Levels of p27\textsuperscript{kip1} determine Aplidin sensitivity

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Abstract

Aplidin (plitidepsin) is a novel anticancer drug isolated from the marine tunicate \textit{Aplidium albicans}. Aplidin shows potent antitumor activity in preclinical models against a wide variety of human tumors. Aplidin is currently in phase II clinical trials in a variety of solid tumors and hematologic malignancies. Moreover, clinical studies of Aplidin in combination with other agents are ongoing because it generally lacks cross-resistance with other known cytotoxic drugs. The mode of action of Aplidin in tumor cells is only partially understood. Aplidin induces an early oxidative stress response, which results in a rapid and sustained activation of the epidermal growth factor receptor, the nonreceptor protein tyrosine kinase Src, and the serine threonine kinases c-Jun NH\textsubscript{2}-terminal kinase and p38 mitogen-activated protein kinase. Here, we show that sensitivity to Aplidin correlates inversely with the levels of expression of the cyclin-dependent kinase inhibitor p27\textsuperscript{kip1} (p27) in a panel of low passaged human sarcoma cell lines. Aplidin induces p27 through an oxidation-dependent mechanism and the reduction of p27 levels by specific short hairpin RNA increases Aplidin sensitivity. We confirmed these results in p27 null mouse embryonic fibroblasts corroborating the specificity of the p27 role in Aplidin response because p21\textsuperscript{waf1} null mouse embryonic fibroblasts do not show this increased sensitivity. We propose a mechanism of action of Aplidin involving p27 and support the analysis of p27 in the response to Aplidin in currently ongoing clinical trials to establish the levels of this protein as response predictor.

clinical trials with Aplidin to evaluate this pharmacogenomic model. In this study, using a panel of low passaged sarcoma cell lines, we have identified p27 as a putative marker of Aplidin sensitivity.

Materials and Methods

Generation of Cell Lines and Culture Conditions

Sterile fragments from resected tumors were minced and then disaggregated by 1- to 2-h incubation in collagenase (100 units/mL) at 37°C. Twenty-four hours later, medium was changed to F-10 Ham (Invitrogen, Carlsbad, CA) supplemented with 1% Ultroser G (Pall Biosepra, East Hills, NY). Cell lines generated (20) were cultured in F-10 Ham (Invitrogen, Carlsbad, CA) supplemented with 1% Ultroser G (Pall Biosepra, East Hills, NY). Cell lines generated (20) were cultured in F-10 Ham supplemented with 1% Ultroser G. A673 cells were cultured in RPMI 1640 (Sigma, St. Louis, MO) and SW872 in Leibovitz L-15 (Sigma). All media were supplemented with 10% fetal bovine serum, fungizone, and penicillin/streptomycin. Once cells became confluent, adherent cells were removed by trypsin treatment and seeded at 1:2 or 1:3 ratio with medium. Throughout the establishment of these cell lines, phenotypic features were followed (20). Additionally, they were routinely checked for Mycoplasma contamination (Invivogen, San Diego, CA). All cell lines used were established immortal tumor cell lines. Mouse embryonic fibroblasts (MEF) either p27(−/−) or p21 (−/−) were generated from heterozygous p27(+/−) or p21(/−) mouse crosses. MEFs were generated as reported previously (21). Once established, MEFs were characterized for p27 or p21 expression by reverse transcription-PCR and Western blot. Wild-type (WT) controls were generated from littersmates in each case.

