Induction of Senescence in Diterpene Ester–Treated Melanoma Cells via Protein Kinase C–Dependent Hyperactivation of the Mitogen-Activated Protein Kinase Pathway

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Abstract

The diterpene ester PEP005 is a novel anticancer agent that activates protein kinase C (PKC) and induces cell death in melanoma at high doses. We now describe the in vitro cytostatic effects of PEP005 and the diterpene ester phorbol 12-myristate 13-acetate, observed in 20% of human melanoma cell lines. Primary cultures of normal human neonatal fibroblasts were resistant to growth arrest, indicating a potential for tumor selectivity. Sensitive cell lines were induced to senesce and exhibited a G1 and G2-M arrest. There was sustained expression of p21WAF/CIP1, irreversible dephosphorylation of the retinoblastoma protein, and transcriptional silencing of E2F-responsive genes in sensitive cell lines. Activation of mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 by PKC was required for diterpene ester–induced senescence. Expression profiling revealed that the MAP kinase inhibitor HRE107 was expressed at a higher transcript level in resistant compared with sensitive cell lines. We propose that activation of PKC overstimulates the Ras/Raf/Mek/Erk pathway, resulting in molecular changes leading to the senescent phenotype.

Introduction

The diterpene ester PEP005, a novel anticancer agent extracted from the sap of Euphorbia peplus, is an activator of protein kinase C (PKC; ref. 1). We have previously shown that high-dose topical application of PEP005 cured 80% of s.c. tumors in mice via PKC–independent necrosis, with a favorable cosmetic outcome (2). At 10,000 times lower dose, a PKC–dependent apoptotic mechanism has been reported for PEP005 in leukemia cell lines (3). Previous work has shown that i.v. administration of phorbol 12-myristate 13-acetate (PMA) to patients suffering from myelocytic malignancies resistant to chemotherapy resulted in remission (4, 5). These results provided support for examining the potential anticancer activity of PEP005 and PKC in the context of systemic rather than topical administration.

PKC encompasses a family of serine/threonine phospholipid-dependent protein kinases, of which 12 isoenzymes have been identified and clustered into four subfamilies according to their cofactor requirements. The “conventional” PKCs include α, β1, β2, and γ, and require binding of calcium, phosphatidylinerine, and diacylglycerol or phorbol esters to render the enzyme competent for activation. Members of the “novel” PKC subfamily, δ, ε, η, and θ, depend on diacylglycerol/phorbol esters for activation but are calcium independent. The “atypical” PKCs ε, η, the murine homologue) and ζ, are independent of calcium and diacylglycerol/phorbol esters, but have been shown to be activated by cis-unsaturated fatty acids. Finally, distant PKC members include PKC-μ/PKD and PKC-τ. These kinases retain certain common structural features that suggest they are members of the PKC family (reviewed in ref. 6). Upon activation, PKC isoforms translocate to the particulate fraction where they access substrates (6) such as members of the mitogen-activated protein kinase (MAPK) family (7–9) and then become depleted by proteolytic degradation (10).

Cytostasis, or growth arrest, has not been widely exploited in the past for the treatment of malignant tumors. However, the rational design of anticancer compounds targeting selective entities unique to cancer cells has been adopted in an attempt to improve efficacy and selectivity, and to minimize toxicity. The pharmacologic outcome of target-based compounds is more likely to be cytostatic than cytotoxic (11, 12). When considering growth arrest in chemotherapy, the irreversibility of this process is essential for therapeutic efficacy (13). The observation that cancer cells have maintained the ability to senesce in vivo (14) has opened a new window of opportunity for cytostatic drugs in chemotherapy. Defined as permanent growth arrest, replicative senescence has been extensively studied in human diploid fibroblasts as a model for aging. Since then, further studies have identified the induction of premature senescence in vitro in tumor cell lines (14–17). Recently, the induction of senescence following chemotherapeutic treatment has been shown in vivo in frozen sections from breast cancer cells, suggesting that senescence of cancer cells in vivo is achievable (14). Furthermore, te Poele et al. (14) found that the induction of senescence in breast cancer following adjuvant therapy is correlated to favorable outcome. These results warrant further investigations of the induction of senescence in cancer by compounds that target specific pathways.

