

Runx2 downregulation, migration and proliferation inhibition in melanoma cells treated with BEL β -trefoil

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Abstract. Malignant melanoma is a lethal form of skin cancer and highly metastatic tumor with poor prognosis; BEL β -trefoil, a lectin, obtained by our group, possesses the ability to act specifically on malignant cells. Therefore, the aim of our study was to investigate the effects of BEL β -trefoil in melanoma cells in an attempt to evaluate its potential usage as anticancer agent. BEL β -trefoil was purified by chromatography and A375 and MeWo melanoma cells were treated. Viability and proliferation were evaluated as well as apoptosis, RUNX2 gene expression and migration ability. The treated tumor cells decreased viability as well as proliferative ability. Flow cytometry analysis showed a lessen effect of the treatment on apoptosis. The gene expression analysis showed a reduction of RUNX2 expression in a dose-dependent manner and migration ability was reduced significantly in both treated cell lines. Our findings suggest that BEL β -trefoil can be considered a useful tool against malignancy due to its effect based on the simultaneous proliferation ability reduction as well as the inhibition of migration capacity on melanoma tumor cells.

Introduction

Lectins can act specifically on malignant cells thanks to the presence on the surface of transformed cells of the Thomsen-Friedenreich antigen or T-antigen, a disaccharide, Gal β 1-3GalNAc. This disaccharide, that is hidden in normal

cells (1), allows selective binding of lectins through serine or threonine of glycoproteins.

Mushroom derived lectins have been considered to this purpose by taking advantage of their ligand-binding specificity (2). For instance, *Agaricus bisporus*, a lectin contained in edible mushrooms, has been shown to selectively inhibit the proliferation of human malignant epithelial cell lines without toxicity for normal cells (3).

Recently, we obtained a lectin with antineoplastic properties from the wild mushroom *Boletus edulis* (4). This protein was named *Boletus edulis* lectin (BEL) β -trefoil and it could represent an interesting tool for therapeutic applications against cancer.

Malignant melanoma is a lethal type of cancer with an incidence that is increased in the last decades (1). It is a highly metastatic tumor generally resistant to apoptosis as well as to chemotherapeutic treatments and local tumor surgery. Radiotherapy and chemotherapy are the standard treatment used. Since metastatic melanoma has a poor prognosis, the challenge is to develop new and more effective therapeutic tools.

Transcription factors have been considered in recent years crucial targets in strategies against this malignancy, as well as other cancer types.

Among other transcription factors, RUNX2 regulates the expression of genes involved in tumor progression, invasion and metastasis such as osteopontin, bone sialoprotein, and collagenases (5). RUNX2 play an important role in tumor progression by regulating proliferation, migration and invasion pathways. In melanoma, RUNX2 has been considered a molecular regulator of epithelial-to-mesenchymal transition (6). In general, we found in patients with bone metastases an increased expression of RUNX2 gene, suggesting that this transcription factor may be considered a mesenchymal stem marker for cancer (7).

The aim of this study was to use BEL β -trefoil against A375 and MeWo melanoma cells, focusing the study on effects in key mechanisms involved in neoplasia, such as apoptosis and Ki67 expression as well as migration properties.

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Materials and methods

BEL β -trefoil purification. Commercial king bolete mushrooms (*Boletus edulis*) were homogenized in a blender with an equal volume of PBS (10 mM NaH_2PO_4 pH 7.5 and 150 mM NaCl) and centrifuged at $7,500 \times g$ for 30 min at 4°C . Proteins in the supernatant were precipitated by adding 50% w/v ammonium sulphate, separated from the supernatant after centrifugation at $12,000 \times g$ for 15 min at 4°C , resuspended and dialyzed in buffer A (20 mM Tris-HCl pH 7.5). The protein solution was loaded onto a DEAE-cellulose column previously equilibrated with buffer A and then washed with buffer A containing 30 mM NaCl to eliminate contaminants. The fraction containing BEL β -trefoil was eluted with 20 mM Tris-HCl pH 7.5, 250 mM NaCl and further purified by size exclusion chromatography using a Superdex G75 column in 20 mM Tris-HCl pH 7.5, 150 mM NaCl.