Cytotoxicity Assessment

The compounds were tested on 96-well trays. Cells growing in a flask were harvested just before they became confluent, counted using a hemocytometer, and diluted down with medium adjusting the concentration to the required number of cells/0.2 mL (volume for each well). Cells were then seeded in 96-well trays at a density between 1,000 and 4,000 cells per well, depending of the cell size. Cells were left to plate down and grow for 24 h before adding the drugs. Drugs were weighed out and diluted with DMSO to get them into solution to a concentration of 10 mmol/L. From here, a ‘mother plate’ with serial dilutions was prepared at 200× the final concentration in the culture. The final concentration of DMSO in the tissue culture medium should not exceed 0.5%. The appropriate volume of the compound solution (usually 2 µL) was added automatically (Beckman FX 96 tip; Beckman Coulter, Fullerton, CA) to medium to make it up to the final concentration for each drug. The medium was removed from the cells and replaced with 0.2 mL of medium dosed with drug. Each concentration was assayed in triplicate. Two sets of control wells were left on each plate, containing either medium without drug or medium with the same concentration of DMSO. A third control set was obtained with the cells untreated just before adding the drugs (seeding control, number of cells starting the culture). Cells were exposed to the drugs for 96 h and then washed twice with PBS before being fixed with 10% glutaraldehyde. Cells were washed twice and stained with 0.5% crystal violet during 30 min. Then, cells were washed extensively and solubilized with 15% acetic acid, and absorbance was measured at 595 nm.

Reverse Transcription-PCR

Total RNA was collected using the Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). Reverse transcription was done (Promega, Madison, WI) with 1 µg RNeasy following the manufacturer’s protocol. The following primers were used to amplify regions: β-actin, 5′-AGGCC-CAACCGCAAGAGATGAC-3′ (forward primer) and 5′-GAAGTCCAGGGAGCGCTAGCA-3′ (reverse primer). cDNA was subjected to PCR, and products were analyzed by electrophoresis on a 1% agarose gel.

Figure 1. A, expression of p27 in a panel of sarcoma cell lines analyzed by reverse transcription-PCR and Western blot. Presence of p27 mRNA in all cell lines analyzed by reverse transcription-PCR (top), levels of protein in all the cell lines (middle), and Western blot of the levels of α-tubulin as loading control for protein determination (bottom). Similar results were obtained in at least other three independent experiments. B, expression levels of p27 protein were quantified by densitometry and normalized by α-tubulin (Aplidin IC50 is shown above each column). C, correlation between Aplidin IC50 and p27 protein expression. Cell lines were grouped according to the relative p27 levels and the correlation with the IC50 was determined. After verifying the statistical significance of the overall ANOVA, the three t tests were carried out to compare the means of the high, medium, and low p27 groups.
Western Blot Analysis
To prepare the whole-cell extract, cells were washed once in cold PBS and suspended in 1 mL lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 1% NP40, 10% glycerol, 150 mmol/L NaCl, 2 mmol/L Complete protease inhibitor cocktail (Roche, Basel, Switzerland)]. The protein content of the lysates was determined by the modified method of Bradford. Samples were separated on 12.5% SDS-PAGE gels, transferred onto Immobilon-P membrane (Millipore, Billerica, MA), and immunostained. To identify p27, we used anti-p27 antibody (Anti-kip1/p27 monoclonal antibody, BD Biosciences, Franklin Lakes, NJ) as primary antibody and horseradish peroxidase–conjugated antiamo (Promega) as secondary antibody. Proteins were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Fairfield, CT).

Apoptotic Nuclei
Apoptosis was visually assessed by staining cells with Hoechst 33258 pentahydrate (Invitrogen) for 5 min. The cells were then examined with a Leica fluorescent microscope (Leica Microsystems, Wetzlar, Germany) and apoptotic cells were distinguished by condensed fragmented nuclear regions. We analyzed a total of 400 cells per treatment and results are given as percentage.

Flow Cytometric Analysis of Apoptosis by Propidium Iodide Staining
Cellular DNA content was determined by flow cytometric analysis of propidium iodide–labeled cells. Cells were grown to exponential phase, seeded at a density of 106 cells/10-cm dish, and treated with the indicated concentrations of Aplidin. Then, cells were harvested, fixed in ice-cold 70% ethanol, stored at 4°C, washed with PBS, treated with RNase A, and stained with 50 μg/mL propidium iodide for 10 min. For flow cytometric analysis, we used a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). A minimum of 10,000 cells per sample were used for the analysis done using CellQuest software.

Apoptosis Assessment by Annexin V/Propidium Iodide Staining
The effect of Aplidin on cells was assessed using flow cytometry. Cells were grown up to ~70% confluence and treated with Aplidin (1–10 nmol/L) for 6 h. Briefly, after treatment, cells were harvested, and cell concentration was adjusted to 3 × 106 cells/mL with binding buffer (Becton Dickinson), stained with 5 μL Annexin V (Becton Dickinson) and 10 μL propidium iodide (Sigma), and incubated in the dark for 15 min. A total of 10,000 size-gated cells were analyzed by FACSCalibur (Becton Dickinson).

