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Bioactive form of resveratrol in glioblastoma cells and its safety for normal brain cells

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ABSTRACT

Background: Resveratrol, a plant polyphenol existing in grapes and many other natural foods, possesses a wide range of biological activities including cancer prevention. It has been recognized that resveratrol is intracellularly biotransformed to different metabolites, but no direct evidence has been available to ascertain its bioactive form because of the difficulty to maintain resveratrol unmetabolized in vivo or in vitro. It would be therefore worthwhile to elucidate the potential therapeutic implications of resveratrol metabolism using a reliable resveratrol-sensitive cancer cells.

Objective: To identify the real biological form of trans-resveratrol and to evaluate the safety of the effective anticancer dose of resveratrol for the normal brain cells.

Methods: The samples were prepared from the condition media and cell lysates of human glioblastoma U251 cells, and were purified by solid phase extraction (SPE). The samples were subjected to high performance liquid chromatography (HPLC) and liquid chromatography/tandem mass spectrometry (LC/MS) analysis. According to the metabolite(s), *trans*-resveratrol was biotransformed *in vitro* by the method described elsewhere, and the resulting solution was used to treat U251 cells. Meanwhile, the responses of U251 and primarily cultured rat normal brain cells (glial cells and neurons) to 100μM *trans*-resveratrol were evaluated by multiple experimental methods.

Results: The results revealed that resveratrol monosulfate was the major metabolite in U251 cells. About half fraction of resveratrol monosulfate was prepared *in vitro* and this *trans*-resveratrol and resveratrol monosulfate mixture showed little inhibitory effect on U251 cells. It is also found that rat primary brain cells (PBCs) not only resist 100μM but also tolerate as high as 200μM resveratrol treatment.

Conclusions: Our study thus demonstrated that *trans*-resveratrol was the bioactive form in glioblastoma cells and, therefore, the biotransforming activity of *trans*-resveratrol would be reversely correlated with the chemosensitivity of the treated cells. The findings from PBCs suggest that an effective anti-glioblastoma dose of resveratrol may not exert side-effect on normal brain cells, providing a strong evidence for practical use of resveratrol in the management of human brain malignancies.

Key words: Resveratrol, glioblastoma, drug metabolism

BACKGROUND:

Glioblastoma multiforme (GM) is the most common primary brain malignancy in human adults [1]. Irrespective to the combination of surgical operation with improved external radiotherapy and adjuvant chemotherapy, the prognosis of GMB remains very poor due to its highly aggressive biological behavior and frequent recurrence rate [2]. Therefore, exploring effective and less toxic chemotherapeutic approaches would be of clinical values in better management of this sort of lethal disease.

Resveratrol, a plant polyphenol existing in grapes and many other natural foods [3], possesses a wide range of biological activities including cancer prevention [4,5]. More importantly, resveratrol has little cytotoxic effect and is able to penetrate blood-brain barrier [6], suggesting its potential therapeutic values in the management of glioblastoma. It has been recognized that resveratrol is intracellularly biotransformed to different metabolites, but no direct evidence has been available to ascertain its bioactivity form, and how normal brain cells respond to this agent. So it would be worthwhile to identify the real biological form of trans-resveratrol and evaluate the resveratrol's safety in the normal glial cells with effective anticancer dose.

MATERIALS AND METHODS:

Primary rat brain cell culture and treatment. A 1-day-old Wistar rats were obtained from the Experimental Animals Center of Dalian Medical University. The rat brains were freshly removed and minced with a scalpel and triturated in high glucose DMEM (Gibico, Invitrogen Corporation, NY, USA). After centrifugation at 2000 rpm for 5 minutes, the brain cells were washed with DMEM and centrifugated for 5 minutes. The cell suspensions were plated to 60mm dishes (Nunc A/S, Rosklide, Denmark) and cultured in high glucose DMEM

supplemented with 10% FBS under 37^{o}C and 5% CO_2 condition, then treated with resveratrol. All experimental protocols had been reviewed and approved by the ethics committee of Dalian Medical University (ECDMU-09066) for protection of human subjects and experimental animals before conducting the project.

Sample preparation and purification. After 100μM resveratrol treatment, the primary brain cells and glioblastoma U251 cells were collected, washed three times with PBS (pH 7.4), and lysed by sonication. Meanwhile, cell-free media were harvested directly by the end of 48-hour resveratrol treatment or after an additional 24-hour normal culture. The collected media and cell lysates were centrifuged at 10,000g for 5 minutes, and the supernatants were purified by SPE [7]. The eluate was dried by nitrogen spraying, and the residues were dissolved in 500μL methanol and a 10μL aliquot was injected onto the liquid chromatography column for HPLC and LC/MS analysis.

