False HDAC Inhibition by Aurone Compound

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Fluorescence assays are useful tools for estimating enzymatic activity. Their simplicity and manageability make them suitable for screening enzyme inhibitors in drug discovery studies. However, researchers need to pay attention to compounds that show auto-fluorescence and quench fluorescence, because such compounds lower the accuracy of the fluorescence assay systems by producing false-positive or negative results. In this study, we found that aurone compound 7, which has been reported as a histone deacetylase (HDAC) inhibitor, gave false-positive results. Although compound 7 was identified by an in vitro HDAC fluorescence assay, it did not show HDAC inhibitory activity in a cell-based assay, leading us to suspect its in vitro HDAC inhibitory activity. As a result of verification experiments, we found that compound 7 interferes with the HDAC fluorescence assay by quenching the HDAC fluorescence signal. Our findings underscore the faults of fluorescence assays and call attention to careless interpretation.

Key words fluorescence assay; histone deacetylase (HDAC); drug discovery screening; enzymatic activity

The evaluation of enzymatic activity is essential for understanding the biology of an enzyme or identifying its inhibitors. At present, in vitro or cell-based fluorescence assays are often used for the estimation of enzymatic activity, or for high-throughput screening for drug candidates.1–4) For example, substrates bearing a fluorophore are available for estimating the activity of trypsin or caspase-3 or searching their inhibitors5–7) (Figs. 1A–C). Trypsin or caspase-3 recognizes a particular amino acid or amino acid sequence in the fluorescent substrates and specifically cleaves the peptide bond of the C-terminus to release a fluorophore, such as 7-amino-4-methylcoumarin (AMC, 1) (Fig. 1A). Because N-acylated AMC (2) does not emit strong fluorescence, the detected fluorescence should correspond to the enzymatic activity (Figs. 1A–C); in this way, the enzymatic activity or inhibitory activity can be evaluated. Such assay systems based on fluorometric methods are frequently utilized in drug discovery studies.

Assay systems based on fluorescent substrates are often used for evaluating the activity of histone deacetylases (HDACs).8–10) HDACs, which catalyze the deacetylation of acetylated lysine residues in histone or non-histone proteins, are an interesting molecular target of cancer therapeutic agents.11) Many HDAC inhibitors have been identified11,12) and some have been approved by the Food and Drug Administration (FDA). Examples include vorinostat (S)13) and romidepsin (E)14) for cutaneous T-cell lymphoma; belinostat (S)15) for peripheral T-cell lymphoma; and panobinostat (E)16) for use in the combination therapy for recurrent multiple myeloma with bortezomib and dexamethasone (Fig. 2). However, problems persist regarding the approved HDAC inhibitors, including adverse effects and limited additional indications. Therefore, explorative studies of novel HDAC inhibitors should be performed. To this end, fluorescence assays will be used in increasing frequency.

Typical HDAC fluorescence assay systems are coupled assays with trypsin or lysyl endopeptidase, as shown in Fig. 1D.8) Firstly, an HDAC removes the acetyl group of lysine attached to a peptide substrate to generate an unmodified lysine residue (Fig. 1D, Step 1). Then, trypsin or lysyl endopeptidase in developer reagents recognizes the unmodified lysine residue and catalyzes the cleavage of the peptide bond to release a fluorophore (Fig. 1D, Step 2). Finally, HDAC activity is estimated by detecting the fluorescence intensity of the fluorophore. Because of the simple operation and the reliability of the results, this system is widely used and has indeed contributed to the discovery of many HDAC inhibitors. However, auto-fluorescent or fluorescence quenching compounds could interfere with the system and the inhibition of trypsin/lysyl endopeptidase could yield false results. Therefore, in this assay system, researchers need to pay attention to the spectroscopic properties and the trypsin/lysyl endopeptidase inhibitory activity of the tested compounds.

In the course of our studies of HDAC inhibitors, we realized that aurone compound 717) (Fig. 3A), which is one of the compounds reported as an HDAC inhibitor, did not show HDAC inhibitory activity in cell-based assays. Therefore, we examined the HDAC inhibitory activity of compound 7 in the reported fluorometric assay and carried out the assay in our laboratory. As a result, we found that the in vitro HDAC inhibitory activity of compound 7 is a false-positive result. Herein we present the processes undertaken to reach this conclusion.

