Plitidepsin Has a Cytostatic Effect in Human Undifferentiated (Anaplastic) Thyroid Carcinoma

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Abstract

Undifferentiated (anaplastic) thyroid carcinoma is a highly aggressive human cancer with very poor prognosis. Although there have been a few studies of candidate treatments, the fact that it is an infrequent tumor makes it very difficult to design clinical trials. A strong association has been observed between undifferentiated thyroid carcinoma and TP53 mutations in numerous molecular genetic and expression studies. Plitidepsin (Aplidin, PharmaMar, Madrid, Spain) is a novel anti-cancer compound obtained from a sea tunicate. This compound has been reported to induce apoptosis independently of TP53 status. We investigated the actions of plitidepsin in human thyroid cancer cells. In initial experiments using primary cultured cells from a differentiated (papillary) carcinoma, we found that 100 nmol/L plitidepsin induced apoptosis, whereas lower doses were cytostatic. Because our aim was to study the effects of plitidepsin at clinically relevant concentrations, subsequent experiments were done with a dosage regimen reflecting plasma concentrations observed in previously reported clinical trials: 100 nmol/L for 4 hours, followed by 10 nmol/L for 20 hours (4100/2010 plitidepsin). This plitidepsin dosage regimen blocked the proliferation of a primary undifferentiated/anaplastic thyroid carcinoma culture obtained in our laboratory and of a commercial cell line (8305C) obtained from an undifferentiated thyroid carcinoma; however, it did not induce apoptosis. The proportion of cells in the G1 phase of the cell cycle was greatly increased and the proportion in the S/G2-M phases greatly reduced, suggesting that plitidepsin blocks G1-to-S transition. Levels of the cyclin D1/cyclin-dependent kinase 4/p21 complex proteins were decreased and, in line with this, the levels of unphosphorylated Rb1 increased. The decrease in cell cycle proteins correlated with hypoacetylation of histone H3. Finally, we did experiments to assess how rapidly tumor cells return to their initial pretreatment proliferative behavior after 4100/2010 plitidepsin treatment. Cells from undifferentiated tumors needed more than 3 days to recover logarithmic growth, and after 7 days, cell number was still significantly lower than in control cultures. 4100/2010 plitidepsin inhibited the growth in soft agar. Together, our data show that plitidepsin is able to block in vitro cell cycle progression at concentrations similar to serum concentrations observed in vivo, and that this effect is persistent for several days after plitidepsin removal. Whether plitidepsin will prove to be clinically useful in the treatment of undifferentiated thyroid cancers remains to be established. However, our results raise the possibility that plitidepsin might be effective alone or in combination with radiotherapy and/or other drug treatments.

Undifferentiated (anaplastic) thyroid carcinoma is a highly aggressive malignant neoplasm typified by rapidly progressive local disease (ref. 1 and references therein). Almost all patients with undifferentiated thyroid carcinoma present with a rapidly expanding neck mass. Surrounding structures are frequently invaded, and cervical adenopathy is present in more than 40% of patients. At least 40% of patients have distant metastasis at time of diagnosis, in 50% involving the lungs, 15% the bones, and 10% the brain. Cardiac metastases have also been described. The overall 5-year survival ranges from 0% to 14%, and the mean survival is 2.5 to 6 months. (1–6). Undifferentiated thyroid carcinoma is considered a radioresistant tumor, but treatment in most clinics is based on radiotherapy plus chemotherapy, together with surgery when appropriate. Radical surgery alone does not improve survival. Patients with tumors that are amenable to complete surgical resection combined with preoperative or postoperative adjuvant doxorubicin-based chemotherapy and irradiation may have prolonged survival.
Paclitaxel seems to have been effective in a few patients (7). However, intensive chemotherapy has not been conclusively shown to improve survival. The very few cases in which chemotherapy was certainly useful had concomitant radical surgery, which is only possible in a small subset of patients who do not show regional dissemination of the tumor (1, 2, 8–10).

