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TRANSCRIPTIONAL ANALYSIS OF ZINC-DEPENDENT HISTONE DEACETYLASES IN SEVERAL HUMAN CANCER CELLS

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***Background*: Histone deacetylases, especially zinc-dependent deacetylases HDACs, are among attractive drug targets for treating cancer in recent years. *Aim*: To explore the expression level of HDACs in several human cancer cell lines and examine the possible association between their expression and the sensitivity/resistance to the selective- or pan-HDAC inhibitors. *Materials and Methods*: The RNA expression of 11 HDACs isoforms was assayed in HeLa, HepG2, AV3, HEK293, A549, and K562 cells by semiquantitative reverse transcription-polymerase chain reaction. The sensitivity/resistance of these cell lines to the pan- or selective- HDAC inhibitors was estimated by MTS assay. *Results*: The relative transcription of *HDACs* genes demonstrated that members of Class I HDAC (HDAC1, 2 and 3) and members of Class II HDAC (HDAC4, 5, 6 and 7) had slight to significant levels of expression in cell lines under study with no dominant *HDAC*-subtype gene transcription. pan-HDAC inhibitor demonstrated superior antitumor activity compared to HDAC isoform-selective inhibitor. *Conclusion*: The absence of the dominant *HDAC*-subtype gene transcription in different human cancer cell lines explains the inferior efficacy of HDAC isoform-selective inhibitors as compared to pan-HDAC inhibitors.**

***Key Words*: histone deacetylase, HDAC inhibitors, transcriptional profile, cancer cell lines.**

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Histone deacetylases (HDACs), which can remove the acetyl groups from the ω-amino lysine residues of histone tails, are among promising drug targets for treating cancer in recent years [1]. There are in general 18 human histone deacetylases grouped into four classes [2, 3]. Class I, Class II and Class IV belong to zinc-dependent deacetylases, so-called HDACs, while Class III are nicotinamide adenine dinucleotide-dependent deacetylases, so-called sirtuins. Class I includes HDAC1, 2, 3 and 8. Class II contains two subclasses: Class IIa includes HDAC4, 5, 6, 7 and 9 and Class IIb includes HDAC6 and 10. Class IV has only one member of HDAC11. Other than the previous Classes, Class III consists of seven members, called SIRT1 to SIRT7. HDACs have been shown to play certain crucial roles in carcinogenesis and development of many human cancers [4–10]. Therefore, sirtuin inhibitors and HDAC inhibitors have been intensively investigated over the last few decades. Compared to sirtuins, HDAC inhibitors is showed to be more successful in treating cancer. There are five approved HDAC inhibitors for clinically treating cancers. Vorinostat (SAHA) [11], romidepsin (FK228) [12] and belinostat (PXD-101) [13] have been approved by Food and Drug Administration in USA for treating cutaneous T cell lymphoma or peripheral T cell lymphoma while panobinostat (LBH-589) [14] has also been approved by the Food and Drug Administration for the treatment of multiple myeloma. Recently, chidamide [15] was approved by China Food and Drug Administration for the treatment of peripheral T cell lymphoma.

Among these approved HDAC inhibitors, most are non-specific, even the "selective" HDAC inhibitor chidamide, which could also inhibit HDAC1, 2, 3 and 10 efficiently under the concentration of submicromolar range [11–15]. On the other hand, since HDACs are important targets for treating cancer, there is continuous interest and demand in developing novel HDAC inhibitors [16–18]. The development of novel HDAC inhibitors requires different cancer cell lines to evaluate *in vitro* antitumor activity. The expression profile of different subtypes of HDAC in human cancer cell lines allow us to choose the suitable one as study tools to facilitate the study of selective- or pan- novel HDAC inhibitors. Meantime, the expression profile of HDACs could probably help to explain why all approved HDAC inhibitors are non-selective. However, there are scarce studies of expression profiles of HDACs in human cancer cell lines [19–23]. In the current study, we attempted to explore the expression level of HDAC1-11 in several human cancer cell lines by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, we examined the possible association between the expression levels of HDAC1-11 and the sensitivity/resistance to the reported selective or pan-HDAC inhibitors.

