Investigation of the Cell Stabilization and the Epithelial to Mesenchymal Transition Effect of Flavopiridol in Mouse Lung Squamous Cell Carcinoma

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Abstract

Background: Lung malignancy is a leading cause of cancer related morbidity and mortality worldwide. The three main subtypes of Non-small cell lung cancer (NSCLC) are adenocarcinoma, squamous cell lung cancers (SqCLCs) and large-cell carcinoma. Flavopiridol is a flavone that inhibits several cyclindependent kinases and exhibits potent growthinhibitory activity, apoptosis and G1 phase arrest in a number of human tumor cell lines. The present study focused on the effect of flavopiridol in cell stabilization, cell adhesion, junctional complex and epithelial to mesenchymal transition (EMT) in mouse lung squamous cell carcinoma cell.

Methods: Cell viability and proliferation of untreated controls and flavopiridol treated cells were determined by using the WST-1 assay. SqCLCs and M. dunni mouse skin fibroblast cells (MSF) cells immunofluorescence analyses were performed for the evaluation of Hsp90β, e-cadherin and occludin. The percentages of CD133+/CD44+ cells in the flavopiridol treated cells when compared with the control untreated cells by flow cytometric analysis.

Results: Flow cytometric analysis showed that the percentages of CD133+ and CD44+ cells did not show significant changes when compared to the untreated cells. Hsp90b expression which is significantly available in SqCLCs was considered as important its significant reduction by following flavopiridol application and also flavopiridol caused the significant increase the E-cadherin expression but there was no significant effect on the occludin expression. SqCLCs cells are uniformly highly sensitive to flavopiridol induced cytotoxicity during prolonged 72 h exposure to clinically achievable concentrations of this drug.

Conclusions: These observations may have translational implications for the use of flavopiridol in the treatment of SqCLCs.

Keywords: Flavopiridol; SqCLCs; EMT; Cell stabilization

Introduction

Lung malignancy is a leading cause of cancer related morbidity and mortality worldwide. The majority (85%) of cases are histologically proven NSCLC. The three main subtypes of NSCLC are adenocarcinoma, SqCLCs and large-cell carcinoma. More than 55% of lung carcinomas harbor at least one genetic alteration, most of them being histologic subtype specific. SqCLCs accounts for approximately 20% of all lung cancers and will cause the death of 90 - 95% of affected individuals [1,2]. One molecular abnormality in SqCLCs that has becomeincreasingly apparent is the loss of cell cycle regulation. It has been known for several years that the G1-S restriction point isdefective, largely because of loss of function of the retinoblastoma gene product, which is mutationaly altered in the vast majority of SqCLCs [3]. Flavopiridol inhibits phosphokinases. Its activity is strongest on cyclin dependent kinases (cdk -1, -2, -4, -6, -7) and less onreceptor tyrosine kinases (EGFR), receptor associates tyrosine kinases (pp60 Src) and on signal transducing kinases (PKC and Erk-1). Although the inhibiting activity of flavopiridol is strongest for cdk, the cytotoxic activity of flavopiridol is not limited tocytling cells. Resting cells are also killed. This fact suggests that inhibition of cdks involved in the control of cell cycle is not the only mechanism of action. Inhibition of cdks with additional functions (i.e. involved in the control of transcription or function of proteins that do not control cell cycle) may contribute to the antitumoral effect. Moreover, direct and indirect inhibition of receptor activation (EGFR) and: or a direct inhibition of kinases (pp60 Src, PKC, Erk-1) involved in the signal transduction pathway could play a role in the antiproliferative activity of flavopiridol [4].

More specifically, flavopiridol effects tumor cells through cytostatic activity and supports cell cycle arrest and apoptosis. This small molecule is an inhibitor of multiple cyclindependent kinases (CDKs), including CDK2, CDK4 and CDK6, which directly compete with adenosine triphosphate at pharmacological doses. This inhibition blocks cell cycle progression and induces G1 phase arrest and apoptosis through negative regulation of the phosphoinositide 3 kinase/protein kinase B signaling pathway [5,6].
Figure 1: Flow cytometric analysis of CD133 and CD44 expression in SqCLCs cell line 48 h after flavopiridol treatment (A) compared with control untreated cells (B). Expression profiles of CD133 and CD44 and their co-expression in SqCLCs cell line was examined. Suspensions cells were labeled with PE-conjugated anti-CD133 and FITC-conjugated anti-CD44 antibodies. The percentage of CD133 and CD44 at 48 h following drug treatment was assessed by flow cytometric analysis. Data were calculated from three independent experiments.

