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Original paper

Role of p38-mitogen-activated protein kinase in modulation of the response to therapy in FaDu Human pharyngeal carcinoma cell

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Abstract

Although cisplatin (CisPt) is used in treating head and neck squamous cell carcinomas (HNSCC), little is known about the cellular mechanisms triggered. We examined the morphological and functional changes in HNSCC cells when natural compounds such as resveratrol (RSV) are added to CisPt treatment of FaDu human pharyngeal carcinoma cell line. In addition, we analyzed the effects of CisPt and/or RSV treatments on cell proliferation and evaluated the ability to modulate the activation of p38 Mitogen-Activated Protein Kinase (p38MAPK). Moreover, we explored whether activation of p38MAPK is associated with p53 phosphorylation status in FaDu cells using SB203580 specific p38MAPK inhibitor. Proliferation vs. cytotoxicity induced by CisPt and/or RSV treatments in FaDu cells were studied using MTS colorimetric assay and evaluating cell viability. Whereas the activation of p38MAPK could be specific to HNSCC therapy, we investigated the phosphorylation status of p38 and p53 proteins by ELISA assays, using antibodies that recognize their dual forms (the active, phosphorylated and the total one). The obtained data showed that RSV enhanced the cytotoxic effect of CisPt in FaDu cells, having a synergic effect and the ability to promote p53 phosphorylation, suggesting a possible link between the p38MAPK and p53 activation pathways.

Keywords : HNSCC, cisplatin, resveratrol, p38MAPK, p53, SB203580

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Introduction

Head and neck squamous cell carcinomas (HNSCC) include cancers of the nasal cavity, oral cavity, larynx, and pharynx (J.P. SHAH, Z. GIL [1]). The development of HNSCC is a multistep progressive process from precancerous lesions to malignant tumors, and despite the chemotherapy advances, the survival rate of HNSCC patients has not considerably changed over the last decades (R. P. TAKES & al. [2] and N. ZHANG & al. [3], BERTESTEANU & al. [4], POPESCU & AL. [5]). Treatment options are available, including chemotherapeutic agents such as cisplatin (CisPt) which is considered to be a standard treatment for patients with advanced HNSCC (R. BELCHER & al. [6]). CisPt is routinely used to treat the hypopharynx cancer, but almost 50% of the patients present tumor relapse or develop drug resistance in time. CisPT is a DNA damage-inducing agent and by its properties could block DNA replication and gene transcription (J.L. FISCHEL, G Milano [7]), but CisPt should not be related exclusively to the inhibition of DNA synthesis because it could also trigger other mechanisms, such as activation of p53 protein or Mitogen-Activated Protein Kinases (MAPKs), and thus could induce the apoptotic process (D. SANO & al. [8], A.C. JOERGER, A.R. FERSHT [9]).

In normal conditions, p53 - a nuclear transcription factor with pro-apoptotic function, is expressed at a low level, which is caused by proteasomal degradation largely mediated by E3 ubiquitin-protein ligaseMdm2 (Mouse double minute 2 homolog) a protein that in humans is encoded by the MDM2 gene. (R. KULIKOV & al. [10]). The cellular stresses like DNA damage might induce p53 accumulation in cell nucleus through post-translational modifications such as phosphorylation and acetylation. These chemical modifications convert p53 from a latent to an active form, which might be due to the dissociation of MDM2 from the former (N.D. MARCHENKO & al. [11]). When cells have serious DNA damages, p53 exerts its pro-apoptotic function to eliminate cells and thereby inhibit the transfer of damaged DNA to the daughter cells, thus, p53 has an important role to maintain genomic integrity (P. BRAGADO & al. [12]). Over 50% of human cancers have mutations in p53 gene and an oncogenic potential, and sometimes p53 mutated cells express a chemo-resistant phenotype. Phosphorylation of p53 in different serine (Ser)/ threonine (Thr) residues appears to be an essential key step, especially in the regulation of the response to some carcinogenic agents (J.J. MUKHERJEE & al. [13]). In addition, depending on the cellular context, CisPt could induce the cell death by mechanisms mediated by p53 activation but not clearly described. Several studies tried to demonstrate that activation of p38 MAPKs could lead to p53 activation, therefore influencing the apoptotic response of the cells. The MAPKs represent a highly conserved group of protein kinases that catalyze the phosphorylation of specific Ser and Thr residues in target substrates. The phosphorylation process is part of the cascade events that converts extracellular signals to intracellular pathways, and controls a wide range of cellular responses, such as proliferation, differentiation and apoptosis (I. DOLADO & al. [14]). MAPKs also play an essential role in signal transduction, and each group of MAPKs can be stimulated by a separate signal transduction pathway in response to different extracellular stimuli (E.F. WAGNER & al. [15]).

