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HSP90 inhibitor CH5164840 induces micronuclei in TK6 cells *via* an aneugenic mechanism

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ABSTRACT

Heat-shock protein 90 (HSP90) is a promising druggable target for therapy of conditions including cancer, renal disease, and chronic neurodegenerative diseases. Despite the possible beneficial effects of HSP90 inhibitors, some of these agents present a genotoxicity liability. We have examined the mode of action of micronucleus formation in TK6 cells by a novel and highly specific HSP90 inhibitor, CH5164840, by means of an *in vitro* micronucleus test with fluorescence *in situ* hybridization (FISH), γH2AX staining to detect DNA damage, and microscopic observation of chromosomal alignment in mitotic cells. The percentage of centromere-positive micronuclei induced by CH5164840 (FISH analysis) was significant, but the percentage of centromere-negative ones was not, suggesting that induction of micronuclei was due to a mechanism of aneugenicity rather than DNA reactivity. This conclusion was further supported by the result of co-staining γH2AX and the apoptosis marker caspase-3; the predominant elevation of apoptotic γH2AX rather than non-apoptotic γH2AX indicated little involvement of DNA-reactivity mechanisms. Microscopic observation revealed asymmetric spindle microtubules and chromosomal misalignment of metaphase cells. These data indicated that CH5164840 causes spindle dysfunction that induces micronuclei. The risk/benefit ratio must be considered in the development of HSP90 inhibitors.

1. Introduction

The molecular chaperone heat-shock protein 90 (HSP90) is evolutionally conserved and ubiquitously expressed in mammalian cells. HSP90 plays an important role in the stabilization and functional activation of client proteins associated with wide-ranging signal transduction processes, including control of cell cycle and proliferation; thus, HSP90 is a potential target for antitumor drug development [1,2]. Several HSP90 inhibitors, including geldanamycin (GM) derivatives, exhibit only modest antitumor effects in clinical studies; therefore, combination therapy trials with antibody therapeutics have been started [3]. HSP90 inhibitors may also have beneficial effects in neurodegenerative disorders, such as Alzheimer's disease or polyglutamine diseases, by reducing the level of the abnormal form of protein that is causing the disease [4,5]. HSP90 inhibitors are also potential drug candidates for treatment of renal fibrosis, because they can suppress TGF-β1 signaling [6]. Better understanding of the toxicological profiles of HSP90 inhibitors is urgently required.

Since many client proteins of HSP90 are involved in regulating cell proliferation, HSP90 inhibitors might perturb normal cell physiology. Niikura et al. [7] demonstrated that 17-allylamino-17-demethoxygeldanamycin (17-AAG) alters the localization of centromere protein (CENP) to induce mitotic arrest, chromosome misalignment, and aneuploidy in HeLa cells and HCT116 human colon carcinoma cells. Micronuclei (MN) were produced in malignant cells in a mouse sarcomatosis model bearing Crocker's sarcoma ascites, after administration of 17-AAG [8]. On the other hand, Hernández et al. [9] reported that 17-AAG did not increase the proportion of aneuploid HCT116 cells.

CH5164840 is a potent non-GM-class HSP90 inhibitor [10]; it has high affinity for HSP90, binding to the N-terminal of the ATP-binding site [10]. Combination treatment of CH5164840 with trastuzumab, lapatinib, or erlotinib exhibited synergistic antitumor activity in animal models [11,12]. CH5164840 appears to be specific for HSP90: there was no inhibitory activity against 22 protein kinases and no significant competitive ATP binding to 400 kinases [11]. Thus, the compound is a useful test case for studying genotoxic risks related to HSP90 inhibition. In this study, CH5164840 was tested in the *in vitro* micronucleus test (MNT) with TK6 cells, and the result was positive. In order to elucidate the mode of action of induction of micronuclei (MN), the treated cells were examined with fluorescence *in situ* hybridization (FISH), γH2AX staining,

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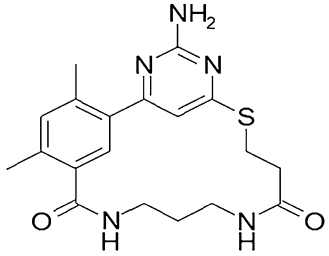

Fig. 1. Chemical structure of CH5164840.

and microscopic observation of chromosomal alignment in mitotic cells.