The protein solution, previously equilibrated in buffer A was loaded onto a stronger anionic exchanger MonoQ to discriminate among isoforms. A linear sodium chloride gradient was performed and the most abundant isoform eluted with NaCl 90 mM was defined as BEL- β trefoil.

Recombinant BEL β -trefoil expression and purification. BEL β -trefoil coding sequence (4) was inserted into pET22b plasmid using *NdeI* and *XhoI* restriction sites. The construct was used to transform *E. coli* BL21 C41 expression strain following a standard heat shock protocol. When cell culture reach an OD_{600} of 0.8 in LB media protein expression was induced by adding 0.25 mM IPTG and shaking overnight at 20°C . Cells were harvested by centrifugation at $7500 \times g$, lysed by sonication and the soluble fraction was loaded onto a Nickel-Sepharose column previously equilibrated with buffer B (20 mM Tris-HCl pH 7.5, 500 mM NaCl and 10 mM imidazole). After washing the column with buffer B BEL β -trefoil was eluted with a linear imidazole gradient from 10 to 350 mM. Next, thrombin was added to cleave the histidine tag. Tag-free protein was separated from the uncut through Nickel-Sepharose column.

BEL β -trefoil was then collected, loaded onto Superdex G75 column and eluted with 20 mM Tris-HCl pH 7.5, 150 mM NaCl. The fluorescent BEL β -trefoil was obtained by cloning the protein coding sequence into pWaldo-GFP plasmid using *NdeI* and *KpnI* restriction enzymes; this protocol allowed the expression of recombinant BEL β -trefoil fused with a C-terminal GFP. After the heat shock transformation of *E. coli* BL21 (DE3) the chimeric protein was expressed and purified following the same protocol of BEL β -trefoil alone.

Cells and BEL β -trefoil treatment. A375 and MeWo melanoma cells were cultured under humidified atmosphere of 5% CO_2 and passaged in growth medium: DMEM/F12 containing 10% FBS (fetal bovine serum) supplemented with antibiotics (1% penicillin and streptomycin) and 1% glutamin. Cells were then harvested using trypsin, washed and counted on a microscope using a Burkert hemocytometer and plated again in growth medium. Once 80% confluence was reached BEL β -trefoil at concentration ranging from 0 to $100 \mu\text{g/ml}$ was added.

Cell viability. Cell viability was evaluated by a colorimetric assay based on the reduction of the tetrazolium salt XTT (sodium 3I-[1-phenylamino-carbonyl-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) by mitochondrial dehydrogenase of viable cells to a formazan dye (Cell proliferation kit II - XTT Roche).

Briefly 100 μl XTT labelling mixture was added to each well and cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 4 h. The spectrophotometric absorbance of the samples was measured every hour using a microtitre plate (ELISA) reader at a wavelength of 450 nm.

Real-time PCR. PCR was performed in a total volume of 20 μl containing 1X Premix Ex TaqTM (2X), 1X Rox Reference Dye (50X) and 20 ng of cDNA; probe sets for gene *Runx2*, (Hs00231692_m1) actin and GAPDH obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems). Real-time RT-PCR reactions were carried out in two-tube system and in multiplex.

The amplification conditions included 30 sec at 95°C (initial denaturation), followed by 50 cycles at 95°C for 5 sec (denaturation) and at 60°C for 31 sec (annealing/extension). Thermocycling and signal detection were performed with ABI PRISM 7000 Sequence Detector (Applied Biosystems). Signals were detected according to the manufacturer's instructions.

As previously reported, the C_t value correlates to the starting quantity of target mRNA (8); we selected the ΔR_n in the exponential phase of amplification plots to determine the C_t values. $\Delta\Delta C_t$ values were then calculated with respect to control.

To normalize mRNA expression for sample-to-sample in RNA input, quality and reverse transcriptase efficiency, we amplified the β -actin and β -2 microglobulin housekeeping genes. β -actin and β -2 microglobulin endogenous/internal control genes were abundant and remained constant proportionally to total RNA among the samples.

