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Mar. Drugs **2013**, *11*, 1-x manuscripts; doi:10.3390/md110x000x

OPEN ACCESS

marine drugs

ISSN 1660-3397

www.mdpi.com/journal/marinedrugs

Article

c-Jun N-terminal kinase phosphorylation is a biomarker of plitidepsin activity

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Received: / Accepted: / Published:

Abstract: Plitidepsin is an antitumor drug of marine origin currently in Phase III clinical trials in AGAINST multiple myeloma. In cultured cells, plitidepsin induces cell cycle arrest or an acute apoptotic process in which sustained activation of JNK plays a crucial role. To optimize its clinical use, we evaluated the possibility of using JNK activation as an *in vivo* biomarker of response. In this study, we show that plitidepsin administration to mice xenografted with human cancer cells increases phosphorylation of JNK in tumors at 4 to 12 h after a single dose of drug. In contrast, no changes were found in other targets of plitidepsin *in vitro* such as the levels of phosphorylated-ERK or -p38MAPK, or of $p27^{KIP1}$ protein. Interestingly, plitidepsin also increased JNK phosphorylation in spleens from xenografted mice with a similar kinetics to that observed in tumors, suggesting that normal tissues might be used to test drug activity. Consistently, the administration of plitidepsin to rats that leads to plasma concentrations achievable in patients also increased JNK

phosphorylation in peripheral blood mononuclear cells. These data suggest that changes in JNK activity is a reliable marker for plitidepsin activity that could be useful to the design of clinical trials in order to maximize the efficacy of plitidepsin.

Keywords: Plitidepsin; Aplidin; JNK; biomarker; xenograft

1. Introduction

Plitidepsin (Aplidin®, APL) is a marine cyclic depsipeptide originally isolated from the tunicate *Aplidium albicans* and currently obtained by synthesis. Plitidepsin is under Phase III clinical development in patients with relapsed/refractory multiple myeloma (ADMYRE trial).

Plitidepsin has demonstrated strong anticancer activity in a large variety of human cancer cell lines *in vitro* and in xenografted mice [1]. In cultured cells from solid tumors, plitidepsin induces dose-dependently cell cycle arrest or an acute apoptotic process mainly through the sustained activation of c-Jun N-terminal kinase (JNK) as revealed by an increased level of phosphorylation [2,3]. Plitidepsin activates also other kinases such as the epidermal growth factor receptor, protein kinase C-δ and the extracellularly regulated and p38 mitogen-activated protein kinases (ERK, p38MAPK) in a cell-dependent context [4,5]. In breast cancer cells, plitidepsin induces several early response genes such as c-*JUN*, *JUNB*, *JUND*, c-*FOS*, *FOSB* and *FRA1*, and also *RELA*/p65, the major component of the transcription factor nuclear factor *kappa* B (NF*k*B), while decreases the cellular content of c-MYC protein [6]. Additionally, a series of candidate plitidepsin target genes have been identified in transcriptomic and proteomic studies in several cell types [7-9]. In sarcoma cells, plitidepsin increases the cellular content of the $p27^{KIP1}$ cell cycle inhibitor [10].

Plitidepsin has strong activity against hematological cancer cells at nanomolar concentration ranges RANGE, in which it activates both the intrinsic/mitochondrial and extrinsic/death receptor apoptotic pathways [9,11-13]. Remarkably, plitidepsin-induced apoptosis is only partially dependent of caspases and independent of the *TP53* tumor suppressor gene status [5,11]. In haematological malignancies (myeloma multiple and leukemia) cell lines, plitidepsin induces JNK activation and translocation from the cytosol to plasma membrane lipid rafts [9,14].

The objective of this study was to identify a potential pharmacodynamic biomarker of plitidepsin. In view of the results obtained in a panel of hematological cancer cell lines, we evaluated JNK phosphorylation as a surrogate of its activation and a biomarker of plitidepsin activity *in vivo*. The level of JNK phosphorylation was examined in tumors from athymic mice xenografted with human leukemia K562 cells following plitidepsin treatment. In K562 tumor-bearing mice, increased phosphorylated-JNK level was observed in tumors at 4 to 12 h after a single dose administration. In spleens of host mice, a similar time course and extent of phosphorylated-JNK was observed. Furthermore, intravenous administration of plitidepsin to rats significantly increased JNK phosphorylation in peripheral blood mononuclear cells 8 h after administration. In contrast, no changes were found in the levels of phosphorylated-ERK or -p38MAPK. Likewise, the expression of $p27^{KIP1}$ was unaffected by plitidepsin. These results validate phosphorylated JNK as a pharmacodynamic biomarker of plitidepsin in xenografted tumors as well as in normal surrogate tissues.

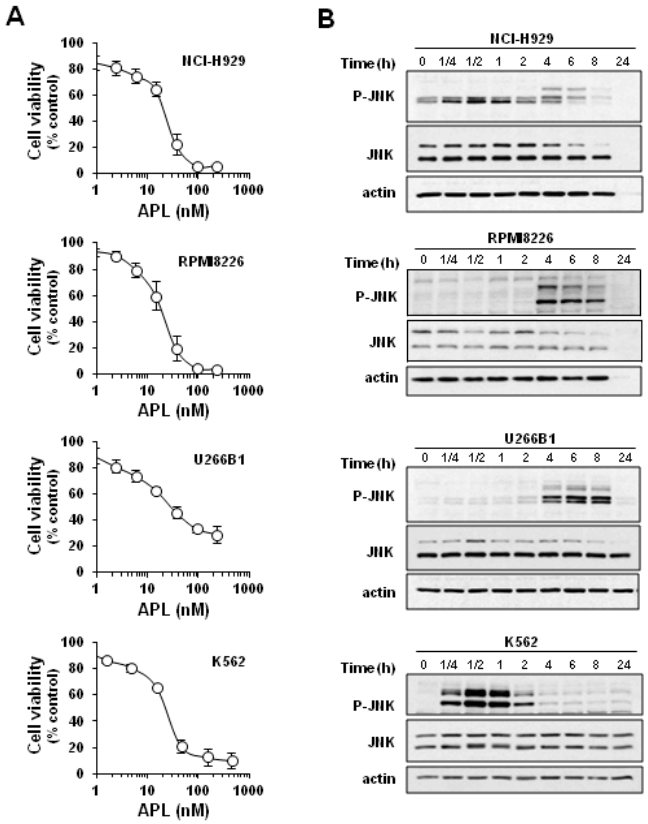
2. Results and Discussion

2.1. Plitidepsin induces JNK phosphorylation in cultured K562 leukemia and other hematological cancer cells

Previous studies have shown that Plitidepsin induces JNK phosphorylation in a variety of cultured solid and hematological cancer cell lines [3-5,9,11,15]. Given the advanced clinical development of Plitidepsin in multiple myeloma and leukemia, we evaluated the effect of Plitidepsin on the viability and the level of JNK phosphorylation in a panel of these cancer cell types.

Plitidepsin showed a strong, concentration-dependent effect on the viability of multiple myeloma NCI-H929, RPMI8226 and U266B1 cells (IC_{50} of ~ 11, 13 and 34 nM at 24 h post-treatment) and of chronic myelogenous leukemia K562 cells (IC_{50} of ~ 20 nM) (Fig. 1A). Western blot analysis revealed a progressive increase of the level of phosphorylated JNK by plitidepsin in the multiple myeloma cell lines, which persisted until cell death (Fig. 1B). In K562 cells, plitidepsin also induced JNK phosphorylation that was extremely rapid (within the first 15 min of treatment) and sustained, reaching a maximum at 30 to 60 min (Fig. 1B). These results suggested that K562 cells are good responders to plitidepsin cytotoxicity and JNK activation and so, a suitable model to assess JNK phosphorylation as a potential biomarker of plitidepsin in xenografted mice. An additional reason to choose K562 cells for *in vivo* studies is their expression of the chimeric BCR-ABL protein, which allows checking the purity of the tumor and host tissue samples.

