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ORIGINAL PAPER

Casiopeinas IIgly and IIIia Induce Apoptosis in Medulloblastoma Cells

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Abstract The medulloblastoma is a tumor of the central nervous system that is expressed in childhood. Casiopeinas® are a family of molecules with an active copper 2^+ core and an amino acid sequence that seem give them tumoral specificity. The mechanism of action is poorly understood; however, it has been reported that some metals such as copper and some of their complexes are toxic due to their high potential to participate in redox reactions which could cause apoptosis in medulloblastoma cells. Cell survival was measured by the MTT method and apoptosis was identified by the presence of condensed nuclei, disruption of the mitochondrial transmembrane potential, and cytoskeleton disorder. In all cases medulloblastoma cells treated with Casiopeinas showed more apoptotic features than untreated cells. Casiopeinas IIgly and IIIia promise to be important compounds for the treatment of medulloblastoma, mainly by their ability to induce apoptosis.

Keywords Cell survival · Cisplatin · MTT method · Mitochondria · Actin · Copper

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Introduction

Medulloblastoma is a very aggressive tumor of the central nervous system that is expressed primarily in childhood. Even after a good response to surgery and radiation, recurrence within 2 years is common. Medulloblastoma first appears in children of 5–9 years and to a lesser extent between 20 and 30 years [2]. It has a high rate of proliferation and potential to metastasize, even to extracranial sites including large bones. Medulloblastoma is treated by surgery, although it is never possible to extirpate 100% of the tumor without additional treatments by radiotherapy and/or chemotherapy [1].

Casiopeinas® (Cas) are a large family of molecules that has an active copper 2^+ core and an amino acid to seem give them tumoral specificity [14]. At date, the action mechanism is poor understanding; however, it has described that some metals such as copper and some complexes of them, are toxics by their high potential to participate in redox reactions. This event generates reactive oxygen species (ROS) [8], which could be responsible of the apoptotic phenomenon. Moreover, there is evidence that Cas IIgly has an apoptotic effect on rat glioma and it could be dependent or independent of caspases in agreement with the dose [18]. Currently, efforts to fight these cancers are focused on improving our understanding of the apoptotic phenomenon. Apoptosis is characterized by morphological and biochemical events such as the activation caspases, chromatin condensation and rearrangement at the nuclear periphery, DNA fragmentation, cell shrinkage, and the formation of apoptotic bodies [15].

In order to determine how Casiopeinas IIgly and IIIia influence apoptosis in medulloblastoma, we analyzed their effects on the medulloblastoma cell line Daoy.

Material and Methods

Reagents

Casiopeinas IIgly and IIIia were synthesized as previously described [14, 17] and dissolved in sterile water. All other reagents were of the highest quality commercially available.

Medulloblastoma Cell Culture

The cell line Daoy (American Type Culture Collection, HTB-186. Rockville, MD, USA) was maintained at 37°C in 5% CO_2 under sterile conditions in Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma) and treated for 24 h with increasing concentrations of cisplatin, Cas IIgly, or IIIia (0.1, 1, 10, 100 μg/ml) in 96 microplates. Cells were stained with sulphorhodamine-B [16], absorbance was quantified in a spectrophotometer at 560 nm (Lab-system Uniskan, Manchester, UK). The IC_{50} was determined using the PROBIT analysis program and the curve was simulated by fitting a first order exponential decay.

Cell Viability

Cell viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Roche Diagnostics, Mannheim, Germany). Briefly, cells (2×10^5 per well) were seeded in 24-well culture plates and preincubated for 24 h. After exposure to cisplatin (25 μg/ml), Cas IIgly (16 μg/ml), or IIIia (10 μg/ml) for 24 h at 37°C, 2 ml of MTT (0.1 mg/ml) was added, and cells were then incubated for 3 h at 37°C in darkness. The tetrazolium crystals were solubilized by the addition of 10% SDS in 0.01 N HCl, and the formazan blue formed from MTT was quantified in a spectrophotometer at 560 nm (Lab-system Uniskan, Manchester, UK).