MATERIALS AND METHODS

We declare that all the research meets the ethical guidelines, including adherence to the legal requirements of China.

Cell culture. Cervical adenocarcinoma (HeLa), human hepatocellular carcinoma (HepG2), AV3 (HeLa contaminated cells) and normal human embryonic kidney (HEK293) cell lines were cultured in DMEM

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Abbreviations used: FBS – fetal bovine serum; HDACs – histone deacetylases; RT-PCR – reverse transcription-polymerase chain reaction.
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with L-glutamine supplemented with streptomycin (500 μg/mL), penicillin (100 units/mL), and 10% fetal bovine serum (FBS) (Gibco, USA). Non-small cell lung carcinoma (A549) and human chronic myeloid leukemia (K562) cell lines were cultured in RPMI-1640 supplemented with streptomycin (500 μg/mL), penicillin (100 units/mL), and 10% FBS (Gibco, USA). Human colorectal adenocarcinoma (HT29) cells were cultured in DMEM/F12 (Gibco) supplemented with streptomycin (500 μg/mL), penicillin (100 units/mL), and 10% FBS (Gibco, USA). All cell lines were cultured in a humidified atmosphere (37 °C, 5% CO_2) with medium exchanges every 2 days.

Cell lines were authenticated using Short Tandem Repeat analysis by Shanghai Biowing Applied Biotechnology Co. Ltd. (http://www.biowing.com.cn/). In general, DNA was extracted by a commercial kit from CORNING (AP-EMN-BL-GDNA-250G). The twenty Short Tandem Repeats including Amelogenin locus were amplified by six multiplex PCR and separated on ABI 3730XL Genetic Analyzer. The signals were then analyzed by the software GeneMapper.

Mycoplasma test was performed for all cell lines under study by Shanghai Biowing Applied Biotechnology Co. Ltd. (http://www.biowing.com.cn/) based on the fluorescent quantitative PCR platform.

Total RNA extraction. The cells were seeded in T25 cell culture flasks. Upon the 90–100% confluence, the cells were lysed according to the TIANGEN Total RNA Extraction Kit (DP419) and RNA was extracted. RNA concentration (A260 nm) and purity (A260 nm/A280 nm and A260 nm/A230 nm) were determined by using NanoPhotometer N50 (IMPLEN, USA). Gold View Type I Nucleic Acid Dyes (Solarbio) was added to 1.5% agarose (BIOWEST) gel solution. The gel was loaded with 1 μg of each RNA sample and ran for 30 min at constant voltage (90 V) for checking RNA quality.

Reverse transcription and semiquantitative PCR reaction. Three micrograms of total RNA were used per sample to generate the first-strand cDNA with the GoScript™ Reverse Transcription System (Promega, A5000). Reverse transcription procedure: initial denaturation at 70 °C for 5 min, annealing at 25 °C for 5 min, extension at 42 °C for 60 min, inactivation at 70 °C for 15 min. After the program is completed, the product of cDNA is obtained and stored at –20 °C.

Semiquantitative PCR mixture contained 2x Pfu PCR MasterMix (TIAN GEN, China), 10 μmol of primers (Table 1) and 50.0 ng of DNA template in a total volume of 25 μl. S1000 Thermal Cycler (Bio-Rad) was programmed as (a) 1 cycle at 94 °C for 3 min, (b) 30 s at 94 °C followed by 30 s at 55 °C followed by 2 min at 72 °C for 30 cycles, (c) 5 min at 72 °C for 1 cycle, and (d) keeping at 4 °C. Gold View Type I nucleic acid dye (Solarbio, China) was added to a 1.5% agarose (BIOWEST, France) gel solution. 5 μl of a semi-quantitative PCR reaction solution was added to the gel and run at a constant voltage (90 V) for 30 min. The results were then photographed and analyzed by the Gel imaging system (G-BOX-Chemi-XL1.4). All experiments were performed in triplicate.

