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Targeting LMW-PTP to sensitize melanoma cancer cells toward chemo- and radiotherapy.

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Targeting LMW-PTP to sensitize melanoma cancer cells toward chemo- and radiotherapy

Running title: Targeting LMW-PTP to fight melanoma

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Summary

In melanoma cells, LMW-PTP modulates sensitivity to chemio- and radiotherapy. LMW-PTP targeting with Morin, a natural inhibitor of this phosphatase, enhances effectiveness of dacarbazine and radiotherapy treatment, and reduce self-renewal ability of melanoma, without affecting viability of normal cells. In conclusion, Morin behaves as a potent, no toxic, sensitizing agent.

Abstract

Melanoma cells are intrinsically resistant to chemo- and radiotherapy. In this paper, we demonstrated that LMW-PTP regulates sensitivity of melanoma cells to apoptosis. We show that knockdown of LMW-PTP enhances cytotoxic effect of dacarbazine and 5-FU, improves effectiveness of radiotherapy, and impairs self-renewal capability of melanoma cells. Similar results were obtained treating melanoma cells with Morin, a small molecules that induces the proteasome-dependent degradation of LMW-PTP. Morin sensitizes melanoma cells to apoptosis, and synergizes with dacarbazine, improving its effectiveness. Interestingly, we showed that Morin treatment does not affect viability of non-cancerous cells, neither alone or when administrated in combination with anticancer drugs. Decrease of endogenous LMW-PTP levels sensitizes PC3 to docetaxel and radiotherapy, confirming the general role of LMW-PTP as regulator of apoptotic process. In summary, we demonstrate that LMW-PTP targeting improves effectiveness of traditional anticancer drugs used for treatment of melanoma. These results suggest also that Morin could be used as adjuvant to improve the outcome of patients affected by metastatic melanoma.

Introduction

Malignant melanoma is one of the most aggressive types of skin cancer, showing a high metastatic potential, and mortality [**Garbe et al., 2011**]. In fact, even if melanoma represents only a small proportion of skin cancer (4%), it accounts for 80% of skin-cancer related mortality [**Geller et al., 2003**]. The main risk factors for development of melanoma include intense exposure to Ultraviolet radiation (UV), familial history of melanoma, or non-melanoma skin cancer [**Gandini et al., 2004 a**; **Gandini et al., 2004 b**]. Different therapeutically protocols are used for treatment of melanoma, depending on the stage of pathology. When melanoma is diagnosed in the early stages, surgical ablation of the tumor is recommended; in other cases, surgery is associated with different types of adjuvant therapies, or with radiotherapy. However, in presence of metastatic melanoma, chemotherapy is often the only therapeutical option. Alkylating agents such as dacabarzine, temozolomide or fotemustine are the most recommended drugs, even if these generally show a modest efficacy, and induce heavy side effects in most of patients [**Dummer et al., 2012**; **Garbe et al., 2011**].

Intrinsic resistance to apoptosis of melanoma cells is one the main cause of anticancer therapy failure. Many factors contribute to the deregulation of the apoptotic mechanism, including the lost or mutation of P53 gene, over-activation of survival pathway, overexpression of anti-apoptotic proteins and of drug extrusion pumps, or DNA repairing proteins [**Kalal et al., 2017**]. There is a wide accord about the fact that the development of new strategies useful to overcome intrinsic drug resistance of melanoma cells could have a significant impact on survival rate of patients affected by this kind of cancer.

In the last decades, compelling evidence suggested that the LMW-PTP could have an important role in modulating response of cancer cells to genotoxic insults. LMW-PTP can be considered an early marker of carcinogenesis. Its expression is increased in pre-neoplastic lesions of colon from rats treated with tumor inducing agents [**Marzocchini et al., 2007**]. Further studies showed that LMW-PTP is overexpressed in different cancers, including colon cancer and neuroblastoma, in that its expression is related with a worse prognosis and a reduced survival rate [**Malentacchi et al., 2005**]. Overexpression of LMW-PTP in NIH-3T3 cells is sufficient to induce neoplastic transformation, and LMW-PTP-transfected NIH3T3 fibroblasts generate large tumors once engrafted in nude mice [**Chiarugi et al., 2004**]. In addition, a study carried out on 481 male patients suffering of prostate cancer, highlighted that LMW-PTP is over-expressed in prostate cancer cells, and its expression correlates with a worse prognosis and with disease recurrence [**Ruela-de-Sousa et al., 2015**].

