

## Original Article

# Serum metabolic changes in rats after intragastric administration of entinostat

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**Abstract:** Entinostat, also known as SNDX-275 and MS-275, is a benzamide histone deacetylase inhibitor undergoing clinical trials for treatment of various cancers. In this study, we developed a serum metabolomic method based on gas chromatography-mass spectrometry (GC-MS) to evaluate the effect of intragastric administration of entinostat on rats. The entinostat group rats were given 12.3, 49 mg/kg of entinostat by intragastric administration each day for 7 days. Partial least squares-discriminate analysis (PLS-DA) revealed that intragastric administration of entinostat induced metabolic perturbations. Compared to the control group, the level of butanedioic acid of entinostat group decreased. The results indicate that metabolomic methods based on GC-MS may be useful to elucidate side effect of entinostat through the exploration of biomarkers (butanedioic acid). According to the pathological changes of liver at difference dosage, entinostat is hepatotoxic and its toxicity is dose-dependent.

**Keywords:** Metabolomics, GC/MS, entinostat, rat

## Introduction

Entinostat, is an orally active bioavailable histone deacetylase inhibitor [1, 2]. In preclinical studies, MS-275 has demonstrated antitumor activity against a variety of human cancer cell lines [3, 4]. As a research method, metabolomics can reflect changes in endogenous substances in different physiological or pathological states. Metabolomics has unique advantages in drug safety evaluation, toxicity prediction, and disease diagnosis because of its high sensitivity, broad coverage, and good reproducibility [5]. In recent years, metabolomics has been widely applied to uncover biomarkers [6] and metabolic fingerprint in drug discovery and clinical toxicology [7], especially to investigating systematic metabolic responses to toxins [8] and the associated mechanisms [9]. The primary goal of this study is to study systematically the metabolic pathway changes induced by entinostat in rats.

## Material and methods

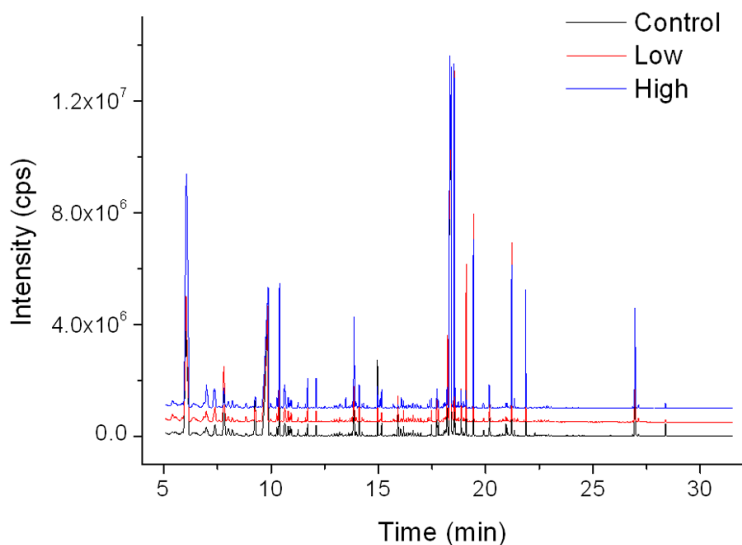
### Chemicals and animals

Trimethylchlorosilane (TMCS) and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich (Shanghai, China). Pyridine and methylhydroxylamine hydrochloride were purchased from Aladdin Industrial, Inc. (Shanghai, China). HPLC-grade n-heptane and acetonitrile were purchased from Tedia Reagent Company (Shanghai, China). Sprague-Dawley rats (male, 220±20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd.

### Instrumentation and conditions

Agilent 6890N-5975B GC/MS, HP-5MS (0.25 mm×30 m×0.25 mm), were from Agilent Company (Santa Clara, California, USA). The GC oven was initially set at 80°C and was kept at this temperature for 5 minutes. The tempera-

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**Figure 1.** Typical GC-MS total ion chromatogram of rat serum after intragastric administration of entinostat.

Eighteen rats (220±20 g) were randomly divided to entinostat group (Low, High) and control group. Entinostat group were give entinostat (12.3, 49 mg/kg, Low, High, each dosage was 6 rats) by continuous intragastric administration for 7 days. Control group were give saline by continuous intragastric administration for 7 days.