Nuclear Condensation by Hoechst

Cells plated on coverslips at a density of 2×10^4 cells per square centimeter were cultured in DMEM medium supplemented with 10% fetal bovine serum. Cells treated with cisplatin (25 μg/ml), Cas IIgly (16 μg/ml), or IIIia (10 μg/ml) for 24 h were fixed in 4% fresh paraformaldehyde at 4°C for 30 min, then incubated with 0.01% Hoechst 33258 (Molecular Probes, Eugene, OR) in PBS for 10 min, and mounted with methanol–glycerol 1:1 (*v*/*v*). Cultures were examined with an UV objective (×100, glycerol immersion, 1.3 N.A.) in an epifluorescence microscope (Nikon, UV BA-2 filter and a barrier filter of 520 nm using 340 nm excitation and 460 nm emission wavelengths).

Mitochondrial Transmembrane Potential

Disruption of the mitochondrial transmembrane potential was evaluated by the fluorescence-based method MitoCapture™Apoptosis Detection Kit (Oncogene, USA). This assay distinguishes between healthy cells, which retain a cationic fluorescent dye that accumulates and aggregates in the mitochondria and gives off a bright red fluorescence, and apoptotic cells, which generate a green fluorescent signal due to their altered mitochondrial membrane potential. Briefly, 1×10^6 cells were treated with cisplatin (25 μg/ml), Cas IIgly (16 μg/ml), or IIIia (10 μg/ml) for 24 h, washed with PBS, and incubated at 37°C for 20 min with 1 μl MitoCapture™ reagent diluted into 1 ml of pre-warmed incubation buffer. After washing the cells twice with PBS, fluorescence was analyzed using a fluorescence microscope.

Cytoskeleton Analysis

Cells were grown on glass coverslips, treated with cisplatin (25 μg/ml) or Cas IIgly (16 μg/ml) for 24 h, rinsed in PBS, and then fixed with 3.7% formaldehyde (ultrapure grade, Tousimis Research Co., Rockville, MD, USA) at room temperature for 20 min. Cells were permeabilized by a 3-min treatment with 0.5% Triton X-100 and then blocked with 0.5% bovine serum albumin in PBS (PBS/BSA) for 30 min. After rinsing with PBS, cells were incubated with the primary antibodies diluted 1:50 in PBS/BSA for 1 h at 37°C. Cells were then rinsed with PBS, and the antibodies goat anti-mouse IgG tagged with FITC (Santa Cruz, CA, USA; sc-2010,) for actin identification (Santa Cruz, Ca; sc-8432) and goat anti-rabbit IgG tagged with Texas Red (1:200 dilution with PBS/BSA; Santa Cruz, CA; sc-2780) for ß-tubulin (Santa Cruz, CA; sc-9104) were added for 60 min at room temperature. After rinsing with PBS, the coverslips were mounted on glass slides in a quenching medium (Vecta Shield H-100, Vector Laboratories Inc. Burlingame, CA, USA) for observation in a confocal laser microscope (Bio Rad MRC-600, Watford, UK).

Results

Casiopeinas Inhibit Cell Proliferation and Produces Morphological Changes

Some morphological alterations were found, such as cells that were contracted, rounded, or showed blebbing and early formation of apoptotic bodies. In contrast, nontreated medulloblastoma cells remained morphologically unchanged (Fig. 1). We established the inhibitory concentrations (IC_{50}) of cisplatin and casiopeinas to be used in

