Inhibition of Mek 1/2 kinase activity and stimulation of melanogenesis by 5,7-dimethoxycoumarin treatment of melanoma cells

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Abstract. In this study, the processes of differentiation and melanogenesis induced by 5,7-dimethoxycoumarin in murine (B16) and human (A375) melanoma cells were investigated. Taking into account the previously demonstrated anti-proliferative and differentiation activities of this compound, we examined Ras/Raf/Mek/Erk mitogen-activated protein kinase activity following treatment; inhibition of Mek 1/2 kinase activity and subsequent reduction in Erk 1/2 activation were detected in both cell types. We observed melanogenesis induction associated to an increase in cAMP-response element-binding protein (CREB) and microphthalmia-associated transcription factor (Mitf) expression, both involved in its regulation. Mitf is fundamental for development, survival and differentiation of melanocyte and melanoma, since it regulates transcription of genes encoding for proteins involved in cell cycle progression or in melanogenesis, such as the enzyme tyrosinase. A significant increase of tyrosinase activity was revealed following treatment in B16 but not in A375 cells, although a strong synthesis of melanin was induced by 5,7-dimethoxycoumarin in both cell lines. The treatment induced protoporphyrine IX accumulation involved in melanogenesis since it promotes stability of cAMP. Finally, the Mek 1/2 inhibitor U0126 significantly potentiated growth inhibition of B16 cells triggered by 5,7-dimethoxycoumarin, suggesting that down-regulation of Raf/Mek/Erk pathway sensitizes melanoma cells to 5,7-dimethoxycoumarin treatment.

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Introduction

Coumarins, a class of compounds widely distributed in the plants (1), possess several biological activities, protect against oxidative damage and exhibit anti-tumorigenic activity (2-5). Coumarins and their synthetic derivative compounds have been used in clinical trials, alone or in combination with traditional drugs such as etoposide, for the treatment of various malignant tumors, including renal, lung and kidney carcinoma as well malignant melanoma (6). A coumarin derivative, the 5,7-dimethoxycoumarin, has been identified in a small group of vegetable species, among which *Citrus limon* L. (7) and *Carica papaya* L. (8). This molecule showed both a differentiating effect on HL-60 cell line (9) and an inhibition activity in DNA adduct formation, induced by carcinogens, in mouse mammary gland (10). Recently, our group investigated its antiproliferative activity on melanoma cell lines, including the murine B16 and human A375 lines (11). The 5,7-dimethoxycoumarin significatively reduced cell proliferation in a time and dose-dependent manner by blocking cell cycle in G_0/G_1, while cell growth inhibition was associated with the occurrence of cell differentiation. Consistent with these findings, we observed that 5,7-dimethoxycoumarin strongly decreased, in the murine murine melanoma cell line, the activation of the mitogen-activated protein kinase extracellular signal-related kinase 1/2 (MAPK Erk 1/2), which has been found to be upregulated in many types of cancer (12). In this study, we further investigated the molecular mechanisms driving the antiproliferative and differentiation activities of the 5,7-dimethoxycoumarin, considering that the inhibition of the Erk phosphorylation is as a key point of its biological outcome, at least in the melanoma cell line model. Thus, we monitored kinase activity of MAP kinase kinase 1/2 (Mek 1/2) following treatment, with the aim to define the point of mitogen-activated protein kinases (MAPKs, serine threonine kinases) pathway where 5,7-dimethoxycoumarin blocks phosphorylation cascade and Ras/Raf/Mek/Erk kinases activation. Besides, the effect of the 5,7-dimethoxycoumarin on the melanogenesis process was evaluated by determining

the expression of p-cAMP-response element-binding protein (p-CREB) and microphthalmia-associated transcription factor (Mitf), and the activity of the tyrosynase enzyme, which catalyses two key steps in the pathway of melanin synthesis (13). Mitf is one of the substrates regulated by p-Erk 1/2 and it is involved in the differentiation, proliferation and survival of melanocytes as well in the activation of the transcription of the tyrosinase gene (14,15). Mitf transcriptional activity is regulated through the cAMP-mediated pathway by the phosphorylation of the transcriptional factor CREB which upregulates the Mitf gene transcription (16). Melanogenesis represents a characteristic phenomenon of melanocyte differentiation (17), a crucial point in the development and progression of melanoma (18). We also explored the expression of porphobilinogen deaminase (PBG-D), the key enzyme of heme synthesis wherein protoporphyrine IX (PpIX) was produced. In our previous study, the accumulation of PpIX was detected following 5,7-dimethoxycoumarin treatment and considered as a further marker of melanoma differentiation (19). Furthermore, PpIX expression induces melanogenesis through the trigger of a cAMP-mediated pathway by activating the soluble guanylate cyclase and increasing the intracellular cGMP (cyclic guanine monophosphate) content which in turn amplifies cAMP content by inhibition of cAMP phosphodiesterase (20,21). Finally, to underscore the role of MAPK signalling pathway Ras/Raf/Mek/Erk in provoking the antineoplastic activity of 5,7-dimethoxycoumarin on melanoma cells, the compound was exploited in combination with the U0126 Mek 1/2 inhibitor (22).

Materials and methods

Test compound. Synthetic 5,7-dimethoxycoumarin was purchased from Sigma-Aldrich (116238). The compound was dissolved in methanol and a solution with a concentration of 20 mM was obtained. For cell treatment, a volume of this solution was added to culture medium and its percentage with respect to the medium was 0.5, 1.25 and 2.5% for 100, 250 and 500 μM, respectively.