Blood samples were collected from the rats from the control group and intragastric administration of entinostat group at 8:00 am after 2 days, respectively. The blood samples were collected and then centrifuged at 8000 g for 10 min at 4°C. The serum was stored at -80°C until measurement.

ture was then gradually increased to 260°C at a rate of 10°C/min, and then kept at 260°C for 10 minutes. Mass detection was conducted first in EI mode with electron energy of 70 eV, then in full-scan mode with m/z 50-550, and finally, by splitless mode injection [10, 11].

### Sample preparation

The 250 µL of acetonitrile was added to 100 µL of serum, kept in an ice-bath for 15 min, and then were centrifuged at 10000 g for 10 minutes at 4°C. The 150 µL of the supernatant was transferred to a GC vial and evaporated to dryness under a stream of nitrogen gas. Methoximation was carried out at 70°C for 24 h after 50 µL of methylhydroxylamine hydrochloride (15 mg/mL in pyridine) was added. The 50 µL MSTFA (with 1% TMCS as the catalyst) was added and kept at 70°C for another hour, and then vortexed after adding 150 µL n-heptane [11].

### Metabolomics study

Rats were housed under a natural light-dark cycle conditions with controlled temperature (22°C). All eighteen rats were housed at Laboratory Animal Research Center of Wenzhou Medical University. All experimental procedures were approved ethically by the Administration Committee of Experimental Animals of Wenzhou Medical University.

### Histopathology

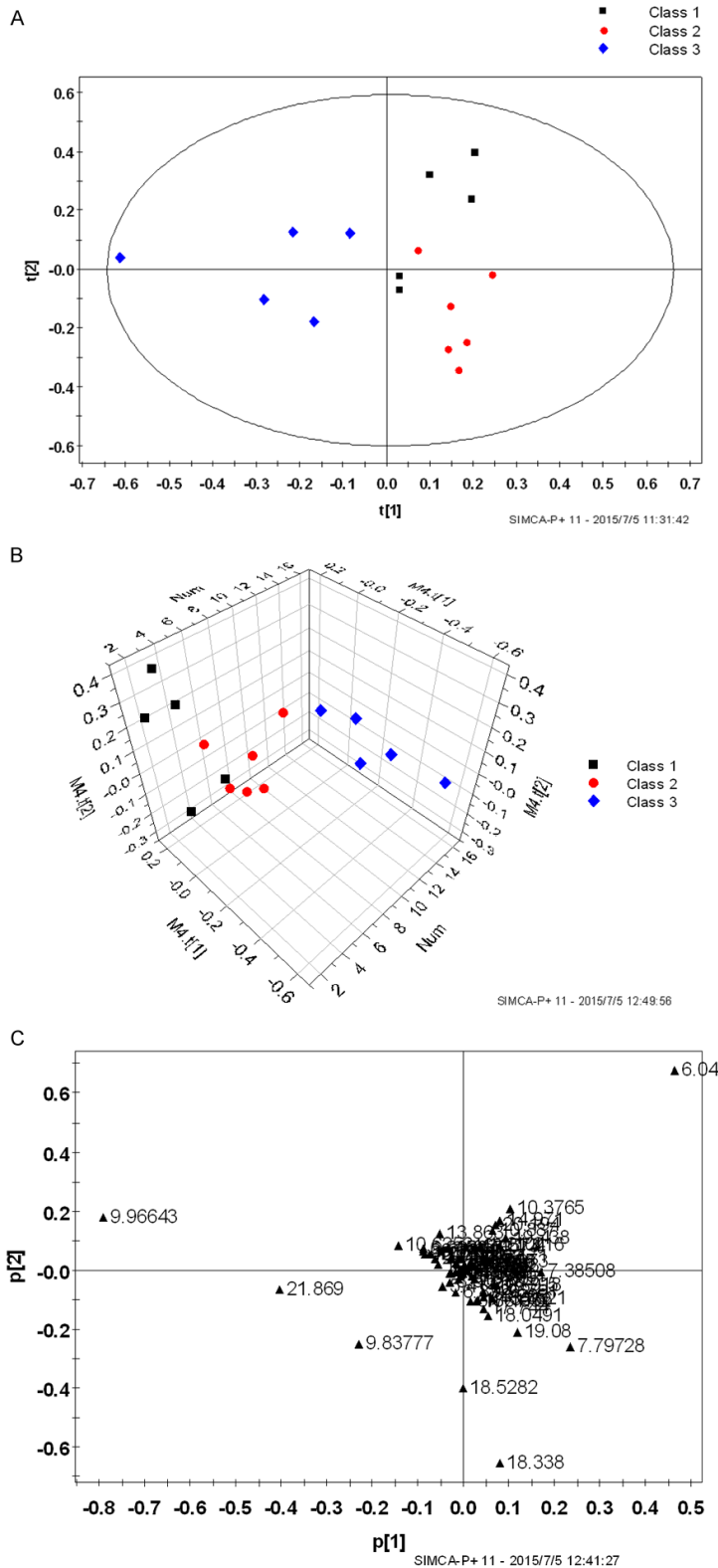
After metabolomics experiment, rats were deeply anesthetized with 10% chloral hydrate (i.p., 20 mg/kg). The liver were rapidly isolated and immersed in freshly prepared 4% w/v formaldehyde (0.1 M phosphate buffers, pH 7.2) for 48 h and then embedded in paraffin. Then 4-µm-thick histologic sections were prepared and stained with hematoxylin and eosin by routine hematoxylin-eosin staining method. The morphological changes were observed under light microscope.