Cell Stabilization and Cancer

Cancerous cells overexpress a number of proteins, including growth factor receptors, such as EGFR, [7] or signal transduction proteins such as PI3K and AKT (Inhibition of these proteins may trigger apoptosis). Hsp90 stabilizes various growth factor receptors [8] and some signaling molecules including PI3K and AKT proteins. Hence inhibition of Hsp90 may induce apoptosis through inhibition of the PI3K/AKT signaling pathway and growth factor signaling generally [9]. Hsp90 is involved in the maturation and stabilization of a wide range of oncogenic client proteins crucial for oncogenesis and malignant progression, making cancer cells particularly dependent on proper Hsp90 function [10-11].

Figure 2: Immunofluorescence staining of e-cadherin in SqCLCs and MSF cell lines following treatment of the half maximal inhibitory concentration (IC$_{50}$) value of the flavopiridol. Selected representative images of immunofluorescence on untreated SqCLCs (A), SqCLCs treated with 172. 16 nM flv (B), untreated MSF (C) and MSF treated with 384.90 nM flv (D) for 48 h. Cellular staining was visualized using FITC-conjugated secondary antibody (green). Nuclear staining was visualized using DAPI (4', 6-diamidino-2-phenylindole) (blue) staining. Images are representative of three independent experiments. Statistical analysis was tested by oneway analysis of variance, followed by Tukey’s or Dunett’s post hoc test. p<0.05 was considered to indicate a statistically significant difference.

Cell adhesion/Cell junction/Epithelial to Mesenchymal transition and Cancer

Tight junction plays an important role in mediating paracellular permeability in epithelia. Coordinated cell proliferation and ability to form intercellular seals are essential features of epithelial tissue function. Tight junctions (TJs) classically act as paracellular diffusion barriers. More recently, their role in regulating epithelial cell proliferation in conjunction with scaffolding zonula occludens (ZO)
proteins has come to light [12]. EMT occurs in normal physiological processes essential for embryogenesis, tissue morphogenesis and wound healing but is also tightly linked to pathological conditions including fibrosis and cancer progression. During EMT, epithelial cells typically lose their epithelial characteristics, including loss of cell polarity and cell–cell contact and acquire a spindle-shaped migrating phenotype [13,14]. The most relevant gene product for the maintenance of the epithelial phenotype is E-cadherin. As it is well known, E-cadherin is required for the formation of adherens junction; however its role is not limited to this since it binds and restrains the transcriptional activity of β-catenin [15] that, as commented above, is required for the expression of EMT-TFs. A similar negative action of E-cadherin has also been reported on NF-κb activity [16]. Accordingly, it is expected that epithelial cancer cells will be much more susceptible to EMT than non-transformed epithelium. Total or partial loss of adherens junctions is a common alteration in neoplastic cells normally due to posttranslational modifications of E-cadherin-associated proteins; for instance β-catenin tyrosine phosphorylation decreases its interaction with E-cadherin and causes adherens junction instability [17].

**Drug Resistance and Cancer**

Resistance to chemotherapy drugs is one of the biggest challenges in the Figure 3 against cancer these days. The increased expression of cell surface proteins has been associated with resistance to chemotherapy in many cancers. CD133, also known as prominin 1 or AC133 (a glycoprotein comprising of five transmembrane domains), has been described as a marker of cancer-initiating cells in different tumor types [18]. It has recently been discovered that CD44, a transmembrane protein, is involved in the drug resistance of multiple types of tumor [19]. Therefore, CD133 and CD44 cells could be potential targets of antitumor therapy in the future.

The aim of the present study was to more rigorously define the effect of flavopiridol on SqCLCs cell lines. Of these effects, revealing specifically of the effects on cell stabilization, and EMT process was considered as significant. In this study, we also investigated the effect of flavopiridol on the expression of two putative stem cell markers, plasma membrane-associated glycoproteins CD133 and CD44 in SqCLCs cell line.

