The critical role of alpha-folate receptor in the resistance of melanoma to methotrexate

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Significance

Melanomas are intrinsically resistant to radiation therapy and to many chemotherapeutic drugs, including antifolates. Unraveling the mechanism of drug resistance in melanomas could help to improve current therapeutics approaches; however, the precise mechanism underlying these refractory properties are still unknown. In this work, we have identified the presence of FRα in normal and pathological melanocytes and demonstrated that MTX is preferentially transported through this receptor in melanoma cells. FRα-induced endocytic transport of MTX, together with drug melanosomal sequestration and cellular exportation, ensures reduced accumulation of this cytotoxic compound in intracellular compartments. The identification of this mechanism of resistance to antifolates in melanoma could be of importance for the development of alternative therapeutic strategies for the treatment of this skin pathology.

Summary

Although methotrexate is an effective drug for several types of cancer, it is not active against melanoma. Experiments following methotrexate treatment indicated a reduced accumulation of the drug in the cytosolic compartment in melanoma cells, suggesting that the mechanisms that control the transport and retention of this drug could be altered in melanoma. For this reason, we analyzed the presence and function of folate receptor-α (FRα) in melanoma cells. Here, we have identified the presence of FRα in normal and pathological melanocytes and demonstrated that MTX is preferentially transported through this receptor in melanoma cells. FRα-induced endocytic transport of MTX, together with drug melanosomal sequestration and cellular exportation, ensures reduced accumulation of this cytotoxic compound in intracellular compartments. The critical role of FRα in this mechanism of resistance and the therapeutic consequences of these findings are also discussed.

Introduction

Cancer remains a frequent cause of death despite the development of many standard and experimental treatments including surgery, chemotherapy, radiotherapy, biotherapy and immunotherapy. In particular, the incidence rates of melanoma have risen faster than that of any other malignancy in the Caucasian populations over the past 30 years (Giblin and Thomas, 2007). According to a World Health Organization estimate, there are 132,000 new cases of melanoma per year worldwide. Light skin, large numbers of nevi and excessive sun exposure, especially in childhood, are the major factors that contribute to melanoma risk (Houghon and Polsky, 2002). Once detected, primary melanomas should be surgically removed, whereas chemotherapy should focus on metastasis control. At present, limited therapeutic options exist for patients with metastatic melanomas, and all standard combinations currently used in metastasis therapy have low efficacy and poor response rates. Therefore, unraveling the mechanism of drug resistance in melanomas could help to improve current therapeutic approaches.

One complication involved in melanoma chemotherapy is the limited effectiveness of antifolates. Although methotrexate (MTX), the most frequently used antifolate, is an effective drug for several types of cancer, it is not active against melanoma (Kufe et al., 1980). The mechanisms of resistance to classical antifolates such as MTX have been extensively studied, mainly in experimental tumors propagated *in vitro* and *in vivo* (Assaraf, 2007; Gaukroger et al., 1983; Kufe et al., 1980; Zhao and Goldman, 2003). However, the specific basis for the resistance of melanoma cells to MTX is unclear, but it seems to be related to (*i*) reduced cellular uptake of this drug; (*ii*) insufficient rate of MTX polyglutamylation, which diminishes long-chain MTX polyglutamates from being preferentially retained intracellularly; and/or (*iii*) high intracellular levels of dihydrofolate reductase (DHFR). Recently, a melanoma-specific mechanism of resistance to cytotoxic drugs has been described (Chen et al., 2006). Melanosomal sequestration of these drugs would facilitate their cellular exportation and, therefore, contribute to the intractability of malignant melanomas.

Although the mechanisms of folate and antifolate transport have been extensively studied in a wide range of normal and cancer cells (Matherly and Goldman, 2003), the transport of these compounds in healthy and pathological skin cells is mostly uncharacterized. Folates and classical antifolates such as MTX are transported into cells via two separate mechanisms. One mechanism involves a transmembrane transporter known as the reduced folate carrier (RFC). This ubiquitously expressed protein uses a bidirectional anion-exchange mechanism to transport folates into the cytoplasm. It has a high affinity for 5-methyltetrahydrofolate (5-MTHF), the predominant folate found in serum, and a much lower affinity for folic acid (Doucette and Stevens, 2001). The other folate uptake system uses a glycosylphosphatidylinositol (GPI)-

anchored protein known as folate receptor-α (FRα), which transports folates into the cytosol via fluid-phase endocytosis (Kelemen, 2006; Matherly and Goldman, 2003). This receptor binds folic acid and 5-MTHF with very high affinity and its expression is limited to normal differentiated epithelial cells (Antony, 1996; Kelemen, 2006). FRα is also overexpressed in malignant tissues of epithelial origin (Parker et al., 2005; Toffoli et al., 1997; 1998) and, in some cell lines, is a necessary component of folate and MTX transport (Theti and Jackman, 2004). Although these studies suggest that the FRα may be expressed to sufficient levels in tumors, it is not known whether the FRα is functional (Jackman et al., 2004). In the present study, we have detected the presence of FRα in normal and pathological melanocytes and demonstrated that MTX is preferentially transported through this receptor in melanoma cells. FRα-induced endocytic transport of MTX, together with drug melanosomal sequestration, reduces the accumulation of this cytotoxic compound in intracellular compartments. The subsequent exportation of MTX-containing melanosomes impedes DHFR inhibition, which contributes to the resistance of melanomas to this classical antifolate. The importance of this mechanism of resistance in melanoma cells was confirmed by experiments designed to transport MTX into the cells by FRα-independent processes or by inhibiting melanosome secretion.

Results

MTX acts as a cytostatic agent on melanoma cells

When we compared the antiproliferative action of MTX on melanoma (SkMel-28) and colon (Caco-2) cell lines, we observed that high doses of this drug (20 μM) inhibited cellular growth (Figure 1A). However, the morphological aspects of these cells after 6 days of treatment, compared with untreated controls, revealed important and significant differences (Figure 1A). Although treating SkMel-28 with MTX considerably reduced the number of cells, the cells showed a healthy aspect similar to untreated control cells. The data indicated that this drug inhibited SkMel-28 proliferation but that these cells were essentially resistant to MTX-induced apoptosis (Figure 1B). In contrast, Caco-2 cells treated with MTX showed clear signs of apoptosis (Figure 1B). To confirm these results, a “growth recovery” experiment was performed. On the sixth day, untreated and MTX-treated cells were collected by trypsinization and seeded at the same number in drug-free medium. SkMel-28 cells that had been treated with MTX recovered their growth capacity, whereas Caco-2 cells treated with the same concentration of the drug were unable to proliferate (Figure 1C). These data indicated that MTX acted as a cytostatic drug on SkMel-28, suppressing cellular growth and multiplication, whereas it was a cytotoxic drug for Caco-2 cells. To confirm that this effect was not associated with a specific melanoma cell line, the specific cytostatic activity of MTX was also probed on different human (SkMel-1 and G361) and mouse (B16/F10) melanoma cell lines (data not shown).

Reduced accumulation of MTX in melanoma cells

To understand why melanoma and colon cancer cells respond differently to MTX, we searched for differences in the cellular accumulation of this drug. Accumulation of [^{3}H]-MTX in several melanoma cell lines was determined as described under "Materials and Methods" and compared with its accumulation in Caco-2 cells. Cells were incubated with 0.5 μCi of [^{3}H]-MTX at 37ºC for 12 h, after which the radioactivity retained in the cytosol was measured. The results showed a reduced accumulation of [^{3}H]-MTX in melanoma cells compared with Caco-2 cells (Figure 2A). Quantification experiments of MTX in cellular extracts of Caco-2 and SkMel-28 cells using LC/MS/MS showed similar results (120 ± 19 and 7.9 ± 1.2 pmol/10^7 cells, respectively; Figure 2B). The results indicated that the reduced accumulation of MTX in melanoma cells could be the reason for their high resistance to death in the presence of this cytotoxic drug and suggested that the mechanisms that control the transport and/or retention of this drug could be altered in melanoma.

