RESEARCH ARTICLE

A New Cell Counting Method to Evaluate Anti-tumor Compound Activity

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Abstract

    Determining cell quantity is a common problem in cytology research and anti-tumor drug development. A simple and low-cost method was developed to determine monolayer and adherent-growth cell quantities. The cell nucleus is located in the cytoplasm, and is independent. Thus, the nucleus cannot make contact even if the cell density is heavy. This phenomenon is the foundation of accurate cell-nucleus recognition. The cell nucleus is easily recognizable in images after fluorescent staining because it is independent. A one-to-one relationship exists between the nucleus and the cell; therefore, this method can be used to determine the quantity of proliferating cells. Results indicated that the activity of the histone deacetylase inhibitor Z1 was effective after this method was used. The nude-mouse xenograft model also revealed the potent anti-tumor activity of Z1. This research presents a new anti-tumor-drug evaluation method.

Keywords: Cell counting method - nucleus image formation - anti-tumor

Introduction

    Determining cell quantity is a common problem in cytology research and anti-tumor drug development. For instance, an anti-tumor compound can incubate with cells in a 96-well plate; the cell quantity then reflects anti-tumor compound activity in the evaluation of the cell-proliferation inhibition rate of the compound. The bromodeoxyuridine (BrdU) method is commonly used to determine the quantity of DNA that reflects cell proliferation. However, this method is tedious and costly. The MTT method is also commonly used to determine cell proliferation; the succinic acid dehydrogenase in cell mitochondria can transform MTT into formazan, and the formazan quantity detected using the multi-function plate reader reflects cell proliferation activity. However, the mitochondrion quantities in the experimental and control groups may vary after drug treatment; thus, the MTT-transformation capability induced varies as well (Supplementary material Figure 1). Therefore, MTT data may not be able to reflect cell proliferation activity.

    A simple and low-cost method has been developed to reflect monolayer and adherent-growth cell quantity as an alternative to the tedious and costly BrdU method and the ineffective MTT method. The adherent-growth cell typically possesses the contact-inhibition property. The cells stop growing when density is heavy. Counting cells through image analysis is difficult after the entire cell is dyed because the cell density is too heavy and the cells are contiguous with one another. The number of cells cannot be accurately calculated after dyeing because the color stains all of the cells as a result of the contiguity. However, the cell nucleus is located in the cytoplasm; thus, it is independent and cannot make contact even if the cell density is heavy. This phenomenon is the foundation of accurate cell-nucleus recognition. The cell nucleus is easily recognizable in the image after dyeing because it is independent. Therefore, this method can be used to detect cell proliferation activity.

    Histone deacetylases (HDACs) are a class of enzymes that catalyze the deacetylation of lysine residues from the ε-N-acetyl lysine on histone. The acetylation and deacetylation of histones and non-histone proteins by histone acetylases and HDACs play important roles in gene expression regulation and transcription (Chueh et al., 2014; Dhar et al., 2014). The effects of acetylation and deacetylation include signal transduction, protein phosphorylation, cell cycle, proliferation, apoptosis, and cardiac development (Wang et al., 2014; Zhang et al., 2014). The overexpression and aberrant recruitment of HDACs play a significant role in tumorigenesis (Okudela et al., 2013). The histone deacetylase inhibitor (HDACi) alters gene expression by blocking HDAC action and inducing the hyperacetylation of histones (Thiagalingam et al., 2003). A number of structurally diverse HDACis have shown potent anti-tumor efficacy in various stages of

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clinical trials. Suberoyl anilide hydroxamic acid (SAHA) (Richon et al., 1998) and FK228 (Ueda et al., 1994) have been approved by the Food and Drug Administration (FDA) for use in the treatment of advanced cutaneous T-cell lymphoma. And HDACi can be potential therapeutic drugs for several cancers, such as cholangiocarcinoma, Osteosarcoma and pancreatic cancer (Cheng et al., 2012; Sriraksa and Limpaiboon, 2013; Feng et al., 2014). Histone deacetylase inhibitor trichostatin A also can enhances antitumor effects of chemotherapeutant docetaxel and erlotinib (Zhang et al., 2012). Therefore, evaluating the potency of HDACi in new drug development is important.