C_t data. C_t values for each reaction were determined using TaqMan SDS analysis software. For each amount of RNA tested, triplicate C_t values were averaged. Because C_t values vary linearly with the logarithm of the amount of RNA, this average represents a geometric mean.

Cell proliferation test. Proliferating cell nuclei were identified by Ki67-positive immunofluorescence on cells cultured in slide glass chambers, acetone fixed and stored at -20°C . Briefly, cells were grown in slides with different BEL β -trefoil concentrations for 48 h under humidified atmosphere of 5% CO_2 at 37°C ; then, cultured cells were fixed with cold acetone and stored at -20°C . For immunofluorescence, cells were firstly rinsed twice in PBS and permeabilized with washing solution (Triton X-100 0.1% in PBS). After incubating the cells with anti-Ki67 primary antibody (clone Ki67, Dako), the secondary antibody (FITC-conjugated rabbit anti-mouse IgG, Dako) was applied. Finally, the nuclei were counterstained with DAPI (Sigma) and observed at 40X objective using Nikon epifluorescence microscope. Semiquantitative analysis of protein expression was achieved by counting fluorescent cells in three fields of each of six slides at magnification of x40. The estimate was

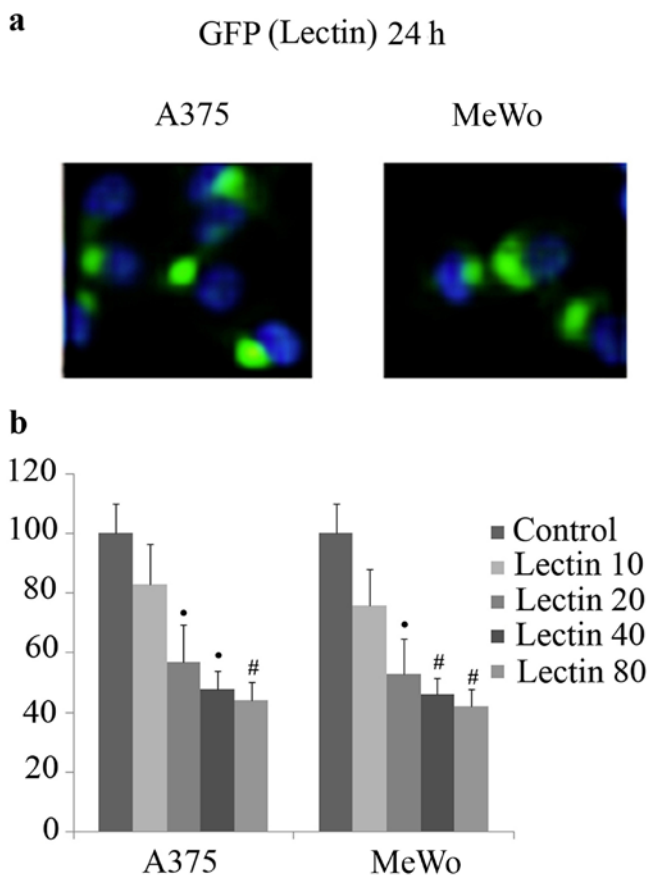


Figure 1. BEL β -trefoil effect on melanoma cell viability. (a) After 24 h of treatment at 20 $\mu\text{g/ml}$, recombinant-fluorescent BEL β -trefoil was endocytosed by almost all cells. Nuclei were counterstained with DAPI. (b) Viability was analyzed by XTT test in both cell lines at increasing concentrations of BEL β -trefoil as indicated. After 48 h a dose-dependent reduction of cell viability was observed. * $p < 0.05$, # $p < 0.01$.

calculated as the percentage of positive cells with respect to the total DAPI-stained nuclei.

Annexin staining. The exposure of phospholipid phosphatidylserine on the plasma membrane of apoptotic cells was detected using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) detection kit (Bender Med System, Vienna, Austria), as previously reported (7). Briefly, cells were treated with lectin for 48 h. After incubation, cells were trypsinized and 250×10^3 cells were centrifuged and re-suspended in 500 μl of 1X binding buffer. Next, cells were stained with Annexin V-FITC for 10 min. Propidium iodide (PI) was added just before the analysis with the FACSCanto™ flow cytometer (BD Biosciences, San Jose, CA, USA). At least 10,000 events were acquired and samples were analyzed by FlowJo software (TreeStar, Ashland, OR, USA). Viable cells were defined as Annexin V-negative and PI-negative; early apoptotic cells were defined as Annexin V-positive and PI-negative; late apoptotic/necrotic cells were defined as Annexin V-positive and PI-positive.