We now report the induction of a senescent phenotype in a subset of human melanoma cell lines treated with PEP005 or PMA. We present evidence supporting PKC–dependent activation of the MAPK pathway leading to dephosphorylation of the retinoblastoma protein (Rb), transcriptional silencing of E2F-1 and induction of an irreversible G1 and G2-M cell cycle arrest, characterizing the senescent phenotype.

Materials and Methods

Reagents. PEP005 was supplied by Peplin, Ltd. (Brisbane, Australia). PMA, PD098059, U0126, and bisindolylmaleimide-1 (BIS-1) were purchased from Sigma (St. Louis, MO). G66976 and rottlerin were from Calbiochem (San Diego, CA).
Cell lines. All melanoma cell lines used in this study were previously described (18), with the exception of LSP.M2 kindly provided by Prof. Andrew Boyd (Queensland Institute of Medical Research, Brisbane, Australia). Primary cultures of neonatal foreskin fibroblasts were established as previously described (19). Cells were routinely checked for Mycoplasma infection (20). Cell lines were grown in RPMI 1640 supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin (CSL, Ltd., Melbourne, Australia).

Cell growth assay. Cells were seeded in triplicate at 5,000 per microtiter well and allowed to attach. A separate plate was seeded for each time point, when cells were fixed and growth estimated as a function of intensity of sulforhodamine B protein staining (21). The experiments were repeated at least twice and the mean ± SE values were determined in Prism 3.0 (GraphPad Software, San Diego, CA).

Cell cycle analysis. Asynchronous cells were harvested, counted, and pelleted by centrifugation at 1,500 rpm for 5 minutes. The pellet was resuspended in PBS, and 4 × 10⁵ to 6 × 10⁵ cells were fixed by the addition of 2:1 100% ice cold methanol/cell suspension in PBS for at least 2 hours at 4°C. The fixed cells were pelleted by centrifugation and resuspended in 500 μL propidium iodide solution (50 mg/mL propidium iodide, 1 mg/mL RNase A, and 0.01% Triton X-100 in PBS). The cell suspension was subsequently filtered through a fine gauze and analyzed on a FACS calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest Pro (Becton Dickinson) and ModFit (Verity, Topsham, ME) analysis software.

Detection of senescence-associated β-galactosidase activity. The detection of β-galactosidase activity at a suboptimal pH of 6.0 was used as a biomarker of senescence (SA-β-GAL), as described by Dimri et al. (22). The nuclei were counterstained in Hoechst 33258 or propidium iodide. Microscopy was done using a Leica microscope (Leica DM IRM, Wetzlar, Germany) under >400 magnification. Two images were acquired per field: one with visible light for SA-β-GAL activity, followed by detection of the nuclei under fluorescence. The percentage of positively staining cells per field was determined. The experiment was conducted in at least duplicate with a total of over 100 cells counted per treatment.

Analysis of telomerase activity. Telomerase activity was detected using the TRAP-PCR method (14). A series of filtering steps were conducted to remove elements that had been flagged as absent or poor and a minimum value of 100 pixel units imposed on the reference samples to determine that the element was of high quality. Genes differentially regulated by at least 2-fold following treatment were identified. A series of Venn diagrams enabled those genes common and unique to sensitive or resistant cell lines to be identified.

Real-time reverse transcription-PCR. CDNA was generated by reverse transcribing 4 μg total RNA as described (18). PCR products were amplified (RotorGene 3000, Corbett Research, Sydney, Australia) using the QuantiTect SYBR Green PCR kit (Qiagen). The conditions for the PCR were as follows: 95°C for 30 sec; 60°C for 30 seconds; and 72°C for 45 seconds for 40 cycles. The forward and reverse primer sequences were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5’-GGGCTTCCAGAACATCATTCCCTGC-3’, reverse 5’-GGGTTGCGTGTGGAAGTCTG-3’; HREV107 forward 5’-GCCCTCCAGAAGTTGCTCG-3’, reverse 5’-GCCCTTACCAGAGTGCTC-3’. To determine the reaction efficiency, dilutions of a CDNA mixture composed of equal amounts of CDNA generated from the A07.RM, D23.BDF, LSP.M2, and MM253 cell lines were PCR amplified alongside the samples. Primer specificity was confirmed by melt curve analysis. The mathematical model described by Pfaffl (24) was used to determine the relative expression of HREV107 to the housekeeper GAPDH. To compare the PCR results to the microarray data, the relative expression of each cell line tested was normalized to the expression of MM329.