Figure 1. Plitidepsin decreases viability and induces JNK activation in human hematological cancer cells. (A) Multiple myeloma NCI-H929, RPMI8226 and U266B1 cells, and leukemic K562 cells were incubated in the presence of the indicated concentrations of plitidepsin (APL) for 24 h and cell viability was determined by the MTT assay. Data are mean ± S.E.M. obtained in three experiments performed in quadruplicate. (B) Western blot analysis showing the levels of total (JNK) and phosphorylated JNK (P-JNK) protein in cells incubated with plitidepsin (100 nM) for the indicated times. Actin was used as loading control.



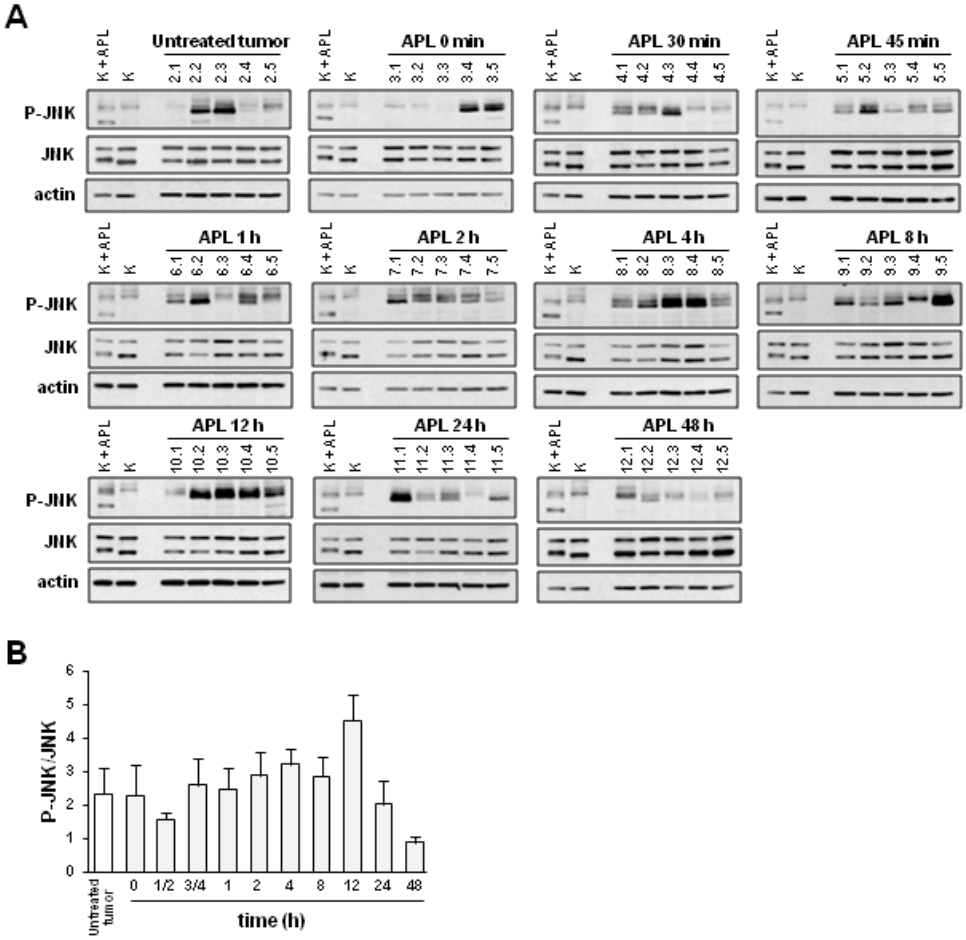
To optimize the detection of phosphorylated JNK in tissue extracts, we examined by immunoprecipitation the specificity of two widely used commercial anti-phospho-JNK antibodies. As shown in Supplementary Figure S1, although both antibodies recognized phosphorylated JNK in these assays, we selected for the *in vivo* study the anti-P-JNK sc-6254 antibody from Santa Cruz that rendered a better signal in whole protein extracts.

2.2. Plitidepsin induces JNK phosphorylation in both tumors and spleens from K562 xenografts

To evaluate the ability of plitidepsin to active JNK *in vivo* we first subcutaneously implanted K562 cells in athymic nude mice (5 x 10^6 cells/mouse). Twenty days after initial tumor detection mice were randomized into treatment and control groups (five mice/group) and treated animals received plitidepsin at 200 μg/kg. Mice in control group were sacrificed immediately after plitidepsin administration (0 h). Tumors and spleens of all animals were harvested at several time points after the treatment.

Western blot analysis of JNK phosphorylation in xenografted K562 tumors showed substantial variability in each group of animals; however, the number of animals with high level of phosphorylated JNK increased in response to 4-12 h plitidepsin treatment (Fig. 2A). No changes in the level of total JNK protein were observed. Samples from plitidepsin- or vehicle-treated K562 cells were included in each gel in order to compare the signals obtained in different membranes. Quantification of phosphorylated JNK upon normalization to total JNK and to differences in inter-membranes signal was performed by optical densitometry (Fig. 2B). A tendency for increased level of phosphorylated JNK was observed in tumors corresponding to 4 to 12 h plitidepsin administration. This effect was transient, as JNK phosphorylation returned to pretreatment levels at 24-48 h after dosing.

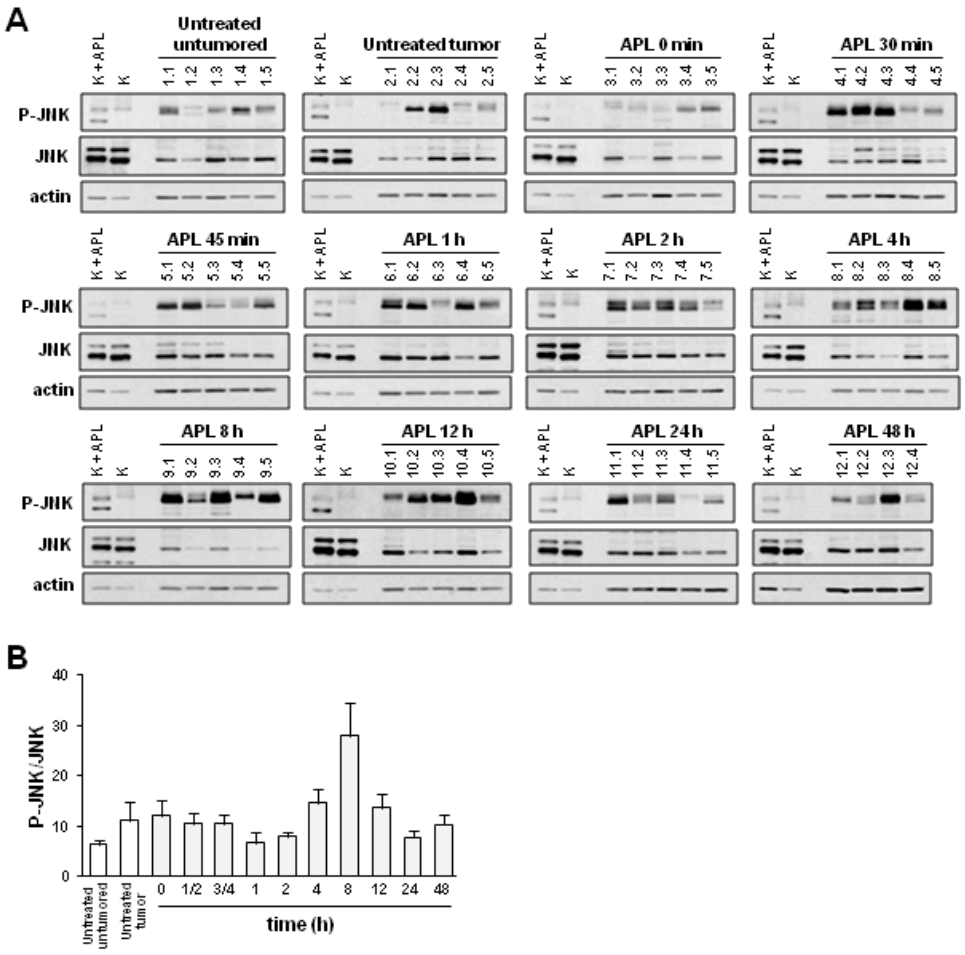
Figure 2. Plitidepsin increases the level of phosphorylated JNK in tumors of K562-bearing mice. Mice xenografted with K562 cells received a single administration of plitidepsin (APL; 200 μg/kg) and either immediately (0 min) or at the indicated times after injection were sacrificed (5 mice/group). (A) Western blot analysis of total (JNK) and phosphorylated JNK (P-JNK) in excised tumors. Actin was used as loading control. K, K562 cells; K + APL, plitidepsin-treated K562 cells. (B) Quantification of P-JNK levels (normalized to those of total JNK) and related to that of vehicle-treated K562 sample at the indicated times after APL administration. Mean ± S.E.M. values of each group of animals are shown.



