Plitidepsin Has a Dual Effect Inhibiting Cell Cycle and Inducing Apoptosis via Rac1/c-Jun NH₂-Terminal Kinase Activation in Human Melanoma Cells

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ABSTRACT

Melanoma is the most aggressive skin cancer and a serious health problem worldwide because of its increasing incidence and the lack of satisfactory chemotherapy for late stages of the disease. The marine depsipeptide Aplidin (plitidepsin) is an antitumoral agent under phase II clinical development against several neoplasias, including melanoma. We report that plitidepsin has a dual effect on the human SK-MEL-28 and UACC-257 melanoma cell lines; at low concentrations (≤5 M), it inhibits the cell cycle by inducing G₁ and G₂/M arrest, whereas at higher concentrations it induces apoptosis as assessed by poly-(ADP-ribose) polymerase cleavage and the appearance of a hypodiploid peak in flow cytometry analyses. Plitidepsin activates Rac1 GTPase and c-Jun NH₂-terminal kinase (JNK). In addition, it induces AKT and p38 mitogen-activated protein kinase (MAPK) phosphorylation. By using inhibitors, we found that JNK and p38 MAPK activation depends on Rac1 but not on phosphatidylinositol 3-kinase (PI3K), whereas AKT activation is independent of Rac1 but requires PI3K activity. Plitidepsin cytotoxicity diminishes by Rac1 inhibition or by the blockage of JNK and p38 MAPK using 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), but not by PI3K inhibition using wortmannin or 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). It is remarkable that plitidepsin and dacarbazine, the alkylating agent most active for treating metastatic melanoma, show a synergistic antiproliferative effect that was paralleled at the level of JNK activation. These results indicate that Rac1/JNK activation is critical for cell cycle arrest and apoptosis induction by plitidepsin in melanoma cells. They also support the combined use of plitidepsin and dacarbazine in vivo studies.

Metastatic melanoma is an aggressive skin cancer whose incidence has rapidly increased over the past five decades. Although patients with early stage melanoma can be treated efficiently by surgical dissection, patients with metastatic melanoma have a very poor prognosis, with a median survival of less than 1 year (Jemal et al., 2005). Effective therapies for advanced stages of this disease have not been defined, because malignant melanoma has proven to be highly resistant to standard antineoplastic treatment. Dacarbazine (DTIC) is an alkylating agent considered the most active agent for treating metastatic melanoma, and it is the only drug approved by both the United States Food and Drug Administration and the European Agency for the Evaluation of Medicinal Products for use in this disease. However, dacarbazine has only a response rate <20%, with complete response observed in <5% of cases (Lev et al., 2003). It is clear that additional therapeutic agents are needed for advanced resistant melanoma.

Aplidin (plitidepsin) is a marine antitumor agent isolated from the Mediterranean tunicate Aplidium albicans that displays a potent activity against human hematological and solid tumors cell lines. The compound has com-

ABBREVIATIONS: DTIC, dacarbazine; JNK, c-Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; GSH, glutathione reduced ethyl ester; NSC23766, 1,2,6,7-tetrathiacyclodecane; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; GST, glutathione transferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; CI, combination index; ANOVA, analysis of variance; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidylinositol 3-kinase; Fa, fraction of cells affected.
receptor, Src, p38 MAPK, extracellular signal-regulated kinase (JNK) (Cuadrado et al., 2003). In accordance with the activation of Rac1, plitidepsin inhibits the low-molecular-weight protein tyrosine phosphatase (Taddei et al., 2006), an enzyme that is inhibited by Rac1-induced reactive oxygen species (ROS) production (Nimnual et al., 2003). Furthermore, in leukemic cell lines, plitidepsin also activates JNK and triggers Fas/CD95 receptor and mitochondrial apoptotic pathway through the recruitment of signaling molecules at membrane lipid rafts (Gajate and Molinedo, 2005).