### Data analysis

The GC/MS data was exported into Microsoft Excel, with the peaks normalized to the total sum of spectrum prior to multivariate analyses. The resulting data was processed through principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) using SIMCA-P 11.5 software (Umetrics, Umea, Sweden).

Statistical analysis was carried out using SPSS software (Version 18.0, SPSS). Independent samples T-test was applied in order to detect significant differences in all metabolites between two groups. A P value of < 0.05 was considered statistically significant.

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**Figure 2.** PLS-DA score results of rat serum samples (A), PLS-DA 3D score results of rat serum samples (B), after intragastric administration of entinostat (12.3, 49 mg/kg, Low, Medium, High), Control (Class 1), Low (Class 2), High (Class 3); the corresponding load diagram (C).

## Results and discussion

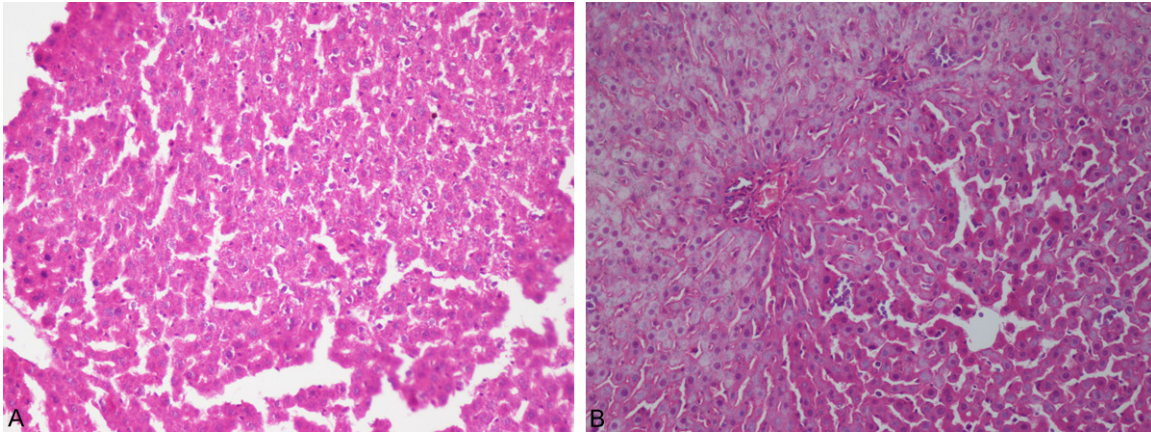
### Metabolomics study

Metabolomics is a newly emerging omics approach to the investigation of metabolic phenotype changes induced by environmental or endogenous factors [12-17]. It has shown promising results in healthcare fields, especially in disease diagnosis and drug-toxicity assessment, as reviewed recently [18, 19]. In drug-toxicity assessment, metabolomics is often concerned with finding toxicity-related biomarkers by investigating the changes in metabolic signatures induced by drug exposure [10, 20].