Structural identification of resveratrol metabolite(s). The HPLC system (Waters Co., Milford, MA, USA) is consisted of a Waters 1525 binary pump and 2487 dual wavelength UV-VIS detector. The detection was carried out at 306nm [8]. Chromatographic separation of resveratrol and its metabolite(s) was performed on a Cosmosil 5C18-AR-Ⅱ column (4.6 ID×250 mm, NACALAI TESQUE, INC. Japan) containing a C18 guard column (5μm, 4.6×10mm), using the mobile phase of 5mM ammonium acetate (phase A) and methanol (phase B) with a flow rate of 1.0 ml/min. The gradient solution conditions were referred elsewhere [9]. Further LC-MS/MS analysis was performed on a Agilent 1200 liquid chromatography series (Agilent Technol Inc., Santa Clara, CA, USA) coupled to an Applied Biosystems API 3200 QTrap tandem mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) equipped with an ESI source, and operated by Applied Biosystem/MDS SCIEX analyst software (Version 1.4.1) to obtain the MS and MS/MS data in a negative ion mode [9]. Further identification and confirmation of the metabolites was performed on a Shimadzu LC/MS-IT-TOF (Shimadzu Co., Kyoto, Japan) instrument equipped with an ESI source in negative ion mode at a resolution of 10000 FWHM. MS data were processed with LC/MS solution ver. 3.4 software (Shimadzu, Japan).

Resveratrol biotransformation and cell treatment. One female specific pathogen free Wistar rat was cultured in a wire cage in a room maintained at 22°C with a 12-hour light/dark period at the Experimental Animal Center of Dalian Medical University. It was sacrificed by an animal expert in accordance with approved Ministerial procedures appropriate to the species. The brain tissue was rapidly obtained and treated to prepare cytosolic sulfotransferase.

For resveratrol sulfonation, 5mM resveratrol, 100μL cytosolic sulfotransferase, 2mM PAPS (Sigma Chem Co., St. Louis, MO, USA), 1mM DTT and 20mM Mops buffer were admixed and incubated at 37°C for 2h. After the product was centrifuged, the supernatant was subjected to HPLC and LC/MS analysis, and the aliquots of the remaining part were used

to treat U251 cells in the total concentration of 100μM. The cells treated by the chemical solution for brain lysate preparation, the brain lysate alone and the combination of resveratrol and brain lysate (without PAPS supplementation) were respectively used as background controls.

RESULTS:

Resveratrol monosulfate as major metabolite. According to the HPLC and LC-MS/MS analysis, the major metabolite in human glioblastoma U251 cells was resveratrol monosulfate Figure 1A. The $[M-H]^-$ ion of the compound (t_R=12.92min) showed the deprotonated molecule ions of m/z 307 and 227, respectively, and the ion corresponding to resveratrol (m/z 227) through the neutral loss of the sulfate unit (m/z 80) from the resveratrol monosulfate, then the m/z 227 was fragmented to m/z 185 for the further loss of 42 amu (C_2H_2O) from resveratrol (Figure 1B), which was concluded to resveratrol monosulfate as reported by other investigators [10]. The compound was further confirmed by HRMS that gave the $[M-H]^-$ molecular ion exact mass as 307.0261 ($C_{14}H_{11}SO_6$, calculated m/z 307.0276), which corresponded to resveratrol monosulfate.