Plitidepsin also has antiangiogenic properties, because it reduces the expression of several angiogenic genes in cancer xenografts (Straight et al., 2006). Moreover, plitidepsin reduces the secretion of VEGF and blocks the stimulatory VEGF autocrine loop in leukemic MOLT-4 cells (Broggini et al., 2003). Likewise, it inhibits the response of endothelial cells to angiogenic stimuli (Tarabotti et al., 2004). Therefore, the in vivo antiangiogenic effect of plitidepsin might result from the induction of tumor or endothelial cell apoptosis (Biscardi et al., 2005).

Another mechanism that may contribute to the antitumoral activity of plitidepsin is its antiproliferative effect. Plitidepsin causes G1 arrest and G2/M blockage in leukemia cells (Erba et al., 2003; Biscardi et al., 2005). In a recent study, it has been reported that therapeutic concentrations of plitidepsin block anaplastic thyroid carcinoma cells in the G1- to-S transition of the cell cycle (Bravo et al., 2005). In contrast, no cell cycle perturbation by plitidepsin has been observed in other human solid tumor cells (García-Fernández et al., 2002; Cuadrado et al., 2003).

Based on initial clinical data indicating activity of plitidepsin as unique agent against advanced or metastatic melanoma in patients that were previously treated with chemotherapy (Eisen et al., 2004), we have for the first time studied its effects on cultured human melanoma cells. Our results show that plitidepsin has a dual, concentration-dependent effect on UACC-257 and SK-MEL-28 human metastatic melanoma cells. Whereas at low concentrations plitidepsin inhibits proliferation by inducing cell cycle arrest at G1 and G2/M phases, at higher concentrations it induces apoptosis through Rac1/JNK activation. Moreover, we report in this study that plitidepsin has a synergistic antiproliferative activity in combination with DTIC. These results support the interest of further studies on the potential use of plitidepsin for melanoma therapy.

Materials and Methods

Cell Culture. Human SK-MEL-28 and UACC-257 melanoma cells were obtained from the American Type Culture Collection (Manassas, VA) and National Cancer Institute-Frederick Cancer, Division of Cancer Treatment and Diagnosis Tumor/Cell Line Repository Ft. Detrick United States Army facility (Frederick, MD), respectively. They were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (all obtained from Invitrogen, Paisley, UK). Plitidepsin is a cyclic depsipeptide (for its chemical structure, see García-Fernández et al., 2002) originally isolated from the tunicate A. albicans, and it is currently obtained by total synthesis by Pharma Mar S.A. (Madrid, Spain) (Sakai et al., 1996). Stock solutions were freshly prepared in dimethyl sulfoxide. The final dimethyl sulfoxide concentration for cultures was always less than 0.05% (v/v), which did not affect either cell proliferation or JNK activation compared with untreated cells. Glutathione reduced ethyl ester (GSH) and DTIC (DTC-Dome, 5-(3,3-dimethyl-1-triazenyl)-1H-imidazole-4-carboxamide) were from Sigma-Aldrich (St. Louis, MO). Rac1 inhibitor NSC23766, wortmannin, LY294002, and SB203580 were from Calbiochem-Merck Biosciences (Darmstadt, Germany).

Flow Cytometry Analyses. Cells were stained with propidium iodide, and then they were analyzed by flow cytometry (FACScan; BD Biosciences, San Jose, CA) equipped with a 488-nm argon ion laser. For staining, 1 million cells were harvested, they were washed in phosphate-buffered saline, and then they were fixed with 70% ethanol. After washing, fixed cells were treated with DNase-free RNase (50 μg/ml; Sigma-Aldrich) for 1 h at 37°C in phosphate-buffered saline containing propidium iodide (50 μg/ml; Sigma-Aldrich). Ten thousand events per sample were acquired for data analysis using CellQuest software (BD Biosciences).

