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Novel Antifolates as Produgs for the Treatment of Melanoma

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1. Introduction

Malignant melanoma is a deadly disease in which standard treatment options have remained remarkably static over the past 30 years (Sullivan & Atkins, 2009). At present, the incidence of melanoma continues to increase despite public health initiatives that have promoted protection against the sun. Thus, during the past ten years, the incidence and annual mortality of melanoma has increased more rapidly than any other cancer and according to an American Cancer Society estimate, there will have been approximately 68,720 new cases of invasive melanoma diagnosed in 2009 in the United States, which resulted in approximately 8,650 deaths (American Cancer Society, 2009). Unfortunately, the increase in incidence has not been paralleled by the development of new therapeutic agents with a significant impact on survival. Although many patients with melanoma localized to the skin are cured by surgical excision, increased time to diagnosis is associated with higher stage of disease, and those with regional lymphatic or metastatic disease respond poorly to conventional radiation and chemotherapy with 5-year survival rates ranging from 10 to 50% (Tawbi & Buch, 2010). Currently, 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. One example of the complications involved in melanoma chemotherapy is the limited effectiveness 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 (Kufe et al., 1980). Undoubtedly, unravelling the mechanism of the resistance of melanomas to this drug could help to improve current therapeutic approaches. Moreover, it could help to develop a novel generation of antifolate drugs that overcome resistance problems and present low toxicity for the prophylaxis and treatment of melanoma.

2. Mechanisms of resistance of melanoma to classical antifolates

Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: NADP+ oxidoreductase, EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) in the
presence of coenzyme NADPH as follows: DHF + NADPH + H+ → THF + NADP+. This enzyme is necessary for maintaining intracellular pools of THF and its derivatives which are essential cofactors in one-carbon metabolism. Coupled with thymidylate synthase (Lockshin et al., 1979), it is directly involved in thymidylate (dTMP) production through a de novo pathway. DHFR is therefore pivotal in providing purines and pyrimidine precursors for the biosynthesis of DNA, RNA and amino acids. In addition, it is the target enzyme (Blakley, 1969) for antifolate drugs such as the antineoplastic drug MTX and the antibacterial drug trimethoprim (TMP). The mechanisms of resistance to MTX have been extensively studied, mainly in experimental tumors propagated in vitro and in vivo (Kufe et al., 1980; Zhao & Goldman, 2003; Assaraf, 2007); however, the specific basis for the resistance of melanoma cells to MTX is unclear. During decades the mechanism of resistance of melanoma to MTX was associated with general mechanisms of resistance detected in other epithelial cancer cell including reduced cellular uptake of this drug, high intracellular levels of DHFR and/or insufficient rate of MTX polyglutamylation, which diminishes long-chain MTX polyglutamates from being preferentially retained intracellularly (Assaraf, 2007). However, recently, a melanoma-specific mechanism of resistance to cytotoxic drugs, including MTX, has been described (Chen et al., 2006; Sánchez-del-Campo et al., 2009a; Xie et al., 2009). Experiments from our laboratory and others provide evidences that melanosomes contribute to the refractory properties of melanoma cells by sequestering cytotoxic drugs and increasing melanosome-mediated drug export. Concretely, we have described that folate receptor α (FRα)-endocytotic transport of MTX facilitates drug melanosomal sequestration and cellular exportation in melanoma cells, which ensures reduced accumulation of MTX in intracellular compartments (Sánchez-del-Campo et al., 2009a).

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 reduced folate carrier (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 (Kufe et al., 1980) 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 in this study indicated that drug exportation was an operative mechanism of resistance to MTX in melanoma cells. 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 (Sánchez-del- Campo et al., 2009a). To test the importance of this process on the resistance of melanoma to antifolates, we silenced the expression of the melanosomal structural protein gp100/Pmel17, which is known to play a critical role in melanosome biogenesis (Theos et al., 2005). Recently, Xie and collaborators (2009) provided the first direct evidence that disruption of the process of normal melanosome biogenesis, by mutation of gp100/Pmel17, increased sensitivity to cisplatin. We also observed that effective silencing of gp100/Pmel17 significantly increased the sensitivity of melanoma cells to MTX, favouring MTX-induced apoptosis. 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.

To explore the relationship between MTX exportation and melanosome trafficking, we studied the possible interaction of MTX with melanin (Sánchez-del-Campo et al., 2009a). Such interaction was confirmed by incubating this drug with synthetic 3,4-dihydroxyphenylalanine (DOPA)-melanin. Importantly, folic acid and 5-methyl-THF, the natural source of cellular folates, did not appear to interact with synthetic DOPA-melanin. A comparison of the interaction of several folates (folic acid and 5-methyl-THF) and antifolates (MTX and aminopterin) with synthetic DOPA-melanin indicated that the double amino group of the pterin ring is an important molecular requirement for the drug-melanin interaction. Therefore, 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.