Migration assay. Transwell plates with 8- μm pore size membranes (Corning, NY, USA) were used to analyze cell migration, according to the manufacturer's instructions.

A375 and MeWo cells in RPMI with or without BEL β -trefoil were added to the top of the Transwell inserts and cultured for 48 h in humidified environment with 5% CO_2 at 37°C. Migrated cells (passed through polyethylene membrane) were fixed with methanol and stained with a 0.1% crystal violet solution and counted in six random fields.

Statistical analysis. Results are expressed as mean \pm SE. The Wilcoxon test was used for non-parametric data. For analysis of treatment responses, multiple measurement ANOVA followed by Bonferroni as post hoc analysis was performed. A probability value of < 0.05 was considered statistically significant. Spearman correlation coefficient and regression curve estimations were performed when indicated. Analyses were applied to experiments carried out at least three times. Statistical analyses were performed using SPSS for Windows, version 16.0 (SPSS Inc, Chicago, IL, USA).

Results

Cells viability, apoptosis and proliferation. Recombinant-fluorescent BEL β -trefoil entered the cells after 24 h at concentration starting from 20 to 80 $\mu\text{g/ml}$ (Fig. 1a). In order to study the effect of the treatment, we analyzed cell viability by XTT test in cells incubated at 37°C and exposed to BEL β -trefoil up to 80 $\mu\text{g/ml}$. After 48 h we observed a dose-dependent reduction of cell viability. The BEL β -trefoil decreased cell viability in a manner of statistical significance versus non-treated cells when it was added at 20 $\mu\text{g/ml}$ ($p < 0.05$), 40 and 80 $\mu\text{g/ml}$ ($p < 0.01$) (Fig. 1b).

To clarify whether BEL β -trefoil effect was pro-apoptotic or/and it inhibited proliferation, we evaluated the exposure of the phospholipid phosphatidylserine on the plasmatic membrane (apoptotic feature) by annexin staining and the proliferation index by *in situ* expression of Ki67 protein (proliferative marker).

We observed an apoptotic induction in treated compared to untreated cells. In fact Annexin V staining confirmed cell reduction showed by XTT assay in both cell lines even if the difference was not statistically significant (Fig. 2).

When proliferating cells were evaluated by Ki67 immunofluorescence, positively stained nuclei were reduced in treated A375 and MeWo cells as compared to non-treated cells. This proliferative reduction was statistical significant in both cell lines treated with 20 and 40 $\mu\text{g/ml}$ of BEL β -trefoil (Fig. 3)

Migration ability and RUNX2 gene expression. RUNX2 expression has been shown to be involved in promoting migration of melanoma cells. To evaluate if BEL β -trefoil treatment would modulate RUNX2 expression, we treated both MeWo and A375 lines with increasing doses up to 80 $\mu\text{g/ml}$. After 24 h of treatment Runx2 gene expression was significantly reduced in treated cells already from 40 $\mu\text{g/ml}$ of BEL β -trefoil ($p < 0.05$) (Fig. 4).

To evaluate the impact of exposing cells to BEL β -trefoil on migration ability, we cultured the cells in Transwell plates. As shown in Fig. 5, the presence of lectin significantly reduced cell migration in both cell lines either at 40 ($p < 0.05$) or at 80 ($p < 0.01$) compared to untreated cells after 24 h of treatment.