More recently, independent studies demonstrate that LMW-PTP is involved in the regulation of apoptosis and in the acquisition of drug resistance. Ferreira PA and colleagues, demonstrate that over-expression of LMW-PTP confers resistance to vincristine in leukemic cells [**Ferreira et al., 2012**]. Similar results have been obtained by a research focused on colorectal cancer, showing that the LMW-PTP over-expression mediates malignant potential of cancer cells, inducing drug resistance, and enhancing cell motility and invasivity [**Hoekstra et al., 2015**]. Together, this evidence suggest that LMW-PTP is a key player in sustained tumor growth and resistance of cancer cells toward traditional anticancer therapies.

This study aims to define the role of LMW-PTP in inducing resistance of melanoma cells to chemo- and radiotherapy. We show that melanoma cells express high LMW-PTP levels in comparison with normal fibroblasts. Moreover, using RNA interfering technology we demonstrate that LMW-PTP downregulation increases sensitivity of melanoma cells to dacarbazine, 5-FU and radiotherapy, and impair cancer cells clonogenic ability. Furthermore, we show that treating melanoma cells with Morin, a natural polyphenol that *in vitro* inhibits both LMW-PTP isoforms, we can reproduce all effects induced by RNA interfering. Interestingly, we find that Morin is active against cancer cells that overexpress LMW-PTP, but is ineffective against non-cancer cells that express low LMW-PTP levels. Finally, we demonstrate that LMW-PTP downregulation sensitize PC3 cells toward docetaxel, suggesting that the role of LMW-PTP as a negative regulator of apoptosis is not limited to melanoma cells. Taken together, these findings confirm the role of LMW-PTP in inducing resistance toward traditional anticancer therapy, and open new opportunities for designing innovative therapeutic approaches based on the use of specific LMW-PTP inhibitors as chemo-sensitizing agents for treatment of melanoma and other type of chemo- or radio-resistant tumors.

MATERIAL AND METHODS

Cell culture and transfections

A375 melanoma cells were purchased from ATCC (Manassas, USA) and cultured in Dulbecco's Modified Eagles Medium (DMEM, Sigma Aldrich, St. Louis, USA), supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin, 100 mg/ml streptomycin (Euroclone), and propagated at 37°C in a 5% CO_2 humidified atmosphere. A375 cells (100000 cells/mL) were grown for 24h and then transiently trasfected with LMW-PTP siRNA (Target sequence CCCATAGTGCACACTTGTATA), using Hiperfect Trafection Reagent (Qiagen) according to the manufacturer's instructions. Briefly, the cells were trasfected for 24, 48 or 72h with siRNA at final concentration 20 nM. To test the specificity of LMW-PTP trasfection, control cells were trasfected with a Scramble Sequence (AllStars Negative Control siRNA, final concentration 20 nM, Qiagen). Western Blotting assessed the efficiency of transfection.

Enzymatic assay

Enzymatic assays were carried out using purified recombinant human LMW-PTPs purified as previously described [Ottanà et al., 2009]. Phosphatase assay was carried out at 37 °C using p-nitrophenylphosphate as substrate dissolved in a solution containing 0.075 M of β,β-dimethylglutarate buffer (pH 7.0), 1 mM EDTA and 5 mM dithiothreitol. All reactions were initiated by addition of enzyme in the solutions and stopped by adding 4 ml of 1 M KOH. The amount of *p*-nitrophenolate released was determined using a spectrophotometer, reading the absorbance of samples at 400 nm (ε = 18,000 M^{-1} cm^{-1}). Km and Vmax were determined by measuring the initial hydrolysis rate of enzyme at different substrate concentrations. Experimental data were analysed using the Michaelis-Menten equation and a non-linear fitting program (Fig-Sys). Inhibition constants were determined measuring initial hydrolysis rates at differing substrate and inhibitor concentrations. The apparent Km values measured at the various inhibitor concentrations were plotted against concentration of the inhibitor to calculate the Ki values. All tests were carried out in triplicate.

