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Report

Antitumor bicyclic hexapeptide RA-VII modulates cyclin D1 protein level

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A bicyclic hexapeptide, RA-VII or *O*-methyl deoxybouvardin, isolated from *Rubia cordifolia*, is known to inhibit protein biosynthesis *in vitro* and *in vivo*. We here demonstrate that the treatment of human colon cancer DLD-1 cells with RA-VII induces cell growth inhibition associated with a partial G_1 arrest and a rapid decrease (below 2 h) in the level of cyclin D1 protein. Since cycloheximide, another protein synthesis inhibitor, neither decreased the amount of cyclin D1 in the cells nor arrested cells in G_1 phase, it is unlikely that this RA-VII-induced reduction of cyclin D1 was fully dependent on its direct inhibitory effect of protein synthesis. Northern blot analysis revealed that RA-VII did not affect the level of cyclin D1 mRNA. Meanwhile, pre-treatment of cells with lactacystin, a proteasome inhibitor, abolished the RA-VII-induced decrease in cyclin D1. Moreover, RA-VII still decreased cyclin D1 protein in the presence of cycloheximide. These results indicate that the RA-VII-induced cyclin D1 decrease depends on cyclin D1 degradation via the ubiquitin–proteasome pathway and does not require additional protein synthesis. RA-VII might actively proceed the degradation process of cyclin D1 via the ubiquitin-proteasome pathway in DLD-1 cells. [© 2001 Lippincott Williams & Wilkins.]

Key words: Cyclin D1, DLD-1, lactacystin, proteasome, RA-VII.

Introduction

The eukaryotic cell cycle is regulated by the sequential activation and inactivation of cyclin-dependent kinases (Cdks), whose activities are determined by the binding of cyclins and Cdk inhibitors (for review, see Sherr[1]). Cdk4 and Cdk6 form active complexes with D-type cyclins in early G_1 phase and promote the progression of the cell cycle by phosphorylating their substrate, retinoblastoma gene product (Rb).[2] Entry into S phase from late G_1 is governed by the cyclin E–Cdk2 complex, followed by cyclin A–Cdk2. The transition from G_1 to S phase, known as the G_1 checkpoint, is strictly regulated by Cdk inhibitors including INK4 families and Cip/Kip families. INK4 families ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) are the specific inhibitors for Cdk4 and Cdk6, while $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ inhibit the cyclin complex of Cdk1 and Cdk2. The G_1 checkpoint seems to be one of the major mechanisms by which cancer development is prevented, as disorders of the G_1 checkpoint are often observed in various cancer types. Overexpression of cyclin D1 is found in breast, pancreatic and esophageal cancers,[3–5] and mutational inactivation of Rb protein is found in breast cancer, lung cancer and osterosarcoma.[6–8] In addition, it has been shown that inactivation of the $p16^{INK4a}$ gene is also found in various cancer types[9] and artificial reconstitution of these genes, in turn, repairs the G_1 checkpoint. The suppression of cyclin D1 expression induced by the antisense of cyclin D1 cDNA inhibits cell proliferation and causes cell-cycle arrest at G_1 phase.[10] It is also known that cyclin D1 forms a complex with estrogen receptors in breast cancer cells and regulates the gene transcription apart from the Cdk-activating system.[11,12] These findings indicate that cyclin D1 plays a pivotal role not only in the activation of Cdks, but also in gene expression. Meanwhile, recent studies have revealed that the proteolysis of cyclins is mediated by ubiquitin-dependent degradation pathways.[13–15] Little, however, is known about the relevance of cyclin degradation in cancer treatment, as no chemicals are found to selectively induce degradation of cyclins.

While a number of drugs have been used for cancer treatment, little is known about their effects on cell-cycle-related proteins, even though some have

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been shown to arrest the cell cycle of cancer cells at certain phases. RA-VII, a bicyclic hexapeptide (Figure 1), is the most potent antitumor compound among its derivative forms isolated from the *Rubiaceae* family.[16] RA-VII inhibits cell growth of various tumor cells at concentrations almost comparable to those of taxol. The molecular mechanisms of RA-VII by which it kills tumor cells are shown to inhibit protein biosynthesis,[17] as is the case with bouvardin, a structurally similar compound.[18,19] RA-VII inhibits *in vitro* protein synthesis in lysate of rabbit reticulocytes and its target molecule is thought to be 60S ribosome.