Cell lines and treatments. The murine melanoma B16 F1 and the human melanoma A375 cell lines, purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), were used in this study. Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% L-glutamine (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown at 37˚C in a humidified atmosphere with 5% CO_2. Cells were treated with 100, 250, 500 μM 5,7-dimethoxycoumarin or with concentration 1, 5, 10 μM of U0126 (Sigma-Aldrich), a specific inhibitor of Mek 1/2 (22); moreover, 5,7-dimethoxycoumarin was used in combination with U0126 using the concentrations specified above.

Trypan blue exclusion assay. B16 cells were seeded at a density of $1x10^4$ cells/well in 24-well plates. After 24 h, cells were treated with different concentrations of either 5,7-dimethoxycoumarin or U0126 alone; or 5,7-dimethoxycoumarin in combination with U0126. After 72 h of treatment, floating cells in the medium were transferred to a centrifuge tube while adherent cells were washed, collected by trypsinization and mixed with the corresponding floating cells before centrifugation. Cells were stained with 0.4% trypan blue and counted in triplicate with an optic microscope. The number of live and dead cells were then determined. Cell viability was expressed as a percentage comparing treated cells with respect to appropriate vehicle-treated control cells.

Melanin synthesis determination. A375 cells were seeded and treated as reported above, in order to measure extracellular and intracellular melanin according to the method of Hill *et al* (23). After 72 h of incubation, the culture medium was removed, centrifuged (700 x g, 10 min) and the supernatant was collected for extracellular melanin quantification. One ml of 0.4 M HEPES buffer (pH 6.8) and EtOH (9:1, v/v) was added to 1 ml of the medium and the optical density (OD) at 475 nm was measured to quantify extracellular melanin by using a calibration curve obtained with synthetic melanin solutions. Cells collected by trypsinization, were pelleted and washed twice with PBS and digested in 1 ml 1 N NaOH for 16 h at room temperature; intracellular melanin was measured as described above. The stimulation of melanogenesis following the treatment was estimated as percentage of total melanin (μg/ml), calculated by the addition of intracellular and extracellular melanin, synthesized in treated cells with respect to vehicle-treated control cells.

Tyrosinase activity assay. Tyrosinase activity was determined according to the method of Lin *et al* (24). B16 and A375 cells were seeded and treated as described above. After 24, 48 and 72 h of treatment, cells were washed, collected by trypsinization, stained with 0.4% trypan blue and counted in triplicate. For each sample, $7x10^4$ cells were then lysed in 1% Triton X-100 in 0.1 M Na phosphate buffer (pH 6.8) for 20 min; lysates were incubate with an equal volume of DOPA, 3 mg/ml in 0.1 M Na phosphate buffer for 3 h at 37˚C. Tyrosinase activity was determined by measuring sample absorbance at 490 nm. The stimulation of tyrosinase activity following the treatment was estimated as fold increase in treated cells with respect to vehicle-treated control cells.

Western blot analysis. B16 and A375 cells were seeded at a density of $1x10^4$ cells/well in 6-well plates and treated as described above. After 24-, 48- and 72-h treatment, cells were washed, collected by trypsinization and suspended in RIPA lysis buffer [1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 NaCl, 0.01 M sodium phosphate (pH 7.2), 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate and 100 U/ml aprotinin]. After 1 h on ice, the cell lysates were cleared by centrifugation at 13000 x g for 15 min and the resultant supernatants collected and used to estimate protein concentration by Bradford assay (25). Then, 40 μg of total protein lysates were resolved on 12% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and the separated proteins were transferred to PVDF membrane. The level of protein expression in each sample was detected using specific mouse monoclonal primary antibodies diluted in TTBS solution containing 1% (w/v) of BSA: antibodies

against phospho-Mek 1/2 (Ser 218/Ser 222), phospho-Erk 1/2 (Tyr-204), Mitf (N-15), PBG-D (A-16), p-CREB-1 (Ser 133) and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The membranes were incubated with a specific HRP-conjugated secondary antibody and developed using the enhanced chemiluminescent substrate from Pierce; then, they were stripped and reprobed with α-tubulin (4G1) primary antibody as a protein loading control.

Mek 1/2 immunoprecipitation and kinase assay. B16 and A375 cells were seeded and treated as described above; after 72 h of treatment, cells were washed, collected by trypsinization and lysed. Equal protein aliquots of precleared cell lysates (300 μg) were incubated overnight with Anti-Mek antibody and Ezview Red Protein A Affinity Gel beads (Sigma-Aldrich) at 4˚C with gentle rotation. The immune complexes were collected by centrifugation, washed three times and incubated for 90 min at 30˚C with not activated Erk substrate solution (Sigma-Aldrich) for kinase assay. Then, the samples were boiled in SDS sample buffer and loaded on 12% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE); the separated proteins were transferred to PVDF membrane. The level of diphosphorylated Erk 1/2 was detected using a monoclonal antibody against the activated MAP kinase (Sigma-Aldrich); the membrane was then incubated with a specific HRP-conjugated secondary antibody (Sigma-Aldrich) and developed using the enhanced chemiluminescent substrate from Pierce.

Statistical analysis. Student's t-test was employed to determine the significance of any reduction in cellular viability, the increase of melanin synthesis and any alteration in cell cycle following the treatment. A $p \leq 0.05$ was deemed statistically significant.