Fortunately, anaplastic thyroid carcinoma is a rare disease, with an annual incidence of only one or two cases in a million (1). Unfortunately, this complicates the design of standardized clinical trials of candidate treatments; there is thus a clear need for reliable models for evaluation of new compounds. Xenografts in nude mice are an established model for many types of tumor; however, heterotopic s.c. xenografts are generally poor models versus homotopic xenografts because they lack the invasive and metastatic potential of the modeled tumor (11–14), whereas homotopic xenografts are problematic in the case of thyroid cancer because of difficult access. An alternative approach is to use human anaplastic cell lines, cultured under conditions reproducing local availability of hormones and factors, because undifferentiated thyroid carcinoma is generally a locally spreading tumor.

In recent years, there have been a few in vitro studies in undifferentiated thyroid carcinoma evaluating promising anticancer molecules useful for other tumors (15–22). Unfortunately, some of these studies have used in vitro doses of questionable clinical relevance, leading to conflicting results as in the case of imatinib mesylate (23–25). Plitidepsin (Aplidin) is a new anticancer agent obtained from a marine animal (see Supplementary Fig. 1; refs. 26, 27) that is currently under phase II clinical trials. In vitro, it has shown a very potent cytotoxic effect due to apoptosis in both leukemic cell lines and primary leukemia cultures (28–31). It has also been shown to have an apoptotic effect in mammary and renal carcinoma cell lines (32).

Our interest in plitidepsin as a possible treatment for undifferentiated/anaplastic thyroid carcinoma is based on the observation that its apoptotic effect in human anaplastic cell lines, cultured under conditions reproducing local availability of hormones and factors, because undifferentiated thyroid carcinoma is generally a locally spreading tumor.

In addition, we studied the 8305C thyroid anaplastic carcinoma cell line obtained from the European Collection of Animal Cell Cultures; this line was derived from a 67-year-old female patient (33). A previous study has examined expression of some cell cycle–related genes in this cell line (36): retinoblastoma (Rb1), antigen-presenting cells (APC), and moth cytochrome c (MHC) were normal, but a C-to-T transition was detected in TP53 gene codon 273. Loss of heterozygosity of tumor suppressor genes was not observed.

We routinely tested thyroglobulin expression in the cultured cells by a very sensitive immunofluorescence technique using HeLa cells as negative control (0% staining) and 4′,6-diamidino-2-phenylindole to counterstain the nuclei (35). T-PC2 showed near 100% positivity whereas both anaplastic cultures maintained a residual staining (around 20%) in line with their follicular origin.

Cells were cultured in a complete defined medium to maintain their phenotype as much as possible: Coon's modified Ham's F-12 medium supplemented with 5% NCS (Life Technologies, Inc.), 2 mmol/L glutamine, the five-hormone mixture SH (1 mmol/L thyroid-stimulating hormone, 10 ng/mL somatostatin, 10 μg/mL insulin, 1 mmol/L hydrocortisone, and 5 μg/mL transferrin), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 ng/mL amphotericin B.

### Table 1. Main Features of the human thyroid tumor cultures used in this study

<table>
<thead>
<tr>
<th>Culture</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Tumor pathology</th>
<th>Thyroglobulin immuno-fluorescence</th>
<th>TP53 mutation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-PC2</td>
<td>M</td>
<td>34</td>
<td>Papillary carcinoma</td>
<td>94%</td>
<td>Negative (IH)</td>
<td>Bravo et al. (35)</td>
</tr>
<tr>
<td>T-AC1</td>
<td>F</td>
<td>59</td>
<td>Undifferentiated (anaplastic) carcinoma PAAF</td>
<td>31% (±5)</td>
<td>Positive (IH)</td>
<td></td>
</tr>
<tr>
<td>8305C</td>
<td>F</td>
<td>67</td>
<td>Thyroid anaplastic carcinoma</td>
<td>20% (±11)</td>
<td>transition C:G to T:A codon 273</td>
<td>Ito et al. (33, 36)</td>
</tr>
</tbody>
</table>