As the phosphorylation of p38MAPK and ERK and the expression level of $p27^{KIP1}$ protein increase in response to plitidepsin in several cultured cell lines [4,5,10], we examined also the activation of these kinases and $p27^{KIP1}$ level by using appropriate antibodies. In contrast to the *in vitro* data, plitidepsin did not change the level of phosphorylated-ERK or -p38MAPK in xenografted K562 tumors (Supplementary Fig. S2). This result discarded these kinases as markers of plitidepsin action *in vivo*. Likewise, no changes were found in the level of $p27^{KIP1}$ expression following plitidepsin administration (Supplementary Fig. S3). Confirming the purity of excised tumors, high level of the fusion BCR-ABL protein expressed in the xenografted K562 cells was found in tumor samples (Supplementary Fig. S4).

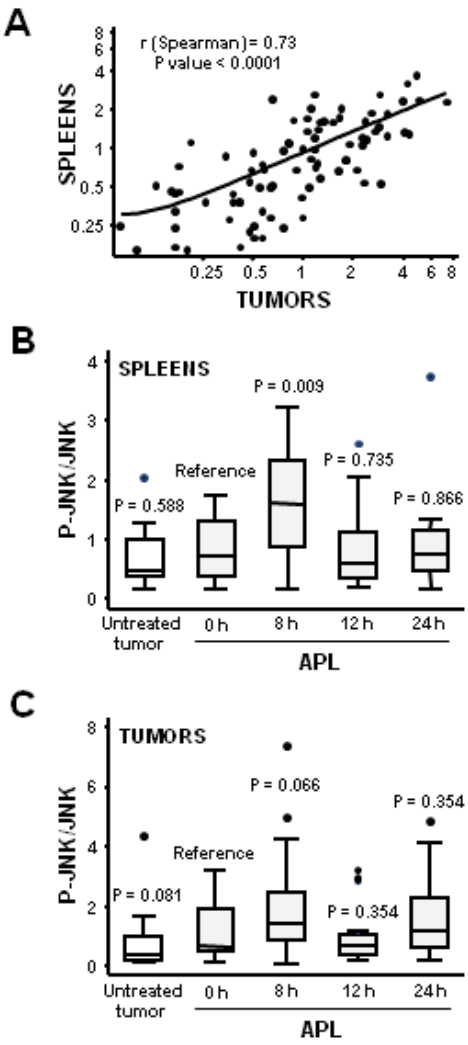
We then sought to compare phosphorylation of JNK in tumor and normal tissues that might be used as a surrogate to analyze changes in level of phosphorylated JNK in response to plitidepsin. For that purpose we chose spleens that are a source of mononuclear cells. Interestingly, JNK phosphorylation increased in spleens from K562 tumor-bearing mice after plitidepsin administration (Fig. 3A). Moreover, both tissues responded similarly to plitidepsin treatment and there was a good correlation between the levels of phosphorylated JNK is tumor and spleen in each animal (Fig. 3A). Consistently with the previous experiment, densitometry analysis confirmed an increase of phosphorylated JNK in spleens of mice treated with plitidepsin for 4 to 12 hours, an effect that reversed also at 24 h post-treatment (Fig. 3B).

Figure 3. Plitidepsin increases the level of phosphorylated JNK in spleens of K562 tumor-bearing mice. Mice xenografted with K562 cells received a single administration of plitidepsin (APL; 200 μg/kg) and either immediately (0 min) or at the indicated times after injection were sacrificed (5 mice/group). (A) Western blot analysis of total (JNK) and phosphorylated JNK (P-JNK) in spleens excised from the animals. Actin was used as loading control. K, K562 cells; K + APL, plitidepsin-treated K562 cells. (B) Quantification of P-JNK levels (normalized to those of total JNK) at the indicated times after APL administration and related to that of sample vehicle-treated K562 at the indicated times after APL administration. Mean ± S.E.M. values of each group of animals are shown.



To confirm JNK phosphorylation as a pharmacodynamic biomarker of plitidepsin allowing a better statistical analysis, we performed a new experiment using higher number of animals per group. Fifteen K562 tumor-bearing mice were treated with a single dose of plitidepsin (200 μg/kg) for 0, 8, 12, or 24 h, and the level of phosphorylated JNK was analyzed in tumors and spleens by Western blot. To allow the comparison of signals in different membranes, samples from four untreated mice were included in all of them. Confirming earlier observations, a transient increase in JNK phosphorylation was found in response to plitidepsin in both, tumors and spleens from xenografted mice (Supplementary Fig. S5). In addition, to include all data in the statistical analysis, samples from the first experiment were analyzed together with control samples from the second one (Supplementary Fig. S6). For statistics evaluation, signals were quantified by densitometry and related to that of control sample #64, which was selected as reference signal. A strong correlation was found between the increase in JNK phosphorylation in tumors and spleens of the same animal, with a Spearman´s correlation coefficient of 0.73 and a P value < 0.0001 (Fig. 4A). In addition, a statistically significant increase in phosphorylated JNK was found at 8 h after plitidepsin treatment in spleens (P value = 0.009) (Fig. 4B) and a strong tendency close to significance in tumors (P value = 0.066) (Fig. 4C). In summary, these data demonstrated that JNK is activated in response to plitidepsin *in vivo* and suggested that JNK activation can be detected in both, tumors and normal mouse tissues.

Figure 4. Statistical analysis of JNK activation by plitidepsin in tumors and spleens from mice xenografted with K562 leukemia cells. (A) Relation between the activation of JNK in tumors and spleens of K562 bearing-mice after plitidepsin administration. Samples corresponding to mice untreated and treated with plitidepsin (200 μg/kg) for 0, 8, 12 or 24 h were pooled. p-JNK/total JNK ratios in tumors and spleens were computed and their correlation statistically assessed following a non-parametric approach. The Spearman´s correlation coefficient (r) and P value are shown. (B, C) Box-plot of JNK activation in tumors and spleens of K562 bearing-mice after plitidepsin administration. The p-JNK/JNK ratio in spleens (B) and tumors (C) at each time point was compared to that of mice that were sacrificed just after plitidepsin injection (0 h) using Wilcoxon rank-sum test. A box-plot of JNK activation in each group (18-20 mice/group) and its statistical significance (p) are presented. Boxes in the plot include values in the 25%-75% interval; internal lines represent the median.