In addition to these cellular mechanisms of resistance to MTX in melanoma, other mechanism that includes liver transformation of the drug has also been reported. A paradoxical response of malignant melanoma to MTX in vivo and in vitro has been described (Gaukroger et al., 1983). The authors observed that MTX showed consistent cytotoxicity for melanoma cells in vitro but was ineffective at equivalent concentrations in vivo. MTX
undergoes oxidation to its primary metabolite 7-hydroxy-MTX (7-OH-MTX) in the liver by the enzyme aldehyde oxidase (Assaraf, 2007) and therefore, this transformation has been proposed as a novel mechanism of resistance to explain this paradox (Gaukroger et al., 1983; Assaraf, 2007). In contrast to the large body of literature available on the multiple modalities of MTX resistance, very little is known regarding the ability of 7-OH-MTX to provoke antifolate-resistance phenomena that may disrupt MTX activity. Recent studies seem to indicate that 7-OH-MTX which exceeds by far MTX in the plasma of MTX-treated patients can provoke distinct modalities of antifolate-resistance that severely compromise the efficacy of the parent drug MTX (Joerger et al., 2006).

3. The antifolate activity of tea catechins

Recent studies have presented data that show a variety of biological activities of tea catechins, compounds which constitute about 15% (dry weight) of green tea (Mukhtar & Ahmad, 2000; Fujiki et al., 2002). Green tea catechins include: (+)-epigallocatechin gallate (EGCG), (+)-epigallocatechin (EGC), (+)-epicatechin gallate (ECG) and (+)-epicatechin (EC). EGCG is the most abundant (one 240 mL cup of brewed tea contains up to 200 mg EGCG), and many health benefits, including antioxidant, antibiotic and antiviral activities, have been attributed to this compound (Yam et al., 1998; Mabe et al., 1999; Hamilton-Miller, 2001). Some authors consider EGCG alone to be the active anticancer component, while others suggest that other tea constituents also have antiproliferative or anticarcinogenic properties (Jung & Ellis, 2001). Green tea extracts have been shown in vitro to stimulate apoptosis of various cancer cell lines, including prostate, lymphoma, colon, and lung (Yang et al., 2000; Mukhtar & Ahmad, 2000; Jung & Ellis, 2001; Gupta et al., 2003). Moreover, EGCG was reported to inhibit tumour invasion and angiogenesis, processes that are essential for tumour growth and metastasis (Jung & Ellis, 2001). Despite great efforts during the last two decades to understand the anticarcinogenic activity of tea, the exact mechanism(s) of action are not well defined. Therefore, deciphering the molecular mechanism by which green tea or its polyphenols impart their antiproliferative effects could be important and may result in improved opportunities for the treatment of cancer.

Based on the observation that classical (MTX) and non-classical (TMP) antifolate compounds possess similar chemical structures to some tea polyphenols (Navarro-Perán et al., 2005a), we started to work on the hypothesis that tea catechins could inhibit DHFR activity. Suppression of DNA synthesis by tea catechins could explain many of the observed effects on cancer inhibition by these compounds. Recently, we have shown that ester bonded gallate catechins isolated from green tea, such as EGCG and ECG are potent inhibitors of DHFR activity in vitro at concentrations found in the serum and tissues of green tea drinkers (0.1–1.0 μM) (Navarro-Perán et al., 2005b). EGCG exhibited the kinetic characteristics of a slow-binding inhibitor of DHF reduction with bovine liver DHFR but of a classical, reversible, competitive inhibitor with chicken liver DHFR. Structural modelling showed that EGCG can bind to human DHFR in a similar orientation to that observed for a number of structurally characterized DHFR inhibitor complexes (Fig. 1) (Navarro-Perán et al., 2005a). These results suggested that EGCG could act as an antifolate compound in the same way as MTX and TMP. Since these first reports describing the inhibition of DHFR by tea polyphenols, several studies by us and other laboratories have reported that EGCG inhibits DHFR from a variety of biological sources (Navarro-Martínez et al., 2005; 2006; Navarro-Perán et al., 2007; Hannewald et al., 2008; Kao et al., 2008; Spina et al., 2008; Sánchez-del-
Campo et al., 2010a). Recently, a screening of DHFR-binding drugs by MALDI-TOFMS demonstrated that EGCG is an active inhibitor of DHFR and has a relative affinity between that of pyrimethamine and MTX (Hannewald et al., 2008).

