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Research Article
N-Acetyltransferase Activity Assay and Inhibitory Compounds Screening by Using Living Human Hepatoma HepaRG Cell Model

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Abstract

Background and Objective: N-acetyltransferases (NATs) were phase II drug-metabolizing enzymes, NATs activity was associated with certain adverse drug reactions. This study aimed to establish a method for NAT1 and NAT2 activity assay by using living human hepatoma HepaRG cells and tried to apply the established method for preliminary screening of the potential inhibitors of NAT1 and NAT2. **Materials and Methods:** Immunohistochemistry and western-blot techniques were used to evaluate the expression of NAT1 and NAT2 in HepaRG cells. Non-cytotoxic concentration of the specific substrates of NAT1 and NAT2, i.e., 4-aminosalicylic acid and isoniazid were incubated with HepaRG cells for 8 h, the culture medium was collected and determined by LC-MS/MS to evaluate the activity of NAT1 and NAT2. The effects of 4-aminosalicylic acid and isoniazid on NAT1 and NAT2 expression in HepaRG cells were also tested by western-blot and the reported NAT inhibitors were also used here to evaluate the sensitivity of the established method for NAT activity assay. **Results:** The results showed that NAT1 and NAT2 were expressed in HepaRG cells and NAT1 and NAT2 activity could be evaluated by quantifying the acetylated metabolites of their specific substrate 4-aminosalicylic acid and isoniazid, respectively. NAT activity detected by using living HepaRG cells was in parallel with that from traditional method for NAT activity assay, i.e., reactions using cells lysate. NAT activity assay by using living HepaRG cells was suitable for high-throughput screening of the potential inhibitors of NAT1 and NAT2, indicating the validity of the established method for NAT activity assay. **Conclusion:** The present study established a low-costing and stable method for screening of compounds with NAT1 and NAT2 inhibitory properties, which was helpful for new drug discovery and development.

Key words: N-acetyltransferase activity, HepaRG cells, high-throughput screening, NATs inhibitors

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Arylamine N-acetyltransferases (NATs) were polymorphic phase II drug-metabolizing enzymes, acetylating arylamine carcinogens and drugs including anti-hypertensive hydralazine, antitubercular isoniazid and anti-bacterial sulfonamides[1]. Up to now, three NAT isoforms had been identified in numerous species[2]. NAT1 had a wide spread distribution in the body, while NAT2 was mainly expressed in the liver and gut[3]. Human NAT3 was a pseudogene and murine NAT3 was considered as a low-activity enzyme compared with NAT1 and NAT2[4]. Human NATs exhibit single nucleotide polymorphisms in the populations that modify drugs and carcinogen metabolisms, which had been found associated with drugs toxicity or an increased risk of certain type of cancers[2]. NATs polymorphisms affecting enzyme activity could lead to "rapid" or "slow" acetylator phenotypes, which had been reported to be associated with adverse drug reactions, such as rapid acetylator phenotype-associated myelotoxicity of amonafide and slow acetylators-associated peripheral neuropathy of isoniazid (INH)[5].

In addition to the genetic processes controlling NAT expression, NAT activity could also be modulated by non-genetic factors, such as substrate-dependent regulation and drugs inhibition[6]. It was found that the activity of NAT1 and NAT2 could be inhibited by flavonoids, such as quercetin and kaemferol[7] and, certain natural products, such as luteolin, ferulic acid and glycyrrhizic acid, showed inhibitory effect on NAT1; whereas curcumin and scopoletin had inhibitory effect on NAT2[8,9]. Concerning the important roles of NATs in drugs metabolism and adverse drug reactions, it was necessary to evaluate the effect of herbal compounds on NATs. NATs activity could be assessed by measuring NATs-selective substrates and the metabolites, p-aminobenzoic acid and 4-aminosalicylic acid (4-ASA) as well as the metabolites acetylated PABA and acetyl 4-ASA (AC-4-ASA) were often used for NAT1 activity assay, while INH and its metabolite acetyl isoniazid (AC-INH) were used for NAT2 activity assay[7,10]. These methods for NAT activity assay were mainly based on *in vitro* reactions containing the extracted-NAT enzyme, enzyme-specific substrate and NAT cofactor Acetyl-CoA. NATs enzymes were commonly acquired from hepatocarcinoma cell line, keratinocyte cell line or bacteria[6,11,12], for its stability, reproducibility and low-cost[13]. However, the preparation and purification of NATs from tissue or culture cells were costly and time-consuming and not suitable for the high-throughput screening of the active compounds on NATs.

