are attractive candidates for novel therapeutic drugs against these diseases.

This study applied a new BiFC assay system recently developed in our laboratory to screen for LIMK1 inhibitors (Ohashi *et al.*, 2012). The assay, which uses a pair of Venus fragments obtained by splitting the Venus fluorescent protein at position 210, can quantitatively measure protein–protein interactions with high specificity and low background fluorescence. Recently it was successfully used to screen for inhibitors that block protein–protein interactions (Shoji *et al.*, 2012). Therefore, the BiFC assay system will provide a useful method for screening inhibitors of various protein kinases, once the BiFC probes that properly detect the phosphorylation-dependent protein–protein interactions can be developed. Protein phosphorylation can negatively and positively regulate protein–protein interactions. For example, as reported here, LIMK1-mediated cofilin phosphorylation negatively regulated the actin–cofilin interaction. Conversely, protein phosphorylation by PKB/Akt or PKD often produces the binding site for 14-3-3 proteins in target proteins (Tzivion and Avruch, 2002), and the cross-phosphorylation of receptor tyrosine kinases produces the binding sites for Src homology region 2 domain–containing or phosphotyrosine-binding domain–containing proteins in receptor proteins (Schlessinger and Lemmon, 2003). Thus it is feasible to develop the BiFC assay system to screen for inhibitors of these protein kinases. Targeted inhibition of protein kinases is considered to be a promising therapeutic strategy in the treatment of relevant diseases (Cohen, 2002). Based on an approach similar to that outlined in this study, the BiFC assay system will provide a useful method for identifying new inhibitors of protein kinases and facilitating the development of attractive therapeutic drugs that target specific protein kinases.

MATERIALS AND METHODS

Materials

SCADS inhibitor kits I and II (each consisting of 95 chemical inhibitors) were provided by the Screening Committee of Anticancer Drugs via a Grant-in-Aid for Scientific Research (priority area "Cancer") from the Ministry of Education, Culture, Sports, Science and Technology, Japan. Other chemical compounds (MO-1 to MO-768) were synthesized as partial structures and analogues of natural products, as well as of enzyme inhibitors (Oikawa *et al.*, 1995, 2005; Kusumoto and Oikawa, 2001; Oikawa, 2010). Dam (Enzo Life Sciences, Farmingdale, NY), emodin (Tokyo Chemical Industry, Tokyo, Japan), and mitoxantrone (Sigma-Aldrich, St. Louis, MO) were purchased commercially. CXCL12 was purchased from PeproTech (Rocky Hill, NJ). Rabbit polyclonal antibodies against LIMK1, cofilin, and P-cofilin were generated as described (Okano *et al.*, 1995; Toshima *et al.*, 2001). Mouse monoclonal antibody against Myc epitope (9E10) and rat monoclonal antibody against hemagglutinin epitope (3F10) were purchased from Roche Diagnostics (Basel, Switzerland). Mouse monoclonal antibodies against Lck (3A5) and Src (B-12) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against MAPK, P-MAPK, and β-actin (AC-15) were purchased from Sigma-Aldrich. FITC-labeled anti-mouse I-Ad antibody was purchased from BioLegend (San Diego, CA).

Plasmid construction

Venus cDNA was provided by A. Miyawaki (Riken, Wako, Japan; Nagai *et al.*, 2002). The cDNA plasmids for LIMK1 and LIMK2 were constructed as described previously (Mizuno *et al.*, 1994; Okano *et al.*, 1995). The cDNA plasmids for GST-ROCKΔ3 (amino acids 1–727) and PAKΔN (amino acids 162–544) were provided by S. Narumiya (Kyoto University, Kyoto, Japan) and H. Sumimoto (Kyushu University, Fukuoka, Japan), respectively. The cDNA plasmids for mouse Lck and human Src were purchased from Open Biosystems (Tokyo, Japan). The cDNA plasmids for PKCα and CaMKIα were constructed by PCR amplification. For the in vitro BiFC assays, expression plasmids for actin-VC210-His_6, VN210-cofilin-His_6, LIMK1-(Myc+His), and GST-ROCKΔ3 were constructed by inserting the cDNAs into the pFastBac1 baculovirus expression vector (Invitrogen, Carlsbad, CA; Ohashi *et al.*, 2012). For the in vitro kinase assays, expression plasmids for (Myc+His)-tagged LIMK1, LIMK2, Lck, SrcΔC (amino acids 1–322), ROCKΔ3, and PAK3ΔN and HA-tagged PKCα were constructed by inserting the cDNAs into the pcDNA3.1/(Myc+His) (Invitrogen)– or pEYFP-C1 (Clontech, Mountain View, CA)–derived pHA-C1 vector. Expression plasmids coding for the kinase-dead mutants were constructed using a site-directed mutagenesis kit (Stratagene, Santa Clara, CA). Expression plasmids for GFP-CaMKIα-(1-293) and -(K49E) were constructed as described previously (Saito *et al.*, 2013). Expression plasmids for YFP-actin, HA-RacV12, and CFP-tagged LIMK1 were constructed as described previously (Ohashi *et al.*, 2011).

