BBA - General Subjects 1862 (2018) 1134–1147

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

The small molecule CS1 inhibits mitosis and sister chromatid resolution in HeLa cells

Xingkang Wu[a], Zhenyu Li[b], Yuemao Shen[a,c,*]

[a] Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, No. 44 West Wenhua Road, Jinan, Shandong 250012, PR China
[b] Department of Pharmacy, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, PR China
[c] State Key Laboratory of Microbial Technology, Shandong University, No. 27 South Shanda Road, Jinan, Shandong 250100, PR China

ARTICLE INFO

Keywords:
CS1
Mitotic arrest
Spindle assembly checkpoint
Sister chromatid resolution
Microtubule inhibitor
Histone phosphorylation

ABSTRACT

Background: Mitosis, the most dramatic event in the cell cycle, involves the reorganization of virtually all cellular components. Antimitotic agents are useful for dissecting the mechanism of this reorganization. Previously, we found that the small molecule CS1 accumulates cells in G2/M phase [1], but the mechanism of its action remains unknown.

Methods: Cell cycle analysis, live cell imaging and nuclear staining were used. Chromosomal morphology was detected by chromosome spreading. The effects of CS1 on microtubules were confirmed by tubulin polymerization, colchicine tubulin-binding, cellular tubulin polymerization and immunofluorescence assays and by analysis of microtubule dynamics and molecular modeling. Histone phosphoproteomics was performed using mass spectrometry. Cell signaling cascades were analyzed using immunofluorescence, immunoprecipitation, immunoblotting, siRNA knockdown and chemical inhibition of specific proteins.

Results: The small molecule CS1 was shown to be an antimitotic agent. CS1 potently inhibited microtubule polymerization via interaction with the colchicine-binding pocket of tubulin in vitro and inhibited the formation of the spindle apparatus by reducing the bulk of growing microtubules in HeLa cells, which led to activation of the spindle assembly checkpoint (SAC) and mitotic arrest of HeLa cells. Compared with colchicine, CS1 impaired the progression of sister chromatid resolution independent of cohesin dissociation, and this was reversed by the removal of CS1. Additionally, CS1 induced unique histone phosphorylation patterns distinct from those induced by colchicine.

Conclusions and significance: CS1 is a unique antimitotic small molecule and a powerful tool with unprecedented value over colchicine that makes it possible to specifically and conditionally perturb mitotic progression.

1. Introduction

Cell division is perhaps the most fundamental characteristic of cells and is essential to growth and development. The cell cycle, which is characterized by a series of events through which a cell produces two genetically identical daughter cells, can be divided into two main phases: interphase and mitosis [2]. Interphase, which is divided into four stages (G0, G1, S and G2) is required to prepare enzymes, chromatin, organelles, and cytoskeletal elements and to accomplish the necessary cellular reorganization for mitosis [3,4]. Although the duration of interphase, which accounts for approximately 90% of the total time occupied by the cell cycle, is highly variable, the duration of mitosis is short and remarkably constant [5]. Mitosis of mammalian cells, which usually occurs over a period of approximately 1 h, can be divided into four stages, namely prophase, prometaphase, metaphase, anaphase and telophase [2,4–6], based on the organization and behavior of the chromosomes [6]. During prophase, the chromatin fibers progressively condense into discrete chromosomes. After completion of prophase, the cells enter prometaphase, during which the chromosomes move back and forth. As prometaphase ends and metaphase begins, chromosome movements result in the congression of chromosomes at the cell equator. Shortly after metaphase alignment, the cell enters anaphase, the stage at which the sister chromatids separate and move to opposite poles of the cell. During telophase, the chromosomes arrive at the cell poles and decondense as the nuclear envelope reassembles around each set of chromosomes. After mitosis is accomplished, the sister cells remain connected by a thin bridge termed the midbody. Finally, the cell is split into two identical daughter cells by cytokinesis, the process

* Corresponding author at: State Key Laboratory of Microbial Technology, Shandong University, No. 27 South Shanda Road, Jinan, Shandong 250100, PR China.
E-mail address: yshen@sdu.edu.cn (Y. Shen).

https://doi.org/10.1016/j.bbagen.2018.01.012
Received 26 October 2017; Received in revised form 1 December 2017; Accepted 18 January 2018

through which abscission of the midbody results in complete separation of the sister cells [7].