Results and Discussion

Compound 7 was identified as an HDAC inhibitor from among a series of natural and synthetic aurone derivatives.17) Compound 7 was reported to show HDAC inhibitory activity in an in vitro fluorometric enzyme assay with HeLa nuclear extract containing HDACs or with recombinant HDAC1,18,19) HDAC2,20,21) or HDAC622,23) (Table 1). Because some of the aurone analogs have unique chemical reactivities24) and show anticancer or neuroprotective activity in cells or in vivo...
studies,25–27) we were interested in the relationship between its HDAC inhibitory activity and its biological effects (Fig. 1D). Therefore, we investigated the biological activity of compound 7 in detail.

We initially tested the activity of compound 7 against HeLa cell proliferation. As shown in Fig. 3B, compound 7 inhibited HeLa cell proliferation strongly, although its activity was lower than that of vorinostat (3). Therefore, compound 7 was expected to penetrate the cell membrane and show some effects in cells. Next, the HDAC inhibitory activity of compound 7 in cells was evaluated by means of Western blot analysis. In general, HDAC inhibition induces histone and non-histone protein acetylation.28–30) Accordingly, we attempted to measure increases in histone H3 lysine 9 acetylation (H3K9Ac) levels in HeLa cells treated with compound 7. However, contrary to our expectation, the treatment with \( \leq 50 \mu M \) of compound 7 for 3 or 6 h did not increase H3K9Ac levels in HeLa cells (Fig. 3C). Then, considering the possibility that compound 7 could inhibit HDAC6 in cells, we examined the effect of compound 7 on acetylated \( \alpha \)-tubulin, one of the HDAC6 substrates.31) As shown in Fig. 3C, compound 7 did not also increase acetylated \( \alpha \)-tubulin levels. In contrast, treatment of HeLa cells with 0.2 \( \mu M \) vorinostat (3), the positive control, induced an increase in the levels of both H3K9Ac and

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*Fig. 1. (A) Chemical Structures of 7-Amino-4-methylcoumarin (AMC, 1) and \( \text{N} \)-Acyl AMC (2); (B) Typical Fluorescence Assay System for Trypsin Activity; (C) Typical Fluorescence Assay System for Caspase-3 Activity; (D) Typical Fluorescence Assay System for HDAC Activity

*Trypsin recognizes basic amino acid residues, arginine or lysine, and cleaves the peptide bond of the C-terminus. *Developer probably contains trypsin or lysyl endopeptidase.
acetylated α-tubulin (Fig. 3C). These results indicate that compound 7 does not strongly inhibit HDACs, including HDAC6, in cells.

From the above-mentioned results, we thought that the reported results in in vitro enzyme assays might be false-positive results. Specifically, we hypothesized that compound 7 might inhibit the developer reagent or quench the fluorescence of a fluorophore (Fig. 1D). To verify our hypothesis, we performed the following experiments.

Firstly, we confirmed the reproducibility of the reported results. According to the reported experimental method, we used a commercially available assay kit, which is a general HDAC fluorescence assay system shown in Fig. 1D. As shown in Table 1, the HDAC inhibitory activity of compound 7 in our test was almost equal to the reported result (reported, HDACs IC$_{50}$=8.2 µM; our study, 10.9 µM). Then, we investigated the possibility that compound 7 inhibits the developer reagent by using a deacetylated standard substrate corresponding to an HDAC assay substrate. The IC$_{50}$ value against the developer reagent was nearly the same as that against HDACs in our study and the reported ones against HDACs, HDAC1, HDAC2, and HDAC6 (Table 1). These indicate that the HDAC inhibitory activity of compound 7 is a false-positive result.

This false-positive result should be caused by the quenching of fluorescence signal and/or by the inhibition of trypsin/lysyl endopeptidase in the developer reagent (Fig. 1D).

Fig. 2. Chemical Structures of Representative HDAC Inhibitors
Vorinostat (3), romidepsin (4), belinostat (5), and panobinostat (6).