Results

Inhibition of Mek 1/2 catalytic activity in melanoma cells upon 5,7-dimethoxycoumarin treatment is preceded by Mek 1/2 phosphorylation. We have previously demonstrated the antiproliferative activity of 5,7-dimethoxycoumarin on murine B16 and human A375 melanoma cells. This activity was associated in the mouse cells with a strong decrease in Erk phosphorylation. To determine whether the antiproliferative activity exerted by 5,7-dimethoxycoumarin on the human melanoma cell line was also dependent on the modulation of the Ras/Raf/Mek/Erk signalling pathway, the activation level of Erk following treatment was determined. By using Western blot analysis, we observed that after 72 h of treatment, 5,7-dimethoxycoumarin was able to inhibit the phosphorylation of Erk in the human melanoma cell line, as well, up to 70% in the case of the higher dose (Fig. 1). Next, the phosphorylation level of the closer upstream kinase Mek 1/2 was determined. The effects of 5,7-dimethoxycoumarin on the Mek 1/2 activation, in B16 and A375 cells, are shown in Fig. 2A and B, respectively. Upon treatment, the level of phospho-Mek 1/2 (p-Mek 1/2) increased in both cell lines as compared with control samples. Densitometric analysis showed in the mouse melanoma cells an increase of p-Mek up to 16, 32 and 85% when stimulated with the dose of 100, 250 and 500 μM, respectively; similarly in the human melanoma cell line, p-Mek 1/2 increased up to 42, 67 and 72%. Our results indicate that 5,7-dimethoxycoumarin treatment induces Mek 1/2 phosphorylation, followed by the simultaneous decline of Erk 1/2 phosphorylation. To corroborate this *in vivo* finding, the kinase activity of Mek 1/2 was determined using Erk 1/2 as the *in vitro* natural substrate. The *in vitro* kinase assay demonstrated lack of phosphorylation of Erk 1/2 by phosphorylated Mek 1/2 from B16 and A375 melanoma cells treated with 5,7-dimethoxycoumarin respect to not treated cells (Fig. 3).

Melanogenesis process is induced in human melanoma cell following treatment with 5,7-dimethoxycoumarin. The significant change in light scatter properties of 5,7-dimethoxycoumarin treated melanoma cells was previously related to an increase of cellular complexity. Furthermore, in the murine

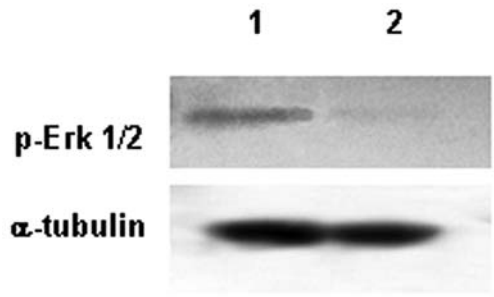

Figure 1. The effect of 5,7-dimethoxycoumarin on p-Erk 1/2 expression in A375 melanoma cells following treatment 72 h. Cells were incubated with or without the compound using 500 μM dose. After treatment, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting as described in Materials and methods. Activation level of Erk 1/2 was analysed with its corresponding phospho-specific antibody.

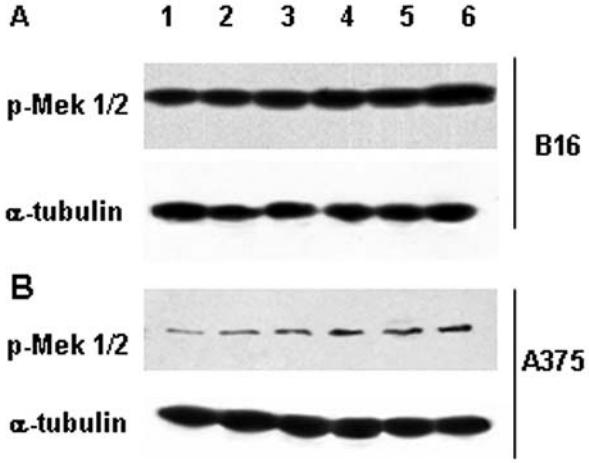

Figure 2. The effect of 5,7-dimethoxycoumarin on p-Mek 1/2 expression in B16 (A) and A375 (B) melanoma cells following treatment 72 h. Cells were incubated with the compound using 100, 250 and 500 μM doses: lanes 2 (100 μM), 4 (250 μM) and 6 (500 μM); or with respective vehicle solvent: lanes 1, 3 and 5. After treatment, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting as described in Materials and methods. Activation level of Mek 1/2 was analysed with its corresponding phospho-specific antibody.

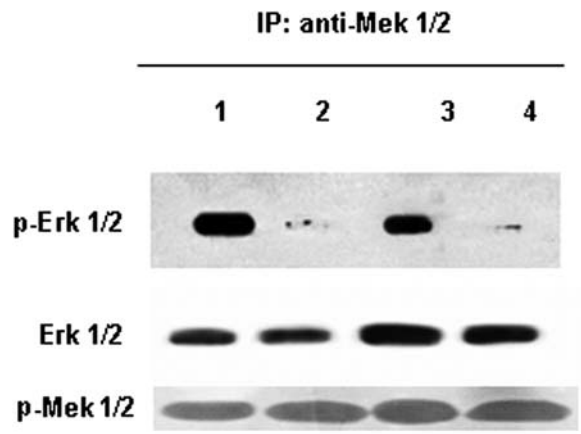

Figure 3. The effect of 5,7-dimethoxycoumarin on *in vitro* kinase activity of p-Mek 1/2 in B16 and A375 melanoma cells. B16 and A375 cells were incubated for 72 h with the vehicle solvent (1 and 3, respectively) or with the compound using 500 μM dose (2 and 4, respectively). Mek 1/2 was immunoprecipitated with anti-Mek 1/2 antibody from equal protein aliquots of cell lysates and immune complexes were incubated with non-activated Erk substrate solution for *in vitro* kinase assay. Then, the samples were subjected to SDS-PAGE followed by Western immunoblotting as described in Materials and methods. Phosphorylation level of Erk 1/2 was analysed with its corresponding phospho-specific antibody.