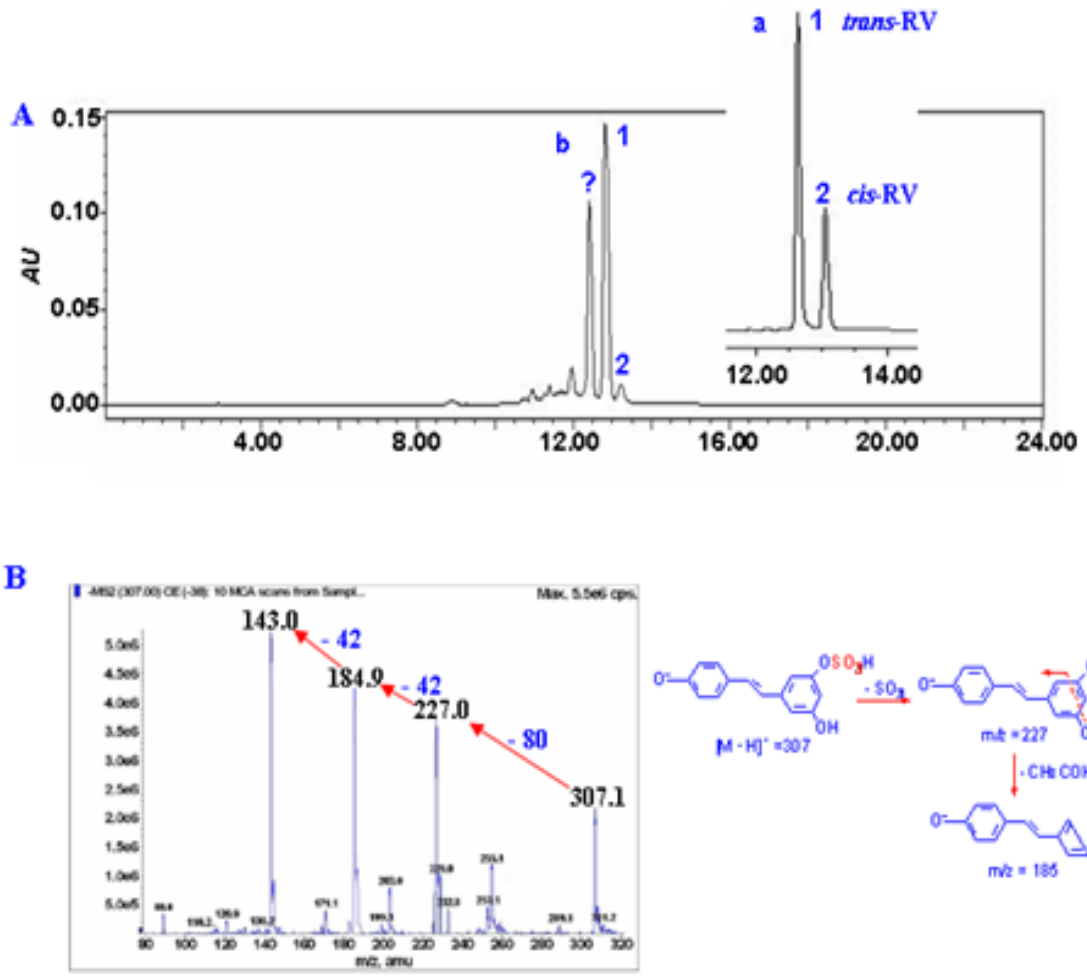

Figure 1. LC-MS analyses of resveratrol metabolites in glioblastoma U251 cells

(A) HPLC-UV chromatography analysis on the metabolites of U251 cells treated with 100μM resveratrol for 48h. 1, 2 represent *trans*-resveratrol and *cis*-resveratrol, respectively. (B) indicates the MS1 and MS2 analysis of the metabolite of resveratrol. ? represents resveratrol monosulfate.

Metabolic pattern of resveratrol in glioblastoma cells. Furthermore, the results showed that the resveratrol monosulfate concentration increased to the platform level at 12-hour time point, but the U251 cells reached apoptotic peak at 48-hour time point, which suggested that the metabolic mechanism pre-existed in the tumor cells apoptosis and its efficiency was enhanced in response to resveratrol treatment. It was also noticed that the amounts of *trans*-resveratrol elevated at 24- and 48-hour time points presumably due to the hydrolytic reaction that reversed resveratrol monosulfate to *trans*-resveratrol as proposed by Walle T. et al [11,12]. Several additional small peaks were observed from the HPLC spectrum, but their chemical features were not further analyzed here because of their low amounts.

***trans*-Resveratrol as the bioactive form in glioblastoma cells.** Resveratrol monosulfate was prepared by incubating *trans*-resveratrol with the brain lysates for 2h, and then was analyzed by HPLC and LC/MS. It was also revealed that the monosulfate was the major metabolite and more than 1/2 of parent *trans*-resveratrol was biotransformed to resveratrol monosulfate. In difference with the situation of 100μM *trans*-resveratrol treatment, U251 cells treated by the same concentration of this mixture for 48 hours showed neither distinct growth suppression nor apoptosis signs (Figure 2).