FRα is expressed in melanocytes and melanoma cells

FRα expression is limited to normal differentiated epithelial cells (Antony, 1996; Weitman et al., 1992) and in many cases it is overexpressed in malignant tissues of epithelial origin (Kelemen, 2006; Parker et al., 2005; Toffoli et al., 1997; 1998). However, to our knowledge, the presence and possible function of this folate receptor in normal and pathological melanocytes have not been reported. To investigate whether FRα could be involved in the resistance of melanomas to MTX, we studied the status of FRα in HeM and melanoma cells. First, the expression levels of the FRα gene, *FOLR1*, were analyzed in these cells using real-time PCR. The results showed that *FOLR1* was expressed in non-pathological human melanocytes (HeM) and that its expression levels were significantly higher in the melanoma SkMel-28 cell line than in HeM (5.8-fold) (Figure 3A). To verify whether FRα mRNA expression correlated with the FRα protein levels, cell extracts were analyzed by western blot. Using this technique, FRα was detected in HeM and was also found overexpressed in SkMel-28 cells (Figure 3B). In both cell types, two immunoreactive FRα bands at 34 and 38 kDa were observed, and immunoprecipitation of cell extracts with anti-FRα, followed by mass spectroscopy of the tryptic digest products, confirmed the identity of this folate receptor (Figure 3B). The presence of FRα in other human and mouse melanoma cell lines and in primary and metastatic tumors from melanoma patients was also confirmed by western blot (Figure 3C). Indirect immunofluorescence localization of FRα also demonstrated that this receptor was expressed in melanocytes and melanoma cells (Figure 3D); however, the fluorescence distribution indicated that FRα localization differs in normal and pathological cells. Thus, the differential polarized distribution of this receptor in HeM was less evident in melanoma cells.

MTX treatment depletes FRα in melanoma cells by receptor melanosomal sequestration and exportation

To investigate the effect of MTX on FRα expression in melanoma, FRα levels of SkMel-28 cells exposed to this drug were analyzed by western blot. Analysis after 48 h of drug exposure revealed that FRα was not present in the cells (Figure 4A). The results acquired after different MTX treatment times indicated that there was a time-dependent decrease in FRα content; by 10 h of MTX exposure, this receptor was completely absent from the cells (Figure 4A). Real-time PCR experiments demonstrated that FRα mRNA expression in SkMel-28 cells was not affected by MTX (Figure 3A); therefore, the depletion of FRα in SkMel-28 cells after MTX treatment must be attributed to other factors, independent of *FOLR1* gene expression. Indirect immunofluorescence localization of FRα during MTX treatment indicated that this receptor was exported out of the cells, as demonstrated by the observation of fluorescent vesicles located at both the cellular membrane and the extracellular medium (Figure 4B). The data indicated that MTX could interfere with FRα-mediated endocytosis, disturbing the normal recycling of this membrane receptor.

Treatment of melanoma with several cytotoxic drugs, including cisplatin, altered melanogenesis and accelerated melanosome exportation (Chen et al., 2006). Here, we observed via sucrose density gradients and electron microscopy that MTX treatment resulted in a significant decrease in the SkMel-28 melanosome content (Figure 4C), which might be related to the cellular exportation of FRα. To verify this hypothesis, the FRα content of melanosome fractions, purified using sucrose density gradients, was evaluated by western blot at different times after MTX exposure. As observed in Figure 4D, MTX treatment mislocalized FRα to the melanosome fraction after short exposure times and the maximum FRα content in this fraction was detected after 3 h of MTX exposure. After longer MTX treatments, FRα was mainly localized in the extracellular medium and was associated with secreted melanosomes (Figure 4D). Together, the results indicated that this receptor may be involved in the transport of (anti)folates in these cells and suggested that FRα modulation may be relevant to MTX cytotoxicity or resistance in melanoma cells.

Melanosomal sequestration and cellular exportation of MTX

Recently, it has been demonstrated that one potential mechanism of multidrug resistance in melanomas is intracellular melanosomal drug trapping and active melanosome-mediated drug export (Chen et al., 2006). To test whether this mechanism is involved in the export of MTX from melanoma cells, we treated SkMel-28 cells with MTX-FITC and then analyzed its subcellular localization. MTX-FITC was efficiently transported into melanoma cells, and the fluorescence was localized to the perinuclear regions of the cells after 1 h (Figure 5A). After 4

h, fluorescence was mainly observed in the cell membrane in fluorescent vesicles, indicating that MTX was being exported out of the cell (Figure 5A). To explore the relationship between MTX export and melanosome trafficking, melanosomes were purified from MTX-FITC-treated cells. After a short exposure to MTX-FITC, we found that fluorescence predominantly accumulated in the melanosome fraction (Figure 5B). Disrupting this fraction in an acidic environment to facilitate MTX dissociation from folate carrier proteins, followed by ultrafiltration through a 3-kDa molecular weight cut-off membrane, demonstrated that MTX interacted with some component of the melanosomes, presumably melanin (Latocha et al., 2000). Although MTX-FITC freely crossed this ultrafiltration membrane, fluorescence associated with melanosomes was highly retained in the membrane filter (Figure 5C). The interaction of MTX with melanin was confirmed by incubating this drug with synthetic DOPA-melanin (Figure 5D). Importantly, folic acid and 5-MTHF, the natural source of cellular folates, did not appear to interact with synthetic DOPA-melanin (Figure 5D). A comparison of the interaction of several folates (folic acid and 5-MTHF) and antifolates (MTX and aminopterin) with synthetic DOPA-melanin (Figure 5D) indicated that the double amino group of the pterin ring is an important molecular requirement for the drug-melanin interaction.

FRα-endocytic transport and melanosomal sequestration of MTX is an active mechanism of resistance of melanoma to this classical antifolate

Although the mechanism by which cytotoxic drugs are sequestered into melanosomes remains unclear, we demonstrated that MTX-melanosome trapping may be a consequence of its FRα-endosomal transport. To test the importance of this process on the resistance of melanoma to antifolates, we carried out two sets of experiments. First, the drug was directly delivered to the cytosol of melanoma cells, thus avoiding MTX receptor-mediated transport. We observed that INTERFERin^{TM} liposomes, which are commonly used for siRNA transfection, could also encapsulate MTX (Figure 6A). Treating SkMel-28 with MTX-loaded liposomes resulted in drug liberation into the cytoplasm (Figure 6A) and, consequently, MTX-induced apoptosis (Figure 6A). Second, we designed a strategy to avoid melanosome exportation. In cultured melanoma cells, melanin secretion depends on the presence of serum in the growth medium because serum contains specific factors capable of inducing melanin secretion (Laskin et al., 1982). To avoid melanosome/melanin secretion in B16/F10 and Skmel-28 melanoma cells, we cultured them in a medium containing 1% charcoal-stripped FBS. Although the inhibition of melanin secretion was more evident in the highly melanotic B16/F10 cell line (containing stage III and IV melanosomes) than in amelanotic SkMel-28 (containing stage I and II melanosomes) (Figure 6B), both cell lines were more sensitive to MTX treatments when grown in serum-free medium. Concentrations of MTX that only induced cell growth inhibition in a normal medium killed the cells via apoptosis in the absence of serum (Figure 6B). We interpret these results to

indicate that FRα-endosomal transport of MTX and its subsequent melanosomal sequestration and export are critical mechanisms of melanoma resistance to this classical antifolate.