Immunoblot analysis. Total cell lysates were prepared as previously described (25). The primary antibodies used were as follows: E2F-1, PKC-α/β/γ/δ/ε/ν/θ/Rb (PharMingen/Becton Dickinson, San Jose, CA), proliferating cell nuclear antigen (PCNA; DAKOCytomation, Carpinteria, CA), phosphorylated and total extracellular signal-regulated kinase 1/2 (ERK1/2)/MEK (MAP/ERK kinase) 1/2 (Cell Signaling, Danvers, MA), and GAPDH (R&D Systems, Minneapolis, MN). Immunodetection was done using the appropriate peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ).

Results

Melanoma cell lines sensitive to diterpene ester treatment are irreversibly arrested in G1 and G2-M. Previous work has shown that several melanoma cell lines treated with PMA were reversibly arrested in G1 and/or G2-M phases of the cell cycle (26–28). However, these studies did not address the question of irreversibility. From a preliminary screen conducted on 39 human melanoma cell lines treated with PMA or PEP005, we selected a panel of four sensitive and four resistant cell lines for further study. Treatment of sensitive melanoma cell lines for 24 hours with doses between 0.1 and 1 μg/mL PMA or PEP005 led to an irreversible growth arrest (Fig. 1A), whereas the growth of resistant cell lines was unaffected (Fig. 1B). To determine whether selectivity was achievable at this dose, we selected neonatal foreskin fibroblasts as a normal cell line because in vitro growth of melanocytes is stimulated by PMA (29). We found that the growth of neonatal foreskin fibroblast cells was unaffected by treatment (Fig. 1C), indicating that at these doses diterpene esters were selective for inhibition of tumor cell lines. To further characterize the growth arrest, we assessed the cell cycle responses of three sensitive and three resistant cell lines. The histograms in Fig. 1D are representative of sensitive (MM455) and resistant (MM253) cell lines. Selective and irreversible induction of a G1 and G2-M arrest occurred in sensitive melanoma cell lines treated for 24 hours with 1 μg/mL PMA or PEP005 (Fig. 1D, top). Conversely, the cell cycle profiles of resistant cell lines remained unaffected by treatment (Fig. 1D, bottom).

Diterpene ester–induced senescence. The irreversibility of the growth arrest led us to evaluate the possibility that sensitive cell lines were induced to senesce when treated with diterpene esters. We chose two markers: SA-β-GAL (pH 6), which has been widely used as a marker for senescence both in vitro and in vivo (22), and telomerase activity, the major enzyme responsible for maintaining telomere length in actively dividing cells (30). To avoid measuring a direct effect of the compounds on the activity of these enzymes, the cells were treated for 24 hours with 1 μg/mL PMA or PEP005, followed by 72 hours recovery in fresh medium. Treatment of

1 Data are available at www.ncbi.nlm.nih.gov/geo (GSE3484).

2 Unpublished data.
sensitive melanoma cell lines with 1 μg/mL diterpene ester selectively induced SA-β-GAL activity (Fig. 2A). Although a faint background staining was observed in all D08 cells, and therefore no change in the percentage staining positive reported, an increase in the staining intensity (activity) was noted, indicating that these cells also exhibit an increase in SA-β-GAL expression in response to treatment with diterpene esters. Conversely, no significant change in SA-β-GAL activity was seen in resistant melanoma cell lines.

Confirming these findings, the activity of telomerase (Fig. 2B) was selectively repressed following treatment of sensitive melanoma cell lines. Together, these results indicate induction of senescence in melanoma cell lines sensitive to diterpene ester treatment.

Silencing of E2F-responsive genes in diterpene ester–induced senescent melanoma cells. To investigate the molecular changes associated with the senescent phenotype, we examined the unique transcriptional changes occurring in sensitive melanoma cell lines treated with PMA or PEP005. Initially, a time course cDNA microarray analysis was conducted on one sensitive and one resistant cell line treated with 1 μg/mL PMA or PEP005 for 24 hours. Points, mean from two independent experiments; bars, SE. Following 0 and 72 hours of recovery, the cells were harvested, fixed, stained with propidium iodide, and analyzed by flow cytometry. Histogram, representative of two independent experiments for representative sensitive (MM455) and resistant (MM253) melanoma cell lines; data, mean and SE.