Since the cooperation between p53 and p38 MAPKs has not yet been established in the CisPt-treated HNSCC cell lines, our study focused on the evaluation of the crosstalk between p53 and p38 MAPKs in FaDu human pharyngeal carcinoma cell line in order to create an “in vitro” model. Moreover, we tested the modulation capacity of several compounds on p38 MAPK functional behavior, previously studied in inhibition assays of p38 MAPK and known for their therapeutic potential in inflammatory diseases (J. H. LOSA & al. [16] and J.L. LEFEBVRE & al. [17]). Like most chemotherapeutic agents, CisPt acts by forming DNA crosslinks within cells, leading to apoptosis and cellular senescence of tumor cells, but remission rates were often associated with severe side-effects, including nephrotoxicity, gastrointestinal or cardiovascular complications and bone marrow suppressive sequelae (E.E. COHEN & al. [18] and S. T. ELIAS & al. [19]). So, the main disadvantages of CisPt treatment are the associated side effects which often require a dose reduction or even discontinuity of CisPt-therapy (A.M. FLOREA & al. [20]). Efforts have been made to achieve a more effective management strategy to reduce this

phenomenon, and find alternative and less toxic cancer treatments like biotherapies, that use natural compounds (e.g. phytochemicals) which might be used in the treatment of various diseases, including head and neck cancers (T. KUNO& al. [21] and S.K. GOSWAMI & al. [22]). Despite the number of publications regarding HNSCC treatment, there are few data regarding the cellular mechanisms of CisPt action in association with natural compounds. One good example of such natural compounds is resveratrol (RSV) or 3,5,4'-trihydroxystilbene, a polyphenol found in red grapes, berries and peanuts, that has biological properties, such as antioxidant, anti-inflammatory, anticancer and anti-aging activities (L. MARZOCCHELLA & al [23]). In order to achieve our goal and further understand how cellular mechanisms might be influenced by CisPt and RSV combined treatment, the present study examined its cytotoxic effects on FaDu human pharyngeal tumor cell line, analyzed the modulation of p53 and p38-MAPK expression, and evaluated the capacity to alter the cell proliferation function. Since activation of p38 MAPK is associated with cancer progression, we further explored whether p38 MAPK activation is associated with p53 phoshorylation status in FaDu cells and might be influenced by CisPt and/or RSV treatments. Moreover, we studied the crosstalk between the two molecules and tried to evaluate the relevance of p53 and/or p38 MAPKs activation in order to improve the survival and quality of life of HNSCC patients, the main goal being the development of novel molecular targeted treatment strategies.

Materials and Methods

Reagents: Cisplatin (Cis-diammineplatinum (II) dichloride, DDP), Resveratrol (3,5,4'-trihydroxystilben), dimethyl sulfoxide (DMSO), etylenediaminetetraacetic acid (EDTA, PBS (Phosphate Buffered Saline), SB2035809 (4-[4-(4-fluorophenyl)-2-[4- methylsulfinyl) phenyl] - 1H-imidazol-5-yl]-pyridine) were purchased from Sigma (St. Louis, MO, USA).

Cell cultures: FaDu human pharynx squamous cell carcinoma cell line was purchased from the American Type Culture Collection (ATCC® HTB-43™). Cells were cultured either in 96-well plates or 25 and 75 cm2 culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-Glutamin and 100 U/ml penicillin/ 100 µg/ml streptomycin (Biochrom, Germany), and incubated at 37°C and in 5% CO2 humid atmosphere. When needed, the cells were grown for 24 hours to achieve around 60% confluence, and then treated with various concentrations of CisPt and/or RSV for different periods of time. Previously, CisPt was dissolved in 0.9% sodium chloride to a stock solution of 3.33mM, while RSV was dissolved in DMSO, and added with DMEM medium to obtain a 1mM stock solution. For inhibition studies of p38 MAPK function, cells were incubated for 2 h with 10 uM of SB203580 before CisPt and/or RSV treatments. After treatments, adherent cells were detached from flasks with a non-enzymatic solution of PBS/1mM EDTA, washed twice in PBS, and cell viability was evaluated using Trypan Blue exclusion test. Then FaDu cells were used for the evaluation of cell proliferation, cytotoxicity capacity or conserved as cell pellets at -80oC for preparation of cell lysates to further be used in ELISA assays. Non-treated cells were used as controls throughout all experiments.