In addition to the inhibition of protein synthesis, we first describe here that a rapid reduction of cyclin D1 protein occurs in human colon cancer cells treated with RA-VII in a degradation-dependent manner.

Materials and methods

Materials

Cycloheximide was purchased from Wako Pure Chemical (Osaka, Japan). Anti-cyclin D1 rabbit polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies against cyclin A (BF683), cyclin B1 (GNS1) and cyclin E (HE12) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St Louis, MO). Lactacystin was purchased from Kyowa Medix (Tokyo, Japan). ^{3}H-labeled thymidine, uridine and leucine were purchased from NEN (Boston, MA). The murine cyclin D1 and Cdk4 cDNAs were provided by Dr H Matsushime (University of Tokyo, Tokyo, Japan). The human cyclin E cDNA and the human Cdk2 cDNA were gifts from Dr D Morgan (UCSF, CA). RA-VII was extracted and characterized by Natural Products Division (Dr Reddy's Research Foundation, Hyderabad, India).

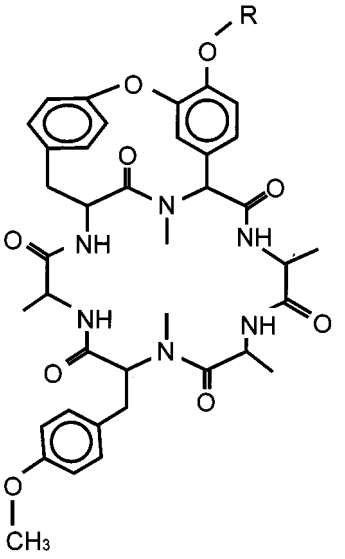

Figure 1. The structure of RA-VII.

Cell culture and MTT assay

Human colon cancer DLD-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). For the MTT assay, cells were seeded in 96-well plates at 5000 cells/well and cultured for 24 h. Various concentrations of RA-VII or cycloheximide were added to the culture and incubated for an additional 72 h. Cells were treated with aliquots of 10 μl MTT (50 mg/ml) for 4 h. After the medium was removed, cells were dissolved in 150 μl of dimethylsulfoxide (DMSO), mixed for 5 min and the OD_{595} of each well was measured.

Protein synthesis detection assay

Cells were seeded in 96-well plates at 5000 cells/well and cultured for 24 h. For [^{3}H]thymidine and [^{3}H]uridine incorporation assay, the medium was exchanged to RPMI 1640 containing dialyzed FBS (10%). For the assay of [^{3}H]leucine incorporation, the medium was exchanged to leucine-free RPMI 1640 containing dialyzed FBS. After 3 h of incubation with RA-VII, ^{3}H-labeled thymidine, uridine or leucine was next added to the culture and incubated for another hour. Cells were disrupted with water and transferred to 96-well glass filter plates (Packard, Meriden, CT). The radioactivity of the filters corresponding to newly synthesized nucleic acids or protein was measured by using a scintilation counter TopCount (Packard).

Cell-cycle analysis

Cell-cycle distribution was analyzed by a flow cytometry as recommended by the manufacturer (CycleTEST Plus; Becton Dickinson, Erembodegen-Aalst, Belgium). Briefly, 5×10^{5} to 2×10^{6} cells trypsinized for 10 min at room temperature, treated with trypsin inhibitor and RNase mixture for 10 min at room temperature were stained with propidium iodide, and filtrated with 50-μm nylon mesh into 12×75-mm tubes. Prepared samples were measured by a FACScan flow cytometer (Becton Dickinson) and their cell-cycle distribution was analyzed by using CellFIT software.

Western blot analysis

Cells treated with RA-VII were harvested by centrifugation. Isotonic buffer containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM EDTA, 1 μM dithiothreitol, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF and 0.1% Tween 20 was added to the cells. After sonication for 30 s, cell lysates were centrifuged at 15 000 r.p.m. for 5 min and the supernatant was normalized with the protein concentration to use as samples for later experiments. The samples were separated by 12.5% PAGE, transferred to nitrocellulose membranes and cell-cycle-related proteins were detected through exposure to their specific antibodies.