Cell culture and transfection

N1E-115, COS-7, and MDA-MB-231 cells were cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum (FCS). These cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen). Jurkat and JCaM1.6 cells were cultured in RPMI medium supplemented with 10% (vol/vol) FCS. Jurkat cells were transfected by electroporation at 280 V and 975 μF using a Gene Pulser II (Bio-Rad, Hercules, CA), as described previously (Nishita *et al.*, 2005).

LIMK1 inhibitor screens

Screening of LIMK1 inhibitors was performed using the in vitro BiFC assay with probes composed of actin-VC210 and VN210-cofilin, as described previously (Ohashi *et al.*, 2012). His_6-tagged actin-VC210, VN210-cofilin, and LIMK1-(Myc+His) were expressed in Sf9 or Sf21

cells, using the Bac-to-Bac baculovirus expression system (Invitrogen). Proteins were resolved on a nickel-nitrilotriacetic acid agarose (Qiagen, Valencia, CA) column and eluted with 0.2 M imidazole buffer, and the elution buffer containing each protein was exchanged for binding buffer using a PD-10 gel-filtration column (GE Healthcare, Piscataway, NJ; Ohashi *et al.*, 2012). GST-ROCKΔ3 was expressed in Sf21 cells, purified using a glutathione–Sepharose (GE Healthcare) column, and eluted with glutathione buffer, and the elution buffer containing GST-ROCKΔ3 was exchanged using a PD-10 column. LIMK1 was incubated with ROCKΔ3 and ATP at 30°C for 10 min and then with one of the small organic compounds at a final concentration of 6.6 μM for known inhibitors or 10 μM for synthetic compounds in 30 μl of screening buffer (40 mM Tris-HCl, pH 7.4, 1 mM ATP, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol) in 96-well microtiter plates. Chemical compounds were used after dissolving in dimethylsulfoxide (DMSO). After incubation for 5 min, the VN210-cofilin probe (3 μM) was added to the mixture and incubated at 30°C for 1 h. Then actin-VC210 (1.5 μM) was added and fluorescence intensity was measured at 0 and 120 min at 545 nm with excitation at 505 nm in a fluorescence microphotometer. Experiments were carried out in duplicate, and recovery of fluorescence intensity was calculated relative to control (in the absence of compound).

In vitro kinase assay

COS-7 cells were transfected with cDNA plasmids coding for LIMK1-Myc, LIMK2-Myc, Src-(Myc+His), Lck-(Myc+His), ROCK-Myc, PAK3-Myc, HA-PKCα, GFP-CaMKIα, or their mutants using Lipofectamine 2000 (Invitrogen). After 48 h, cells were washed with phosphate-buffered saline (PBS) and lysed by resuspension in lysis/kinase buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 150 mM NaCl, 0.5% [vol/vol] Nonidet P-40, 5% [vol/vol] glycerol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). After centrifugation to remove debris, supernatants were incubated for 4 h at 4°C with anti-Myc, anti-HA, or anti-GFP antibody and protein A–Sepharose (GE Healthcare). The immunoprecipitates were washed three times with the lysis/kinase buffer and subjected to an in vitro kinase reaction. In vitro kinase reactions were performed in 20 μl of lysis/kinase buffer containing 100 μM ATP and 185 kBq of [γ-^{32}P]ATP (110 TBq/mmol), supplemented with 2 μg of substrate (either recombinant cofilin or MBP), in the presence or absence of chemical inhibitors (Dam or MO-26 in 1 μl of DMSO) at 30°C for 40 min. The reaction mixture was boiled in SDS sampling buffer, and aliquots were separated by SDS–PAGE. Proteins were transferred to polyvinylidene fluoride membranes and analyzed by autoradiography using BAS1000 Bio-image analyzer (Fuji Film, Tokyo, Japan), amido black staining, and immunoblotting with anti-Myc, anti-HA, or anti-GFP antibody. Kinase activity was measured via ^{32}P incorporation into the substrate protein and expressed as relative activity using control activity, in the absence of inhibitor, as 100%. The IC_{50} values were calculated using Prism software (GraphPad, La Jolla, CA).

Measurement of the rate of actin retrograde flow using FRAP time-lapse imaging

N1E-115 cells were cotransfected with the cDNA plasmids for YFP-actin, Rac-V12, and LIMK1-CFP or control vector and cultured for 24 h. Cells were pretreated with Dam or the control (drug vehicle, 1% [vol/vol] DMSO) at 37°C for 30 min and then subjected to FRAP time-lapse assays. FRAP analysis was performed using a laser-scanning confocal microscope (LSM 510; Carl Zeiss, Jena, Germany) equipped with a PL Apo 63× oil-immersion objective lens (numerical aperture 1.4), as described previously (Ohashi *et al.*, 2011). N1E-115 cells were plated on a 35-mm glass-bottom dish and maintained in DMEM containing 10 mM HEPES, pH 7.4, and 10% FCS, at 37°C in a heat insulation chamber. Before photobleaching, a fluorescence image of the cell in a rectangular region (512 × 300 pixels) was acquired by irradiation with 0.5–1% of power from a 514-nm argon-ion laser. Photobleaching was performed in a rectangular region (45 × 225 pixels, 101 μm^2) of partially overlapping lamellipodium by 30 times irradiation for a total time of 3.1 s with full power of a 30-mW argon-ion laser at 458, 488, and 514 nm. Immediately after photobleaching, fluorescence images of the foregoing cell areas were acquired every second for 30 s by weak irradiation with a 514-nm argon-ion laser. The rate of actin retrograde flow was measured by kymograph analysis. Kymograph analysis was conducted with a customized macro in ImageJ (http://rsb.info.nih.gov/ij/). The kymograph image was constructed using stacked images of 3 × 100–pixel (0.3 × 10 μm) areas, which were taken by FRAP time-lapse analysis. The rate of actin retrograde flow was measured as the rate at which the boundary between the bright and dark areas of the recovering YFP-actin fluorescence signal migrated inward from the initial cell margin.

