Biochemical and Biophysical Research Communications 438 (2013) 468–472

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Acyldepsipeptides inhibit the growth of renal cancer cells through G1 phase cell cycle arrest

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ARTICLE INFO

Article history:
Received 27 July 2013
Available online 6 August 2013

Keywords:
Acyldepsipeptide
Renal cell carcinoma
Cell cycle
Antitumor
Cyclin D1

ABSTRACT

Acyldepsipeptides are a group of potent antibiotics discovered in the secondary metabolites of *Streptomyces* species. However, besides the function of antibiotics, no other activities have been reported about these important compounds so far. In the course of searching the natural products as chemotherapeutic agents for renal cell carcinoma, we found that ADEP1, a major metabolic component of *Streptomyces hawaiiensis* NRRL 15010, could effectively inhibit the growth of 786-O, 769-P, and ACHN renal carcinoma cells in MTT assay. Flow cytometric analysis demonstrated that ADEP1 could block the cell cycle arrested at G1 phase. Moreover, it was found that ADEP1 down-regulated the expressions of cyclin D1, CDK4 and PCNA and inhibited activity of MAPK–ERK pathway by detection of decreased expression of phosphorylated ERK1/2 and c-Fos in 786-O and 769-P cells by Western blotting. To our knowledge, this is the first report concerning to the antitumor activities of acyldepsipeptides. Based on these results, ADEP1 may become a promising lead compound to be developed a novel chemotherapeutic agent for treatment of renal carcinoma.

1. Introduction

Renal cell carcinoma (RCC) accounts for 90–95% of neoplasm of the kidney and 20–30% patients present with metastatic disease at the time of diagnosis [1,2]. Once metastatic disease develops, the prognosis is extremely poor. Clear cell RCC, which is often associated with mutations of the Von Hippel–Lindau (VHL) tumor suppressor gene, is the most common sporadic subtype constituting 70–80% of RCC [3,4]. Clear cell RCC is considered refractory to the chemotherapy because of expression of the multidrug resistance transporters in proximal tubules and it is also resistant to the immunomodulatory therapies because the tumors themselves induce immunosuppression, meanwhile, some immunomodulatory agents had significant clinical toxicities [1,5]. Due to the current status of RCC treatment, developing the non-toxic natural products which specifically target the unique characteristics of the RCC could be a promising approach to obtain the effective agents for treatment of the advanced RCC.

Natural products and their derivatives are the important origins of antitumor agents, especially secondary metabolites of microorganisms have recently been found to provide potential and some potent antitumor activities and are a very valuable source of chemotherapeutic agents of tumors.

In particular, actinomycetes and streptomyces can produce a large number of metabolites with antitumor properties, many of which also have antimicrobial activity and were usually first discovered as antibiotics [6]. Acyldepsipeptides (ADEPs), produced by *Streptomyces hawaiiensis* NRRL 15010 [7], were found as a new class of antibiotics. They killed bacterial cells by indiscriminately increasing the activity of ClpP *in vivo*, redirecting its activity away from its physiological substrates, targeting it to nascent polypeptide chains, and finally resulting in inhibition of cell division and cell death [8,9]. Their antibacterial potency makes ADEPs promising lead compounds for future antibiotic development.

Although the antimicrobial activities of ADEPs have been explored in depth, there have been no reports regarding to any other activities so far. In the present study, we investigated the effect of ADEP1, a major metabolic component of *S. hawaiiensis* NRRL 15010, on the growth of renal cancer cells *in vitro*. Study of the mechanism of action revealed that ADEP1 inhibited the renal carcinoma cells growth by blocking the cell cycle arrested at G1 phase. Western blotting results showed that ADEP1 inhibited the expression of cyclin D1 and phosphorylated ERK1/2 protein in 786-O and 769-P cells. Taken together, we propose that ADEP1 may become a promising lead compound for developing a novel chemotherapeutic agent of renal carcinoma.

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http://dx.doi.org/10.1016/j.bbrc.2013.07.119

2. Materials and methods

2.1. Fermentation of strain and isolation of acyldepsipeptides

The strain *S. hawaiiensis* NRRL 15010 was obtained from the American Type Culture Collection (ATCC). Fermentations of the strain were carried out according to [7] with some modifications. Briefly, the media consisting of 0.5% glucose, 0.5% yeast extract, 0.8% malt extract, 0.05% calcium carbonate and 1% D101 resin in deionized water (pH 7.2) in 250 mL flasks was cultivated for 5 days at 30 °C on a rotary shaker (300 rpm).

