Taurine But Not Proline Protects Cells from Cisplatin, Activates p21, and Fibronectin Expression

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ABSTRACT

Amino acids are macromolecules forming proteins. This makes them essential for life, as proteins are needed for many cell functions. Amino acids that are not part of a protein, but rather freely floating, are called free amino acids (FAAs). Cells can use FAAs to support metabolism, growth, and survival. We have previously determined the intracellular concentration of various FAAs in cells isolated from ovarian cancer (OC) malignant fluids. Taurine and proline were among the most enriched free intracellular amino acids in these patient cell samples. In contrast to malignant samples, a tissue culture model representing OC precursor cells contained significantly less intracellular taurine and proline. Taurine but not proline supplementation induced p21 activation, fibronectin deposition, cell adhesion, and resistance to DNA-damaging agent cisplatin. Our data suggest the possibility that accumulation of intracellular taurine but not proline can protect cells from cisplatin and this protection is associated with the activation of p21 and fibronectin protein expression.

INTRODUCTION

Amino acids are the macromolecule building blocks of proteins, which are necessary for all functions of life. However, not all amino acids are incorporated into a protein: some remain freely floating and are known as free amino acids (FAAs)[1]. Non-transformed and cancer cells use FAAs present within the tissue microenvironment to support metabolism, proliferation, and survival [2]. In this manuscript, we provide evidence that supplementation of tissue culture cells, representing ovarian cancer (OC) precursor cells, with the FAA taurine but not proline support cell survival.

OC can originate from the fallopian tube non-ciliated epithelial cells that carry mutations in the tumor suppressor gene *TP53*[3]. Under stress, activation of wild-type p53 protein, a product of the TP53 gene, can induce expression of the cell cycle inhibitor CDKN1A (p21), arresting proliferation and promoting stress resolution[4]. Mutation in the *TP53* gene can suppress the expression of p21 leading to uncontrolled proliferation and cancer[5]. Mutations in the *TP53* gene are ubiquitous in OC precursor cells, the fallopian tube non-ciliated epithelial (FNE) cells [6]. Here, we used FNE cells expressing mutant p53 (FNE-m-p53) to determine whether FAA (taurine or proline) supplementation modulates p21 activity.

Fibronectin, as a component of the extracellular matrix (ECM), is involved in the regulation of developmental biological processes such as cell adhesion, migration, proliferation, and survival[7]. Cells interact with fibronectin through the engagement of integrin molecules including integrin $\alpha5\beta1$[8]. We previously provided evidence that mutant p53, through regulation of fibronectin and integrin $\alpha5\beta1$ expression, supported the survival of anchorage-deprived FNE cells[9] . In this report, we demonstrate that supplementation of FNE-m-p53 cell monolayer cultures with taurine but not proline induces fibronectin expression and increases cell adhesion area.

Cisplatin is a DNA-damaging agent that is commonly used in the treatment of cancer[10]. While cisplatin is initially very effective in killing most OC cells, some cell populations are resistant[11]. Therefore, understanding the mechanisms of cisplatin resistance is important for the success of treatment. Here we demonstrate that taurine and proline are enriched in an intracellular pool of FAAs in cells isolated from OC patients. Furthermore, taurine but not proline supplementation of OC precursor cell cultures evoked cisplatin resistance, indicating the possibility that accumulation of taurine could help OC cells survive during treatment.

MATERIALS AND METHODS

Preparation of Cell Media

FNE-m-p53 cells were cultured in a 1:1 ratio of DMEM/F12 (HiMedia), and Medium 199 (HiMedia), 2% heat-inactivated Fetal Bovine Serum (HI-FBS; Sigma-Aldrich), 1% v/v penicillin-Streptomycin (VWR), 0.5 ng/mL of beta-estradiol (US Biological), 0.2 pg/mL of triiodothyronine (Sigma-Aldrich), 0.025 µg/mL all-trans retinoic acid (Beantown Chemical), 14 µg/mL of insulin (Sigma-Aldrich), 0.5 ng/mL of EGF (Peprotech), 0.5 µg/mL hydrocortisone (Sigma-Aldrich), and 25 ng/mL of cholera toxin (Calbiochem). All cell lines were cultured in a humidified incubator at 37°C and with 5% carbon dioxide. Cell cultures were tested for the presence of mycoplasma every 3-6 months using the Uphoff and Drexler detection method.