HepaRG, a unique human hepatoma cell line, was the first cell line able to differentiate *in vitro* into mature hepatocyte-like cells and was capable of expressing both drug metabolizing enzymes and transporters[14,15]. The accumulating evidence showed that HepaRG cell line was an *in vitro* model for drug metabolism, disposition and toxicity studies[16,17]. Previous studies showed that the gene expression of NAT1 and NAT2 in HepaRG cells was comparable to primary human hepatocytes[18,19], while low mRNA level of NAT1 and NAT2 was found in the widely used HepG2 cells[20], indicating that HepaRG cell line was a promising alternative to human primary hepatocytes for NATs study. This present study aimed to investigate the protein expression of NAT1 and NAT2 in HepRG cells and tried to establish a novel method for NATs activity assay using living HepaRG cells. The purpose of this study was to establish a low-costing and stable method for high-throughput screening of compounds with inhibitory properties on NAT1 and NAT2, which will be beneficial for researchers on new drug discovery and development.

MATERIALS AND METHODS

The cell culture and NAT activity assay were carried out in the Lab of Department of Pharmacy in First Hospital of Lanzhou University from August-December in 2017; NAT inhibitors screening were carried out in the Lab of Department of Pharmacy in First Hospital of Lanzhou University from February to March, 2018.

Materials: 4-ASA, AC-4-ASA, INH, AC-INH and Acetyl-CoA were purchased from Sigma-Aldrich (St. Louis, USA). Chloral hydrate was purchased from Bangjing Scientific Supplies (Shanghai, China). Curcumin and ferulic acid were purchased from HaoXuan Pharmaceutical Co. Ltd. (Xi'an, China).

Cell culture: Human hepatoma HepaRG cells (HPRGC10, Invitrogen, USA) were maintained according to procedures from the supplier. To obtain stable expression of drug-metabolizing enzymes, HepaRG cells were cultured according to previously reported methods[21].

Immunocytochemistry: HepaRG cells were seeded on coverslips in 24-well plate and cultured for 72 h, then fixed with 4% paraformaldehyde. The procedure for immunocytochemistry was carried out according to previous report[22]. Briefly, cells were incubated with primary antibodies (anti-NAT1, 1:1000, anti-NAT2, 1:500, Proteintech, Wuhan, China) overnight, further incubated with secondary antibodies (Anti-rabbit Fluor® 488, 1:500; anti-rabbit Fluor® 568, 1:500, Thermo Fisher) and then stained with DAPI (20 μg mL^{-1}, Sigma).

Western blot analysis: Cells were collected at 24, 48 and 72 h after passage, respectively. After washing in PBS, the cells were collected for protein detection by western-blot technique as previously report[23]. The PVDF membrane was incubated with primary antibody (anti-NAT1, 1:1000, anti-NAT2, 1:500, Proteintech, Wuhan, China) overnight. The immunoreaction was detected using ECL Western blotting kit.

Cell viability assessment: Cell viability was assessed as previous report[24]. Briefly, cells were cultured overnight. NAT1-specific substrates (4-ASA, 0-500 μM), NAT2-specific substrates (INH, 0-100 mM), or the reported NAT specific inhibitors (curcumin and ferulic acid, 0-300 μM) were added and further cultured for 48 h. The absorbance of the plates was read at 570 nm using a microplate ELISA reader (Bio-Rad, Hercules, CA, USA).

NAT activity assay on living cells: HepaRG cells were seeded in 24-well culture plates at a density of 50,000 cells per well and cultured for 72 h. Fresh medium containing 2% DMSO ornon-cytotoxic concentration of 4-ASA (0-100 μM) or INH (0-10 mM) was added, 20 μL/well culture medium was collected per hour for a total of 8 h and the samples were proceeded for LC-MS/MS assay. NAT activity was expressed as nanomoles of AC-4-ASA or AC-INH per milli gram of protein per minute of reaction time.

NAT activity assay using cells lysate: HepaRG cells were suspended in 0.2 mL lysis buffer (20 mM Tris, 1 mM EDTA, 1 mM DTT, pH 7.4). The cell lysate was centrifuged and the supernatant was collected for NAT activity assay[23]. Briefly, the reaction vials contained cell lysate protein (10 μg) and NAT-specific substrates 4-ASA (25-100 μM) or INH (1.25-10 mM)[11]. AC-4-ASA or AC-INH in the supernatant was quantified by LC-MS/MS. NAT activity was normalized to lysate protein concentration. NAT activity was given in nanomoles of AC-4-ASA or AC-INH per milligram of protein per minute of reaction time.