Expression analysis. The expression levels of HDAC1-11 in different cell lines were calculated by comparing the gray values of HDAC1-11 to the gray values of GAPDH as internal reference using Gray value calculation software (Gene Tools from Syngene, USA).

Cell cytotoxicity assay. Cells were kept at logarithmic growth phase in 5% CO_2 at 37 °C with the corresponding medium supplemented with 10% FBS, streptomycin (500 μg/mL) and penicillin (100 units/mL). In cell viability assays, 5,000 cells for HeLa, A549, HepG2, AV3, HT29 and HEK293 cells, or 20,000 cells for K562 cells were seeded in each well of 96-well plate. After attachment for 12 h, these cells were treated with indicated inhibitor for 48 h. Then, the medium was refreshed. After incubation with 20 μl of 3-(4, 5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium (MTS) for 2.5 h, the absorbance was measured using a Model 680 (Bio-Rad, USA) at 490 nm. The optical density of the MTS assay was directly proportional to the number of viable cells. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Optimal amount of cDNA and PCR cycles. Typically, the PCR cycles are between 25–40, which should give a significant amount of PCR product to be visualized on the gels. Too many cycles can increase the amount of PCR products to some extent; however, non-specific amplification may also arise correspondingly. In this case, when higher concentration of cDNA is required to reach enough PCR products to be visualized on the gels, higher concentration as the same as the target gene is also applied to housekeeping gene, resulting that the PCR products of housekeeping gene may be out

Table 1. Primers used for RT-PCR

№	Gene	Forward primer sequence	Reverse primer sequence	Length (bp)
1	*HDAC1*	TGCCACAGAACCACCAGTAGA	GCTCCATCCGTCCAGATAACA	128
2	*HDAC2*	GGACACCAGGTGCATGAGGTA	CAGTTGCCCTTGATTGTGAGA	189
3	*HDAC3*	CTGAGGTTAAAGCAGCCCAAT	CGGTGTCCTTCCACAAATACG	250
4	*HDAC4*	GAAGCCTGATGACACCAGCACC	ACCGCTACGACGATGGGAACT	212
5	*HDAC5*	CTGCGTTGATGTTGGGCTTTT	TCCTCTGGGTGGCTACTCTGTCA	218
6	*HDAC6*	AGGGCAGTGGTGTCACAGCATAAAA	ACCTTGGAGCTAGGCAGCGAATC	172
7	*HDAC7*	CTTCAGCCAGGATGCCCACAG	AACAGAAACCCAACCTCAATGCC	192
8	*HDAC8*	AAGACCACTGCTTTGGGATTA	CATTCAGGATGGCATACAAGA	96
9	*HDAC9*	TCTGCTGCTGGTATTGCTTCT	TTCCCTTACATCCTCAGTCTCC	198
10	*HDAC10*	CATCCAGCATCCCATCTAAGAGGTA	TCATCCAACAGGAAGCGTCAGC	132
11	*HDAC11*	ACACCAGCTCATCCCGCTTCA	GTCTACAACCGCCACATCTACCC	250
12	*GAPDH*	GGACCTGACCTGCCGTCTAG	GTAGCCCAGGATGCCCTTGA	100

of the linear range. On the other hand, different isoforms of target genes may have different optimal amounts of cDNA and optimal cycles. The current study was the exploratory one for assessing the relative expression of HDAC isoforms. Since we chose *GAPDH* gene as an internal quality control, we first selected the optimal amount of cDNA for *GAPDH* by making several dilutions of cDNA of GAPDH from 12.5 to 500 ng to perform the amplification of PCR and then quantify the bands by their intensity of gray scale. The results were shown in Fig. 1, *a*, indicating that the linear range of cDNA was between 12.5 and ~100 ng. Therefore, we chose 50 ng as the starting amount of cDNA.

Besides, considering the PCR cycles is extremely important to identify the amount of product no longer depending on the initial amount of cDNA, we selected the optimal number of PCR cycles. We have set the cycles from 20 to 50 and then quantify the bands of GAPDH by their intensity of gray scale. As shown in Fig. 1, *b*, the cycles from 20 to 30 demonstrated the linear increase while the cycles over 30 reached the plateau stage. Therefore, we chose 30 cycles as the optimal PCR cycles for the following study.

