



Research Article

## D4476, a cell-permeant inhibitor of CK1, potentiates the action of Bromodeoxyuridine in inducing senescence in HeLa cells

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### Abstract

To elucidate the mechanism of bromodeoxyuridine (BrdU) induced cellular senescence, we treated HeLa cells with D4476, a potent and specific inhibitor of casein kinase 1(CK1). We found that D4476 (10 $\mu$ M) treatment could arrest cell growth at G1 stage and induced cellular senescence when treated together with BrdU (10 $\mu$ M). However neither D4476 nor BrdU can induce cellular senescence alone, at a concentration of 10 $\mu$ M. These results suggest that the targets of CK1 may be involved in maintaining normal cellular process and their inactivation potentiates BrdU to induce senescence like phenomena in HeLa cells.

**Keywords:** Senescence, BrdU, D4476, CK1, HeLa cell line

## INTRODUCTION

Senescence, also termed as biological aging, is a state of permanent growth arrest, during which cells are unable to re-enter the cell cycle (Rufini et al., 2013). During senescence, cells lose their capability to proliferate in response to growth factors or mitogens (Sherwood et al., 1988, Kuilman et al., 2010). Cellular senescence can be induced by various means such as oxidative stress, DNA damages, cell cycle perturbation, chromatin destabilization, and signaling imbalances (Herbig et al., 2005). CK1 (Casein kinase 1), included in the family of monomeric serine-threonine protein kinases, is found in eukaryotic organisms from yeast to human (Eide et al., 2001). To justify the senescent condition on cell cycle, CK1 is widely chosen due to its versatile physiological roles in living organisms. It is involved in many diverse and important cellular functions related to development processes, such as regulation of membrane transport, cell division, DNA repair and cell signaling (Knippschild et al., 2005, Gross and Anderson, 1998 and Price, 2006).

Among several CK1 inhibitors, D4476 is more potent and specific than IC261 or CKI-7. D4476 (4-[4-(2,3-dihydro-benzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl]benzamide) is identified as inhibitor of activin receptor-like kinase (ALK) 5, a member of the family of type-I TGF- $\beta$  receptors (Callahan et al., 2002, Rena et al., 2004 and Lehner et al., 2011). On the other hand, Bromodeoxyuridine (BrdU), a synthetic analog of thymidine, can cause mutation because of its ability to replace thymidine during DNA replication. Therefore, it is considered as potential health hazard but it is neither radioactive nor myelotoxic at labeling concentrations. It is widely preferred for *in vivo* studies of cancer cell proliferation and senescence (Fujimaki et al., 2006, Hoshino et al., 1985, Romagosa et al., 2011 and Michishita et al., 2002). Cell culture technique is widely observed *in vivo* in cancer lesions and physiological aging (Collado et al., 2005, Krishnamurthy et al., 2004, Liu et al., 2009, Sharpless, 2004, Nogueira et al., 2011 and Caldwell et al., 2012). HeLa cell line is widely used in the research of cancer, AIDS, the effects of radiation and toxic substances, gene mapping, and countless other scientific pursuits. In this experiment, D4467 and BrdU were applied individually and then jointly on HeLa cell

line to evaluate their effect on cellular growth, specifically on senescence. It is noteworthy that combined application of D4467 and BrdU provided synergistic effect on the inhibition of G1 stage of cell cycle resulting inducing cellular senescence whereas their individual administration did not show such stimulation on cell cycle inhibition determined by flow cytometry.

## MATERIALS AND METHODS

### *Cell culture and transfection*

HeLa cells were cultured at 37°C in plastic dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum under 5% CO<sub>2</sub> and 95% humidity (Michishita et al., 1999). The HeLa cells were treated with BrdU (10 $\mu$ M) or D4476 (10 $\mu$ M) alone or co-treated together with BrdU (10 $\mu$ M) and D4476 (10 $\mu$ M) for 4 days and then assayed.

### *Cell growth curve*

Twelve wells were plated with 2x10<sup>4</sup> cells/well. Cells from each well of the triplicate were trypsinized and counted daily. Then the mean number of cells/well was obtained every day from the triplicate average.

### *Northern blot analysis*

Total RNA samples (15 $\mu$ g per lane) were subjected to electrophoresis in 1% formaldehyde agarose and transferred to a nylon membrane (Hybond-N, Amersham). The blots were hybridized with <sup>32</sup>P labeled cDNA probes, washed twice at 65°C for 30 min in 2X SSC and 0.1% SDS and twice in 0.1X SSC and 0.1% SDS, and subjected to autoradiography (Michishita et al., 1999).

