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Significant decrease of ADP release rate underlies the potent activity of dimethylenastron to inhibit mitotic kinesin Eg5 and cancer cell proliferation ☆

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ABSTRACT

Eg5 is a mitotic kinesin that plays a crucial role in the formation of bipolar mitotic spindles, by hydrolyzing ATP to push apart anti-parallel microtubules. Dimethylenastron is potent specific small molecule inhibitor of Eg5. The mechanism by which dimethylenastron inhibits Eg5 function remains unclear. By comparing with enastron, here we report that dimethylenastron prevents the growth of pancreatic and lung cancer cells more effectively, by halting mitotic progression and triggering apoptosis. We analyze their interactions with ADP-bound Eg5 crystal structure, and find that dimethylenastron binds Eg5 motor domain with higher affinity. In addition, dimethylenastron allosterically blocks the conformational change of the "sandwich"-like ADP-binding pocket more effectively. We subsequently use biochemical approach to reveal that dimethylenastron slows ADP release more significantly than enastron. These data thus provide biological, structural and mechanistic insights into the potent inhibitory activity of dimethylenastron.

1. Introduction

Anticancer drugs that interfere with mitosis, such as taxanes and vinca alkaloids, have been widely used in the clinical therapy of human malignancies [1,2]. They target tubulin, a key protein component of the mitotic spindle. However, clinical use of these microtubule inhibitors often leads to dose-limiting toxicities, because microtubules are also involved in many other cellular processes, such as cell motility, maintenance of cell shape, and intracellular transport [3].

The kinesin family of motor proteins is emerging as an attractive target for specific anti-mitotic cancer therapies [4]. These kinesins are abundant in hyper-proliferative cells, i.e. cancer cells, and barely expressed in non-dividing cells [5]. As a member of the kinesin family, Eg5 (also known as kinesin-5 or kinesin spindle protein, KSP) plays essential roles in bipolar spindle assembly, by hydrolyzing ATP to push apart anti-parallel microtubules and separate the duplicated centrosomes [6–8]. Similar to other kinesins, Eg5 contains a motor domain, which mediates interactions with microtubule and ATP hydrolysis.

Specific small molecule inhibitors of Eg5 represent a new generation of chemotherapeutic compounds [9–11], some of which have been examined in phase I and II clinical trials [12,13]. Dimethylenastron, the dihydropyrimidine (DHPM)-derived analog, is developed as a potent inhibitor of Eg5 by Gartner et al. [14] in an initial SAR study. However, its biologic effects and mechanism of action are not well understood. In this study, by comparing with the analog enastron, we sought to determine the molecular basis for the potent Eg5 inhibition by dimethylenastron.

2. Materials and methods

2.1. Materials

Dimethylenastron and enastron were provided by Drs. Vasiliki Sarli and Athanassios Giannis (University of Leipzig, Germany).

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Sulforhodamine B (SRB), 4′-6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) were purchased from Sigma–Aldrich (St. Louis, MO, US). The Rabbit antibody against PARP was from Cell Signaling Technology (Beverly, MA, US) and the mouse monoantibody against GAPDH was from Santa Cruz Biotechnology (Santa Cruz, CA, US).

2.2. Cell culture

EPP85 human pancreatic cancer cells, PC9 and A549 human lung cancer cells (ATCC) were cultured in RPMI 1640 medium (Gibico, USA) supplemented with 10% fetal bovine serum (Gibico, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. In vitro cell proliferation assay

Cells grown in 96-well culture plates were treated with gradient concentrations of dimethylenastron or enastron for 48 h and were fixed with 10% trichloroacetic acid and stained with 0.4% SRB dissolved in 1% acetic acid. The cells were then washed with 1% acetic acid to remove unbound dye. The protein-bound dye was extracted with 10 mM Tris base to determine the optical density at 490 nm wavelength. The percentage of cell proliferation as a function of drug concentration was plotted to determine IC50, which stands for the drug concentration needed for 50% inhibition of cell proliferation.