NOTE: A very sensitive thyroglobulin immunofluorescence assay was used to test for maintenance of the thyroid phenotype, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Although clinical samples of the original undifferentiated tumors were negative for thyroglobulin, we could still see residual staining in cultures of these tumors in complete defined medium (see Material and Methods).
All experiments were done in this complete medium, except the initial experiments described in Fig. 2C, which were initiated in deprived medium (2 days in 0.5% NCS without 5H). Plitidepsin (Aplidin, PharmaMar, Madrid, Spain) was dissolved in DMSO at 10 mg/mL (10 nM/L). In most experiments (Figs. 3–5), the cells were treated for 4 hours with 100 nM/L plitidepsin, and for the next 20 hours with 10 nM/L plitidepsin (4100/2010 plitidepsin). In the recovery experiments shown in Fig. 5, cells were treated in this way between day −1 and day 0; on day 0, plitidepsin was removed by washing thrice with PBS, and fresh plitidepsin-free medium was then added and changed every 2 days, or cells were seeded in soft agar.

**Flow cytometry.** To monitor the effects of plitidepsin on the cell cycle, cells were fixed and stained with propidium iodide and analyzed in a FACS Scan (Excalibur, Becton Dickinson). To show G1/early S-phase arrest, a 7-amino-actinomycin D (AAD)-BrdUrd double-labeling kit was used (Becton Dickinson). Following the instructions of the manufacturer, arrest, a 7-amino-actinomycin D (AAD)-BrdUrd double-labeling kit was used after incubating the cells with 10 nM/L BrdUrd for the last 4 hours of culture.

**Proliferation assays.** Initial 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done as described (37), but because 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide indicates cell activity and is only an indirect measure of cell number, we decided to count cells directly. In all experiments, cells were counted in a Neubauer cytometer chamber. An ELISA kit for detecting BrdUrd uptake (Biotrack kit, Pharmacia) was used after incubating the cells with 10 μM/L BrdUrd for the last 4 hours of culture.

**Immunohistochemistry.** For these assays, 50 nM/L Hoechst 33258 and 1.250 Annexin V-FITC (Becton Dickinson) plus 20 nM/L HEPES (pH 7.4) were added to the medium. After 45 minutes in the incubator, at least 200 cells/well were evaluated under the microscope with Annexin V-FITC or Hoechst 33258 positivity taken to indicate early or late apoptotic events, respectively. Because we did not observe any difference between the results obtained with these two techniques (35), we show the results for Hoechst staining only. In flow cytometry, cells with less than 2N DNA content were considered as apoptotic.

**Immunoblotting.** Total extracts were obtained with lysis buffer as described (35). Immunodetection was carried out with antibodies to p21Cip1, p27kip1, cyclin D1, cyclin-dependent kinase (cdk) 4, cyclin E, and cdk2 (Santa Cruz Biotechnology) at dilutions between 1:250 and 1:1,000. Rb1 (Becton Dickinson) at 1:200, β-actin (Becton Dickinson) at 1:2,000, and α-tubulin at 1:100 (Sigma). Acetylated histone H3 was detected using hot-SDS extracts (38) at 1:1,000 dilution (Cell Signaling). A kit with alkaline phosphatase–labeled secondary antibodies (Tropix, Bedford, MA) was used for detection.

**Apoptosis assays.** For these assays, 50 nM/L Hoechst 33258 and 1:250 Annexin V-FITC (Becton Dickinson) plus 20 nM/L HEPES (pH 7.4) were added to the medium. After 45 minutes in the incubator, at least 200 cells/well were evaluated under the microscope with Annexin V-FITC or Hoechst 33258 positivity taken to indicate early or late apoptotic events, respectively. Because we did not observe any difference between the results obtained with these two techniques (35), we show the results for Hoechst staining only. In flow cytometry, cells with less than 2N DNA content were considered as apoptotic.