HDACs gene expression in human cancer cell lines. As shown in Fig. 2, all tested *HDAC* and *GAPDH* transcripts were detected in several cell lines and all bands of *GAPDH* showed the similar intensity under the optimal PCR conditions: 50 ng as the amount of cDNA and 30 cycles as the PCR cycles for all tested samples. A panel of six human cancer cell lines (HT-29, K562, HeLa, AV3, HepG2 and A549) was studied. As an internal control, a housekeeping gene, the *GAPDH* transcription showing similar signals in all analyzed cell lines confirmed that the mRNA isolated from the cells was intact. As shown in Fig. 2, in most of cell lines under study, significant levels of gene transcription were observed for members of class I (*HDAC1, 2, 3*) and class II (*HDAC7*) genes while members of class II (*HDAC9* and *10*) only showed the slight levels of gene transcription. Comparison of transcript profiles in various cancer cell lines by RT-PCR analysis indicates that members of some *HDAC* genes are expressed at different levels. It was found that A549 cells demonstrated the least level of *HDAC2* RNA while AV3 cells showed the least level of *HDAC6* RNA among all tested

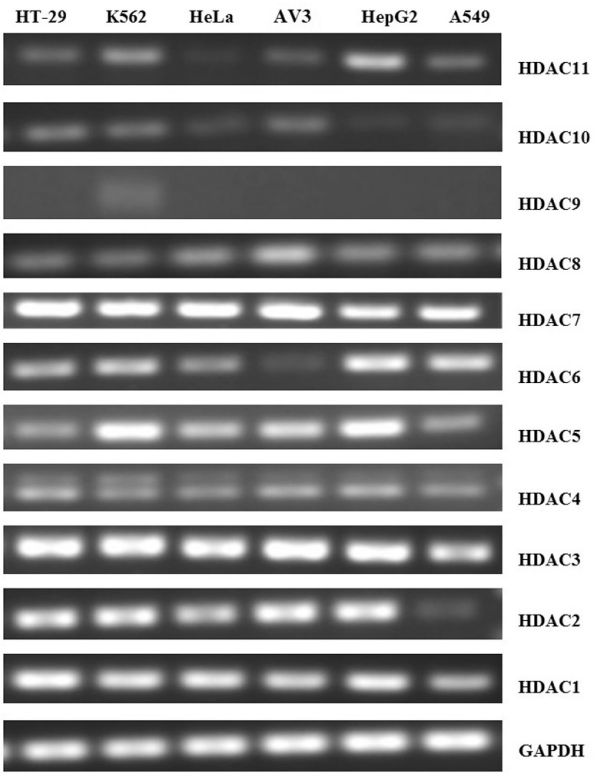

Fig. 2. RT-PCR analysis of *HDAC* RNA from human cancer cell lines: HT-29, K562, HeLa, AV3, HepG2, A549. The total RNA was isolated from cells and then was reverse transcribed to cDNA. After PCR amplification of cDNA, the products were analyzed on 1.5% agarose gel and visualized with ethidium bromide. *GAPDH* transcript was used as an internal control. The gene primers used are shown in Table 1

cancer cell lines. Interestingly, K562 and HepG2 cells presented the relatively high level of *HDAC5* and *HDAC11* RNA compared to the other cancer cell lines. Notably, we could not detect the *HDAC9* transcripts in all tested cancer cell lines but K562 cells.

The relative levels of *HDAC1-11* RNA in several cell lines is shown in Fig. 3. In A549 cells, *HDAC3* and *7* RNA demonstrated the highest level as compared to other *HDACs*. On the contrary, *HDAC9* was hardly detected in A549 cells. In HeLa cells, the highest level was detected for *HDAC7* transcripts while the bands of *HDAC9, 10* and *11* were not detectable. Significant levels of gene transcripts were shown