Other studies have been focused in understand weather the antifolate activity of tea catechins could explain their anti-inflammatory and antitumoral properties (Navarro-Perán et al., 2007; Navarro-Perán et al., 2008). The most common use of MTX is as an anticancer drug, although the drug is also considered to have anti-inflammatory and immuno-suppressive properties with accompanying activity against autoimmune disorders (Cutolo et al., 2001). Inflammation is central to our fight against pathogens, but if it is not ordered and timely the resulting chronic inflammation may contribute to diseases such as arthritis, heart attacks and Alzheimer’s disease. A functional link between chronic inflammation and cancer has long been suspected (Aggarwal, 2004; Balkwill & Coussens, 2004). This link is of great interest in the context of this chapter because green tea has shown remarkable anti-inflammatory activity (Sueoka et al., 2001). Understanding the mechanisms by which EGCG imparts this effect could be of importance for explaining the epidemiological data on the prophylactic effects of diets high in gallate polyphenols for certain forms of cancer. Most solid tumors contain many non-malignant cells, including immune cells and blood-vessel cells, which are important in inflammation, although the crucial molecular pathways that permit communication between abnormally growing cancer cells and these inflammatory cells remain unknown. A mouse model of inflammation-associated cancer now points to the involvement of the gene transcription factor NF-κB and the inflammatory mediator known as TNF-α in cancer progression (Pikarsky et al., 2004). Several of the anti-inflammatory effects of MTX and other antifolates can be explained by the suppression of NF-κB activation, a multisubunit factor known to play a role in inflammation, immune modulation and cell proliferation.
Although the mechanism by which antifolates modulate NF-κB activation has remained unclear for some time, recent investigations have demonstrated that MTX could inhibit the TNF-α-induced NF-κB activation though the release of adenosine (Cutolo et al., 2001; Majumdar & Aggarwal, 2001). By lowering THF cofactors, MTX inhibits two steps of the purine synthesis pathway: the conversion of GAR to FGAR and the conversion of AICAR to FAICAR. Excess AICAR inhibits the conversion of AMP to IMP by AMP deaminase, while AMP is rapidly converted to adenosine by surface expressed ecto-5’ nucleotidase. Adenosine is a potent endogenous regulator of a variety of physiological processes through specific receptors on the cell surface and binds to four different types of G protein-coupled cell surface molecules, termed the A₁, A₂A, A₂B, and A₃ adenosine receptors (Linden, 2001). After binding to the cell surface receptors, adenosine alters the immune cell production of soluble mediators such as cytokines, free radicals, and arachidonic acid metabolites (Majumdar & Aggarwal, 2001). Although MTX is widely used for the treatment of inflammatory and autoimmune diseases, its use as a chemopreventive agent is precluded, even at low doses, due to its adverse side effects. However, there is no evidence for such side effects as a result of the regular consumption of tea. The finding that EGCG shares mechanisms of action with MTX could be of interest, and suggests that the regulation of chronic inflammation by EGCG could represents a strong possibility to explain the epidemiological data concerning the green tea’s prophylactic effects on certain forms of cancer (Yang et al., 2000). To check our hypothesis that the anti-inflammatory properties of EGCG could be related to its antifolate action and whether adenosine and its receptors are involved in EGCG action, we investigated the EGCG-induced suppression of NF-κB in Caco-2 cell monolayer, which acted as a model of the human intestinal epithelium (Navarro-Perán et al., 2008). We observed that EGCG, by inhibiting DHFR, can disturb the metabolism of this vitamin in Caco-2 cells, producing the release of adenosine and the suppression of NF-κB. The data suggest that by modulating NF-κB activation, EGCG might not only combat inflammation, but also cancer. Since by reducing chronic inflammation there is a strong possibility of modulating tumorogenesis, these results could be of importance for explaining tea’s cancer preventive effects.

4. Synthetic catechins as antifolate prodrugs against melanoma

Despite the excellent properties of tea catechins, they have at least one limitation, their low bioavailability (Nakagawa & Miyazawa, 1997). Factors influencing this low bioavailability could be related to their low stability in neutral or slightly alkaline solutions and their inability to cross cellular membranes (Hong et al., 2002). In an attempt to solve such bioavailability problems, we first synthesized a 3,4,5-trimethoxybenzoyl analogue of ECG [3-O-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin, TMECG] (Sánchez-del-Campo et al., 2008). This compound was successfully synthesized following the five-step reaction sequence shown in Fig. 2, starting from the commercially available catechin. In comparing the antiproliferative activity of TMECG on several human and mouse cancer cell lines, we noticed that this compound was much more active on melanoma cells than on normal human melanocytes and other epithelial cancer cell lines from breast, lung and colon cancers (Sánchez-del-Campo et al., 2008; Sánchez-del-Campo et al., 2009b). Next, we designed experiments to throw light on the elevated activity of TMECG on melanoma cell lines. As one of the most striking differences between melanoma and other epithelial cells is the presence of tyrosinase in melanoma, we wondered whether TMECG cytotoxicity against melanoma might be mediated by cellular tyrosinase activation. The results indicated that tyrosinase oxidized TMECG to its corresponding o-quinone, which quickly evolved through a series of chemical reactions to a quinone methide (QM), which showed high stability over a wide pH range (Fig. 3).
Fig. 2. Synthesis of TMECG (6) and TMCG (8). Reagents and conditions: (a) benzyl bromide, K$_2$CO$_3$, N,N-dimethylformamide, $-10^\circ$C to rt; (b) Dess-Martin periodinane, moist CH$_2$Cl$_2$, rt; (c) L-Selectride, n-Bu$_4$NCl, THF, $-78^\circ$C; (d) 3,4,5-trimethoxybenzoyl chloride, 4, CH$_2$Cl$_2$, DMAP, rt; (e) H$_2$, 20% Pd/C, THF/MeOH, rt.