**Soft agar.** 4100/2010 plitidepsin was added on day −1. On day 0, plitidepsin was removed by washing thrice with PBS, and cells were trypsinized and counted. Cells at 50,000/35 mm well were seeded in 0.3% top agar (0.9% bottom agar) using the complete medium (see above). Every 4 days, 1 mL of fresh top agar was added. Fifteen days later, colonies bigger than four cells were counted.

**Statistical analysis.** All experiments were repeated at least thrice with four replicates when possible. Results were compared by a nonparametric

![Fig. 1. Histology of the tumors from which the primary cultures were obtained.](https://example.com/fig1.png)
Cytostatic Effect of Plitidepsin in Thyroid Carcinoma

A

B

C

D

E

% apoptosis

5 10 15 20 25 30 35

0 8h 12h 14h 24h 34h

Control

1nM Plitidepsin

10 nM Plitidepsin

100 nM Plitidepsin

Proliferating

Oh

60%/61% S=14% G2/M=16%
Apoptosis=0%

60%/61% S=5% G2/M=83%
Apoptosis=0%

60%/61% S=7% G2/M=12%
Apoptosis=0%

60%/61% S=9% G2/M=14%
Apoptosis=0%

60%/61% S=13% G2/M=10%
Apoptosis=0%

60%/61% S=15% G2/M=15%
Apoptosis=4%

60%/61% S=48% G2/M=2%
Apoptosis=16%

60%/61% S=53% G2/M=3%
Apoptosis=34%

60%/61% S=65% G2/M=3%
Apoptosis=29%

60%/61% S=67% G2/M=1%
Apoptosis=22%
Results

To evaluate the effects of plitidepsin in human thyroid carcinoma, we first cultured the papillary carcinoma T-PC2 line (35) in growth medium containing different concentrations of plitidepsin. We did not detect any significant effect of 1 nmol/L plitidepsin on cell growth over the first 24 hours, either in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (results not shown) or BrdUrd uptake assay (Fig. 2A). However, higher plitidepsin doses (10 and 100 nm) caused significant declines in cell growth (Fig. 2A). Because plitidepsin has been reported to have a strong apoptotic effect in human cells, we evaluated apoptosis by Hoechst staining assay. Only 100 nmol/L plitidepsin strongly induced apoptosis, and after 72 hours, no cells could be seen (Fig. 2B); 10 nmol/L plitidepsin induced significant apoptosis only after 72 hours and only in a small proportion of cells (Fig. 2B). Direct cell counting indicated that both 10 and 100 nmol/L plitidepsin significantly reduced the number of cells at 24, 48, and 72 hours (Fig. 2C).

The main reported effect of plitidepsin in human leukemia cells is apoptosis induction, even with low doses of 1 to 10 nmol/L (28–30, 39). In epithelial cells, high doses of plitidepsin (100–500 nmol/L) have apoptotic effects (32, 40, 41). To assess possible dose dependence in thyroid tumor cells, we did a dose-response experiment using synchronized cells that had been maintained for 2 days in deprived medium. More than 90% of these cells were in G0/G1 phase (Fig. 2D). Cells
were then restimulated with complete medium alone or in the presence of different concentrations of plitidepsin. Control cells were slowly reentering the cell cycle so that by 34 hours after the start of the experiment, the cell-phase percentages were the same as before deprivation. Plitidepsin at 1 nmol/L had no effect on recovery after deprivation; 10 nmol/L plitidepsin completely blocked entry into the cell cycle but had no apoptotic effect; 100 nmol/L plitidepsin had a surprising effect, apparently accelerating incorporation into the cell cycle but driving the cells to apoptosis. Figure 2E shows the percentage of apoptotic cells in each treatment. Together with the other data shown in Fig. 2, these results indicate that plitidepsin has a dual effect in thyroid follicular cancer cells: cytotoxic at high doses, cytostatic at low doses.