LC-MS/MS determination: NAT activity was determined using the substrate 4-ASA (selective substrate of NAT1) and INH (selective substrate of NAT2), which were determined by LC-MS/MS[25,26]. The analysis was performed on Agilent 1260 infinity HPLC coupled to Agilent 6460 Tripe-Quadrupole mass spectrometer. Chromatographic separations were performed on an Agilent HC-C18 column (4.6×250 mm, 5 μm particles), guarded by an Agilent Eclipse XDB-C18 4.6×12.5 mm analytical guard column (Agilent Technologies, USA).

Screening of NAT inhibitor using living cells and cell lysate: HepaRG cells were pre-treated with ferulic acid (0-50 μM) or curcumin (0-10 μM) for 30 min and then 4-ASA (75 μM) or INH (5 mM) were added, respectively. After incubation for another 6 h, culture medium was collected for LC-MS/MS assay. The methods for NAT inhibitor screening was summarized in Table 1. For traditional NAT activity assay[7], a total of 40 μL cells lysate (20 μg mL^{-1}) was pre-incubated with ferulic acid (0-50 μM) or curcumin (0-10 μM) for 10 min before the start of the reaction.

Statistical analysis: Data were presented as Mean±SEM. Statistical analysis was conducted using SPSS 24.0 Software. Differences between two groups were analyzed by Student t-test. When multiple groups were compared, data were analyzed using one-way ANOVA followed by Student-Newman-Keuls (SNK) test. Differences were considered significant when $p<0.05$.

Table 1: Method for NAT activity assay in living HepaRG cells and the procedures for NAT inhibitor screening

Steps	NAT1 activity assay	NAT2 activity assay	NAT1/2 inhibitor screen
1	HepaRG cells were seeded in 24-well culture plates at a density of 50,000 cells per well and culture for 72 h		
2	Culture medium is replaced by 400 μL/well fresh medium		
3	-	-	Non-cytotoxic concentration of the tested compound is added and culture for 30 min
5	75 μM 4-ASA or 0.01% DMSO is added and culture for 6~8 h	5 mM INH or 0.01% DMSO is added and culture for 6~8 h	75 μM 4-ASA or 5 mM INH or 0.01% DMSO is added and then culture for 6~8 h
5	20~40 μL/well culture medium is collected and stored at -80 for LC-MS/MS assay and the cells were also harvested for protein determination and western blot analysis		
6	Con. AC-4-ASA is quantified by LC-MS/MS	Con. AC-INH is quantified by LC-MS/MS	Con. AC-4-ASA or AC-INH is quantified by LC-MS/MS
7	NAT1/2 activity is expressed as nanomoles of AC-4-ASA or AC-INH per milligram of protein per minute of reaction time		

RESULTS

Protein expression of NAT1 and NAT2 in HepaRG cells: As shown in Fig. 1a, at 24, 48 and 72 h post cell passage, the protein expression of NAT1 and NAT2 in HepaRG cells was time-dependently increased, the expression of NAT1 and NAT2 was both significantly increased after 48 and 72 h cells culture when compared to that of 24 h ($p<0.05$). Results from immunocytochemistry study also showed that the protein expression of NAT1 and NAT2 both could been identified in the cytoplasm of HepaRG cells (Fig. 1b).

Effect of specific substrates of NAT1 and NAT2 on cell viability: In Fig. 2, after 48 h exposure to NAT1-specific substrate 4-ASA, the cells viability was significantly decreased when 4-ASA concentration reached 250 and 500 μM ($p<0.05$). After 48 h exposure to NAT2-specific substrate INH, the cells viability was markedly decreased when INH concentration reached 25, 50 and 100 mM ($p<0.05$). From the results, the non-cytotoxic concentration of 4-ASA and IHN in HepaRG cells was determined as that below 100 and 10 mM, respectively.