### *Flow cytometry*

Cells were harvested by trypsinization, washed with PBS, fixed in 70% ethanol, and incubated with 0.5 mg/ml RNase A for 30 min. The cells were stained with 50  $\mu$ g/propidium iodide for 15 min and analyzed by an EPICS XL flow cytometer (Coulter). Two types of signals, flashes of fluorescence indicative of relative DNA content per cell and forward light scattering indicative of relative cell size, were collected by the detector. Data were processed with installed software.

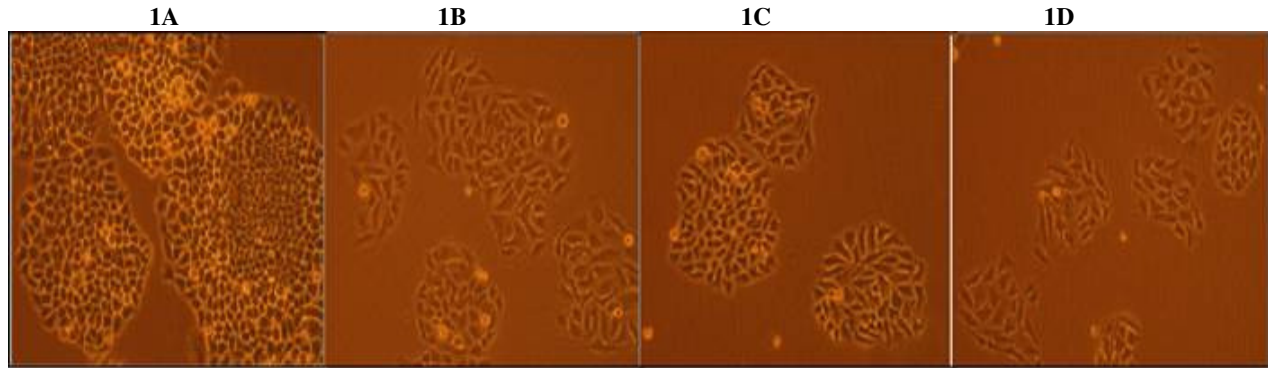
***β-Galactosidase assay***

Assay was performed as described previously (Michishita et al., 1999). Cells were fixed in 2% formaldehyde/0.2% glutaraldehyde at room temperature for 5 min, and incubated at 37 °C with a fresh staining solution [1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside, 40 mM citric acid-sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mMNaCl, and 2 mM MgCl<sub>2</sub>].

**RESULTS**

***Morphology and growth***

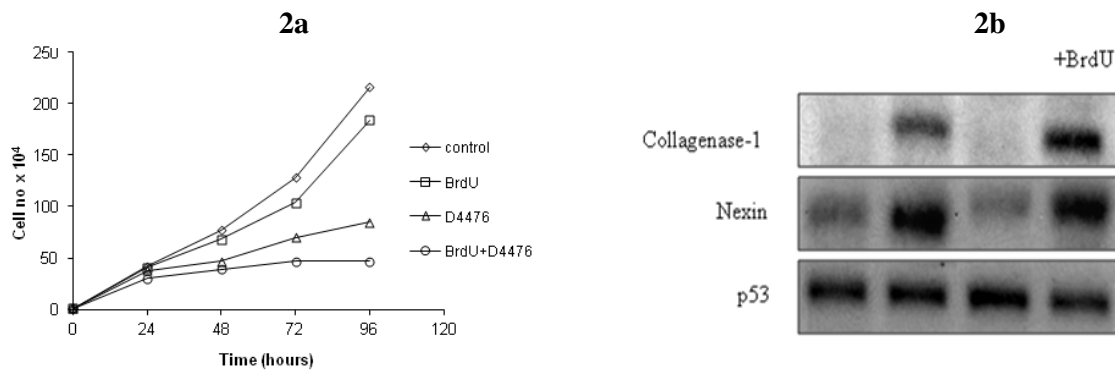
To test the effect of D4476 on growth, we treated HeLa cells with D4476 alone or together with BrdU. Untreated cells were more than 80% confluent after 96 hours, where as the growth of D4476 treated cells was strongly diminished with an overt morphological alterations (Figure 1).



**Figure 1.** Comparison of cell growth of HeLa cell line under treatment of BrdU, D4476, combined BrdU and D4476 as well as untreated control. Combined treatment of BrdU and D4476 showed more enlarged, flattened, and senescent like morphology of cells compared to only D4476 and BrdU treated cells.

However, the morphology of D4476 treated cells was different from BrdU treated or BrdU, D4476 double treated cells. BrdU, D4476 double treated cells showed more enlarged, flattened, senescent like morphology compare to only D4476 treated cells. To confirm the clear difference between untreated and D4476, BrdU

treated cells; we monitored cell number over time. Treatment of HeLa cells with D4476 led to a time dependent decrease in the growth rate of the cells over a period of 96 hours (Figure 2a).