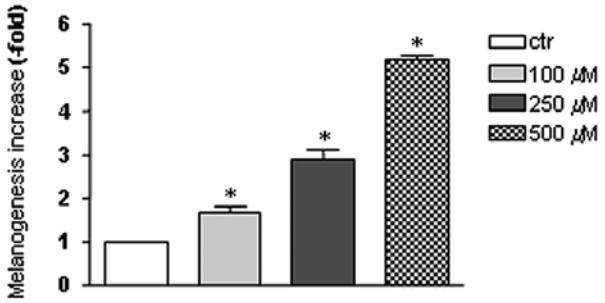

Figure 4. The effect of 5,7-dimethoxycoumarin on melanin synthesis in A375 melanoma cells following 72 h treatment. Cells were incubated with or without the compound using 100, 250 and 500 μM doses. The melanin concentrations (μg/ml) and the increase in melanin production were estimated as described in Materials and methods. Results are the mean ± SD from three separate experiments. *P<0.05 versus vehicle control treated cells.

melanoma cell line, a strong melanogenesis process following 5,7-dimethoxycoumarin treatment was observed (11). In the present study, we investigated if the treatment affects melanogenesis in the human melanoma cell line as well (Fig. 4). After 72 h of incubation, 5,7-dimethoxycoumarin induced melanin synthesis in treated cells. The extracellular melanin amount significantly increased in respect to the control through all doses, while the intracellular increase appeared to be significant only when the higher dose was used. Total melanin calculation revealed that the melanogenesis significantly increased in a dose-dependent manner, being 1.7-, 2.9- and 5.2-fold compared to the control at the concentration of 100, 250 and 500 μM, respectively. This result further supports the evidence of melanoma cells differentiation upon 5,7-dimethoxycoumarin treatment. Next, the activity of the tyrosinase enzyme, catalyzing the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) followed by the oxidation of L-DOPA to dopaquinone, was analyzed after treatment of melanoma cells (Fig. 5). Our results indicated in the murine cells a significant 2.59-, 2.64- and 3.3-fold increase of the enzymatic activity, compared to the control, at the concentration 100, 250 and 500 μM, respectively, after 72 h of incubation. This increase appears to be dose-dependent, although significant differences were not detected between the effects observed at the dose of 100 and 250 μM. The tyrosinase activity increase was also significant after treatment of the human melanoma cells (Fig. 5B). After 72 h the increase was 1.13-, 1.20- and 1.23-fold for 100, 250 and 500 μM dose, respectively.

CREB phosphorylation and expression of Mitf in melanoma cells following 5,7-dimethoxycoumarin treatment. It has been demonstrated that the transcription of the gene encoding for the tyrosinase is activated by the microphthalmia-associated transcription factor (Mitf) which is a substrate for p-Erk 1/2 and is involved in differentiation, proliferation and survival of melanocytes (26,27). Because, during melanogenesis, the Mitf activity is regulated by the activation of protein kinase A (PKA), through phosphorylation of the transcriptional factor CREB (cAMP-response element-binding protein) (26). CREB phosphorylation, and Mitf expression were investigated in melanoma cells following treatment with 5,7-dimethoxycoumarin.

Western blot analysis of B16 cell lysates detected an increase of CREB phosphorylation in the melanoma cells incubated with 5,7-dimethoxycoumarin for 24 h (Fig. 6A). This increase was 1.4- and 2.3-fold in respect to the control at the dose 250 and 500 μM, respectively. No phosphorylation modification was detected when using lower doses or following treatment for 72 h (data not shown). Moreover, an increase of 3.0- and 2.1-fold of Mitf expression was detected in B16 cells in respect to the control when using the 250 and 500 μM doses, after 72 h of incubation (Fig. 6A). Conversely, the expression level of this protein remained unchanged after lower dose treatment. Of note, in A375 cell line, Mitf expression remained almost unchanged through extensive incubation with the compound (Fig. 6B). CREB phosphorylation increased in moderate manner also in A375 treated cells respect to methanol control after 24-h treatment, as shown in Fig. 6B. These results are consistent with activation of melanogenesis process caused in melanoma cells by 5,7-dimethoxycoumarin, probably through induction of tyrosinase activity.

Expression of porphobilinogen deaminase (PBG-D) in melanoma cells after treatment with 5,7-dimethoxycoumarin. We have previously demonstrated that melanoma cells treated with 5,7-dimethoxycoumarin, react with the production of protoporphyrine IX (PpIX), a compound synthesized during heme biosynthesis and considered a differentiation marker for several cancer cell lines including B16 cells (19). Treated melanoma cells emitted higher PpIX fluorescence than untreated cells, owing intracellular accumulation of PpIX (11). In this study, the expression of porphobilinogen deaminase (PBG-D), a key enzyme in heme synthesis that catalyses the combination of four molecules of PBG-D into hydroxy-