Small Interfering RNA Knockdown of p27
To target human p27, we used short hairpin RNAs supplied by Centro Nacional de Investigaciones Oncológicas (CNIO) short hairpin RNA library. Three different small interfering RNAs (siRNA) were selected against p27 sequences by the bioinformatics unit: GCACCTGCAGAGA (122 bases downstream the start codon), CCGACGATTCTTCTACTCA (451 bases downstream the start codon), and GAGCCACAGAACAGAGA (477 bases downstream the start codon). As vector, we used pA70 Retro derived from pSuperRetro. Cells from lines A673, AA, and AW were plated in 10-cm dishes at 50% confluency and transfected 24 h later by calcium phosphate precipitation using 20 μg pA70 Retro vector. The clones were selected in puromycin (1 μg/mL; Sigma).

Statistical Analysis
Prisma 4 statistical software was used for the statistical analysis. Determination of statistical significance was done by ANOVA (one-way ANOVA). Post-hoc comparison was completed using Bonferroni’s multiple comparison test. All data are reported as the mean ± SE. Statistical significance was considered as P < 0.05, P < 0.01, and P < 0.001.

Results
Response to Aplidin of Different Low Passaged Cell Lines
With the aim of identifying new markers of sensitivity and resistance to Aplidin, a panel of low passaged human tumor cell lines, mainly from mesenchymal origin, was treated with Aplidin. All cell lines were treated under similar conditions and IC50 s were calculated as an average of three independent experiments done in triplicate (Table 1). Response to Aplidin varied from <1 nmol/L, such as in CNIO BG, 1455, and CNIO AA cell lines, to >30 nmol/L, such as in CNIO BC and SW872 cell lines, more than a 100-fold difference between the most sensitive and the most resistant cell lines. The response was independent of the tumor type.

Table 1. Aplidin sensitivity of a panel of low passaged sarcoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor origin</th>
<th>IC50 (nmol/L)</th>
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<tbody>
<tr>
<td>CNIO BC</td>
<td>MPNST</td>
<td>35.65 ± 6.11</td>
</tr>
<tr>
<td>SW672</td>
<td>Liposarcoma</td>
<td>33.14 ± 3.08</td>
</tr>
<tr>
<td>CNIO BJ</td>
<td>Osteosarcoma</td>
<td>17.21 ± 4.2</td>
</tr>
<tr>
<td>A673</td>
<td>Ewing sarcoma</td>
<td>17.09 ± 5.69</td>
</tr>
<tr>
<td>CNIO BM</td>
<td>Hibernoma</td>
<td>12.67 ± 5.33</td>
</tr>
<tr>
<td>CNIO BB</td>
<td>MPNST</td>
<td>8.22 ± 2.28</td>
</tr>
<tr>
<td>CNIO BP</td>
<td>Osteosarcoma</td>
<td>7.86 ± 3.61</td>
</tr>
<tr>
<td>CNIO BN</td>
<td>Fibrohistiocytoma</td>
<td>6.44 ± 2.73</td>
</tr>
<tr>
<td>CNIO AY</td>
<td>Leiomyosarcoma</td>
<td>3.85 ± 0.37</td>
</tr>
<tr>
<td>CNIO AZ</td>
<td>Fibrous tumor</td>
<td>2.83 ± 0.73</td>
</tr>
<tr>
<td>CNIO AW</td>
<td>Liposarcoma</td>
<td>2.33 ± 0.66</td>
</tr>
<tr>
<td>CNIO AX</td>
<td>Liposarcoma</td>
<td>2.25 ± 1.53</td>
</tr>
<tr>
<td>SAOS-2</td>
<td>Osteosarcoma</td>
<td>1.93 ± 0.75</td>
</tr>
<tr>
<td>CNIO BF</td>
<td>Osteosarcoma</td>
<td>1.59 ± 0.09</td>
</tr>
<tr>
<td>CNIO CE</td>
<td>Rhabdomyosarcoma</td>
<td>1.21 ± 0.6</td>
</tr>
<tr>
<td>CNIO BI</td>
<td>GIST</td>
<td>1.0 ± 0.44</td>
</tr>
<tr>
<td>CNIO BG</td>
<td>Myxoid fibrosarcoma</td>
<td>0.92 ± 0.21</td>
</tr>
<tr>
<td>1455</td>
<td>Liposarcoma</td>
<td>0.89 ± 0.5</td>
</tr>
<tr>
<td>CNIO AA</td>
<td>Leiomyosarcoma</td>
<td>0.27 ± 10.08</td>
</tr>
</tbody>
</table>