Figure 1. Irreversible G1 and G2-M growth arrest of melanoma cell lines sensitive to diterpene esters. Growth curves following 24-hour treatment with PMA or PEP005 of (A) a representative sensitive melanoma cell line (MM455); (B) a representative resistant melanoma cell line (MM253); and (C) normal human neonatal fibroblasts. Exponentially growing cell lines were treated with 1 μg/mL PMA or PEP005 for 24 hours. Points, mean from two independent experiments; bars, SE.

The unique transcriptional changes occurring in sensitive melanoma cell lines treated with PMA or PEP005. Initially, a time course cDNA microarray analysis was conducted on one sensitive and one resistant cell line treated with 1 μg/mL PMA for 6 and 24 hours, and 24-hour recovery following 24-hour treatment, to determine the earliest time point at which the most significant changes in transcription occurred. The results1 provided support for conducting array experiments with 24-hour treatment, in which three sensitive and four resistant melanoma cell lines were treated with 1 μg/mL of either diterpene ester. Our primary objective was to identify those genes that were uniquely up- or down-regulated in sensitive or resistant cell lines in response to treatment, and which could determine the phenotypic outcome. Through applying a series of stringent selective criteria (see Materials and Methods), we found that the most significant changes occurred in the

silencing of E2F-responsive genes in diterpene ester–induced senescent melanoma cells.
transcriptional repression of genes required for DNA synthesis and mitosis in cell lines sensitive to treatment (see Supplementary Table S1). To confirm that these changes were reflected at the protein level, immunoblot analysis was conducted on three sensitive and three resistant cell lines following 6 and 24 hours treatment with PMA or PEP005. We also included a 24-hour recovery time point to determine the irreversibility of the change. Typical results for a sensitive (Fig. 2C) and a resistant (Fig. 2D) cell line are shown. The microarray evidence was confirmed at the protein expression level for E2F-1 and PCNA, supporting the hypothesis that gene silencing resulted in loss of protein.

Given the changes in genes required for cell cycle progression, the role of other key molecules involved in cell cycle progression was determined. Although induction of p16\textsuperscript{INK4a} has been widely reported in senescence, mutational data showed that, with the exception of LSP.M2 for which no data was available, the expression of p16\textsuperscript{INK4a} was silenced by methylation, mutation, or homozygous deletion (31). Hence, the role of other cell cycle regulators was investigated. As shown in Fig. 2C, treatment of sensitive melanoma cell lines with 1 \(\mu\)g/mL PMA or PEP005 led to the dephosphorylation of Rb and the sustained induction of p21\textsuperscript{WAF1/CIP1}. No change in the phosphorylation status was observed in resistant melanoma cell lines; treatment of the particular cell line depicted in Fig. 2D, MM253, transiently induced expression of Rb.

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3 S. Pavey, personal communication.
Lack of correlation between induction of senescence and PKC isoform expression or diterpene ester–induced depletion of PKC. We initially examined whether differences in expression levels of PKC isoforms correlated with sensitivity or resistance to diterpene ester–induced senescence, by immunoblot analysis. We looked at the protein expression levels of members of the classic (α, β, and γ) and novel (δ, ε, η, and θ) subfamilies, which possess the two zinc finger binding motifs in their C1 domain responsible for binding diterpene esters. The results (Fig. 3A) showed that all of the melanoma cell lines expressed high levels of isoforms α, δ, and ε. PKC-η, which has not been widely examined in melanoma, was detectable in most lines. PKC-γ was expressed at very low levels in most of the cell lines, whereas PKC-β was found to be expressed exclusively in MM253 cells. No expression of PKC-θ was observed in any of the melanoma cell lines tested. There was no correlation between the constitutive expression of any particular PKC isoform and sensitivity to treatment.