Effect of substrates on the activity and expression of NAT1 and NAT2: As shown in Fig. 3a, with the increase of incubation time, the production of AC-4-ASA in the medium of HepaRG cells was increased in a concentration-dependent manner. However, after 6 h exposure to 25 μM and 100 μM 4-ASA, the production of AC-4-ASA was slowed down. When HepaRG cells were exposure to INH (1.25~5 mM), AC-INH production was time-dependently increased. However, after 10 mM INH exposure, AC-INH production was slowed down and markedly lower than that of 5 mM INH. As shown in Fig. 3b, at 8 h post different concentration of 4-ASA or INH exposure, no significant difference was found about the protein expression of NAT1 and NAT2 in HepaRG cells ($p>0.05$).

Comparison on NAT activity in living cells and cell lysate: As shown in Fig. 4a, in the presence of different concentration of

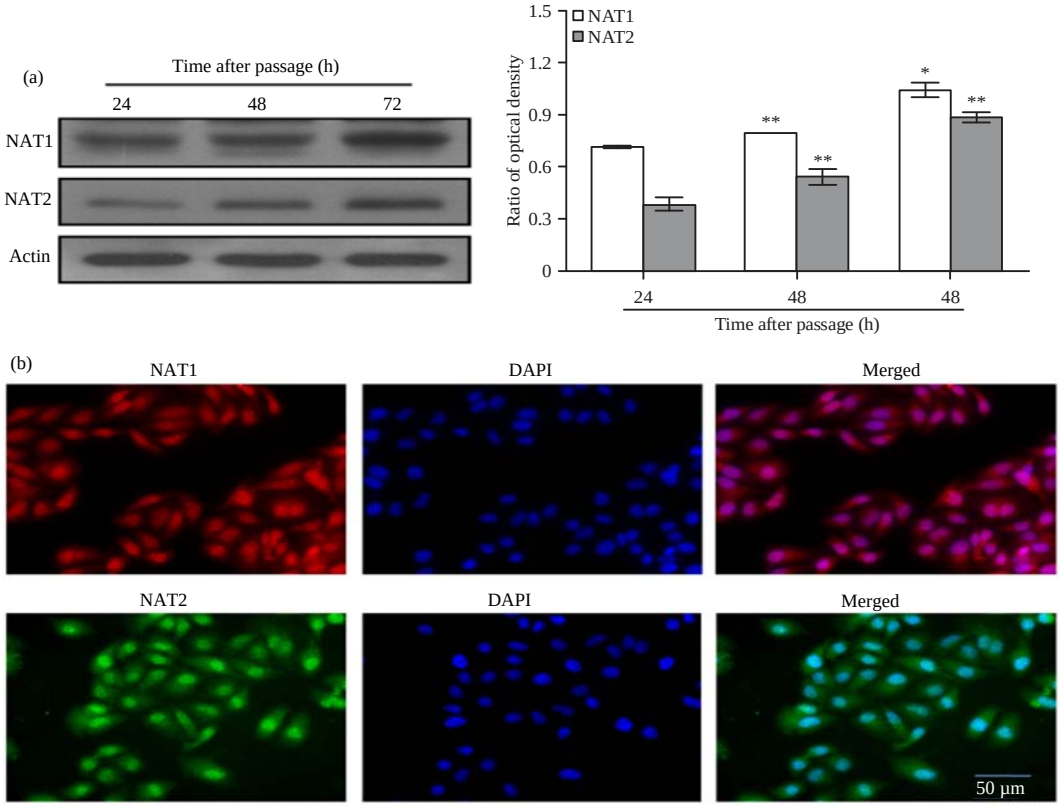

Fig. 1(a-b): Expression of NAT1 and NAT2 in HepaRG cells, (a) Protein expression of NAT1 and NAT2 in HepaRG cells at 24, 48 and 72 h after cell passage, (b) Immunostaining of NAT1 and NAT2 in the cytoplasm of HepaRG cells at 72 h after cell passage (scale bar = 50 μm)

Data were presented as Mean±SEM (n = 4). *$p<0.05$, *$p<0.01$ versus 24 h post cell passage

