Chapter 14

Cellular and Molecular Mechanisms of Methotrexate Resistance in Melanoma

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1. Introduction

Melanoma is a cancer that develops in melanocytes, the pigment cells present in the skin. It can be more serious than the other forms of skin cancer because it may spread to other parts of the body (metastasize) and cause serious illness and death. For malignant melanomas standard treatment options have remained remarkably static over the past 30 years [1,2]. 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 the American Cancer Society estimate, there will have been approximately 76,250 new cases of invasive melanoma diagnosed in 2012 in the United States, which resulted in approximately 9,180 deaths [3].

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% [4]. 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. For instance, the only approved chemotherapy for metastatic melanoma, dacarbacine, has a response rate of about 10% and a median survival of 8-9 months.

The other approved agent for advanced melanoma is high dose interleukin-2, which can induce dramatic complete and durable responses [2]. However, only one patient in twenty derives lasting benefit. These data indicate the needed for alternative therapies for this disease and recent results indicated that combined therapies could became an attractive strategy to fight melanoma [2].

Other 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 [5-7]. Undoubtedly, unravelling the mechanisms of melanoma resistance to MTX could yield important information on how to circumvent this resistance and could have important pharmacological implications for the design of novel combined therapies. Thus, although an old drug, MTX could become a valuable tool with which to improve melanoma therapy.

2. General mechanisms of resistance to classical antifolates

The antifolate methotrexate was rationally-designed nearly 70 years ago to potently block the folate-dependent enzyme dihydrofolate reductase (DHFR). DHFR (5,6,7,8-tetrahydrofolate: NADP+ oxidoreductase, EC 1.5.1.3) catalyses 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 (TS) [8], 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 [9] 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 tumours propagated *in vitro* and *in vivo* [5,10,11]; 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 [11]. However, recently, a melanoma-specific mechanism of resistance to cytotoxic drugs, including MTX, has been described [6,12,13].

Antifolate resistance in cancer cells is believed to be a multifactorial process in which dysregulation of apoptosis, insufficient rates of MTX polyglutamylation, and enhanced DNA repair play important roles [11,14]. In melanoma, another classical mechanism of resistance to MTX, the upregulation of endogenous dihydrofolate reductase (DHFR) activity, has been described [5]; however, the contribution of this mechanism to the overall resistance of melanoma to MTX as well as its possible impact on DNA damage response pathways in cells is unknown. 'Thymineless' death, which occurs upon the depletion of cellular dTTP pools, has been proposed

as a mechanism by which antifolate drugs promote apoptosis in cancer cells [15,16]. Although the mechanism of dTTP depletion-induced apoptosis is yet to be determined, Pardee's group recently postulated that dTTP controls E2F1, which regulates both DNA synthesis and apoptosis. This hypothesis was based on the observation that MTX increased E2F1 levels in sensitive cancer cells, resulting in an increase in the E2F1-mediated apoptotic cascade.

Eukaryotic cells have developed complex checkpoint pathways that monitor DNA for damage or incomplete replication. Checkpoint pathways are amplified upon detection of aberrant DNA structures and lead to a delay in cell cycle progression during which damage can be repaired or replication be completed. Alternatively, in case of heavily damaged or seriously deregulated cells, checkpoint activation can result in apoptosis. As such, checkpoint mechanisms are essential for the maintenance of genomic integrity [17]. When vertebrate cells experience replication arrest or undergo DNA damage by UV irradiation, the ATR kinase [ataxia telangiectasia mutated (ATM)- and Rad3-related kinase] phosphorylates and activates the Chk1 protein kinase. Activated Chk1 inhibits Cdc25 phosphatases, which control inhibitory phosphorylation sites on cyclin-dependent kinases, the latter being critical regulators of cell cycle transitions [18,19]. Because the ability of cells to delay cell cycle progression and halt DNA synthesis represents a defensive mechanism that spares potential toxicity [20], the activation of Chk1 by MTX could constitute a key event in the resistance of melanoma to MTX.

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 [21]. 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 [11] and therefore, this transformation has been proposed as a novel mechanism of resistance to explain this paradox [11,21]. 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 [22].

3. Melanoma-specific mechanisms of resistance to MTX

3.1. The critical role of alpha-folate receptor in the resistance of melanoma to MTX

Experiments from our laboratory and others provide evidence that melanosomes contribute to the refractory properties of melanoma cells by sequestering cytotoxic drugs and increasing melanosome-mediated drug export [6,12,13]. 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 [6]. 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 [23]. 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 [5] 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 [23].

A defect in intracellular folate retention is another recognized mechanism of drug resistance [5,10,11,21]. In addition to a decrease in antifolate polyglutamylation, melanoma cells may also export cytotoxic drugs by melanosome sequestration [12]. 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 [6]. 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 [24]. Recently, Xie and collaborators [13] 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 [25,26]; 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 [26] and/or induces a failure of lysosomal acidification, which is essential for normal endocytosis [27], remains to be determined.