Cell culture media containing taurine or proline were prepared by dissolving the required amount of amino acid in the culture media. The necessary mass was first weighed out and transferred to a sterile 30-mL conical tube. Cell culture media was then added. The mixture was then placed in a water bath heated to 37℃, vortexing periodically until all solids dissolved. The mixture was then passed through a 0.22µm micropore filter to sterilize the media and remove any remaining particles.

Patient Malignant Fluids

For a study from 2021 to 2022, we enrolled Caucasian patients with histologically confirmed high-grade serous OC and ascites in the Department of Gynecologic Oncology, Poznań University of Medical Sciences. Ovarian tumors were staged according to the FIGO (International Federation of Gynecology and Obstetrics) system. Ascites fluid cell samples were collected from patients (i) at laparoscopy before starting neoadjuvant chemotherapy. Ascites fluid (10 mL) was centrifuged within 2 hours after collection in a falcon tube at 1,100 × *g* for 10 minutes at room temperature to separate a cell pellet. The supernatant and malignant cell pellets were stored at -80°C and analyzed collectively.

Mass-spectrometry-based quantification of taurine levels in cell lysates

The panel of amino acids was quantified based on a TRAQ kit for amino acid analysis (SCIEX, Framingham, MA, USA) and liquid chromatography coupled to a triple quadrupole tandem mass spectrometry technique. The samples (cell lysates) were thawed at room temperature, and 40 µL of a matrix was transferred to a 0.5 mL Eppendorf tube. Then, 10 µL of sulfosalicylic acid was added to precipitate the proteins, and the vial contents were mixed and centrifuged. Subsequently, 10 µL of supernatant was transferred to a clean tube, and 40 µL of borate buffer was added, mixed, and centrifuged. In the next step, the 10 µL of the obtained mixture was transferred to a clean tube and mixed with 5 µL of amino-labeling reagent (aTRAQ Reagent Δ8®). After centrifugation, samples were incubated for 30 minutes at room temperature. The incubation was followed by the addition of 5 µL of hydroxylamine solution, mixing, and centrifugation. Then the samples were incubated for 15 minutes at room temperature. In the next step, 32 µL of freshly prepared internal standards solution was added, mixed up, and centrifuged. The contents of the tubes were concentrated (temperature 50°C for about 15 minutes) to a volume of about 20 µL using a vacuum concentrator (miVac

Duo, Genevac, Stone Ridge, NY, USA). In the last step, 20 µL of ultrapure water was added to each vial and mixed. The contents of the tubes were transferred to amber-glass autosampler vials with inserts. Samples were analyzed in random order by chromatographic separation followed by tandem mass spectrometry detection LC-MS/MS. The analytes were separated on a Sciex C18 column (4.6 mm × 150 mm, 5 µm) maintained at 50°C using a 1260 Infinity HPLC instrument (Agilent Technologies, Santa Clara, CA, USA). A gradient flow of the mobile phase was applied. The mobile phase consisted of 0.1% formic acid (FA) and 0.01% heptafluorobutyric acid (HFBA) in water—phase A, and 0.1% FA and 0.01% HFBA in methanol—phase B, maintained at a flow rate 800 µL/min. Total runtime was 18 minutes per sample, with injection volume equal to 2 µL. Detection and quantitation of analytes were performed by means of a quadrupole tandem mass spectrometer with an electrospray ionization (ESI) TurboV ion source operated in positive-ion mode. All results were generated in a scheduled multiple-reaction monitoring mode. Raw data from amino-acid assays was acquired and analyzed using the Analyst software version 1.6.3 (Sciex, Framingham, MA, USA). The method validation and sample preparation were described in detail before [19].

Cisplatin Response Assay

Cells were counted and plated (75,000 cells) on a cell culture-treated 6-well plate with 1mL of the relevant medium and left for 12 hours to adhere. After this period, the media was aspirated and 1mL of culture-grade phosphate-buffered saline (PBS) was added to each well. This too was aspirated, followed by the addition of 2mL of either unsupplemented or supplemented media where relevant. Cells were left to acclimate to this treatment for 3-4 hours, followed by treatment with cisplatin to a final concentration of 10µM. Treatment lasted 72 hours.