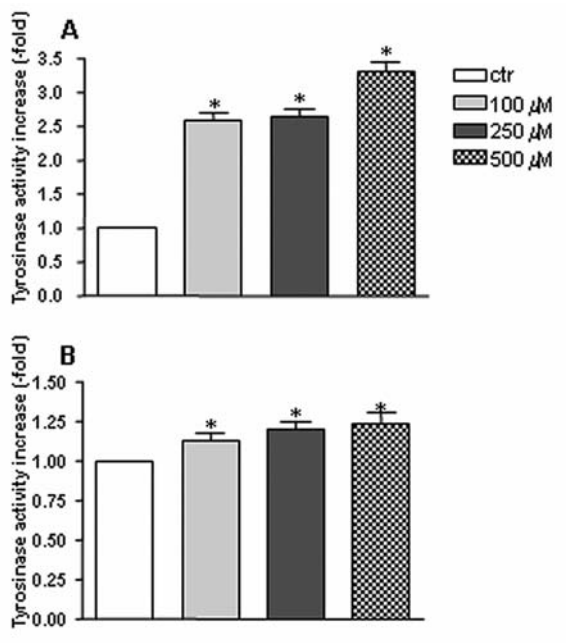

Figure 5. The effect of 5,7-dimethoxycoumarin on tyrosinase activity in B16 (A) and A375 (B) melanoma cells following treatment 72 h *in vitro*. Cells were incubated with or without the compound at 100, 250 and 500 μM dose. The tyrosinase activity was estimated as described in Materials and methods. Results are the mean ± SD from three separate experiments. *P<0.05 versus vehicle control treated cells.

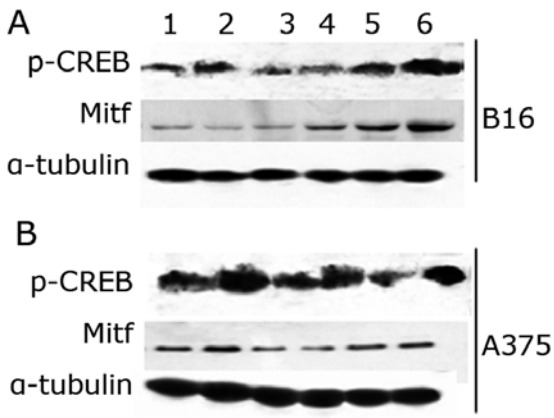

Figure 6. The effect of 5,7-dimethoxycoumarin on CREB phosphorylation and Mitf expression in B16 (A) and A375 (B) melanoma cells following 24 h (for p-CREB) and 72 h (for Mitf) treatment. Cells were incubated with the compound using 100, 250 and 500 μM doses: lanes 2 (100 μM), 4 (250 μM) and 6 (500 μM); or with respective vehicle solvent: lanes 1, 3 and 5. After treatment, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting as described in Materials and methods. Expression levels of p-CREB and Mitf were analysed with corresponding phospho-specific antibodies.

methylbilane, which is turned into uroporphyrinogen III was investigated. As shown in Fig. 7, Western blot analysis revealed a dose-dependent increase in total PBG-D protein both in B16 and A375 melanoma cell lines after a 72 h of 5,7-dimethoxycoumarin treatment. In B16 cells, the increase of enzyme expression was estimated to be 8.0- and 2.8-fold higher with respect to the control for 100 and 500 μM doses.

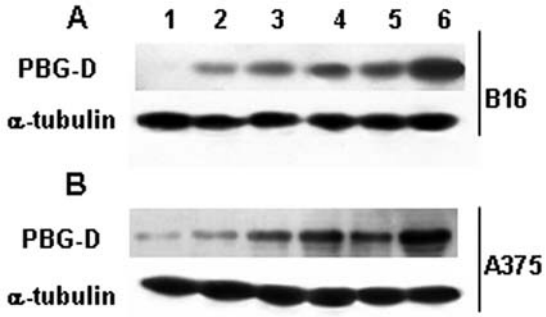

Figure 7. The effect of 5,7-dimethoxycoumarin on PBG-D expression in B16 (A) and A375 (B) melanoma cells following treatment 72 h. Cells were incubated with the compound using 100, 250 and 500 μM doses: lanes 2 (100 μM), 4 (250 μM) and 6 (500 μM); or with respective vehicle solvent: lanes 1, 3 and 5. After treatment, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting as described in Materials and methods. Expression level of PBG-D and was analysed with corresponding phospho-specific antibody.

Conversely, no significant difference was observed at the 250 μM dose; in the case of A375 cells, it was 1.9-, 1.5- and 2.1-fold for 100, 250 and 500 μM dose, respectively.

Effect of Mek 1/2 inhibitor on 5,7-dimethoxycoumarin activity in B16 cell line. Our finding that 5,7-dimethoxycoumarin treatment induces Mek 1/2 phosphorylation, followed by the simultaneous decline of Erk 1/2 phosphorylation, prompted us to investigate the use of the compound in combination with the Mek 1/2 inhibitor U0126. After 72 h treatment, the U0126 reduced cell growth with a percentage of 8, 29 and 43% for 1, 5 and 10 μM dose, respectively (Fig. 8A). When used in combination with 5,7-dimethoxycoumarin, the inhibitor increased growth inhibition induced by 5,7-dimethoxycoumarin (Fig. 8B-D). When the 5,7-dimethoxycoumarin 100 μM dose was combined with increasing amounts of U0126, cell viability was reduced in respect to the control from 52.1 to 45.4 and to 22.3%; for the 250 μM dose, from 16.2% to 10.8 and to 4.1% and for the 500 μM dose, from 11% to 5.1 and to 2.7%. These result suggests that Mek 1/2 inhibition can potentiate the antiproliferative effect of 5,7-dimethoxycoumarin on B16 melanoma cells in a synergic or additive mode. In the case of 100 μM dose, U0126 showed an additive effect for the concentration 1 and 5 μM and a synergic effect for higher concentration; while, when we treated B16 cells with 5,7-dimethoxycoumarin at 250 and 500 μM doses, the effect of Mek 1/2 inhibitor was additive only for its lower dose and it became synergic for the ather two doses.