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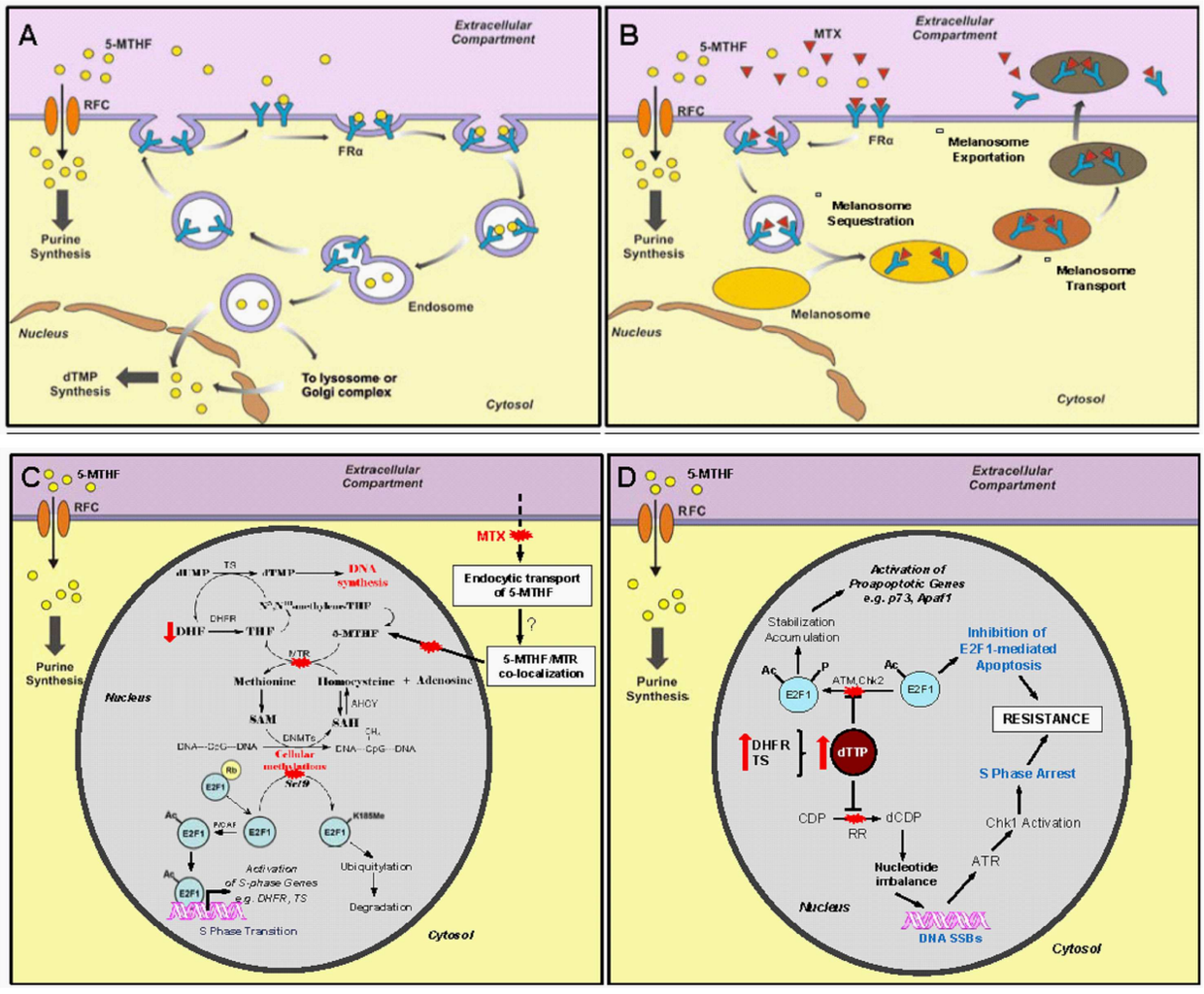


Figure 1. A) Possible mechanisms for transport and trafficking of folates in melanoma cells. (B) Mechanisms to explain the MTX-induced depletion of DHF in melanoma cells. (C) Folate deficiency induces DHF depletion and enhances the transactivational potential of E2F1. (D) Excess of dTTP inhibits E2F1-mediated apoptosis and activates Chk1 in melanoma cells. High levels of DHFR and TS could reactivate *de novo* dTMP biosynthesis impeding depletion of dTTP. Excess of dTTP would prevent apoptosis by several mechanisms. First, dTTP is an allosteric inhibitor of ribonucleotide reductase (RR), the enzyme which reduces cytidine diphosphate (CDP) and uridine diphosphate to dCDP and dUDP.

To explore the relationship between MTX exportation and melanosome trafficking, we studied the possible interaction of MTX with melanin [6]. Such interaction was confirmed by incubating this drug with synthetic 3,4-dihydroxyphenylalanine (DOPA)-melanin. Importantly, folic acid and 5-methyl-THF (5-MTHF), 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-MTHF) 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 [28]. 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.

3.2. MTX disrupts folate trafficking in melanoma cells

Although MTX is exported within a few hours in contact with cells, in this short time, MTX is capable of inducing important changes in folate metabolism by depleting dihydrofolate (DHF) early on and by inducing the expression of folate-dependent enzymes later on [7]. The increased expression of DHFR is a common occurrence in melanoma and other cancer cells in response to MTX treatment; however, the observed depletion of DHF was completely unexpected. The pathways that comprise folate-mediated one-carbon metabolism have been suggested to function in a metabolic network that interconnects the three biosynthetic pathways, namely *de novo* purine biosynthesis, *de novo* dTMP biosynthesis, and homocysteine remethylation. Recent studies provide direct evidence for cell cycle–dependent nuclear dTMP biosynthesis in the nucleus [29]. However, there are many unanswered questions regarding the role and regulation of nuclear *de novo* dTMP biosynthesis. Nothing is known about the transport, processing, and accumulation of folates into the nucleus, the one-carbon forms of folate present in the nucleus, and the relationship between cell cycle dependency of *de novo* dTMP biosynthesis and cell cycle-dependent accumulation of nuclear folate [29]. Although there is no data of how the homocysteine remethylation cycle is compartmentalized, the observation that MTX affected both DHF synthesis and E2F1 methylation (see below) seem to indicate that both the *de novo* dTMP biosynthesis and the homocysteine remethylation cycles might operate simultaneously in the nucleus.