Drug cytotoxicity assays: All assays were performed in triplicate using colorimetric CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA). The method is based on the reduction of yellow MTS tetrazolium salt by the viable cells and generation of colored formazan product that is soluble in the culture medium. Developed color can be spectrophotometrically quantified by measuring the absorbance at λ=490nm (J.A. BARLTROP & al. [24]). Briefly, 1 x 104/100µL/well of FaDu cells were seeded in 96-well microplates with flat bottom (Greiner, Frickenhausen, Germany), and incubated for 24 h at 37°C/5% CO2. After 24 h, supernatants were discarded, adherent cells treated with different concentrations of compounds under study in a final volume of 100 µL/well, and incubated for additional 12, 24, or 48 h. Then, 20 µl/well of MTS reagent containing (a) [3-(4,5 -dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl) -2-(4-sulfophenyl) -2H-tetrazolium] and (b) phenazine ethosulfate were added into each well, plates were further incubated for 0.5-4 h/ 37°C, and absorbance of color developed in each well was measured at λ=490 nm using a Dynex plate reader (DYNEX Technologies – MRS, USA). Results were expressed as mean values of three determinations

± standard deviation (SD). Untreated cells served as control and considered to have 100% viability.

Viability % = (T-B)/ (U-B) x 100, where T = O.D. of treated cells; U = O.D. of untreated cells, and B = O.D. of blank.

ELISA assay: Sandwich ELISA kits were used to measure both human P53 or p38 MAPK total proteins, and phospho-p53 or phospho-p38 α-MAPK proteins in cell lysates using a standard Streptavidin-HRP system. The kits consisted of: DuoSet_IC Human Total p53 ELISA [Cat. No DYC1043]; DuoSet_IC Human Phospho-p53 (S15) ELISA [Cat. No DYC1839]; DuoSet_IC Human/Mouse/Rat Total p38 ELISA [Cat. No DYC8691B] DuoSet_IC Human/ Mouse/ Rat Phospho-p38α (T180/Y182) ELISA [Cat. No DYC869B], and were purchased from R&D Systems Inc. (USA). In order to obtain the cell lysates, the untreated or CisPt and/or RSV treated FaDu cells were suspended in lysis buffer (1mM EDTA, 0,5% Triton X-100, 5mM NaF, 6M urea, 10ug/ml leupeptin, 10ug/ml pepstatin, 100uM PMSF, 3ug/ml aprotinin, 2,5mM sodium pyrophosphate, 1mM sodium orthovanadate in PBS, pH7.2-7.4), the Eppendorf tubes kept 30 min on ice with stirring every 5 min, then sequentially centrifuged 5 min/2000xg, and 15 min/14000xg (J.R. CROWTHER [25]). Protein concentration in lysates was evaluated using the Bradford assay. All experiments were performed in triplicate and sample O.D. was measured at λ=450 nm using Dynex plate reader.

Statistical Analysis. Data were analyzed using Student's t Test. One-way analysis of variation with p values of < 0.05 was considered statistically significant.

Results and Discussions

CisPt and/or RSV inhibition of human FaDu cell viability

CisPt or RSV concentration response curves in FaDu cells were generated by 6 h, 24 or 48 h incubation with 2-100μM CisPt and 0.5 - 200μM RSV treatments. Effects of CisPt or RSV monotherapy on FaDu cells were dose and time-dependent, and had statistical significance as compared with control untreated cells (Figure 1 A and B). The time-dependent effects of treatment with CisPt or RSV on FaDu cells viability indicated the 24h treatment as optimal.

The main objective of the present study was to investigate the potential synergistic effect of CisPt and RSV phytochemical combination on FaDu cells. The cell viability rate after 24 h treatment with 50μM RSV was 75% ($p<0.02$), while treatment with 10μM CisPt showed a cell viability rate of 64% ($p<0.003$) (Fig. 2A). After the simultaneous treatment of the FaDu cells with 50uM RSV and 10μM CisPt it was observed a reduction in cell viability till 52% after 24h treatment ($p<0.007$) as shown in Figure 2A. Moreover, combined CisPt-RSV treatments were compared to the single ones, and statistical significance were obtained: $p<0.005$ when compared to CisPt treatment, and $p<0.0045$ when compared to RSV, which demonstrated that combination could significantly reduce FaDu cell survival rate.

