Targeting the Methionine Cycle for Melanoma Therapy with 3-*O*-(3,4,5-Trimethoxybenzoyl)-(-)-Epicatechin

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The higher expression of methionine cycle genes in melanoma cells than in normal melanocytes may be related with increased protein synthesis and transmethylation reactions and the subsequent need for high levels of methionine. 3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin (TMECG), a trimethoxy derivative of epicatechin-3-gallate, effectively suppressed proliferation of melanoma cells in cultures by inducing apoptosis. TMECG modulates the expression of genes involved in methionine metabolism, cellular methylation and glutathione synthesis in melanoma cells. TMECG treatment of melanoma cells resulted in the downregulation of anti-apoptotic Bcl-2, the upregulation of proapoptotic Bax and the activation of caspase-3; however, it did not induce the expression of the apoptosis protease-activating factor-1 (Apaf-1). Having elucidated the effects of TMECG on the melanoma methionine cycle, we designed therapeuthical strategies to increase its effectiveness. Combinations of TMECG with S-adenosylmethionine or compounds that modulate the intracellular concentration of adenosine strongly increase the antiproliferative effects of TMECG. The ability of TMECG to target multiple aspects related with melanoma survival, with a high degree of potency, points to its clinical value in melanoma therapy.

Key words: methionine; 3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin; DNA methylation; melanoma therapy; glutathione.

Malignant tumors are characterized by a high rate of growth. Tumor cells drain the energy of the host, particularly glucose but also amino acids. Methionine is an essential amino acid with at least four major functions (Fig. 1).[1] First, methionine participates in protein synthesis. Second, methionine is a precursor of glutathione, a tripeptide that reduces reactive oxygen species, thereby protecting cells from oxidative stress.[2] Third, it is required for the formation of polyamines, which have far-ranging effects on nuclear and cell division.[3] Fourth, methionine is the major source of the methyl groups necessary for the methylation of DNA and other molecules.[1] It is important to bear in mind the well-established connection of the methionine cycle with two crucial cell metabolites, folic acid and adenosine (Fig. 1). Folic acid acts as the fuel for the methionine cycle, which, after transformation by folate cycle enzymes such as dihydrofolate reductase (DHFR), thymine synthase (TS) and 5,10-methylene-tetrahydrofolate reductase (MTHFR), forms N^5-methyl-tetrahydrofolate (N^5-CH_3-THF), the cofactor of methionine synthase (MS), the enzyme responsible for methionine synthesis. Adenosine, in contrast, is a product of the methionine cycle, and is produced at high concentrations in tumor cells. The efficient intracellular elimination of this product by adenosine-transforming enzymes, such as adenosine deaminase (ADA), or its transport out of the cells by specific adenosine transporters, including the equilibrate nucleoside transporters (ENTs), is of vital importance for cancer cell survival.

Melanoma, a malignant neoplasm of melanocytes, is the most deadly form of skin cancer.[4] The incidence of melanoma continues to increase despite public health initiatives that have promoted protection against the sun. A light skin type, large numbers of nevi and excessive sun exposure, especially in childhood, are the major factors in melanoma risk.[5] When melanoma is detected in its early stages it is curable, but once advanced it is very difficult to treat. Currently, limited therapeutic options exist for patients with metastatic melanoma, and

all standard combinations for metastasis therapy have low efficacy and low response rates.[6] An example of the difficulty involved in melanoma chemotherapy is the limited effect of antifolates. Although methotrexate (MTX), the most frequently used antifolate, is an efficient drug for several types of cancer it is not active against melanoma.[7] The mechanisms of resistance to classical antifolates such as MTX have been extensively studied, mainly in experimental tumors propagated *in vitro* and *in vivo*. The resistance of melanoma cells to MTX is unclear but it seems to be related with (*i*) reduced cellular uptake of this drug, (*ii*) insufficient rates of MTX polyglutamylation, which diminishes the accumulation of long-chain MTX polyglutamates that are preferentially retained intracellularly and/or (*iii*) high intracellular levels of DHFR.[7-9] It will, therefore, be of interest to develop combined therapies that overcome these problems and present low toxicity for the prophylaxis and treatment of melanoma.

Tea polyphenols have shown anti-proliferative and proapoptotic effects on human melanoma.[10] Recently, we have shown that the ester-bonded gallate catechins isolated from green tea, epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG), are potent inhibitors of DHFR activity.[11,12] In our search to improve the stability and bioavailability of green tea polyphenols for cancer therapy, we synthesized a trimethoxy derivative of ECG which showed high antiproliferative activity against malignant melanoma.[13] 3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin (TMECG) efficiently binds to DHFR and down-regulates the expression of folate cycle enzymes, such as DHFR, TS and MTHFR in SkMel-28 melanoma cells.[13] In the present work, we concentrate on the effects that TMECG has on the methionine cycle and its connection with other metabolic and survival pathways in pathological skin cells in a search for alternative and efficient therapeutic strategies against melanoma.

Materials and Methods

Synthesis

TMECG was synthesized from catechin with the subsequent inversion of the stereochemistry at C-3 and by reaction with 3,4,5-trimethoxybenzoyl chloride.[13]

Cell culture experiments

Human (SkMel-1, SkMel-28 and G361) and mouse (B16/F10) melanoma cell lines were obtained from the American Type Tissue Culture Collection (ATCC) and were maintained in appropriate culture media under standard tissue culture conditions. Human epidermal melanocytes (HeM) were supplied by Gentaur (Brussels, Belgium) and were cultured in HAM-F10 medium supplemented with 10% FBS, antibiotics and the human melanocyte growth supplement (Gentaur). Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assays for adherent (HeM, SkMel-28, G361 and B16/F10) and suspension (SkMel-1) cells, respectively. For these assays, cells were plated in 96-well plates at a density of 2000 cells/well and treated when they reached 50% confluence. Co-treatments of SkMel-28 cells with TMECG and other reagents were carried out by supplementing medium cultures with S-adenosylmethionine (SAM), adenosine or dipyridamole (all from Sigma, Madrid, Spain).

RNA isolation and RT-PCR

SkMel-28 and HeM 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, extracted samples were digested with a DNase I kit (Sigma) to avoid genomic DNA contamination. mRNA (200 ng) was used to synthesize cDNA using SuperScript First-Strand Synthesis System (Invitrogen, Barcelona, Spain). PCR amplification of 1 μL of the cDNA

strand generated was carried out in a total volume of 50 μL using an Eppendorf Mastercycler Thermal Cycler (Eppendorf Ibérica, Madrid, Spain). Samples were amplified by 40 cycles at 95ºC (1 min), 62ºC (1 min) and 72ºC (1 min). The amplified PCR products were subjected to electrophoresis in 3% agarose gel and stained with ethidium bromide.

Quantitative real time PCR analysis

cDNA samples (0.1 μL) were used for real time PCR in a total volume of 20 μL using SYBR Green Reagent (Applied Biosystems, Foster City, CA) and specific primers in a 7500 Real Time PCR System of Applied Biosystems. The PCR amplification cycles included denaturation at 95ºC for 15 min (to activate HotStar Taq DNA polymerase and to minimize primer-dimer contribution) and amplification [over 40-50 cycles including denaturation (94ºC; 30 s), annealing (55ºC; 30s), and extension (72ºC; 1 min)]. Non-RT control and negative control samples (without template) were processed in the same manner. The specificity of the amplification was verified by melting curve analysis of all samples, and occasionally by agarose gel electrophoresis. Amplification of target gene sequences were compared against serial dilutions of known quantities of their purified cDNA fragments, and normalized to the abundance of the house-keeping gene β-actin. The sequences of the primers are presented in the legend of the figures.

Susceptibility of genomic DNA to endonucleases

Genomic DNA was isolated from SkMel-28 cells using the DNeasy Tissue kit from Qiagen (Valencia, CA). Genomic DNA samples (0.2 μg) were digested with 3 units of restriction endonuclease *Msp* I (Sigma) or *Hpa* II (Invitrogen) for 90 min at 37ºC, according to the protocols provided for the supplier. *Msp* I and *Hpa* II can distinguish between the unmethylated and methylated cytosine in the nucleotide sequence 5'-CCGG. *Msp* I is insensitive to methylation status, whereas *Hpa* II will digest only if the internal cytosine is

unmethylated. Digested and undigested samples were subjected to electrophoresis in 0.8% agarose gel and stained with ethidium bromide.