Fig. 3. Reaction sequences indicating the oxidation of TMECG and TMCG by tyrosinase and the formation of quinone methide (QM) species.

\[ \text{TYR} + O_2 \rightarrow \text{TYR}^* \]

\[ \text{TMECG} \rightarrow \text{QM}_{\text{TMECG}} \]

\[ \lambda_{\text{max}} = 275, 412 \text{ nm} \]

\[ \text{Neutral pH} \]

\[ \text{GM} \rightarrow \text{QG}_{\text{GM}} \]

\[ \lambda_{\text{max}} = 275, 470 \text{ nm} \]

\[ \text{Acidic pH} \]
The TMECG-QM was found to be a potent irreversible inhibitor of human DHFR, and this highly stable product may be responsible for TMECG’s high activity against melanoma cells (Sánchez-del-Campo et al., 2009b). To explain the irreversibility on the binding of TMECG-QM to human DHFR, we performed in silico molecular modelling experiments. TMECG bound to human DHFR in a similar way to that described for EGCG binding (Navarro-Perán et al., 2005a), with specific hydrogen bonding interactions, most notably involving Glu-30 (Fig. 4). However, the open structure of QM increases its molecular flexibility, and it adopts a different conformation in the active site of human DHFR (Fig. 4). QM maintained the hydrogen bond with the Glu-30 side chain (O · · · ··O distance 1.99 Å), but three new interactions were detected. The other phenolic group of ring A forms a hydrogen bond with Ile-7, whereas the other two hydrogen bonds formed between two oxygens of the methoxy groups of ring D and Ser-59 and Ile-60. This strong interaction between QM and different residues of the protein could explain the irreversibility of the inhibitor-protein complex.

Prodrugs are compounds that must be transformed before exhibiting their pharmacological action. They are often divided into two groups: those designed to increase bioavailability to improve the pharmacokinetics of antitumor agents, and those designed to deliver antitumor agents locally (Rooseboom et al., 2004). TMECG could, therefore, be considered an anticancer prodrug on melanoma since it showed both of these characteristics. Therapies with TMECG would increase bioavailability and would achieve high melanoma drug concentrations. The soft antifolate character (Graffner-Nordberg et al., 2004) of the prodrug (TMECG), its specific activation on melanoma cells and the fact that antifolates are more active on fast-dividing cancer cells make this compound ideal for the prevention and treatment of this skin pathology.

However, the synthesis of TMECG is difficult and results in low yields. Therefore, we synthesized a related-epimer compound with catechin configuration [3-O-(3,4,5-trimethoxybenzoyl)-(−)-catechin (TMCG)] (Fig. 2) (Sáez-Ayala et al., 2011). As observed in this figure TMECG and TMCG share the first synthesis step, but the yields of the other synthetic steps were significantly different. The overall yield of TMCG in the two steps of alkylation and deprotection was 88%; however, the overall yield of TMECG in the four steps of epimerisation of C-3 (oxidation and reduction), alkylation and deprotection was 16%. The difference between these yields was due to the limiting stereoselective reduction of compound 2 (Fig. 2), which gives moderate yield and purity and requires further purifications lowering the yield. Because of the absence of the limiting reduction step, the synthesis of TMCG was simpler (only three steps) and more economical (only common reagents). Since the active product of TMECG in melanoma is its QM derivative, we hypothesized that both TMECG and TMCG should have similar activity against these cancer cells (Sáez-Ayala et al., 2011). Oxidation of TMCG and TMECG by tyrosinase is predicted to generate the same final product because proton-catalysed hydrolysis of ring C would generate a freely rotating carbon (C-3), which should prevent epimeric differences in the QM product (Fig. 3). To confirm that TMECG and TMCG generate the same quinonic product after tyrosinase oxidation, both substrates were oxidised in vitro using mushroom tyrosinase as a catalyst. The final products of the corresponding oxidations were analysed and compared using several spectroscopic techniques. Tyrosinase oxidised TMECG and TMCG to stable final products, which varied in colour from yellow to orange depending on pH. The products had similar spectroscopic properties, with \( \lambda_{\text{max}} \) at 275/412 nm at acidic pH and 275/470 nm at higher pH values (pKa = 6.9). Thus, the UV-Vis spectroscopy data indicated that, as represented in Fig. 3, both TMEGC and TMCG generated the same QM...
product after tyrosinase oxidation. Mass spectroscopy confirmed these results, and the spectra of both final oxidation products exhibited the same molecular ion peak. High-performance liquid chromatography-mass spectrometry (HPLC-MS) revealed that the molecular weights of the compounds were 498.7 (for TMECG) and 498.8 (for TMCG), which correspond to the calculated mass of the QM product depicted in Fig. 3. Both molecules were analysed by MS/MS and produced the same daughter ion peaks at m/z 363 and m/z 287, corresponding to the loss of the dihydroxybenzoyl moiety and the trimethoxybenzoyl moiety, respectively.