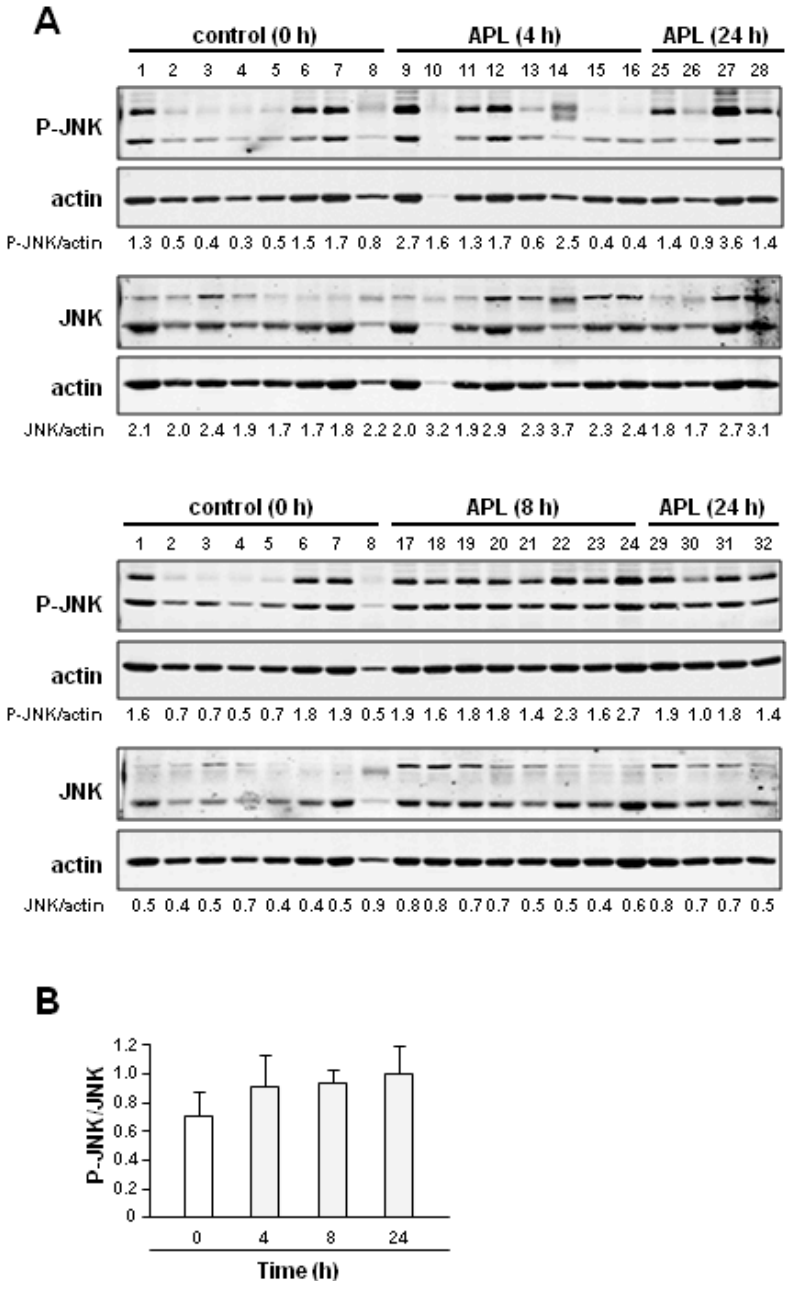


2.3. Plitidepsin induces JNK phosphorylation in peripheral blood mononuclear cells

As the spleen is not an achievable tissue in humans for regular pharmacodynamic biomarker measurement, we next decided to determine whether plitidepsin could also affect JNK phosphorylation of mononuclear cells (PBMCs) in peripheral blood of healthy animals. To this end, PBMCs were prepared from healthy rats following a single intravenous administration of plitidepsin (1 mg/kg), which yielded plasma concentrations of around 2 ng/mL that are similar to those achieved in clinical trials [16,17] (Supplementary Table S1). As above, the levels of total and phosphorylated JNK were examined by Western blot. In order to improve quantification accuracy, we used the Odyssey Infrared Imaging System (Li-Cor Biosciences). In an initial time course analysis, we found a tendency for increased phosphorylated JNK in PBMCs at all post-treatment times studied (Fig. 5).

Figure 5. Plitidepsin increases JNK phosphorylation in rat peripheral blood mononuclear cells. (A) Blood mononuclear cells (PBMCs) were isolated from non-tumor-bearing rats after 4, 8 and 24 h i.v. administration of plitidepsin (1 mg/kg) and the levels of total (JNK) and phosphorylated JNK (P-JNK) were analysed by Western blot. Control group were treated only with vehicle (control (0 h)). Actin was used as loading control for normalization. Immunoblots were incubated with infrared-labeled secondary antibodies, and the signals were visualized and quantified using the Odyssey Infrared Imaging System

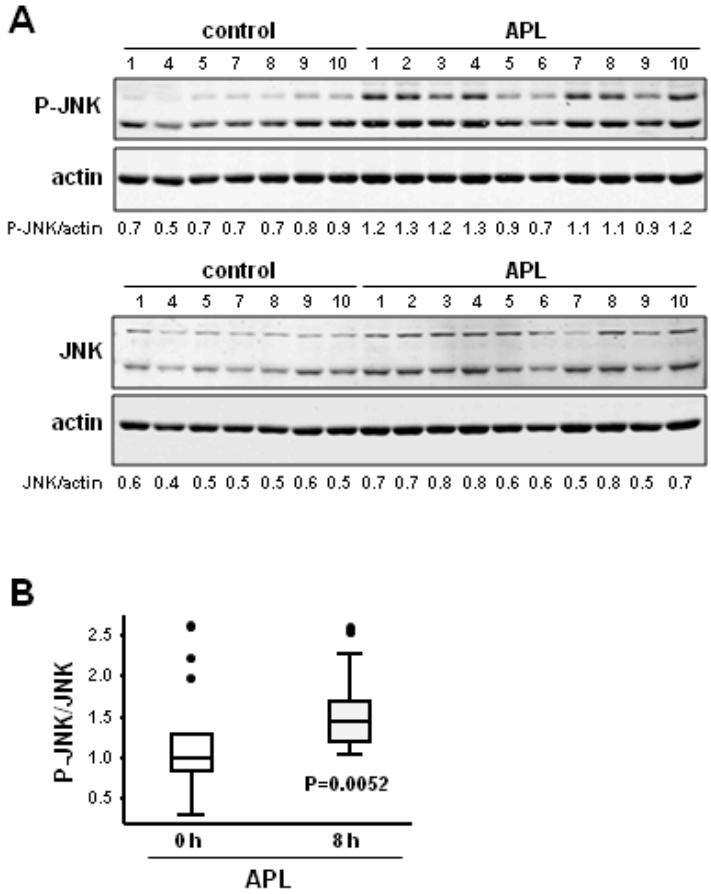
(Li-Cor Biosciences). (B) Quantification of P-JNK/JNK ratio upon normalization to actin level at the indicated times after plitidepsin administration in PBMCs derived from non-tumor-bearing rats. Mean ± S.E.M. values of each group of animals are shown.