Preparation of melanoma cells granular fractions and electron microscopy

SkMel-28 cells were subjected to different treatments, harvested with a mixture of 0.25% trypsin and 0.25 mM EDTA, and washed once in 0.25 M sucrose by centrifugation at 700*g* for 5 min at 4ºC. Melanosome-enriched granular fractions were obtained as described elsewhere[14] and examined by electron microscopy (Zeiss EM10 electron microscope; Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Determination of intracellular ROS

Intracellular ROS were measured using 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA; Sigma) as a fluorescent probe. DCHF-DA diffuses readily to the intracellular compartment, where it is deacetylated to the non-membrane-permeable DCHF. Then, during the cellular production of ROS, DCHF is oxidized and emits a fluorescent signal. SkMel-28 cells were grown to 90% confluence in a black clear bottom 96-well plate and treated with vehicle, 50 µM TMECG or 50 µM ECG for 48 hours. Cells were washed and incubated with 5 µM DCHF-DA for 2 h at room temperature in the dark. After incubation, cells were washed twice with PBS and the fluorescence was measured (excitation and emission wavelengths at 485 and 530 nm, respectively; Fluostar Galaxy, BMG-Labtech, Durham, NC).

Apoptosis assays

Apoptosis induction in TMECG-treated melanoma cells was assessed by analysis of cytoplasmic histone-associated DNA fragmentation using a kit from Roche Diagnostics (Barcelona, Spain). Cells were treated during 7 days with different concentrations of TMECG and ELISAs were revealed with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) during 15 min, according to the manufacturer's instructions. The specific enrichment

of mono- and oligonucleosomes released into the cytoplasm (cytosolic nucleosome enrichment factor) was calculated dividing the absorbance at 414 nm of treated samples by the absorbance at 414 nm of untreated controls.

Caspase-3 activity assay

Caspase-3 activity was measured using the caspase-3 colorimetric activity assay kit (Sigma). Briefly, the cells were washed with cold PBS and lysed with the cell lysis buffer included in the kit. The cell lysates were centrifuged (10,000*g* for 5 min at 4ºC), and the supernatants were collected. Equal amounts of protein (100 μg), 30 μg of colorimetric caspase-3 substrate (acetyl-Asp-Glu-Val-Asp *p*-Nitroanilide; Ac-DVED-*p*NA) and assay buffer were added to each reaction mix, which were then incubated for 4 h at 37ºC. Caspase-3 activity was determined by measuring the absorbance at 405 nm. One unit of caspase-3 activity was expressed as 1 pmol of *p*NA formed per hour, and its specific activity was that unit divided by micrograms of protein. Caspase-3 inhibitor (Ac-DVED-CHO) was used as negative control.

Western blot analysis

Cells were lysed in PBS, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. Soluble proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting (ECL Plus, GE Healthcare). Antibodies against Bax and caspase-3 (p20) were from Santa Cruz Biotechnology (Santa Cruz, CA), Apaf-1 from BD Biosciences (San Jose, CA) and Bcl-2 from Sigma.

Confocal microscopy

SkMel-28 cells were cultured over 35 mm glass bottom microwell dishes, grown to 90% confluence and treated with vehicle, 50 μM TMECG or 50 μM ECG for 48 hours. Cells were washed and incubated with 5 μM DCHF-DA for 2 h at room temperature in the dark. After

incubation, preparations were visualized by a Leica TCS 4D confocal scanning laser fluorescence inverted microscope (Leica Microsystems, Wetzcar, Germany) at 750-fold magnification. The immunohistochemistry for localization of Bax protein was carried out as described elsewhere.[15]

Statistical analysis

In all experiments, the mean ± standard deviations (SD) for 5 determinations in triplicate were calculated. Statistically significant differences were evaluated using the Student's *t*-test. Differences were considered statistically significant at the levels of $p<0.05$.

Results and Discussion

The methionine cycle in melanoma cells

The synthesis of methionine in all mammalian tissues is mainly catalyzed by MS (Fig. 1). The levels of expression of MS mRNA in non-pathological human epithelial melanocytes (HeM) and in the melanoma SkMel-28 cell line were analyzed by real time PCR (Fig. 2). SkMel-28 showed a much higher content of MS mRNA (Fig. 2). Methionine adenosyltransferase (MAT) is an essential cellular enzyme that catalyzes the formation of SAM, the principal biological methyl donor and the ultimate source of the propylamine moiety used in polyamine biosynthesis.[16] MAT is the product of two different genes, MAT1A and MAT2A, which code for two different enzymes, MATI/III and MATII respectively, with distinct kinetic and regulatory properties that display a tissue-specific pattern of expression.[17] MAT1A is expressed mostly in liver, whereas MAT2A shows a wider distribution and is believed to be responsible for SAM synthesis in extra-hepatic tissues.[18,19] The levels of MAT2A mRNA, analyzed by real time PCR, were also found to be highly increased in melanoma cells compared with normal melanocytes (Fig. 2). Next, we analyzed the expression of S-adenosylhomocysteine hydrolase (AHYC), the only enzyme that cleaves *S*-

adenosylhomocysteine (SAH). AHYC was found to be overexpressed in melanoma cells, where its mRNA levels were 6-fold higher than in HeM (Fig. 2). Taken together, the results obtained for methionine cycle gene expression indicated that this cycle is highly activated in melanoma cells.

Expression of methylases and methylation status in melanoma cells

A methylase reaction is needed to complete the methionine cycle (Fig. 1). SAM formed by MAT action is used as a methyl donor in the methylation of phospholipids, proteins, small molecules, DNA and RNA. To evaluate the methylation status in melanoma cells, we studied the expression levels of two important methylase genes: catechol-*O*-methyltransferase (COMT) and DNA-methyltransferase-1 (DNMT1), whose enzyme products catalyze the methylation of catechols and DNA, respectively. In normal melanocytes, COMT is believed to play an important role in protecting these cells against reactive dihydroxyphenolic intermediates of melanogenesis leaking from the melanogenic compartments.[20] In human, COMT protein exists in two different forms; the soluble form (S-COMT) resides in the cytoplasm and possibly also in nuclei, whereas the membrane-bound form (MB-COMT) is an integral membrane protein of the rough endoplasmic reticulum.[21] Due to the importance of this enzyme for melanocyte survival, we decided to analyze the levels and distribution of COMT mRNAs in human melanoma cells compared with normal melanocytes. Fig. 3*a* shows that the relative abundance of mRNAs for both COMT isoforms was similar in HeM cells. However, the higher abundance of (S+MB)-COMT compared with MB-COMT mRNA in SkMel-28 indicates that most of the COMT are in soluble form in these tumor cells. The high over-expression of S-COMT with respect to MB-COMT in melanoma cells could be related with the different mechanisms involved to regulate their expression. Recently, it has been found that the promoters S and MB of COMT are differently modulated by methylation in

endometrial cancer cell lines and tissues, where MB-COMT is selectively inactivated and methylated and S-COMT is expressed and unmethylated.[22] Although it is well documented that MB-COMT is more catalytically active than S-COMT in different detoxification processes, the membrane form is strongly repressed in the highly methylated environment of cancerous cells. Therefore, up-regulation of S-COMT enzyme may represent a protection mechanism of melanoma cells against their own reactive compounds generated during melanogenesis. Human MB-COMT has a higher affinity for catechol substrates than S-COMT, but the catalytic constant has been found to be slightly higher for S-COMT than for MB-COMT;[23] therefore, in melanoma environments, with their high production of melanin related products, the soluble enzyme could be even more catalytically efficient than the membrane form for melanogen detoxification.

The high methylase activity of SkMel-28 was indicated by the high levels of expression of DNMT1 mRNA in these cells, ranging from 7 to 9-times higher than in HeM (Fig. 3*b*). DNA methylation patterns are frequently altered in human cancer, and include genome-wide hypomethylation as well as regional hypermethylation at CpG islands.[24-26] To investigate whether DNMT1 overexpression is involved in the overall level of DNA methylation, the susceptibility of genomic DNA from SkMel-28 cells to the methylation-sensitive restriction endonuclease *Hpa* II was studied. The methylation-insensitive isoschizomer *Msp* I was used as a control. As shown in Fig. 3*c*, DNA from DNMT1-overexpressing cells (SkMel-28) was highly resistant to digestion with *Hpa* II, suggesting that DNMT1 mediates aberrant methylation of multiple genes in these tumor cells.