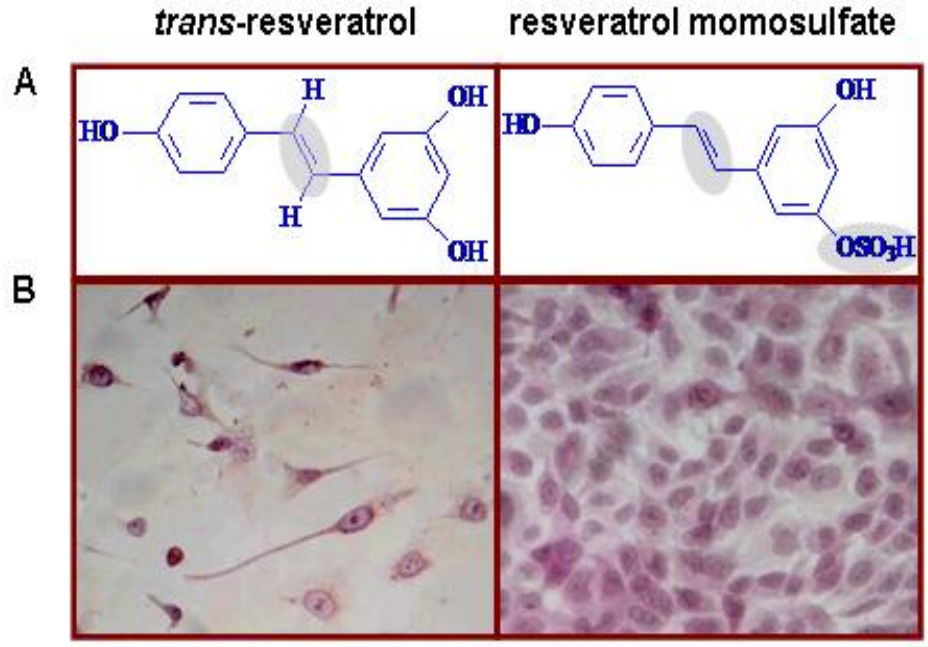

Figure 2. Biotransformation and bioacitve form evaluation in U251 cells (A) The chemical structures of *trans*-resveratrol and resveratrol monosulfate; (B) Morphologic evaluation of U251 cells incubated with 100μM of *trans*-resveratrol and *trans*-resveratrol/resveratrol monosulfate mixture for 48 h by H&E staining (40X).

No side-effect of resveratrol on normal rat brain cells. After 100μM resveratrol treatment, the glioblastoma U251 cells exhibited distinct apoptosis and growth inhibition, but the primarily cultured rat glial cells and neurons kept growth and showed no sign of cell

apoptosis and death, even incubated with 200μM resveratrol. Trypan blue staining revealed that the percentage of nonviable cells was 0.11%, 12.26%, 31.79%, 34.96%, and 52.73% in U251 cells, but 0.17%, 1.09%, 0.95%, 1.61%, and 1.99% in PBC cells after 100μM resveratrol treatment for 0, 12, 24, 36, and 48 hours, respectively. FCM analyses further demonstrated that G1 and S fractions were 37.7% and 25.7% in normal U251 cells and changed to 37.9% and 47.2% in the resveratrol-treated cells. In PBC cells, G1 and S fractions were 54.5% and 32.9% in normal cells and remained almost unchanged (53.7% and 35.0%) after resveratrol-treatment. The percentages of apoptotic cells in 100μM resveratrol-treated U251 and PBC cells were 24.3% and 1.1%, respectively.

DISCUSSION:

An ideal cancer therapeutic agent should have minimal cytotoxicity to normal tissues, meanwhile, exerts crucial effects on cancer cells. Our results showed that resveratrol could induce glioblastoma U251 cells growth arrest and apoptosis, on the contrary resveratrol was insensitive to the PBC cells. Which suggested that resveratrol had no side effect on normal brain cells, but exerted significantly anti-glioblastoma bioactivity, furtherly, resveratrol could penetrate the blood-brain-barrier (unpublished data). Therefore, resveratrol would be an ideal cancer-therapy drug for clinical brain tumors therapy.

trans-Resveratrol has been recognized as an ideal cancer preventive and therapeutic agent, and it can generate one or more metabolites in cells [10,13]. However, the pharmaceutical potentials of those metabolic products have not yet been well ascertained. Some researchers proposed that resveratrol metabolite(s) such as resveratrol sulfates or resveratrol glucuronides were the bioactive forms because of the low bioavailability of parent *trans*-resveratrol in vivo [11], while others considered that *trans*-resveratrol by itself was sufficient to cause vital cellular and molecular consequences in the treated cancer cells [14,15]. Apparently, determination of the bioactive form(s) of resveratrol in individual cell and cancer types becomes a fundamental issue for the successful application of resveratrol in clinical therapy.

In this study, it was found that the metabolite of *trans*-resveratrol was mainly resveratrol monosulfate, and rather than its metabolite, resveratrol parent form exhibited anti-tumor activity in the human glioblastoma U251 cells. It is therefore demonstrated that the efficiency of metabolic reduction of trans-resveratrol is the major determinant of the fate of resveratrol-treated glioblastoma cells. These findings would be of translational values in exploring tumor-selective and personalized application of resveratrol in cancer prevention and treatment.

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