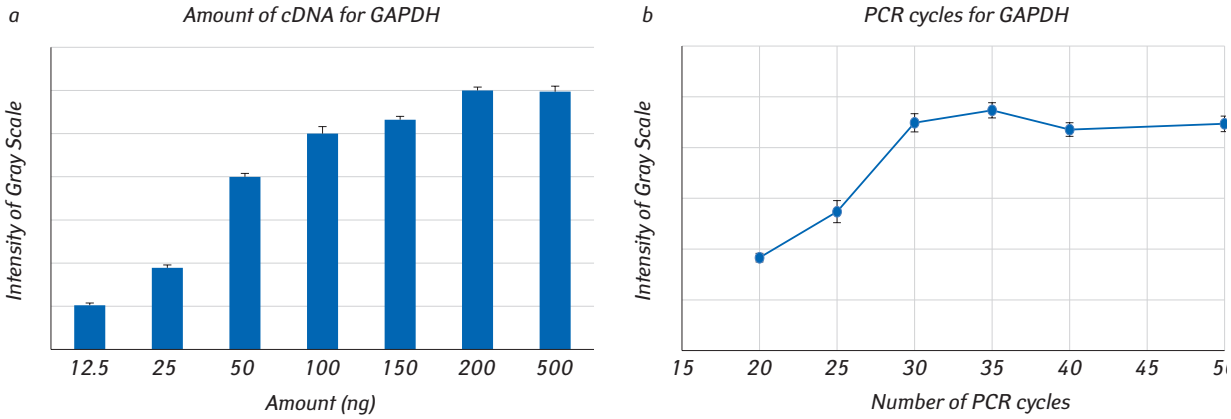

Fig. 1. Selection of the optimal PCR conditions for GAPDH: amount of cDNA *(a)* and number of cycles *(b)*

in *HDAC1, 2, 3* and *7* in HT-29 cells. Again, the gene transcripts of *HDAC9* were traceless in HT-29 cells. In K562 cells, the gene transcripts of *HDAC1, 2, 3, 5, 6* and *7* were more prominent than *HDAC4, 8, 9, 10* and *11*. In AV3 cells, the level of *HDAC2, 3* and *7* transcripts were roughly equal without the obvious gene transcription of *HDAC9*. The levels of *HDAC2, 3* and *5* were similar in HepG2 cells with no obvious *HDAC9*.

Furthermore, the relative levels of *HDAC1-11* transcription in A549, HeLa, HT-29, K562, AV3 and HepG2 cells were arbitrarily compared to those obtained in HEK293 cells. As shown in Fig. 4, the gene transcription of *HDAC8* in tested six human cancer cell lines has commonly higher level compared to those obtained in HEK293 cells. Additionally, the gene transcription of *HDAC10* and 11 in HepG2 cells was also higher than those in HEK293 cells while HT-29 and K562 cells demonstrated the higher level of gene transcription of *HDAC10* by contrast to those in HEK293. Notably, the *HDAC9* transcription was extremely high in K562 cells.

Cytotoxicity of HDAC inhibitors. Overall, the relative transcription of *HDACs* genes demonstrated that members of Class I HDAC (*HDAC1, 2* and *3*) and members of Class II HDAC (*HDAC4, 5, 6* and *7*) had slight to significant levels (Fig. 3 and 4). However, there were no dominant *HDAC*-subtype gene transcription found in all tested human cancer cell lines. To further confirm these findings, we performed the cytotoxicity assay of these human cancer cell lines using some reported HDAC inhibitors. A549, HeLa, HT-29, K562, AV3 and HepG2 cells were subjected to SAHA, RGFP966 and bufexamac treatment. Unlike SAHA as a pan-HDAC inhibitor, RGFP966 is a HDAC3 inhibitor while bufexamac is a HDAC6/HDAC10 dual inhibitor. The calculated IC_{50} were shown in Table 2. SAHA, a classical inhibitor of Class I and II HDACs, was shown to be the most effective compound compared to RGFP966 and bufexamac in all tested human cancer cell lines. These results agreed with the expression profile of *HDAC* genes in these cancer cell lines. This also explains why all approved HDAC inhibitors are non-selective [11–15].