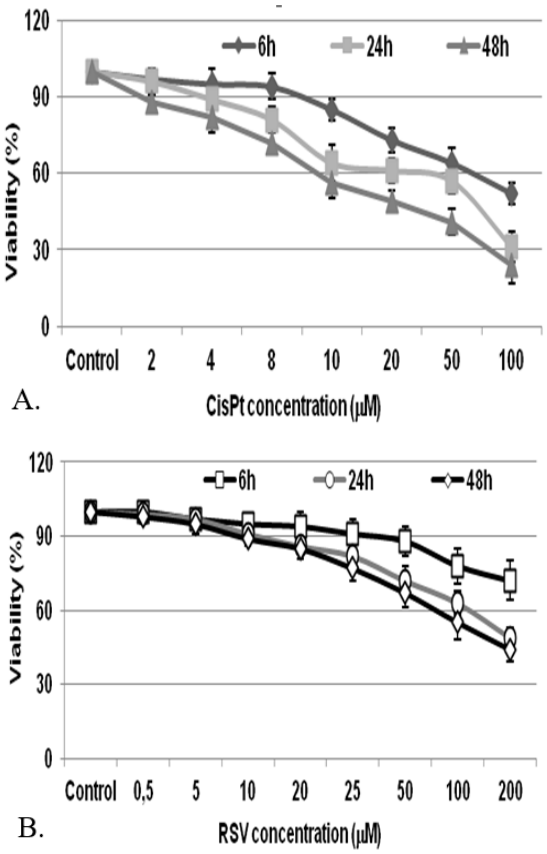

Figure 1. Sensitivity of the FaDu cells to CisPt and RSV treatments. Cell viability was measured by MTS assay after cell incubation with different doses of CisPt (A) or RSV (B) for distinct periods of time - 6, 24 or 48 hours. Data represent the mean ± SD values from three experiments.

In order to test whether p38 MAPK activation could control the cell proliferation in HNSCC, we blocked p38 signaling using SB203580 specific inhibitor. In this regard, we pretreated the FaDu cells

with SB203580 (10μM) for 2 h, followed by incubation with CisPt (10μM) and/or RSV (50μM) during 6, 24 or 48h. When the cells were pretreated with SB203580 for 2 hours, there were no significant differences in proliferation process of the cells treated with CisPt or RSV alone; however, the proliferation process of the cells simultaneously treated with CisPt and RSV was more suppressed in the presence of SB 203580 inhibitor, as shown in Figure 2 (CisPt+RSV+SB203580 versus CisPt+RSV-SB203580, $p<0.0095$), after 24h treatment.

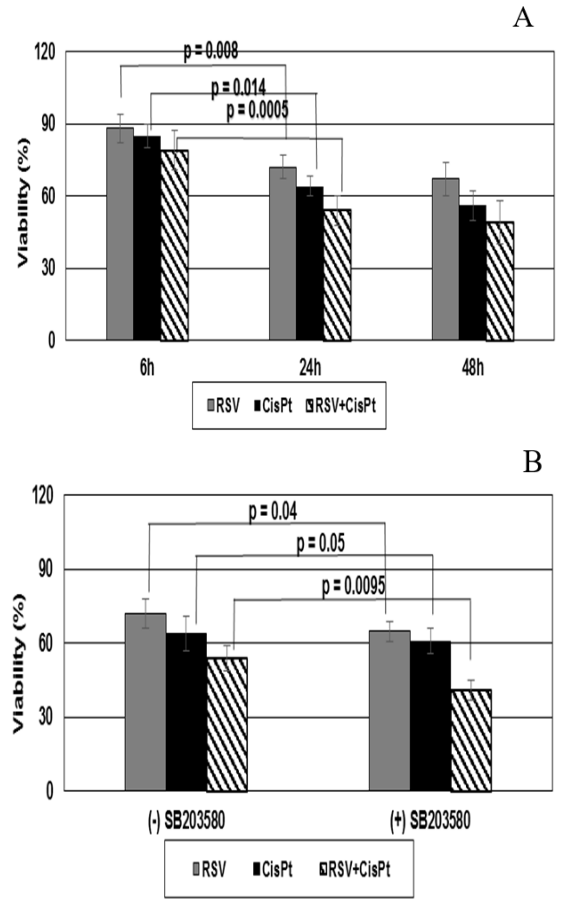

Figure 2. Effects of combined CisPt-RSV treatments on FaDu cells viability, in the presence or absence of SB203580 inhibitor of p38 MAPK.

(A) FaDu cells were incubated for 6, 24 or 48h with 10μM CisPt and/ or 50 μM RSV, then cell viability measured by MTS assay;

(B) Alternatively, cell viability was measured after preincubation of FaDu cell line with SB203580 inhibitor of p38 MAPK, and then with 10 μM CisPt and/or 50 μM RSV for 24h. Data represent the mean ± S.D. values of three experiments.