Discussion

We have previously demonstrated the anti-proliferation activity of 5,7-dimethoxycoumarin on murine (B16) and human (A375) melanoma cells (11). Indeed, the compound significantly reduced cellular proliferation in a time- and dose-dependent manner, blocking the cell cycle in G_0/G_1 phase. Morphological changes with dendrite outgrowth and melanogenesis were observed following treatment, suggesting that 5,7-dimethoxycoumarin induced a terminal

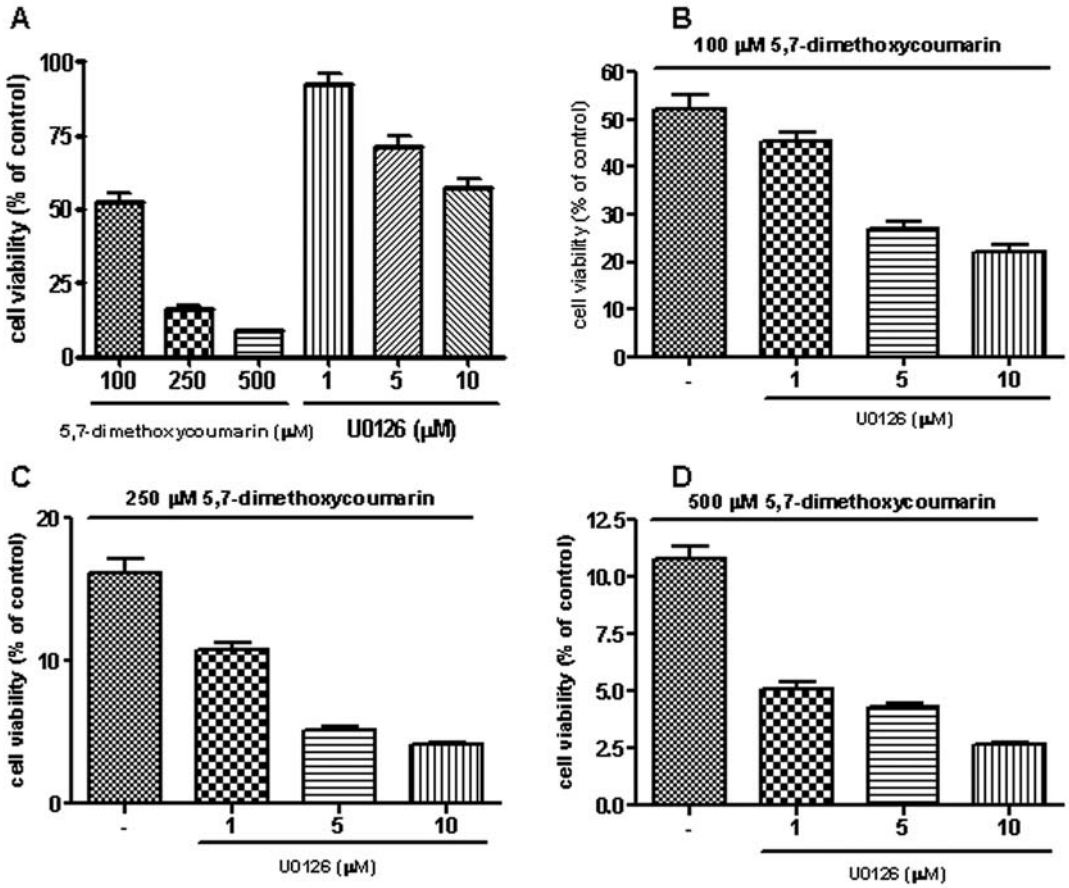

Figure 8. The effect of Mek 1/2 inhibitor U0126 on 5,7-dimethoxycoumarin activity in murine melanoma cell line. B16 cells were incubated with the compound using 100, 250 and 500 μM doses or with U0126 using 1, 5 and 10 μM doses (A); and in combination treatment with 5,7-dimethoxycoumarin 100 (B), 250 (C) and 500 μM (D) and U0126 (1, 5 and 10 μM). After 72-h incubation, trypan-blue exclusion test was carried out. Data are expressed as % of cell viability respect to vehicle control as described in Materials and methods. Results are the mean ± SD from three separate experiments. *P<0.05 versus vehicle control treated cells.

differentiation in melanoma cells after blocking cellular cycle in G_0/G_1 phase. These events were associated with a strong reduction of the extracellular signal-related kinase 1/2 (Erk 1/2) phosphorylation in the murine cells. In this study we extended this observation to the human melanoma cell line. Our results indicate that Erk phosphorylation is inhibited in the human melanoma cell line to a lesser extent than the murine; and this different behaviour could partially explain the differences in values of IC_{50}, i.e., 109 μM for B16 and 142 μM for A375 cells (11). It is known that Erk 1/2, member of Ras/Raf/Mek/Erk mitogen-activated protein kinase (MAPK) signalling pathway, regulates cell survival, proliferation and differentiation processes following transmission of a wide variety of extracellular stimuli from the cell surface to the nucleus initiating transcription of specific genes promoting cell cycle progression through G_0/G_1 phase and from G_0/G_1 to S phase (28). Since Mek 1/2 is a key regulator in the MAPK pathway (29) and phosphorylates Erk 1/2, its phosphorylation form (p-Mek) was examined. Surprising, the treatment with 5,7-dimethoxycoumarin was shown to increase p-Mek in a moderate manner both in B16 and A375 cells. The increase in Mek phosphorylation, followed by Erk disactivation, promped us to analyze the kinase activity of p-Mek following treatment. Our results of immunoprecipitation following kinase assay of Mek 1/2, by using Erk as substrate, demonstrated for the first time that the 5,7-dimethoxycoumarin inhibited enzymatic activity of the kinase derived from melanoma treated cells respect to not treated cells, triggering a significant reduction of Erk phosphorylation.