Using HeLa and MCF-7 cells, Stover and coworkers observed that cytoplasmic serine hydroxymethyltransferase (SMTH), TS, and DHFR are all translocated into the nucleus during S and G_2/M phases following their modification by the small ubiquitin-like modifier (SUMO) [30,31]. This finding indicated that the folate cycle may be compartmentalized and that dTMP and DHF synthesis may occur in the nucleus during DNA synthesis. In a recent study, Wollack et al. [32] characterized 5-MTHF uptake and metabolism by primary rat choroid plexus epithelial cells *in vitro*. They distinguish two different processes for 5-MTHF transport, one that was FRα dependent and the other that was independent of this receptor and mediated by the proton couple folate transporter or reduced folate carrier (RFC). This investigation revealed that cellular metabolism of 5-MTHF depends on the route of folate entry into the cell. Thus, 5-MTHF taken up via a non-FRα–mediated process was rapidly metabolized to folylpolyglutamates, whereas 5-MTHF that accumulates via FRα remained non-metabolized and associated to endocytic compartments. The observation that MTX induces the overall depletion of FRα in

melanoma cells [6] would suggest that MTX might also induce depletion of reduced folate coenzymes associated to endocytic compartments (Figure 1A and 1B). Therefore, a possible explanation for the depletion of DHF during MTX exposure could be that this drug diminishes the required supply of folates to the nucleus for the maintenance of both dTMP and DHF synthesis; however, how melanoma cells can control endocytic pathways to supply their own nucleus with folates is unknown. Recent studies have indicated that some endocytic proteins are also involved in direct signaling pathways from membranes to the nucleus, and mechanisms for the nuclear translocation of intact or fragmented endosome-localized proteins have been identified [33]. Another possibility is the existence of a late endosome-lysosome transport mechanism for folate [34]. The proximity of lysosomes to the nucleus suggests that folates could be released into the perinuclear region of the cytoplasm, perhaps facilitating their nuclear entry during cell division following the disassembly of the nuclear membrane [29].

Although the uptake of 5-MTHF into mammalian cells is mainly mediated by the RFC, the expression of FRα in several epithelial tissues and especially its overexpression in cancerous cells indicate that this receptor may confer a growth advantage to these cells [35]. The high affinity of FRα for 5-MTHF suggest that this GPI-anchored receptor may play an important role in maintaining nuclear folates even at low extracellular concentrations of this vitamin. This hypothesis is supported by the finding that induction of FRα expression in cells that normally do not express this receptor allows the cells to grow in low nanomolar folate concentrations [36]. On the other hand, the observation that methionine synthase was localized in the nucleus of melanoma cells could explain many of the unanswered questions on the role and regulation of the folate metabolism in the nucleus of these cancer cells. The methionine synthase -mediated catalysis of 5-MTHF would first supply THF and methionine to maintain both dTTP synthesis and the methylation reactions in the nucleus of the cells (Figure 1C) and second would prevent the nuclear accumulation of 5-MTHF, a potent inhibitor of SHMT [29]. Therefore, in melanoma, the existence of a specific folate transport pathway from the plasma membrane to the nucleus, mediated by FRα, is possible [37] and could shed light on the unknown function of overexpressed FRα in cancer cells [38].

4. Melanoma coordinates general and cell-specific mechanisms to promote MTX resistance

4.1. MTX induces E2F1 demethylation and prevents dTTP depletion in melanoma

MTX acts as a cytostatic agent in melanoma cells [6]. To discriminate between the mechanisms by which MTX could induce cell growth arrest without inducing apoptosis, the effect of this drug on the cell cycle of several melanoma cell lines was analysed [7]. The results indicated that, in all the tested melanoma cell lines, MTX conferred an arrest in early S phase; the G_1 peak shifted toward the G_1/S border, and cells were arrested with a minimal increase in their DNA content. Because S phase arrest has been recognized as a major mechanism of resistance in response to non-toxic concentrations of drugs that induce DNA replication stress, these pre-

liminary results suggest that moderate DNA damage could be responsible for the cytostatic effect of MTX on melanoma cells.