A new cell counting method has been developed recently according to the idea that the cell nucleus is independent in adherent cells. The evaluation of HDACi activity in the Z1 compound using this method is effective; thus, it is worth further development. However, this method reflects cell quantity based on the cell nucleus; therefore, it can only be used in monolayer and adherent-growth cells.

Materials and Methods

Chemicals

The chemical structures of Z1, Z2 and SAHA are shown in Figure 2A. Compound Z1 and Z2 were synthesized in our project group. SAHA was obtained from Apeloa Kangyu Phar-maceutical Co. Ltd., Zhejiang, China.

Supplementary material Figure 1. Images of Various Groups of Cells After MTT Incubation. The MTT-transformation capabilities in the control group. **A)** and the drug-treated group. **B)** are different. Bar=50 μm

Cell culture

Human prostate carcinoma cells PC3, human colon carcinama cells HCT116, human ovarian carcinoma cells 3AO and MDA-MB-231, human hepatocarcinoma cell line H7402, and human leukemic monocyte lymphoma cell line U937 were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS). These cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.
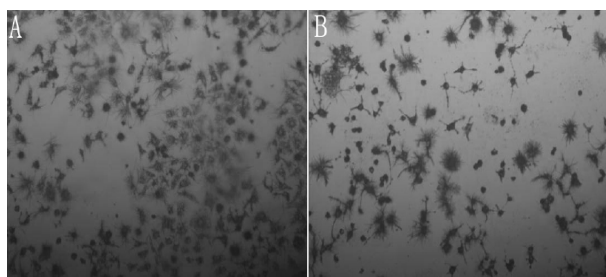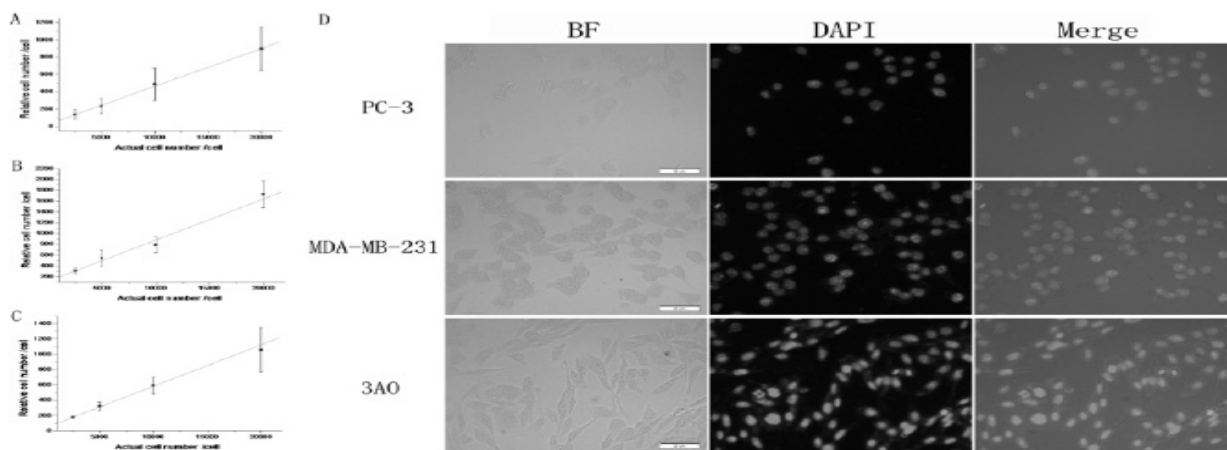
Cell counting method

The cell culture was removed from 96-well plates and 100 μL alcohol was added. After 10 min, the cells in each well were incubated with 1 μg/mL 4', 6-diamidino-2-phenylindole (DAPI) for an additional 5 min. Five random field images (100×) were taken using the Nikon microscope X51. Image-Pro Plus software was used to count the number of cells.