Abbreviations: MPNST, malignant peripheral nerve sheath tumor; GIST, gastrointestinal stromal tumor.
Expression of protein and mRNA levels from different genes was analyzed and correlated with the in vitro sensitivity to Aplidin. The following genes involved in tumor progression, cell adhesion, cell cycle control, and cell signaling were analyzed: Apaf-1, APC, CDK4, c-kit, cyclin D1, E-cadherin, MDM2, MLH-1, MSH-2, p14ARF, p15INK4b, p16INK4a, p21cip1, p27kip1, p53, p73, p85, PDGFR, p60src, PTEN, and β-catenin. Expression of p27 protein and sensitivity to Aplidin was found to be correlated. Levels of p27 protein were analyzed by Western blot with cells actively proliferating under the same conditions. Cells were seeded at 40% saturation and grown until 80% confluence was reached and then harvested, and total protein was extracted; p27 was detected in total lysates by Western blot immunodetection (Fig. 2A). The experiment was repeated three independent times with similar results. p27 levels were quantified by densitometry and normalized with α-tubulin levels in the same membrane. Then, we compared these p27 levels to the sensitivity to Aplidin. There is a highly significant correlation between the IC_{50} to Aplidin and the relative levels of p27. A Spearman’s rank correlation test shows a P value of 6.835e−05 between IC_{50} and p27 levels.

Three different p27 levels were selected: low levels, when p27 levels are <50% of those of α-tubulin; moderate levels, when they vary from 50% to 150%; and high levels, when p27 levels are >150% of those of α-tubulin. The different expression levels of p27 protein were correlated with the sensitivity to Aplidin in the panel of sarcoma cell lines (Fig. 1B). The mean IC_{50} in each of the three subgroups of cell lines was calculated and correlated with Aplidin sensitivity (Fig. 1C). Cell lines with high levels of p27 (SW872, BC, A673, and BP) showed higher IC_{50}. Cell lines with moderate levels of p27 (AX, AZ, BB, BM, BN, BJ, SAOS2, and CE) showed intermediate IC_{50} with a significant statistical difference when compared with cells with high levels of p27 (P = 0.0031). Cells with lower levels of p27 (AA, AW, AY, BF, BG, and 1455) showed the highest sensitivity to Aplidin (P = 0.0072 when compared with cells with high levels of p27; P = 0.13 when compared with cells with medium levels of p27). In summary, cell lines with high levels of p27 were more resistant to Aplidin treatment, whereas cell lines with lower levels of p27 were more sensitive to Aplidin.

**Absence of p27 Increases Sensitivity to Aplidin in MEFs**

MEFs lacking p27 gene were treated with Aplidin and their sensitivity was compared with that of the corresponding WT MEFs. Sustaining the correlation between p27 levels and Aplidin sensitivity found in the panel of sarcoma cell lines, p27^{−/−} MEFs were more sensitive to Aplidin compared with isogenic MEFs from WT littermates. When WT and p27^{−/−} MEFs were treated with other drugs, such as vinblastine and flavopiridol, p27^{−/−} cells were equal or even less sensitive to the antitumor treatment than the corresponding WT cells (Table 2).

It is possible to argue that the effect of Aplidin is due to absence of cell cycle inhibition, making p27^{−/−} cells more sensitive to Aplidin treatment. To study this specificity, p21^{−/−} cells from p21 knockout mice were treated with Aplidin under the same conditions as above. In this case, p21^{−/−} cells were even less sensitive to Aplidin than WT cells (data not shown).