Discussion

Here, we have analyzed and identified several possible cellular mechanisms that explain the strong resistance of melanoma to MTX. A global mechanism for MTX resistance is summarized as follows. After MTX exposure, this drug is transported into melanoma cells through FRα, where both are trapped into melanosomes and then exported out of the cells. This process reduces the accumulation of cytotoxic MTX in the cytosol of melanoma cells and impedes FRα recycling to the cell membrane, which makes the melanoma impermeable to this drug and to other folate coenzymes. The depletion of intracellular folate coenzymes decreases cell proliferation; cells enter a latent state in which they stop dividing but maintain their basic functions, thus avoiding some apoptotic processes. Although the uptake of hydrophilic antifolates like MTX and reduced folates, including 5-MTHF, into mammalian cells is mediated by the RFC, the expression of FRα in several epithelial tissues and especially its overexpression in cancerous cells indicate that this receptor may confer a growth advantage to these cells (Antony, 1996). The different affinities for folic acid and 5-MTHF between FRα and RFC (~ 10^5-fold for folic acid and 100-fold for 5-MTHF) suggest that the GPI-anchored receptor may play an important role in transporting folates when extracellular concentrations are in the nanomolar range (Doucette and Stevens, 2001). This hypothesis is supported by the finding that induction of FRα expression in cells that normally do not express this receptor allows the cells to grow in low nanomolar folate concentrations (Luhrs et al., 1992). Because cancerous cells require a good supply of this vitamin, it is reasonable to suggest that these cells would drain surrounding folates; therefore, we may speculate that the presence of FRα may be an advantage for the growth of cancer cells. This advantage could explain the commonly observed overexpression of FRα in tumor cells (Antony, 1996; Kelemen, 2006; Parker et al., 2005; Toffoli et al., 1997; 1998). In this report, we observed that FRα is a critical element for MTX transport in melanoma cells. The results that demonstrated that FRα is exported from and depleted in these cancer cells during MTX treatment indicate that a transport-mediated resistance mechanism could be indirectly operating under these conditions. As observed in other human cancer cells, a decrease in FRα content has been identified as a mechanism of resistance to MTX (Saikawa et al., 1993).

An important observation in this study was that MTX was a cytostatic agent on melanoma cells. These cells were resistant to MTX-induced apoptosis but responded to the drug by arresting their growth. A similar response was observed when the murine B16/F10 melanoma cell line was grown in low folate. After 3 days in folate-deficient medium the cells had

restricted proliferative activity and also increased their metastatic potential (Branda et al., 1988). Taking this into consideration, the results indicate that MTX might also induce depletion of intracellular reduced folate coenzymes by reducing their transport though the FRα and/or competing with them for the RFC. Melanoma cells may be highly sensitive to intracellular depletion of folate coenzymes, and in this situation may enter into a "latent" state. This form of melanoma should indeed be highly resistant to MTX, since antifolate drugs are more effective on fast-dividing cells, which require continuous DNA synthesis. Most likely, the high increases of DHFR expression in cells treated with MTX[3] would represent an adaptation mechanism that allows cells to survive with low intracellular concentrations of folate coenzymes. Increasing the recycling of folate molecules the cells would maintain other cellular functions that are dependent on folate coenzymes, such as the synthesis of purines, pyrimidines, amino acids and methylation reactions. The presence of this "latent" form of melanoma should be critical for the resistance to MTX during *in vivo* therapies. Although MTX chemotherapy could initially halt the development of the tumor, after clearance of the drug from the body the melanoma cells may reinitiate their progression, possibly with an increased metastatic potential (Branda et al., 1988).

A defect in intracellular folate retention is another recognized mechanism of drug resistance (Assaraf, 2007; Gaukroger et al., 1983; Kufe et al., 1980; Zhao and Goldman, 2003). In addition to a decrease in antifolate polyglutamylation, melanoma cells may also export cytotoxic drugs by melanosome sequestration (Chen et al., 2006). The results presented here indicate that export is an operative mechanism of resistance to MTX in melanoma cells. Although an increase in melanogenesis is a general stress-response mechanism in melanoma cells, the processes by which cytotoxic drugs are trapped in melanosomes have not been delineated. Here, we demonstrate that the trapping of MTX in melanosomes occurs as a consequence of its endocytic trafficking through FRα. This observation strongly supports the hypothesis which indicates that melanosome biogenesis is a specialization of the endocytic pathway (Raposo and Marks, 2002; 2007); however, the exact mechanism by which MTX induces abnormal trafficking of early endosomes in melanoma cells, favoring the exportation of melanosomes, is still unclear. Whether MTX blocks the formation of carrier vesicles operating between early and late endosomes, inhibits the delivery of endocytosed material from endosomes to lysosomes, promoting, thus, the generation of exosomes (Raposo and Marks, 2007) and/or induces a failure of lysosomal acidification, which is essential for normal endocytosis (Liang et al., 2003), remains to be determined. The physiological importance of the high affinity of melanin for antifolates, such as MTX and aminopterin, for drug melanosomal sequestration is also another important issue that remains to be addressed. Endocytic transport of molecules involves several processes, including the fusion of early and late endosomes and

the dissociation of receptor-ligand complexes through the acidic pH of preformed vesicles (Sabharanjak and Mayor, 2004). After melanosome biogenesis from MTX-loaded endosomes, dissociated MTX could be trapped in the melanosomes by its interaction with melanins. In contrast, folate substrates would not be sequestered in melanosomes due to their low affinities for melanin; facilitated by the acidic pH of this organelle, uncharged reduced folates would leave the melanosome by passive diffusion and reach the cytosol, where they would become available for cellular functions. Therefore, elucidation of the molecular basis for the (anti)folate interaction with melanins could have important therapeutic implications, and this study might be used as a guide for the synthesis of new antifolates or for using existing antifolates in ways that escape melanin trapping.

Other key questions that remain are the involvement of FRα in the transport of folates in normal melanocytes and the differences (if any) between normal and pathological melanocytes with respect to the mechanism of FRα endocytosis. Although we observed that FRα was overexpressed and differently localized in melanoma cells in comparison with normal melanocytes, the physiological importance of these findings should be further investigate. Nevertheless, comparison of trafficking pathways between normal and malignant cells will provide insights into the delivery of (anti)folates into cells, and may help in understanding the functional role of FRα in the mammalian epidermal system. Meanwhile, we conclude in this report that FRα has an important role in the resistance of melanoma to MTX. FRα-induced endocytic transport of MTX, together with its melanosomal sequestration and cellular export, ensures reduced accumulation of this cytotoxic drug in intracellular compartments. The results also indicated that these events might constitute a general resistance mechanism for melanomas. Primary melanoma tumors are highly melanotic, but pigmentation loss is very common in advanced and metastatic lesions (Ackermann et al., 2005; Potterf et al., 1996). In addition, a change in cell mobility is also one of the preconditions of tumor metastasis (Ackermann et al., 2005). Here, we observed that the mechanisms that result in high resistance to MTX were similar in several melanoma cell lines with different origins (human or mouse) and different characteristics (melanotic/amelanotic or adherent/suspension). Therefore, the information presented here may direct the development of alternative therapeutic strategies for the treatment of melanoma. Strategies to direct delivery of MTX into the cytosol of melanoma cells and/or to inhibit either melanogenesis or the export of melanosomes in melanoma cells represent attractive approaches to dramatically increase cytosolic trapping of MTX, thus enhancing its cytotoxicity.