To allow a statistical analysis with higher number of animals studied, we performed a new experiment at 8 h of plitidepsin administration. As shown in Figure 6A, plitidepsin increased the level of JNK phosphorylation in PBMCs at 8 h after administration. No significant changes in the level of total JNK protein were observed. Quantitative analysis using Odyssey software revealed a highly significant statistical increase in JNK phosphorylation (P = 0.005) in PBMCs of rats 8 h after a single administration of plitidepsin (Fig. 6B). This effect parallels the increase of JNK phosphorylation found in tumors and tissues of xenografted mice (Fig. 4). Together, these data support the notion that phosphorylated JNK may serve as a pharmacodynamic marker for plitidepsin activity and suggest that

changes in JNK activity in PBMCs could perhaps be used as a biomarker when assessing plitidepsin treatment schedules in clinical trials.

Figure 6. Plitidepsin induces a statistically significant increase of the level of phosphorylated JNK in PBMCs of healthy rats after 8 hours of administration. (A) Western blot analysis of JNK and P-JNK proteins in PBMCs obtained from rats treated with plitidepsin (1 mg/kg) for 8 h. Actin was used as loading control. Immunoblots were incubated with infrared-labeled secondary antibodies, and the signals were visualized and quantified using the Odyssey Infrared Imaging System (Li-Cor Biosciences). (B) Statistical analysis of JNK activation in PBMCs obtained from rats at 8 h after plitidepsin administration. Comparison between the P-JNK/JNK ratio in PBMCs of rats treated with plitidepsin or vehicle for 8 h using the Wilcoxon rank-sum test. A box-plot of JNK activation in each group (15-18 mice/group) and its statistical significance using Wilcoxon rank-sum test (p) are presented. Boxes in the plot include values in the 25%-75% interval; internal lines represent the median.



2.4. Discussion

In this study we demonstrate that plitidepsin suppresses cell proliferation in a panel of hematological cancer cell lines with an IC_{50} in the nanomolar range, confirming its anti-tumor activity [1]. This growth inhibition is accompanied by a sustained activation of JNK that, together with data

obtained in other cultured cells [3-5,9,11,15], led us to investigate JNK as a potential surrogate marker for plitidepsin activity. In agreement with this hypothesis, we found that plitidepsin increases the level of phosphorylated JNK *in vivo*, both in xenografted tumors generated by human leukemia K562 cells in immunodeficient mice and in the spleens of host animals. Importantly, the effect of JNK in tumors and spleens parallels and is concomitant. Although this does not ensure an equal effect against tumor or toxicity against a surrogate tissue, it supports that the drug concentration used is appropriate to induce a robust biological response in the host animals.

Our data show that plitidepsin treatment that leads to plasma concentrations achieved in cancer patients also increases JNK phosphorylation in PBMCs of healthy rats. This finding further suggests that normal tissues can be reliable markers of plitidepsin activity *in vivo*. This observation is particularly relevant because obtaining tumor biopsies is not usually feasible in patients, and the use of human PBMCs preparation allows sequential sampling for assessing plitidepsin treatment schedules in the clinical setting. Our data showing that JNK activation upon plitidepsin administration might be used *in vivo* as a biomarker of response is consistent with the evidence that JNK is a protein critical for the anti-tumor activity of plitidepsin *in vitro* [6].

The mitogen-activated protein kinase pathway is a well accepted target for cancer therapy. Although JNK is implicated in oncogenic transformation and tumorigenesis due to its known ability to promote proliferation, a large number of studies have also linked JNK to tumor suppression. One mechanism of tumor suppression is mediated by the pro-apoptotic effects of JNK activation [18,19]. It seems that the extent and/or duration of activation of JNK is important to determine the cellular response; extent or long activation leads to apoptosis, whereas low or transient activation leads to cell growth and differentiation [20]. Phosphorylation of JNK at residues (Thr^{183}, Tyr^{185}) that are recognized by the antibodies used in this study is linked to the activation of this enzyme and to the induction of apoptosis in many systems [21,22]. In cultured cells, sustained activation (hours) of JNK, but not transient (minutes), causes apoptosis in response to diverse stimuli that include anti-tumor drugs in clinical use [23,24]. Thus, cisplatin, 5-fluorouracil, etoposide, vinblastine and paclitaxel, among others, increase JNK phosphorylation in different human cancer cell types [25-29]. In our *in vivo* system the increase in JNK phosphorylation was first detected a few hours after administration of plitidepsin to mice and lasted for at least 8 h to then decline to basal level. Although temporal correlations between *in vitro* and *in vivo* systems are difficult, this kinetics may be considered a sustained activation of JNK. Notably, at least in human melanoma cells JNK mediates both dose-dependent plitidepsin-induced cell cycle arrest and apoptosis [3].

In contrast to JNK, the kinases p38MAPK and ERK, and the cell cycle inhibitor $p27^{KIP1}$ that had been shown to be targets of plitidepsin in several cultured cell types did not response to administration of the drug *in vivo* in our studies. This could be due to differences in cell specificity and/or to the *in vitro* conditions (culture media, plastic dishes…). Alternatively or additionally, changes in p38MAPK, ERK and $p27^{KIP1}$ by plitidepsin may require higher doses and/or longer times.

Clearly, the identification of biomarkers of response in preclinical models is essential to the clinical development of novel drugs. In the clinic, biomarkers may help to identify the optimal dose and regimen, predict and monitor drug sensitivity, and select patients most likely to respond to a therapeutic agent. Indeed, extensive preclinical biomarker studies have facilitate the development of numerous anticancer agents that have undergone clinical evaluation, including imatinib (Gleevev),

trastuzumab (Herceptin), gefitinib (Iressa), sunitinib malate (Sutent), dasatinib (Sprycel) and everolimus (Afintor) [30-37]. Plitidepsin has successfully advanced to Phase II/III clinical trials for the treatment of hematological malignancies [38] (ADMYRE trial), but molecular targets that predict its response have not been identified yet. In this report, we show that plitidepsin suppresses the proliferation of cultured hematological cancer cells in parallel with a sustained activation of JNK, a protein associated with cancer. Importantly, plitidepsin induces the phosphorylation of JNK *in vivo* and support the potential use of phosphorylated JNK as a pharmacodynamic biomarker to predict and monitor drug sensitivity in cancer patients. Furthermore, the results obtained show that phosphorylated JNK in spleens and in PBMCs is a reliable surrogate for phosphorylated JNK in tumors, and suggest that JNK activation by plitidepsin can be detected in normal tissues. Therefore, blood sampling could provide a source of material for biomarker evaluation of plitidepsin in the clinic.

3. Experimental Section

3.1. Cell culture and drug solutions

Human K562 chronic myelogenous leukemia, and RPMI8226, U266B1 and NCI-H929 multiple myeloma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% FCS and penicillin and streptomycin (all from GIBCO-Invitrogen, Paisley, UK). For tumor implantation, K562 cells obtained directly from the *in vitro* culture were suspended in 50% Matrigel®/RPMI 1640 media without serum or antibiotics. Passage 17 cells were implanted into the mice. Plitidepsin (Aplidin®, APL) was obtained from PharmaMar (Madrid, Spain). Stock solutions were freshly prepared in dimethylsulfoxide (DMSO). For *in vivo* studies, lyophilized vials of plitidepsin were reconstituted with vehicle (cremophor/ethanol/water) and further dissolved in PBS to reach the target dose.