![Fig. 4. Molecular modelling for the binding of TMECG and TMECG-QM to human DHFR.](image)

5. Antitumoral activity of TMECG in melanoma

5.1 TMECG inhibits folate metabolism and transport in melanoma

The folate cycle plays a central role in cell metabolism. Among its important functions are the delivery of one-carbon units to the methionine cycle for use in methylation reactions, and the synthesis of pyrimidines and purines. Several enzymes, including DHFR, TS and methyltetrahydrofolate reductase (MTHFR), participate in the activation and regeneration of folic acid coenzymes. Several studies have shown that the protein and mRNA levels of TS and DHFR are higher in tumor tissues and cancer cells than in their normal counterpart (Kufe et al., 1980; Rahman et al., 2004). Tumors with high levels of these enzymes are thought to undergo more active cellular proliferation, which, in turn, is associated with tumor invasiveness and metastasis. Having demonstrated the strong in vitro inhibition of human DHFR by QM, further experiments were designed to test its antifolate activity in culture systems. To investigate whether TMECG could interfere with folate metabolism in cancer cells, the levels of expression of these folate cycle genes were analyzed in melanoma SK-MEL-28 cells using real time PCR (Sánchez-del-Campo et al., 2008). As observed in other tumor tissues and cells, the genes involved in the metabolism of folic acid were highly overexpressed in melanoma cells compared with normal melanocytes. The levels of DHFR, TS and MTHFR mRNAs were calculated to be about 400-, 22- and 4-fold higher, respectively, in melanoma cells than in normal melanocytes. Treatment of SK-MEL-28 with TMECG produced a substantial and rapid downregulation of these genes, involving significant changes in the mRNA levels of the genes at 24 h of treatment. DHFR, TS and
MTHFR mRNA levels were similar to that detected in normal melanocyte cells after 5 days of TMECG treatment. The data indicated that TMECG disturbs the folate metabolism in melanoma cells and suggest that this might be the mechanism by which TMECG induces cell growth inhibition and death. It is widely accepted that antifolates block the de novo biosynthesis of thymine, purines and pyrimidines by inhibiting the synthesis of THF, an essential cofactor in these biosynthetic pathways, and that the administration of exogenous reduced folates, such as leucovorin (5-formyl-THF), or growing the cells in an HT-medium, effectively prevent antifolate cytotoxicity in mammalian cells. We observed that TMECG was more active on cells growing in a normal culture medium than in an HT medium (Sánchez-del-Campo et al., 2009b); however, leucovorin did not “rescue” SK-MEL-28 cells from TMECG-induced death. Recently, it has been reported that natural tea catechins inhibit folate transport in Caco-2 cells, which might be partly responsible for their antifolate activity (Alemdaroglu et al., 2007). Therefore, to understand the different response of SK-MEL-28 to leucovorin- and HT-treatments, and to prove or discard the in vivo antifolate activity of TMECG, the status of the RFC, the major protein involved in the transport of reduced folates, was analyzed in this cell line. RFC mRNA expression was significantly higher in SK-MEL-28 than in normal human melanocytes (8.9 times), but treating SK-MEL-28 with TMECG strongly downregulated RFC gene expression. The time-dependent effect of TMECG on SK-MEL-28 was studied using RT PCR, and the data indicated that cells responded quickly to TMECG treatment with a more than 80% reduction in RFC expression 24 h after treatment. Protein levels of this transporter correlated with gene expression (Sánchez-del-Campo et al., 2009b). This finding could explain why leucovorin did not affect TMECG treatments. As demonstrated, TMECG highly down-regulated the RFC and, therefore, SK-MEL-28 cells became practically impermeable to leucovorin during TMECG treatment and can not, therefore, restore the reduced folate levels in the cells.