Evaluation of synergism between Morin and dacarbazine

Synergism between Morin and dacarbazine was determined using method proposed by Ting-Chao Chou [Chou 2006]. Data obtained were analysed using Compusyn computer program [Chang et al., 1985].

Western Blotting

Cells were lysed on iced in 1X Laemli Buffer (0,5 M TrisHCl pH 6,8, 10% SDS, 20% Glicerol, βMercaptoetanol, 0,1% bromophenol blue) and samples were boiled for 10 minutes. Cell extracts were resolved by SDS-PAGE and transferred to PVDF membranes (BioRad). Membranes were incubated overnight at 4°C with the appropriate primary antibody: rabbit polyclonal anti-LMW-PTP were produced in our laboratory, Bcl-2, Bim, Caspase3, Actin were obtained from Santa Cruz Biotechnology. After washing in TPBS-Tween 20 (0,1%) membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1h. Proteins were detected using Clarity Western ECL (Biorad) by UVP.

Cell viability assay

$2x10^4$ cells were seeded in 24 wells plate: after 24, 48 or 72h 5mM MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added and incubated for 2h at 37°C. Cells were resuspended in 200 µL of Dimethyl sulfoxide: wavelength measuring was performed at 595 nm using a spectrophotometer.

Apoptosis evaluation

Apoptosis was determined using Annexin-V-FLUOS Staining kit from Roche according to manufacturer's instructions. $1x10^6$ cells were detached with Acutase, washed in PBS and centrifuged at 1000 rpm for 5 min. Cell pellet was resuspended in 100 µL of Annexin-V-FLUOS labelling solution and incubated for 10-15 min at room temperature in the dark. 500 µL of incubation buffer was added and cells were analyzed by flow cytometry BDFACS Canto.

Colony Formation Assay

Briefly cells were seeded in six well plate and treated with Morin or trasfected with siRNA at the indicated concentration. After 24h cells were detached and re-plated 1000 cells/well and incubated at 37°C for 10 days. Subsequently cells were fixed and stained with a solution containing 1% Crystal Violet and 20% Methanol. Colonies were counted and photographed using NIKON Digital Sight. Plating Efficiency and Surviving Factor were extrapolated using the following formulas:

PE= (n° of colonies/n° of cells seeded) X 100

SF= PE of treated sample / PE of control sample

SF = (n° of colonies/n° of cells seeded) X PE

Adhesion test

A375 cells were seeded on 35 mm Petri dishes. When cells reached appropriate density (70% confluence), these are washed with PBS and incubated in the presence of 0.05% trypsin solution. After 10 minutes, dishes were washed with PBS containing 0.5 ml/ml of soyben trypsin inhibitor to stop proteolytic trypsin activity. Then, cells were incubated for 5 minutes at 37°C with 0.5 % crystal violet solution. After, extensive washing was carried out using PBS to remove the excess of dye that not penetrated into the cells. Releasing of dye absorbed by cells was obtained incubating cells with 0.1M Citrate buffer, pH 4.2, for 1h at 37°C. After this time, citrate solution was withdrawn, to allow quantification of dye released from cells using a spectrophotometer. All sample were read at 595 nm using a 1-cm pathlength cuvette.

Gelatin zimography

Conditioned media were collected and subjected to electrophoresis on 7,5% PAGE gels containing 0,1% gelatin. After electrophoresis the gel was washed twice with 2,5% Triton X-100 and once with Reaction Buffer (50mM Tris HCl, pH 7,5, 200 mM NaCl, 5mM $CaCl_2$). The gel was incubated overnight at 37°C with fresh Reaction Buffer. Then gel was stained with 0,25 Coomassie Brilliant Blue and destained (30% methanol and 10% acetic acid).