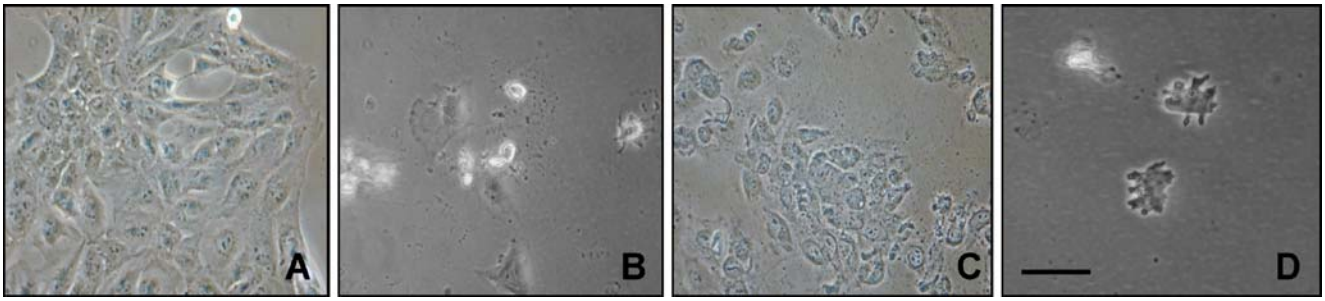

Fig. 1 Casiopeinas induce morphological alterations. *A* Control cells showing the typical morphology of medulloblastoma cells. *B* Cells treated with cisplatin exhibit changes such as contraction or loss of adherence. *C* Treatment with Cas IIgly showed mainly rounded cells. *D* Finally, blebbing was observed on cells were treated with Cas IIIia. Images from a light microscope at ×10. The *bar* shown indicates 10 μm

further experiments. Working concentrations for Daoy cells treated with the different drugs for 24 h were 25 μg/ml for cisplatin, and 16 μg/ml and 10 μg/ml for Cas IIgly and IIIia, respectively (Fig. 2). These results suggest that Cas IIgly and IIIia have an antineoplastic effect on medulloblastoma cells through their ability to inhibit cell proliferation, apparently by an apoptotic mechanism.

Viability by MTT Assay

Figure 3 shows the effect on cell survival tested by means of the MTT reduction assay. Cells with cisplatin had a lower survival rate (47.78%) than cells treated with Cas IIgly (52.75%) or Cas IIIia (59.24%).

The Hoechst Assay Showed Nuclear Condensation

Apoptosis was determined by nuclear morphology in cells treated with Hoechst 33258, a blue dye that intercalates between DNA bases. We studied the morphology of nuclei by means of fluorescence microscopy. Nuclei were considered apoptotic when they were brilliant, shrunken, and occasionally fragmented. As expected, both Casiopeinas increased the number of nuclei displaying apoptotic features; this phenomenon was observed mainly in cells treated with Cas IIgly (Fig. 4).

Casiopeinas Induced the Loss of Mitochondrial Membrane Potential

Because mitochondria manifest signs of outer and/or inner membrane permeabilization when exposed to a variety of proapoptotic drugs, we determined the mitochondrial membrane potential in medulloblastoma cells using the fluorescent dye Mitocapture® and analyzed it by fluorescence microscopy. For control cells (Fig. 5, A) there were not a markedly difference between healthy (Fig. 5, B), and apoptotic cells (Fig. 5, C). However, the majority of Daoy cells treated with cisplatin (Fig. 5, D–F) and casiopeinas IIgly (Fig. 5, G–I) and IIIia (Fig. 5, J–L) had reduced

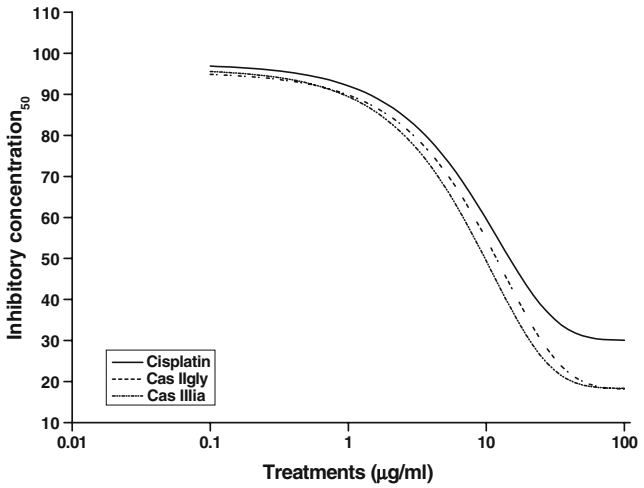

Fig. 2 Determination of inhibitory concentration (IC_{50}) for Casiopeinas. Cisplatin, Cas IIgly, and Cas IIIia were assayed for 24 h at 0.1 to 100 μg/ml in Daoy cells. The mean from three independent experiments is shown