5.2 TMECG downregulates DHFR expression in melanoma

As described for other cancer cell lines, DHFR is overexpressed in melanoma (Kufe et al., 1980). The level of DHFR polyA+ mRNA in these melanoma cells was estimated to be 400 to 500 times higher than in normal human melanocytes, resulting in increased DHFR protein content (Sánchez-del-Campo et al., 2009b). An increase in DHFR expression/activity after antifolate treatment has been recognized as a mechanism of resistance of cancer cells to antineoplastic drugs. As expected, treatment of SK-MEL-28 with MTX resulted in a significant increase of DHFR mRNA and protein. However, treatment of SK-MEL-28 with TMECG rapidly reduced DHFR mRNA and protein to normal levels. The efficient down-regulation of DHFR by TMECG is evidence of its proposed antifolate activity. The lack of reduced folate coenzyme recycling by DHFR could be one of the reasons for its in vivo activity. The mechanism by which TMECG-QM downregulates DHFR expression in melanoma has been studied in more detail (Sánchez-del-Campo et al., 2010b). TMECG-QM has a dual action on these cells. First, it acts as a potent antifolate compound, disrupting folate metabolism and increasing intracellular oxidized folate coenzymes, such as DHF, which is a non-competitive inhibitor of dihydropteronine reductase, an enzyme essential for tetrahydrobiopterin (H_{4}B) recycling. Such inhibition results in H_{4}B deficiency, endothelial nitric oxide synthase (eNOS) uncoupling and superoxide production. Second, TMECG-QM
acts as an efficient superoxide scavenger and promotes intra-cellular $H_2O_2$ accumulation. We presented evidence that TMECG markedly reduces melanoma $H_2B$ and NO bioavailability and that TMECG action is abolished by the eNOS inhibitor $\text{NO}_\text{o}$-nitro-$L$-arginine methyl ester or the $H_2O_2$ scavenger catalase, which strongly suggested $H_2O_2$-dependent DHFR downregulation (Sánchez-del-Campo et al., 2010b). It is this duality that may be of importance for TMECG action. Classical antifolate compounds are very effective in inhibiting DHFR but the lack of an antioxidant property may be the cause of the lack of DHFR downregulation. In cells treated with antifolates, the common effect is the overexpression of DHFR, which may be responsible for cell resistance to antifolates and is probably the reason that these compounds fail to treat melanoma (Zhao & Goldman, 2003). In addition, antioxidant therapies have little effect on melanoma. It is interesting to speculate that unsatisfactory outcomes of some of these antioxidant therapies are partially due to their ineffectiveness in uncoupling the eNOS reaction (Chalupsky & Cai, 2005).

5.3 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 (Sánchez-del-Campo & Rodríguez-López, 2008). The results indicated that the reduced viability of melanoma cells in the presence of TMECG 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 SK-MEL-28 cell line. SK-MEL-28 cells exposed to 50 $\mu$M 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. 5). Another feature of apoptotic cell death, the activation of caspase-3, was evaluated by a colorimetric activity assay and Western blot analysis. The cells treated with TMECG showed significantly higher caspase-3 activity. Immunoblot analysis confirmed caspase-3 activation (Fig. 5). 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 (Nihal et al., 2005), 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. Immunohistochemistry results also indicated that TMECG treatment caused mitochondrial translocation of Bax, which is a common response of cancer cells subject to certain apoptotic stimuli (Choi & Singh, 2005).

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 (Soengas et al., 2001). 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 (Soengas et al., 2001). To
evaluate the participation of Apaf-1 in SK-MEL-28 apoptosis during TMECG treatment, its mRNA and protein levels were determined by PCR and Western blot, respectively (Sánchez-del-Campo & Rodríguez-López, 2008). Apaf-1 mRNA and protein were detected in SK-MEL-28 and their levels were essentially the same than those detected in normal human melanocytes. 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 SK-MEL-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 (Zanon et al., 2004).

5.4 TMEGC inhibits growth and metastasis of induced melanoma tumors in mice
To check whether TMECG was pharmacologically active in in vivo situations and to study the possible inactivation of TMECG in the body, we performed experiments to test the effectiveness of this compound in induced melanoma tumors in mice. The group receiving TMECG therapy showed significantly longer survival times than the control group (Fig. 6). Moreover, we observed that tumor growth was significantly reduced by the treatment with TMECG but not with MTX (Fig. 6). The observation that TMECG-treated animals survived with larger tumors indicated that treatment could also reduce the metastasis of primary tumors. To confirm this, a third group was inoculated with B16 melanoma cells and treated with TMECG for 21 days (median survival time of the control group). After this time the
animals were sacrificed and a post-mortem examination of the lungs was performed to search for metastatic lesions. Secondary metastasis in the lungs was more frequent in control mice (Fig. 6), while treatment with TMECG drastically reduced lung metastasis. The lungs of the control animals showed prominent tumor nodules around the terminal bronchioles. These tumor nodules were composed of polygonal tumor cells with a prominent nucleolus. Intracellular melanin deposition and clear areas of necrosis were also detected. The lungs of the TMECG-treated tumor-bearing animals showed no significant tumor mass. The alveoli and pleura were tumor free, and the alveolar passage was lined with healthy ciliated columnar epithelial cells.

Fig. 6. Inhibition of melanoma growth and metastasis by TMECG: (a) Median survival time following tumor implantation; (b) Mean tumor size in C57/B16 mice bearing B16 melanomas. (c) Rate of metastasis in post mortem and histological examination of lungs showing general aspect and histopathological analysis of lungs from C57/B16 mice bearing the B16 melanoma in control and TMECG-treated animals (Sánchez-del-Campo et al., 2009b).