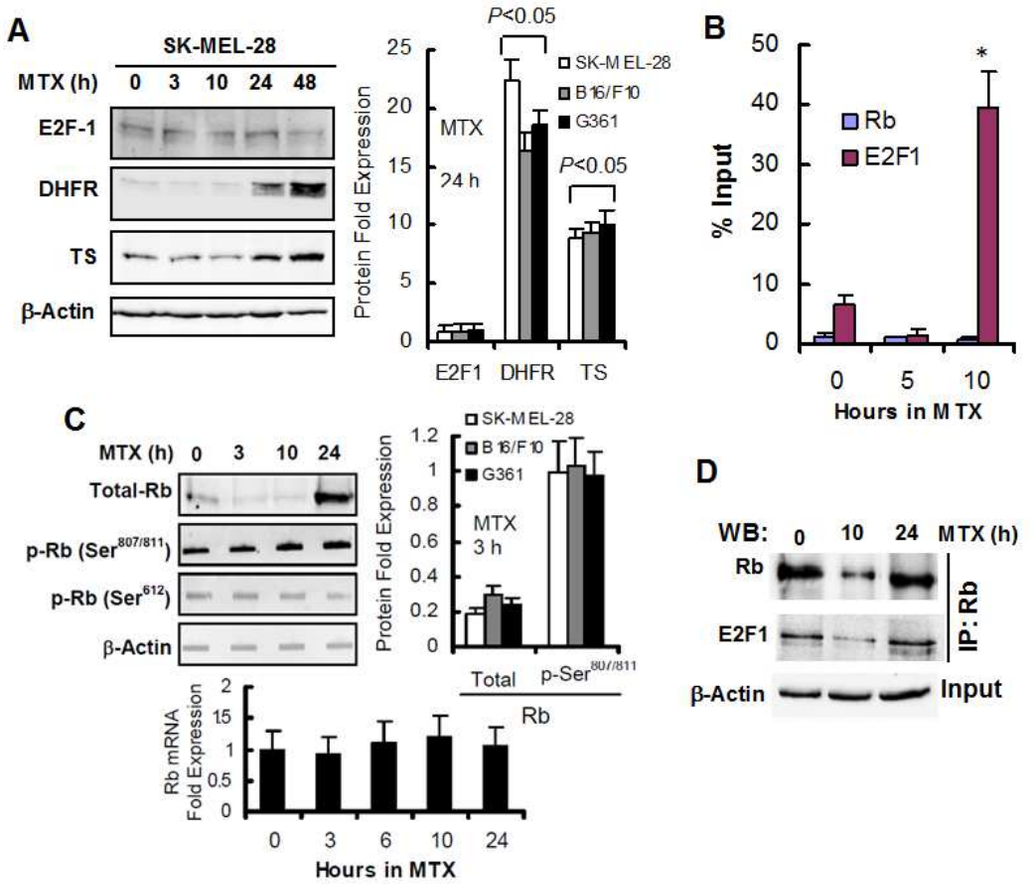


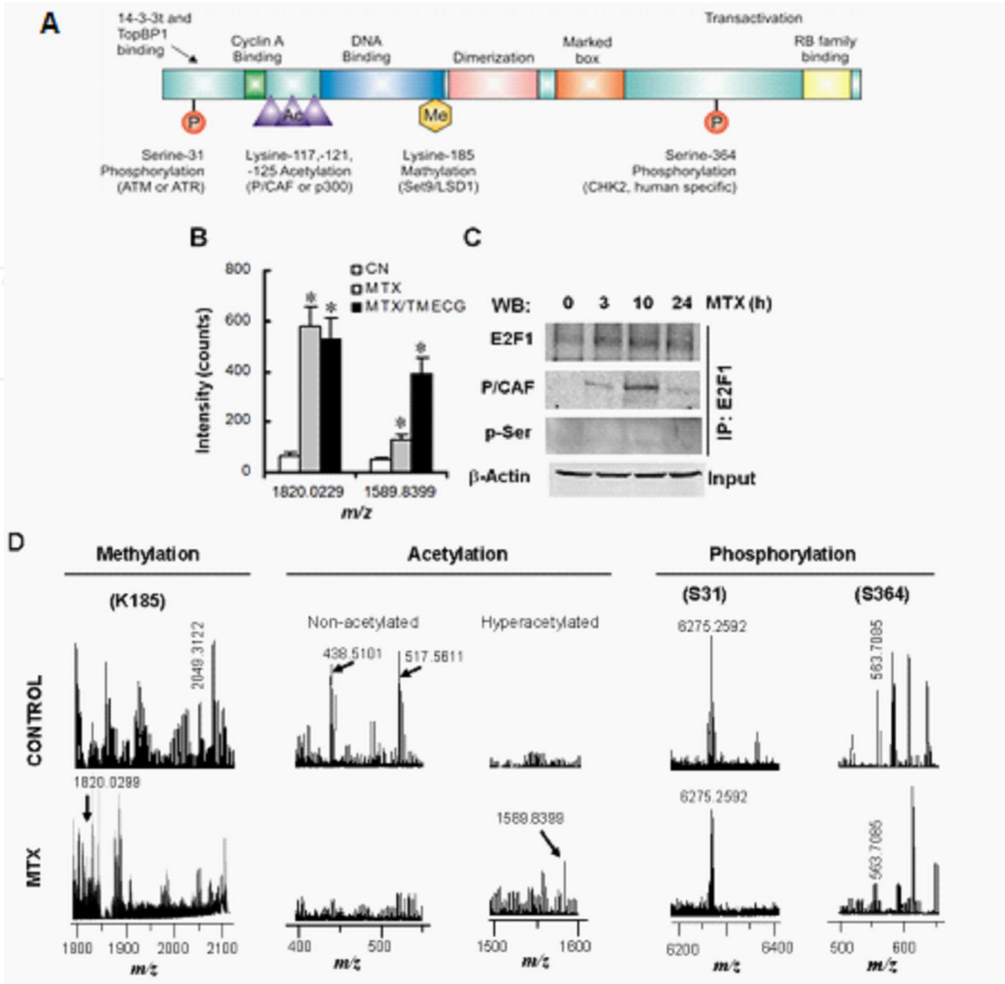
Figure 2. MTX enhances the transactivation potential of E2F1 in melanoma cells. (A) The time-dependent effect of MTX treatment (1 μM) on the expression of E2F1, DHFR, and TS proteins as assayed by western blot (WB). (B) ChIP experiments showing the occupancy of E2F1 and Rb on the DHFR promoter of B16/F10 melanoma cells (*$P < 0.05$). (C) The upper panels represent the time-dependent effects of MTX (1 μM) treatment on the expression and phosphorylation state of the Rb protein as assayed by WB. The lower panel depicts the Rb mRNA expression as assayed by qRT-PCR. The changes observed after MTX treatment were not statistically significant. (D) Co-immunoprecipitation assays were performed to test the interaction between Rb and E2F1.

To understand the mechanisms involved in G_1 cell cycle progression in MTX-treated melanoma cells, the effect of this drug on several G_1 cell cycle components was analysed. Although the protein levels of E2F1 were not affected by MTX (Figure 2A), this drug significantly increased the protein levels of DHFR and thymidylate synthase (TS), two E2F1-target genes involved in folate metabolism and required for G_1 progression and DNA synthesis (Figure 2A). Chromatin immunoprecipitation (ChIP) experiments that were designed to analyze the occupancy of E2F1 on the DHFR promoter of B16/F10 melanoma cells indicated that MTX stimulated the transcriptional activity of E2F1 (Figure 2B). First, we observed that MTX induced a transient decrease in the hypophosphorylated Rb protein in melanoma cells (Figure 2C) as evidenced by a noticeable lack of Rb co-immunoprecipitation with E2F1 in 10 h MTX-treated SK-MEL-28 cells when compared to untreated controls (Figure 2D). In addition, mass