Materials and methods

Cell lines, proliferation and apoptosis assays

Melanoma cell lines from human (SkMel-1, SkMel-28 and G361) and mouse (B16/F10) origin and the colon adenocarcinoma cell line, Caco-2, were obtained from the American Type Tissue Culture Collection (ATCC) and maintained in the appropriate culture medium supplemented with 10% FBS and antibiotics. Experiments that required growing cells in low-serum medium were carried out in the appropriate culture medium supplemented with 1% charcoal-stripped FBS (Invitrogen, Barcelona, Spain). Human epidermal melanocytes (HeM) were supplied by Gentaur (Brussels, Belgium) and cultured in Ham's F10 medium supplemented with 10% FBS, antibiotics and human melanocyte growth supplement (Gentaur). Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Madrid, Spain) and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega Biotech Iberica, Barcelona, Spain) cell viability assays for adherent (Caco-2, HeM, SkMel-28, G361 and B16/F10) and suspended (SkMel-1) cells, respectively. Apoptosis induction was assessed by analysis of cytoplasmic histone-associated DNA fragmentation using a kit from Roche Diagnostics (Barcelona, Spain). Apoptosis was defined as the specific enrichment of mono- and oligonucleosomes in the cytoplasm and was calculated by dividing the absorbance of treated samples by the absorbance of untreated controls after correcting for the number of cells.

Patients

Melanoma specimens corresponding to primary and metastatic lesions were obtained from Cambridge Bioscience Ltd. (Cambridge, UK). This company supply high quality and ethically sourced biospecimens which have been sourced with full donor consent from sites exclusively in Europe and the USA, following all regulations relevant to human tissue collection. A primary tumor was surgically extracted from the back of a 69-year-old female patient. Metastatic melanoma in the left lateral thigh muscle was surgically removed from a 70-year-old male patient. The presence of neoplastic cells in tumors was assessed by standard pathological techniques.

Preparation of melanoma cell granular fractions, sucrose-purified cellular melanosomes and secreted melanosomes

Control untreated SkMel-28 cells and those subjected to treatments with MTX (Sigma) were harvested with a mixture of 0.25% trypsin and 0.25 mM EDTA and washed once in 0.25 M sucrose with centrifugation at 1,000 ×*g* for 10 min at 4ºC. Specimens were then homogenized on ice using 20 strokes of a Potter homogenizer and centrifuged at 1,000 ×*g* for 10 min at 4ºC. The supernatant was recovered and further centrifuged at 19,000 ×*g* for 30 min at 4ºC (Potterf et

al., 1996). The pellet containing melanosome-enriched granular fractions was resuspended in ice-cold 0.25 M sucrose in 10 mM HEPES (pH 7.0) and examined by electron or confocal microscopy. Highly purified melanosomes were obtained as described previously (Watabe et al., 2004). Briefly, purified SkMel-28 granular fractions were resuspended in 2.0 M sucrose and layered at the bottom of a 1.0-2.0 M sucrose step (1.0, 1.2, 1.4, 1.5, 1.6, 1.8 and 2.0 M) gradient. The gradient was centrifuged at 100,000 ×g in a Beckman SW 41 swinging-bucket rotor for 1 h at 4ºC and the various layers of the fraction were carefully recovered. Samples layering in the 1.0-1.2 M interface of sucrose gradients were analyzed by western blot using FRα antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To isolate the secreted melanosomes, the cell culture medium was collected in the presence of 1 mM PMSF and protease inhibitor cocktail and centrifuged at 503 ×g to remove cell debris (Chen et al., 2006). The supernatant was collected and ultracentrifuged at 157,800 ×g for 1 h at 4ºC. The melanosome-containing pellets were then resuspended in PBS and analyzed by western blot using FRα antibodies.

Western blot

For whole-cell extract preparation, cells were washed twice with PBS, scraped and centrifuged (300 ×*g*, 5 min). The supernatant was then removed, and the pellet was resuspended in denaturation buffer [10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.4, 0.2% SDS (w/v), and protease inhibitor cocktail]. Samples were denatured at 100ºC for 5 min and centrifuged (10,000 ×*g*, 1 min) to remove insoluble material. The total protein concentration was determined and proteins (50 μg/well) were resolved on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and analyzed by immunoblotting (ECL Plus, GE Healthcare, Barcelona, Spain) using FRα and β-actin antibodies. Melanoma tissues were subjected to the same extraction procedures but homogenized using polytron and Potter homogenizers. In both cases, the relative FRα content was calculated with respect to the amount of β-actin. Cellular and extracellular melanosome fractions were mixed with 2× SDS electrophoresis sample buffer and proteins (5 μg of protein/well) were electrophoretically separated and analyzed as described above.

FRα trypsin digestion and HPLC/MS analysis

SkMel-28 membrane proteins were solubilized as described elsewhere (Kim et al., 2002) and the soluble fractions were immunoprecipitated with anti-FRα and protein G-agarose (Sigma). Bound proteins were electrophoresed on an SDS-PAGE gel, which was then stained with ProteoSilver Plus® (Sigma). Selected bands were excised and destained using the same kit, and the samples were digested with trypsin using standard procedures (Shevchenko et al., 1996). Separation and analysis of the tryptic digests were performed with an HPLC/MS system consisting of an Agilent 1100 Series HPLC connected to an Agilent Ion Trap XCT Plus MS

using an electrospray interface. Dry samples from in-gel digestion were resuspended in 10 μL water/acetonitrile/formic acid (94.9:5:0.1) and injected onto a thermostatted Zorbax SB-C18 HPLC-column (5 μm, 150 × 0.5 mm) at a flow rate of 10 μL/min at 40°C. The digested peptides were eluted using a linear 0-80% gradient of water/acetonitrile/formic acid (10:89.9:0.1) for 120 min. The MS was operated in the positive mode with a capillary spray voltage of 3,500 V and a scan speed of 8,100 (m/z)/s from 300-2,200 m/z. The nebulizer gas pressure was set to 15 psi, and the drying gas flow was set to 5 L/min at 350°C. MS/MS data were collected in an automated data-dependent mode. The most intense ions were sequentially fragmented using helium collision-induced dissociation with an isolation width of 2 and relative collision energy of 35%. Data processing was performed with the DataAnalysis program for LC/MSD Trap v.3.2 (Bruker, Madrid, Spain) and Spectrum Mill MS Proteomics Workbench (Agilent Tech., Santa Clara, CA, USA).

Quantitative real-time PCR

PolyA$^+$ mRNA was extracted from 5×10^6 cells using the Illustra Quick Prep Micro mRNA purification kit (GE Healthcare, Barcelona, Spain). Prior to cDNA synthesis, samples were digested with DNase I to avoid genomic DNA contamination. mRNA (200 ng) was used to synthesize cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR was performed as described previously (Sánchez-del-Campo and Rodríguez-López, 2008). Primers included FRα (forward: 5'-GTC GAC CCT GGA GGA AGA AT-3'; reverse: 5'-GCC ATC TCT CCA CAG TGG TT-3') and β-actin (forward: 5'-AGA AAA TCT GGC ACC ACA CC-3'; reverse: 5'-GGG GTG TTG AAG GTC TCA AA-3').