5.5 Proposed mechanism for the activation and antitumoral activity of TMEGC on melanoma

Taken in consideration all these observations a mechanism for the activation, cellular distribution and action of TMECG and its products may now be proposed (Fig. 7). Although TMECG efficiently binds to DHFR, we hypothesized that this hydrophobic compound would cross the cell membrane without needing to bind to folate transporters. Therefore, the most plausible transport mechanism for this lipophilic drug is passive diffusion across the plasma membrane in a manner driven solely by the concentration gradient (Fig. 7a). Its independence from folate transporters means that TMECG may avoid transport-mediated resistance mechanisms (Ma et al., 2000). In fact, TMECG downregulated RFC but, far from...
Fig. 7. Schematic mechanism underlying activation, cellular distribution and action of TMECG in melanoma cells (Sánchez-del-Campo et al., 2010b).

being a disadvantage, this could represent an important advantage for its antiproliferative action. Downregulation of RFC would reduce intracellular folate pools, reducing competition reactions with the active form of TMECG and the molecular target, DHFR. Subsequent transport to the melanosome by a concentration gradient would facilitate its tyrosinase-catalyzed oxidation and transformation to the corresponding QM (Fig. 7b). Observations from our laboratory indicated that TMECG or TMECG-QM did not interact with DOPA-melanins, which may avoid melanosomal trapping. Because of the low pH of this organelle, the predominant form is QMH, which, due to its high stability and the absence of formal charge, would exit the melanosome and enter the cytosol (Fig. 7c). Under the slightly basic pH of the
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cytosol, the anionic form of QM would be the predominant form, and it would be trapped in
this compartment due to its formal negative charge. This retention mechanism would
represent another advantage with respect to antifolates that require polyglutamylation for
cellular retention. This would be even more essential in cancer cell environments, in which
folate requirements lead to an increase in the cellular THF-cofactor pools. As the THF-cofactor
pool is increased, there is a feedback inhibition of folate polyglutamylation, which results in
the accumulation of easily exported compounds (Tse & Moran, 1998). Once in the cytosol,
TMECG-QM may inhibit DHFR activity and, in addition, lead to the downregulation of DHFR
(Fig. 7d-h). The finding that tyrosinase is highly overexpressed in melanoma cells with respect
to normal melanocytes might also influence the degree, the specificity and the duration of the
antifolate inhibitory effect of TMECG-QM. Finally, the data on the effectiveness of TMECG in
mouse melanoma models indicates that TMECG is bioavailable for cancer cells and avoids not
only cellular mechanisms of resistance to antifolates but also mechanisms related with body
drug inactivation.

6. Combined therapies to improve the activity of synthetic catechins in melano

The combination of several drugs to improve the clinical efficiency of the treatments is a
common strategy for the fighting of pathological bacteria. For instance, TMP is used in
combination with sulphonamides as a therapy against multiple bacterial infections. These
are treatment based in the metabolic attack of the bacteria, where TMP inhibits the recycling
of folic acid coenzymes and the sulphonamide inhibits the synthesis of folic acid.
Conventional chemotherapy treatments against cancer have shown important clinical
limitations and, therefore, it would be of interest to develop new and more efficient
strategies for the treatment of this fatal pathology. The metabolic attack of cancer cells could
be an interesting perspective. The identification of metabolic and signalling pathways
essential for tumour growth and dissemination, but less important for healthy cells, could
guide the development of new cancer therapies. The combination of several drugs to target
several metabolic and/or signalling pathways could increase the efficiency of the
 treatments. Recently, it has been observed that the major catechin presented in green tea,
EGCG, showed synergic activity with other drugs commonly used for the treatment of
several cancers and in some cases EGCG sensitizes cancer cells to other treatments (Liu et
al., 2001; Farabegoli et al., 2007). The identification of synthetic catechins as antifolate
compounds can help the design of more directed combined therapies against melanoma
since the folic acid has multiple connections with metabolic and signalling pathways. Thus,
we started this application targeting the methionine cycle in melanoma using TMECG in
combination with other drugs (Fig. 8).
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. 8) (Cellarier et al., 2003). 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 (Anderson, 1998). Third, it is required for the formation of polyamines, which have
far-ranging effects on nuclear and cell division (Thomas & Thomas, 2001). Fourth,
methionine is the major source of the methyl groups necessary for the methylation of DNA
and other molecules (Cellarier et al., 2003). 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. 8). Folic acid acts as the fuel for the methionine cycle, which, after transformation by folate cycle enzymes such as DHFR, TS and MTHFR, forms 5-methyl-THF, the cofactor of methionine synthase (MS), the enzyme responsible for methionine synthesis (Fig. 8). 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 equilibrative nucleoside transporters (ENTs), is of vital importance for cancer cell survival.

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. We observed that TMECG modulated the expression of genes involved in methionine metabolism, cellular methylation and glutathione synthesis in melanoma cells (Sánchez-del-Campo & Rodríguez-López, 2008). Having elucidated the effects of TMECG on the melanoma folate and methionine cycles, we designed therapeutical strategies to increase its effectiveness. Combinations of TMECG with S-adenosylmethionine (SAM) 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.

Fig. 8. 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.