Western Blotting. The preparation of cell protein extracts and Western blotting analysis were as described previously (Cuadrado et al., 2004; Gonzalez-Santiago et al., 2006). In brief, cells were lysed with radioimmunoprecipitation assay buffer, and 20 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked at room temperature for 1 h in Tris-buffered saline (25 mM Tris-HCl, pH 7.4, 136 mM NaCl, 2.6 mM KCl, and 0.5% Tween 20) containing 5% bovine serum albumin and incubated overnight at 4°C with the appropriate antibody. Antibodies used were as follows: anti-JNK1, anti-p88 MAPK, anti-actin, anti-PARP, anti-cyclin B1, and anti-cyclin A were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-JNK, anti-phospho-p38 MAPK, anti-phospho-AKT (Ser473), and anti-AKT were from New England Biolabs (Ipswich, MA)/Cell Signaling Technology Inc. (Danvers, MA); and anti-Rac1 monoclonal antibody was from Transduction Laboratories/BD Biosciences (Heidelberg, Germany). After washing, blots were incubated with horseradish peroxidase-secondary antibodies for 1 h at room temperature, and they were developed by peroxidase reaction using the enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein expression levels were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

Rac1 Activity Assays. Bacterial expression of fusion proteins and in vitro binding assays were as described previously (González-Santiago et al., 2006). The plasmid pGEX-PAK-CRIB containing the
Rac1-binding domain fused to glutathione transferase was kindly provided by J. G. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). For in vitro binding assay, the glutathione transferase-fusion protein on glutathione-Sepharose beads (purified from Escherichia coli BL21(DE3) harboring this plasmid) was incubated with cell extracts and analyzed as described previously (González-Santiago et al., 2006).

**Cell Proliferation and CalcuSyn Analysis.** Cell proliferation was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assays that were performed following the manufacturer’s instructions (MTT Cell Proliferation Kit I; Roche Diagnostics, Mannheim, Germany). Analysis of synergistic, additive, or antagonistic effects of drug combination studies was determined according to the median-effect principle analysis of Chou and Talalay (1983) using CalcuSyn software (Biosoft, Ferguson, MO). The program returns the values of the dose required for 50% inhibition of cell proliferation (IC$_{50}$) and the combination index (CI), which reflects the nature of the interaction between drugs. A CI value of 1 indicates an additive effect between two drugs, whereas a CI < 1 or CI > 1 indicates synergism and antagonism, respectively. The degree of synergism is proportional to the value of CI. CI values <0.1 represent a very strong synergism, whereas a range of CI values of 0.1 to 0.3, 0.3 to 0.7, 0.7 to 0.85, and 0.85 to 0.9 represent strong synergism, synergism, moderate synergism, and slight synergism, respectively.

**Statistical Analysis.** Results are expressed as mean ± S.E.M. Statistical significance of differences between values was calculated by one-way ANOVA and Dunnett’s post-hoc test using the Instat3 program (GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant when $P < 0.05$. When $P > 0.05$, the data were considered not significant (N.S.). The single asterisk indicates $P < 0.05$, the double asterisk indicates $P < 0.01$, and the triple asterisk indicates $P < 0.001$.

**Results**

To determine the effect of plitidepsin against melanoma, we first analyzed its antiproliferative activity in two cell lines, SK-MEL-28 and UACC-257. SK-MEL-28 are nonpigmented melanoma cells that harbor mutations in $B$-RAF, PTEN, and TP53 genes, and they express moderate levels of apoptotic protease-activating factor-1 protein. In contrast, UACC-257 are melanotic cells with normal, nonmutated PTEN and TP53 genes, whereas the absence of mutated $B$-RAF is controversial (Davies et al., 2002; Shields et al., 2007), and they show reduced apoptotic protease-activating factor-1 expression. We chose these two cell lines to explore whether the response to plitidepsin is cell-specific and whether melanin content or the most common melanoma-associated mutations can compromise the response to the drug.