Table 1. HDAC, HDAC1, HDAC2, HDAC6, and Developer Inhibitory Activities of Compound 7

<table>
<thead>
<tr>
<th></th>
<th>HDACs</th>
<th>HDAC1</th>
<th>HDAC2</th>
<th>HDAC6</th>
<th>Developer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reported$^a$</td>
<td>8.2±1.2</td>
<td>11.4±0.7</td>
<td>5.1±0.10</td>
<td>26.9±6.9</td>
<td>N.D.$^b$</td>
</tr>
<tr>
<td>Our study$^c$</td>
<td>10.9±0.11$^d$</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>17.0±0.21$^e$</td>
</tr>
</tbody>
</table>

$^a$ Taken from the literature (ref. 17). $^b$ N.D.=no data available. $^c$ Values are means of at least three experiments. $^d$ Vorinostat (3) was used as positive control (IC$_{50}=0.9947±0.0035$ µM). $^e$ Vorinostat (3) was inactive.
Effects of Several Aurora Kinase Inhibitors on HeLa Cell Proliferation

We studied the activity of compound 7, which was reported to be an HDAC inhibitor. However, we found that compound 7 did not induce histone acetylation by HDAC inhibition in a cell-based assay. Therefore, we hypothesized that the reported HDAC inhibition by compound 7 should be a false-positive result and attempted to confirm our hypothesis experimentally. By investigating the effects of compound 7 on the HDAC assay system, we found that compound 7 disturbed the HDAC assay system by quenching the emission of the fluorophore released by the HDAC substrate. Our study is expected to alert researchers to false-positive results in fluorescence assays.

Experimental

Chemistry Compound 7 was prepared using the reported procedure in ref. 17.

Cell Proliferation Assay Human cervical cancer HeLa cells (JCRB) were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin mixture at 37°C in a humidified atmosphere of 5% CO₂ in air. The HeLa cells were plated in 96-well plates at the initial density of 1×10⁵ cells/well and incubated at 37°C. After 24 h, the cells were exposed to the test compounds by adding solutions (50 μL/well) of the compounds at various concentrations in medium at 37°C under 5% CO₂ in air for 72 h. The mixtures were then treated with 10 μL of AlamarBlue® (AbD Serotec, U.S.A., #BUF012A), and incubation was continued at 37°C for 3 h. The fluorescence in each well was measured with an ARVO X3 microplate reader (excitation at 540 nm, emission at 590 nm). The % cell number versus the control was estimated from the fluorescence readings.

Western Blotting HeLa cells (5×10⁵ cells/2mL/dish) were treated for the indicated period with the test compounds at the indicated concentrations in 10% FBS-supplemented culture medium. Then, the cells were collected and extracted with sodium dodecyl sulfate (SDS) buffer. Protein concentrations of the lysates were determined by bicinchoninic acid (BCA) protein assay. Equivalent amounts of protein from each lysate were resolved in 5–20% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with Tris-buffered saline with Tween 20 (TBS-T) containing 5% skimmed milk, the transferred membranes were probed with rabbit monoclonal H3K9Ac antibody (CST, #9649) (1:2000 dilution), rabbit polyclonal H3 antibody (Abcam, U.S.A., #ab791) (1:200000 dilution), mouse monoclonal acetyl-α-tubulin antibody (Sigma, U.S.A., #T6793) (1:2000 dilution), or mouse monoclonal α-tubulin antibody (Sigma, #T8203) (1:1000 dilution). The probing membranes were washed three times with TBS-T, incubated with enhanced chemiluminescence (ECL) rabbit immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked whole antibody (GE Healthcare Life Sciences, U.S.A., #NA934) (1:2500 dilution) or ECL mouse IgG, HRP-linked whole antibody (GE Healthcare Life Sciences, #NA931) (1:2500 dilution), and again washed three times with TBS-T. The immunoblots were visualized by enhanced chemiluminescence with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, U.S.A., #P90718).

HDAC, Developer, and AMC Assays The assay for HDAC activity and the developer assay were performed using a FLUOR DE LYS® HDAC fluorometric activity assay.
kit (Enzo Life Sciences, U.S.A., #BML-AK500-0001) and a FLUOR DE LYS® deacetylated standard (Enzo Life Sciences, #BML-KI142), according to the supplier’s instructions. The AMC assay was performed by detecting the fluorescence of 5 μM AMC in the presence of vorinostat (3) or compound 7 at the indicated concentration. The fluorescence for each condition was measured on an ARVO X3 microplate reader with excitation set at 360 nm and emission detection set at 460 nm, and the % inhibition was calculated from the fluorescence readings of inhibited wells relative to those of control wells. The concentration of a compound that resulted in IC₅₀ was determined by plotting log[Inh] versus the logit function of % inhibition. IC₅₀ values were determined by regression analysis of the concentration/inhibition data.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

References