Northern blot analysis

Total cellular RNA was extracted from DLD-1 cells by guanidine isothiocyanate and purified by the silica-gel-membrane spin method (Qiagen, Hilden, Germany). RNA samples (10 μg) were separated by 1% formaldehyde/agarose gel electrophoresis and transferred to nylon membranes (Hybond-N; Amersham, Little Chalfont, UK). RNA was then cross-linked to membranes by exposure to UV light. Hybridization was performed for 24 h at 65°C. Complementary DNA fragments of murine cyclin D1, human cyclin E and human cdk2 were α-^{32}P-labeled by randomly primed cDNA synthesis using a random primer kit (Amersham). Membranes were washed with 2 × standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) and with 0.5 × SSC, 0.1% SDS at room temperature, followed by a final wash with 0.1 × SSC, 0.1% SDS at 65°C. The radioactivity left on the membranes was scanned by using a bioimage analyzer (Fujix BAS 2000; Fuji Photo Film, Kanagawa, Japan).

Results

RA-VII inhibits cell growth of DLD-1 cells with protein synthesis inhibition

When DLD-1 cells were treated with RA-VII or cycloheximide for 3 days, 50% inhibition of cell growth was observed at 13 and 94 ng/ml, respectively (Table 1). This strong growth inhibitory effect of RA-VII was also observed in many other human cancer cell lines including A549 (lung), T-47D (breast), Saos-2 (osteosarcoma) and TE671 (brain) at similar concentrations (data not shown). Growth of DLD-1 cells was completely blocked by 100 ng/ml RA-VII or 1 μg/ml cycloheximide treatment (data not shown). The growth arrest effect of RA-VII was dismissed by changing to RA-VII-free RPMI 1640+10% FBS medium (data not shown). The growth arrest of cells was parallel to the inhibition of protein synthesis induced by RA-VII and cycloheximide in DLD-1 cells. As shown in Figure 2, both RA-VII and cycloheximide inhibited incorporation of [^{3}H]leucine in a dose-dependent manner, and 50% inhibition of [^{3}H]leucine incorporation occurred at similar concentrations to that of cell growth inhibition (Figure 2 and Table 1). DNA and RNA syntheses, determined by incorporation of [^{3}H]thymidine and [^{3}H]uridine into cells, were not affected by either treatment with 1 μg/ml RA-VII or 10 μg/ml cycloheximide (data not shown), at which the protein synthesis was completely inhibited.

RA-VII induced partial G_1 arrest of DLD1 cells with rapid reduction of cyclin D1 protein

Despite their known similar physiological effects, RA-VII and cycloheximide arrested cell growth differently. The G_1 population of cells treated with 100 ng/ml RA-VII was 22%, whereas that of cells treated with 1 μg/ml cycloheximide was 5% (Table 2). The G_2/M population of both RA-VII- and cycloheximide-treated cells increased to 43 and 54%, respectively. A similar tendency was observed in the other human cancer cell lines described above. As RA-VII treatment resulted in a different pattern of distribution in the cell cycle compared to that of cycloheximide treatment, we measured the amount of cell cycle-related proteins by Western blot. Treatment of DLD-1 cells with RA-VII resulted in the specific reduction of cyclin D1 protein (Figure 3a). The decrease of the cyclin D1 protein level was observed from 2 h and reached an undetectable level at 8 h. The expression levels of cdk4, cyclin E and cdk2 (Figure 3b), and other cyclins A, B and cdk1 were not affected by RA-VII treatment after 8 h of culture (data not shown). Parallel with the reduction of cyclin D1 protein, an unphosphorylated form of Rb was also observed (Figure 3b). These data suggest that RA-VII induces the partial G_1 arrest via a decrease in cyclin D1-dependent kinase activity due to the lack of cyclin D1 protein. In this experiment, treatment with

Table 1. Cell growth inhibition of DLD-1 cells

Chemical	IC_{50} of drugs (ng/ml)
RA-VII	13
Cycloheximide	94

Cells were treated with chemicals for 72 h and IC_{50} values were obtained graphically as the dose of drug causing 50% reduction of the control values. This experiment was performed in triplicates