The previously reported phase II clinical trials have indicated that plitidepsin concentrations in plasma and blood depend on the duration of infusion. After infusion for 1 or 3 hours, plitidepsin concentration peaks at around 50 to 100 nmol/L, then after a few hours declines to 10 nmol/L; by contrast, after infusion for 24 hours, plitidepsin concentration remains above 10 nmol/L for more than 24 hours, but it is not known when it achieves the peak and what concentration it has (42). Concentrations above 100 nmol/L were rarely seen and never for long. We wanted to test an effective concentration to compare with other studies in epithelial carcinoma cells where they used 100 to 500 nmol/L during 24 hours (32, 40, 41), but we had the aim of modeling real clinical conditions. Preliminary time-dependent experiments with 100 nmol/L plitidepsin showed an apoptotic effect after 24-, 12-, and 8-hour exposure but not after only 4 hours of incubation (Fig. 2 and data not shown). For that, in the subsequent experiments, we used a combined dose of 100 nmol/L for 4 hours, followed by 10 nmol/L for 20 hours (henceforth 4100/2010 plitidepsin). We used three cell lines:

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Fig. 4. Exposure to plitidepsin for 24 hours (100 nmol/L for 4 hours, 10 nmol/L for 20 hours; 4100/2010 plitidepsin) blocks progression from G1 to S phase in human thyroid tumors. A, a representative experiment using double-labeled cells (BrdUrd for active S-phase labeling and AAD for total DNA content) maintained in the absence (control) or presence of 4100/2010 plitidepsin. B, statistical comparisons for four independent experiments, indicating a very significant decline in the proportion of S-phase cells after plitidepsin treatment. C, the levels of G1 cell cycle proteins, such as cyclin D1, cdk4, and p21Cip1, were decreased by 4100/2010 plitidepsin. p27kip1 levels were very low as expected because these are cycling cells, but even so, a reduction was observed after plitidepsin treatment. Cdk2 was also affected. Cyclin E levels were reduced in T-PC2 and T-AC1 but unaffected in 8305C cells. The cytoskeleton proteins β-actin and α-tubulin were unaffected. Unphosphorylated Rb1 was consistently increased in all tumors after plitidepsin whereas phosphorylated Rb1 levels decreased. In the T-AC1 culture, total Rb1 levels were increased. As a control for phosphorylated Rb1, we used U2OS cells, which have a mutated Rb1 gene. D, histone H3 acetylation was abolished by 4100/2010 plitidepsin in the three thyroid tumors. As a control, we used 3 mmol/L butyrate for 24 hours (6). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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6 L. Lopez-Lazaro, personal communication.
Fig. 5. The cytostatic effect of 4\(^{10^5}/20^5\) plitidepsin remains for some days after washing the drug. Cells were treated with plitidepsin from day \(-1\) to day 0, then washed thoroughly and left in complete medium over the following week. Cell numbers are shown for T-PC2 (A), T-AC1 (B), and 8305C (C). Insets, data are expressed on a semilogarithmic scale. D, the same treatment with plitidepsin from day \(-1\) to day 0 was applied, then cells were washed, trypsinized, and seeded in soft agar. Fifteen days later, colonies bigger than four cells were counted. Pictures of T-PC2 tumor that made big colonies in the agar (\(>40\)). T-AC1 grew poorly whereas 8305C presented many small colonies. In the three cases, the differences were very significant. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
T-PC2, obtained in our laboratory from a primary culture of a papillary carcinoma (35), as used in the initial experiments; T-AC1, obtained in our laboratory from an undifferentiated thyroid carcinoma (see Material and Methods), and bearing a TP53 mutation; and 8305C, a commercial cell line that was likewise obtained from an undifferentiated thyroid carcinoma and that has a TP53 mutation but otherwise normal cell cycle–related genes (33, 36). Figure 3A shows the marked effect of 4100/2010 plitidepsin on growth, with cell proliferation blocked in all three cultures. Figure 3B shows that 4100/2010 plitidepsin had no apoptotic effect, although 100 nmol/L plitidepsin for 24 hours caused strong apoptosis. Figure 3C compares the 4100/2010 plitidepsin effects on growth in the T-PC2 thyroid cells with other epithelial carcinoma cells, such as MCF-7 (pleural metastasis of a breast carcinoma) and HeLa (endometrial carcinoma). In MCF-7, plitidepsin blocked cell proliferation as in PC-2 without inducing apoptosis (data not shown). The number of HeLa cells after 4100/2010 plitidepsin was significantly lower than previous to the treatment and around 20% to 30% were apoptotic cells (data not shown and see below). Figure 3D shows that 4100/2010 plitidepsin significantly reduced the proportion of cells in the S and G2–M phases in all three thyroid cultures (i.e., in both papillary and undifferentiated carcinomas). No significant percentage of cells with less than 2N DNA was detected, opposed to HeLa cells where the percentage was around 20% (apoptotic cells). Taken together, these results indicate that 4100/2010 plitidepsin blocks proliferation of human thyroid carcinoma cells independently of their TP53 status, and more specifically suggest that this treatment blocks the transition from G1 to S phase (resulting in accumulation in G1 phase).