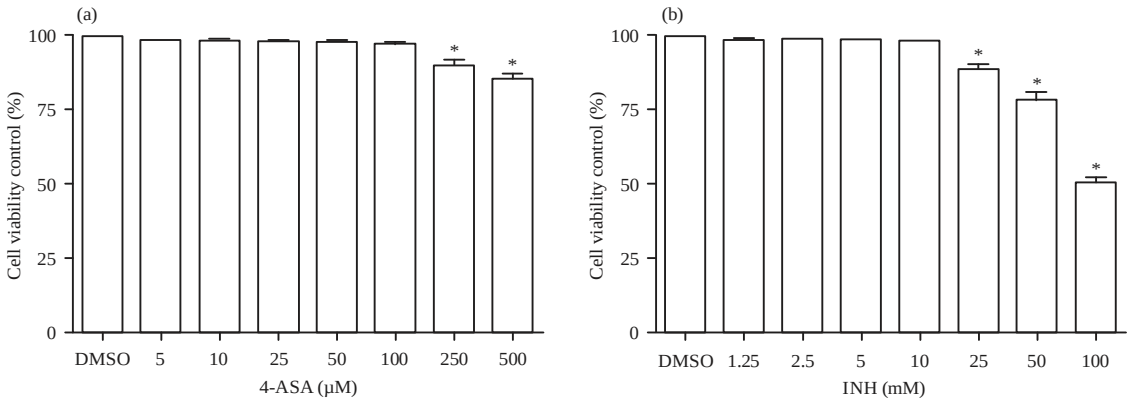

Fig. 2(a-b): Effect of the substrates of NAT1 and NAT2 on cells viability in HepaRG cells
Data were presented as Mean±SEM (n = 4), *p<0.05 vs. DMSO group