Microtubules (MTs) are cytoskeletal polymers that are assembled from α/β tubulin heterodimers that are stacked head-to-tail into polar protofilaments that associate laterally to form single microtubules [8,9]. The ends of microtubules can dynamically switch between growing and shrinking phases, a hallmark behavior known as dynamic instability [8,9]. This property is essential for the function of microtubules, most notably during mitosis [6]. Microtubules initiate mitotic spindle formation during prophase. Following nuclear envelope breakdown at the beginning of prometaphase, the spindle microtubules gain access to chromosomes and attach to the kinetochores [10]. During prometaphase, the growing microtubule plus-ends contribute to the force by which the chromosomes are moved to the spindle equator [11]. During metaphase, every chromosome has at least two microtubules extending from its two kinetochores to each pole of the cell [12]. This prominent arrangement of MTs is referred to as the metaphase spindle [12] and was identified under the light microscope as early as the 19th century by Flemming [13]. At late anaphase, astral microtubules are anchored to the cell membrane and pull the two sets of chromosomes apart [14]. During telophase, the mitotic spindle disassembles [6]. Hence, in mitosis, microtubules form a highly dynamic apparatus that organizes and builds the spindle, a protein machine that segregates the duplicated chromosomes into two genetically identical parts.

Due to the short duration of mitosis and the dynamic events that occur during this phase of the cell cycle, dissection of the temporal and spatial mechanisms of this process poses a great challenge. Many methods of perturbing the progress of mitosis have been used in attempts to understand cell division. Although genetic approaches to this problem have been used most frequently, small molecules offer some distinct advantages. Genetic approaches commonly remove a protein in its entirety, whereas small molecules can be used to modulate protein function in a dose- and time-dependent manner without altering protein levels [15]. In particular, because many mitotic events occur on time scales of seconds to minutes, small molecules offer greater potential for rapid, temporal and reversible regulation of mitotic progression than genetic approaches. Indeed, the use of small molecules that target mitosis has been shown to be an extremely powerful approach to transient manipulation of mitotic processes [16–19]. Most antimitotic agents are microtubule-targeting agents (MTAs) that stabilize or destabilize MTs. The earliest-used antimitotic agent, colchicine (Col) [20], a microtubule-destabilizing agent, opened the path to the discovery of spindle fiber dynamics [16]. In addition, colchicine is widely used to arrest cells in metaphase for the detection of chromosome instability [21]. Another popular microtubule inhibitor, nocodazole (Noco), is frequently used to synchronize cells in mitosis to permit the identification of mitosis-specific signaling cascades [16,22,23]. Paclitaxel (PTX), a microtubule-stabilizing agent, has been used to dissect spindle checkpoint signals in conjunction with other antimitotic agents [16,24]. Fluorescent paclitaxel conjugates are being developed by Invitrogen Corporation as tools for imaging microtubule formation and motility in living cells. Overall, antimitotic agents have proven to be valuable tools in the elucidation of mitotic progression. Despite the great success of those agents, however, our understanding of mitotic progression is still limited. Therefore, there is an urgent demand for novel antimitotic small molecules that control the complicated events that occur during mitotic progression.