To further study the effects of 4100/2010 plitidepsin on the cell cycle, we double labeled cells with AAD to determine stage in the cell cycle, and with BrdUrd to reveal the passage through S phase. Figure 4A shows the results of a representative double-labeling experiment of this type. 4100/2010 plitidepsin greatly reduced the number of cells that took up BrdUrd, with a peak in the lower left quadrant of the figure (i.e., low BrdUrd uptake, low AAD staining). Figure 4B shows that the number of cells that passed through the S phase (i.e., cells taking up BrdUrd) was markedly and significantly lower in the presence of 4100/2010 plitidepsin. These results again indicate that this treatment blocks the transition from G1 to S phase. The S phase can be defined as the period between the last third of the AAD-defined G1 peak and the first third of the AAD-defined G2–M peak; within this period, we will consider the first half as “early S” and the second half as “late S.” 4100/2010 plitidepsin greatly reduced the proportion of BrdUrd-labeled cells in both early and late S phase, confirming blockage of the transition from G1 to S phase, and ruling out a block in early S phase (see Supplementary Fig. 2).

The transition from G1 to S phase is regulated by a sequential activation of cyclin D1-cdk4 and cyclin E-cdk2 to obtain a fully phosphorylated Rb1 and initiate DNA synthesis. We studied these cell cycle–related proteins in lysates of the three tumors incubated with 4100/2010 plitidepsin (Fig. 4C). Levels of cyclin D1-cdk4, cdk2, and p21Cip1 were reduced to similar extents in the lysates of all three tumors. Some proteins were affected differently in the three tumors: p27kip1 levels were reduced by 4100/2010 plitidepsin in all cultures, although this reduction appeared less marked in T-PC2 than in T-AC1 and 8305C; cyclin E levels were reduced by 4100/2010 plitidepsin in T-PC2 and T-AC1, but were unaffected in 8305C. The levels of unphosphorylated Rb1 (the active form) were increased in all three tumors by 4100/2010 plitidepsin, notably in T-AC1 in which even total Rb1 levels were increased. Cytoskeleton proteins such as β-actin and α-tubulin were not affected. Taken together, these results suggest that the proteins of which levels were most markedly and consistently reduced by 4100/2010 plitidepsin were those involved in the transition from G1 to S phase before the “start” point. There was a similar reduction in proteins promoting proliferation than in cell cycle inhibitors. Some anticancer drugs act through histone deacetylase inhibition, up/down-regulating the transcription of specific cell cycle proteins (reviewed in refs. 43 and 44), whereas others inhibit histone acetyltransferase, inducing a silencing state (45, 46). We studied the levels of histone H3 acetylation after 4100/2010 plitidepsin, using as a control 3 mmol/L sodium butyrate, a well-described histone deacetylase inhibitor. A low basal H3 acetylation status is the result of a histone deacetylase/histone acetyltransferase balance for any given gene that is being transcribed plus the hypoacetylated heterochromatin (Fig. 4C; ref. 47). Whereas sodium butyrate induced hyperacetylation, plitidepsin induced a strong hypoacetylated H3 status, suggesting a transcriptional repressive state.