Plitidepsin inhibited the proliferation of both SK-MEL-28 and UACC-257 cell lines in a concentration-dependent manner and with a very similar IC$_{50}$ value range of 12 to 14 nM at 48 h after treatment (Fig. 1A). To analyze whether, similarly to what happens in other cell types, plitidepsin induced apoptosis in melanoma cells, we studied by Western blotting the expression of poly(ADP-ribose) polymerase (PARP), whose proteolytic cleavage by caspases is a hallmark of the apoptotic process. PARP cleavage was found as soon as 3 to 6 h after drug exposure at 450 nM (Fig. 1B, top) and only at high concentrations of plitidepsin in both cell types (Fig. 1B, bottom). Flow cytometry analysis confirmed a dual, concentration-dependent effect of plitidepsin. At low concentrations (15–45 nM), plitidepsin arrested cells in the G$_1$ cell cycle phase, as shown by a slight accumulation of cells in the G$_1$/G$_0$ phase and a marked decrease in the percentage of cells in the S phase (Fig. 1C). However, there was also a persistent population of cells with a G$_2$/M phase DNA content, which probably represents G$_2$/M-arrested cells, as observed in other systems (Niculescu et al., 1998). In contrast, at higher concentrations (150–450 nM), plitidepsin induced the formation of a hypodiploid sub-G$_1$ peak indicative of apoptosis (Fig. 1C). The same results were observed in UACC-257 cells (data not shown). Consistent with the effects on the cell cycle, Western blot analysis revealed that exposure to plitidepsin for 24 h resulted in a significant concentration-dependent reduction of the levels of cyclin A and cyclin B, two cyclins whose expression are dependent on cell cycle progression through S phase and that peak at G$_2$ and G$_2$/M, respectively (Fig. 1D).

Next, we examined whether plitidepsin affected JNK activity in melanoma cells. Figure 2A shows that plitidepsin induced a rapid, progressive, and sustained activation of JNK in SK-MEL-28 and UACC-257 melanoma cells, as measured by Western blotting analysis of phosphorylated JNK levels consistent with changes noted in other cell types (García-Fernández et al., 2002; Cuadrado et al., 2003; González-Santiago et al., 2006). JNK activation was concentration-dependent, being first detected at 15 nM (Fig. 2, B and C). Plitidepsin also activated Rac1 GTPase with short kinetics (Fig. 3, A and B), and this effect was abolished by exogenous GSH (Fig. 3C). These results parallel those recently reported for breast, renal, and cervical carcinoma cells (González-Santiago et al., 2006; Suárez et al., 2006). In addition, pretreatment with NSC23766, a specific Rac1 inhibitor, prevented the cytostatic/cytotoxic effect of plitidepsin in a concentration-dependent manner (Fig. 3D).

To obtain further insight the mechanism of action of plitidepsin in melanoma cells, we explored putative actions on other kinases involved in cell survival/death, such as AKT and p38 MAPK. Unexpectedly from the prosurvival role of AKT, plitidepsin induced AKT activation as seen by an increase in the phosphorylation at residue Ser$^{473}$ (Fig. 4A, middle). This effect was not blocked by the Rac1 inhibitor NSC23766. In contrast, p38 MAPK was also activated but in a Rac1-dependent manner (Fig. 4A, bottom). As in previous results with MDA-MB-231 or HeLa cells (González-Santiago et al., 2006), JNK activation was sensitive to Rac1 inhibition (Fig. 4A, top). The putative role of phosphatidylinositol 3-kinase (PI3K) in the activation of these kinases by the compound was investigated by using wortmannin and LY294002, two PI3K inhibitors. Although neither of these inhibitors prevented the activation of JNK or p38 MAPK by plitidepsin, they abolished that of AKT (Fig. 4B). In untreated cells, wortmannin and LY294002 reduced the basal levels of phosphorylated AKT, but they had no effect on those of phosphorylated JNK or p38 MAPK (Fig. 4B). Likewise, neither of these two inhibitors affected cell viability per se, nor did they change the cytostatic/cytotoxic effect induced by plitidepsin (data not shown).