A total 10 L amount of whole fermentation broth was filtered with gauze. The filtrate was extracted with ethyl acetate for three times. The residue (resin and mycelia) was extracted with ethyl acetate for four times. The ethyl acetate extracts were combined and evaporated in vacuum. The resulted residue was dissolved in methanol and subjected to silica gel column eluting with chloroform/methanol (1:0 → 0:1). Fractions were subjected to C-18 reversed phase silica gel (ODS) chromatography eluting with methanol/water (4:1 → 1:0) and then purified by HPLC (Shimadzu LC-6AD, column: Shimadzu Shim-pack Prep-ODS, 20 × 250 mm, 10 μm. flow rate: 8 mL/min, UV 298 nm) eluting with methanol/water (73:27). The retention time of acyldepsipeptide 1 (ADEP1) and acyldepsipeptide 2 (ADEP2) was 16.3 min and 11.5 min, respectively. Other acyldepsipeptides were also purified at the same time, but their yields were comparatively low in the metabolites of *S. hawaiiensis* NRRL 15010. The structures of ADEP1 and ADEP2 were determined on the basis of their physicochemical properties and IR, NMR and MS data.

2.2. Cell culture and ADEP1 and ADEP2 treatment

Human renal cancer cells 786-O, 769-P and ACHN were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Gibco) supplemented with 10% (*v*/*v*) fetal bovine serum (Hyclone) at 37 °C in a humidified 5% CO_2 incubator. For ADEP1 and ADEP2 treatment, appropriate volumes of stock solution of ADEP1 and ADEP2 were added to the medium to achieve the indicated final concentrations (0, 1, 10, 20, 50, and 100 μM) and were then incubated with cells for the indicated periods of time (48 h and 72 h).

2.3. MTT assay

786-O, 769-P and ACHN cells were seeded in each well of a 96-well plates, and various concentrations of ADEP1 and ADEP2 (0, 1, 10, 20, 50, and 100 μM) were added and cultured for 48 and 72 h at 37 °C in 5% CO_2 atmosphere, respectively. 20 μL MTT (5 mg/mL in PBS) was added into each well and incubated for 4 h. Then, the medium containing MTT was removed and 150 μL DMSO was added to dissolve the formazan crystals. The absorbance at 490 nm was then measured by a Microplate Autoreader (Bio-Tek Instruments). The growth inhibitory rate was calculated by the following formula: growth inhibitory rate = (average OD value in the control group−average OD value in the treatment group)/average OD value in the control group × 100%.

2.4. Clone and clonogenic assay

786-O, 769-P and ACHN renal cancer cells were seeded in each well of a 24-well plates (100 cells/well), cultured in medium containing ADEP1 (50 μM) for 5 days. After that, cells were stained with crystal violet. The colony formation capacity of the cells was tested by using 2-dimensional (2D) culture and the number of clones was counted and plotted.

2.5. Western blotting analysis

Total cellular protein was extracted from cells with radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40, and 0.5% sodium deoxycholate] containing protease inhibitors, 1% Cocktail and 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma, St Louis, MO). 30 μg of protein was separated on SDS–PAGE and blotted onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (pH 7.6, TBST) at room temperature for 1 h and then incubated with total-ERK (Bioss 1:200), phospho-ERK (Bioss 1:400), c-Fos (Santa Cruz 1:400), cyclin D1 (Santa Cruz 1:200), CDK4 (Santa Cruz 1:400), and PCNA (SCT 1:2000) antibodies at 4 °C overnight. The membrane was then treated with an appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The bands were visualized using Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare, UK) on X-ray film. Immunoblotting against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Shanghai Kangchen 1:10,000) was performed as an internal control.

2.6. Cell cycle analysis

Human renal cancer cells, 786-O and 769-P were grown in RPMI-1640 medium supplemented with 10% FBS in 6 cm-dishes, and treated with 50 μM of ADEP1 and ADEP2 for 48 and 72 h. Subsequently, cells were harvested, washed with PBS, and fixed overnight in 70% ethanol at −20 °C. Then cells were incubated with propidium iodide at room temperature for 30 min and were analyzed by flow cytometry using a FACS Calibur flow cytometer (BD Bioscience). The data were analyzed using cell fit software.