2. Materials and methods

2.1. Chemicals

CH5164840 (Fig. 1; molar mass 385.5) was synthesized by Chugai Pharmaceutical Co. The compound was ≥98% pure (liquid chromatography/mass spectrometry analysis). Methylmethanesulfonate (MMS, CAS 66-27-3, Sigma, St. Louis, MO, USA) was used as a reference compound. The chemicals were dissolved in dimethyl sulfoxide (DMSO, analytical grade, Wako Pure Chemical Industries, Osaka, Japan).

2.2. Cells

Human lymphoblastoid TK6 cells were purchased from American Type Culture Collection. The doubling time of the cells used in this experiment was approximately 13–14 h and the modal chromosome number was 47. Cells were cultured in RPMI-1640 medium (Gibco® Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco® Invitrogen), 1 mM sodium pyruvate (Gibco® Invitrogen), 100 units/mL penicillin–streptomycin (Gibco® Invitrogen) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Gibco® Invitrogen) at 37 °C in a humidified atmosphere with 5% CO_2. All experiments were performed using the cells in the exponential growth phase.

2.3. In vitro MNT combined with FISH analysis

In vitro MNT were conducted without using cytochalasin B. Due to low level induction of MN after treatment for 3 h with metabolic activation, in a preliminary study with L5178Y cells, the experiment was performed with 24 h treatment only. The experimental procedure was in accordance with the method for long-exposure treatment described in OECD Test Guideline 487 [13]. The cells were seeded in 12-well culture plates (Corning Costar 3513, Corning, NY) at a density of 6×10^5 cells/1.4 mL in each well. DMSO (vehicle control), MMS (1.5 mg/mL), or test compound was added (14 μL/well). Duplicate cultures were used for each concentration. After incubation for 24 h, the number of cells in each well was counted, and relative cell counts (RCC) and population doublings (PD) were calculated. Further MNT with FISH analysis was performed at the final concentration of 0.1, 0.3, or 0.5 μg/mL, which showed 63–51% RCC compared to the solvent controls.

For MNT, the cells were washed, centrifuged to attach them to glass slides, dried, fixed with methanol, and stained with acridine orange, 40 μg/mL. More than 1000 cells per culture well, a total of more than 2000 cells per concentration, were observed under a fluorescence microscope (BX60, Olympus, Tokyo, Japan). FISH analysis was performed as previously described [14]. A portion of the cells from the culture plate for MNT was centrifuged and re-suspended in KCl solution, 75 mM, fixed with methanol/acetic acid (3:1), and dropped onto glass slides. The cell specimens were treated with RNase (Nippon Gene, Tokyo, Japan), 0.2 mg/mL, and denatured in 2× SSC containing 0.5% Igepal CA-630 (Sigma). After denaturation at 75 °C for 7 min, specimens were hybridized with human pancentromeric α-satellite DNA probe conjugated with FITC (MP Biomedicals-Qbiogene, Irvine, CA) overnight at 37 °C. The slides were washed in 0.5× SSC containing 0.1% SDS at 72 °C for 5 min, stained with Hoechst-33257 (Sigma), dried, and mounted with anti-fade reagents (ProLong Gold, Molecular Probes, Eugene, OR). More than 60 MN for each concentration were examined under a fluorescence microscope (BX60, Olympus) for FISH signals.

2.4. Immunofluorescent staining of α-tubulin

Cells were treated with CH5164840, 0.5 μg/mL, under the same conditions as for the *in vitro* MNT. After being fixed with 4% paraformaldehyde followed by methanol and permeabilized with 0.2% Triton-X100, the cells were reacted with FITC-conjugated anti-α-tubulin antibody (1:500, Sigma) overnight at 4 °C. The cells were stained with Hoechst-33257 and centrifuged onto glass slides. The slides were dried and anti-fade reagents were added. The specimens were observed under a confocal laser microscope (FV1000, Olympus).