3.2. Cell proliferation analysis

Cell proliferation was studied by [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assays that were performed following the manufacturer's instructions (MTT Cell Proliferation Kit I, Roche Diagnostics, Mannheim, Germany).

3.3. Xenograft tissues studies

For the generation of K562 leukemia xenografts, we used 12 to 13 week-old (18-24 g) female athymic *nude* mice (Harlan, Madison, WI, USA). Mice were house in ventilated racks and provided with irradiated food and sterilized water ad libitum. On Day 1, mice were implanted with 5 x 10^6 K562 cells per mouse subcutaneously. Tumor size measurements were recorded twice weekly beginning on Day 5 using vernier calipers. The formula to calculate volume for a prolate ellipsoid was used to estimate tumor volume from 2-dimentional tumor measurements: tumor volume (mm^3) = (length x width^2) ÷ 2. On Day 20, tumors reached an average volume of 532.8 mm^3 ± 125.1 (mean ± SD) and mice were randomized into treatment and control groups. Treatments were initiated and administered

on an individual body weight basis. Animals received a single bolus intraperitoneal dose of plitidepsin at 200 μg/kg and dosed at a volume of 10 mL/kg. Control groups were treated with vehicle alone. Then, at 0.5, 0.75, 1, 2, 4, 8, 12, 24, and 48 h post-dosing, mice were CO_2 euthanized and exsanguinated via cardiac puncture. Tumors and spleens were harvested from each animal, individually identified and stored at -80^{o}C until further processed.

All animal protocols were reviewed and approved according to regional Institutional Animal Care and Use Committees.

3.4. Protein lysates from cells and in vivo tissues

Total protein extracts from cultured cell were prepared as described elsewhere [3,6]. To obtain protein lysates from mouse tissues (tumors and spleens) collected at various time points after dosing, samples were fragmented and pulverized in liquid nitrogen using a mortar. The resultant powder was transferred into a Potter-Elvehjem and homogenized with chilled lysis buffer (50 mM Tris-HCl (pH 7.5), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 100 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 30 nM GM6001 MMP inhibitor, 1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerolphosphate and 0.5 mM DTT). Homogenates were centrifuged at 14,000 *g* at 4ºC for 20 min and the supernatants were transferred to new tubes. Protein extracts were quantified using the DC Protein Assay Kit (Bio-Rad). To obtain peripheral blood mononuclear cells (PBMCs) from rats treated with plitidepsin, Sprague Dawley male rats (Harlan) received a single bolus dose of either plitidepsin (1 mg/kg) or placebo. Blood samples were collected at 4, 8 and 24 hours post-dosing by cardiac puncture from deeply isofluorane-anesthetized animals. The blood was transferred into BD Vacutainer® CPT Mononuclear Cell Preparation Tubes (Becton Dickinson) and processed according to Manufacturer`s instructions. After the centrifugation (1,800 *g*, 20 min, 25ºC), lymphocytes portion was isolated and cell pellets were resuspended in complete lysis buffer and processed for JNK expression as described for mouse tissues. The resulting plasma samples were aliquoted and frozen at -80ºC until bioanalysis. Plitidepsin quantification was performed by a sensitive LC/MS-MS method previously described [39].

3.5. Western blot analysis

Twenty μg protein were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were blocked at room temperature for 1 h in TBS-T (20 mM Tris pH 7.4, 136 mM NaCl, 0.1% Tween-20) containing 5% bovine albumin and incubated overnight at 4ºC with the appropriated antibody. Antibodies used were: anti-phosphorylated-JNK (sc-6254), anti-JNK (sc-474), anti-p38MAPK (sc-535), anti-phosphorylated-ERK (sc-7383), anti-ERK (sc-154) and anti-$p27^{KIP1}$ (sc-776) from Santa Cruz Biotechnology, anti-phospho-JNK (#9251) and anti-phosphorylated-p38MAPK (#9211) from Cell Signaling and anti-c-Abl (Ab-3) from Calbiochem. Alexa-Fluor anti-mouse 680, Alexa-Fluor anti-rabbit 680 and Alexa-Fluor anti-goat 800 were from Li-Cor Bioscience. HPR-conjugated anti-mouse IgG (H+L) was from Promega, and HPR-conjugated anti-rabbit IgG (H+L) from MP Biomedicals. As a loading control, membranes were re-probed for actin (sc-1616) provided by Santa Cruz Biotechnology. After washing, blots were incubated with HPR-labeled or infrared-labeled secondary

antibodies for 1 h at room temperature and developed by a peroxidase reaction using the ECL detection system (Amersham-G.E. Healthcare) or visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

Immunoprecipitation of JNK was conducted by incubating 500 µg of protein extracts from tumors with 1 µg of the following antibodies: anti-JNK (sc-474) from Santa Cruz Biotechnology and anti-phosphorylated-JNK (#9251) from Cell Signaling, and collected on Gammabind-Sepharose beads (Amersham-Pharmacia Biotech). Immunoprecipitated proteins were separated by SDS-PAGE and subjected to immunoblot analysis using anti-phosphorylated-JNK (#9251) from Cell Signaling or anti-phosphorylated-JNK (sc-6254) from Santa Cruz Biotechnology.

3.6. Statistical Analysis

Overall differences in JNK expression at different times in the same tissue were evaluated using Kruskal Wallis test. Two-by-two comparisons were also performed using Wilcoxon rank-sum test and taken the expression at time 0, that is, at the moment of plitidepsin injection, as reference. Correlation between the level of phosphorylated JNK measured in matched tumor and spleen samples from the same animal was assessed by computing the Spearman's coefficient and its statistical significance. The data were analysed by using STATA software (StataCorp LP, College Station TX).

4. Conclusions

Plitidepsin increases JNK phosphorylation in xenografted tumors and in surrogate rodent tissues. Our data support that phosphorylated-JNK may serve as a reliable marker for plitidepsin activity and suggest that changes in JNK activity of peripheral blood cells could be used as a pharmacodynamic marker for this agent.

Acknowledgments

This work has been partially supported by grants (Programa Cenit, CEN-20091016 and SAF2010-18302) from Ministerio de Economía y Competitividad of Spain.

Conflict of Interest

The authors declare that MJMA, MJGN, PA and CMG are employees and shareholders of PharmaMar. EA was an employee of PharmaMar and, AM is consultant of PharmaMar. No potential conflicts of interest were disclosed for MP.

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Supplementary Material

Figure S1. Specificity of antibodies against phosphorylated JNK. JNK protein was immunoprecipitated from tumor extracts (#2.1 and #7.1; see Figure 2) using either anti-JNK antibody (sc-474, Santa Cruz; IP-1) or anti-P-JNK (#9251, Cell Signaling; IP-2). Two aliquots of each immunoprecipitate were subjected to SDS-PAGE followed by immunoblotting using anti-P-JNK sc-6254 antibody from Santa Cruz (panel A) or anti-P-JNK #9251 from Cell Signaling (panel B). For comparison, overexposed images are shown (low in both panels) to detect the signal in whole protein extracts.