Boyden Chamber assay

Cell invasion was performed with 1 x 10^5 cells on 8-μm-pore Transwells (Corning) coated with 50 μg/cm^2 of reconstituted Matrigel as described in [**Giannoni et al., 2011**] (Chemotaxis was evaluated by counting the cells migrated to the lower surface of the filters (six randomly chosen fields).

Cell Irradiation

Cell cultures were irradiated with a 6MV XRay beam delivered by a clinical linear accelerator ELEKTA Synergy Beam Modulator. Single doses of 0-4 Gy were delivered to the cell cultures seeded in well plates that were embedded in water equivalent medium to provide a uniform irradiation.

Statistical analysis

Statistical analysis of the data was performed by unpaired student's t-test for pairwise comparison of groups unpaired t-test. All data were expressed as the mean +/- S.E.M. A P-value less then 0.05 was considered statistically significant.

Results

Melanoma cells express high LMW-PTP levels

The expression levels of LMW-PTP in melanoma cells were evaluated using specific antibodies able to recognize both LMW-PTP isoforms. Western blot analysis show that A375 cells express significantly higher levels of LMW-PTP in comparison with Human Dermal Fibroblasts, which represent an example of non-cancerous cells (**Fig. 1A, B suppl.**). As expected, high levels of LMW-PTP were detected in other cancer cell lines, such as PC3 (prostate carcinoma), HT29 and HCT8 (from colon carcinoma), and SHS5Y (neuroblastoma) (**Fig. 1A, B suppl.**). To confirm the clinical significance of LMW-PTP in melanoma cancer, we analysed data concerning mRNA expression levels of LMW-PTP obtained from Oncomine database [**Talantov et al., 2005**]. We found that levels of LMW-PTP are significantly higher in melanoma cells with respect to normal skin cells ($p = 1.72186E-5$) (**Fig. 1C suppl.**). Together, these findings confirm that LMW-PTP is commonly over-expressed both in A375 cells, as well as in melanoma cells derived from patients.

Silencing of LMW-PTP influences morphology, invasivity, resistance to cytotoxic drugs and radiotherapy, as well as self-renewal ability of melanoma cells

In order to clarify the role of LMW-PTP in melanoma cells, we analyzed the behavior of A375 cells silenced for LMW-PTP (**Figure 2A suppl**). We observed that silenced cells acquire a spindle-shape, and develop several plasma membrane protrusions upon transfection (**Fig. 2C suppl**). This evidence suggested that loss of LMW-PTP could affect the adherence of melanoma cells on plastic support. To verify this hypothesis, we performed specific tests to evaluate the adhesive strength and invasive ability of silenced cells. We found that silenced cells adhere stronger on plastic support, secrete lower levels of MMP-9, and result less invasive respect to control cells (**Fig 2D-G suppl.**). Then, we evaluated whether loss of LMW-PTP could influence the resistance of cells to cytotoxic insults. To this aim, control and silenced cells were treated with dacarbazine or 5-FU for 24 hours, and then analyzed to evaluate the apoptotic rate. We found that silenced cells treated with dacarbazine or 5-FU show an apoptotic rate considerably higher than control cells (**Fig. 1A-B, and 3C, D suppl**). In keeping with this evidence, we observed that LMW-PTP silencing causes, per se, a decrease of Bcl2 expression levels and a weak activation of caspase3. When silenced cells are treated with 5-FU, levels of Bcl2 become quite undetectable, while the fraction of active caspase3 increases (**Fig.1C**). Finally, long-term effects of LMW-PTP down-regulation were evaluated

through the colony formation assay. Interestingly, we found that silenced cells generate fewer colonies with respect to control cells, and, most importantly, that combination of silencing and drug treatment completely inhibits generation of new colonies (**Fig. 1D-G**). In conclusion, our results demonstrate that LMW-PTP expression increases invasivity and colony forming ability of melanoma cells, and decreases A375 sensitivity toward drug-induced apoptosis.