TMECG inhibits the growth of melanoma cell and efficiently blocks the methionine cycle in SkMel-28

TMECG, which efficiently inhibited human DHFR, showed a high antiproliferative effect on SkMel-28 melanoma cells.[13] To confirm that this effect was not associated to a specific type of melanoma cell line, the antiproliferative activity of TMECG was assayed on different human and mouse melanoma cell lines (Fig. 4*a*). This compound was seen to inhibit the growth not only of adherent cell lines but also of the suspension SkMel-1 melanoma cell line, which could represent a model for melanoma metastatic cells. The efficacy of antifolates in treating cancer is widely attributed to the subsequent decrease in nucleotide production, but in addition to these effects, antifolate treatment has also been linked to a decrease in cellular methylation. Thus, the effect of this new antifolate drug on methionine cycle gene expression was studied after five days of exposing SkMel-28 to 50 μM TMECG. As can be observed in Fig. 2, the mRNA levels for MS and AHYC were similar to those observed in HeM and significantly lower than those detected in untreated SkMel-28 cells. Although the levels of MAT2A mRNA showed a significant decrease after TMECG treatment with respect to those in SkMel-28, they still slightly augmented when compared with normal melanocytes. The data indicated that TMECG-treatment had efficiently blocked the methionine cycle in this melanoma cell line. In addition to the effects on the methionine cycle, TMECG treatment had an important effect on the expression of methylases (Fig. 3). DNMT1 recovered to reach normal levels in treated cells and, as a consequence, the overall DNA methylation of the TMECG-treated cells was reduced (Fig. 3*c*). TMECG treatment also modulated COMTs mRNA levels (Fig. 3*a*), producing a strong decrease on S-COMT and a slight increase on MB-COMT, which could be related with the unmethylation of its MB promoter.[22]

TMECG activates glutathione synthesis in melanoma cells

Although the pathogenesis of human melanocyte transformation is still incompletely understood several studies have suggested that a change in melanin status from antioxidant to pro-oxidant could explain melanoma formation.[27,28] The high levels of reactive oxygen species (ROS) in SkMel-28 melanoma cells were effectively evaluated in our laboratory by measurements of intracellular DCFH fluorescence (Fig. 5*a*). The treatment of these cells with TMECG highly reduced their intracellular ROS content (Fig. 5*a*). Associated with this finding, SkMel-28 subjected to TMECG treatment underwent significant cell whitening, and electron microscopy studies revealed important differences in the content of melanosome melanin between TMECG-treated and untreated cells (Fig. 5*b*). Although the direct antioxidant activity of TMECG would explain, in principle, these findings, two experimental observations suggested that other effect/s may account for this TMECG action. First, we observed that its catechin analogue, ECG, which presented higher antioxidant activity against radicals generated in the aqueous phase,[13] was less effective in reducing melanoma intracellular ROS (Fig. 5*a*) and in inhibiting melanin formation; in fact, melanoma cells treated with ECG presented a higher melanin content that untreated controls (Fig. 5*b*). To demonstrate that the observed TMECG effects could be associated with its described antifolate activity, the experiment was carried out in the presence of MTX instead TMECG. As observed in Fig. 5*b,* MTX also produced a reduction in the content of melanin. By analyzing the scheme of Fig. 1, the connection between the folate cycle and the melanin synthesis pathway could be established though the methionine cycle and the synthesis of glutathione. An increase in cellular glutathione levels after TMECG treatment could explain both the decrease of ROS and the inhibition of melanogenesis. To evaluate the effect of TMECG on glutathione synthesis, we analyzed the levels of γ-glutamylcysteine synthetase

(GCS), the rate-limiting enzyme in the biosynthesis of this compound, in Skmel-28 cells. The GCS mRNA ranged from 2600 to 4200 copies per million copies of β-actin mRNA in untreated SkMel-28 (Fig. 5*c*). Treatment of these cells with TMECG increased the number of copies of GCS mRNA to a mean value of 26300 (Fig. 5*c*). Glutathione is an important metabolite for normal or tumor cells because it is involved in many cellular functions.[29] Melanoma cells are no exception, and strict control of their glutathione levels is of vital importance for these cells. Excessive glutathione production could interfere with the melanin biosynthesis pathway. Glutathione has been implicated in skin lightening and its proposed mechanisms of action include direct inactivation of the enzyme tyrosinase, mediating the switch mechanism from eumelanin to phaeomelanin production, quenching free radicals and peroxides that contribute to tyrosinase activation and melanin formation, and modulation of depigmenting abilities of melanocytotoxic agents. Therefore, the data presented here suggest that the homocysteine accumulated as a consequence of methionine cycle disruption by TMECG can serve as a substrate for glutathione synthesis in TMECG-treated cells.

TMECG induces apoptosis in melanoma cells

To investigate whether the metabolic changes induced by TMECG resulted in the apoptosis of melanoma cells, melanoma cell lines were treated for seven days with different concentrations of this compound and the degree of apoptosis induction was evaluated using a DNA fragmentation assay (Fig. 4*b*). The results indicated that the reduced viability of melanoma cells in the presence of TMECG (Fig. 4*a*) was indeed due to apoptosis induction. The data also indicated that normal melanocytes were highly resistant to TMECG-induced apoptosis, which is a highly desirable feature for potential antitumoral agents. TMECG-induced apoptosis was studied in greater detail using the SkMel-28 cell line. SkMel-28 cells exposed to 50 μM TMECG for seven days showed evident signs of cellular damage.

Morphological changes included cell shrinkage, loss of cell-cell contact and the fragmentation of plasmatic and nuclear membranes (Fig. 6*a*). Another feature of apoptotic cell death, the activation of caspase-3, was evaluated by a colorimetric activity assay and Western blot analysis (Figure 6*b*). The cells treated with TMECG showed significantly higher caspase-3 activity, which was inhibited by Ac-DVED-CHO (Fig. 6*b*). Immunoblot analysis confirmed caspase-3 activation (Fig. 6*b*). The Bcl-2 family proteins play a critical regulatory role through their interacting pro- and anti-apoptotic members, which integrate a wide array of upstream survival and distress signals to decide the fate of cells. Bax and Bcl-2 proteins are the key elements of this protein family. As reported for EGCG,[10] TMECG treatment resulted in a decrease in anti-apoptotic Bcl-2 and an increase in proapoptotic Bax at the levels of mRNA and protein, thereby resulting in a significant increase in the Bax/ Bcl-2 ratio that favors apoptosis (Fig. 6*c*). Immunohistochemistry results (Fig. 6*d*) also indicated that TMECG treatment caused mitochondrial translocation of Bax, which is a common response of cancer cells subject to certain apoptotic stimuli.[15]

In general, melanoma cells are quite resistant to apoptosis and it has recently been shown that these cells can avoid suicide by inactivating the apoptosis protease-activating factor-1 (Apaf-1) gene, which is one step further on from p53 in the apoptosis pathway.[30] It was proposed that Apaf-1 inactivation involves the addition of methyl groups to cytosine nucleotides in DNA and the removal of acetyl groups from the hystone proteins that bundle DNA into the compressed form seen in the nucleus. In vivo, loss of expression of Apaf-1 has been associated with tumor progression, suggesting that Apaf-1 inactivation may provide a selective survival advantage to neoplastic cells.[30] To evaluate the participation of Apaf-1 in SkMel-28 apoptosis during TMECG treatment, its mRNA and protein levels were determined by PCR and Western blot, respectively. Apaf-1 mRNA and protein were detected in SkMel-

28 and their levels were essentially the same than those detected in HeM (Fig. 6*e*). This data indicated that, although methylation of Apaf-1 has been proposed as a mechanism for controlling its expression, TMECG, which decreased cellular methylation in SkMel-28 cells, was not able to produce Apaf-1 activation. These results are in accordance with others which found that drugs acting on different mechanisms did not induce or upregulate the expression of Apaf-1 at the levels of mRNA and protein.[31] Due to the importance of elucidating the apoptotic mechanisms operating in melanoma, the sequence of events leading to TMECG-induced apoptosis in this melanoma cell line is currently being investigated in our laboratory.