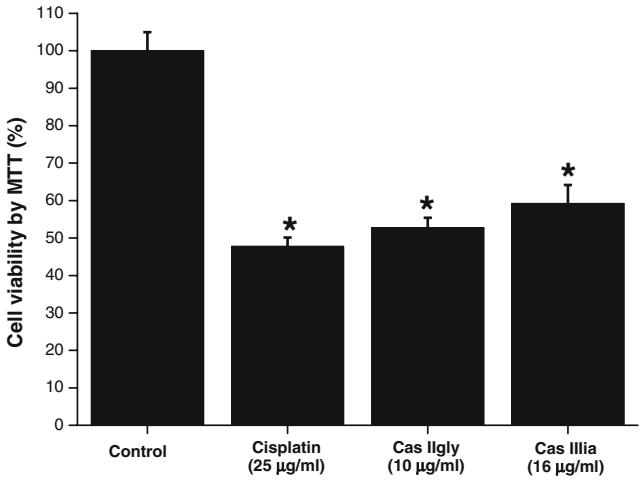

Fig. 3 Inhibition of cell viability by MTT assay. Each chemotherapeutic agent was applied at a concentration equal to its IC_{50}, and cell viability was determined. All treated cells showed lower survival than the control cells. Each *bar* represents the mean ± SD (*asterisks*, $p < 0.05$) of three independent experiments

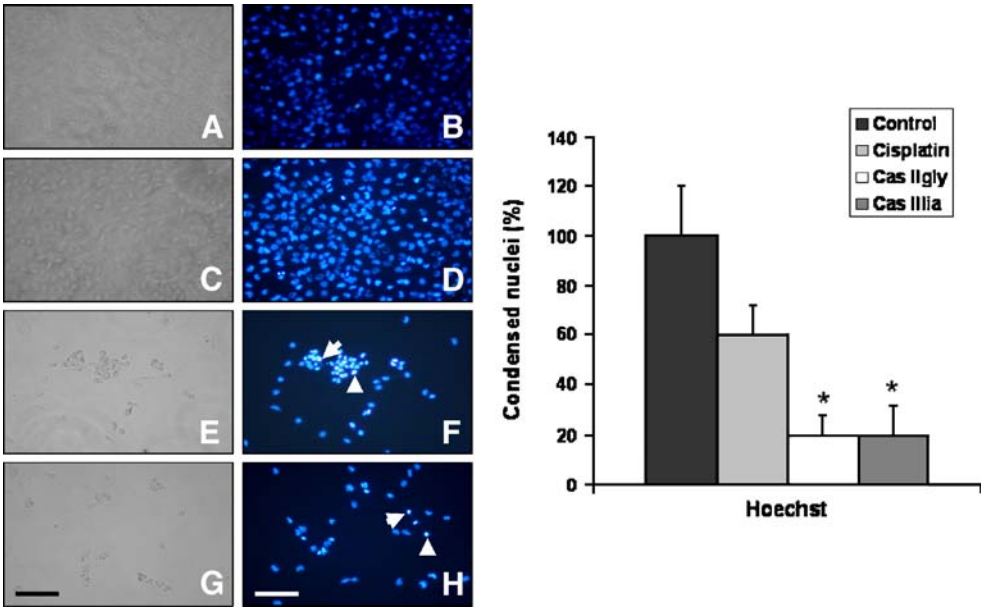

Fig. 4 Nuclear condensation in medulloblastoma cells. Cell morphology was visualized by light microscopy (*left line*) and fluorescence microscopy (*right line*) at ×10. *A*, *B* Control cells (without treatment) don't show condensed nuclei. All treatments caused chromosomal derangement with nuclear condensation. *C*, *D* cells with cisplatin (25 μg/ml); *E*, *F* Cas IIgly (10 μg/ml); *G*, *H* Cas IIIia (16 μg/ml). Images are representative of three independent experiments. The mean ± SD (*asterisks*, $p<0.05$) is shown for each condition