Effect of CisPt and/or RSV treatment on cell morphology

Cell morphology changes after 24h treatment with 10 μM CisPt and/or 50 μM RSV were recorded under light microscopy. The untreateated cells (CTR) showed a polygonal shape with a good appearance, typical for cell growth (Figure 3A). Treatment with 50 μM RSV induced morphological cell changes including shrinkage and a shift to a rounded shape, but they still adhered to the bottom (Figure 3B), while after 24 h treatment with 10 μM CisPt many cells rounded up, with some floating in the culture medium (Figure 3C). The combined treatment with CisPt and RSV induced a greater loss of cell attachment to ground matrix, membrane blebbing and more floating cells (Figure 3D), suggesting much stronger effects than single treatments.

Effects of CisPt and/or RSV on the activation of p38MAPK pathway in FaDu cell line.

The status of phosphorylation of p38MAPK in FaDu cells was investigated and data obtained showed a limited activity of p38 MAPK in FaDu untreated cells (control cells). These results indicate an association between a lower p38 MAPK activation with a high proliferative capacity of FaDu cells. In order to determine whether CisPt alone or in combination with RSV might activate a pathway leading to phosphorylation of p38MAPK, FaDu cells were treated with 10 μM CisPt and/or 50 μM RSV for 6, 24 or 48h. Then, cell lysates were prepared and analyzed by DUO IC ELISA method. CisPt and/or RSV induced a time-dependent phosphorylation of p38 MAPK of the incubation time (6, 24 h or 48h) (Fig. 4A). The results showed that p38 MAPK activity was significantly modified in treated cells when compared to control cells; thus the highest phosphorylation level of p38 MAPK was recorded after 24h treatments of FaDu cells with CisPt ($p=0.025$) or RSV ($p=0.005$). When cells were simultaneous treated with CisPt and RSV, it was noticed a significant enhancement of p38 MAPK phosphorylation (Figures 4B) as compared to control ($p=0.002$), CisPt ($p=0.003$) or RSV ($p<0.001$) treatments. After 48h, all single or combined treatments induced a significant decrease of phosphorylation level of p38 MAPK levels in FaDu cells as compared to 24 h treatments.

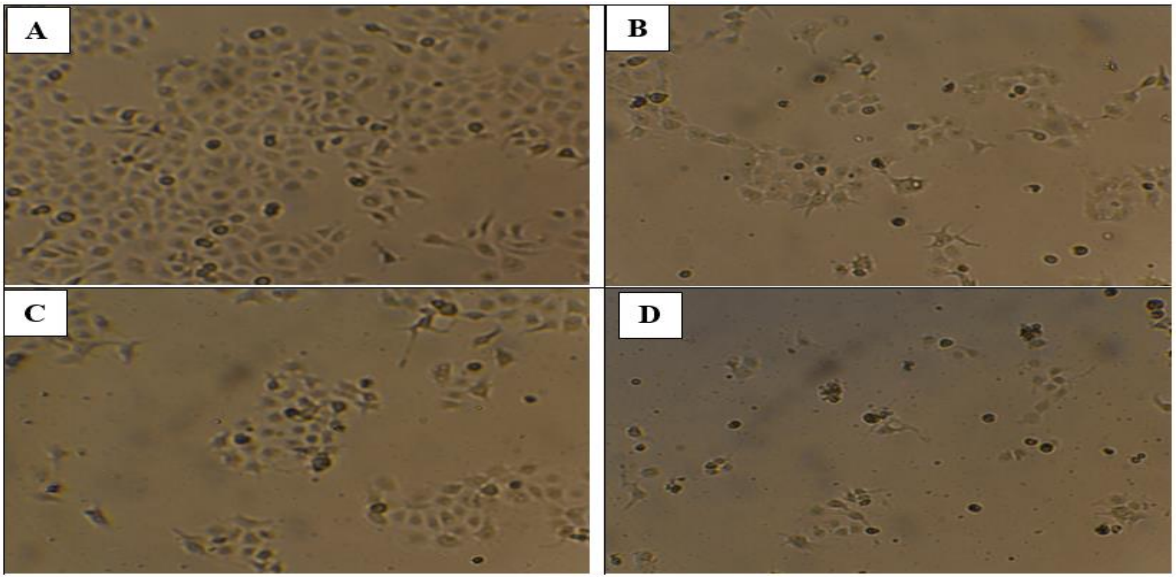

Figure 3. Effects of CisPt and/or RSV on morphology of FaDu cells.
FaDu cell line was cultured for 24h in complete DMEM medium (A), or added with 50μM RSV (B), 10μM CisPt (C), or 10μM CisPt and 50μM RSV (D), then morphological changes were examined under light microscopy and images taken with a digital camera (100x magnification).