Up to now, it has been reported that some compounds, in the class of coumarins, inhibit the activation of non-phosphorylated Mek 1 binding to an allosteric site in the inactive conformation of this kinase; but they can not inhibit the activity of the activated Mek 1 unlike our tested compound (30). According to the literature, equol, a natural compound of the flavonoid class, greatly inhibited Mek 1 kinase activity in mouse epidermal cells, binding directly to the kinase; moreover, also this compound suppressed phosphorylation of Erk, but did not show effect on phosphorylation of Mek (31).

It is known that cyclin-dependent protein kinase 5 (cdk5), involved in neuronal development and neurite outgrowth, can down-regulate the Map kinase pathway by inhibiting Mek 1 activity: cdk5 targeted specifically the Raf-phosphorylated Mek 1 or a constitutively active form of Mek 1 through phosphorylation of a threonine residue (Thr^{286}), triggering to inactivation of enzymatic activity (29). We can presume

either a direct or an indirect modulation of Mek 1/2 by 5,7-dimethoxycoumarin; the compound could bind to one site of the activated kinase Mek 1 and inhibit its enzymatic activity; alternatively it could up-regulate other kinases, such as cdk5, that in turn will phosphorylate and inactivate Mek 1/2 in irreversible manner. Previously, we determined that 5,7-dimethoxycoumarin induced a terminal differentiation in B16 cells, characterized by morphological changes and melanogenesis process. A similar change in cellular shape, although less marked, was observed also in treated A375 cell line, and in this study we demonstrated a dose-dependent increase of melanogenesis process also in the human melanoma cells. It is known that 60% of melanoma cells carry an activating mutation in the catalytic domain of the serine-threonine kinase B-Raf, as a consequence of a substitution of a glutamate for valine at position 600 (V600E); this mutation triggers a constitutive activation of Mek and Erk with a subsequent induction of cell proliferation and a strong reduction of dendricity and pigmentation (27). Our results suggested for the first time that 5,7-dimethoxycoumarin, by targeting activated Mek 1/2 in melanoma cells and reducing its kinase activity, led to the development of dendrite-like projections and induced cellular differentiation with activation of melanogenesis, following cell cycle block.

Next, we tried to determine whether 5,7-dimethoxycoumarin induced melanin synthesis in melanoma cells through modulation of cAMP pathway which plays a key role in the regulation of melanogenesis stimulated by some agents, including α-melanocyte (α-MSH) stimulating hormone and forskolin (32). Infact, cAMP activates protein kinase A (PKA), that in turn translocates to the nucleus and phosphorylates cAMP-response element-binding proteins (CREB) that finally upregulates transcription of specific genes (16). Among the others, there is the gene encoding for microphthalmia-associated transcription factor (Mitf), which is involved in differentiation, proliferation and survival of melanocyte (14,15). It promotes the transcription of the gene encoding for tyrosinase, which catalyses two key steps in the pathway of melanin synthesis (33); for this reason, it is known that Mitf is required for the cAMP-induced melanogenesis (26). We observed, by using Western blot analyses, that in B16 cells the treatment enhanced CREB phosphorylation associated to an augmentation of Mitf expression; furthermore, a subsequent stimulation of tyrosinase activity, which finally led to increase of melanin synthesis. Since p-Erk 1/2 down-regulates Mitf by phosphorylation at serine 73 and its subsequent degradation (34), it can be hypothesized that in treated B16 cells, Mitf expression is up-regulated, in addition to transcriptional factor CREB, also through inhibition of Erk1/2 phosphorylation induced by the tested compound. Furthermore, it is known that Mitf can control cell cycle progression in melanoblasts and melanoma cells, since it induces a G_0/G_1 cell-cycle arrest through upregulation of $p21^{Cip1}$ (cyclin-dependent kinase inhibitor gene) expression and this will contribute to activation of the melanogenesis and the differentiation program (35). Previous studies showed that ^{V600}EB-Raf triggers Mitf degradation in mouse and human melanocytes and that its re-expression suppresses melanoma cell proliferation (27). Considering this evidence, we demonstrated that the treatment of B16 with 5,7-dimethoxycoumarin inhibited Mek 1/2 kinase activity and consequent Erk phosphorylation, which is fundamental for transcription of the genes encoding for proteins involved in cellular proliferation; at the same time, 5,7-dimethoxycoumarin increased transcriptional activity of Mitf, reducing its phosphorylation raised by p-Erk and promoting CREB phosphorylation. Then, it might be possible that Mitf promoted cell cycle arrest in G_0/G_1 and differentiation, probably through p21 expression, and melanin synthesis following the increase of tyrosinase gene transcription. We suggest that the moderate augmentation in Mek 1 phosphorylation, observed both in B16 and A375 cells, could be explained with a parallel increase in cAMP pathway activation; the cAMP and Ras-Raf-Mek-Erk signalling pathways interact through cross-talk such as the PKA-induced activation of Erk 1/2 interceded through B-Raf (36,37).