peptide analysis of immunoprecipitated E2F1, after trypsin digestion (Figure 3), indicated that MTX promoted the demethylation of E2F1 at Lys185 (Figures 3B and 3D). A negative crosstalk between methylation and other posttranslational modifications of E2F1, such as acetylation and phosphorylation, has been recently described [39]. We observed that MTX induced the transient co-immunoprecipitation of E2F1 with p300/CBP-associated factor (P/CAF) (Figure 3B), an interaction that has been associated with the transcriptionally active hyperacetylated form of this transcription factor [40]. The hyperacetylated status of E2F1 after MTX treatment was also confirmed by MALDI-TOF mass spectrometry (Figures 3B and 3D). In response to severe DNA damage, the E2F1 protein is stabilized through distinct mechanisms, including direct phosphorylation by Chk2 at Ser^{364} [41] or ATM kinase at Ser^{31} [42]. As we did not observe phosphorylation of E2F1 after MTX treatment (Figures 3C and 3D), these data further suggest that MTX induced moderate DNA damage without inducing double strand breaks (DSBs) [43].

MTX increased E2F1 levels in sensitive cancer cells [16]. However, we did not observe an MTX-mediated increase in E2F1 levels in melanoma cells (Figure 2A) [7], a result that could be explained, at least in part, by the results obtained after determination of dNTP pools in melanoma cells (Figure 4). Contrary to the effects of MTX in most cancer cells [16], this drug increased the levels of dTTP in melanoma. Increased levels of dTTP were accompanied by a decrease in dCTP levels, which resulted in a nucleotide imbalance that favored thymidine excess. The MTX-induced expression of DHFR and TS (Figure 2A) and the low levels of MTX accumulated in melanoma cells [6] could explain this paradoxical response of melanoma cells to a cytotoxic drug that typically depletes dTTP levels.

The data obtained in our study indicate that melanoma cells respond to the lack of folate coenzymes by enhancing the transactivational potential of E2F1. We observed that treatment of melanoma cells with MTX transiently affected the stability of Rb and the posttranslational state of E2F1 [7]. A crosstalk between the methylated and acetylated forms of E2F1 has been suggested [39]. Methylated E2F1 is prone to ubiquitination and degradation, whereas the demethylation of E2F1 favors its P/CAF-dependent acetylation. Together, the results suggest a model whereby the MTX-induced degradation of Rb and the demethylation of E2F1 would result in the accumulation of E2F1 in its 'free' state, and in the absence of DNA damage, free E2F1 would be acetylated, leading to the transcription of genes required for S phase (Figure 1C). The activation of E2F1 by MTX would allow S phase transition in melanoma cells, and importantly for melanoma survival, cells would recover an operative folate cycle, thereby restoring the original status of the Rb/E2F1 system. In the absence of exported MTX, high levels of TS and DHFR would impede the lethal depletion of dTTP and in turn, would produce a nucleotide imbalance that would favor a dTTP excess. Contrary to thymidine depletion, excess thymidine stops cells in S phase by blocking synthesis of DNA, an effect known as 'thymidine block' (Figure 1D) [15]. Recently, a mechanism by which dTTP allosterically feedback controls E2F1 has been proposed [15,16]. According to this mechanism, excess of dTTP inhibits E2F1 accumulation acting either upon production of E2F1 or its degradation. Because control of E2F1 is essential for cell survival, this mechanism would prevent E2F1 accumulation, which would result in activation of apoptosis through a process that involves p53 or p73, cytochrome c, and caspases (Figure 1D) [44].



Modification	E2F1 Status	Peptide Sequence	Measured (m/z)	Theoretical (m/z)
Methylation (K185)	Non-methylated	(K)NHIQWLGSHTTVGVGGR(L)	1820.0299	1820.0331
	Methylated	(K)SK$_{Me}$NHIQWLGSHTTVGVGGR(L)	2049.3122	2049.3140
Acetylation (K117,120,125)	Non-acetylated	(R)HPGK(G)	438.5101	438.5097
		(K)SPGEK(S)	517.5611	517.5625
	Hyperacetylated	(R)HPGK$_{Ac}$GVK$_{Ac}$SPGEK$_{Ac}$SR(Y)	1589.8399	1589.8394
Phosphorylation (S31)	Non-phosphorylated	(R)LLDSSQIVIISAAQDASAPPAPTGPAAPAAGPC(Carbamidomethyl)DPDLLLFATPQAPRPTPSAPRPALGRPPVK(R)	6275.2592	6275.2600
	Phosphorylated	(R)LLDSS$_P$QIVIISAAQDASAPPAPTGPAAPAAGPC(Carbamidomethyl)DPDLLLFATPQAPRPTPSAPRPALGRPPVK(R)	6355.2361	6355.2398
Phosphorylation (S364)	Non-phosphorylated	(R)MGSLR(A)	563.7085	563.7006
	Phosphorylated	(R)MGS$_P$LR(A)	643.6799	643.6804

Figure 3. MTX induces demethylation and hyperacetylation of E2F1 in melanoma cells. (A) Schematic representation of the E2F1 protein. Residues susceptible to methylation (K185), acetylation (K117, K120, and K125), and phosphorylation (S31 and S364) are shown. (B) Relative intensity of unmethylated [(K)NHIQWLGSHTTVGVGGR(L); m/z 1820.0229] and hyperacetylated [(R)HPGKAcGVKAcSPGEKAcSR(Y); m/z 1589.8399] peptides in E2F1-trypsin digested samples. Peptides were analyzed in untreated SK-MEL-28 cells (CN) or treated for 10 h with 1 μM MTX (*$P < 0.05$). Intensities were normalized with respect to an internal matrix control. (C) Cell lysates from SK-MEL-28 cells that had been treated with 1 μM MTX were used for IP assays with E2F1 to test the co-immunoprecipitation of E2F1 with P/CAF and the phosphorylated state of E2F1. (D) MALDI-TOF mass spectra of tryptic digests of immunoprecipitated E2F1. The characteristics peptides involving posttranslational modifications of E2F1 (methylation = Me, acetylation = Ac, and phosphorylation = P), as well as their measured and theoretical m/z are shown.