After 72 hours, cells were collected for flow cytometry according to the following protocol. Cell media was collected into a separate 15mL conical tube for each well by micropipette, followed by the addition of 1mL of PBS to each well. This too was collected into the same tube, followed by the addition of 1mL of trypsin in PBS, leaving the 6-well plate in an incubator at 37℃ for 5 minutes. Following this, trypsin was inactivated via the addition of 1mL of PBS containing 2% fetal bovine serum (FBS). The entire suspension was then transferred to the same respective tubes as were previously used. The tubes containing the cells were then centrifuged at room temperature at a speed of 300xg for 3 minutes. The supernatant was aspirated and the pelleted cells were resuspended in 1mL of PBS/2% FBS, followed by a second identical centrifugation. Again, the supernatant was aspirated and the cells were resuspended in 2mL of PBS/2% FBS, adding propidium iodide to a final concentration of 2 µg/mL. The suspensions were kept on ice and immediately analyzed by flow cytometry. Data acquisition was performed using an Attune NxT Flow Cytometer from ThermoFisher. Data were then analyzed using FlowJo software.

Western Blot

Cells were counted and (300,000 cells) plated on 6 cm^2 tissue culture-treated dishes, to which were added 3mL of relevant culture medium. The cells were left to adhere to the plates for 12 hours in an incubator at 37℃. Following this, media was aspirated, and 1mL of PBS was added to each plate. This too was aspirated and 4mL of either unsupplemented or supplemented media was added to each plate. Cells were treated with amino acid for 72 hours in an incubator at 37℃, followed by lysis. This was performed by first placing the plates on ice, and aspirating all media. 4-5mL of PBS cooled to 4°C was added to each plate. This was then aspirated and 250mL of a 1X RIPA buffer with 1X protease inhibitor cocktail was added. The cells were removed by a cell scraper which was previously sterilized with 70% ethanol, and pipetted into pre-chilled 1.5mL tubes.

Lysed cells were allowed to stand on ice for 15 minutes to accommodate the lysis reaction. They were then centrifuged at 17,900xg for 15 minutes at 4℃. The supernatant was removed and transferred into a new set of pre-chilled 1.5 mL tubes. Protein quantification was done via BCA assay, measured using a plate reader. Following this, a solution of lamellae buffer with beta-mercaptoethanol was added and the proteins were boiled in a heat block at 95℃ for 15 minutes and stored at -20℃ until later use.

The vertical transfer was performed using a 10% SDS-PAGE. Horizontal transfer was performed onto a PVDF membrane. This membrane was blocked with 5% milk in 1X TBS-T, followed by overnight incubation in the respective primary antibody, diluted 1:2000 in 5% non-fat milk. The membrane was then transferred to a solution of secondary antibody, diluted 1:10,000 in TBS-T. After incubation in the secondary antibody for 1 hour, the membrane was left to wash in TBS-T for 30 minutes, replacing the wash solution with fresh Tris-buffered saline containing 0.1% v/v Tween-20 (TBST) every 10 minutes. Membranes were developed using ImmobilonTM Forte enhanced chemiluminescent substrate and visualized using an iBright CL1500.

Cell Area Quantification

Cells were counted and (200,000) plated on a 6-well tissue-culture treated plate, to which was added 1mL of relevant culture medium. The cells were left to adhere to the plates for 12 hours in an incubator at 37C. Following this, media was aspirated, and 1mL of PBS was added to each plate. This too was aspirated and 2mL of either unsupplemented or supplemented media was added to each plate. Cells were treated with amino acid for 6 days in an Agilent BioTek Lionheart FX automated fluorescence microscope, taking images every 12 hours under both Phase Contrast and GFP channels. After 6 days, cells were disposed of and the images were analyzed using Fiji ImageJ software. The mean cell area was quantified.

Cell Viability Assay

Cells were counted and (200,000) plated on a 6-well tissue-culture treated plate, to which was added 1mL of relevant culture medium. The cells were left to adhere to the plates for 12 hours in an incubator at 37C. Following this, media was aspirated, and 1mL of PBS was added to each plate. This too was aspirated and 2mL of either unsupplemented or supplemented media was added to each plate. Cells were treated with amino acid for 72 hours and then analyzed by flow cytometry according to the following protocol.