SAM enhances the antiproliferative effects of TMECG

Having analyzed the ability of TMECG to disrupt the methionine cycle, we designed several strategies to completely block this pathway. The first strategy was to inhibit MAT2A enzyme. As described above, the expression of MAT2A gene was not completely reversed after treatment with TMECG, and this MAT2A residual expression could represent a mechanism of cells for escape from TMECG treatment. MAT2A shows a very low K_m for methionine (4-10 μM) and, in tissues that predominantly express MAT2A, the rate of SAM synthesis is near maximal and relatively unaffected by fluctuations in methionine concentration and, thus, MAT2A can work even a very low concentration of methionine. Another kinetic characteristic of MAT2A is that this enzyme is strongly inhibited by SAM, its reaction product (IC_{50} = 60 μM).[32] We hypothesized that, by increasing intracellular SAM concentration in the presence of TMECG, two consecutive steps in the methionine cycle, the synthesis of methionine and the synthesis of SAM, could be blocked (Fig. 1). The effect of adding SAM alone on the growth of SkMel-28 can be visualized in Fig. 7*a*. Only high concentrations of SAM (up to 100 μM) affected SkMel-28 growth in accordance with its calculated IC_{50} towards MAT2A. However, in the presence of TMECG, lower concentrations

of SAM showed a synergistic behavior with this antifolate compound. Thus, the combination 20 μM SAM with 50 μM TMECG efficiently inhibited the growth of SkMel-28 (Fig. 7*a*). The results indicated that when MAT2A is working at limiting concentrations of methionine in the presence of TMECG, it is highly susceptible to inhibition by low SAM concentrations.

Strategies to increase the intracellular concentration of adenosine in the presence of TMECG.

As observed in Fig. 1, adenosine is a direct product of the methionine cycle, and is produced in high concentration when the cycle is highly active. Any resulting excess of adenosine may not be a problem for cancer cells. Adenosine is efficiently metabolized by specific enzymes such as ADA and adenosine kinase (ADK) before being used for purine nucleotide synthesis, which is even more necessary for DNA synthesis in these highly proliferating cells. Finally, excess adenosine can be transported out of the cells by ENTs, bidirectional transporters that allow adenosine release and uptake by facilitating diffusion along its concentration gradient. However, in the presence of an antifolate compound, adenosine accumulation might represent a severe problem for the cell. Depletion of N^5-CH_3-THF would result in the production of high concentrations of SAH, which strongly inhibits cellular methyltransferases. This inhibition would produce an accumulation of SAM, which would inhibit the MAT2A reaction. We hypothesized that accumulation of adenosine in the presence of TMECG may block the methionine cycle at three levels: the synthesis of methionine, the methylase reaction and the synthesis of SAM (Fig. 1). Adenosine flux across the cellular membrane depends on the concentration gradients between extra- and intracellular nucleoside levels.[33] Therefore, we first tried to increase the extracellular concentration of adenosine. Adenosine alone had no detectable effect on SkMel-28 growth at the studied concentrations (up to 500 μM) but, in the presence of TMECG, adenosine had a significant

synergistic effect, enhancing the antiproliferative action of this antifolate compound. A combination of 50 μM adenosine with 50 μM TMECG was seen to completely inhibit the growth of SkMel-28 (Fig. 7*b*).

Although adenosine is currently used for the treatment of several cardiovascular diseases, its use as a therapeutic agent is restricted, since it is rapidly metabolized to inosine and AMP, which limits its ability to exert a systemic effect. Therefore, we planned other strategies to accumulate adenosine in melanoma cells using dipyridamole. This drug suppresses adenosine transport by inhibiting ENTs. Moreover, it also inhibits the enzyme ADA, which normally breaks down adenosine into inosine. We hypothesized that inhibition of ENTs and ADA in melanoma cells would result in an intracellular accumulation of adenosine, which, in the presence of TMECG-accumulated homocysteine, would produce an effective blockage of the methionine cycle. The effect of dipyridamole alone or in combination with TMECG on the growth of SkMel-28 cells can be observed in Fig. 7*c*. Dipyridamole alone inhibited SkMel-28 growth with a calculated IC_{50} (at 5 days) of 20 μM. However, in the presence of 50 μM TMECG, the IC_{50}-value fell to less than 1 μM. The deadly effect on melanoma cells of the combination of 5 μM dipyridamole with 50 μM TMECG can be observed in Fig. 7*d*. The results pointed to the possibility of using this combination treatment against malignant melanoma.

Methionine depletion for melanoma treatment: conclusions

Conventional chemotherapy treatments have shown their limits, especially for patients with advanced cancer. New therapeutic strategies must be identified, and the metabolic abnormalities of cancer cell open up such opportunities.[1] Many human cancer cells lines and primary tumors have an absolute need for methionine, an essential amino acid. In contrast, normal cells are relatively resistant to exogenous methionine restriction. Here, we show that

melanoma cells are highly dependent on methionine. The resistance of melanomas to general chemotherapies and their avoidance of cellular suicide or resistance to apoptosis is primarily related with the high activity of the methionine cycle in these cells, which permits the methylation of specific genes and activation of different survival pathways. Here, we show that TMECG modulates multiple aspects of melanoma metabolism and survival, including the folic acid and the methionine cycles and the methylation status of the cells. The specific activity of TMECG on the melanoma methionine cycle was confirmed by the high synergy found with compounds that uncoupled adenosine metabolism in these cells. Although the effectiveness, the pharmacokinetics and toxicology of TMECG need to be further investigated in animal experiments, our results indicated that TMECG alone or in combination with other compounds that modulate the melanoma methionine cycle, such as SAM, adenosine or dipyridamole, could represent an effective therapy against melanoma.

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References

1. Cellarier E, Durando X, Vasson MP, Farges MC, Demiden A, Maurizis JC, Madelmont JC, Chollet P. Methionine dependency and cancer treatment. Cancer Treat Rev 2003;29:489-99.
2. Anderson ME. Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 1998;111-112:1-14.
3. Thomas T, Thomas TJ. Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 2001;58:244-58.
4. Chudnovsky Y, Khavari PA, Adams AE. Melanoma genetics and the development of rational therapeutics. J Clin Invest 2005;115:813-24.
5. Houghon AN, Polsky D. Focus on melanoma. Cancer Cell 2002;2: 275-8.
6. Koon H, Atkins M. Autoimmunity and immunotherapy for cancer. N Engl J Med 2006;354:758-60.
7. Kufe DW, Wick MM, Abelson HT. Natural resistance to methotrexate in human melanomas. J Invest Dermatol 1980;75:357-9.
8. Gaukroger J, Wilson L, Stewart M, Farid Y, Habeshaw T, Harding N, Mackie R. Paradoxical response of malignant melanoma to methotrexate in vivo and in vitro. Br J Cancer 1983;47:671-9.
9. Zhao R, Goldman ID. Resistance to antifolates. Oncogene 2003;22:7431-57.
10. Nihal M, Ahmad N, Mukhtar H, Wood GS. Anti-proliferative and proapoptotic effects of (-)-epigallocatechin-3-gallate on human melanoma: possible implications for the chemoprevention of melanoma. Int J Cancer 2005;114:513-21.
11. Navarro-Perán E, Cabezas-Herrera J, García-Cánovas F, Durrant MC, Thorneley RNF, Rodríguez-López JN. The antifolate activity of tea catechins. Cancer Res 2005;65:2059-64.
12. Navarro-Peran E, Cabezas-Herrera J, Sánchez-del-Campo LS, Rodríguez-López JN. Effects of folate cycle disruption by the green tea polyphenol epigallocatechin-3-gallate. Int J Biochem Cell Biol 2007;39:2215-25.
13. Sánchez-del-Campo L, Otón F, Tárraga A, Cabezas-Herrera J, Chazarra S, Rodríguez-López JN. Synthesis and biological activity of a 3,4,5-trimethoxybenzoyl ester analogue of epicatechin-3-gallate. J Med Chem 2008; 51:2018-26.
14. Potterf SB, Muller J, Bernardini I, Tietze F, Kobayashi T, Hearing VJ, Gahl WA. Characterization of a melanosomal transport system in murine melanocytes mediating entry of the melanogenic substrate tyrosine. J Biol Chem 1996;271:4002-8.