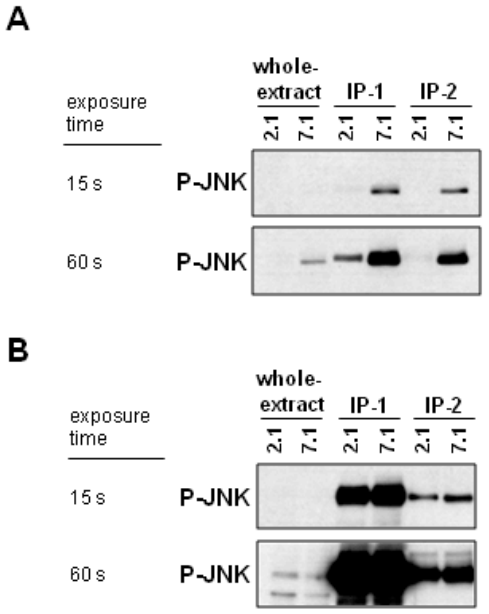

Figure S2. Levels of phosphorylated-p38MAPK and phosphorylated-ERK in xenografted tumors after plitidepsin treatment. K562 tumor-bearing mice were treated with plitidepsin (200 μg/kg) for the indicated times and tumors were excised, processed, and subjected to immunoblot analysis for total (p38) and phosphorylated p38MAPK protein (P-p38) and total (ERK) and phosphorylated ERK protein (P-ERK).

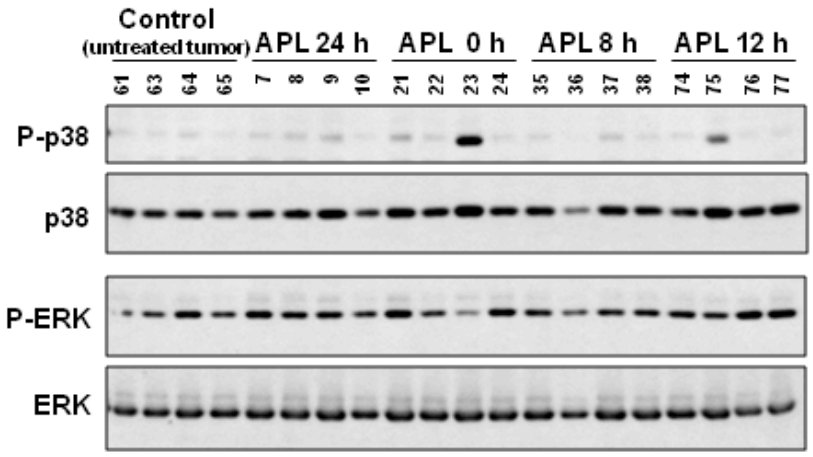

Figure S3. Levels of $p27^{KIP1}$ in tumors of K562-bearing mice after plitidepsin treatment. K562 xenografted mice were treated with plitidepsin (200 μg/kg) for the indicated times and tumors were excised, processed, and subjected to immunoblot analysis for $p27^{KIP1}$. Actin was used as loading control.

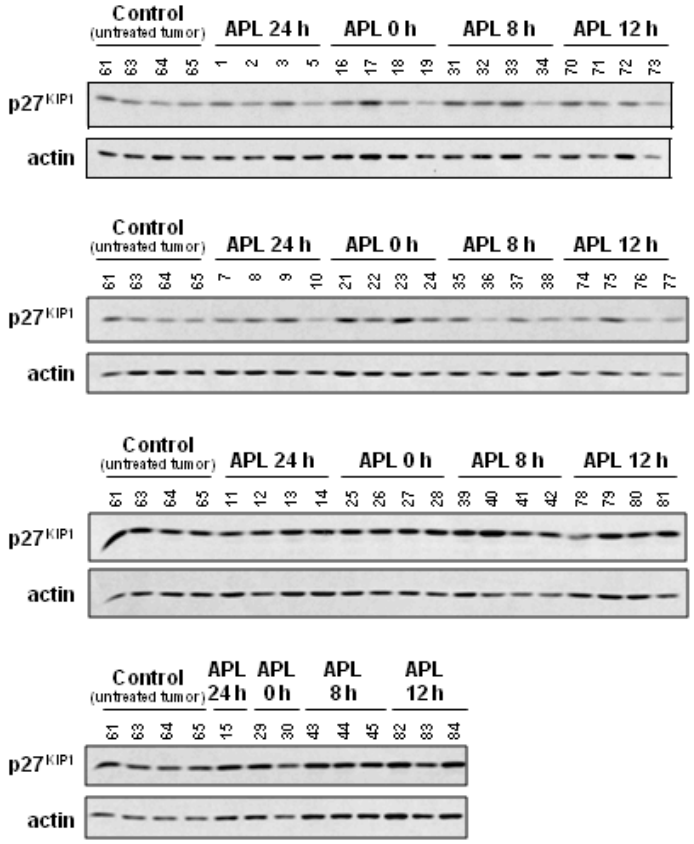

Figure S4. Levels of c-Abl and BCR-ABL proteins in tumors from mice xenografted with K562 cells. Western blot analysis of the expression of c-Abl and BCR-ABL (Bcr/Abl) proteins in tumors excised from mice xenografted with K562 cells that were treated with plitidepsin (200 μg/kg) for the indicated times. K, K562 cells; K + APL, plitidepsin-treated K562 cells.

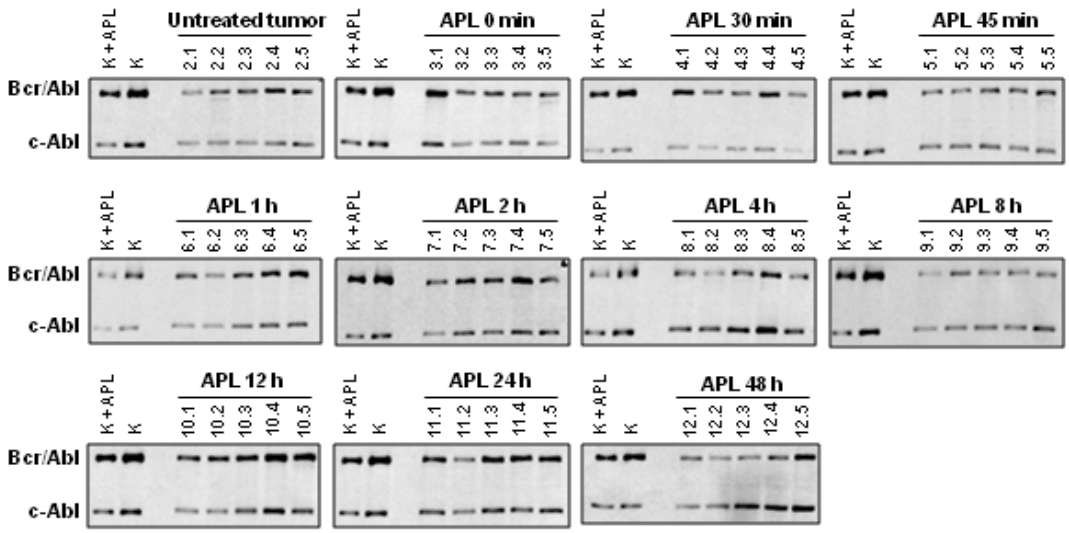

Figure S5. Levels of phosphorylated JNK in tumors and spleens of K562 tumor-bearing mice after plitidepsin treatment. Tumors (A) and spleens (B) from plitidepsin-treated xenografted mice (study #2) were excised, processed and subjected to Western blot analysis of total (JNK) and phosphorylated JNK (P-JNK). Actin was used as loading control. Values (Arbitrary Units, A.U.) correspond to P-JNK/JNK ratio in each sample normalized to actin and related to the same ratio in control sample #64.