After 72 hours, cells were collected for flow cytometry according to the following protocol. Cell media was collected into a separate 15mL conical tube for each well by micropipette, followed by the addition of 1mL of PBS to each well. This too was collected into the same tube, followed by the addition of 1mL of trypsin in PBS, leaving the 6-well plate in an incubator at 37℃ for 5 minutes. Following this, trypsin was inactivated via the addition of 1mL of PBS containing 2% fetal bovine serum (FBS). The entire suspension was then transferred to the same respective tubes as were previously used. The tubes containing the cells were then centrifuged at room temperature at a speed of 300xg for 3 minutes. The supernatant was aspirated and the pelleted cells were resuspended in 1mL of PBS/2% FBS, followed by a second identical centrifugation. Again, the supernatant was aspirated and the cells were resuspended in 2mL of PBS/2% FBS, adding propidium iodide to a final concentration of 2 µg/mL. The suspensions were kept on ice and immediately analyzed by flow cytometry. Data acquisition was performed using an Attune NxT Flow Cytometer from ThermoFisher. Data were then analyzed using FlowJo software.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 9.0 (Graphpad Software, San Diego, CA, USA). Statistical significance was determined using an unpaired, two-tailed, parametric t-test, ordinary one-way ANOVA with Tukey's *post hoc* multiple comparisons test, or two-way ANOVA with Sidak's *post hoc* multiple comparisons test, with $p \leq 0.05$ considered statistically significant.

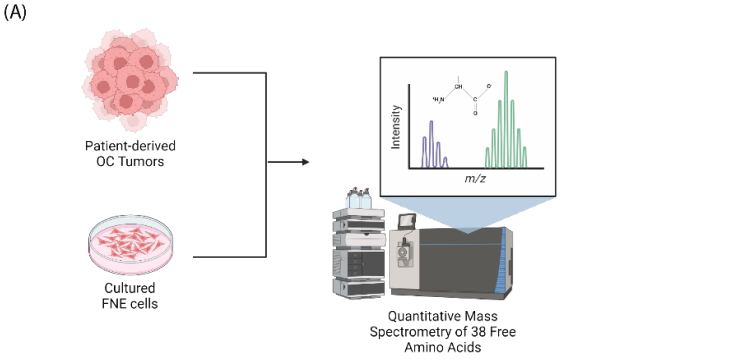
RESULTS

Taurine but not proline supports the survival of OC precursor cells under cisplatin culture conditions.

To start addressing whether supplementation of OC precursor cells with FAAs supports survival from cisplatin treatment, we first used mass spectrometry to determine the intracellular levels of 38 FAAs in cells isolated from OC patients and in FNE-m-p53 cells cultured as monolayers (**Fig. 1A**). Our experiment revealed that cells isolated from OC patients contained significantly more intracellular taurine or proline (**Fig. 1B-C**). Based on our previous report[12], demonstrating that 160 mM taurine supplementation of tissue culture cells increases intracellular taurine to comparable levels found in cells isolated from OC patients, we used 160 mM taurine or proline supplementation protocol. We found that under these culture conditions, taurine but not proline significantly protected FNE-m-p53 cells from cisplatin (**Fig. 1D**). Our results are consistent with the idea that intracellular accumulation of taurine but not proline can protect cells from DNA damaging cisplatin treatment.

Taurine but not proline supplementation activates p21 protein expression in OC precursor cells.

Expression of cyclin-dependent kinase inhibitor, CDKN1A, also known as p21 can be associated with cisplatin resistance[13]. We have previously demonstrated that taurine induces p53 binding to DNA and p53-dependent activation of p21[12].



(B)

Intracellular Concentration (μM)		
	Taurine	L-Proline
Patient 1	401.67	79.06
Patient 2	42.34	56.63
Patient 3	384.45	193.94
Patient 4	262.93	687.92
Patient 5	1292.43	480.23
Patient 6	145.89	123.86

(C)

Intracellular Concentration (μM)		
	Taurine	Proline
Sample 1	3.18	12.21
Sample 2	3.22	12.28