LMW-PTP downregulation increases sensitivity of A375 cells to radiotherapy

Radiotherapy represents a valuable therapeutic alternative to systemic chemotherapy for treatment of metastatic melanoma. Thus, we investigated whether reduction of LMW-PTP levels could increase sensitivity of melanoma cells to radiotherapy. Figure 2 shows that dishes containing silenced cells exposed to 2Gy radiation contain many rounded cells and many cells fragments (**Fig 2A**). MTT test confirm that most of radied cells silenced for LMW-PTP are not viable (**Fig 2B**). In addition, combination of LMW-PTP silencing and irradiation strongly impair the ability of cancer cells to generate new colonies, while irradiation alone does not (**Fig. 2C-D**). In summary, our results demonstrate that LMW-PTP downregulation makes melanoma cells more sensitive to radiotherapy and, in the same moment, strongly impairs self-renewal capability of melanoma cells exposed to radiation.

Morin strongly enhances the cytotoxic activity of dacarbazine

To verify whether pharmacological inhibition of LMW-PTP could reproduce, *in vivo*, the same effects of the LMW-PTP silencing, we tested the effect of Morin in our experimental setting. Morin is a polyphenol that behaves as a mixed type non-competitive inhibitor of LMW-PTP, showing a Ki value in the micromolar range (**Fig. 4 suppl**). Melanoma cells were treated with Morin, dacarbazine or combination of both, and then analysed to evaluate their viability. We observed that only when administrated in combination with Morin, dacarbazine reduces melanoma cells viability. It is noteworthy that in the presence of Morin, 0.5 μM dacarbazine leads to a 70 % reduction in cells viability (**Fig. 3A**). Moreover, using Annexine-V/Pi method we confirmed that only morin-dacarbazine combination boosts apoptosis, while Morin and dacarbazine alone do not. (**Fig 3 B and Fig. 5 suppl**). Long-term effects of Morin were evaluated using the colony assay method. We found that co-treatment of A375 cells with Morin and dacarbazine strongly inhibits the ability of cancer cells to form new colonies (**Fig. 3C**), while smaller effects were observed using Morin or dacarbazine alone. Together, our results demonstrate that Morin acts as a non-toxic sensitizing agent, which is able to enhance cytotoxic effects of dacarbazine, and to inhibit self-renewal ability

of melanoma cells. To evaluate whether Morin synergizes with dacarbazine we used the method proposed by T-C Chou [Chou 2006]. CI-Fa plot generated by Compusyn software clearly shows that all CI values calculated for combination are comprised between 0.071 and 0.14 (**Fig. 6A suppl**), thereby confirming that the presence of a strong synergy between Morin and dacarbazine. The isobologram plot generated by computer program confirms these results (**Fig. 6B suppl**). Most importantly, F_a-log(RDI) plot shows that using Morin as sensitizing agent it is possible to reduce the dose of dacarbazine without loss to its cytotoxic effects (**Fig. 6C suppl**).

Morin sensitizes A375 cells towards different chemotherapeutic agents

We next investigated whether Morin sensitized melanoma cells toward 5-FU. We found that Morin strongly decreases the IC_{50} of 5-FU for A375 cancer cells (**Fig. 4A**). Sensitizing effect of Morin is strictly dose dependent, and a significant decrease of cancer cell viability was observed already at 0.1 μM Morin (**Fig. 4B**). In keeping with this evidence, we found that Morin/5-FU co-treatment boost apoptotic events, while treatment with 5-FU alone cause only a slight increase of apoptosis rate. Similar results were obtained treating melanoma cells with Morin alone (**Fig. 4C and Fig. 7 suppl.**). Finally, colony forming assay demonstrate that co-treatment with 5-FU and Morin strongly impairs colony forming ability of melanoma cells, whereas a partial or no inhibition was observed using 5-FU or Morin alone, respectively (**Fig. 4D, E**). Together, this evidence suggests that Morin: i) acts by modulating the expression of antiapoptotic proteins and the activity of caspase3, ii) acts as chemosensitizing agents only on cancer cells that express high LMW-PTP levels; iii) sensitizes melanoma cells toward different cytotoxic drugs, suggesting that it could be used to expand the list of chemotherapy drugs useful for treating melanoma.

Co-treatment with Morin and 5-FU does not impair cell viability of non-cancer cells.