15. Choi S, Singh SV. Bax and Bak are required for apoptosis induction by sulforaphane, a cruciferous vegetable-derived cancer chemopreventive agent. Cancer Res 2005; 65:2035-43.

16. Mato JM, Alvarez L, Ortiz P, Pajares MA. S-adenosylmethionine synthesis: molecular mechanisms and clinical implications. Pharmacol Ther 1997;73:265-80.

17. Garcia-Trevijano ER, Latasa MU, Carretero MV, Berasain C, Mato JM, Avila MA. S-adenosylmethionine regulates MAT1A and MAT2A gene expression in cultured rat hepatocytes: a new role for S-adenosylmethionine in the maintenance of the differentiated status of the liver. FASEB J 2000;14:2511-8.

18. Cai J, Sun WM, Hwang JJ, Stain SC, Lu SC. Changes in S-adenosylmethionine synthetase in human liver cancer: molecular characterization and significance. Hepatology 1996;24:1090-7.

19. Cai J, Mao Z, Hwang JJ, Lu SC. Differential expression of methionine adenosyltransferase genes influences the rate of growth of human hepatocellular carcinoma cells. Cancer Res 1998;58:1444-50.

20. Smit N, Tilgmann C, Karhunen T, Slingerland R, Ulmanen I, Westerhof W, Pavel S. O-methylation of L-dopa in melanin metabolism and the presence of catechol-O-methyltransferase in melanocytes. Pigment Cell Res 1994;7:403-8.

21. Tenhunen J, Heikkila P, Alanko A, Heinonen E, Akkila J, Ulmanen I. Soluble and membrane-bound catechol-O-methyltransferase in normal and malignant mammary gland. Cancer Lett 1999;144:75-84.

22. Sasaki M, Kaneuchi M, Sakuragi N, Dahiya R. Multiple promoters of catechol-O-methyltransferase gene are selectively inactivated by CpG hypermethylation in endometrial cancer. Cancer Res 2003;63:3101-6.

23. Mannisto PT, Kaakkola S. Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacol Rev 1999;51:593-628.

24. Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet 1999;21:163-7.

25. Widschwendter M, Jones PA. DNA methylation and breast carcinogenesis. Oncogene 2002;21:5462-82.

26. Bestor TH. The DNA methyltransferases of mammals. Hum Mol Genet 2000;9:2395-402.

27. Meyskens FLJr, Farmer PJ, Anton-Culver H. Etiologic pathogenesis of melanoma: a unifying hypothesis for the missing attributable risk. Clin Cancer Res 2004;10:2581-3.

28. Picardo M, Maresca V, Eibenschutz L, De Bernardo C, Rinaldi R, Grammatico P. Correlation between antioxidants and phototypes in melanocytes cultures. A possible link of physiologic and pathology relevance. J Invest Dermatol 1999;113:424-5.

29. Obrador E, Carretero J, Ortega A, Medina I, Rodilla V, Pellicer JA, Estrela JM. γ-Glutamyl transpeptidase overexpression increases metastatic growth of B16 melanoma cells in the mouse liver. Hepatology 2002;35:74-81.

30. Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, McCombie R, Herman JG, Gerald WL, Lazebnik YA, Cordón-Cardó C, Lowe SW. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 2001;409:207-11.

31. Zanon M, Piris A, Bersani I, Vegetti C, Molla A, Scarito A, Anichini A. Apoptosis protease activator protein-1 expression is dispensable for response of human melanoma cells to distinct proapoptotic agents. Cancer Res 2004;64:7386-94.

32. Sullivan DM, Hoffman JL. Fractionation and kinetic properties of rat liver and kidney methionine adenosyltransferase isozymes. Biochemistry 1983;22:1636-41.

33. Tabrizchi R, Bedi S. Pharmacology of adenosine receptors in the vasculature. Pharmacol Ther 2001;91:133-47.

Legend to Figures

FIGURE 1. The methionine cycle and its connections with several metabolic and survival cell pathways. Abbreviations: ADA, adenosine deaminase; AHYC, S-adenosylhomocysteine hydrolase; COMT, catechol-*O*-methyltransferase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DNMT, DNA methyltransferase; ENT, equilibrative nucleoside transporter; GCS, γ-glutamylcysteine synthetase; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TS, thymine synthase.

FIGURE 2. Semiquantitative determination of MS, AHYC and MAT2A mRNAs. The estimated relative levels of the mRNA with respect to that of β-actin were calculated and then compared with respect to the expression levels in HeM. SkMel-28 cells were treated with 50 μM TMECG for 5 days. Asterisks show the statistically significant differences ($p<0.05$) between the treated SkMel-28 cells and untreated controls. Primers: *MS* (forward: 5'-GAGAACCACTCTACATTGGA-3'; reverse: 5'-GGAAGACCTGCATTGGGATA-3'); *AHYC* (forward: 5'-TGGACATTGCTGAGAACGAG-3'; reverse: 5'-CTCCACGGTCA-TGTGCAG-3'); *MAT2A* (forward: 5'-CCACGAGGCGTTCATCGAGG-3'; reverse: 5'-CAGCAGCTCTGGATGTAATTT-3').

FIGURE 3. Expression of methylases and methylation status in SkMel-28 cells. (*a*) Semiquantitative determination of (S+MB)-COMT, (MB)-COMT and (S)-COMT mRNAs. Histograms represent the number of copies of mRNA for every 1 x 10^3 copies of β-actin. SkMel-28 cells were treated with 50 μM TMECG for 5 days. Asterisks show the statistically significant differences ($p<0.05$) between the treated SkMel-28 cells and untreated controls. Because S-COMT has no unique sequences compared with MB-COMT mRNA, a first set of primers was designed from the carboxyl terminal region to amplify both the soluble and membrane-bound forms, (S+MB)-COMT. The second set of the primers is specific for the amino terminal portion and provides a measurement of only the membrane bound form, MB-COMT. Primers: *(S+MB)-COMT* (forward: 5'- GAACGAGTCATCCTGCAGCCCATC-3'; reverse: 5'-CTGCTCGCAGTAGGTGTCAA-3'); *(MB)-COMT* (forward: 5'-GTCGCGGGA-

GAGAAATAACA-3'; reverse: 5'-CTGCTCGCAGTAGGTGTCAA-3'). (*b*) Semiquantitative determination of DNMT1 mRNA. The estimated relative levels of the mRNA with respect to that of β-actin were calculated and then compared with respect to the expression levels in HeM. SkMel-28 cells were treated with 50 μM TMECG for 5 days. **p*<0.05 when compared with SkMel-28 untreated controls. Primers: *DNMT1* (forward: 5'-CCCCTGAGCCCTACCGAAT-3'; reverse: 5'-CTCGCTGGAGTGGACTTGTG-3'). (*c*) Sensitivity of genomic DNA from SkMel-28 cells to restriction endonucleases. SkMel-28 cells were treated for 72 h with vehicle (Sk28) or 50 μM TMECG before DNA extraction and digestion.

FIGURE 4. Concentration-dependent effect of TMECG on the viability and the apoptosis of normal melanocytes and several melanoma cell lines. Normal human melanocytes (HeM) and the human (SkMel-28, SkMel-1 and G361) and mouse (B16/F10) melanoma cell lines were exposed to vehicle (control), 1, 5, 10, 20 or 50 μM TMECG for seven days. At this time, the number of viable cells (*a*) and the induced apoptosis (*b*) were determined as described in the Material and Method section.

FIGURE 5. Effect of TMECG on intracellular ROS content, cell whitening and glutathione synthesis in melanoma cells. (*a*) ROS content in melanoma cells after ECG or TMECG (both at 50 μM) 48-h treatments was determined by DCHF fluorescence in black clear bottom 96-well plates (left panel) and confocal microscopy (right panel). **p*<0.05 when compared with SkMel-28 untreated controls. (*b*) Electronic microscopy of SkMel-28 cells before and after TMECG (50 μM), ECG (50 μM) and MTX (10 μM) treatments. (*c*) Semiquantitative determination of GCS mRNA. Histograms comparing the estimated relative levels of the mRNA with respect to that of β-actin. Values represent the number of copies of mRNA for every 1 x 10^6 copies of β-actin. Primers: *GCS* (forward: 5'-GGCGATGAGGTGGAA-TACAT-3'; reverse: TGTCCTTTCCCCCTTCTCTT-3').