Finally, we did two experiments to assess the extent to which cells recover their initial pretreatment proliferative phenotype after 4100/2010 plitidepsin treatment. First, small numbers of cells were seeded, allowed to attach for 24 hours, then treated with 4100/2010 plitidepsin (day –1 to day 0); the plitidepsin was then removed by thorough washing and fresh medium was added. Cells were counted on several days over the subsequent week. For all three tumors, the total number of cells after 4100/2010 plitidepsin treatment and removal remained markedly and significantly lower than in the corresponding controls (i.e., cells not exposed to plitidepsin) for 5 days (Fig. 5A-C). This result indicates that the effects of plitidepsin are persistent. On day 5, different patterns were observed for the different tumors: in the case of the T-PC2 papillary carcinoma culture, cell numbers remained significantly lower than in the corresponding control culture, but the difference was small; in the T-AC1 undifferentiated carcinoma culture, there was no significant difference with respect to the corresponding control; whereas in the 8305C undifferentiated carcinoma, cell numbers remained much lower than in the corresponding control. Tumor growth rates were analyzed by plotting these data as semilogarithmic curves (Fig. 5A-C, insets). T-PC2 recovered exponential growth after the second day without plitidepsin, whereas the two undifferentiated tumors recovered exponential growth only after the third day. This result may have important implications for in vivo efficacy and may be related to the results of Gajate et al. (30), who found that the apoptotic effect of plitidepsin was stronger in leukemic lymphocytes than in normal resting peripheral lymphocytes. Second, we tested the effect of 4100/2010 plitidepsin treatment to prevent anchorage-independent growth. After treatment, cells were seeded in soft agar in the complete medium. The number of colonies was counted after 15 days. Untreated cells presented some differences in their colonies after that time as T-PC2 showed very big colonies, T-AC1 showed low number of small colonies, and 8305C had a big number of small colonies. However, in the three tumors, plitidepsin was able to reduce very significantly the number of colonies.
Discussion

We have studied the effects of plitidepsin on human thyroid undifferentiated (anaplastic) tumor cells, one of them cultured directly from a fine needle aspiration biopsy. In these cultures, at doses similar to plasma concentrations observed in vivo in plitidepsin-treated patients, plitidepsin had a potent and stable cytostatic effect and did not induce apoptosis. Moreover, its effect was independent of the presence of a mutation in TP53.

We also studied a papillary carcinoma culture, T-PC2 (35), which has been reported that differentiated thyroid carcinoma can transform into undifferentiated thyroid carcinoma. In fact, both our own primary undifferentiated culture (T-AC1) and the commercially available 8305C cell line showed a stable 20% of cells weakly positive for thyroglobulin when cultured with the thyroid-conditioned medium. It is known that undifferentiated thyroid carcinoma express thyroglobulin mRNA (48), but in clinical routine immunohistochemistry, undifferentiated thyroid carcinomas are negative for thyroglobulin expression. The higher sensitivity of immunofluorescence versus immunohistochemistry could be the explanation for this percentage of positive cells in our cultures, while being very specific with a completely negative control of nonthyroid cells (see Material and Methods).

It has previously been reported that plitidepsin has a widespread apoptotic effect in tumor cells. The fact that we needed surprisingly long incubation times and high concentrations to achieve an apoptotic effect in our tumor cell cultures could be due to an intrinsic resistance to apoptosis of thyroid neoplastic cells. Conversely, it could be due to a particular sensitivity of leukemic cells to apoptosis induction by plitidepsin because plitidepsin showed marked apoptotic effects in these cells with very low doses (20 nmol/L) over short incubation times (29, 31). In epithelial tumor cells, the apoptotic effects of plitidepsin have been explored with very high doses to investigate biochemical mechanisms, not with a view to assessing clinical efficacy (32, 40, 41). Using the combined dose, we could see apoptosis in HeLa cells as previously described with bigger doses (41), but not in the breast carcinoma cells MCF-7, a caspase 3–negative cell line.