The induction of apoptosis by Aplidin in p27^{−/−} cells was analyzed. Treatment of p27^{−/−} MEFs with different concentrations of Aplidin (1, 5, or 10 nmol/L) induced an increase of apoptosis measured as apoptotic nuclei (Fig. 2A) or by Annexin V staining (Fig. 2B). The effect of Aplidin on the cell cycle was also evaluated by measuring the DNA content (data not shown). Neither a cell cycle arrest nor a sub-G_{1} population was induced after treatment of WT MEFs during 24 h with different
concentrations of Aplidin. However, p27−/− cells showed a clear G2 arrest with a concomitant induction of a sub-G1 population, which increased with the concentration of Aplidin (see also Fig. 3D; data not shown). The results confirm the sensitivity of p27−/− cells to Aplidin treatment and its induction of apoptosis with a concomitant G2-M arrest.

Functional Relationship between p27 Levels and Response to Aplidin

To evaluate the correlation between sensitivity to Aplidin and p27 levels, human isogenic cell lines differing only in the levels of p27 were analyzed. Three different siRNAs against p27 were selected and analyzed for their ability to produce a significant reduction of p27 protein levels. A673, AW, and AA cells either expressing the parental vector or constitutively expressing each of the different siRNAs were generated. One of them (Hs960) reduced the levels of p27 in 80% to 90% (Fig. 3A). Moreover, we have done a further functional validation (Fig. 3B). p27 overexpression in AA cells (low p27 levels) causes growth arrest, whereas the joint expression of p27 siRNA overcomes this arrest (Fig. 3B), confirming the functional validity of the siRNA. Transfection of siRNA has no appreciable effect in the normal growth of any of the cell lines. Cells selected for siRNA expression (Hs960) were tested for their sensitivity to Aplidin and compared with cells expressing the parental vector (Fig. 3C). Reduction of p27 levels yielded cells more sensitive to Aplidin in cell lines with high (A673) and moderate (AW) levels of p27; on the contrary, the sensitivity of the AA cell, with low p27 levels, was not affected by the siRNA.

The p27-dependent effect of Aplidin on apoptosis was evaluated by measuring the DNA content (Fig. 3D). Neither a cell cycle arrest nor a sub-G1 population was induced after treatment of parental A673 cells during 24 h with 10 μmol/L Aplidin. However, A673-Hs960 cells showed a clear G2 arrest with a concomitant induction of a sub-G1 population. The results confirm the sensitivity of cells with lower levels of p27 to Aplidin treatment and its induction of apoptosis with a concomitant G2-M arrest.

Because Aplidin had an antiproliferative effect depending on the p27 levels, this effect should be through cell cycle regulation. We examined the levels of the cyclin-dependent kinase (CDK) inhibitor p27 after Aplidin treatment. Aplidin induced the up-regulation of p27 (Fig. 4A and B). This induction of p27 occurs through an early oxidative stress response because treatment of cells with antioxidants, such as 10 mmol/L reduced glutathione, inhibits this response (Fig. 4C). However, it is possible that Aplidin and reduced glutathione have opposite but independent effects on p27 induction. However, the response to 5'-acetylcysteine (other reactive oxygen species scavenger) was similar to reduced glutathione (data not shown), decreasing the probability of this last hypothesis.

Table 2. Sensitivity to Aplidin, vinblastine, and flavopiridol of WT and p27−/− MEFs

<table>
<thead>
<tr>
<th>Drug</th>
<th>WT (IC50 nmol/L)</th>
<th>p27−/− (IC50 nmol/L)</th>
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<tr>
<td>Aplidin</td>
<td>2.9 ± 0.7*</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>7.8</td>
<td>14</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>1.1 × 10^3</td>
<td>&gt;100 × 10^3</td>
</tr>
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</table>