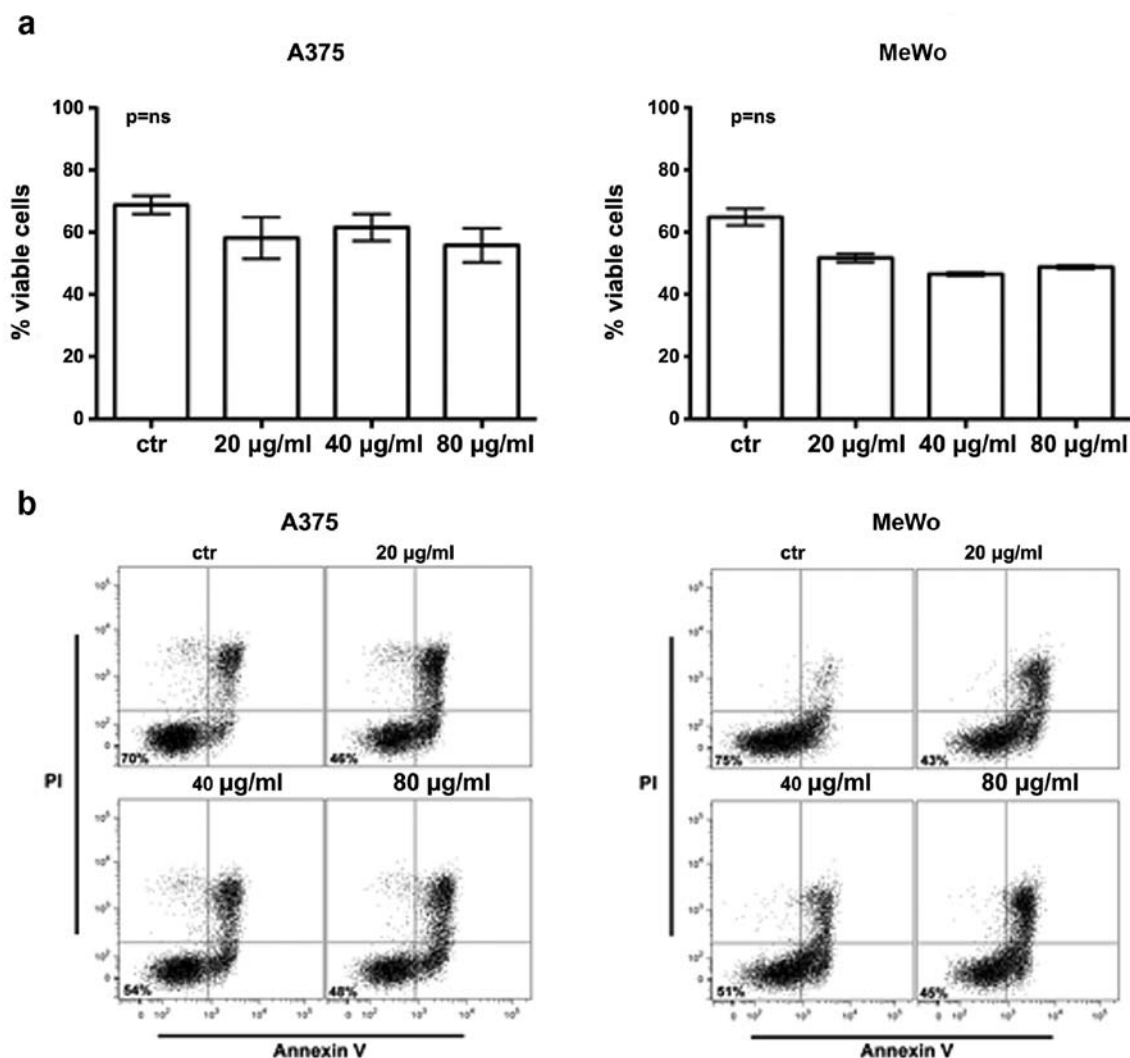


Figure 2. BEL β -trefoil effect on melanoma cell apoptosis. (a) Apoptosis was analyzed by Annexin V/PI flow cytometry assay. Cell lines A375 and MeWo were stimulated with 20, 40 or 80 μ g/ml of lectin, stained with Annexin V/PI and then analyzed by flow cytometry. Comparison between treatments was performed using the Kruskal-Wallis test with Dunn's post hoc test. NS, not significant. (b) Representative example of Annexin V/PI flow cytometry analysis.