FIGURE 6. Apoptosis of SkMel-28 induced by TMECG. (*a*) Morphological aspect (magnification ×100) of untreated SkMel-28 cells compared with those subject to 7-days'

treatment with 50 μM TMECG. (*b*) The effect of TMECG on the activity and protein content of caspase-3 in SkMel-28. Cells were treated for 7 days with 50 μM TMECG. Caspase-3 activity was determined in the absence or the presence of the inhibitor Ac-DEVD-CHO. The results are expressed as the mean ± SD of three cultures. The data are representative of duplicate activity determinations. *$p < 0.05$, significantly different from non-treated group. Western blot analysis of caspase-3 in control cells (Sk28) and those treated with TMECG as described above. (*c*) Semiquantitative determination of Bcl-2 and Bax mRNAs and proteins. SkMel-28 cells were treated with 50 μM TMECG during 5 days. The ratios between Bax and Bcl-2 mRNA and protein are presented. Primers: *Bcl-2* (forward: 5'-GGAT-TGTGGCCTTCTT-TGAG-3'; reverse: 5'-CCAAACTGAGCAGAGTCTTC-3'); *Bax* (forward: 5'-TTTGCTTCAGGGTTTCATCC-3'; reverse: 5'-GCCACTCGGAAAAAGACC-TC-3'). (*d*) Immunohistochemistry for analysis of Bax localization in SkMel-28 cells and those treated 3 days with 50 μM TMECG. Cells were stained with anti-Bax (*green fluorescence*), and MitoTracker Red (*red fluorescence*). Merge images are shown, which indicate *yellow-orange* staining of mitochondria in TMECG-treated SkMel-28 cells due to merge of *green* and *red fluorescence*. Mitochondria in SkMel-28 control cells were stained *red*. Various components of the cells (Nu, nucleus, Cyt, cytosol and Mito, mitochondria) are also indicated. (*e*) RT-PCR and Western blot analysis of Apaf-1 in HeM, SkMel-28 and SkMel-28 treated with 50 μM TMECG during 5 days using β-actin as an internal control. Histograms represents the relative Apaf-1 mRNA and protein content, normalized to β-actin, in untreated and TMECG-treated SkMel-28 cells with respect to the content determined in normal melanocytes (HeM; 100%). The data shown here are from a representative experiment repeated five times with similar results. Primers: *Apaf-1* (forward: 5'-GCTCTC-CAAATTGAAAGGTGAAC-3'; reverse: 5'-ACTGAAACCCAATGCACTCC-3').

FIGURE 7. Effects of combinational treatments with TMECG on the growth of SkMel-28 cells. Viability was determined by the MTT assay. The number of surviving cells is directly proportional to the level of the formazan product created and the color can then be quantified at 570 nm. (*a*) Cells were treated with vehicle only (control), with SAM (20 μM), with TMECG (50 μM) or with a combination of the same concentration of both SAM plus TMECG. (*b*) Cells were treated with vehicle only (control), with adenosine (Ado; 50 μM),

with TMECG (50 μM) or with a combination of the same concentration of both Ado plus TMECG. (*c*) Effect of different dipyridamole concentrations on SkMel-28 cell growth after 5 days of treatment in the absence (- TMECG series) or the presence (+ TMECG series) of 50 μM TMECG. The data was expressed assuming 100% of growth for untreated cells for the series – TMECG or for cells treated with 50 μM TMECG in the series + TMECG. (*d*) Cells were treated with vehicle only (control), with dipyridamole (5 μM), with TMECG (50 μM) or with a combination of the same concentration of both dipyridamole plus TMECG.

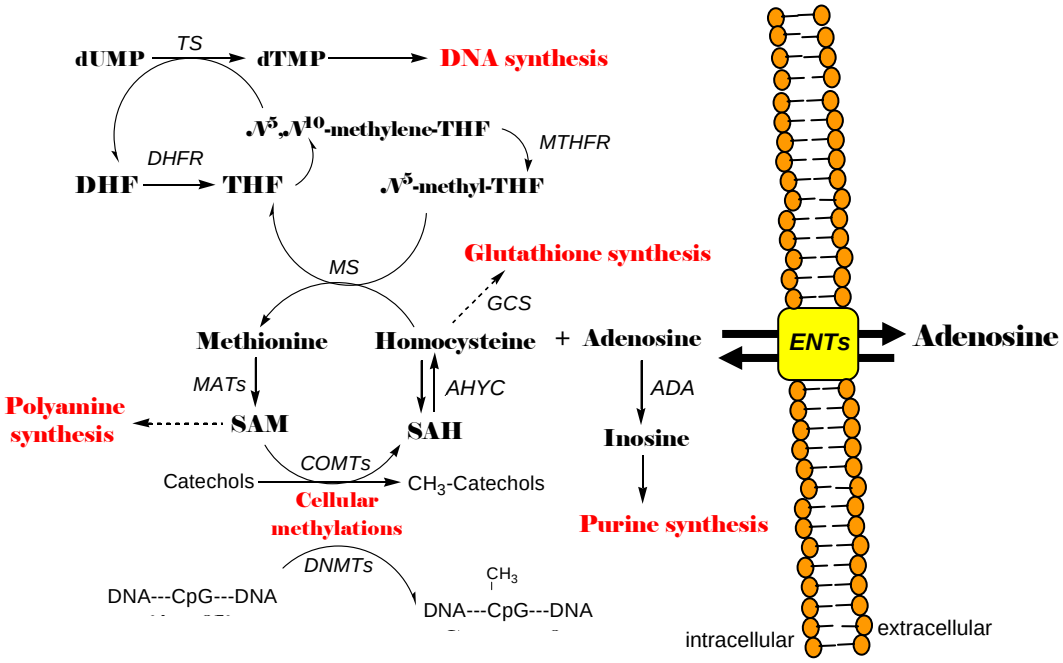

FIGURE 1. Sánchez-del-Campo et al.

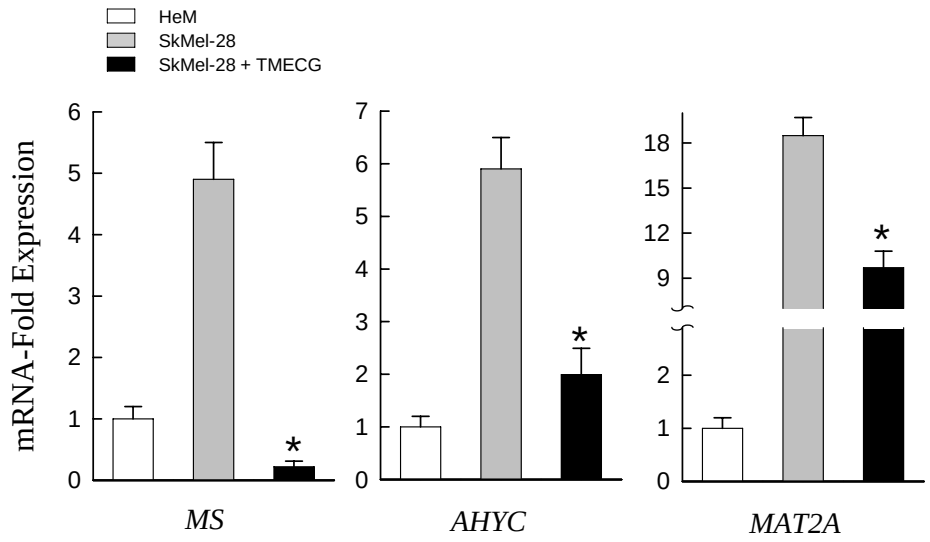

FIGURE 2. Sánchez-del-Campo et al.