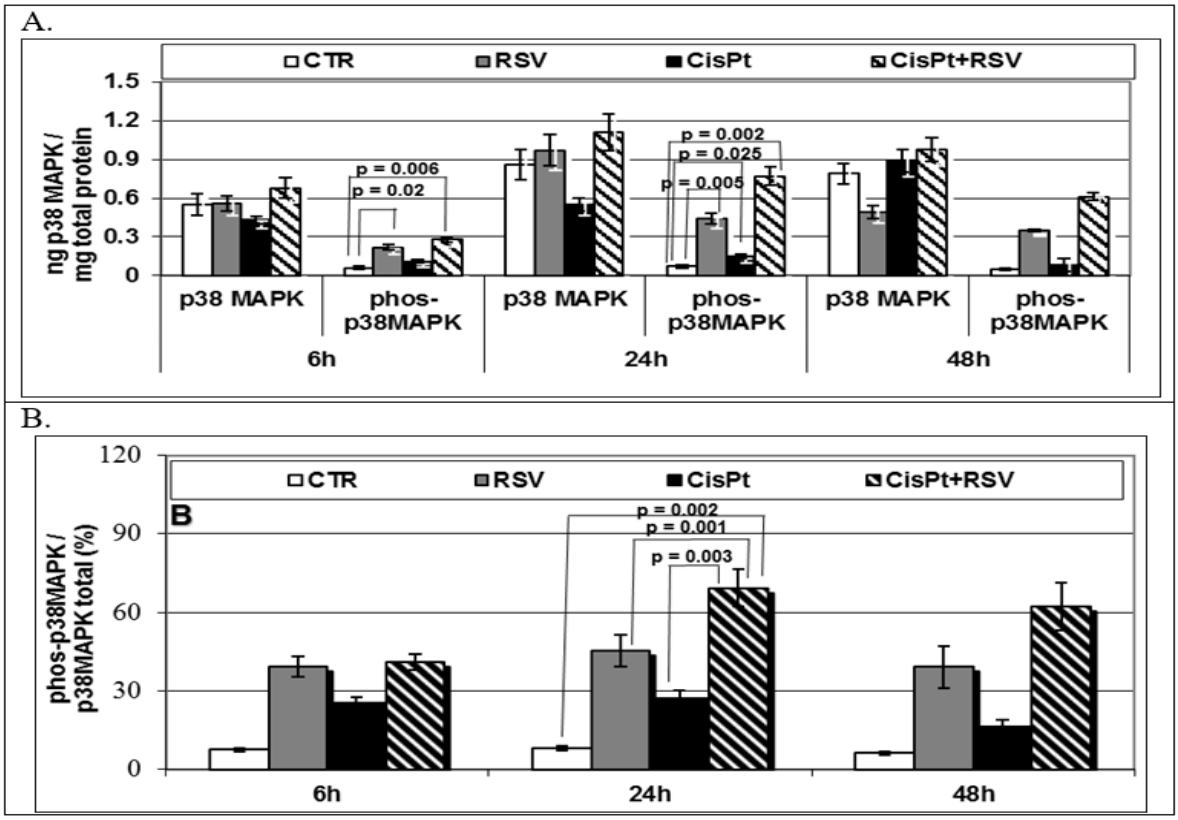

Figure 4. Effect of RSV and/or CisPt on p38MAPK activation in FaDu cell line.
Phosphorylated (phos-) and total p38MAPK levels were measured by ELISA assay in lysates of FaDu cells, untreated (CTR) or treated with RSV50μM and/or CisPt10μM for different periods of time (A). Raw quantitative data were used to calculate phosphorylated-p38MAPK / total p38MAPK ratios (B). Results are presented in graphs as mean ± S.D. values from three independent experiments.