3. Results

3.1. The structural characterization of ADEP1 and ADEP2

ADEP1 was obtained as yellow powder, ESI-MS (*m*/*z*): 741.8 $(M+Na)^+$, 718.9 (M^+), The IR spectrum of ADEP1 showed absorption bands at 3310, 1748, 1668, 1523 and 1450 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) spectra of ADEP1 was almost identical with previous published data of A54550 factor A except for the chemical shift of active hydrogen.

ADEP2: slightly yellowish powder, ESI-MS (*m*/*z*): 727.8 $(M+Na)^+$, 704.9 (M^+), IR (KBr): 3280, 1728, 1640, 1499, 1431 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) spectra of ADEP2 was identical with previous published data of A54550 factor B except for the chemical shift of amino groups.

The chemical structures of ADEP1 and ADEP2 were shown in Fig. 1.

3.2. ADEP1 and ADEP2 inhibit the growth of three renal cancer cells

To investigate the effect of ADEP1 and ADEP2 on cell growth of RCC cells, the 786-O, 769-P, and ACHN renal cancer cells

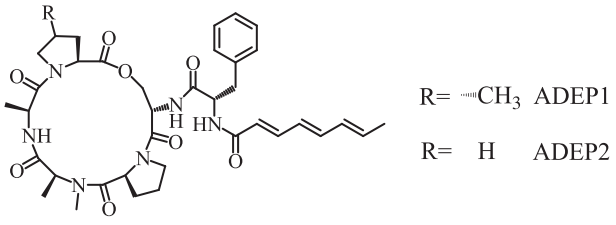

Fig. 1. The chemical structures of ADEP 1 and ADEP 2.

were treated with ADEP1 and ADEP2 (1, 10, 20, 50, 100 μM). As shown in Fig. 2A, the cell growth of 786-O, 769-P, and ACHN cells was obviously inhibited by ADEP1 or ADEP2, comparing to vehicle treated control groups. In concentration-dependent experiments, 1 μM of ADEP1 could not effectively reduce the cell viabilities in 786-O and ACHN cells, whereas only 16.6% growth inhibition was observed in 769-P cells after treatment for 48 h. When treated with ADEP1 at 50 μM concentration, the cell growth was inhibited 63.0%, 61.4%, and 53.5% in 786-O, 769-P, and ACHN cells, respectively. Similarly, treatment with ADEP2 at 50 μM for 48 h only led to 42.4%, 37.5%, and 29.5% growth inhibition in 786-O, 769-P, and ACHN cells, respectively. On the other hand, different treatment time of ADEP1 and ADEP2 (48 h and 72 h) showed almost same cell growth inhibition in three cell lines. These results indicate that ADEP1 has a higher inhibitory effect than ADEP2 on the growth of renal cancer cells. For further determining the inhibition of ADEP1 and ADEP2 on renal cancer cell growth, colony formation assays were performed in 786-O, 769-P, and ACHN cells. As shown in Fig. 2B and C, the treatment of ADEP1 and ADEP2 dramatically decreased the number of colonies in three cell lines, indicating that the capacity of clone formation of RCC cells was significantly inhibited by ADEP1 and ADEP2.

3.3. ADEP1 and ADEP2 block the cell cycle arrested at G1 phase

To determine the detailed mechanism of action by which ADEP1 and ADEP2 inhibited the growth of renal cancer cells, DNA cell cycle analysis was performed in 786-O and 769-P cells after treatment with 50 μM of ADEP1 or ADEP2 for 48 h. As shown in Fig. 3, 786-O and 769-P cells in G0–G1 phase were 54.4% and 51.4% in vehicle (DMSO) treated groups, respectively. In contrast, in ADEP1 treated group 85.0% 786-O cells and 80.7% 769-P cells in G0–G1 phase were observed and 64.8% 786-O cells and 61.8% 769-P cells in ADEP2 treated group were measured. The increase of cell percentage in G0–G1 phase was accompanied by a concomitant decrease of cell number of 786-O and 769-P cells in the S phase and G2–M phases. These results demonstrate that growth of RCC cells was inhibited by ADEP1 and ADEP2 due to G1 cell cycle arrest.