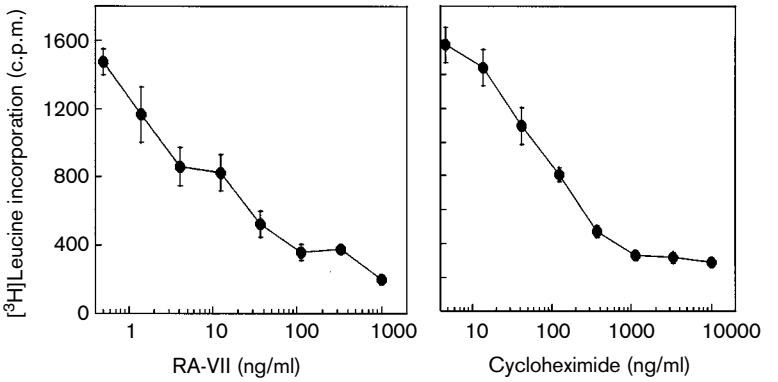

Figure 2. Protein synthesis inhibition by RA-VII and cycloheximide. DLD-1 cells were cultured in ^{3}H-labeled leucine in the presence of various concentrations of RA-VII or cycloheximide for 1 h. This experiment was performed in triplicate.

Table 2. Cell-cycle distribution of DLD-1 cells treated with RA-VII

Chemical	Cell-cycle distribution (%)		
	G_1	S	G_2/M
None	32	41	27
RA-VII (100 ng/ml)	22	35	43
Cycloheximide (1 μg/ml)	5	41	54

Cells were treated with chemicals for 48 h and cell cycle profile was analyzed by using a Cycle TEST Plus (Becton Dickinson) with the manufacturer's recommended procedure

1 μg/ml cycloheximide did not reduce the amount of cyclin D1 protein, despite its sufficient inhibitory activity on protein synthesis (Figures 2 and 3a).

Mechanism of Cyclin D1 reduction by RA-VII treatment

To elucidate the mechanisms by which RA-VII induced reduction of the cyclin D1 protein level, first, the mRNA levels of RA-VII treated cells were detected. As shown in Figure 4(a), the levels of cyclin D1 mRNA were not affected by RA-VII treatment. This result indicated that RA-VII did not have any influence on the cyclin D1 production signaling system such as the mitogen-activated protein kinase (MAPK)-dependent pathway. Next, we co-treated DLD-1 cells with RA-VII and a proteasome inhibitor lactacystin to examine the effect of RA-VII on the degradation process of cyclin D1 protein. With the increasing concentrations of lactacystin, the reduction of cyclin D1 protein was rescued (Figure 4b). This result indicated that the degradation process via the proteasome pathway mediated the RA-VII-induced cyclin D1 decrease and

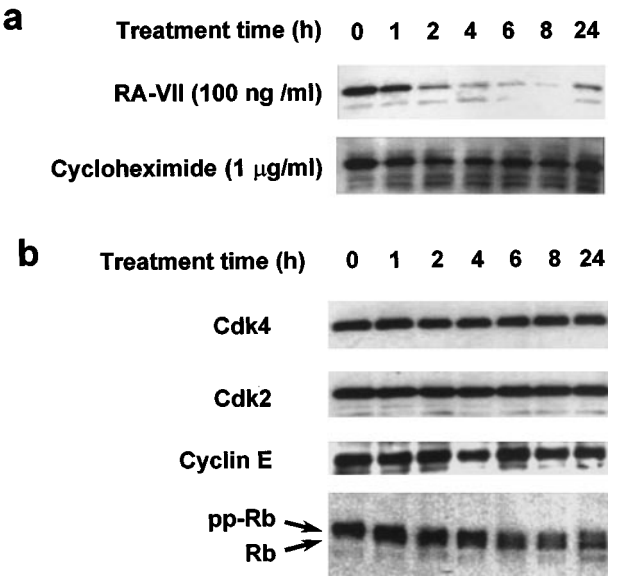

Figure 3. (a) Western blot analysis of cyclin D1 in DLD-1 cells. Cells were exposed to 100 ng/ml RA-VII or 1 μg/ml cycloheximide for different amounts of time and the total amount of cyclin D1 was detected by Western blot. (b) Western blot analysis of cdk4 and Rb in 100 ng/ml RA-VII-treated DLD-1 cells. The same samples obtained from (a) were used for cdk4, cdk2, cyclin E and Rb detection.