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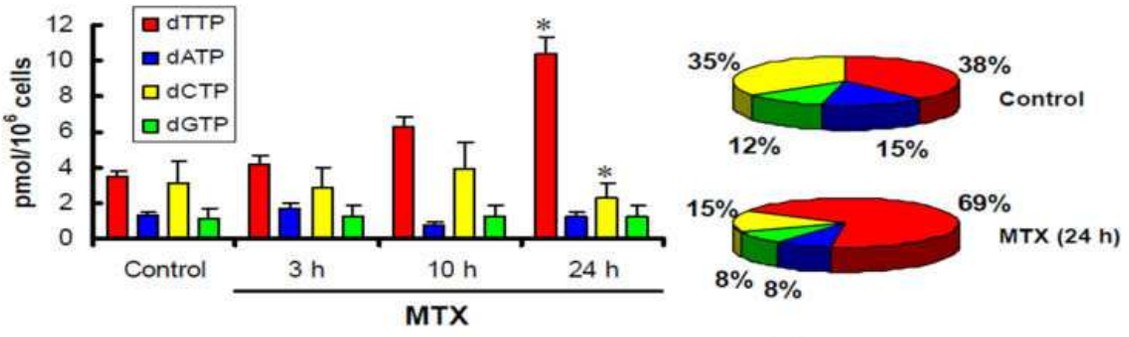

Figure 4. MTX does not deplete dTTP levels in melanoma cells. dNTP quantification in SK-MEL-28 control cells and cells subjected to MTX (1 μM) treatment (*P < 0.05). Data collected from the left panel was used to determine the total amounts of each dNTP at each time point. The percent contribution of each dNTP to the total pool after 24 h of treatment is represented.

4.2. Excess of dTTP favours Chk1 activation in melanoma after MTX treatment

Excess thymidine induces little detectable DNA damage in the form of DSBs. The ATR-mediated response appears to play a more prominent role under these cellular conditions [45]. As it is known that the central mechanism responsible for Chk1 activation upon DNA damage is the distribution of ATR into nuclear foci [46], the effects of MTX on the localization of ATR and the phosphorylation of Chk1 at Ser^{345} were analyzed by confocal microscopy and western blot, respectively (Figures 5A and 5B). Time- and dose-dependent experiments clearly indicated that MTX induced Chk1 phosphorylation in melanoma cells. Because Chk1 phosphorylation may not directly correspond to Chk1 activation, we next analyzed the dose-dependent effects of MTX on the stability of Cdc25A (Figure 5B). We found that Chk1 phosphorylation led to a corresponding decrease in Cdc25A abundance, indicating that MTX not only conferred Chk1 phosphorylation, but it also activated Chk1. Conversely, phosphorylation of Chk2 was not observed in melanoma cells that had been treated with MTX for as long as 48 h (Figure 5B), indicating that this drug specifically induced Chk1 activation in response to DNA single strand breaks (SSBs). To determine the extent to which Chk1 activation affected the resistance of melanoma to MTX, we took two independent experimental approaches. First, we silenced the expression of Chk1 in SK-MEL-28 (p53 mutant) cells and studied the sensitivity of the cells to MTX (Figure 5C). The results indicated that the downregulation of Chk1 increased the sensitivity of SK-MEL-28 cells to MTX and led to apoptosis. As a second approach, we evaluated the ability of Chk1 to protect B16/F10 murine cells (p53 wild-type) from MTX-induced apoptosis by first inducing an S phase arrest with MTX and then treating the S-arrested cells with a combination of MTX and 7-hydroxystaurosporine (UCN-01). We observed that B16/F10 S phase-arrested cells were sensitive to MTX treatment after the effective inhibition of Chk1 (Figure 5C).