We also used SB203580, a compound usually considered a specific inhibitor of p38 MAPK. However, by concentration-curve analyses we found that SB203580 prevented JNK and p38 MAPK activation by plitidepsin with similar efficacy (Fig. 5A), as has been reported previously in breast and renal cancer cells (Cuadrado et al., 2003). Extending the crucial role that these enzymes, particularly JNK, play in other cell types (Cuadrado et al., 2004), SB203580 reverted the antiproliferative action of plitidepsin (Fig. 5B). Likewise,
SB203580 inhibited the induction of SK-MEL-28 cell apoptosis by the drug as assessed by flow cytometry (Fig. 5C). Because SB203580 itself caused G1 arrest, no effective estimation of its effect on the inhibition of cell cycle by plitidepsin can be done, although a moderate recovery of cells in the S phase was observed in cells pretreated with SB203580 before incubation with 45 nM plitidepsin (Fig. 5C).

Finally, we studied the effect of the combined treatment
of melanoma cells with plitidepsin and DTIC. As with plitidepsin (Figs. 1A and 6A), DTIC alone inhibited the proliferation of SK-MEL-28 and UACC-257 cells in a concentration-dependent manner, although with distinct potency: IC$_{50}$ value of 843 and 127 $\mu$g/ml (according to CalcuSyn analysis), respectively, at 48 h after treatment (Fig. 6A). We found that the combination of plitidepsin and DTIC was more effective at inhibiting cell proliferation than each compound alone in both cell lines at all tested concentrations. Data analysis by the Chou and Talalay (1983) method using CalcuSyn software defined plitidepsin and DTIC to act synergistically (CI $< 1$) across a broad range of concentrations. Figure 6B illustrates the CI/fractional effect plots, showing the CI values versus the fraction of cells affected (Fa) by plitidepsin and DTIC in combination. In SK-MEL-28 cells, the CI values for Fa $< 0.75$ were less than 0.3, indicating a strong synergism between plitidepsin and dacarbazine. Likewise, in UACC-257 cells a synergistic effect of plitidepsin and DTIC was found, but the degree of synergism was lower than that in SK-MEL-28 cells, with CI values ranging from 0.3 to 0.7 at the majority of tested Fa. It is remarkable that the level of JNK phosphorylation induced by suboptimal concentrations (45 nM) of plitidepsin was higher when cells were cultured in the presence of DTIC (Fig. 6C, top). This effect was specific, because no such increase was observed in the case of AKT or p38 MAPK (Fig. 6C, middle and bottom).
modulate the cell cycle, at low concentrations it causes G1 arrest. In contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induce apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not induces G2/M arrest, SB203580 reverted the antiproliferative action of low concentrations of plitidepsin, suggesting that JNK is also involved in the plitidepsin-mediated cell cycle arrest. Our data also demonstrate that Rac1 acts upstream of JNK in melanoma cells, because a specific inhibitor of this GTPase abrogates the cytotoxicity and phosphorylation of JNK by plitidepsin. These findings are consistent with published data (González-Santiago et al., 2006) and studies showing that Rac1 triggers apoptosis via JNK in response to tumor necrosis factor α (Jin et al., 2006) and ceramide (Brenner et al., 1997). A large number of anticancer drugs currently in clinical use cause cell death by JNK-mediated apoptosis. In addition, the involvement of JNK in the regulation of cell cycle progression has also been noted in previous reports; JNK mediates G2/M arrest induced by sulforaphane (Cho et al., 2005) and diallyl trisulfide (Antosiewicz et al., 2006) in human prostate cancer cells, and by thiazolidin compounds in human non-small-cell lung and colon cancer cells (Teraishi et al., 2005). It is interesting to note that glial cell line-derived neurotropic factor induces G2/M cell cycle delay via the Rac1/JNK pathway (Fukuda et al., 2005). Likewise, JNK has been shown to contribute to G1 arrest mediated by a ginseng metabolite, compound K, in human monocytic leukemia cells (Kang et al., 2005).