**Materials and Methods**

**Cell culture conditions and reagents**

The mouse lung squamous cell carcinoma cell line (SqCLCs, ATCC-CRL-145) and A. dani mouse (Clone H18C, ATCC-CRL-2017™) skin fibroblasts cells (MSF) were supplied by American Type Culture Collection (Manasas, VA, USA). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEMF12; Biological Industries, Kibbutz BetHaemek, Israel) and McCoy’s 5a Medium Modified respectively. Mediums were supplemented with 10% heat-inactivated fetal calf serum(Gibco, Invitrogen Life Technologies, Paisley, UK), 100 units of penicillin– streptomycin/ml (SigmaAldrich, St Louis, MO, USA) and 1% L-glutamine 37°C in humidified atmosphere of 5 % CO2. Cells in semiconfluent flasks were harvested using 0.05% trypsin (SigmaAldrich), centrifuged (Nuve NF200; Laboratory and Sterilization Technology, Ankara, Turkey) following the addition of DMEMF12 for trypsin inactivation, and resuspended in culture medium. Flavopiridol (Sigma) was dissolved in dimethyl sulfoxide (DMSO), and the final volume of DMSO did not exceed 0.1% of the total incubation volume. The antibodies used were antiHsp90β (1:100 diluted; bs-0135R, Bios, China), antiE-cadherin (1:100 diluted; sc-7870, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), antioccludin (1:100 diluted; sc-271842, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and goat antirabbit immunoglobulin Gfluorescein isothiocyanate (FITC) (1:100 diluted; ab98692, Abcam, Cambridge, MA).

**Figure 3:** Immunofluorescence staining of Hsp90β in SqCLCs and MSF cell lines following treatment of the half maximal inhibitory concentration (IC50) value of the flavopiridol. Selected representative images of immunofluorescence on untreated SqCLCs (A), SqCLCs treated with 172.16 nM flv (B), untreated MSF (C) and MSF treated with 384.90 nM flv (D) for 48 h. Cellular staining was visualized using FITC conjugated secondary antibody (green). Nuclear staining was visualized using DAPI (4’, 6-diamidino-2-phenylindole) (blue) staining. Immunofluorescence staining of Hsp90β decreased in SqCLCs following treatment of flavopiridol, which was applied to cells at the IC50 dose. Images are representative of three independent experiments. Statistical analysis was tested by oneway analysis of variance, followed by Tukey’s or Dunnet’s post hoc test. p<0.05 was considered to indicate a statistically significant difference.

**Culturing conditions of cells and flavopiridol treatment**

Cell viability and proliferation of untreated controls and flavopiridol treated cells were determined by using the WST-1 assay (Roche...
Applied Science, Mannheim, Germany). SqCLCs and MSF cells were placed into 96-well plate at a density of $30 \times 10^3$ cells in 100 μl medium per well. Then, cells were treated with increasing doses of flavopiridol for 24, 48 and 72 h. After having performed the proliferation assay, absorbance of each sample was measured spectrophotometrically at 450 nm with an ELISA reader (Thermo, Vantaa, Finland).

Figure 4: Immunofluorescence staining of occludin in SqCLCs and MSF cell lines following treatment of the half maximal inhibitory concentration (IC$_{50}$) value of the flavopiridol. Selected representative images of immunofluorescence on untreated SqCLCs (A), SqCLCs treated with 172.16 nM flv (B), untreated MSF (C) and MSF treated with 384.90 nM flv (D) for 48 h. Cellular staining was visualized using FITC-conjugated secondary antibody (green). Nuclear staining was visualized using DAPI (4',6-diamidino-2-phenylindole) (blue) staining. There was no significant effect of flavopiridol on the occludin expression. Images are representative of three independent experiments. Statistical analysis was tested by one-way analysis of variance, followed by Tukey’s or Dunnett’s post hoc test. p<0.05 was considered to indicate a statistically significant difference.

Immunofluorescence staining

SqCLCs and MSF cells were treated as indicated above and were harvested and fixed in 4% paraformaldehyde for 30 min. Subsequently, the cells were rendered permeable with 0.1% Triton X 100 for 10 min at room temperature, and blocked with phosphate-buffered saline containing 5% bovine serum albumin for 1 h. Following incubation with antibodies against Hsp90β, E-cadherin, occludin overnight at 4°C, the cancer cells were treated with FITC-conjugated secondary antibody for 1 h at room temperature. The cells were counterstained with 4', 6-diamidino-2-phenylindole and assessed by a fluorescence microscope equipped with a camera (Olympus BX51 and the Olympus CS5050 digital test). Statistical analysis was tested by one-way analysis of variance, followed by Tukey’s or Dunnet’s post hoc test. p<0.05 was considered to indicate a statistically significant difference.