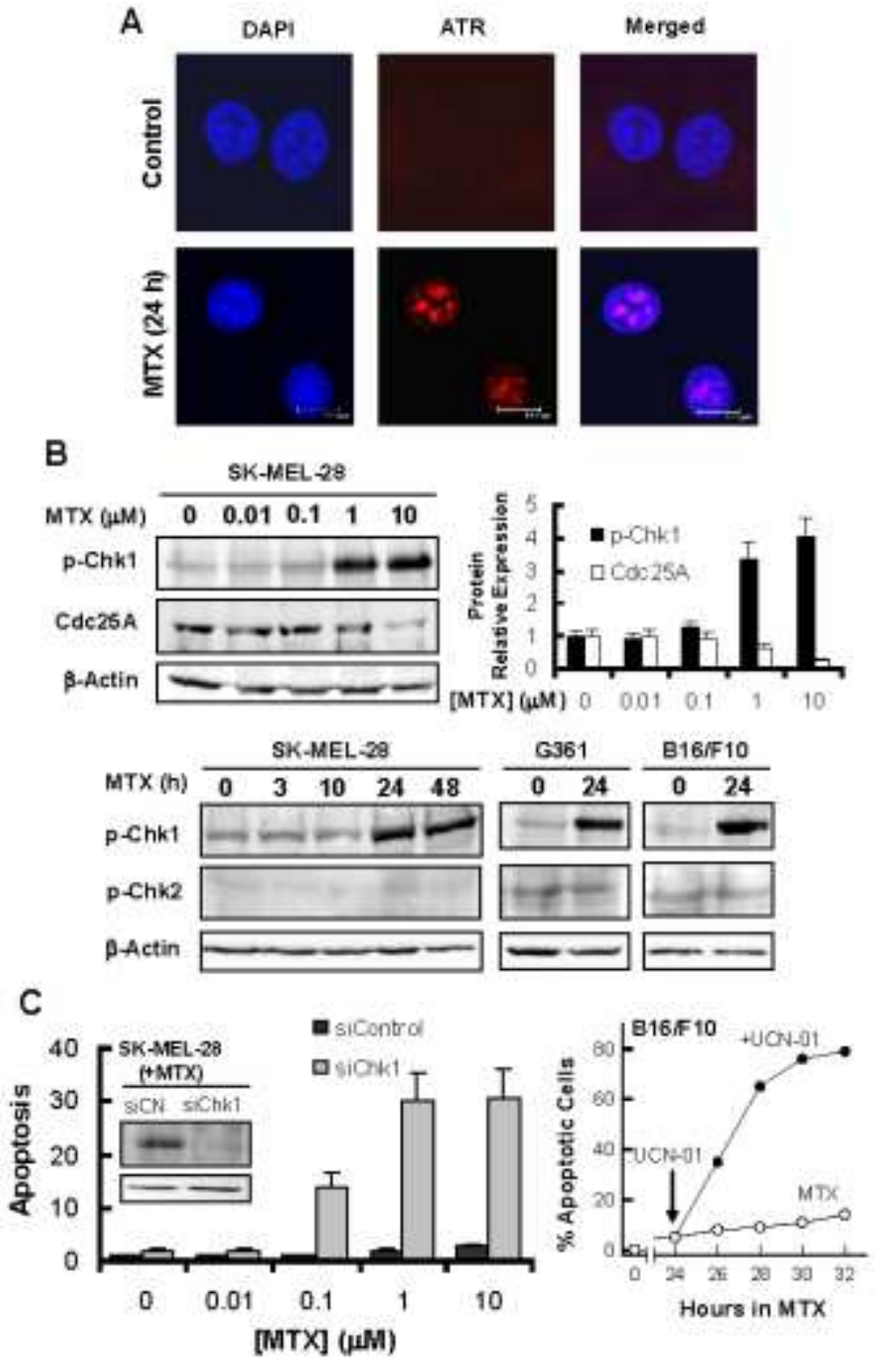

Figure 5. MTX activates Chk1 in melanoma cells. (A) SK-MEL-28 cells were treated with 1 μM MTX for 24 h and then examined for ATR nuclear foci. Nuclei were stained with DAPI. (B) The dose-dependent effects of MTX on Chk1 phosphorylation and Cdc25A degradation in SK-MEL-28 after 24 h of drug exposure (*$P < 0.05$). MTX (1 μM) induced the time-dependent phosphorylation of Chk1, but not Chk2, in different melanoma cell lines. (C) Chk1 siRNA sensitizes SK-MEL-28 cells to MTX-induced toxicity (left panel). siControl (siCN)- and siChk1-transfected cells were treated with increasing doses of MTX for 48 h (*$P < 0.05$). The effective silencing of Chk1 was tested by WB. The induction of the phosphorylated form of Chk1 was analyzed after 24 h of MTX treatment (1 μM). The induction of apoptosis by UCN-01 in MTX-arrested B16/F10 cells is shown in the right-side panel. Cells were incubated with 1 μM MTX continuously for 32 h, and 50 nM UCN-01 was added at 24 h to one group of cells following splitting of the culture. As a control experiment, SK-MEL-28 cells were treated with 50 nM UCN-01 only for 32 h.

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Inhibitors of DNA synthesis, such as excess thymidine, hydroxyurea, and camptothecin, are normally poor inducers of apoptosis; however, these agents become potent inducers of death in S phase cells upon the small interfering RNA-mediated depletion of Chk1 [45]. Here, we observed that MTX activated Chk1 and induced an early S phase arrest in melanoma cells lines that were harboring either wild-type or mutant p53. The impact of MTX on the survival of Chk1-silenced melanoma cells and cells co-treated with UCN-01 indicates that MTX provokes a 'thymidine block'-like effect and that S phase arrest, as a result of Chk1 activation, might constitute a major and general p53-independent mechanism that is responsible for the resistance of melanomas to MTX. However, it would be difficult to understand this extreme resistance without taking into account the melanosome-mediated exportation of MTX. The activation of the DNA damage response pathway reflects the magnitude and extent of DNA damage that occurs in response to a specific genotoxic agent, and a dual role of Chk1, depending on the extent of DNA damage, has been proposed [45]. Thus, Chk1 may play an anti-apoptotic role in response to weaker replication fork stresses, whereas more catastrophic damage, such as the accumulation of DNA strand breaks, may result in the activation of apoptosis by Chk1. Together, the results indicate that low intracellular levels of MTX in melanoma induce moderate DNA damage that favors the anti-apoptotic role of Chk1 (Figure 1D).

5. Therapeutical implications

Although melanoma resistance to MTX was initially thought to be due to the classical mechanisms of resistance that have been observed in other epithelial cells, recent discoveries indicate that the resistance of melanoma to MTX might be due to the idiosyncrasies of these cancer cells [6,12] where drug melanosomal sequestration and its subsequent cellular exportation may have a marked protagonist. Unravelling the mechanisms of melanoma resistance to MTX could, therefore, yield important information on how to circumvent this resistance and could have important pharmacological implications for the design of novel combined therapies. Taking into account these observations, uses of combined treatments with MTX, to prevent melanosomal drug sequestration [6,12] or to avoid MTX-induced S phase arrest [19], are rational therapeutical approaches. The observation that MTX induces cellular depletion of DHF in melanoma [7] could generate novel combined therapies to efficiently inhibit DHFR with antifolates transported into the cells by FRα-independent processes. Also, of great interest is the observed effect of MTX on the posttranslational status of E2F1 in melanoma (Figure 3). Various studies have suggested that E2F1 plays dual roles in cell survival/apoptosis [47-50]. Therefore, the MTX-induced demethylation and acetylation of E2F1 could favour melanoma cell death when combined with E2F1-stabilizing drugs. In addition to E2F1 phosphorylation, acetylation has also been recognized to play a role in the activation and stabilization of the E2F1 protein during DNA damage and apoptosis [40]. A possible strategy to favour E2F1 apoptosis in melanoma by the combination of MTX with E2F1-stabilizing drugs is depicted in Figure 6.