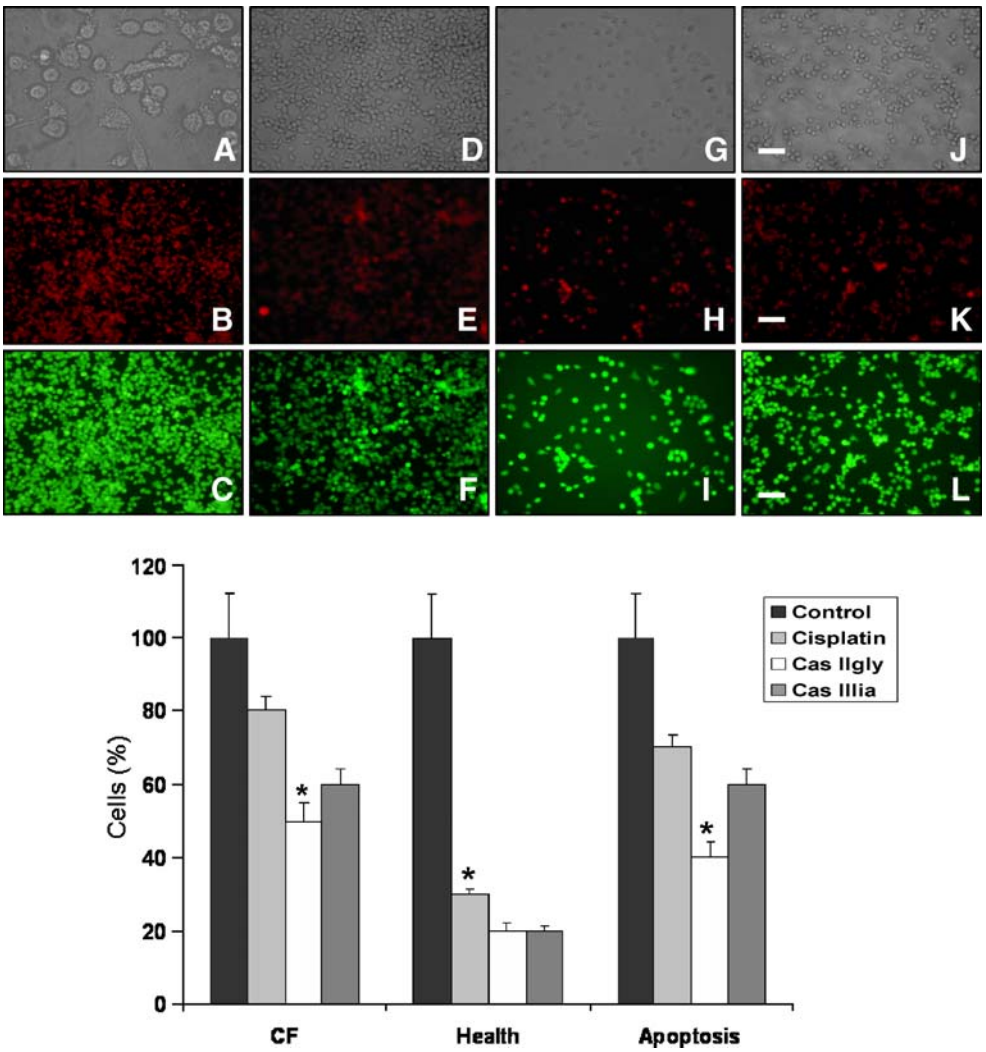

Fig. 5 Loss of mitochondrial membrane potential. Control (*A–C*) and 24-h-treated Daoy cells were visualized with a light microscope (*upper panels*) after treatment with a fluorescent dye sensitive to mitochondrial membrane potential. Healthy cells (middle panels) were identified by a red fluorescence, and a green color was present in apoptotic cells as condensed material (*lower panels*). *D–F* Treatment with 25 μg/ml cisplatin; *G–I* cells with Cas IIgly (10 μg/ml); *J–L* Cas IIIia (16 μg/ml). Images at ×10 are representative of three independent experiments (*bar* indicates 10 μm). The mean ± SD (*asterisk*, $p<0.05$) is shown for each condition

mitochondrial membrane potential, indicating that they were apoptotic (Fig. 5, F, I and L).

Casiopeina IIgly may Disturb the Cytoskeleton

The apparent close association between microtubules and condensed chromatin in late-apoptotic cells prompted us to assess whether microtubules contribute to apoptotic chromatin remodelling. We analyzed the apoptotic phenomenon by means of two cytoskeleton elements: actin and tubulin. In untreated medulloblastoma cells actin was present in the cytoplasm (Fig. 6*A*), whereas in cells treated with cisplatin or Cas IIgly actin was present mainly in apical deposits (Fig. 6*B*, *C*, respectively). On the other hand, tubulin was deposited along a central path in the cytoplasm in medulloblastoma cells, independently of time and treatment (data not shown).