Flow cytometric analysis

SqCLCs cells in the log phase were collected and seeded in semiconfluent flasks. Twenty-four hours later, cells were treated with the half maximal inhibitory concentration (IC$_{50}$) value of the
flavopiridol. For flow cytometry analysis, untreated and flavopiridol treated cells were detached using nonenzymatic cell dissociation solution (SigmaAldrich) and approximately $5 \times 10^4$ cells were incubated with an antibody (diluted 1:100 in FACS wash with 0.5% bovine serum albumin; 2 mM NaN$_3$ and 5 mM EDTA) for 15 min at 4°C. An isotype and concentrationmatched phycoerythrin (PE) labeled control antibody (Miltenyi Biotec Ltd., Woking, Surrey, UK) was used and the samples were labeled with PElabeled CD133/1 (clone AC133/1; Miltenyi Biotec Ltd.) and FITC-labeled CD44 (clone G44-26, BD Bioscience, San Jose, CA, USA). After 35 min, the cells were washed with the FACS wash. The percentages of CD133$^+$ and CD44$^+$ cells were determined by flow cytometry.

Figure 6: Concentration- and time-dependent inhibition of the viability of SqCLCs and MSF cells following flavopiridol treatment. Cytotoxicity was determined by the WST cytotoxicity assay. WST cytotoxicity assay results of flavopiridol treated (A) SqCLCs and (B) MSF cells Dotted lines indicate calculated flavopiridol IC50 concentration as 172.16 nM and 384.90 nM for SqCLCs and MSF cell lines.

Results

The percentages of CD133$^+$/CD44$^+$ cells in the flavopiridol treated cells when compared with the control untreated cells

In order to evaluate the relative percentage of CD133$^+$/CD44$^+$ cells in the total SqCLCs cells population following exposure to flavopiridol, cells were labeled with CD133 and CD44. Flow cytometric analysis showed that the percentages of CD133$^+$ and CD44$^+$ cells did not show significant changes when compared to the untreated cells (Figure 1). After the flavopiridol treatment, SqCLCs cells maintained a higher fraction of CD133$^+$/CD44$^+$ cells (14.4%) compared with that (13.3%) in the control untreated cells.

Increasing cytotoxicity of SqCLCs cells with flavopiridol

To evaluate the effect of flavopiridol on the cell viability of SqCLCs and MSF cells were exposed to increasing concentrations of flavopiridol for 24, 48 and 72 h, and the percentage of viable cells determined by WST-1 assay. Treated cells were subjected to flavopiridol. Flavopiridol decreased the cell viability of SqCLCs and MSF cells in a dose and time-dependent manner. According to the cell inhibition curve, the 50 % inhibitory concentration (IC50) values of flavopiridol were found to be 172.16 n M and 384.90 nM for SqCLCs and MSF cells, respectively at 48 h.

E-cadherin immunoexpression (Figure 2) of SqCLCs decreased significantly from SqCLCs+Flv (p<0.00). Hsp90 beta immunoexpression (Figure 3) of SqCLCs increased from SqCLCs+Flv (p<0.00). Occludin immunoexpression (Figure 4) of SqCLCs no different from SqCLCs +Flv (p>0.05). E-cadherin, Hsp90beta and Occludin immunoexpression of MSF no different from MSF+Flv (Figures 5 and 6).

Discussion

Cell stabilization and epithelial/mesenchymal transition play a critical role on oncogenesis, differentiation of cell and formation of cell hierarchy. The current study aimed to investigate the effects of flavopiridol on lung cancer. Basic areas to be examined; occludin, one of the tight junctional complexes and EMT marker proteins, E-cadherin which is one of the cell adhesion molecules and EMT markers, Hsp90 protein which is an important regulator for cell stabilization are identified as proteins.