In vitro Antiproliferative Assay. Briefly, 2, 000 cells per well of PC3, HCT116, H7402 and MDA-MB-231 cells were seeded in 96 well plates and allowed to grow for 4 h, and the compounds were added. After treatment for two days, the number of cells was counted using the cell counting method.

In vitro HDAC Enzyme Inhibition Assay. In vitro activity assay against Hela cell nucleus extract has been described in our previous work (Zhang et al., 2010). Briefly, Hela cell nucleus extract solution (10 μL) was mixed with various concentrations of compound sample (50 μL). Fluorogenic substrate (40 μL) was added, and after 30 min of incubation at 37°C, the reaction was stopped by addition of developer (100 μL) containing trypsin and TSA. Fluorescence intensity was measured after 20 min of reaction using a microplate reader at excitation and emission wavelengths of 390 nm and 460 nm. The IC_{50} values were determined based on the fluorescence intensity readings.

In Vivo Anti-tumor Assay against U937 Xenograft. In vivo anti-tumor efficacy study was performed using nude mice xenagraft model as described previously. Briefly, 2*107 U937 cells were inoculated subcutaneously in the right shoulder of female athymic nude mice (4-5 weeks old, Slac Laboratory Animal, Shanghai). Six days

Figure 1. Cell Counting Method. Different numbers of PC3 **A)**, MDA-MB-231 **B)**, and 3AO. **C)** cells per well were seeded in 96-well plates. The cell counting method was used to obtain the relative number of cells. A linear dependence between the actual number of cells and the relative number of cells was observed, and a one-to-one relationship between the nucleus and the cell was present (D)

after injection, tumors were palpable and mice were randomized into treatment and control groups (5 mice per group). The treatment groups were administrated with 100 mg/kg/d intragastricly, the control group was administrated with equal volume of PBS solution. During treatment, subcutaneous tumors were measured with a vernier caliper every three days, and body weight was monitored regularly. Tumor volumes (V) were estimated using the equation ($V = ab^2/2$, where a and b stand for the longest and shortest diameter, respectively). After 16 days of administration, the mice were executed and the tumor weight was measured by an electronic balance. This work was in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Results and Discussion

Cell quantity is essential in numerous assays, especially in anti-tumor drug development. Anti-tumor compound activity is reflected in the effective determination of cell quantity through nucleus dyeing according to the idea that the nucleus is independent in this study. A one-to-one relationship between the nucleus and the cell is observed; thus, this method can reflect cell proliferation activity and can be used in anti-tumor drug development.

Different numbers of PC3, MDA-MB-231, and 3AO cells per well were seeded in 96-well plates. The cell counting method was used to determine the number of cells after adherence. Five random field images of the well (100×) were taken to count the number of cells because an image of the entire well could not be obtained. The mean represents the relative number of cells. However, a linear dependence between the actual number of cells and the relative number of cells was observed (Figure 1A), and a one-to-one relationship between the nucleus and the cell exists (Figure 1B).

The MTT method is based on MTT disoxidation. The fumarate reductase in live cells deoxidizes yellow MTT and causes it to become insoluble amethyst formazan. MTT cannot be deoxidized in dead cells because fumarate reductase is not present. The MTT method is commonly used in several laboratories to determine cell growth curve at present. However, the MTT method has an insuperable weakness: Formazan production is affected by treatment time. The absorption value changes with time when the number of samples is excessive, thus increasing the occurrence of experiment error. The procedure for suspension cells after MTT dyeing involves the removal of supernatant after centrifugation, formazan lysing with dimethyl sulfoxide (DMSO). Agents such as DMSO are toxic to humans when used in experiments (Tesei et al., 2005), and MTT deoxidation capabilities are varied in groups treated with different drugs (Supplementary material Figure 1). Therefore, MTT data may not reflect actual cell-growth conditions. DNA detection is used to reflect cell proliferation activity in methods such as BrdU. However, the procedure is tedious and costly. The method proposed in this research has a simple procedure, the agent used is inexpensive, and cell counting is convenient.