2.4. Fluorescence microscopy

Cells grown on glass coverslips were fixed with cold (−20 °C) methanol for 5 min and then washed with phosphate-buffered saline (PBS), followed by staining with DAPI for 5 min. Coverslips were then mounted with 90% glycerol in PBS and examined with an Olympus fluorescence microscope.

2.5. Flow cytometry

$2 * 10^6$ cells were collected, washed twice with ice-cold PBS, and fixed in 70% ethanol for 24 h. Cells were washed again with PBS and incubated with PI (20 μg/ml) and RNaseA (20 μg/ml) in PBS for 30 min in the dark. Samples were analyzed on a BD FACSCalibur flow cytometer.

2.6. Western blot analysis

Proteins were resolved by polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). The membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 and 5% fat-free dry milk and incubated first with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce Biotechnology, USA).

2.7. Structural analysis

The coordinates of ADP–Eg5 structure (PDB: 1II6), ADP–Eg5–dimethylenastron structure (PDB: 2X7D) and ADP–Eg5–enastron structure (PDB: 2X7C) were obtained from the Protein Data Base [15,16] and subsequently analyzed.

2.8. Measurement of Eg5 ATPase activity

The ATPase activity of Eg5 was examined with the pyruvate kinase–lactate dehydrogenase detection system as described previously [5]. Changes in optical density were measured at a wavelength of 340 nm using a microplate reader (Molecular Devices, USA). The percentage of ATPase activity as a function of drug concentration was plotted to determine IC50, which stands for the drug concentration needed for 50% inhibition of ATPase activity.

2.9. Analysis of ADP release rate

The ADP release rate of Eg5 motor domain was examined with the MANT-ADP reagent (Molecular Probes), by measuring the changes in MANT-ADP fluorescence as described previously [5,17]. Briefly, purified Eg5 motor domain was incubated with the MANT-ADP racemate at a 1:1 stoichiometry. The Eg5–MANT-ADP complex was then mixed with 1 mmol/L MgATP in the absence of microtubules. The decrease in MANT-ADP fluorescence was examined over time at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The observed exponential rate constant was subsequently fit to a hyperbola to obtain the ADP release rate.

2.10. Statistical analysis

Experiments were repeated 3 times, and error bars represent standard deviations between experiments.

3. Results

3.1. Dimethylenastron displays higher anti-proliferative activity than enastron

To investigate the effects of dimethylenastron or enastron on the growth of pancreatic cancer cells, EPP85 human pancreatic cancer cells were treated with gradient concentrations of the two compounds respectively. Cell proliferation was measured by sulforhodamine B staining assay [18]and the IC50 values were then determined. As shown in Fig. 1A, dimethylenastron (DIMEN), with an IC50 of 0.37 μM, was about 10 times more potent than enastron (IC50 = 4.57 μM) in inhibiting the growth of EPP85 cells. To directly investigate the anti-proliferative activity of the two chemicals in lung cancer cells, we used phase contrast microscopic analysis to examine the cell morphology after 48 h drug treatment. We found that most of the PC9 lung cancer cells turned round and appeared fragmented in the presence of 1 μM dimethylenastron, whereas they proliferated normally upon 1 μM enastron treatment (Fig. 1B). Thus, dimethylenastron inhibits the growth of cancer cells more potently than enastron.

3.2. Dimethylenastron halts mitotic progression and triggers apoptosis more significantly than enastron

To test whether the dimethylenastron-induced growth inhibition was mediated by mitotic arrest, we examined cellular DNA in the drug-treated EPP85 cells with the DNA dye (DAPI) by fluorescence microscopy. As shown in Fig. 2A, 24-h dimethylenastron treatment led to a higher percentage (32.43%) of EPP85 cells with condensed chromosomes, while enastron exposure resulted in fewer cells blocked at mitosis (7.40%). Similar result was achieved by flow cytometric analysis of cellular DNA content in A549 lung cancer cells. As shown in Fig. 2B, 1 μM dimethylenastron promoted a significant increase in the number of G2/M phase cells with duplicated DNA content. In contrast, 1 μM enastron induced no obvious changes of cell cycle distribution. We used a higher concentration (5 μM) of enastron to achieve the similar effect of 1 μM dimethylenastron treatment. Therefore, these results show