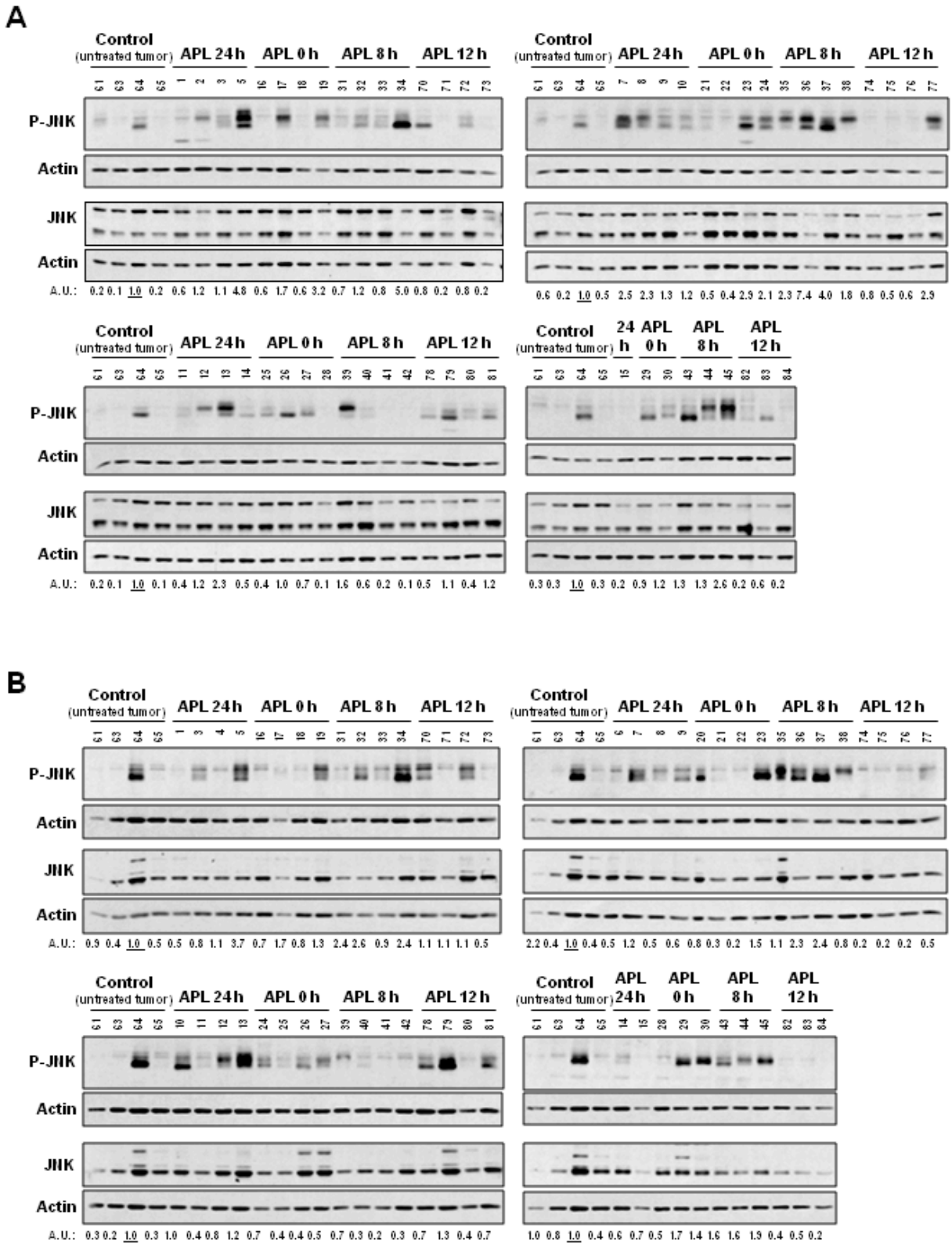

Figure S6. Levels of phosphorylated JNK in tumors and spleens from mice xenografted with human K562 leukemia cells after plitidepsin administration. Tumors (A) and spleens (B) from plitidepsin-treated xenografted mice (study #1; see Figure 2) were excised, processed and subjected to Western blot analysis of JNK and P-JNK together with control samples from study #2. Actin was used as loading control. Values (Arbitrary Units, A.U.) correspond to P-JNK/JNK ratio in each sample normalized to actin and related to the same ratio in control sample #64.

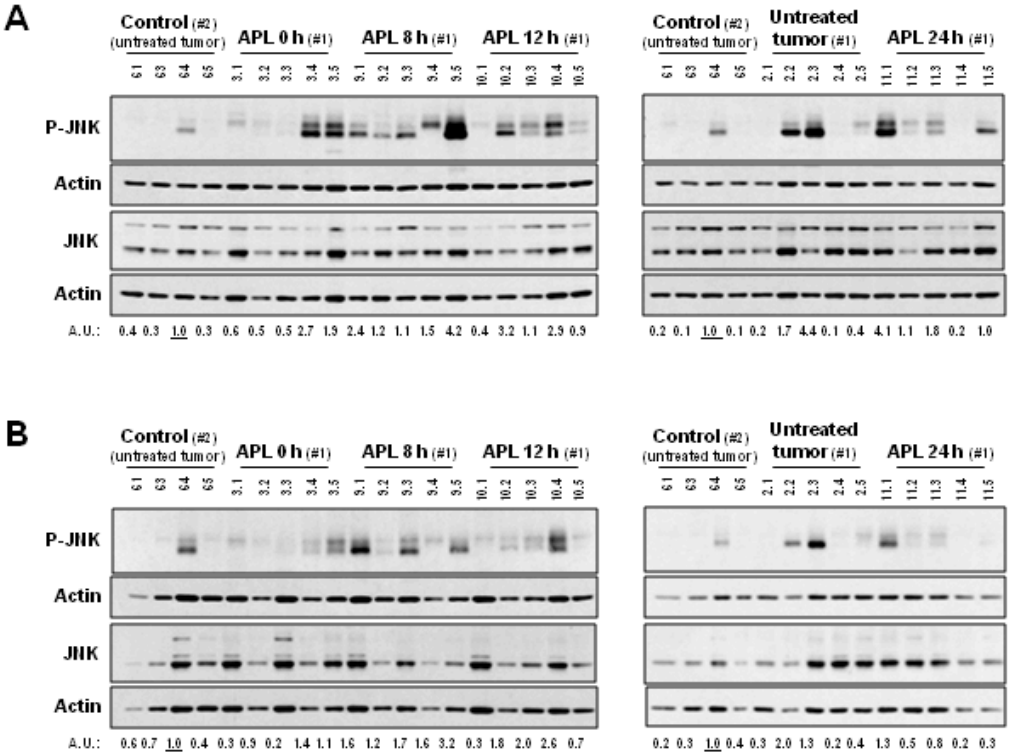

Table S1. Levels of plitidepsin in plasma from healthy rats after plitidepsin administration. Blood was isolated from non-tumor-bearing rats after 8 h i.v. administration of plitidepsin (1 mg/kg) (APL group; #APL1-10) or vehicle (control group; #C1-10), and the levels of plitidepsin in plasma were measured by a sensitive LC/MS-MS method [39]. Undetected; below limit of quantification, 0.05 ng/mL.

Animal (No.)	Treatment	Plasma [APL] (ng/mL)
#C1	Placebo	undetected
#C2	Placebo	undetected
#C3	Placebo	undetected
#C4	Placebo	undetected
#C5	Placebo	undetected
#C6	Placebo	undetected
#C7	Placebo	undetected
#C8	Placebo	undetected
#C9	Placebo	undetected
#C10	Placebo	undetected
#APL1	Plitidepsin	2.00
#APL2	Plitidepsin	1.47
#APL3	Plitidepsin	1.60
#APL4	Plitidepsin	1.71
#APL5	Plitidepsin	2.61
#APL6	Plitidepsin	2.50
#APL7	Plitidepsin	2.07
#APL8	Plitidepsin	2.22
#APL9	Plitidepsin	1.76
#APL10	Plitidepsin	3.76