Microscopy

Electron microscopy was performed as described previously ((Watabe et al., 2004) using a Zeiss EM10 electron microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Confocal microscopy was carried out using a Leica TCS 4D confocal microscope (Wetzlar, Germany). Indirect immunofluorescence localization of FRα was performed as described elsewhere (Doucette and Stevens, 2004) using primary FRα antibodies and Alexa Fluor Dyes (Invitrogen, Barcelona, Spain) as secondary antibodies.

Measurement of [^{3}H]-MTX uptake

Cells were seeded 48 h before assay at 1×10^5 cells/mL of medium per well in each well of a 24-well culture dish. [3',5',7-^{3}H]-MTX (0.5 μCi; GE Healthcare) was added in 0.3 mL of medium per well. Cells were immediately incubated at 37ºC for 12 h. At the end of the incubation, melanoma cells were subjected to removal of their melanosome-enriched granular fractions, as described above, and cytosolic radioactivity was determined by liquid scintillation

counting (Winspectral 1414, Perkin-Elmer, Wellesley, MA, USA). Caco-2 cells were subjected to the same extraction procedure.

Liquid chromatography-tandem mass spectrometry (LC/MS/MS)

Confluent monolayers of SkMel-28 and Caco-2 cells were incubated for 24 h with 20 μM MTX. Cytosolic concentrations of MTX were evaluated by LC/MS/MS as described previously (Guo et al., 2007).

MTX-FITC melanosome trapping

SkMel-28 cells were cultured on 35-mm glass-bottom microwell dishes and treated with 20 μM MTX-fluorescein isothiocyanate (MTX-FITC; Molecular Probes, Invitrogen) when they reached 80% confluence. After being extensively washed with PBS, cells were visualized at different treatment times using a confocal microscope (750× magnification). Intact melanosome fractions were used for MTX-FITC detection by quantitative fluorimetry and confocal microscopy. For other assays, melanosomes from MTX-FITC-treated cells were disrupted by sonication in PBS containing 40 mM acetic acid (pH 4.0) and filtered through a Centricon-3 centrifuge filter. Retained and filtered fluorescence was evaluated by quantitative fluorimetry.

Melanin synthesis and interactions with (anti)folates

The interaction of folates (folic acid and 5-MTHF) and antifolates (MTX and aminopterin) with synthetic melanin was assessed as follows. Melanin was synthesized using a modified method (Latocha et al., 2000). Briefly, the oxidative polymerization of 3,4-dihydroxy-L-phenylalanine (L-DOPA) was carried out in phosphate buffer (10 mM, pH 8.0) for 12 h at room temperature using mushroom tyrosinase as a catalyst (0.12 mg/mL). The obtained pigment was acidified by adding concentrated HCl to reach pH 2.0, washed with deionized water to remove Cl^- and dried over phosphorous pentoxide. Interaction of (anti)folates with synthetic melanin in 50 mM phosphate buffer (pH 5.5) was studied using a procedure described previously (Wilczok et al., 1990). Five milligrams of dry melanin was placed in 2 mL Eppendorf tubes and 1.5 mL of the buffer containing (anti)folates at a concentration of 20 μM was added. After 2-h incubations at room temperature, samples were filtered and the concentration of the (anti)folates remaining in each filtrate was calculated by taking the corresponding molar absorption coefficient. Control samples contained identical amounts of (anti)folates but not melanin.

MTX-liposome encapsulation

MTX or MTX-FITC (both at 20 μM) in Opti-MEM medium (Invitrogen) were incubated with INTERFERin™ (Polyplus Transfection, Cedex, France) for 30 min at room temperature. The liposomes were separated from the MTX solution by centrifuging four times at 220,000 ×*g* for 30 min at 25°C. After each spin, the supernatant was removed, replaced with 1 mL OptiMEM,

and vortexed to resuspend the pellet. MTX-loaded liposomes were used for confocal microscopy or to treat SkMel-28 cells.

Statistics

Values presented are the mean ± SD for at least three experiments. Comparisons between two groups were performed using Student's *t*-test. *P* values <0.05 were considered significant.

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Legends to Figures

Figure 1. Anti-proliferative activity of MTX on SkMel-28 and Caco-2 cells. (A) Time-dependent effect of MTX (20 μM) on the proliferation of SkMel-28 and Caco-2 cells and morphological aspect (400x magnification) of untreated cells (CN) compared with those subjected to 6 days of treatment with 20 μM MTX. (B) Apoptosis induction after 6 days of 20 μM MTX treatment. Differences in apoptosis between treated and untreated groups were significant in Caco-2 but not significant (*ns*) in SkMel-28 cells. (C) Growth potential of SkMel-28 and Caco-2 cells after of 6 days of preincubation with 20 μM MTX compared with untreated SkMel-28 controls.

Figure 2. Reduced accumulation of MTX in melanoma cell lines. (A) Accumulation of [^{3}H]-MTX in cytosolic fractions of Caco-2 and the melanoma cell lines SkMel-28 (Sk28), B16/F10 (B16), G.361 and SkMel-1 (Sk1). *A *t*-test was used to compare [^{3}H]-MTX accumulation in Caco-2 with each of the melanoma cell lines, and statistical analysis demonstrated reduced accumulation in all melanoma cells ($P<0.05$). (B) Chromatograms from cytosolic fractions of Caco-2 and SkMel-28 treated for 24 h with 20 μM MTX, monitored at the transition (*m/z*) 455.4 → 308.0.

Figure 3. FRα expression in melanocytes, melanoma cell lines and melanoma tissues. (A) Semiquantitative determination of FRα mRNA in HeM and SkMel-28 untreated cells (expression at time zero) and those subjected to different treatment times with 10 μM MTX. Histograms represent the number of copies of mRNA for every 1 x 10^6 copies of β-actin. Differences between FRα mRNA levels in HeM and SkMel-28 were statistically significant. (B) The upper panel is a western blot of FRα content in HeM and SkMel-28 (Sk28) cells. This blot is representative of five repeated experiments with similar results. The lower panels are MS results for tryptic digests of FRα-immunoprecipitated proteins. For the 38 kDa protein, the detected double-charged ion 450.30 m/z and its fragmentation ions corresponded to the theoretical tryptic peptide (K)EKPGPEDK(L) (predicted mass of 450.23 m/z) from human FRα (NCBInr code 1483627). The 34 kDa protein was also identified as FRα, with the double-charged ion 598.71 m/z and its fragmentation ions (theoretical tryptic peptide (K)GWNWTSGFNK(C) with a predicted mass of 598.78 m/z). (C) Western blot analysis of FRα content in human (Sk1 and G-361) and mouse (B16) melanoma cell lines and in primary (PT) and metastatic (MT) tumors of melanoma origin. (D) Indirect immunofluorescence to localize FRα in HeM and SkMel-28 cells. The arrow was used as a template for fluorescence intensity determination.