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6.1 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 methionine adenosyltransferase-2A (MAT2A) enzyme (Fig. 8). MAT2A shows a very low $K_m$ for methionine (4-10 $\mu$M) 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 at very low concentrations of methionine. Another kinetic characteristic of MAT2A is that this enzyme is strongly inhibited by SAM, its reaction product ($IC_{50} = 60 \mu$M) (Sullivan & Hoffman, 1983). 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. 8). Only high concentrations of SAM (up to 100 $\mu$M) affected SK-MEL-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 $\mu$M SAM with 50 $\mu$M TMECG efficiently inhibited the growth of SK-MEL-28. 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.

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

As observed in Fig. 8, 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 5-methyl-THF would result in the production of high concentrations of 5-adenosylhomocysteine (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. 8). Adenosine flux across the cellular membrane depends on the concentration gradients between extra- and intracellular nucleoside levels (Tabrizchi & Bedi, 2001). Therefore, we first tried to increase the extracellular concentration of adenosine. Adenosine alone had no detectable effect on SK-MEL-28 growth at the studied concentrations (up to 500 $\mu$M) but, in the presence of TMECG, adenosine had a significant synergistic effect, enhancing the antiproliferative action of this antifolate compound. A combination of 50 $\mu$M adenosine with 50 $\mu$M TMECG was seen to completely inhibit the growth of SK-MEL-28. 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 strategy 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. Dipyridamole alone inhibited SK-MEL-28 growth with a calculated IC_{50} (at 5 days) of 20 \mu M. However, in the presence of 50 \mu M TMECG, the IC_{50}-value fell to less than 1 \mu M. A combination of 5 \mu M dipyridamole with 50 \mu M TMECG had a deadly effect on melanoma cells. The results pointed to the possibility of using this combination treatment against malignant melanoma.

6.3 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 (Cellarier et al., 2003). 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. 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. Blockage of the methionine cycle by the new antifolate, TMECG, is an effective therapy for melanoma. The specific activity of TMECG on melanoma methionine cycle was confirmed by the high synergy found with compounds that uncoupled adenosine metabolism in these cells. In conclusion, our results show that TMECG is a potent antitumor agent that modulates multiple aspects of melanoma metabolism and survival, including the folic acid and the methionine cycles and the methylation status of the cells. This broad spectrum of antitumor activities in conjunction with low toxicity underlies the translational potential of TMECG and suggests it may be used as part of a therapeutic strategy against melanoma. A combination with other compounds that modulate melanoma methionine cycle, such as SAM, adenosine or dipyridamole, represents another promising strategy to combat this malignant skin pathology.

7. Conclusions
Several investigations indicate that natural and synthetic polyphenols may well be beneficial, not only in the prevention but also in the treatment of cancer. Antifolates are usually used as chemotherapeutic agents for certain types of cancer. Although antifolates such as MTX attack proliferating tissues selectively, they are also toxic to normal cells. Deleterious side effects are seen against tissues that proliferate as part of their normal function; such tissues include intestinal mucosa, hair cells, and components of the immune system. The “soft” character of polyphenols could be developed for use in the treatment of cancer with significantly reduced side effects compared to those of the DHFR inhibitors currently in use in chemotherapy such as MTX. Moreover, we have demonstrated that synthetic derivatives of ECG, TMECG and TMCG, might be considered a treatment for melanoma. Disruption of the cell folate cycle by these compounds and their active products may explain many of the molecular and cellular effects described for these synthetic compounds.
polyphenols on melanoma cells because antifolates exert their action by disturbing the nucleic acid metabolism of cancer cells, including its synthesis, methylation and stability (Navarro-Perán et al., 2007; Sánchez-del-Campo & Rodríguez-López, 2008). We define them as new generation antifolates because they maintain the ability to inhibit DHFR and disrupt folate metabolism but show significant and important differences from other classical and non-classical antifolates. TMECG was seen to be active not only in melanoma cells in culture but was also effective in an animal model, where it inhibited growth and metastasis of preformed tumors. An additional advantage of these compounds is their prodrug character, which would favour its specific activity against melanoma cells and prevent unspecific side effects in rapidly dividing healthy cells. In addition, the multiple connections of the folic acid cycle with many metabolic and signalling pathways in melanoma cells could open the way for the development of new combined and directed therapies against this elusive skin pathology.

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9. References


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The book Research on Melanoma: A Glimpse into Current Directions and Future Trends, is divided into sections to represent the most cutting-edge topics in melanoma from around the world. The emerging epigenetics of disease, novel therapeutics under development and the molecular signaling aberrations are explained in detail. Since there are a number of areas in which unknowns exist surrounding the complex development of melanoma and its response to therapy, this book illuminates and comprehensively discusses such aspects. It is relevant for teaching the novice researcher who wants to initiate projects in melanoma and the more senior researcher seeking to polish their existing knowledge in this area. Many chapters include visuals and illustrations designed to easily guide the reader through the ideas presented.

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