this process did not require additional protein synthesis. The molecular targets of RA-VII and cycloheximide were slightly different. Hence, there was a possibility that cycloheximide, unlike RA-VII, might have interfered with the proteasome-dependent protein degradation mechanism with its physiological activity and therefore that cycloheximide did not fully reduce levels of cyclin D1 in DLD-1 cells. In order to address this question, DLD-1 cells were co-treated

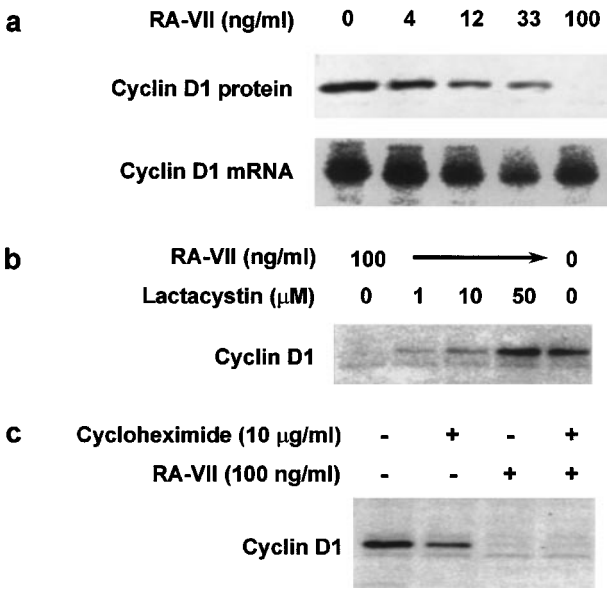

Figure 4. (a) Western blot and Northern blot analysis of cyclin D1 in DLD-1 cells. Cells were treated with various concentrations of RA-VII for 48 h. (b) Inhibition of cyclin D1 degradation by lactacystin. DLD-1 cells were co-treated with 100 ng/m ml RA-VII and various concentrations of lactacystin for 48 h. (c) Reduction of cyclin D1 by RA-VII in the presence of cycloheximide. DLD-1 cells were cultured in the presence of 100 ng/ml RA-VII and/or 10 μg/ml cycloheximide for 48 h.

with RA-VII and cycloheximide. In the presence of cycloheximide, RA-VII-treated cells still reduced protein levels of cyclin D1 (Figure 4c, right end lane). This result suggests that cycloheximide does not interfere with the cyclin D1 degradation process in DLD-1 cells.