Currently, the approved HDAC inhibitors in clinical use are non-selective as they can inhibit almost all HDACs. Even benzamide-type inhibitor, chidamide, which has been approved for treating relapsed or refractory periph-

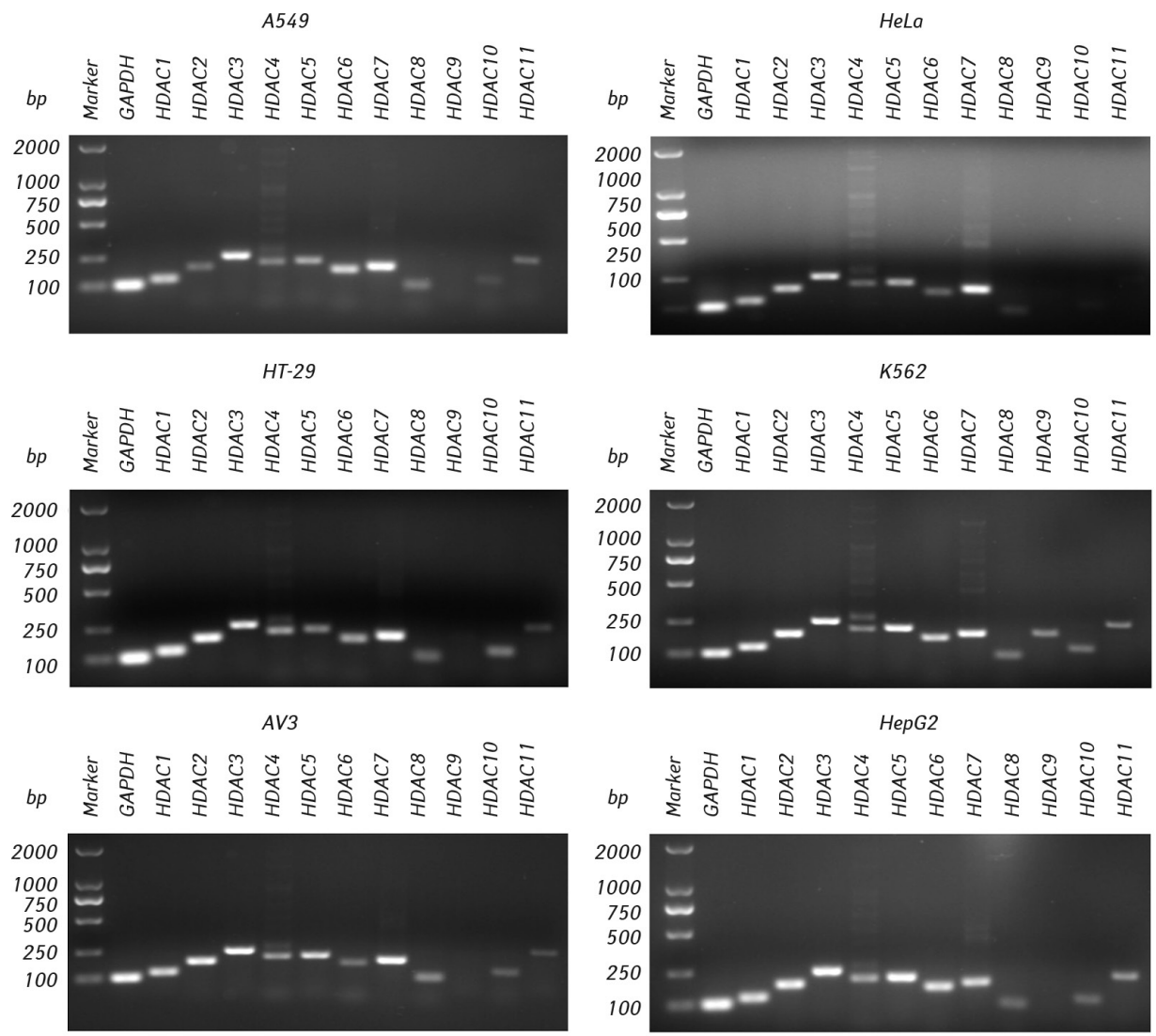

Fig. 3. Analysis of *HDACs* transcription levels by RT-PCR in A549, HeLa, HT-29, K562, AV3 and HepG2 cells