**Figure 1** provides the typical metabolic profiles of serum acquired through GC-MS technique. Metabolic profile data pretreatment resulted in a final dataset consisting of seventy-four metabolic features from GC-MS analyses. The endogenous metabolites in the serum were identified using the NIST 2005 mass spectrometry database.

In order to explore the metabolic profile changes of entinostat in rats after different dose (12.3, 49 mg/kg, Low, High), we compared the GC-MS spectrum of PLS-DA of the entinostat group (Low, High) with the rats in the control group (**Figure 2**). **Figure 2A** PLS-DA score chart showed that the first principal components of the rats in the entinostat group (Low, High) were distinguished from the rats in the control group. PLS-DA 3D (**Figure 2B**) score chart showed that the rats in entinostat group were distinguished from the rats in the control group clearer than 2D **Figure**

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**Figure 3.** Morphological changes of liver in entinostat-group at low (A), high (B) dosage (hematoxylin-eosin,  $\times 200$ ).

**Table 1.** Summary of the changes in relative levels of metabolites in rat serum after intragastric administration of entinostat

NO.	Retention time/min	Metabolite	VIP	Dose group	
				Low	High
1	21.869	Galacturonic acid	3.21217	-	$\uparrow^{**}$
2	9.96643	Serine	3.01332	-	-
3	7.79728	Ethanedioic acid	2.58685	$\downarrow^{**}$	-
4	18.338	d-Glucose	2.44393	-	-
5	6.04492	Propanoic acid	2.41983	-	-
6	14.971	Butanoic acid	1.81271	$\downarrow^{**}$	-
7	19.438	Hexadecanoic acid	1.54104	-	$\downarrow^{**}$
8	19.08	Maltose	1.38711	-	-
9	7.38508	Glycine	1.31725	-	-
10	21.216	Octadecanoic acid	1.2362	-	$\downarrow^{**}$
11	26.969	Benzoic acid	1.21837	-	-
12	9.83777	Urea	1.15607	-	-
13	6.98034	L-Alanine	1.04957	-	-
14	10.887	Butanedioic acid	1.04736	$\downarrow^{**}$	$\downarrow^*$
15	10.6253	L-Isoleucine	1.01635	-	$\uparrow^{**}$

Note: Variable importance in the projection (VIP) was acquired from the PLS-DA model with a threshold of 1.0. Marks indicate the direction of the change, i.e.  $\downarrow$  for decrease,  $\uparrow$  for increase, - for no change. Compared control group with entinostat group (20, 80 mg/kg, Low, High), \* $P < 0.05$  and \*\* $P < 0.01$ , as indicated by the statistical analysis T-test.

**2A**, the corresponding load diagram was shown in **Figure 3C**.

### Morphological changes of liver

Hepatocytes around the central vein disorder, liver plate portion streak structure disappeared, in low dosage group (**Figure 3A**). Cytoplasm of hepatocytes around the central vein lighter color, liver sinusoidal space disappears,

in high dosage group (**Figure 3B**). According to the pathological changes of liver at difference dosage, entinostat is hepatotoxic and its toxicity is dose-dependent.

### Changes in metabolite

Metabolomics comprises the measurement of endogenous metabolites, including amino acids, nucleic acid precursors, lipids, and degradation products of chemical intermediates in catabolism and biosynthesis. The advantage of metabolomics is that it provides the most functional measure of cellular status and can help to describe an organism's phenotype [21].

In this study, the changes of metabolites between entinostat groups and their control group were shown in **Table 1**. Compared to the control group, the level of ethanedioic acid, butanoic acid, butanedioic acid of the low dosage entinostat group decreased. Compared to the control group, the level of galacturonic acid and L-Isoleucine of the high dosage entinostat group increased, while hexadecanoic acid, octadecanoic acid butanedioic acid decreased.

These finding may be useful for new evidences in entinostat study [22, 23]. Additional prospective studies will be required to better understand these observations.

### Conclusion

These biomarkers (butanedioic acid) were the additional evidence. According to the pathological changes of liver at difference dosage, enti-



nostat is hepatotoxic and its toxicity is dose-dependent. We demonstrated that metabolomic methods based on GC/MS could provide a useful tool for exploring biomarkers to elucidate drug-toxicity.

#### Disclosure of conflict of interest

None.

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