Also in human A375 cells, an inhibition of Mek 1/2 kinase activity and p-Erk 1/2 expression was detected following treatment with 5,7-dimethoxycoumarin as described above. However, although the compound stimulated CREB phosphorylation and the melanogenesis process, considerable increases in Mitf expression and tyrosinase activity were not observed in A375 cells. We could partially justify this discrepancy with the assumption that the regulation of Mitf expression and activity is controlled by several agents, both at the transcriptional and post-translational level. For example, GSK3ß can phosphorylate MITF on serine 298, enhancing the binding activity of MITF to the tyrosinase promoter, whereas MAPK and Rsk-1 phosphorylates it at serine 73 and 409, respectively, and promote its activation followed by its degradation (38). Furthermore, MITF may be a direct target of the p38 MAPK signaling pathway that is necessary, in example, for osteoclast differentiation and lipopolysaccharide (LPS)-induced melanogenesis (14,39). Then, 5,7-dimethoxycoumarin could stimulate melanogenesis process in A375 cells through regulation of Mitf similarly to B16 cells, although significant alterations in its expression were not detected. The several phosphorylation states of the transcriptional factor need investigating, since probably in this cell line the treatment controls it in post-translational level.

Furthermore, during the experimental procedure it was evident that cell pellet of A375 was much clear than that of B16 (data not shown); this characteristic was observed in both untreated and treated A375 cells, although the latter appeared to be slightly darker. According to the literature, mammalian melanocytes produce two types of melanin pigments, the black eumelanin and the yellow pheomelanin, and their common precursor is dopaquinone, synthesized from tyrosine through activity of tyrosinase (40). Basal levels of tyrosinase expression lead to pheomelanin synthesis, while elevated levels of tyrosinase switch the pathway to production of eumelanin; indeed, some biochemical studies demonstrated that the first step of pheomelanogenesis, leading to the production of cysteinyldopa by the addition of cysteine to dopaquinone, is preferred to that of eumelanogenesis (33). The switch between pheomelanogenesis and eumelanogenesis is regulated by α-melanocyte stimulating hormone (α-MSH), which activates eumelanogenesis through cAMP pathway, and agouti signal protein (ASP), that on the contrary down-

regulates it, thus triggering instability of melanogenic protein, such as tyrosinase (41). Based on this, we could hypothesized that in A375 the proportion of pheomelanin to eumelanin is higher compared to B16 cells, before and also following treatment with 5,7-dimethoxycoumarin, because the compound did not stabilize enough the Mitf transcriptional factor and tyrosinase enzyme expression, acting through Erk inactivation and modulation of α-MSH and/or ASP signalling.

Treated melanoma cells, both B16 and A375, showed an increase in total PBG-D protein, the key enzyme involved in heme synthesis wherein PpIX (protoporphyrine IX) is synthesized and accumulated in mithocondria, as intermediate molecule. PpIX is considered a differentiation marker for some cancer cell lines including B16 cells, since the induction of differentiation enhanced accumulation of the compound (42-44), although the relationship between tumor cell differentiation and photosensitizer accumulation is poorly defined (45). Furthermore, it is known that PpIX induces melanogenesis through the cAMP-mediated pathway; it activates the soluble guanylate cyclase and elevates intra-cellular cGMP (cyclic guanine monophosphate) content which was demonstrated to increase cAMP content by inhibition of cAMP phosphodiesterase (20,21). We suggest that 5,7-dimethoxycoumarin treatment of melanoma cells stabilizes the cAMP signalling pathway following production of PpIX; then, CREB phosphorylation and Mitf expression increased, thus triggering to differentiation and melanogenesis process, in harness with Ras/Raf/Mek/Erk modulation.

In combination treatment of B16 melanoma cells, the Mek 1/2 inhibitor U0126 significantly potentiated growth inhibition induced by 5,7-dimethoxycoumarin, in synergic or additive mode; moreover, the development of dendrite-like projections was more evident respect to the treatment with the 5,7-dimethoxycoumarin alone (data not shown). It is known that Mek 1/2 inhibitors, such as U0126 or PD098059, cause inhibition of the cell growth, induce a differentiation process in melanoma cells and reduce tumor cell invasion; Mek 1 and Mek 2 are the most critical downstream mediators of Raf signalling and for this reason, they appear a valuable target of new drugs to block the Raf/Mek/Erk pathway in cancer (46). Our results indicate that down-regulation of the Ras/Raf/Mek/Erk pathway, through Mek 1/2 inhibitor, sensitizes melanoma cells to 5,7-dimethoxycoumarin treatment, suggesting a possible regimen for preclinical and clinical therapy; a combination therapy could allow to lower the compound dose at clinically achievable limit. The use of combinations of anticancer agents is in clinical trials and appears an effective strategy to strengthen their anticancer effect, since this could reduce the doses of each agent and thus their toxicity (47).

Overall, in this study, we clarified some molecular mechanisms involved in antineoplastic and differentiation program activated by 5,7-dimethoxycoumarin in melanoma cells. Regulation of switch in melanogenesis during treatment with compound should be further explained, to understand the discrepances between human and murine melanoma cells. It will be necessary to carry out *in vivo* studies in preclinical trials, on the combination treatment with Mek inhibitors, with the aim to analyse cancer antigrowth and antimetastatic properties of the compound in animal models.

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