eral T cell lymphoma in China, can inhibit class I HDACs (HDAC1, 2 and 3) as well as class IIb HDAC10 at low nanomolar concentrations [24]. Besides, there are emerging many pan-HDAC inhibitors with great therapeutic potentials in clinical trials [25]. On the other hand, some HDAC isoform-selective inhibitors such as HDAC3, HDAC6 and HDAC9 selective inhibitors also have demonstrated a promising clinical potential [25]. Therefore, systematic analysis of the expression profiles for different HDACs in human cancer cell lines will add not only theoretical but also practical values into this field because there are few reports on expression profile of HDACs in human cancer cell lines [19–23].

To evaluate the expression profiles of different HDACs, we provided the RT-PCR analysis of total RNA from six human cancer cell lines at transcriptional levels. Similar to the previously reported results [20], class I HDACs (HDAC1, 2, 3 and 8) are ubiquitously and transcriptionally expressed in all cancer cell lines under study, while the level of these *HDACs* seems to be slightly different. In class II HDACs, *HDAC4, 5, 6, 7* and *10* are also transcriptionally expressed at different levels in the cell lines under our study; and so as the only member of class IV HDAC, *HDAC11*, too. However, *HDAC9* gene transcription seems to be visualized only in K562 cell lines. The relative analysis of *HDAC1-11* transcription in an individual cancer cell line against a non-cancer cell line (HEK293) reveals that the gene transcription of *HDAC8* is ubiquitously higher in all tested cancer cell lines and that of *HDAC9* is extremely higher in K562 cells.

To explore the sensitivity of these cancer cell lines to HDAC inhibitors, we have chosen a pan-HDAC inhibitor, a HDAC3 selective inhibitor and HDAC6/HDAC10 dual inhibitor to test their IC_{50} values in cancer cell lines. Our data are in agreement with the reported results that pan-HDAC inhibitors demonstrate superior antitumor activity compared to HDAC isoform-selective inhibitors although the toxicity profile of pan-HDAC inhibitors sometimes limits their use [25].

In summary, the current study has examined the gene expression of HDAC1-11 in six human cancer cell lines (HT-29, K562, HeLa, AV3, HepG2 and A549). Our findings have shown that members of Class I HDAC (HDAC1, 2 and 3) and members of Class II HDAC (HDAC4, 5, 6 and 7) are commonly increased in all tested cancer cell lines. The reason for non-specificity of all approved HDAC inhibitors is that there was no dominant *HDAC*-subtype gene transcription found in all tested human cancer cell lines. Therefore, pan-HDAC inhibitors are clinically more effective than specific HDAC inhibitors [11–15]. If we would like to study pan-HDAC inhibitors, any of these six human cancer cell lines will be a suitable model. Additionally, K562 may be the most suitable cancer cell line for investigating HDAC9 specific inhibitors. Considering the current study is based on semi-quantitative PCR at the transcriptional levels, more accurate quantitative RT-PCR and proteomics analysis are expected to further confirm these results. Nevertheless, these findings will somehow facilitate the development of either pan-HDAC inhibitors or subtype-specific HDAC inhibitors.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