In order to investigate the effects of Morin on non-cancerous cells, further tests were carried out using differentiated mouse myoblasts, human dermal fibroblasts, and MCF10A human breast cells. Differentiated C2C12 cells, were pre-incubated for 4h with different Morin concentrations, and then treated with 20 μM 5-FU for 24 hours. After this time, cells viability was assayed using MTT test. We found that 5-FU has no relevant toxic effects on these cells, and that, most importantly, combined treatment does not impair cell viability (**Fig. 5A**). Similar results were obtained using both HDF and MCF10A cells (**Fig. 5B, C and Fig. 8A, B suppl.**). These results suggest that Morin, alone or in combination with 5-FU, does not impair viability of non-cancerous cells.

Morin induces a proteasome-dependent LMW-PTP degradation

To gain insight in the mechanism of action of Morin, we evaluated whether it modulates LMW-PTP expression. We found that Morin triggers a transient downregulation of LMW-PTP levels (**Fig. 6A, B**). Moreover, we observed that the effect of Morin is strictly dose-dependent, and a significant decrease of LMW-PTP levels was observed already at the concentration of 1μM (**Fig. 6 C, D**). Interestingly, we found that pre-incubation of A375 cells with MG132, a specific proteasome inhibitor, prevents LMW-PTP down-regulation (**Fig. 6E, F**), and almost completely abrogates the sensitizing effect of Morin (**Fig. 9A, B suppl**). This evidence strongly suggest that Morin triggers LMW-PTP degradation through a proteasome-dependent mechanism.

Next, to evaluate the specificity of Morin action, we monitored also the expression levels of PTP1B and SHP2, two other phosphatases that are clearly involved in tumor progression [**Chen et al., 2016; Yip et al., 2010**]. We observed that levels of PTP1B and SHP2 do not change after treatment with Morin (**Fig. 10 A, B suppl.**). In addition, SDS-PAGE analysis of whole sample extracts shows that there are no differences between protein profiles of untreated cells with respect to that of cells treated with Morin (**Fig. 10 C suppl.**). Together, these results suggest that Morin triggers degradation of specific proteins, and does not causes a massive and non-specific degradation of cellular proteins.

Morin treatment increases sensitivity of A375 cells to radiotherapy

Radiotherapy represents a valuable therapeutic alternative to systemic chemotherapy for treatment of metastatic melanoma. Thus, we investigated whether Morin is able to increase sensitivity of A375 cancer cells to radiotherapy. MTT test reveals that a single 4Gy dose does not impair melanoma cells viability. However, pre-treatment with Morin strongly increases radio-sensitivity of A375 cells in a dose dependent manner, being 0.8 μM the IC_{50} value of Morin (**Fig. 7A**). By treating melanoma cells with lower radiation dose, 1 or 2 Gy, we obtained similar results (**Fig. 7B**). Noteworthy, when treating cells with 2.5 μM Morin concentration before the exposition to a 2 Gy radiation dose, we observed a reduction of about 70% of cell viability (**Fig. 7C, D**).

To evaluate the effects of radiation-Morin combined treatment on self-renewal ability of melanoma cells, we used the colony forming assay method. We found that radiated cells pre-treated with Morin generate fewer colonies with respect to control cells, thereby confirming that Morin synergizes with radio-therapy and that combined treatment strongly impairs the ability of melanoma cells to regenerate new colonies (**Fig. 7E, F**).

Morin treatment and LMW-PTP silencing sensitize PC3 cancer cells towards docetaxel

According to our results on melanoma cells we found that LMW-PTP silencing enhances sensitivity of PC3, prostate cancer cells to docetaxel (**Fig. 11 suppl**). In particular, we found that Morin: i) induces a transient down-regulation of the LMW-PTP levels (**Fig. 12A suppl**), ii) in combination with Docetaxel reduces cells viability (**Fig. 12B suppl**), and boosts apoptosis rate (**Fig. 12C and E suppl**); iii) increases radio-therapy effectiveness (**Fig. 12D suppl**). Finally, colony assay tests confirm that by combining Morin with docetaxel treatment it is possible to almost totally inhibit the colony forming ability of PC3 cells (**Fig. 13A, B suppl**). These results confirm that the LMW-PTP reduces sensitivity to apoptosis of PC3 cells, and that using LMW-PTP inhibitor it is possible to reprogram cancer cells toward a phenotype more sensitive to apoptosis.