*Results are the mean ± SD of five independent experiments.
Discussion

Aplidin, a new antitumoral drug presently in phase II clinical trials, has shown both in vitro and in vivo activity against human cancer cells. Aplidin effectively inhibits cell viability by triggering a canonical apoptotic program resulting in alterations in cell morphology, caspase activation, and chromatin fragmentation. Proapoptotic concentrations of Aplidin induce early oxidative stress, which results in a rapid and persistent activation of both c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase and a biphasic activation of extracellular signal-regulated kinase (12). Inhibition of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase blocks the apoptotic program induced by Aplidin, showing its central role in the integration of the cellular stress induced by the drug. c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase activation results in downstream cytochrome c release and activation of caspase-9 and caspase-3 and poly(ADP-ribose) polymerase cleavage, showing the mediation of the mitochondrial apoptotic pathway in this process. There seems to be a series of pathways that lead to this phosphorylation. It has been shown that Aplidin acts both at the cell membrane and intracellularly. At the membrane, Aplidin caused direct oxidative damage and Rac-1 activation. Inside the cell, the molecule reduces levels of reduced glutathione, thus increasing oxidative stress. The addition of antioxidants attenuates Aplidin response (11). Aplidin was also found to be cytostatic at nanomolar concentrations inducing both a G1 arrest and a G2-M blockade. The drug-induced cell cycle perturbations and subsequent cell death do not seem to be related to macromolecular synthesis (protein, RNA, and DNA) because the effects occur at concentrations (e.g., 10 nmol/L), in which macromolecule synthesis was not markedly affected (10).

We have found that the levels of p27kip1 determine Aplidin sensitivity in human tumor cells and that the elimination of p27 in these cells (by siRNA) or in MEFs (p27 knockout) increases sensitivity to Aplidin. We have also found that the increase in p27 protein levels, but not mRNA (18, 19), is a component of the Aplidin response. Therefore, p27 might act as a hinge protein determining the cytostatic or cytotoxic response to Aplidin.

Cell cycle progression depends on the regulated expression of cyclins, which affect the activation of CDKs. Members of the CDK inhibitor CIP/KIP family (including p21^waf1, p27^kip1, and p57^kip2) promote cell cycle arrest by binding and inhibiting cyclin-CDK protein complexes during differentiation, in response to either mitogen deprivation or toxic stress (reviewed in refs. 22, 23). These proteins preferentially associate with the cyclin-CDK complex rather than with the individual CDK subunits. The overexpression of these proteins arrests cells in different states of the cell cycle according to their biochemical function. p27^kip1 can bind and inactivate CDK1, CDK2, CDK4, and CDK6, providing the basis for G1 and G2-M arrest.

Aplidin triggered a very early and pronounced increase in reactive oxygen species production. In this context, it is well known that disruption of the mitochondrial function under conditions of oxidative stress is an important contributor to the apoptotic response (24–26). Significantly, blockade of reactive oxygen species production by the free radical scavengers inhibited both mitochondrial damage and apoptosis (11, 12). On the other hand, reactive oxygen species might trigger the accumulation of p27 protein levels through p38 mitogen-activated protein kinase (27) or c-Jun NH2-terminal kinase, two kinases described previously involved in the sensitivity to Aplidin. p27 phosphorylation triggers the inhibition of its ubiquitination and degradation through Skp2 (28). An accumulation of p27 will inhibit cyclin-CDK complexes acting at G1 and G2-M boundaries (22, 23). Inhibition of cell cycle will result in decreased Aplidin sensitivity as has been broadly reported in similar contexts for p27 and p21 (29–34) and correlating with previous works reporting that Aplidin has higher cytotoxic activity against proliferating than quiescent cells (11, 12). Therefore, absence of p27 will result in increased sensitivity to Aplidin and can be used as a marker for treatment in vivo.
As a member of the CIP/KIP family of CDK inhibitors, p27kip1 is also a potential tumor suppressor. The levels of p27kip1 protein decrease during tumor development and progression in some epithelial, lymphoid, and endocrine tissues. This decrease occurs mainly at the posttranslational level with protein degradation by the ubiquitin-proteasome pathway. A large number of studies have characterized p27kip1 as an independent prognostic factor in various human cancers, including leukemia, breast, colon, and prostate adenocarcinomas (reviewed in ref. 22). Therefore, these evidences indicate that tumors with low levels of p27 may constitute good targets for Aplidin response.

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References
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