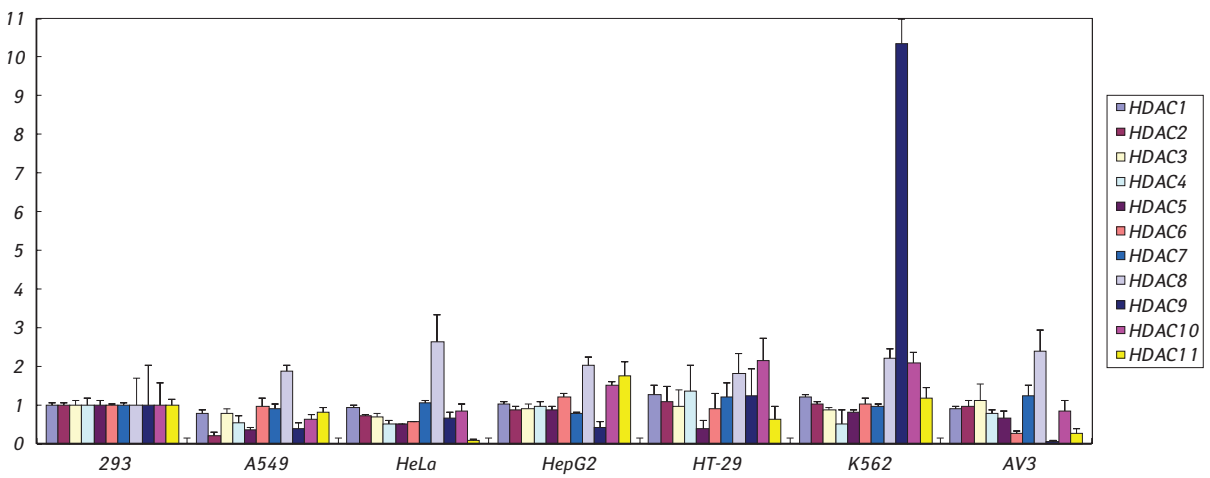

Fig. 4. The relative levels of HDAC1-11 transcription in A549, HeLa, HT-29, K562, AV3 and HepG2 cells were arbitrarily compared to those obtained in HEK293 cells

Table 2. Cytotoxicity of HDAC inhibitors against several cancer cell lines

	IC_{50} (μM)					
	HeLa	MCF7	A549	HepG2	K562	HT29
SAHA	54.7	30.1	8.8	13.1	18.0	7.5
RGFP966	>100	>100	>100	>100	>100	>100
Bufexamac	>100	>100	>100	>100	>100	>100

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АНАЛІЗ ТРАНСКРИПЦІЇ ЦИНК-ЗАЛЕЖНИХ ГІСТОНДЕАЦЕТИЛАЗ У КЛІТИНАХ В ЛІНІЯХ ПУХЛИННИХ КЛІТИН ЛЮДИНИ

*І. Лі[§], З. Цзоу[§], Х. Чен, Б. Хе**

Державна ключова лабораторія з функцій та застосування лікарських рослин інженерного дослідницького центру з розвитку та запровадження етнічної медицини та міністерства освіти, ключова фармацевтична лабораторія провінції Гуйчжоу, Медичний університет Гуйчжоу, Гуйян 550004, Китай

Стан питання: Гістондеацетилази, особливо цинк-залежні гістондеацетилази (ГДАЦ), останнім часом розглядають як одні з привабливих мішеней для спрямованої дії протиракових препаратів. ***Мета***: Визначити рівень експресії ГДАЦ у деяких лініях пухлинних клітин людини та можливий зв'язок між їх експресією та чутливістю до селективних або панінгібіторів ГДАЦ. ***Матеріали і методи***: Експресію РНК 11 ізоформ ГДАЦ досліджували в клітинах ліній HeLa, HepG2, AV3, HEK293, A549 та K562 за допомогою напівкількісної зворотнотранскриптазної полімеразної ланцюгової реакції. Чутливість до селективних або панінгібіторів ГДАЦ визначали в тесті з MTS. ***Результати***: Ізоформи ГДАЦ класу I (ГДАЦ 1, 2, 3) та класу II (ГДАЦ 4, 5, 6, 7) експресуються тією чи іншою мірою в усіх досліджених лініях пухлинних клітин, причому не виявлено домінантної транскрипції якихось певних субтипів генів ГДАЦ. Протипухлинна активність панінгібітора ГДАЦ вища за таку інгібітора, що виявляє селективну активність щодо ізоформ ГДАЦ. ***Висновок***: Відсутність домінантної транскрипції гена певного субтипу ГДАЦ пояснює факт вищої цитотоксичної активності панінгібітора ГДАЦ у порівнянні з інгібіторами із селективною активністю щодо ізоформ ГДАЦ.

Ключові слова: гістондеацетилази, інгібітори ГДАЦ, профіль транскрипції, лінії пухлинних клітин людини.