Discussion

Cancer is a very complex pathology and it represent one the most common cause of death throughout the world. Statistics on cancer incidence, and survival data revealed that melanoma represent one the most lethal type of cancer. This is mainly due to the resistance of melanoma cells to traditional anticancer therapies. Thus, only through the understanding of the complex mechanisms that contribute to survival of melanoma cells, we will get useful information to defeat this kind of tumor.

Studies conducted in the past demonstrate that several factors contribute to resistance of melanoma cancer cells to anticancer therapies, including constitutive activation of pro-survival pathways, overexpression of DNA repairing enzymes, drug extrusion pumps, and antiapoptotic proteins [**Kalal et al., 2017**].

Recent studies identify the LMW-PTP as a new interesting target to fight cancer. This enzyme is overexpressed in many type of cancer [**Malentacchi et al., 2005**]. Moreover, it has been demonstrated that its overexpression is sufficient to induce cells transformation and to increase the resistance of cancer cells toward apoptosis induced by cytotoxic agents [**Chiarugi et al., 2004; Hoekstra et al., 2015; Ferreira et al., 2012**]. To date, the role of this protein in skin cancer and melanoma has not yet been clarified.

Our study confirms that LMW-PTP is essential to modulate sensitivity of melanoma cancer cells toward apoptosis induced by cytotoxic drugs. Using RNA interfering , we demonstrate that depletion of the LMW-PTP in A375 cells triggers deep changes in the morphology of the melanoma cells, which acquire a spindle-shape form, develop stress fibres, and long plasmamembrane protrusions. In addition, silenced cells are more adhesive and less invasive with respect to control cells. On the other hands, we observed that LMW-PTP silencing determines a strong decrease of Bcl2 levels, an increase of Bim expression, and the activation of caspase3. Finally, we observe that melanoma cells silenced for LMW-PTP become more sensible to dacarbazine, 5-FU, and radiotherapy. Taken together, all these findings suggest that LMW-PTP is able to regulate, cell morphology, motility and sensitivity to apoptosis.

Previous studies demonstrated that LMW-PTP targets and inactivates p190Rho-GAP, thereby promoting Rho activation [**Chiarugi et al., 2000**]. On the other hand, a recent study demonstrates that Rho overexpression favours synthesis of ABC transporters, and reduces sensitivity of colon cancer cells to apoptosis induced by treatment with irinotecan. Conversely, it has been reported that Rho silencing suppresses the expression of Bcl-xl and Bcl2, and promotes the expression of Bax

[**Ruihua et al., 2016**]. Together, these findings suggested that LMW-PTP-mediated Rho activation could be the mechanism that drive cancer cells toward the acquisition of a motile and drug-resistant phenotype. Further studies will be needed to clarify the role of Rho and the mechanisms by which Rho contribute to Bcl2 degradation and overexpression of the proapoptotic protein Bim in melanoma cells.

Recently, it has been demonstrated that the effects obtained with RNA interfering could be reproduced using specific inhibitors of LMW-PTP [**Hoekstra et al., 2015**]. In this paper, we illustrated how the polyphenol Morin could be used to this scope. We demonstrated that *in vitro* Morin is a non-competitive inhibitor of LMW-PTP, showing a Ki value in the micromolar range. Our results show that Morin is well tolerated by either non-cancerous and cancer cells. Surprisingly, we observed that, when administrated to cancer cells, Morin triggers the specific and transient down regulation of LMW-PTP, through a proteasome-dependent mechanism. Downregulation of LMW-PTP is associated with a decrease of Bcl2 levels, and with the increase of expression of proapoptotic proteins, suggesting that Morin reprograms melanoma cells toward an apoptosis-sensitive phenotype. In keeping with this evidence, we find that Morin synergizes with dacarbazine and 5-FU, strongly enhancing their cytotoxic effects. Similar effects were observed using radiotherapy as treatment. These results suggest that the decrease of LMW-PTP levels drive the conversion of melanoma cells from a drug-resistant, toward a drug-sensitive phenotype. In keeping with this hypothesis, we demonstrated that inhibiting proteasome activity, it is possible to avoid LMW-PTP depletion and completely revert the sensitizing effects of Morin, thereby confirming that LMW-PTP degradation contributes to sensitize cancer cells to cytotoxic drugs. These findings is in agreement with the evidence that Morin can exert its sensitizing effect on cancer cells that express high LMW-PTP levels, but not on cells that express low LMW-PTP levels, such as fibroblast or other normal cells. Finally, we observed that using Morin as sensitizing agent it is possible to impair self-renewal ability of cancer cells.