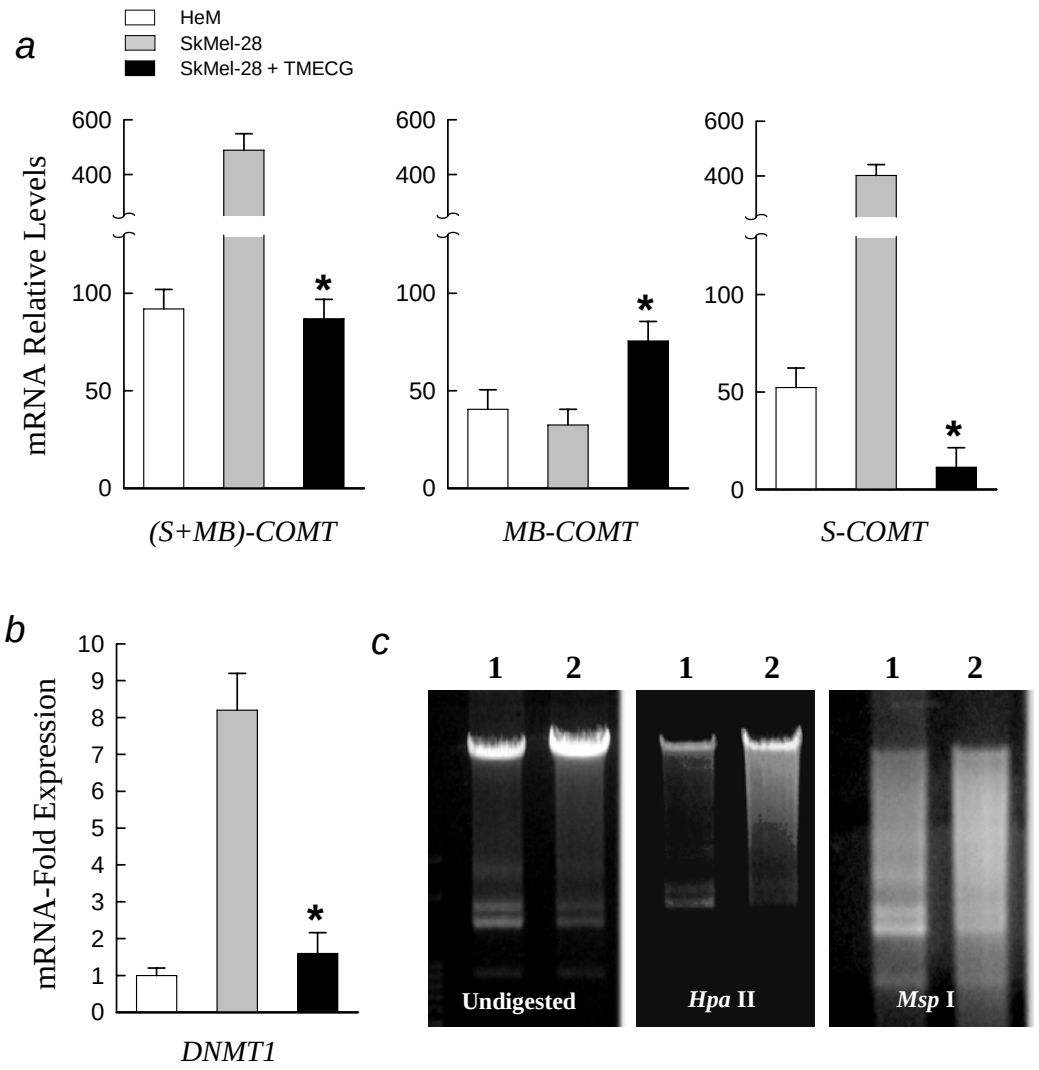

FIGURE 3. Sánchez-del-Campo et al.

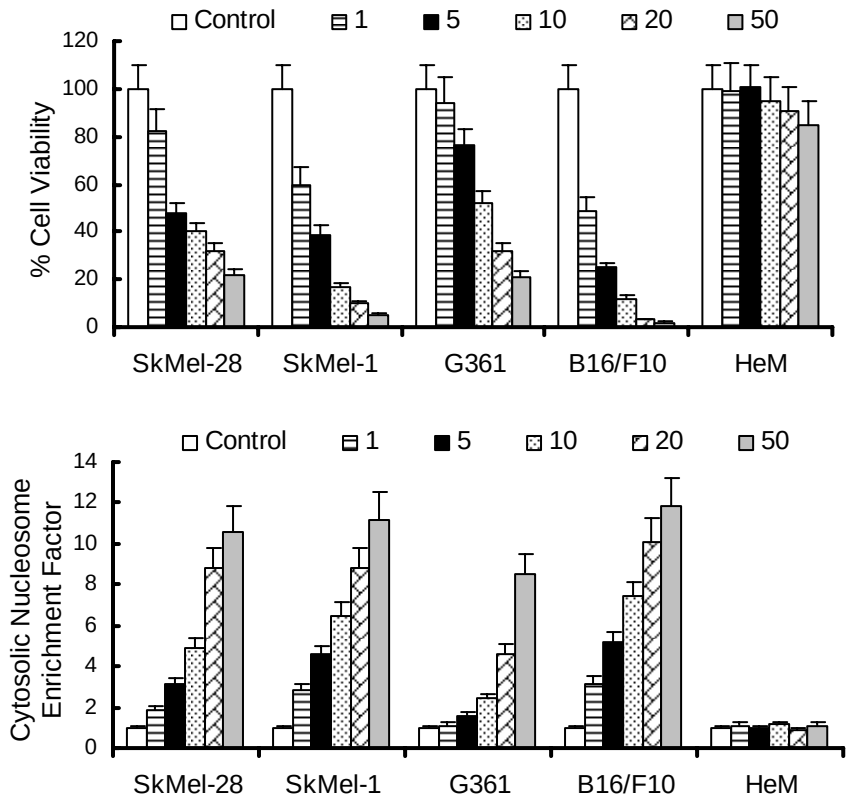

FIGURE 4. Sánchez-del-Campo et al.