The cell counting method was used to evaluate HDACi activity. The relative number of cells was obtained using the cell counting method after the PC3, HCT116, H7402, and MDA-MB-231 cells were incubated for 2 d. The inhibition rate indicated that the compound Z1 was active (Figure 2B). An enzymatic inhibition assay was also performed on a Hela cell-nucleus extract that mainly contained HDAC1 and HDAC2. The IC_{50} value indicated that the inhibitory activity of Z1 was potent (Figure 2C). A nude-mice xenograft model was used to evaluate anti-tumor activity in vivo. The mice were executed after 16 days of intragastric administration, and the tumors were weighed. The tumor weights and volume data indicated that Z1 was more active compared with Z2 and SAHA (Figures 3A, 3B, 3C, and 3D). The lack of body weight loss observed in the mice reveals that all of the compounds are safe (Figure 3E). No evident toxicity was detected in the livers and spleens (data not shown).

However, this method involves nucleus dyeing and image formation to determine the number of cells based on the number of nuclei. Therefore, this method can be used on adherent cells but not on suspension cells. Moreover, this method cannot be used on multilayer-growth cells. Nucleus morphology in some cell lines may also change after drug treatment, as observed during the experiment. This phenomenon may affect the resultant count. The cells
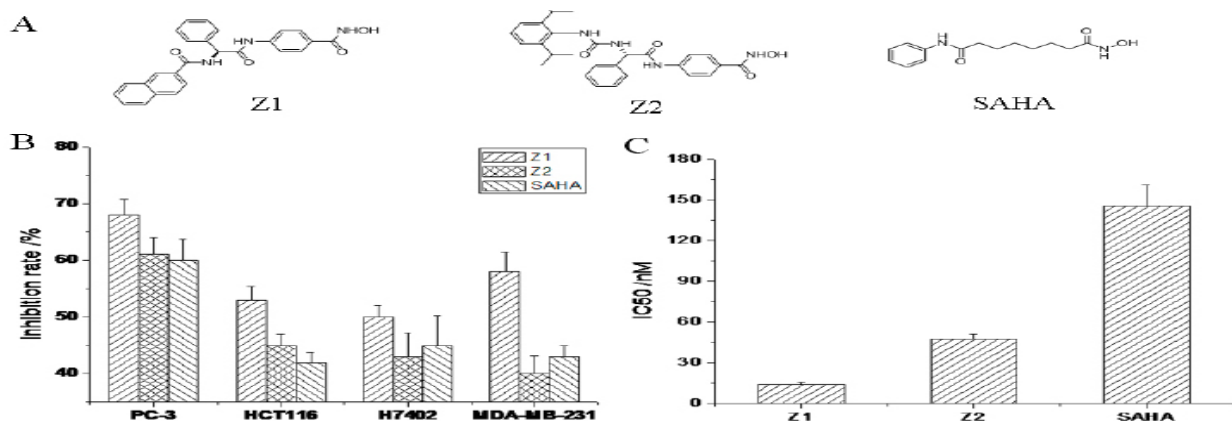