Our observation that plitidepsin blocks anaplastic cells in the G1 phase of the cell cycle, despite the TP53 mutation, is a particularly interesting finding. It is known that TP53 alterations confer resistance to chemotherapy-induced apoptosis, at least in many preclinical models (49). However, the importance of apoptosis in chemotherapy in vivo has been questioned recently. It seems that in real tumors, the main pathway is cell cycle arrest dependent on (e.g., doxorubicin) or independent of (e.g., paclitaxel) intact TP53 (50, 51). In the present study, plitidepsin completely blocked S phase of the cell cycle and led to an accumulation of cells in G1. In line with this, cyclin D1, cdk4, and p21 protein levels were markedly reduced. We cannot rule out the possibility of the effects on these proteins being consequences of arrest, not causal. However, because cyclin E levels remained unchanged in one tumor, and the levels of p27 or cdk2 were also little affected, it seems that the effects are specific for G1 regulatory proteins. To date, the only signal transduction pathway that has been implicated in the effects of plitidepsin action is the protein kinase C-6/c-jun NH2-terminal kinase pathway (30, 32, 40, 41). All these studies have investigated the apoptotic actions of plitidepsin, and the results cannot be directly extrapolated to cytostatic actions. However, it is interesting to speculate a possible involvement of this pathway because protein kinase C-6 represses transcription from the cyclin D1 promoter (52). Although initially p21Cip1 was considered a straightforward tumor suppressor, it has subsequently been established that it promotes the association and stability of cyclin D1-cdk4 complexes (53). In fact, cyclin D1 binding prevents the degradation of p21Cip1 on the proteasome (54), and the oncogene Ras promotes the accumulation of the complex cyclin D1/p21Cip1. It would be very interesting if plitidepsin proved to counteract the effects of Ras, decreasing cyclin D1 (and consequently p21Cip1 and cdk4), because the most frequent mutations found in aggressive papillary carcinomas are in genes of the Ras/Raf-B pathway (55). In any case, we found that after plitidepsin treatment, p53 appears in its unphosphorylated/active state and the cell cycle stops. No mutations of p53 have been reported in undifferentiated/anaplastic thyroid tumors. The hypoacetylated H3 levels following plitidepsin could be a direct effect activating histone deacetylases/inhibiting histone acetyltransferases or could be a result of the G1-S phase arrest. Two other natural anticancer drugs (curcumin and gancinol) directly inhibit histone acetyltransferase activity in cultured cells (45, 46). Histone acetyltransferase–related chromosomal translocations are a recurrent finding in acute leukemias (56, 57). At very low doses, plitidepsin had a strong apoptotic effect in leukemias. It would be interesting to investigate a histone acetyltransferase inhibitory action in responsive leukemias or carcinomas.

In accordance with the data from human leukemia cells, we found that plitidepsin effects were prolonged even after removing the drug and thoroughly washing the plate: even 1 week after plitidepsin, cell number remained significantly lower than in untreated cells, despite the fact that the controls were reaching a confluent state and slowing their growth. After plitidepsin removal, the undifferentiated tumor cultures required 3 days to reattain logarithmic growth, versus only 2 days for the papillary tumor culture. The fact that the main action of plitidepsin was to block the cell cycle and thus synchronize the cells suggests that it may be useful to combine plitidepsin with other therapies that destroy cells when DNA is being synthesized, such as radiotherapy or other chemotherapies.

Finally, in the present study, we have used an in vitro plitidepsin dosage regimen (100 nmol/L for 4 hours, 10 nmol/L for 20 hours) roughly corresponding to the plasma concentrations observed in vivo in phase I/II clinical trials (42). In these clinical trials, however, the length of the plitidepsin infusion period has varied (1, 3, or 24 hours), and for this and other reasons, extrapolation of our in vitro findings to the in vivo situation can only be tentative. If plitidepsin is indeed effective in vivo, it may be necessary to adapt dosage regimens depending on the particular type of tumor (e.g., leukemia versus solid tumors).

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