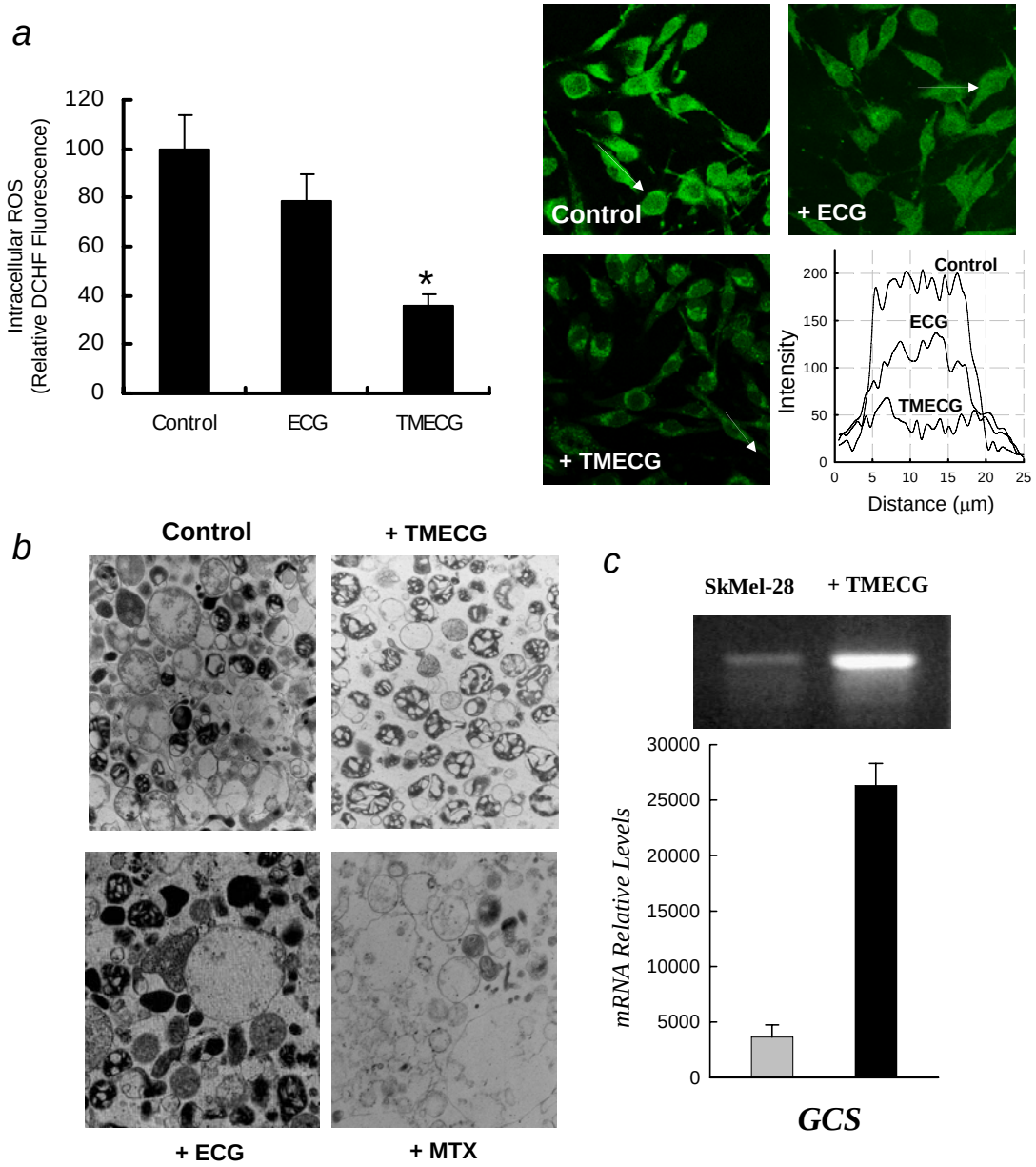

FIGURE 5. Sánchez-del-Campo et al.

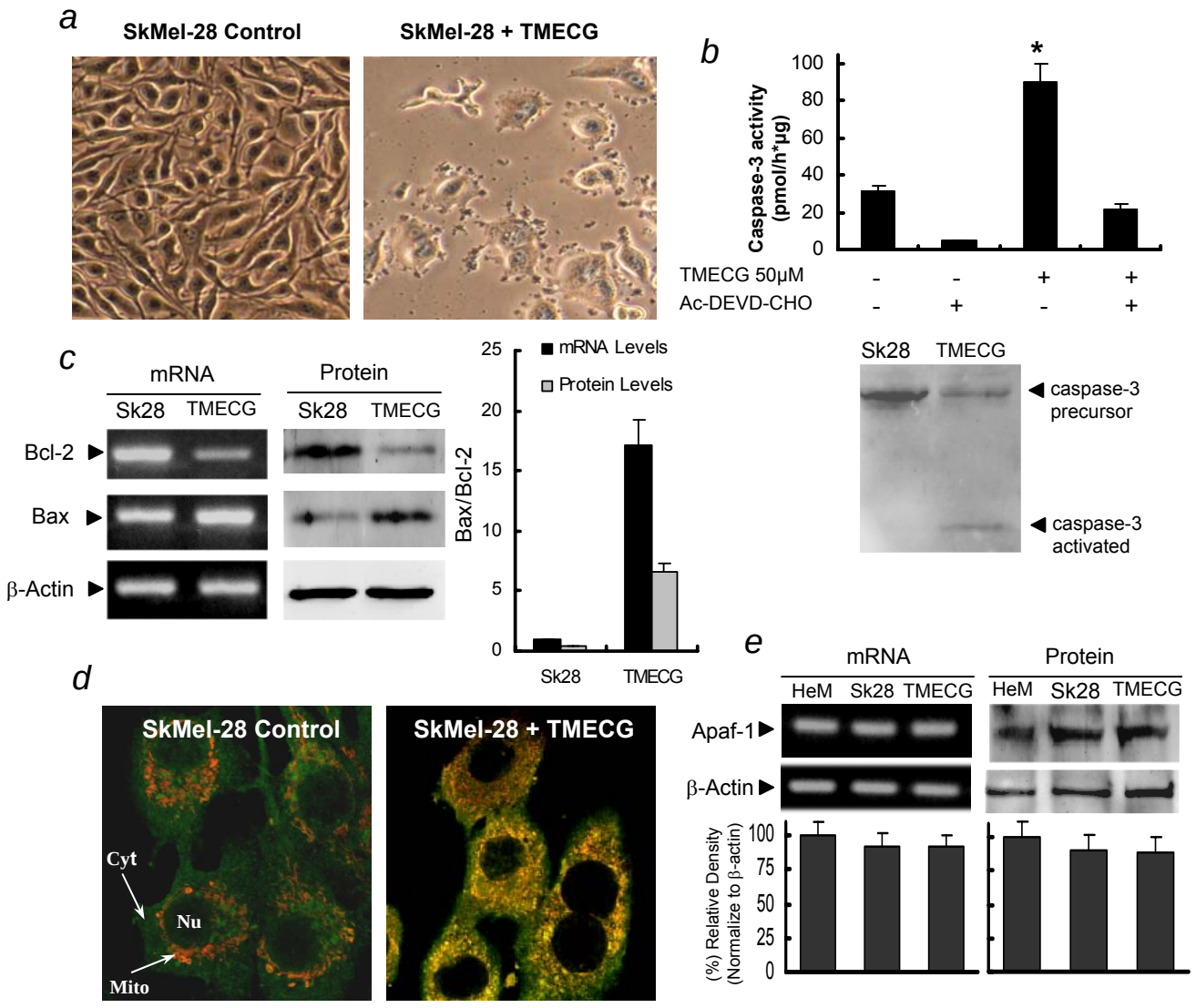

FIGURE 6. Sánchez-del-Campo et al.

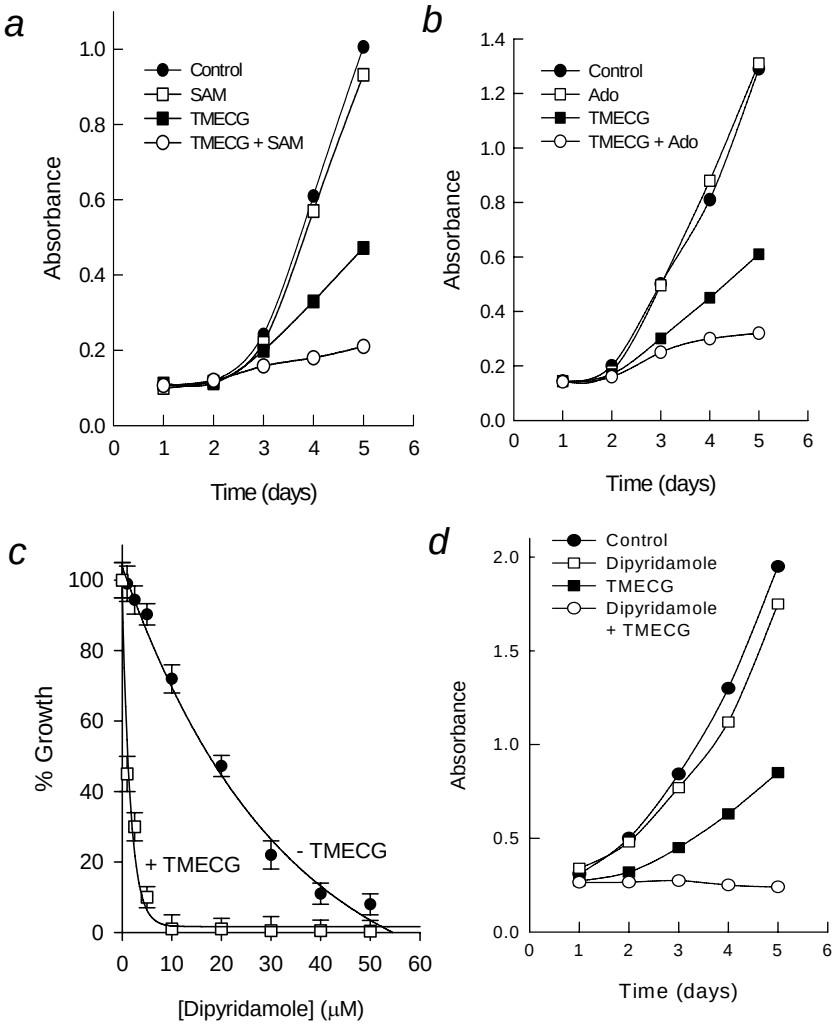

FIGURE 7. Sánchez-del-Campo et al.