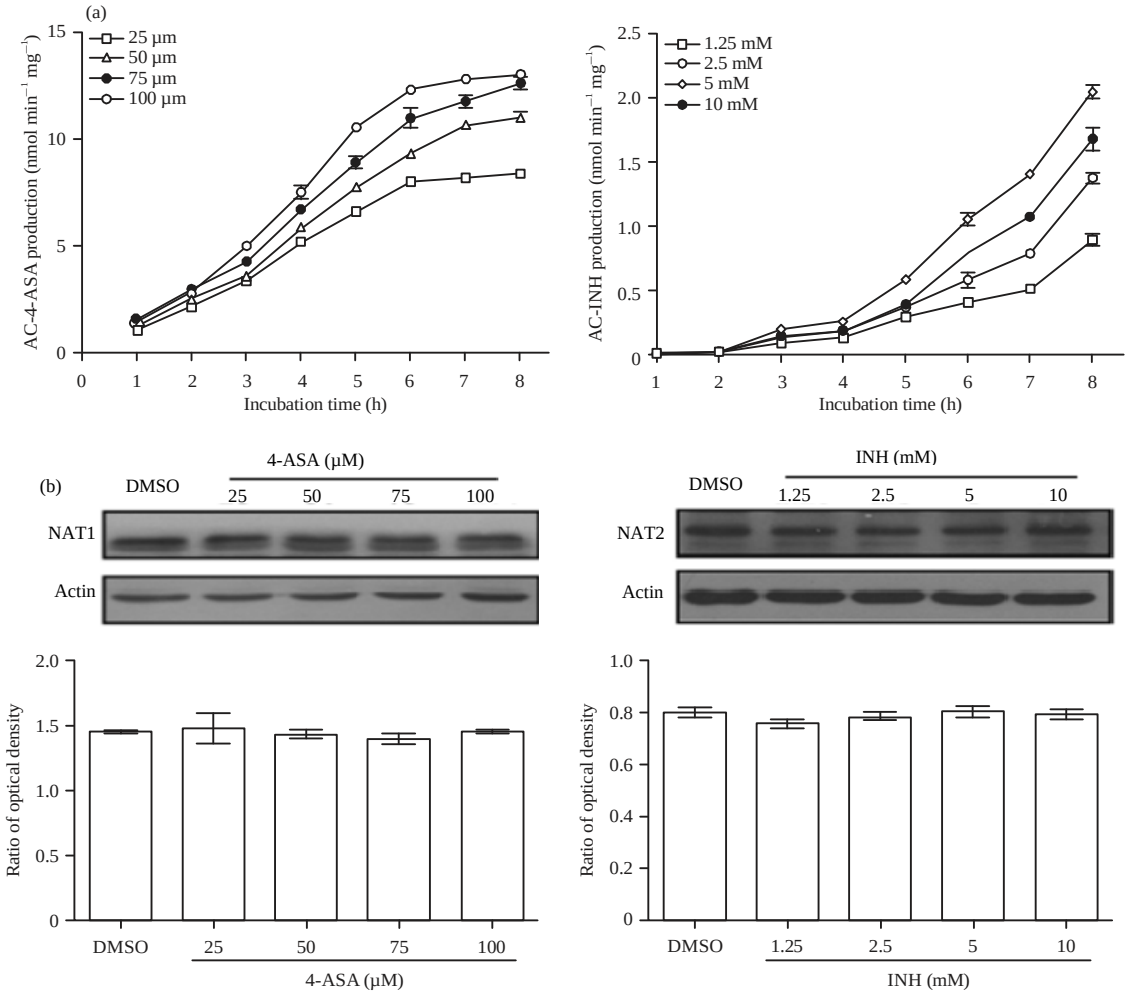

Fig. 3(a-b): Effect of substrates on the activity and expression of NAT1 and NAT2 in HepaRG cells. The activity and protein expression of (a) NAT1 and (b) NAT2
Data were presented as Mean±SEM (n = 4)

increased both in living cells and cell lysate and the NAT1 activity in cell lysate with 4-ASA (50~100 μM) was significantly higher substrate 4-ASA (25~100 μM), NAT1 activity was markedly than that in living cells ($p<0.05$). As shown in Fig. 4b, with the presence of different concentration of substrate INH (1.25~10 mM), NAT2 activity was dramatically increased in cell

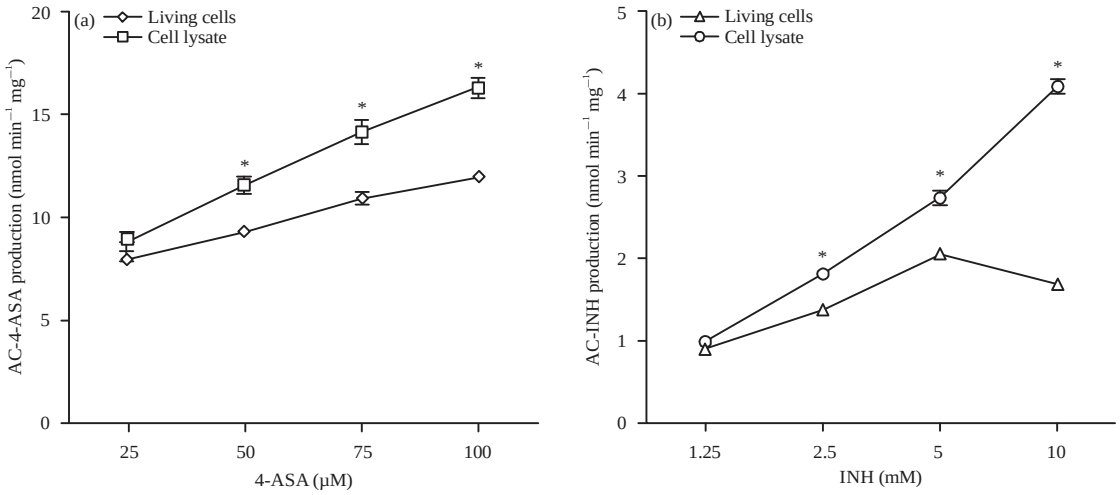

Fig. 4(a-b): NAT activity assay in (a) Living cells and (b) Cell lysate of HepaRG cells
Data were presented as Mean±SEM (n = 4). *p<0.05 vs. cell lysate group

lysate, while it only increase in living cells incubated with 1.25, 2.5 and 5 mM INH but not 10 mM INH. The NAT2 activity of cell lysate with INH (2.5~10 mM) was significantly higher than that of living cells ($p<0.05$). Based on the results, 75 μM 4-ASA and 5 mM INH was selected as the substrates, respectively, for further NAT1 and NAT2 activity assay in living cells.

NAT activity assay for screening of NAT inhibitors: To compare the sensitivity of NAT activity assay methods that based on living cells and cell lysate, ferulic acid and curcumin, the reported specific inhibitor of NAT1 and NAT2 were used in this study, respectively. In Fig. 5a, after 48 h exposure to ferulic acid (100~200 μM) and curcumin (25~200 μM), the cells viability was significantly decreased ($p<0.05$). Therefore, the non-cytotoxic concentration of ferulic acid and curcumin in HepaRG cells was determined as that below 50 and 10 μM, respectively. in Fig. 5b, in NAT1 activity assay, 25 and 50 μM ferulic acid exposure significantly reduced the production of AC-4-ASA in living cells ($p<0.05$), while 50 μM ferulic acid in cell lysate markedly reduced the production of AC-4-ASA ($p<0.05$). For NAT2 activity evaluation, 5 and 10 μM curcumin pretreatment significantly reduced the production of AC-INH both in living cells and cell lysate ($p<0.05$). Moreover, the protein expression of NAT1 and NAT2 in living HepaRG cells was not altered after ferulic acid and curcumin treatment (Fig. 5c).