Discussion

Only by understanding the biology of this tumor can the goal of curing children with medulloblastoma be achieved. Recent developments in cancer research suggest that a number of apoptotic stimuli share common mechanistic pathways characterized by the loss of mitochondrial membrane potential, with subsequent changes in outer mitochondrial membrane permeability and the release of apoptogenic factors [10, 19].

Casiopeinas are novel, copper-based chemotherapeutic agents that increased cell death by apoptosis in Daoy cells, and various mechanisms seem to be involved. Previous studies suggested that Casiopeinas can induce ROS [18] to bind DNA through interactions with adenine and thymine and can also block oxidative phosphorylation [11]. Together, these data indicate that there is more than one mechanism of action for Casiopeinas.

The IC_{50} determination showed that both Casiopeinas are effective at lower doses than cisplatin. Cellular viability, as determined by the MTT assay showed that Casiopeina IIgly and Casiopeina IIIia had antineoplastic effects on medulloblastoma cells; they inhibited cell proliferation, apparently by inducing apoptosis. Apoptosis also occurred in cells treated with cisplatin.

Apoptosis was first demonstrated by means of chromatin condensation with Hoechst 33258 staining. Only a few of the Daoy control cells were condensed; however, most of the cells treated with casiopeinas IIgly and IIIia showed condensed nuclei.

We found that mitochondrial membrane potentials were disrupted principally in cells treated with the two Casiopeinas. Mitochondria play a critical role in apoptosis caused by drugs such as chemotherapeutic and DNA-damaging agents [6, 7]. The elevated plasma and mitochondrial membrane potentials of tumor cells [3, 9] may enhance the selective targeting of Cas IIgly and Cas IIIia to tumor cells and mitochondria.

The generation of apoptotic bodies is considered to be an important step in the safe clearance of apoptotic corpses. Apoptotic cell fragmentation has previously been shown to require actin [5]. This was demonstrated by the apical localization of actin in all treated cells (including those treated with cisplatin and Casiopeinas). The data indicate that microtubules contribute to the process of apoptotic body formation by helping to sustain the peripheral localization of chromatin [12]. Apoptotic surface blebbing is driven by myosin-II-actin contractility, initiated by caspase cleavage of ROCK I [4]. For tubulin, however, we found that its condensed cytoplasmic distribution was maintained and was independent of time and treatment duration. This suggests that microtubule disruption allows chromatin to retreat back to the cell center, but some retractile forces are acting here. Recent evidence suggests that in actively blebbing cells, myosin-II-driven contraction of newly assembled cortical actin bundles also cooperates with plasma membrane tension and extracellular osmotic forces to withdraw surface blebs [13].

In conclusion, Casiopeinas IIgly and IIIia promise to be important compounds for the treatment of medulloblastoma, mainly by the induction of the apoptotic phenomenon.

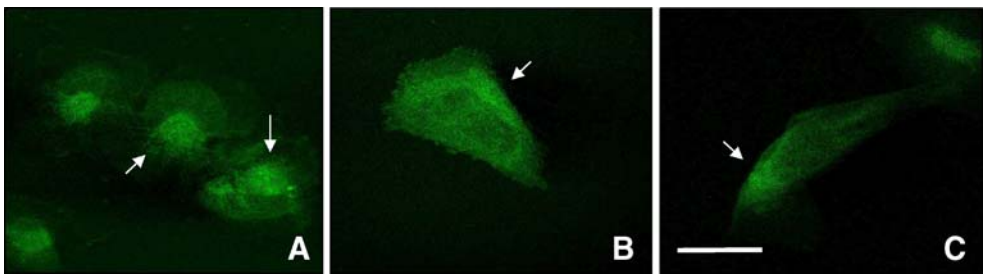

Fig. 6 Disruption of the cytoskeleton in Daoy cells. Actin was measured at 24 h in control and treated cells. *A* Actin distribution was a preferentially cytoplasmic in control cells. *B* Cells treated with cisplatin (25 μg/ml) *C* and Cas IIgly (10 μg/ml) exhibited an apical distribution of actin, as shown by *arrows*. Images by confocal laser microscope (Bio Rad MRC-600, Watford, UK) are representative of three independent experiments. *Bar* indicates 1 μm

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