LMW-PTP is not the only target of Morin *in vivo*. In a recent study, Gupta and colleagues showed that Morin treatment sensitizes myeloma cells to thalidomide and bortezomib. The authors demonstrate that the sensitizing activity of Morin depends on the ability of polyphenol to stimulate the expression of SHP1 phosphatase that, in turn, deactivates STAT3, a transcription factor that stimulates expression of antiapoptotic proteins [**Gupta et al., 2013**]. To date, we cannot exclude that both mechanisms described above could contribute to increase sensitivity of melanoma cells to cytotoxic drugs. Further studies will be needed to clarify the exact Morin action mechanism.

To extend our analysis, we performed further tests to evaluate the radio-sensitizing activity of Morin. Our results show that Morin treatment increases sensitivity of melanoma cells toward radiotherapy. Sensitizing activity of Morin can be observed already at low radiation doses (1 Gy), suggesting that this compound may be used to reduce radiation doses without affecting their efficacy. Finally, colony assay demonstrates that combining Morin treatment with radiotherapy, it is possible to strongly inhibit the colony forming ability of melanoma cells, suggesting that Morin could be used to decrease the risk of relapse. We have extended our analysis also to PC3 cells (a prostate cancer cell line), using Docetaxel or irradiation, obtaining essentially the same results.

For long time, phosphotyrosine protein phosphatases (PTPs) have been considered "undruggable enzymes" [**Caselli et al., 2016**]. The main obstacles that impede the development of potent and specific inhibitors against PTPs are the similarity of active site between all members of the family, and the fact that the active site contains positive charged amino acids. Insertion of highly negative charged groups into inhibitors structure favors the targeting of enzyme active site, but, in the same moment, drastically reduce their bioavailability. Moreover, these inhibitors show low selectivity against all members of PTP family, thereby resulting highly toxic. However, results of a recent study demonstrate that potent and specific inhibitors for LMW-PTP can be obtained designing a molecule able to target aminoacids lining the active site of enzyme. Such a molecule behave as a non-competitive inhibitor, shows a good oral bioavailability, and is useful to in vivo studies [**Stanford et al., 2017**]. Results of our study, for the first time, show that a different strategy, namely transient protein degradation, could be used to control activity of LMW-PTP in cells-based systems. We show that Morin, a natural molecule that *in vitro* behaves as non-competitive inhibitor of this enzyme, possess peculiar properties triggering, *in vivo*, transient and specific degradation of LMW-PTP through a proteasome-dependent mechanism. Despite the facts that the exact action mechanism of Morin remain to be elucidated, our results suggest the existence, *in vivo*, of a specific mechanism deputed to the control of LMW-PTP expression, thereby reinforcing the hypothesis about the central role of this enzyme in the control of cells physiology.

In summary, results showed in this paper demonstrate for the first time that in melanoma and in PC3 cells, LMW-PTP is directly involved in the control of apoptosis and that by regulating its expression levels it is possible to improve the sensitivity of melanoma cells toward anticancer therapies. Moreover, we identify the polyphenol Morin as a LMW-PTP inhibitor able to induce transient down-regulation of this enzyme. These results could have important implications in the field of anticancer therapies because they demonstrate the importance of this enzyme as a target to control melanoma cell growth, and highlight the possibility to use LMW-PTP inhibitors to develop

new adjuvant-based anticancer therapies useful for treatment of metastatic melanoma and other kind of drug-resistant cancers.

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