Effects of CisPt and/or RSV on p53 phosphorylation in FaDu cell line

Phosphorylation is an important modification involved in the control of p53 activity. We hypothesized that phosphorylation of p53 could affect the sensitivity of cells to treatment. To test the effect of CisPt and/or RSV on the expression and phosphorylation of p53 in FaDu cells, we treated cells with 10μM CisPt and/or 50μM RSV for 6, 24 or 48h. We found that RSV treatment induced the phosphorylation of p53 in a time dependent manner, reaching a peak of maximum activation after 24 hours, while CisPt treatment slightly inhibited p53 phosphorylation (Figure 5). In addition, to analyze if phosphorylation of p53 depends on the activation of MAPKs, cells were pretreated with 10μM of SB203580 specific p38MAPK inhibitor for 2h before cells treatments with CisPt and/or RSV. We found that CisPt increased p38 MAPK phosphorylation, but not p53 levels in FaDu cells. In contrast, RSV induced p53 phosphorylation, as shown in Fig. 5 (Panel A and B), that was reduced when the cells were pretreated (2h) with SB203580 (p=0.002). Furthermore, the most significant changes were observed when the cells were simultaneously treated with CisPt and RSV (p=0.0006). Taken together, these evidences indicate that p38MAPK mediates phosphorylation of p53 protein in response to RSV treatment, the effect being even higher than in the cells simultaneously treated with CisPt and RSV.