DISCUSSION

HepaRG cell line was capable of expressing drug metabolizing enzymes of the liver, which had been considered as a novel cell model for drug metabolism studies[27]. Results from this study showed that NAT1 and NAT2 could be identified in HepaRG cells, which was in accordance with the finding showing NATs gene expression in HepaRG cells[18]. It was notable that the protein levels of NATs were gradually increased after cell passage, suggesting that NATs might mainly reside in mature HepaRG cells. This finding was consistent with previous study showing increased b-galactosidase activity in HepaRG cells that had reached confluence[28] and NATs expression reached steady state in the mature HepaRG cells[19].

In this study, 4-ASA and INH were selected as the specific substrates for NAT1 and NAT2 activity assay, respectively. Non-cytotoxic concentration of 4-ASA and INH in HepaRG cells were determined as that below 100 and 10 mM, respectively. Results showed that the produced AC-4-ASA in living cells was substrate dependent during 1~8 h exposure to 50 and 75 μM of 4-ASA but not after 6~8 h exposure to 25 and 100 μM of 4-ASA. It speculated that high 4-ASA (100 μM) might activate substrate-dependent regulation or cause saturation in enzyme activity, for NAT1 activity could be regulated by its substrate in living cells[23]. Low 4-ASA (25 μM) caused lowered AC-4-ASA production, which may result from the insufficient substrate. For NAT2 activity assay, AC-INH production was time-dependently increased when cells were exposed to 1.25~5 mM INH. It was notable that relatively less AC-INH was produced after 10 mM INH exposure, this might be related to the reduced enzyme activity, for 13 mM INH was found to reduce G6PDH activity in HepG2 cells[29].

The sensitivity and validity of NATs activity assay in living cells were compared to that of cells lysate. In the presence of