Figure 4. Effects of MTX on cellular export of FRα. (A) Effect of MTX on FRα protein content. The upper panel is a western blot of FRα content in SkMel-28 (Sk28) and SkMel-28 cells after 48 h of treatment with 10 μM MTX. This blot is a representative experiment that was repeated

five times with similar results. The lower panel represents the time-dependent effect of MTX (10 μM) on SkMel-28 FRα protein (assessed by western blot analysis, with β-actin to normalize the FRα content). (B) Confocal microscopy detection of subcellular localization of FRα after SkMel-28 cells were treated for 3 h with 10 μM MTX. (C) The upper panel shows the sucrose density gradients of enriched melanosomal fractions obtained from untreated SkMel-28 cells (CN) and cells treated with 10 μM MTX for 5 h. The highly purified melanosomal fraction, which pelleted in the 1.0 – 1.2 M sucrose step, is indicated by an arrow. In the lower panel, the electron microscopy experiments for enriched melanosomal fractions obtained from untreated SkMel-28 cells (CN) and cells treated with 10 μM MTX for 48 h are presented. (D) Time-course of the presence of FRα protein (assessed by western blot) in cellular melanosome fractions (CMF) (obtained by sucrose density gradients) and extracellular melanosomes (EM) after cells were treated with 10 μM MTX. The same concentration of protein (10 μg/well) was loaded in the gel, and the relative intensity was calculated by densitometry, assuming that 100% of relative intensity is the maximal density for each experiment. The pictures show representative experiments repeated five times with similar results, and a graphical representation of the calculated relative intensity ± SD is presented.

Figure 5. Melanosomal sequestration of MTX and melanin-drug interaction. (A) Subcellular localization of MTX-FITC in SkMel-28 melanoma cells after 1 and 4 h of drug exposure. (B) Melanosomal localization of MTX-FITC after 4 h of drug exposure. After cell fractionation, MTX-FITC fluorescence was measured in the cytosolic (CF) and melanosomal fractions (MF). Melanosomal fractions of MTX-FITC treated cells were disrupted by sonication and the fluorescence content was determined (100% was the initial fluorescence). Disrupted melanosomal fractions were filtered through a Centricon-3 centrifuge filter and the fluorescence was determined in the retained (RF) and filtered fractions (FF). (C) Interaction of (anti)folates with synthetic DOPA-melanin. The figure also presents the structure of the folate and antifolate compounds studied, where FOL and AMPT represent folic acid and aminopterin, respectively.

Figure 6. Strategies designed to avoid MTX resistance in melanoma cells. (A) INTERFERin/MTX treatment of SkMel-28 melanoma cells. The images represent confocal microscopy of MTX-FITC encapsulated in liposomes and the fluorescence distribution after SkMel-28 INTERFERin/MTX-FITC treatment. Cell proliferation and apoptosis were determined after 5 days of exposure to 20 μM MTX in the absence or the presence of INTERFERin. (B) Serum effects on melanin secretion and MTX activity on B16/F10 and SkMel-28 cells. The effect of serum on melanin secretion is presented by a picture of cell medium collected seven days after cell implantation. For other experiments, cells were grown in 24-well plates in culture medium supplemented with 10% FBS (+FBS) or in culture medium supplemented with 1% charcoal-stripped FBS (-FBS) until they reached approximately 17,000 cells/well. Then, the cells

were treated with vehicle (CN) or 10 μM MTX. Cells were counted every day and apoptosis and cellular morphology were analyzed on the third day of treatment.

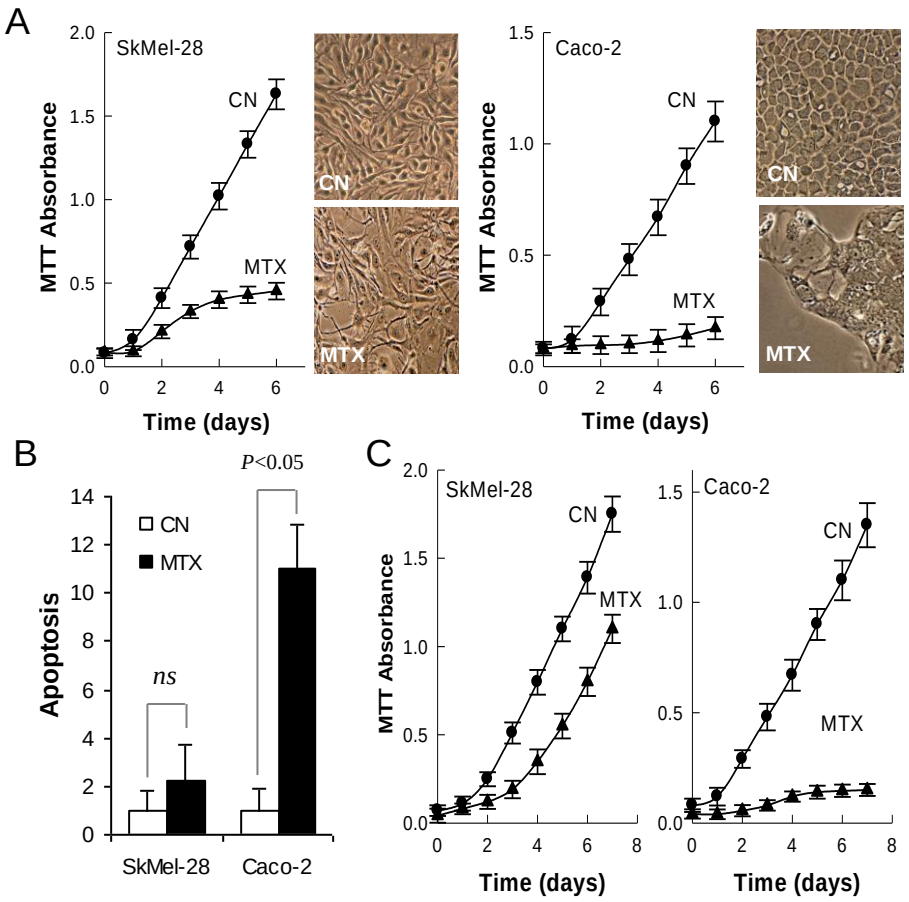
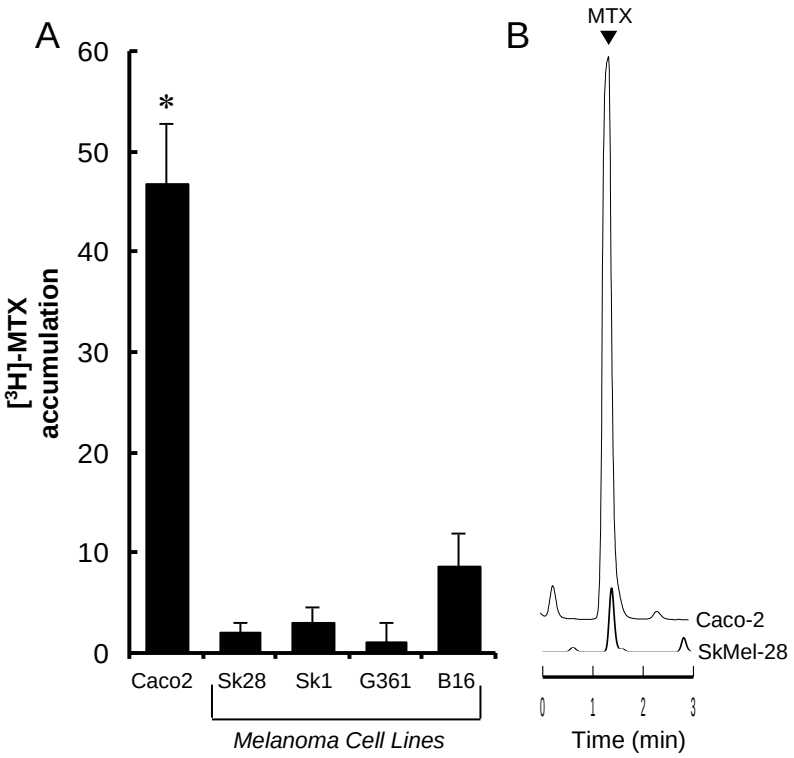


Figure 1. Sánchez-del-Campo et al.