CS1 (Fig. 1A) was originally designed and synthesized by Chen et al. [25] and was initially identified as a potential inhibitor of human DNA topoisomerase IIα in a cell-free assay [1]. CS1 inhibits cancer cell growth with G2/M arrest when applied at concentrations in the 1–20 μM range and significantly suppresses MDA-MB-231 breast cancer xenograft growth, showing an inhibition rate of 62.3% in nude mice at a dose of 20 mg/kg [1]. Those results motivated us to determine the fundamental effect of CS1 on cell division. Strikingly, in this study, we found that CS1 acts as a reversible antimitotic agent in HeLa cells. CS1 induces mitotic arrest in a manner that is dependent on the spindle assembly checkpoint (SAC), which is activated by the perturbation of the spindle equator. This effect is attributed to the fact that CS1 inhibits microtubule polymerization in vitro and in cells by interacting with the colchicine-binding pocket of tubulin. We also evaluated the modulatory effect of CS1 on chromosomes in mitosis. CS1 was found to inhibit sister chromatid resolution and to induce histone phosphorylation patterns that are distinct from those produced by exposure of cells to colchicine, leading to unexpected mechanistic insights.

2. Materials and methods

2.1. Chemicals and antibodies

CS1 was synthesized according to the method of Chen et al. [25]. Detailed information on the antibodies, chemicals, dyes and commercial reagents used in this study is provided in the Supplementary Information (Tables S1–S3).

2.2. Cell culture and synchronization

HeLa cells were purchased from the Shanghai Institute of Cell Resource Center Life Science (Shanghai, China) and maintained in DMEM (Gibco) supplemented with 25 mM glucose, 10% fetal bovine serum (Biological Industries), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 37 °C incubator with 5% CO_2.

The method of cell synchronization was adapted from previous work [26]. For G1/S phase arrest, the cells were treated as follows, which is referred to as the double thymidine block (DTB) method. In brief, HeLa cells were cultured to ~30% confluency. After the first block for 14–16 h with 2 mM thymidine, the cells were released for 8–10 h. The cells were then blocked for 14–16 h with thymidine and arrested in G1/S phase. For mitotic phase arrest, cells were sequentially cultured after release from the second thymidine block and incubated with antimitotic agents to induce mitotic arrest. Mitotic cells were collected by mitotic shake-off and centrifugation.

2.3. Plasmids and siRNA transfection

EB3 cDNA was purchased from Vigenebio and subcloned into the *Nhe*I site of the pLL3.7 lentiviral vector using the Exo III-assisted ligase-free cloning method. For lentivirus production, 293T cells were transfected with lentiviral vectors and lentivirus-packing plasmids (PMDL/REV/VSVG) using the LipoFiter™ reagent. The virus-containing medium was collected 72 h after transfection and concentrated by PEG 8000 precipitation. For infection, a mixture of virus and fresh medium containing 10 μg/mL protamine sulfate was added to cells. The infectious medium was replaced with fresh medium 24 h later.

The siRNA transfections were performed using Oligofectamine™ according to the manufacturer's instructions. Control siRNA (ACGUGA CACGUUCGGAGAAdTdT) and Mad2 siRNA (GGAACAACUGAAAGAU UGGdTdT) were purchased from GenePharma.

2.4. Tubulin polymerization in vitro assay

Tubulin polymerization assays were performed using a Tubulin Polymerization Assay Kit according to the manufacturer's instructions. Standard assays were conducted at 37 °C in PEM buffer (80 mM PIPES, 0.5 mM EGTA, 2 mM $MgCl_2$, pH 6.9) containing 6.5 μM DAPI, 1 mM GTP, 15% glycerol, 30 μM tubulin, 1% DMSO and the indicated concentrations of agents in a total volume of 50 μL. Polymerization reactions were monitored in kinetic mode using a microplate reader fluorimeter (Synergy H1, BioTek, USA) to measure time-series fluorescence intensity (excitation at 360 nm, emission at 450 nm). The tubulin polymerization rate was calculated as $(F_C - F_S) / F_C \times 100\%$, where F_C is the fluorescence in the presence of 1% DMSO and F_S is the fluorescence in the presence of the indicated agents.