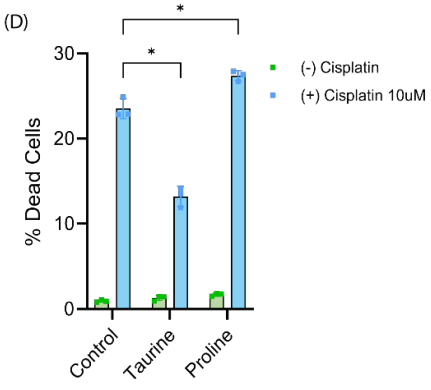


Figure 1. Modelling intracellular amino acid concentrations in OC precursor cells. (A) Experimental design for measuring intracellular amino acid concentration in patient-derived (n=6) OC ascites cells and cultured FNE-m-p53 cells using mass spectrometry. (B-C) Intracellular taurine and proline concentrations in (B) patient-derived OC ascites cells and (C) cultured FNE-m-p53 cells. (D) Chemotherapy resistance in FNE-m-p53 cells measured by flow cytometry-based propidium iodide (PI) staining. Y-axis refers to the percentage of PI(+) cells . (*Indicates $p < 0.05$, compared to control. Data are reported as ± SD and were analyzed using one-way ANOVA.)

Here we report on our experiment testing the activation of p21 in response to taurine or proline supplementation in FNE-m-p53 cells (**Fig. 2A**). Our experiment revealed that 160 mM taurine but not 160 mM proline supplementation of FNE-m-p53 monolayer cell cultures activated p21 (**Fig. 2B**). Our results are consistent with the idea that taurine activates p53/p21 to restrict the cell cycle, and through these mechanisms promotes cisplatin resistance.

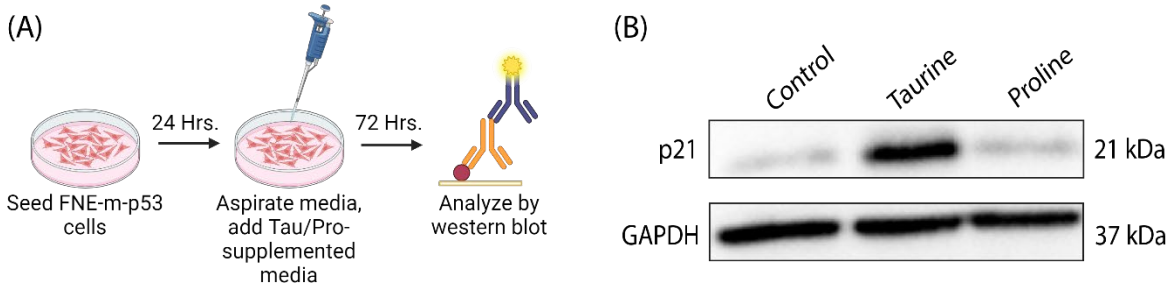


Figure 2. Determination of p21 activation by taurine and proline in FNE-m-p53 cells. (A) Experimental design. Supplemented cell media was prepared at a final concentration of 160mM taurine or proline, respectively. (B) Western blot of p21 expression in FNE-m-p53 cells treated with supplemented medium.

Taurine but not proline supplementation promotes fibronectin expression in OC precursor cells.

Literature supports the idea that prolonged activation of p21 can be associated with increased cell size[14]. Thus, we wondered whether taurine increases cell size, as defined by measuring cell adhesion area. First, we used fluorescent microscopy imaging of FNE-m-p53 expressing green fluorescent protein (GFP) to demonstrate that taurine but not proline supplementation increased cell adhesion area (**Fig. 3A,C**). Second, we probed the expression levels of fibronectin, a major ECM protein involved in cell adhesion[15]. Our experiment revealed that taurine but not proline-induced expression of fibronectin in FNE-m-p53 cells (**Fig. 3B**). These results support the model whereby accumulation of intracellular taurine activates p21 leading to cell cycle arrest and increased deposition of fibronectin.