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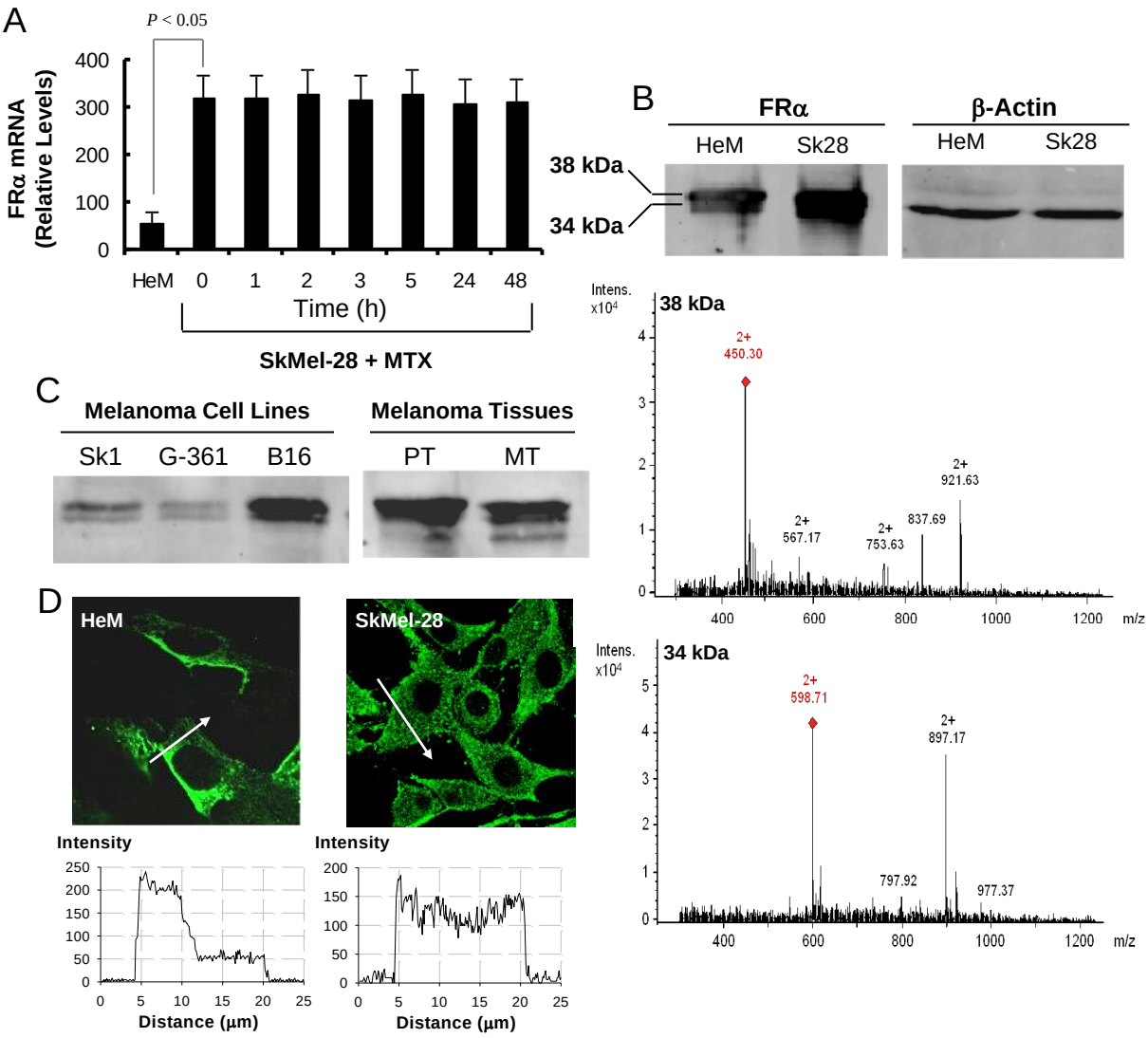

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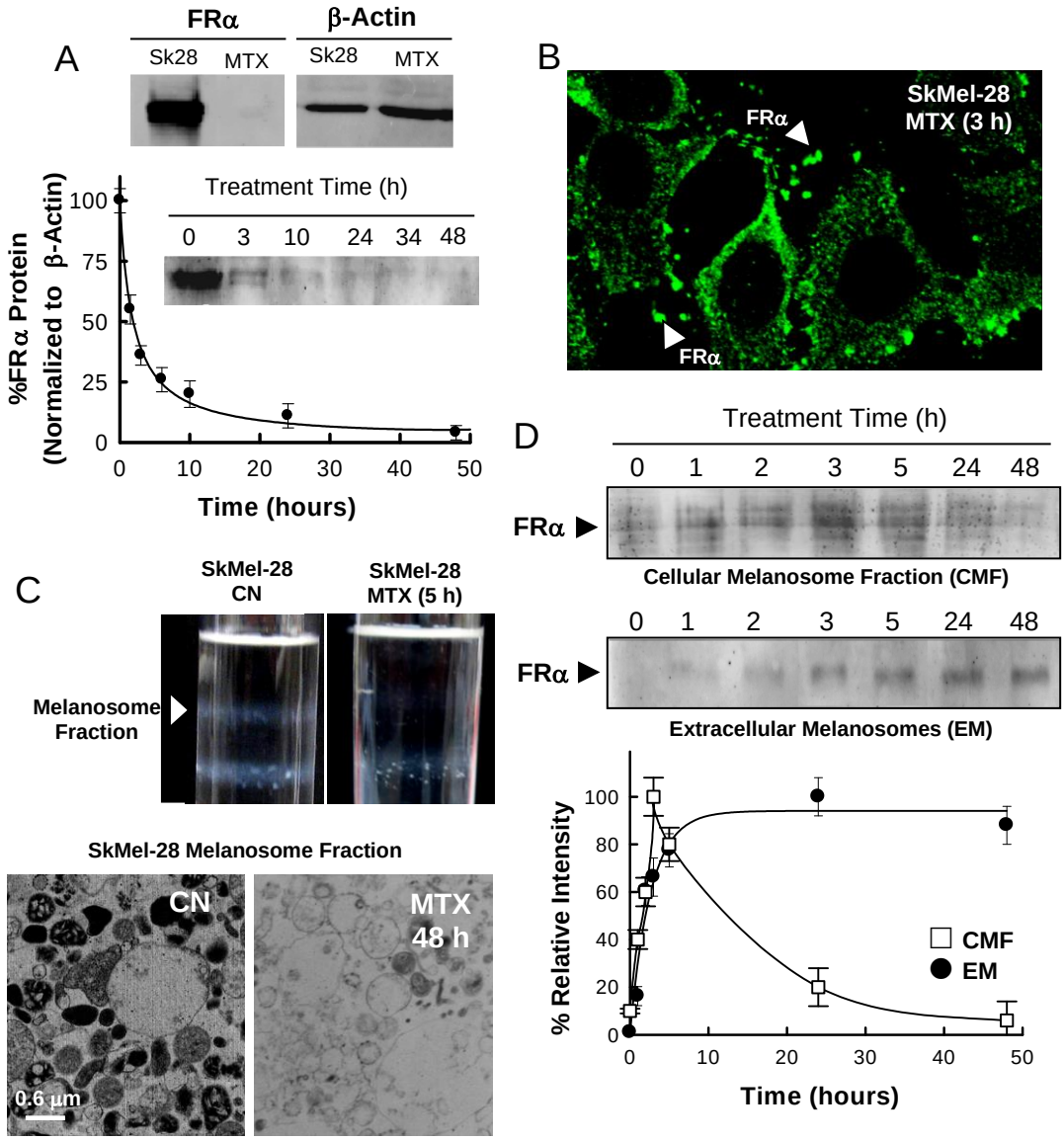