Our results demonstrate that plitidepsin induces an early oxidative stress, an upstream activator of the Rac1/JNK pathway (González-Santiago et al., 2006), the nature of which remains to be characterized. One possibility is that an initial generation of ROS causes the activation of Rac1, that in turn induces more ROS (Nimnuan et al., 2005) causing a positive feedback loop that may lead to apoptosis. Alternatively, the amplified ROS signaling might be due to JNK, similar to that JNK-dependent ROS formation by tumor necrosis factor (Ventura et al., 2004) and diallyl trisulfide (Antosiewicz et al., 2006). In addition, plitidepsin-mediated JNK activation might induce stabilization of JNK pathway components, thereby leading to feedback up-regulation of apoptotic signaling as described for other apoptotic stimuli (Xu et al., 2005).

The activation of AKT by plitidepsin has not been detected in other human cancer cells that have high basal levels of this protein (Cuadrado et al., 2003), and it is somehow puzzling giving the prosurvival role of this enzyme. It is remarkable that AKT activation in response to other anticancer drugs has also been described. Thus, in NIH 3T3 cells doxorubicin, etoposide, and staurosporine activate AKT preceding the onset of apoptosis (Tang et al., 2001; Kim et al., 2006; Lee et al., 2006). Moreover, activation of AKT by daunorubicin has been observed in human acute myeloid leukemia cell lines (Plo et al., 1999). It is possible that the survival signal due to AKT activation by plitidepsin signaling is overridden by its pro-

**Fig. 4.** Plitidepsin induces Rac1-dependent JNK and p38 MAPK activation and PI3K-dependent AKT activation. A, Western blot analysis showing the levels of activated JNK, AKT, and p38 MAPK in SK-MEL-28 cells upon plitidepsin treatment alone or in combination with the Rac1 inhibitor. Cells were pretreated overnight with 100 μM NSC23766 or vehicle before addition of 450 nM plitidepsin for the indicated times. B, effect of PI3K inhibitors on the activation of AKT, JNK, and p38 MAPK by plitidepsin. Cells were pretreated with 10 μM LY294002 (LY), 1 μM wortmannin (W), or vehicle for 1 h, and then they were treated with 450 nM plitidepsin for an additional hour. The levels of phosphorylated and total AKT, JNK, and p38 MAPK were analyzed by Western blot using appropriate antibodies.

**Discussion**

This is the first molecular characterization of the action of plitidepsin on human melanoma cells. We report that plitidepsin has a potent antiproliferative effect by inhibiting cell cycle progression at pharmacologically relevant concentrations (IC50 ~ 15 nM) that are similar to the circulating plasma concentrations of plitidepsin observed in phase II clinical trials (Celli et al., 2004). In addition, at higher concentrations plitidepsin induces cell death by apoptosis.

Our results demonstrate that in melanoma cells plitidepsin causes a rapid activation of Rac1 linked to a strong activation of JNK and p38 MAPK, as has been noted in breast, renal, and cervical carcinoma cells, which leads to a rapid induction of apoptosis (Cuadrado et al., 2004; González-Santiago et al., 2006; Suárez et al., 2006). However, in contrast to these other tumor types, in which plitidepsin does not modulate the cell cycle, at low concentrations it causes G1 arrest and G2/M blockage in SK-MEL-28 and UACC-257 melanoma cells. In agreement with this observation, cyclin A and cyclin B, which are associated to progression of the cycle to the G2 phase, are reduced by plitidepsin. Cell-type differences in plitidepsin action are presumably due to the specific alterations acquired by each type of cancer cells during the neoplastic process.

Although SB203580 lacks specificity of JNK versus p38 MAPK inhibition, it seems that JNK is the main mediator of plitidepsin activity, because cells lacking p38 MAPK, but not those lacking JNK, display normal plitidepsin sensitivity (Cuadrado et al., 2004). By using specific inhibitors, we demonstrate that JNK participates in the apoptosis induced by plitidepsin, because pretreatment with SB203580 decreased the number of cells in the sub-G1 fraction. Furthermore, despite that it induces G1 arrest, SB203580 reverted the antiproliferative action of low concentrations of plitidepsin, suggesting that JNK is also involved in the plitidepsin-mediated cell cycle arrest.