We next addressed the possibility that proteolytic depletion of PKC isoforms (10) accounted for sensitivity or resistance to senescence by examining expression levels in one sensitive (MM455; Fig. 3B) and one resistant (MM253; Fig. 3C) cell line. Immunoblot analysis conducted after 6 and 24 hours of treatment with 1 μg/mL PMA or PEP005 revealed loss of PKC isoform protein expression regardless of cell line sensitivity to treatment. Furthermore, the protein levels were not reestablished to original levels after 24-hour recovery following 24-hour treatment, indicating that the loss was prolonged. We did an identical experiment on a further two diterpene ester–resistant (A07.RM and D23.BDF) and diterpene ester–sensitive (D04 and MM127) melanoma cell lines. In each case, original expression levels were not restored or observed for any of the tested PKC isoforms following 24-hour treatment with 24-hour recovery (data not shown). Our next objective was to determine whether PKC activation and/or the reported PKC-targeted MAPK pathway were responsible for the irreversible growth arrest and the G1 and G2-M cell cycle arrests described in sensitive cell lines.

PKC-dependent activation of the MAPK pathway is required for senescence. One of the signaling pathways targeted by PKC is the RAS/RAF/MEK/ERK arm of the MAPK pathway. Due to the activating mutations of NRAS and BRAF frequently identified in melanoma (32, 33), we compared the activation of ERK1/2 in sensitive and resistant cell lines, following 1-hour treatment with 1 μg/mL PMA or PEP005. ERK1/2 was found to become phosphorylated in response to treatment in five of six cell lines tested, regardless of their sensitivity to diterpene ester treatment (Fig. 4A). There was no apparent correlation with sensitivity to diterpene ester treatment and constitutive level or level of activation of ERK1/2 following 1-hour treatment with PMA (Fig. 4B). Analysis of the magnitude of ERK1/2 activation with increasing concentrations of diterpene ester also showed no correlation with resistance or sensitivity to treatment (Fig. 4C). Importantly, the concentration of diterpene ester that significantly activated ERK1/2 phosphorylation correlated with the growth-inhibiting dose in the sensitive cell lines. These results suggest that sensitive cell lines are more susceptible to enhanced ERK activity leading to senescence. To confirm that the activation of the MAPK pathway resulted from activation of PKC, levels of ERK1/2 phosphorylation were examined following treatment with the nonselective PKC inhibitor BIS-1. ERK1/2 phosphorylation was found to be blocked in MM455 cells pretreated with BIS-1 and then cotreated with either PMA or PEP005 in the presence of the inhibitor (Fig. 4D). Surprisingly, pretreatment of MM455 cells with the MEK1/2 inhibitor PD098059 followed by cotreatment of the inhibitor in the presence of PMA or PEP005 diminished, but did not completely inhibit, ERK1/2 phosphorylation induced by diterpene ester treatment.

Next, we examined whether activation of PKC or MEK1/2 activity was required for the irreversible cell cycle arrest. Sensitive cell lines were pretreated with BIS-1 or PD098059, followed by 24-hour cotreatment of the inhibitor in the presence of 1 μg/mL PMA or PEP005. The diterpene ester was removed and the inhibitors were reapplied for a further 3 days. The inhibitors were subsequently removed and the cells were allowed to grow in inhibitor-free medium for a further 3 days. Cell growth was assayed 0, 4, and 7 days following diterpene ester treatment. A representative growth curve for a sensitive cell line (Fig. 5A) shows...
that pharmacologic inhibition of PKC or MEK1/2 activity prevents the irreversible growth arrest. To establish whether PKC and MEK1/2 were involved in both the G1 and G2-M arrest, cell cycle profiling was examined in sensitive cell lines pretreated with BIS-1 or PD098059 followed by diterpene ester treatment for 24 hours in the presence of the inhibitor. The representative histograms (Fig. 5B) show that in the presence of BIS-1 or PD098059, the loss in S phase and increase in G2-M was prevented. These results suggest that PKC activation of MEK1/2 is required for the phenotypic changes associated with diterpene ester–induced senescence. BIS-1 treatment was found to be slightly more effective than PD098059 treatment at inhibiting diterpene ester–induced cell cycle arrest, which may suggest that a PKC-dependent, MEK-independent pathway could contribute to PMA/PEP005–induced cell cycle arrest. To determine the molecular relevance of PKC and MAPK activity in the induction of senescence, we examined the expression of E2F-1 and PCNA as well as the phosphorylation status of Rb in MM455 cells treated with PMA or PEP005 in the presence of BIS-1 or PD098059. Immunoblot analysis confirmed the requirement for both PKC and MEK1/2 activity for the repression of diacylglycerol-targeted PKC isoforms. As the dophasphorylation of Rb (Fig. 5C), Cotreatment with a pan-PKC inhibitor (BIS-1) or specific MEK1/2 inhibitors (PD098059 and U0126) dramatically reduced the number of cells exhibiting SA-β-GAL activity induced by diterpene ester treatment (Fig. 5D). A partial reduction in SA-β-GAL staining was obtained in the presence of the isoform-specific PKC inhibitors Gö6976 (PKC-α and PKC-β) and rotterlin (PKC-δ). As the β isoforms are not expressed in any of the sensitive melanoma cell lines examined, these results suggest that at least PKC-α and PKC-δ are involved in diterpene ester–induced senescence and may indicate a functional redundancy in this process. These results confirm a role for both pathways in diterpene ester–induced senescence.