Discussion

RA-VII is one of the rare small molecule chemicals from a plant extract that possesses strong growth inhibitory activity. As well as taxol and its derivatives, RA-VII and its derivatives have a potency to be novel anticancer medicines. In fact, RA-VII and one of its derivatives bouvardin are currently in phase I clinical trials. In the process of characterizing RA-VII, we confirmed the inhibitory activities of RA-VII on cell growth and on protein synthesis in human cancer cell lines including DLD-1 human colon cancer cells (Table 1 and Figure 2), which were consistent with previous reports.[16,17] The comparison of RA-VII with another protein synthesis inhibitor, cycloheximide, highlighted the new function of RA-VII. All the cell lines that we examined indicated the rapid reduction of cyclin D1 protein with RA-VII treatment and partial G_1 arrest was also observed in some of the cell lines. The partial G_1 arrest seemed to be a consequence of the reduction of cyclin D1, as we also observed an increase in the unphosphorylated form of Rb in parallel with the reduction of cyclin D1 (Figure 3). These findings implied that Cdk4 activity was diminished by a lack of cyclin D1 protein. As a result, the unphosphorylated form of Rb accumulated and interfered with the G_1–S phase transition. Meanwhile, cycloheximide did not decrease cyclin D1 protein levels as strongly as RA-VII and it did not cause G_1 arrest. This result revealed that protein synthesis was not critical for the cell-cycle progression at early G_1 as far as cyclin D1-dependent kinase activity was sustained. Furthermore, cells treated with RA-VII or cycloheximide were both arrested at G_2/M phase at which cells required more protein synthesis than the G_1 phase did. There are several possible explanations for how effectively RA-VII decreases cyclin D1 levels. The level of cyclin D1 protein is controlled by mRNA transcription, mRNA stability, protein translation and protein degradation. First, we focused on the signaling pathways that mediate the production of cyclin D1 such as the MAPK pathway, since MEK inhibitors PD098059 and U0126 block the MAPK pathway and decrease the levels of cyclin D1 mRNA and protein.[20] However, RA-VII did not change the levels of cyclin D1 mRNA (Figure 4a). This result suggested the RA-VII's target for cyclin D1 reduction was in protein stability or degradation of cyclin D1 rather than the modulation of mRNA. Recent reports demonstrate that there are some independent pathways involved in cyclin D1 degradation; a calpain-dependent pathway,[21,22] an ubiquitin proteasome-dependent pathway that is initiated by GSK-3β phosphorylation[13,23] and a stress-induced RxxL motif-dependent degradation.[24] The dominant pathway for cyclin D1 degradation seems to depend on cell lines and conditions, as the proteasome inhibitor lactacystin prevents cyclin D1 degradation in BEAS-2B cells,[21] but not in NIH 3T3 cells.[22] In our experiments, DLD-1 cells showed sensitivity to lactacystin (Figure 4b) but not to the calpin inhibitor leucyl-leucyl-norleucinal (LLnL) (data not shown). Choi *et al.* reported that cyclin D1 in several cell lines was decreased to undetectable levels with cycloheximide treatment,[21] which implied cyclohexmide did not interfere with cyclin D1 degradation mechanisms. Our results also indicated that RA-VII-induced cyclin D1 degradation was not influenced by cycloheximide (Figure 4c), which implied cycloheximide, at least, did not interfere with the proteasome-dependent cyclin D1 degradation mechanism. Chitnis *et al.* reported that an RA-VII derivative, bouvardin, did

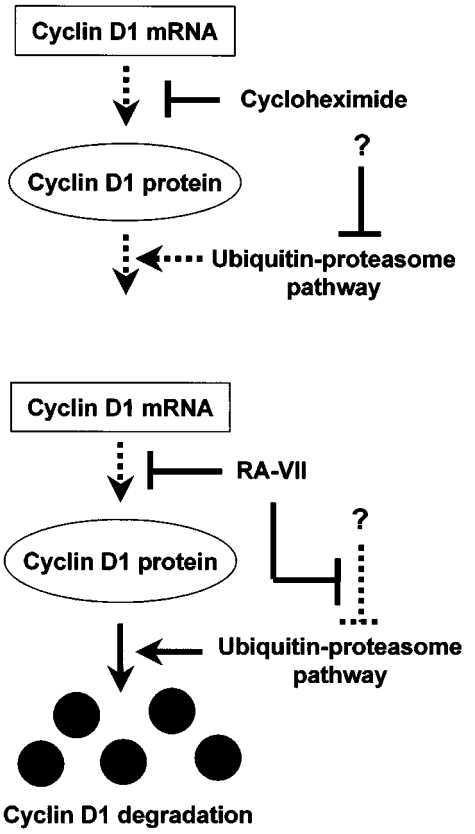

Figure 5. A possible mechanism by which RA-VII reduces the cyclin D1 levels.

not interfere with protein degradation in the P388 mouse leukemia cell line,[25] which was consistent with our result. The fact that the cyclin D1 degradation in DLD-1 cells was not fully induced by protein synthesis inhibition revealed that there might be unknown mechanisms that interfere with cyclin D1 degradation in the cells. In fact, β-catenin, another substrate for GSK-3β, is accumulated in the cytoplasm of DLD-1 cells,[26] and that might have some influence on cyclin D1 degradation efficiency. RA-VII may have a novel function in stimulating the ubiquitin proteasome-dependent protein degradation mechanism of cyclin D1 in colon cancer (Figure 5).

Our results also encourage the possibility of an anti-cancer drug that could enhance the specific degradation of cyclin D1 and RA-VII helps to study the degradation mechanisms of cyclin D1 as a cancer therapy target.

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