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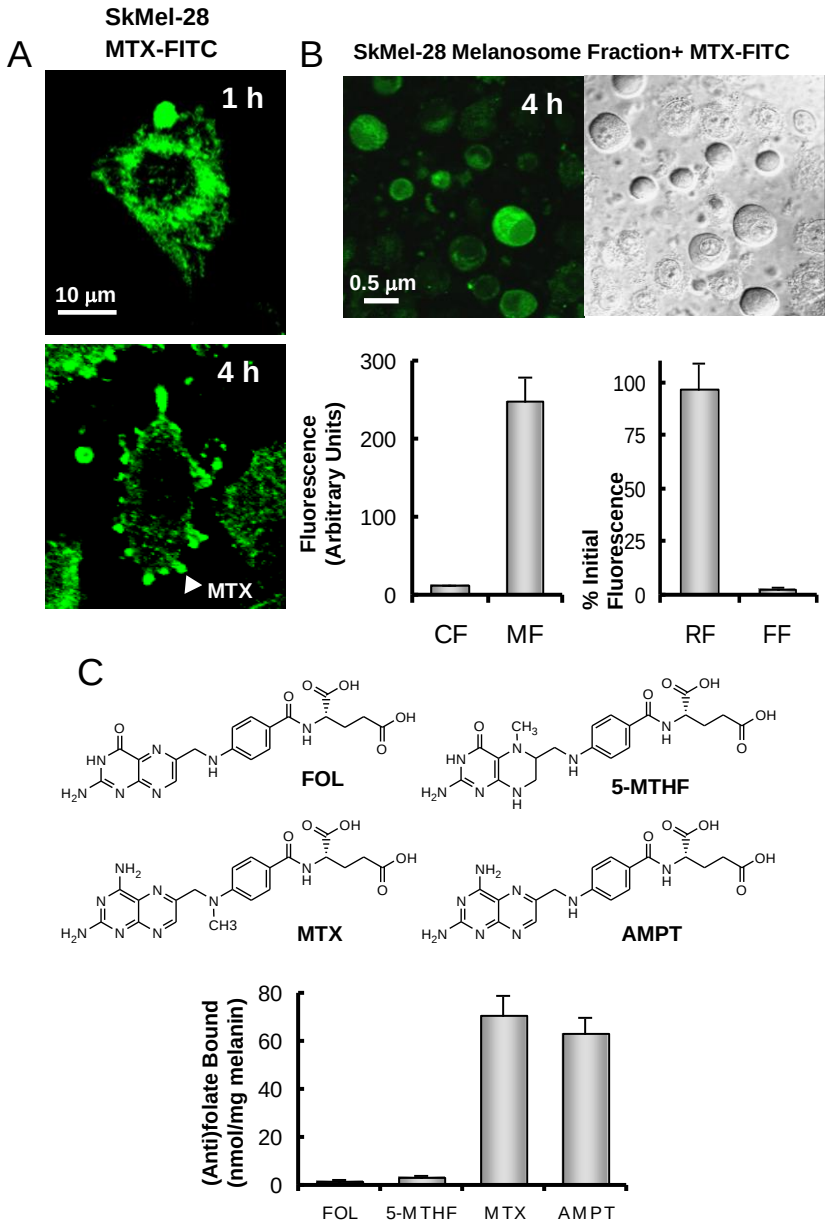

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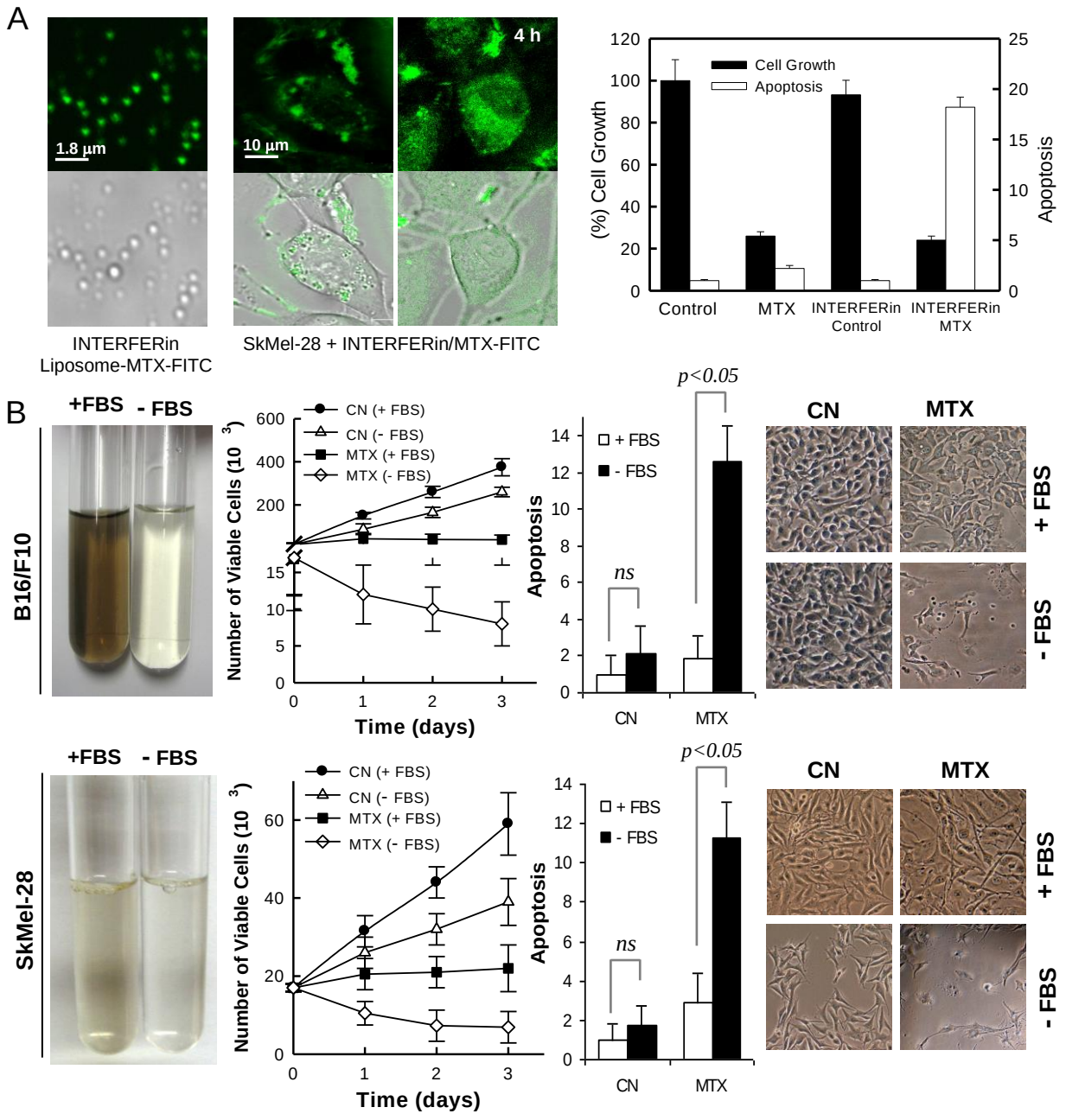

Figure 6. Sánchez-del-Campo et al.