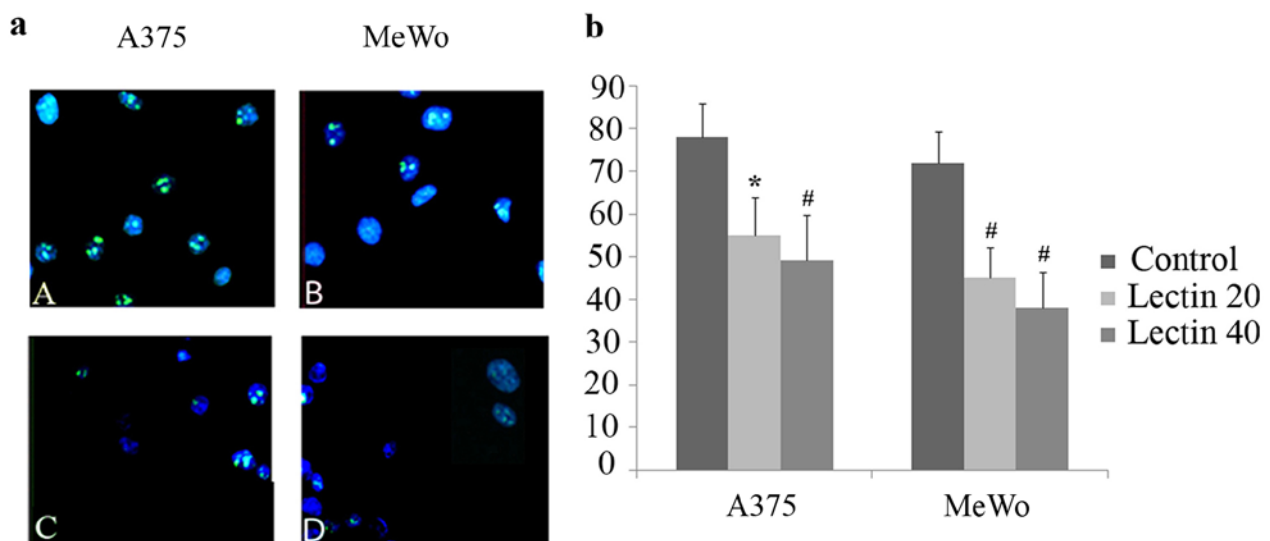


Figure 3. BEL β -trefoil effect on the proliferative rate of melanoma cells. (a) Proliferating cells were evaluated by Ki67 immunofluorescence stained nuclei in both cell lines treated with 40 μ g/ml of BEL β -trefoil. The Ki67 expression was lower in treated A375 (C) and MeWo (D) cells than in untreated cells (A and B). (b) The graph shows the proliferative reduction in both cell lines treated with 40 μ g/ml of BEL β -trefoil. The reduction of Ki67-positive nuclei was statistically significant ($p < 0.05$) in both cell lines.

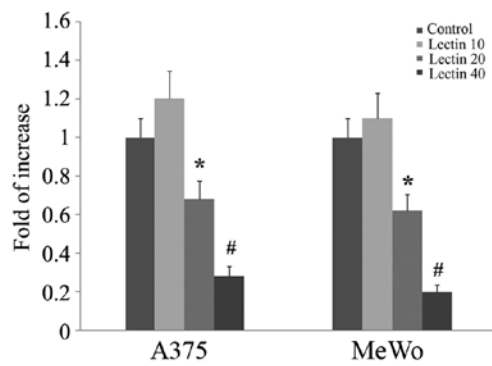


Figure 4. BEL β -trefoil effect on Runx2 expression in melanoma cells. After 24 h of treatment, RUNX2 gene expression was lower in treated versus control cells. Reduced expression was dose-dependent and statistically significant at concentrations ranging from 40 to 80 μ g/ml of BEL β -trefoil in both treated cell lines. * $p < 0.05$, # $p < 0.01$.