Expression levels of HREV107 distinguishes sensitive from resistant cell lines. The identification that constitutive expression of diacylglycerol-targeted PKC isoforms did not correlate with sensitivity or resistance to diterpene ester–induced senescence led us to investigate whether constitutive differences in the MAPK pathway correlated with phenotypic outcome. Initially, we looked at the mutational status of NRAS or BRAF previously published by Pavey et al. (ref. 18; Table 1). The results showed that although there was no correlation between sensitivity to treatment and either a BRAF or NRAS mutation, all sensitive cell lines had a mutation at either loci. Conversely, although most of the melanoma cell lines had a mutation at either locus, the MM329 primary melanoma cell line that was used as a reference in the cDNA study was resistant in the melanoma cell line screen2 and was wild-type at both the NRAS and BRAF loci.

Expression profiles of untreated cells were used to investigate whether there were any significant differences in constitutive expression between sensitive and resistant cell lines within the transcripts present on the cDNA microarray. We did a supervised clustering based on a nonparametric Wilcoxon-Mann-Whitney ANOVA statistical analysis to identify genes statistically different in expression between sensitive and resistant cell lines (P < 0.02). The analysis yielded 165 genes that were significantly different in their expression profile between the groups, whereas only 65 were statistically expected by chance. Due to the limitations associated with small sample sizes, further restrictions were applied to the gene list to increase stringency: first, a minimum of 2.5-fold difference between the mean expression of sensitive and resistant cell lines was imposed. To ensure that the mean difference was not driven by a few cell lines, the genes were subsequently filtered to ensure that the two closest cell lines from each group differentially expressed the genes by at least 1.5-fold. Twelve genes passed these criteria. Of particular interest was HRASL3 (also known as...
HREV107), previously described as a type II tumor suppressor highly expressed in rodent fibroblasts resistant to RAS-induced transformation (34, 35), and found here to be more highly expressed in the resistant melanoma cell lines compared with sensitive lines. To validate the results from the microarray analysis, quantitative reverse transcription-PCR (qRT-PCR) was done. The senescence-sensitive cell line D08 was also included. Concordance between the microarray and qRT-PCR results was confirmed using Spearman’s ranking correlation (Spearman test, \( r = 0.98, P < 0.0004; \) Table 1), supporting a role for HREV107 as part of a molecular signature for sensitivity or resistance to diterpene ester treatment.

**Discussion**

Transcriptional silencing of E2F-responsive genes in senescing melanoma cells. We have shown for the first time that treatment of a subset of melanoma cell lines with PKC activators leads to irreversible G1 and G2-M arrest and senescence, characterized by the induction of SA-\( \beta \)-GAL and loss in telomerase activity. Importantly, cultured human neonatal fibroblasts were resistant to treatment at these doses, indicating the potential of these compounds for cancer treatment.