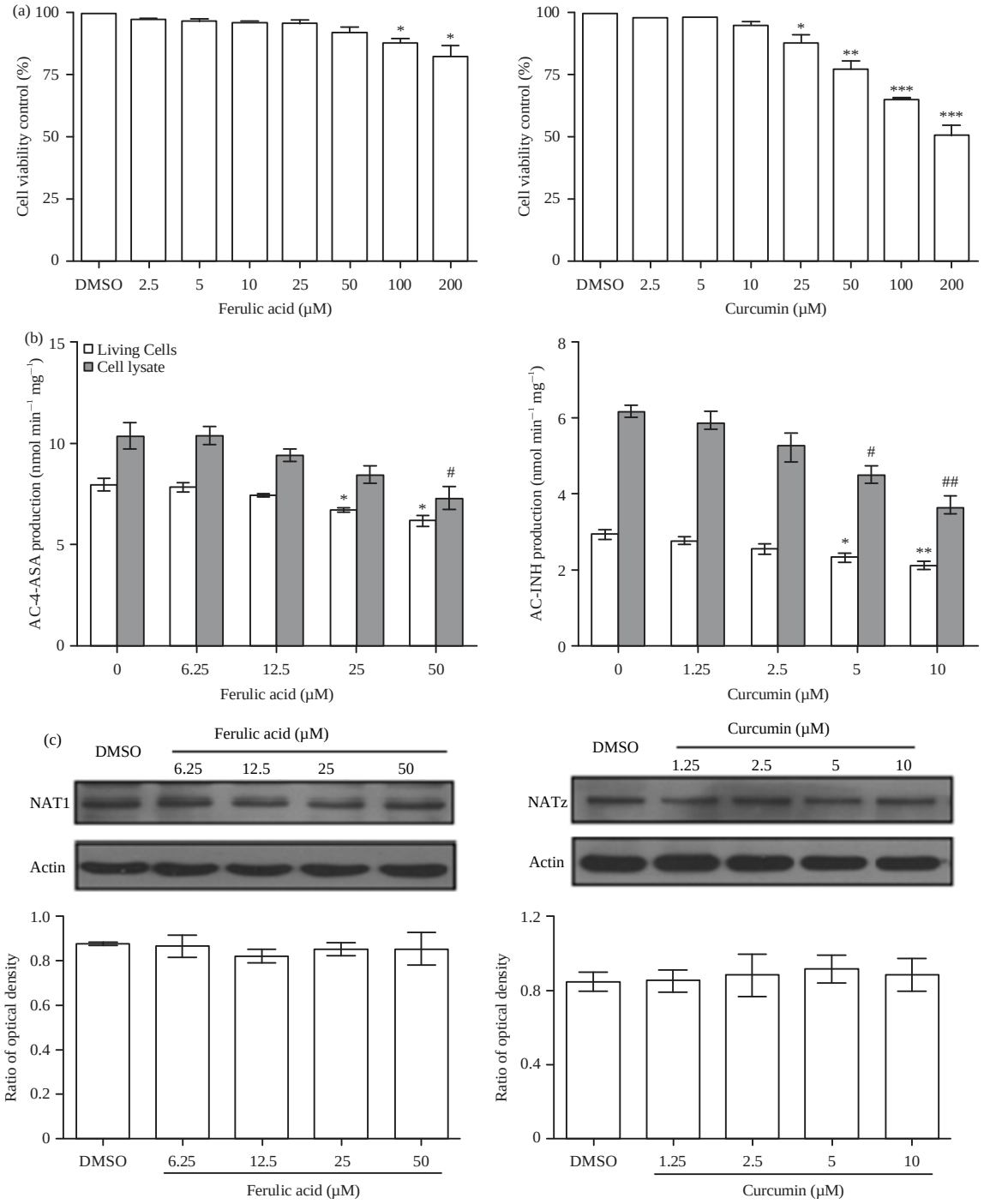

Fig. 5(a-c): Comparison on the results of NAT inhibitor screening using living cells and cell lysate, (a) Effect of ferulic acid and curcumin on cells viability in HepaRG cells, data were presented as Mean±SEM (n = 4) *p<0.05, **p<0.01, ***p<0.001 versus DMSO group. (b) Effect of ferulic acid and curcumin on NAT1 and NAT2 activity, data were presented as Mean±SEM (n = 4) *p<0.05, **p<0.01, versus the control of living cells #p<0.05, ##p<0.01 vs. the control of cell lysate and (c) Effect of ferulic acid and curcumin on the protein expression of NAT1 and NAT2

4-ASA (25~100 μM), substrate dependent AC-4-ASA production was both presented in cells lysate assay and living cells, even though the value of NAT1 activity in cell lysate was relatively higher. The result indicated that NAT1 activity could be evaluated in living HepaRG cells by using 4-ASA (25~100 μM). In the presence of INH (1.25~10mM), substrate

dependent AC-INH production was presented in cells lysate assay; while it was only found upon 1.25~5 mM INH exposure; indicating that NAT2 activity could be determined in living HepaRG cells by using INH (1.25~5 μM). The advantage of the novel method for NATs activity assay using living HepaRG cells may lie in the dynamic process of enzymatic reaction in living organisms.

Ferulic acid and curcumin, the reported specific inhibitor of NAT1 and NAT2, were also tested for NATs activity assay. The results from living cells and cells lysate both showed that ferulic acid had inhibitory effect on NAT1, while curcumin had inhibitory effect on NAT2, which were consistent well with previous report[7]. Above findings indicated that NATs activity assay based on living HepaRG cells could be used for screening potential NATs inhibitors. As 4-ASA was a substrate of multiple transporters that controlling cellular uptake and efflux of 4-ASA[30], the vital role of transporters in 4-ASA disposition. Therefore, to screen NATs inhibitors by determining AC-4-ASA concentration in HepaRG cells may have a limitation, because it couldn't exclude the effect on transporters. Even though, this study provides an approach for preliminary screening of NATs potential inhibitors.

CONCLUSION

This present study showed that NAT1 and NAT2 could be identified in HepaRG cells and NAT1 and NAT2 activity could be respectively evaluated by measuring the acetylated metabolites of their specific substrates 4-ASA or INH in living HepaRG cells. The established method for NAT activity assay could also be used for screening the potential inhibitors of NATs.

SIGNIFICANCE STATEMENT

This present study provided a stable, reproducible and low-cost method for NAT activity assay by using living HepaRG cells. This novel method was suitable for high throughput screening of the NAT inhibitors, for it could provide valuable biological information about the tested NAT inhibitors, such as bio-membrane permeability and cytotoxicity, which was useful for new drug discovery and development.

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