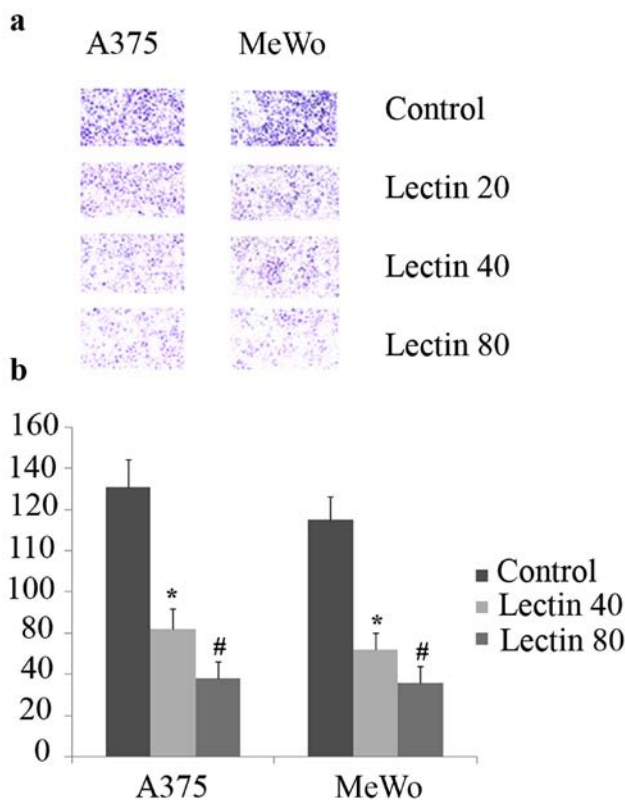


Figure 5. BEL β -trefoil effect on the migration ability of melanoma cells. (a) Cell migration was analyzed after 24 h of treatment with BEL β -trefoil. As compared to untreated cells, the number of migrated cells was reduced either at 40 ($p < 0.05$) or at 80 ($p < 0.01$) μ g/ml. (b) The graph shows the number of cells stained with 0.1% crystal violet solution and counted in six random fields at magnification of $\times 20$.

Discussion

BEL β -trefoil *in vitro* treatment affected viability of both melanoma cell lines: A375 and MeWo. In order to clarify the underlying mechanism, we assayed the treated and untreated cells for Ki67 expression as well as for Annexin V-FITC and PI staining.

The Ki67 protein is a marker of proliferative activity and its overexpression has been correlated with a poor prognosis

in various tumors, including cutaneous melanoma where Ki67 immunohistochemical staining is considered a specific and sensitive method to diagnose and to predict prognosis (9). Our results showed Ki67 expression significantly decreased in both treated cell lines, respect to the untreated controls. On the contrary, Annexin V-FITC and PI staining performed to assess cell apoptosis showed reduced ability of BEL β -trefoil in inducing cellular death, in both melanoma cells. Taken together, these results demonstrated that BEL β -trefoil treatment reduces cell viability mainly by affecting proliferative activity rather than by inducing apoptosis. On the other hand, a pro-apoptotic effect should not be completely ruled out since melanomas are thought to redundantly prevent apoptosis by upregulating multiple signaling pathways. Constitutive signaling mediated by Ras, MAPKs (10,11) and involving PI3K could be acting on Fas cascade (12) minimizing pro-apoptotic signaling mediated by the lectin. Yet, the inhibitory effect on cell proliferation was more striking in both, A375 and MeWo cells.

Recently, the involvement of the transcription factor RUNX2 in melanomagenesis was demonstrated at multiple levels, including cell proliferation (13). RUNX2 was overexpressed by melanoma cell lines and its down modulation by siRNA, inhibited migration ability.

Interestingly, the treatment with lectin decreased RUNX2 expression in both cell lines and the effect was dose-dependent. The role of RUNX2 in the different steps of malignancies has been largely demonstrated for inducing gene expression of molecular targets associated to tumor progression, invasion, metastasis as well as migration and invasion. Additionally, RUNX2 regulates transcription factors such as SOX9, SNAI2 and SMAD3 involved in epithelial to mesenchymal transition (EMT) process (14).

In accordance with the role of RUNX2 on cell migration demonstrated in other systems, we observed that treating melanoma cells with BEL β -trefoil simultaneously and dose-dependently affected RUNX2 expression and migration ability in both melanoma cell lines.

Even if in recent years melanoma treatment has improved by the development of immunological approaches, a certain percentage of melanoma affected patients are unresponsive to immunotherapy (15).