Time-lapse fluorescence imaging to detect changes in actin organization in Jurkat cells

For time-lapse imaging, Jurkat cells were electroporated with plasmids coding for YFP-actin. Images of stacked optical sections were collected every 30 s for 20 min after CXCL12 stimulation using a laser-scanning confocal microscope and objective lens, as described previously (Nishita *et al.*, 2005).

Cell migration and invasion assays

Jurkat or JCaM1.6 cells (2 × 10^6 cells/ml) were resuspended in RPMI1640 medium containing 0.5% bovine serum albumin (BSA) and pretreated with Dam or drug vehicle (final concentration 0.3% [vol/vol] DMSO) for 30 min at room temperature. Cells (2 × 10^5 cells) in 100 μl of medium were loaded into the upper well of the 24-well Transwell culture chamber (5-μm pore size; Corning, Lowell, MA), and 400 μl of RPMI medium containing 0.5% BSA, 5 nM CXCL12, and Dam or vehicle (final 0.3% DMSO) was added to the lower well. After incubation for 3 h at 37°C, the cells that had migrated into the lower well were counted. MDA-MB-231 cells (2 × 10^5 cells/ml) were serum starved for 3 h, resuspended in serum-free DMEM, and pretreated with Dam or vehicle (final 0.3% DMSO) for 30 min at room temperature. Aliquots of cells (2 × 10^4 cells) in 100 μl of medium were loaded into the upper well of Transwell chambers (8-μm pore size). The lower wells were filled with 400 μl of DMEM containing 10% FCS and Dam or vehicle (final 0.3% DMSO). After incubation for 3 h at 37°C, cells were fixed with 3.7% formaldehyde and stained with 4′,6-diamidino-2-phenylindole. The nonmigrating cells on the top of the membrane were gently removed by wiping and rinsing, and the migrating cells on the lower face of the membrane were counted. For invasion assays, 50 μg/50 μl of Matrigel (Becton-Dickinson, San Diego, CA) were loaded into the upper well of Transwell chambers (8-μm pore size) and incubated for 1 h at 37°C. After washing the Matrigel with DMEM three times, MDA-MB-231 cells (2 × 10^4 cells) were loaded onto Matrigel and cultured for 15 h, and the cells invading on the lower face of the membrane were counted, as described.

Immunoprecipitation and immunoblot analysis

Cells were lysed with lysis/kinase buffer, and the lysates were subjected to immunoprecipitation or immunoblot analyses, as described previously (Okano *et al.*, 1995).

In vivo migration assays of epidermal Langerhans cells

BALB/c mice at age 8–10 wk were painted on the ears with 20 µl of 20 µM Dam in 2% DMSO or vehicle (2% DMSO) in 1:4 (vol/vol) acetone/olive oil. After 30 min, the ears were painted with 20 µl of 3% (wt/vol) TNCB in 1:4 (vol/vol) acetone/olive oil or vehicle (acetone/olive oil) with or without 20 µM Dam. After 12 h, the ears were painted again with 20 µl of 20 µM Dam in 2% DMSO or control vehicle. Control and TNCB-painted ear skins were obtained 24 h after TNCB painting. Using standard techniques, epidermal sheet preparations were made and stained with a monoclonal antibody with specificity for I-Ad (Caughman *et al.*, 1986; Aiba and Katz, 1990). Epidermal sheets were obtained after incubation in 0.5 M NH_4SCN at 37°C for 30 min. The sheets were fixed in acetone for 10 min and blocked with PBS containing 0.2% (wt/vol) bovine serum albumin and 0.02% (wt/vol) sodium azide. After incubation with FITC-labeled anti-mouse I-Ad monoclonal antibody (BioLegend) for 3 h, the sheets were washed with PBS, mounted with nonfluorescent glycerol, and analyzed using confocal fluorescence microscopy.

Statistical analysis

Statistical data are expressed as the means ± SD of more than three independent experiments. All statistical analyses were performed by Prism 6 (GraphPad). The *p* values were calculated using unpaired two-tailed Student's *t* test for pairwise data comparisons (in Figure 6 and Supplemental Figure S5) or one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple data set comparison (in Figures 4, 5, 7, and 8 and Supplemental Figure S4). $p < 0.05$ was considered to be significant.

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