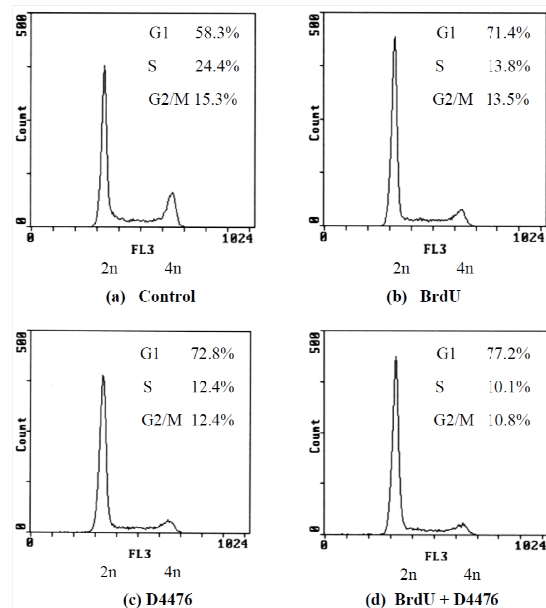
**Figure 2.** (2a) Comparison of cell count of HeLa cell line between control, BrdU, D4476 and double treated BrdU and D4476 sample. Cell counts were observed as follows: control (216 x 10<sup>4</sup>), BrdU (184 x 10<sup>4</sup>), D4476 (85 x 10<sup>4</sup>), Double treatment of BrdU and D4476 (47 x 10<sup>4</sup>), over a period of 96 hours. (2b) The inhibition of HeLa cell proliferation by D4476

or D4476 and BrdU double treatment by trypan blue exclusion. Cell growth inhibition was prominent while D4476 and BrdU treated combindly compared to single BrdU or D4476 treated cells.

The inhibition of growth was much more pronounced in D4476, BrdU double treated cells compare to only BrdU or D4476 treated cells. The inhibition of HeLa cell proliferation by D4476 or D4476 and BrdU double treatment was not caused by cytotoxicity because the cells remained viable as determined by trypan blue exclusion (Figure 2b).

#### *D4476 induces G1 growth arrest*

PI staining and FACS analysis were used to investigate cell cycle distribution of untreated and D4476, BrdU treated HeLa cells. D4476 treatment increased the fraction of cells in G1 phase from 58.3 to 72.8% after 96 hours (Figure 3). BrdU treatment also achieved similar percentage as 71.4% of G1 phase cells. However, when HeLa cells were double treated with D4476 and BrdU, 77.2% of the cells showed G1 phase arrest, whereas cells in the S and G2/M phase decreased to 14.3% and 4.5% compare with the untreated control.



**Figure 3.** Effect of the treatment of BrdU, D4476, combined BrdU and D4476 with control on cell cycle of HeLa cell line analyzed by Flow cytometry. Individual treatment of BrdU and D4476 increased the cell fraction in G1 phase from 58.3 to 71.4 to 72.8% after 96 hours, respectively (Figure 3a & 3b), whereas their combined

application showed 77.2% increase in G1 phase cell fraction in same duration (Figure 3d). Moreover, double treatment BrdU and D4476 decreased cells in the S and G2/M phase compared to untreated control.

## DISCUSSION

Senescence is inevitable for all living cells. Several factors including the efficacy of DNA repair mechanism and antioxidant enzymes as well as the rates of free radical production are considered as significant parameters of aging. The work was designed to confirm the role of D4476 to improve the function of BrdU in the induction of senescence. This paper demonstrates that the combined treatment of BrdU and D4476 on HeLa cells induces senescence more profoundly where their single treatments only induced cell cycle arrest at G1. A synergistic effect was observed in inducing senescence due to their combined treatment. Although, the use of BrdU in cellular senescence related research has been widely established but together with D4476 provided a new dimension in aging research. How can BrdU and D4476 jointly induce cellular senescence at a low concentration? Firstly, the multifaceted roles of CKI in DNA repair, cell signaling as well as cell cycle, should be counted. By inhibiting CKI, any CKI inhibitor like D4476 can increase G1 phase cell fractions and induce the inhibition of cellular growth. On the other hand, as a thymidine analog, BrdU can manipulate normal cell cycle activities at different stages. Therefore, the synergistic effect due to their collective application is an interesting one in senescence based study. Although their exact mechanism is still unknown but the unrevealed mechanism will initiate a new aspect in this field. Finally, we can propose that co-treatment of BrdU and D4476 may cause a defect in the DNA replication and gene expression systems and hence induce cellular senescence but further studies are needed to elucidate the actual mechanism.

## CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests in this paper.

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