2.5. Immunofluorescent staining and flow cytometry analysis of γH2AX, cleaved caspase-3, and HSP70

Cells were seeded in 12-well culture plates (Corning Costar 3513) at a density of 7.5×10^5 cells/2.5 mL in each well. Test compound or MMS (1.5 mg/mL) was added, 25 μL/well. Duplicate cultures were used for each concentration. After incubation for 24 h, RCC was calculated and the doses of 0.05, 0.1, 0.3, 0.5, and 0.7 μg/mL, which showed 82–51% RCC compared to the solvent control, were selected for further assay. Some cells were used for concurrent MN observation. The remaining cells were divided into two: one to detect γH2AX and the other to measure HSP70, a marker of HSP90 inhibition [15]. Both cell samples were washed, fixed in 70% ethanol, and kept at −30 °C until use.

γH2AX immunostaining and flow cytometry analysis was performed as previously described [16]. Briefly, the fixed cells were first stained with anti-γH2AX mouse antibody (1:1000, Anti-phospho-Histone H2A.X [Ser139], clone JBW301; Upstate Biotechnology, Lake Placid, NY) and anti-caspase-3 rabbit antibody (1:200, Cleaved Caspase-3 Asp175, Cell Signaling Technology, Tokyo, Japan), secondary stained with anti-mouse antibody conjugated with R-PE (1:3000, Polyclonal Goat Anti-Mouse Immunoglobulins, Dako Cytomation, Glostrup, Denmark) and anti-rabbit antibody with Alexa Fluor 488 (1:500, Polyclonal Goat anti-Rabbit Immunoglobulins, Invitrogen), and finally with 4′,6-diamidino-2-phenylindole (DAPI). Approximately 5000 cells were analyzed using FACSAria (Becton Dickinson and Company, NJ) with filter sets of 530/30 nm bandpass for Alexa Fluor 488, 575/25 nm for R-PE, and 450/50 nm for DAPI. The percentage of cells with γH2AX fluorescence was determined by FACSDiva 6.3.1 (Becton Dickinson and Company) with a positive gate set to approximately 5% of the basal value of vehicle control. HSP70 immunostaining was conducted according to the same procedure as for γH2AX, except that fluorescein-conjugate HSP70 antibody (Stressgen Bioresgents, Kampenhout, Belgium) without a second antibody, was used instead of anti-γH2AX antibody. Fluorescence intensity was measured using FACSAria with a filter set of 530/30 nm, and the mean fluorescence intensity of each sample was determined by FACSDiva 6.3.1.

2.6. Statistics

Count of MN, centromere-positive MN (CMN), non-centromeric MN (NMN), mitotic cells, and the calculated frequency of CMN and NMN cells at each treatment dose were compared to those of the solvent control with the Chi-square test. Dose dependency was evaluated using the Cochran–Armitage trend test when a significant difference was observed.

3. Results

The results of *in vitro* MNT with FISH analysis are shown in Table 1. CH5164840 significantly induced MN at all concentrations used. Percentages of CMN to total MN were 51% in the vehicle control cells and 50% in the MMS-treated positive control cells. The percentage of CMN was 85%, 93%, and 94% at 0.1, 0.3, and 0.5 μg/mL of CH5164840, respectively, which was significantly higher than that of the vehicle control. CH5164840 increased the frequency of CMN cells per 2000 cells from 9.2 cells in the vehicle control to 40.8, 100.4, and 94.0 cells at 0.1, 0.3, and 0.5 μg/mL of the treatment concentration, respectively. No significant difference was seen in the incidence of NMN cells between the vehicle control and CH5164840 treatment. MMS increased the frequency of both CMN and NMN cells to 42 per 2000 cells. Percentages of mitotic cells were markedly increased from 1.3% to 6.2% after treatment with CH5164840, 0.5 μg/mL.

Metaphase cells were observed under a microscope to evaluate chromosome alignment. A few chromosomes remained from chromosomal alignment on the metaphase plate after treatment with CH5164840, whereas such misalignment was rarely seen in the control cells (Fig. 2). In order to examine the effects of CH5164840 on mitotic spindle formation, α-tubulin was stained with immunofluorescence. Representative images of α-tubulin in metaphase cells are shown in Fig. 3. Asymmetric spindle formation and blurry centrosomal foci were observed after treatment with CH5164840. An asymmetric spindle looks like a loosely-packed assembly of relaxed microtubules.

The cells treated with CH5164840 were stained for γH2AX, caspase-3, and HSP70 to examine direct DNA damage relating to the inhibition of HSP90. Each datum represents the mean