To gain insight into the molecular mechanism underlying the induction of senescence, we investigated the unique transcriptional changes in sensitive melanoma cell lines, using cDNA microarray analysis and immunoblotting. Our results showed that in sensitive melanoma cell lines, the induction of senescence by diterpene ester treatment was accompanied by sustained expression of p21\(^{WAF1/CIP1}\), dephosphorylation of Rb, and selective repression of genes required for G1 progression, DNA synthesis, and mitosis. Down-regulation of E2F-1 was of particular interest, given its central role in entry into S phase and reports of some E2F-responsive genes being down-regulated in other models of quiescence (36). This is also consistent with the findings that human fibroblasts and keratinocytes undergoing replicative senescence lose expression of E2F-1 mRNA (37, 38). Extensive studies on the promoter-binding activity of E2F-1 and E2F-4 have shown that human fibroblasts and keratinocytes undergoing replicative senescence lose expression of E2F-1 mRNA (37, 38). Extensive studies on the promoter-binding activity of E2F-1 and E2F-4 have shown that in quiescent cells, E2F-1 is predominantly found associated on E2F-responsive gene promoters, including E2F-1. As cells are stimulated into S phase, E2F-4-p130 complex is dislodged and binding of E2F-1 is concomitant with increased expression of E2F-responsive genes (39).

Based on these studies, we postulate that the transcriptional repression of E2F-1– and E2F-responsive genes in senescing melanoma cells reflects the binding of transcriptionally repressive complexes to the promoter sequences of these genes as well as the lack of transcriptionally active complexes within the cell.

**Figure 5.** PKC and ERK1/2 activation are required for the molecular and phenotypic changes required for senescence. A, the sensitive melanoma cell line MM455 was pretreated with 5 \( \mu \)M BIS-1 or 20 \( \mu \)mol/L PD098059 for 1 hour followed by cotreatment of the inhibitor with 1 \( \mu \)g/mL PMA or PEP005 for 24 hours. The treatments were removed and the inhibitors alone reapplied for 3 days. The inhibitors were removed and the cells were allowed to recover. Points, mean of two independent experiments; bars, SE. B, MM455 cells were pretreated with 5 \( \mu \)mol/L BIS-1 or 20 \( \mu \)mol/L PD098059 for 1 hour followed by cotreatment of the inhibitor with 1 \( \mu \)g/mL PMA or PEP005 for 24 hours. The cells were harvested, fixed, stained with propidium iodide, and their cell cycle profile examined by flow cytometry. Histogram, representative of two independent experiments; data, mean and SE. C, MM455 cells were pretreated with 5 \( \mu \)mol/L BIS-1 or 20 \( \mu \)mol/L PD098059 followed by cotreatment of the inhibitors with 1 \( \mu \)g/mL PMA or PEP005. The cells were harvested and the lysates were examined by immunoblot analysis. Individual blots were probed with anti-E2F-1, anti-PCNA, and anti-Rb followed by anti-GAPDH. D, the sensitive melanoma cell line MM127 was pretreated with 5 \( \mu \)mol/L BIS-1, 5 \( \mu \)mol/L Go6976, 5 \( \mu \)mol/L rottlerin, 20 \( \mu \)mol/L PD098059, or 10 \( \mu \)mol/L U0126 for 1 hour followed by cotreatment of the inhibitor with 1 \( \mu \)g/mL PMA for 24 hours. The inhibitors alone were reapplied for 3 days, before inhibitors were removed and replaced with medium alone for a further 3 days. The cells were then assayed for SA-\( \beta \)-GAL activity. Columns, mean of two independent experiments; bars, SE.
PKC-dependent activation of the MAPK pathway results in senescence. No correlation was found between sensitivity to growth arrest and particular PKC isoforms, with respect to either their constitutive or depleted levels following treatment. Hence, targets downstream from PKC were considered. Several PKC isoforms have been found associated with members and regulators of the MAPK pathway. Specifically, different PKC isoforms, including PKC-α and PKC-ε, are known to directly phosphorylate Raf (40, 41). Activation of the MAPK pathway is generally associated with increased proliferation (42). In serum-starved quiescent murine fibroblasts, for example, c-N-Ras, c-Raf-1, and PKC-α were found in a latent ternary complex that became activated upon stimulation with PMA and was associated with proliferation (43).