Cytotoxic and apoptotic effects of flavopiridol have been shown previously in a bladder cancer cell line [20], rhabdoid tumors [21]. Flavopiridol is one of the novel agents and is also currently under clinical investigation with expectations at the treatment outcomes for chronic lymphocytic leukemia [22]. Cyclin D1 and CDK4/6 have alternate roles in stemlike cell activity and regulation of migration. In addition, these effects are highly dependent of estrogen receptor (ER) expression. Inhibition of cyclin D1 or CDK4/6 increases or decreases migration and stemlike cell activity in ERnegative and positive breast cancer, respectively [23]. Understanding the molecular mechanisms by which flavopiridol may reveal its biological effects on lung squamous cancer is important to detect if its efficiency is dependent on alterations of apoptosisrelated gene expression and/or induction of apoptosis. The study by Yao et al. [24] demonstrated that the caspase3 and Bax proteins were increased significantly in cells treated with flavopiridol, whereas radiation and Bcl2 protein were significantly decreased. Regarding this, it is believed that flavopiridol promotes Bax and inhibits Bcl2, thereby promoting caspase3 and resulting in apoptosis and G2/M arrest in the esophageal cancer cell line [24]. According to previous studies, flavopiridol downmodulates cyclin D1 and inhibition of its pathway by various mechanisms leads to G1 arrest in various cell lines [25,26]. Cimica et al. [27] demonstrated that flavopiridolinduced G2 arrest was correlated with downregulation of cyclin B1 and upregulation of p53 and p21.

The molecular chaperone Hsp90 (heat shock protein 90) is a promising target in cancer therapy. Preclinical and clinical evaluations of a variety of Hsp90 inhibitors have shown antitumor effect as a single agent and in combination with chemotherapy [28]. Hsp90 and its client proteins were overexpressed in several esophagus squamous cell carcinoma cells and patient tissues [29]. Hsp90 contributes to a number of processes important to tumor development, including cell...
proliferation, apoptosis, angiogenesis, and metastasis [30]. Sufficiency in growth signals which is the main change managed by Hsp is conducted by Hsp90. In the fragile formation of many receptors, this molecular chaperone is indispensable in order to stabilize the protein kinases and transcription factors in cell growth. The degradation of receptor proteins by targeting Hsp90 with chemical inhibition fulfilled the inhibition of growth of tumor which is G1 stopping-sourced and the activation of apoptosis. These observations indicate that Hsp 90 is the major component for the growth signal. The second role of Hsp 90 in cancer cell is the ensuring of stability of mutant proteins by allowing mutant protein aggregation [31]. Our findings clearly demonstrate that Hsp90b expression which is significantly available in lung cancer cell line was considered as important its significant reduction by following flavopiridol application in the formation of treatment strategy.

EMT, which occurs at the invasive front of many metastaticcancers, has been shown to play a crucial role in tumorinvasion and metastasis [32]. During thisprocess, epithelial cancer cells reduce intercellular adhesion, acquire mesenchymal characteristics, and increase invasiveand migratory properties [33]. Theexpression profiles of EMT were correlated with the metastasis and grades of various cancers [34]. In vitro migration of lung cancer cells via induction of EMT, which was confirmed by the down regulation of epithelialmarkers E-Cad and ZO-1, up regulation of mesenchymal markers Vim and N-Cad, and increased expression of transcriptionfactors Snail [35]. EMT is characterized by an extensive down-modulation of the key epithelial gene E-cadherin and the induction of mesenchymal markers, such as Fibronectin or Vimentin [36]. The most relevant gene product for the maintenance of the epithelial phenotype is E-cadherin. As it is well known, E-cadherin is required for the formation of adherens junction; however its role is not limited to this since it binds and restrains the transcriptional activity of β-catenin [37] that, as commented above, is required for the expression of EMT-TFs. Accordingly, it is expected that epithelial cancer cells will be much more susceptible to EMT than non-transformed epithelium. Total or partial loss of adherens junctions is a common alteration neoplastic cells normally due to post-translational modifications of E-cadherin-associated proteins; for instance β-catenin tyrosine phosphorylation decreases its interaction with E-cadherin and causes adherens junction instability [38]. In our study, the application of flavopiridol caused the significant increase the E-cadherin expression butthere was no significant effect of flavopiridol on the occludin expression. SqCLCs cells are uniformly highly sensitive to flavopiridol induced cytotoxicity during prolonged 72-hrs exposure to clinically achievable concentrations of this drug.

In our study; it was clear that E-cadherin and occludin protein expressions significantly decreased in fibroblastic somatic cell line used as the control group when compare to the squamous cell lung cancer. E-cadherin, plays an important role in the process of EMT, significantly increased with flavopiridol theropyosquamous cell lung cancer. Flavopiridol also provided a significant reduction in the cell stabilization via Hsp90b was evaluated as a positive progression. These observations may have translational implications for the use of flavopiridol in the treatment of SqCLCs.

References