Figure 2. Activity Evaluation of HDAC Inhibitors. A) HDACi chemical structure. **B)** Determination of tumor-cell proliferation using the cell counting method after compound treatment. The treatment concentration for PC3 cells was 5 μM, and the treatment concentration for other cells was 2 μM. **C)** An enzymatic inhibition assay was performed on a Hela cell-nucleus extract that mainly contained HDAC1 and HDAC2. The IC_{50} values were determined based on the fluorescence-intensity readings
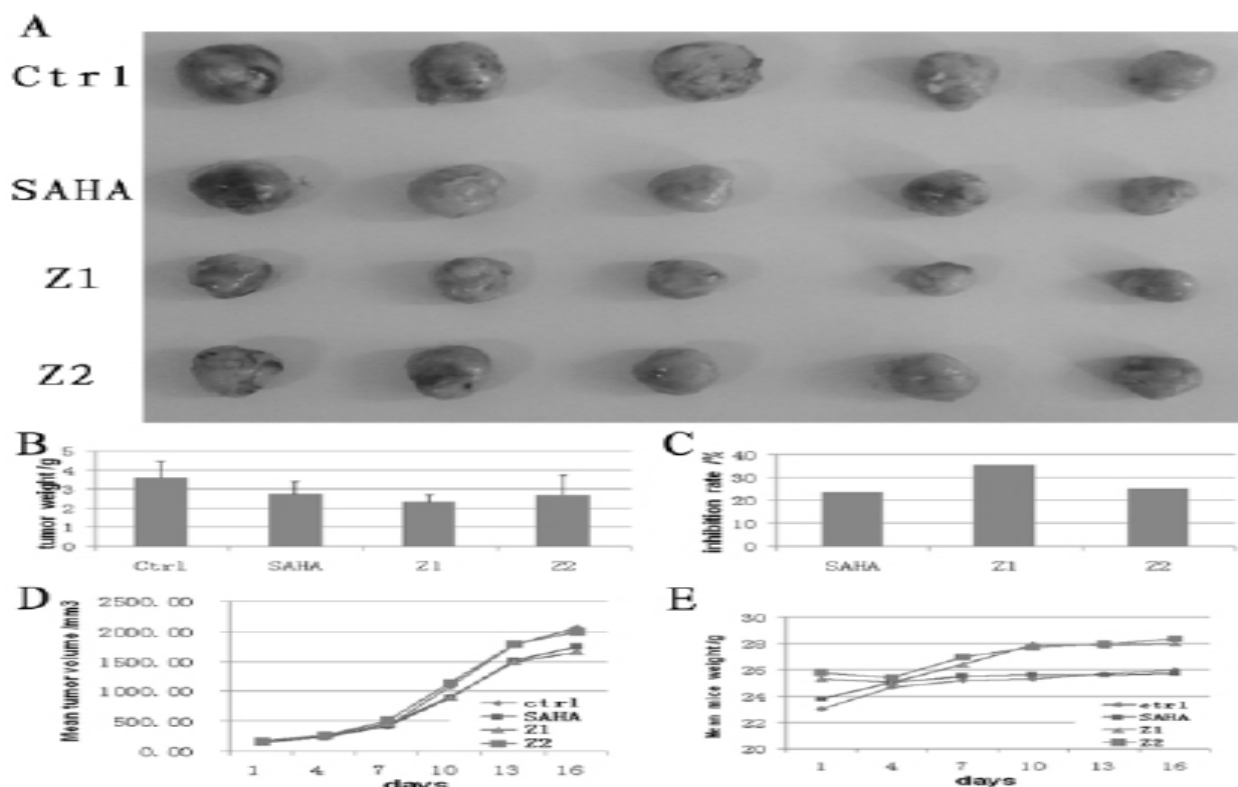
Figure 3. HDACi Anti-tumor Activity Against U937 Human-tumor Xenografts Implanted in Nude Mice. The mice were executed after 16 days of intragastric administration, and the tumors were weighed. The tumor volumes and body weights were measured every three days. **A)** Dissected tumor tissues. **B)** Tumor-weight plot. **C)** Inhibitory-ratio plot. **D)** Tumor-volume plot. E: Nude-mice-body-weight plot. Supplementary material Figure 1 Images of various groups of cells after MTT incubation. The MTT-transformation capabilities in the control group (A) and the drug-treated group (B) are different. Bar=50 μm

are counted manually at present mainly with the help of the Image-Pro Plus software; thus, this method is very convenient if software is used to perform image analysis and cell counting.

In conclusion, this research presents a cell counting method that reflects cell proliferation activity. This method can be used in the evaluation of HDACi activity. The results indicated that Z1 compound activity was effective when this method was used. The nude-mice xenograft model also revealed the potent anti-tumor activity of Z1. This research presents a new anti-tumor drug evaluation method.

Acknowledgements

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