Table 1. Relationship between gene expression and mutational status of NRAS and BRAF in human melanoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Melanoma type</th>
<th>Sensitivity to PMA/PEP005</th>
<th>NRAS/BRAF mutation (18)</th>
<th>HREV107 Expression normalized to MM329</th>
<th>Microarray</th>
<th>qRT-PCR</th>
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<tbody>
<tr>
<td>D04</td>
<td>Secondary</td>
<td>Sensitive</td>
<td>Q61L</td>
<td>WT</td>
<td>1.5 ± 0.03</td>
<td>2.6 ± 0.31</td>
</tr>
<tr>
<td>D08</td>
<td>Secondary</td>
<td>Sensitive</td>
<td>Q61L</td>
<td>WT</td>
<td>ND</td>
<td>1.2 ± 0.12</td>
</tr>
<tr>
<td>MM127</td>
<td>Secondary</td>
<td>Sensitive</td>
<td>G13R</td>
<td>WT</td>
<td>1.2 ± 0.07</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>MM455</td>
<td>Secondary</td>
<td>Sensitive</td>
<td>WT</td>
<td>V600E</td>
<td>1.3 ± 0.01</td>
<td>3.1 ± 0.73</td>
</tr>
<tr>
<td>A07.BM</td>
<td>Secondary</td>
<td>Resistant</td>
<td>G12S</td>
<td>WT</td>
<td>3.1 ± 0.35</td>
<td>5.7 ± 0.25</td>
</tr>
<tr>
<td>D23.RDF</td>
<td>Secondary</td>
<td>Resistant</td>
<td>WT</td>
<td>V600E</td>
<td>2.2 ± 0.32</td>
<td>6.9 ± 0.85</td>
</tr>
<tr>
<td>LSP.M2</td>
<td>Secondary</td>
<td>Resistant</td>
<td>Unknown</td>
<td>Unknown</td>
<td>3.7 ± 0.19</td>
<td>5.0 ± 0.61</td>
</tr>
<tr>
<td>MM253</td>
<td>Secondary</td>
<td>Resistant</td>
<td>WT</td>
<td>V600E</td>
<td>3.9 ± 0.02</td>
<td>7.7 ± 0.95</td>
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<tr>
<td>MM329</td>
<td>Primary</td>
<td>Resistant</td>
<td>WT</td>
<td>WT</td>
<td>R</td>
<td>1.0 ± 0.22</td>
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</tbody>
</table>

NOTE: Results are given as mean ± SD.
Abbreviations: WT, wild type; ND, not detected; R, used as reference.

 Constitutive molecular signatures of sensitivity to treatment. Statistical comparison of constitutive expression profiles of over 4,000 genes identified several genes differentially expressed between sensitive and resistant cell lines. We found and confirmed by qRT-PCR that HrasL3/Hrev107 was more highly expressed in melanoma cell lines resistant to diterpene ester–induced senescence.

Hrev107 was originally discovered by cDNA subtraction in rodent fibroblasts resistant to oncogenic transformation by Ras (35). Classed as a type II tumor suppressor, Hrev107 expression is down-regulated in tumor cell lines, whereas it is almost ubiquitously expressed in normal tissue. Although its exact function has not been described, forced expression of Hrev107 in Ras-transformed cell lines resulted in growth arrest and inhibition of tumorigenic potential, indicating an ability of Hrev107 to inhibit Ras-driven tumorigenesis (52). Our findings that high levels of Hrev107 expression was associated with resistance to diterpene ester–induced senescence in cell lines mutated at either NRAS or BRAF loci (18).

Model for diterpene ester–induced senescence. We therefore speculate that activation of the MAPK pathway results in the induction of senescence in a subset of human melanoma cell lines treated with diterpene esters. To achieve susceptibility to diterpene ester–induced senescence, two constitutive requirements must be met: (a) the presence of an activating NRAS or BRAF mutation and
(b) low transcriptional levels of the MAPK inhibitor HREV107. This hypothesis is supported by the fact that normal human fibroblasts were resistant to diterpene ester–induced senescence and that the resistant MM329 melanoma cell line, used for normalization purposes in the expression profiling studies, expressed low levels of HREV107, both being well-type for NRAS and BRAF.

Our work not only reveals the potential use of PKC activators for the induction of senescence in a solid tumor model but also opens a window of opportunity for the potential use of MAPK activators to induce senescence in melanoma. Preclinical studies of i.v. administered PEP005 are due to be completed, and an investigational new drug application with the Food and Drug Administration will be submitted by Pепlin Ltd.

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References

Induction of Senescence in Diterpene Ester–Treated Melanoma Cells via Protein Kinase C–Dependent Hyperactivation of the Mitogen-Activated Protein Kinase Pathway

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