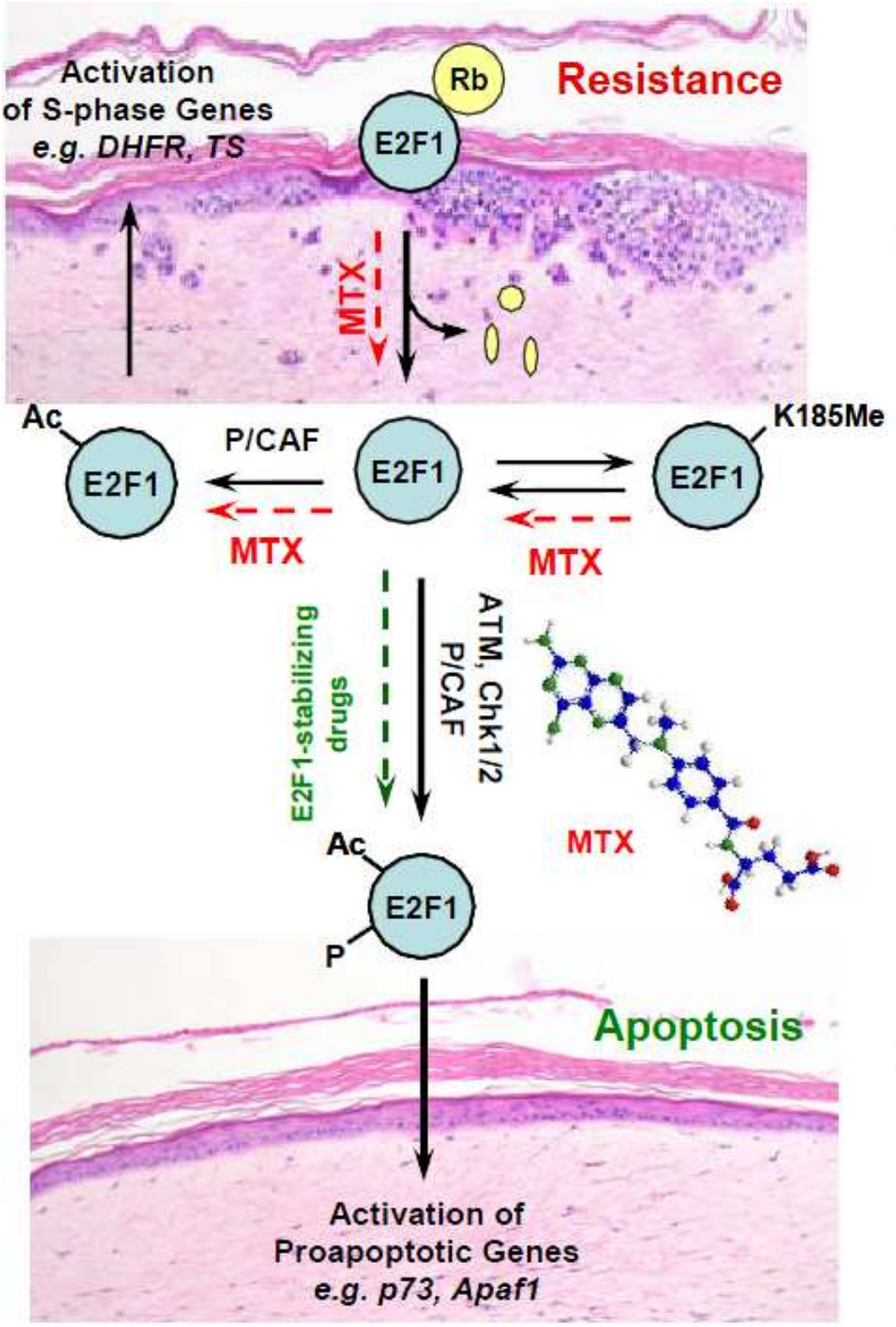

Figure 6. Proposed mechanism for the regulation of E2F1 by MTX. E2F1 is regulated by its interaction with Rb and by several posttranslational modifications, including methylation (Me), acetylation (Ac) and phosphorylation (P) [39]. The effects of MTX (red dashed line) on E2F1 status and that result in melanoma resistance are shown. A possible strategy to stabilize E2F1 (green dashed lines) to induce apoptosis in melanoma cells is also displayed.

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6. Conclusions

Melanoma, the most aggressive form of skin cancer, is notoriously resistant to all current modalities of cancer therapy, including to the drug MTX. Melanosomal sequestration and cellular exportation of methotrexate have been proposed to be important melanoma-specific mechanisms that contribute to the resistance of melanoma to methotrexate. In addition, other mechanisms of resistance that are present in most epithelial cancer cells are also operative in melanoma. This chapter reviews how melanoma orchestrates these mechanisms to become extremely resistant to methotrexate, where both E2F1 and Chk1, two molecules with dual roles in survival/apoptosis, play prominent roles. The results indicated that MTX induced the depletion of DHF in melanoma cells, which stimulated the transcriptional activity of E2F1. The elevate expression of DHFR and TS, two E2F1-target genes involved in folate metabolism and required for G_1 progression, favoured dTTP accumulation, which promoted DNA single strand breaks and the subsequent activation of Chk1. Under these conditions, melanoma cells are protected from apoptosis by arresting their cell cycle in S phase. Excess of dTTP could also inhibit E2F1-mediated apoptosis in melanoma cells. In addition, these discoveries could open the way for the development of new combined and directed therapies against this elusive skin pathology.

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