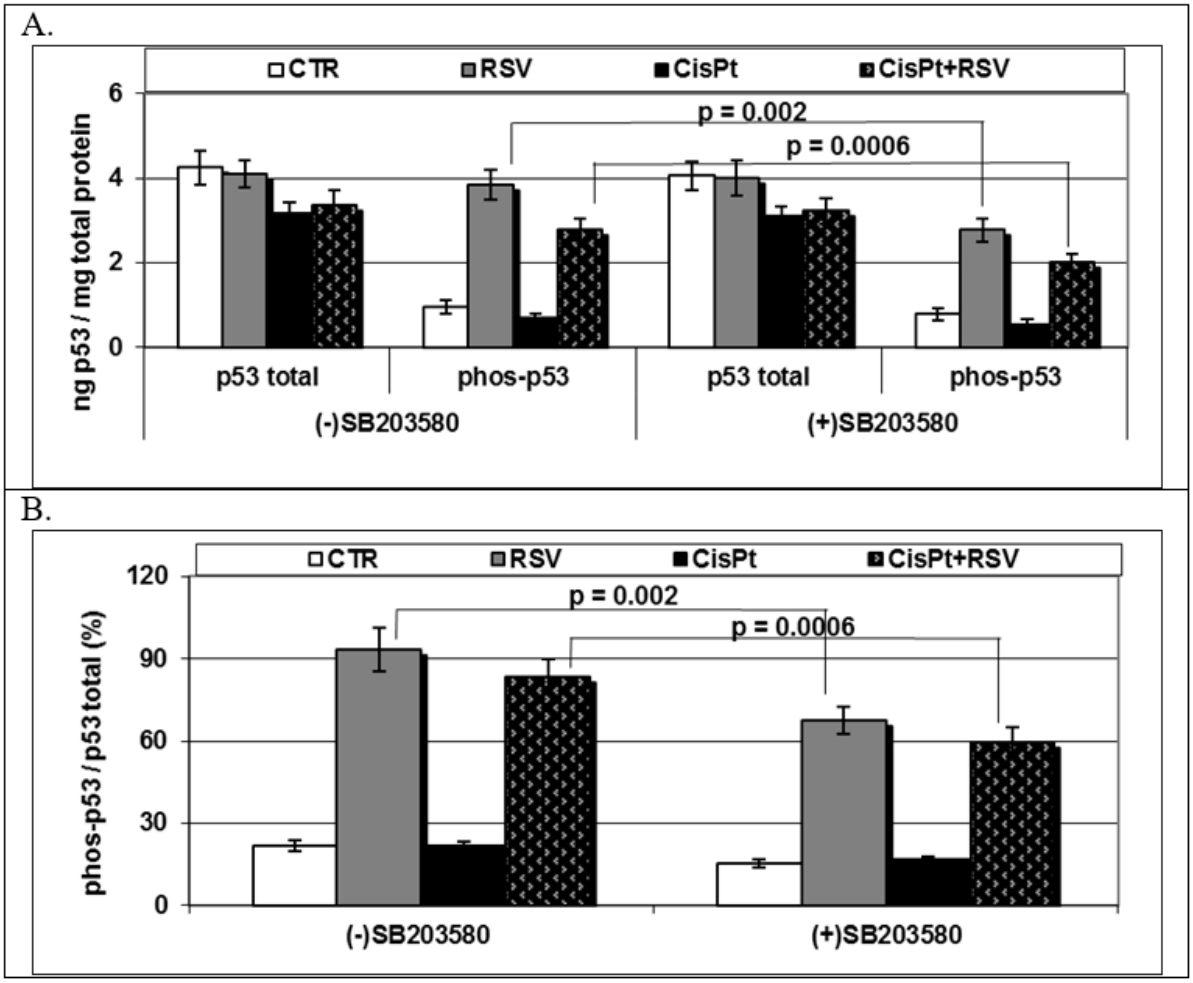

Figure 5. Effect of RSV and/or cisplatin on p53 phosphorylation in FaDu cell line.
Phosphorylated (phos-) and total p53 protein expression in FaDu cell line treated with RSV 50μM and/or CisPt 10μM for 24h in presence or absence of SB203580 inhibitor
(A). The results were analyzed and used to calculate the phospho-p53 /total p53 ratios
(B). Results are presented in graphics as mean ± standard errors from three independent experiments.

Discussions

Cisplatin is commonly used as a cytotoxic drug in chemotherapeutic approaches of several solid tumors, including neck and head cancers. Its mode of action has been linked to the ability to crosslink with the purine bases in DNA, interfering with DNA repair mechanisms and causing DNA damage. Therefore, DNA replication is blocked as well as gene transcriptions. The liaisons that CisPt forms with DNA are believed to be essential for the cytotoxic activity of the drug (T. C. HSIEH, & al. [26]). Following exposure to different agents implicated in DNA damage, cells show an increase in p53 expression levels as a consequence of its stabilization by post-transcriptional modifications. These modifications protect p53 from rapid degradation and lead to its activation as a fully active transcriptional factor able to regulate a set of p53 target genes (R. VIELBA & al. [27]). Phosphorylation at different Ser/Thr residues is a key step in the regulation of p53 function in response to various stimuli. Experimental data demonstrated elevated p38MAPK protein levels in the serum of HNSCC patients, which correlate with resistance to radiotherapy. The p38MAPKs were implicated in complex biological processes, such as cell proliferation, cell death, cell migration, and invasion. Abnormal p38MAPK levels in cancer patients were associated with advanced stages and short survival (K. GILL, & al. [28] and A. CUENDA, S. ROUSSEAU, [29]). The p38 MAPK plays a dual role as a regulator of apoptosis, mediating either cell survival or cell death, which depends not only on the stimulus type, but also of the cell type.

The study was focused on the activation status of p38MAPKs in FaDu cell line, and its relationship to other cell signaling events. To test whether p38MAPK signaling could control cell proliferation and p53 activation, we carried out experiments in the presence of a highly specific p38 MAPK inhibitor (SB203580) in order to block p38MAPK signaling in FaDu cells. SB203580 was effective to block p38MAPK functions when 10 μM concentration was used, similar to those reported by other research groups (N. YOSHIZUKA, & al. [30]). RSV, a potential cancer chemoprevention agent with no toxic or mutagenic effects, was previously reported that could be used to inhibit the growth of cancer cells because of its capacity to affect the activity of multiple targets involved in carcinogenesis (H. K. KOUL, & al. [31] and A. C. YUMUSAKHUYLU, & al. [32]). In addition, RSV seems to induce anti-tumor effects through activation of signal transduction pathways in various cancer types (M.R. ABEDINI, & al. [33] and S.R. CHANDANA, & al. [34]), and several reports mention the use of RSV in cancer treatment in combination with CisPt (E.M. VARONI, & al. [35] and L. MA, & al. [36].

All these data prompted us to analyze the role of RSV and/or CisPt in the inhibition of FaDu cell proliferation, and the possible cooperation between p38MAPK and p53 activation process. We found that exposure of cells to RSV induces activation of p38MAPK in association with an increase of p53 phosphorylation. The obtained results showed that FaDu cell line treatment with both RSV and CisPt has a higher effect than treatment with single drugs. We conclude that RSV treatment could amplify the antitumor activity of CisPt in FaDu cell line, reducing the proliferation rate after 24h of treatment, data being sustained also by the morphological changes observed in tumor cells treated with CisPt and/or RSV for 24 hours. In addition, RSV alone or in combination with CisPt treatment induced p53 phosphorylation which was abolished when the cells were pre-treated with SB203580 for 2 h.

Conclusions

Taken together, the obtained evidences suggest that p53 activation is linked to the activation of p38 MAPK pathway, and provide evidences that cell chemosensitivity could be associated with p53 activation. Therefore, the combined treatment of tumor cells with conventional chemotherapeutic drugs and RSV provides a promising direction for cancer chemotherapy. A better understanding of the role of p38MAPK in response to cancer therapy could lead to new therapeutic approaches in which modulating the activity of p38MAPK could be a cornerstone.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article, and there was no financial support that could have influenced the outcomes. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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