In conclusion, this study showed that BEL β -trefoil inhibited cell viability through cytostatic effects and reduced migration ability in melanoma cells.

Further studies are required to elucidate BEL β -trefoil molecular mechanism, nonetheless it could represent a valid alternative therapeutic approach to treat melanoma based on its inhibitory effects on proliferation and migration.

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References

- Bovi M, Carrizo ME, Capaldi S, Perduca M, Chiarelli LR, Galliano M and Monaco HL: Structure of a lectin with antitumoral properties in king bolete (*Boletus edulis*) mushrooms. *Glycobiology* 21: 1000-1009, 2011.

2. Singh RS, Bhari R and Kaur HP: Mushroom lectins: Current status and future perspectives. *Crit Rev Biotechnol* 30: 99-126, 2010.
3. Yu L, Fernig DG, Smith JA, Milton JD and Rhodes JM: Reversible inhibition of proliferation of epithelial cell lines by *Agaricus bisporus* (edible mushroom) lectin. *Cancer Res* 53: 4627-4632, 1993.
4. Bovi M, Cenci L, Perduca M, Capaldi S, Carrizo ME, Civiero L, Chiarelli LR, Galliano M and Monaco HL: BEL β -trefoil: A novel lectin with antineoplastic properties in king bolete (*Boletus edulis*) mushrooms. *Glycobiology* 23: 578-592, 2013.
5. Blyth K, Vaillant F, Jenkins A, McDonald L, Pringle MA, Huser C, Stein T, Neil J and Cameron ER: Runx2 in normal tissues and cancer cells: A developing story. *Blood Cells Mol Dis* 45: 117-123, 2010.
6. Riminucci M, Corsi A, Peris K, Fisher LW, Chimenti S and Bianco P: Coexpression of bone sialoprotein (BSP) and the pivotal transcriptional regulator of osteogenesis, Cbfa1/Runx2, in malignant melanoma. *Calcif Tissue Int* 73: 281-289, 2003.
7. Valenti MT, Zanatta M, Donatelli L, Viviano G, Cavallini C, Scupoli MT and Dalle Carbonare L: Ascorbic acid induces either differentiation or apoptosis in MG-63 osteosarcoma lineage. *Anticancer Res* 34: 1617-1627, 2014.
8. Heid CA, Stevens J, Livak KJ and Williams PM: Real time quantitative PCR. *Genome Res* 6: 986-994, 1996.
9. Kim DK, Kim DW, Kim SW, Kim DY, Lee CH and Rhee CS: Ki67 antigen as a predictive factor for prognosis of sinonasal mucosal melanoma. *Clin Exp Otorhinolaryngol* 1: 206-210, 2008.
10. Ivanov VN, Bhoumik A and Ronai Z: Death receptors and melanoma resistance to apoptosis. *Oncogene* 22: 3152-3161, 2003.
11. Herr I and Debatin KM: Cellular stress response and apoptosis in cancer therapy. *Blood* 98: 2603-2614, 2001.
12. Lu B, Wang L, Stehlik C, Medan D, Huang C, Hu S, Chen F, Shi X and Rojanasakul Y: Phosphatidylinositol 3-kinase/Akt positively regulates Fas (CD95)-mediated apoptosis in epidermal C141 cells. *J Immunol* 176: 6785-6793, 2006.
13. Boregowda RK, Olabisi OO, Abushahba W, Jeong BS, Haenssen KK, Chen W, Chekmareva M, Lasfar A, Foran DJ, Goydos JS, *et al*: RUNX2 is overexpressed in melanoma cells and mediates their migration and invasion. *Cancer Lett* 348: 61-70, 2014.
14. Cohen-Solal KA, Boregowda RK and Lasfar A: RUNX2 and the PI3K/AKT axis reciprocal activation as a driving force for tumor progression. *Mol Cancer* 14: 137, 2015.
15. Ott PA, Hodi FS and Buchbinder EI: Inhibition of immune checkpoints and vascular endothelial growth factor as combination therapy for metastatic melanoma: An overview of rationale, preclinical evidence, and initial clinical data. *Front Oncol* 5: 202, 2015.