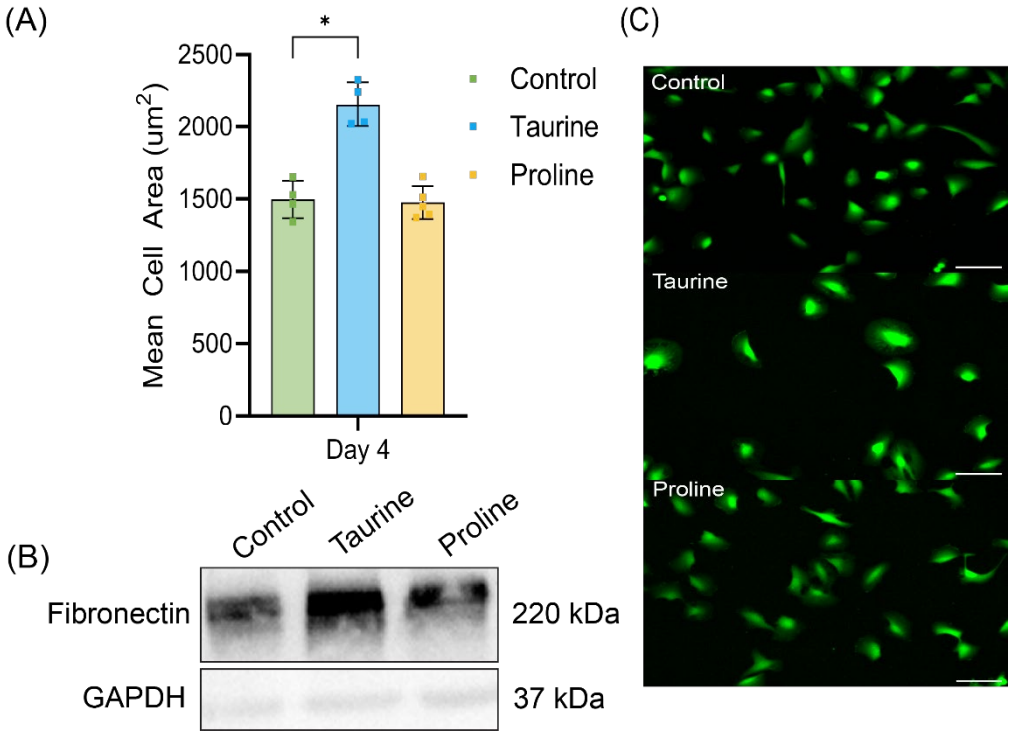


Figure 3. Activation of cell adhesion mechanisms by amino acid supplementation. (A) Mean cell area in FNE-m-p53 cells with respective media supplementation at 160mM. Quantification was performed from live fluorescent (GFP) imaging. (*Indicates $p < 0.05$, compared to control). Data are presented as mean ± SD and were analyzed by one-way ANOVA. (B) Western blot of fibronectin in FNE-m-p53 cells treated with supplemented media. (C) Live cell images of FNE-m-p53 cells. From top to bottom: control, taurine, and proline-treated cells (160mM). Scale bar represents 100µm.

DISCUSSION

These studies were initiated after observing significantly higher levels of intracellular taurine and proline in cells taken from OC fluids compared to OC precursor cells (FNE-m-p53) grown in the laboratory. Prompted by these observations we incorporated taurine or proline supplementation in FNE-m-p53 cell cultures. We found that taurine but not proline supplementation resulted in FNE-m-p53 cell protection from DNA-damaging agent cisplatin. Taurine's cell protective activities were associated with stimulation of p21 and fibronectin expression as well as increased cell adhesion area.

Both p21 and fibronectin can support cell adhesion area increase and cell survival[16,17]; thus, it is possible that taurine's effects on cell size and cell survival depend on the expression of p21 and/or fibronectin. Although we have previously demonstrated that taurine can activate p21 through p53, taurine's ability to protect cells from cisplatin was not dependent on p53[12]. These results raised the possibility that targeting p21 directly (not through p53) could sensitize taurine-supplemented FNE-m-p53 cell cultures to cisplatin. In the follow-up experiments, we will use the shRNA approach to determine the role of p21 in taurine-mediated cell protection from cisplatin.

How taurine activates fibronectin expression in FNE-m-p53 cells is not clear. However, previous investigations[18] including ours provided evidence[9] that expression of mutant p53 can induce transcription of mesenchymal transcription factors involved in fibronectin expression. In another study, we also found[12] that taurine could induce

mutant p53 binding to DNA, supporting the idea of transcriptional activation of fibronectin. In the upcoming experiments, we will perform chromatin precipitation of mutant p53 to determine whether taurine induces mutant p53 binding to promoters representing known regulators of fibronectin or fibronectin promoters.

In summary, this report provides further evidence that taurine but not proline protects OC precursor cells from cisplatin. Taurine-dependent cell protection appears to involve the activation of p21 and fibronectin expression. Future studies will be needed to determine how taurine activates p21 and fibronectin, and whether these mechanisms are involved in cell protection from cisplatin in OC cells.

FUNDING

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