A large number of antitumor drugs currently in clinical use cause cell death by JNK-mediated apoptosis. In addition, the involvement of JNK in the regulation of cell cycle progression has also been noted in previous reports; JNK mediates G2/M arrest induced by sulforaphane (Cho et al., 2005) and diallyl trisulfide (Antosiewicz et al., 2006) in human prostate cancer cells, and by thiazolidin compounds in human non-small-cell lung and colon cancer cells (Teraishi et al., 2005). It is interesting to note that glial cell line-derived neurotropic factor induces G2/M cell cycle delay via the Rac1/JNK pathway (Fukuda et al., 2005). Likewise, JNK has been shown to contribute to G1 arrest mediated by a ginseng metabolite, compound K, in human monocytic leukemia cells (Kang et al., 2005).
apoptotic effects. Supporting this view, the synergistic effect of plitidepsin and DTIC are paralleled by their cooperation activating JNK but not AKT or p38 MAPK.

Consistent with our observation that its antiproliferative activity as a single agent in melanoma cell lines, plitidepsin induces long-lasting objective remissions and tumor control in a subset of advanced resistant melanoma patients (Eisen et al., 2004). DTIC is the only approved therapy for patients with metastatic melanoma, but the response rates are <20%, and the complete responses rarely exceed 5%. It is noteworthy that we found a stronger synergism between plitidepsin and DTIC in SK-MEL-28 (IC_{50} of DTIC ~ 843 μg/ml) than in UACC-257 (IC_{50} of DTIC ~ 127 μg/ml) cells, suggesting that this combination may be promising in the treatment of DTIC-resistant patients. The synergy between plitidepsin and DTIC may well result from their different signaling leading to apoptosis. Thus, although DTIC induces methylation of nucleic acids or direct DNA damage resulting in cell death (Kyrtopoulos et al., 1997), plitidepsin activates apoptosis initially acting from the cell membrane (Suárez et al., 2006). Because JNK is crucial for plitidepsin activity (Cuadrado et al., 2004) and its degree of phosphorylation/activation correlates with the antiproliferative potency of plitidepsin (Figs. 1 and 2), the increased JNK activation by the combined treat-
ment with plitidepsin and DTIC in comparison with plitidepsin alone may contribute to explain the synergistic activity of these drugs. In addition, the inhibition of VEGF secretion by plitidepsin could potentiate the therapeutic effect of DTIC, which has been shown to up-regulate VEGF expression (Broggini et al., 2003; Lev et al., 2003). These results support the ongoing phase I/II trial combining plitidepsin and DTIC in first line against advanced melanoma.

The differential response of melanoma cells to various concentrations of plitidepsin may have potential implications for the in vivo activity of this agent. Once plitidepsin reaches a tumor site in vivo, it is plausible that the cellular response to the agent can fall into one of two pathways; apoptosis at higher tissue concentrations and cell cycle arrest in tumor areas with a more limited exposure to plitidepsin. Because tumors are heterogeneous, and physicochemical and physiological barriers can lead to heterogeneous accumulation of the drug in solid tumors (Jain, 1999), a compound that has the ability to affect cell proliferation at different concentrations via distinct pathways could better allow for local tumor control.

In conclusion, this work shows that plitidepsin has a potent antitumoral activity against human melanoma cells in vitro. This effect seems to be dual, with a cytostatic response at low concentrations and a cytotoxic response at the higher concentration range. The work also reiterates the importance of the activation of Rac1/JNK pathway by plitidepsin as a major operating mechanism for this agent. In addition, it provides a rationale for the clinical evaluation of plitidepsin in combination with DTIC in advanced melanoma patients.

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References