3.4. ADEP1 down-regulates ERK1/2 phosphorylation and expression of cyclin D1 protein in renal cancer cells

To elucidate the growth inhibitory mechanisms of ADEP1 in human renal cancer cells, the expression levels of cyclin D1 and CDK4 proteins, which play important roles in the progression of

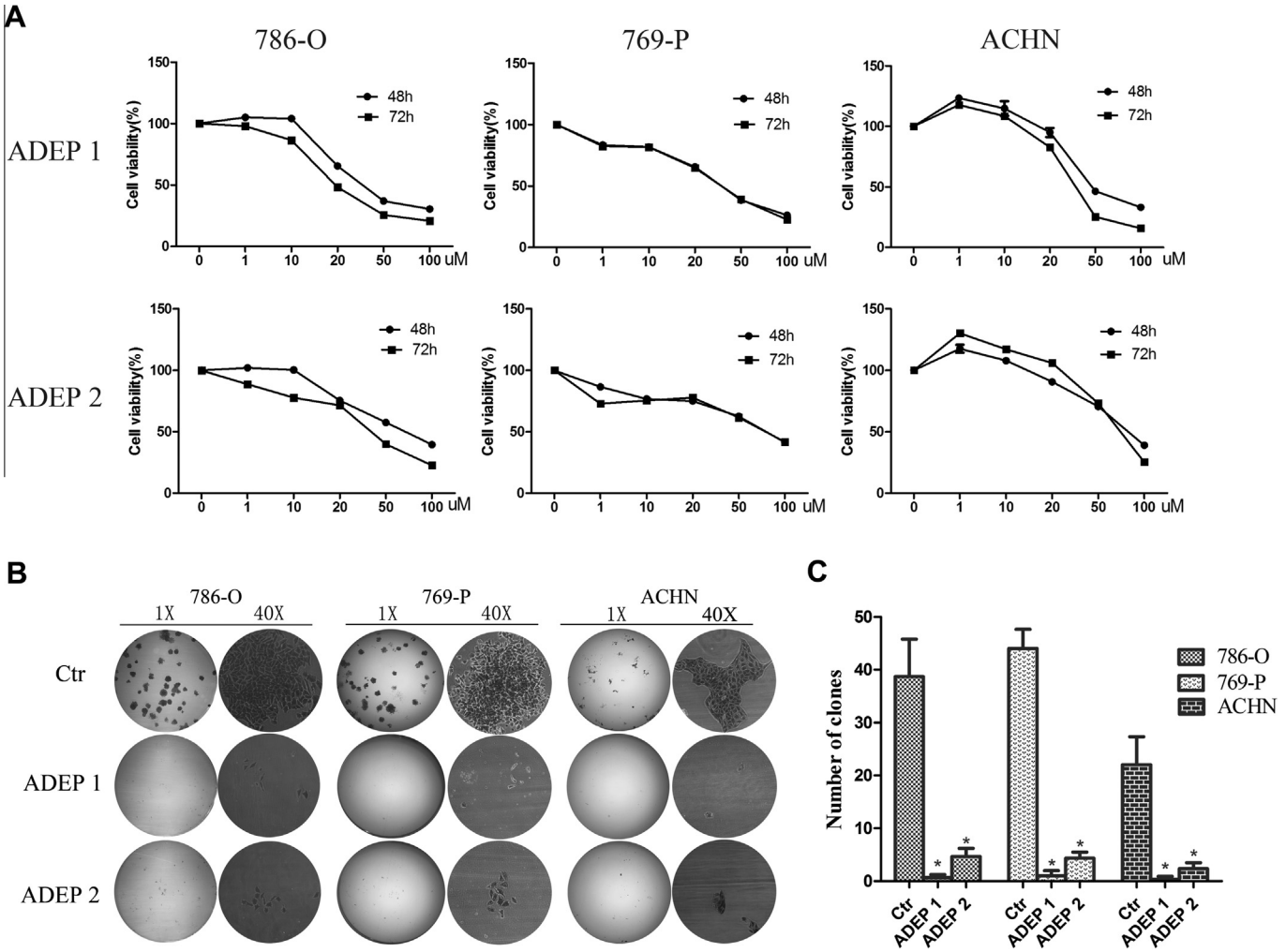

Fig. 2. ADEP 1 and ADEP 2 inhibit the growth of renal cancer cells. (A) ADEP 1 and ADEP 2 inhibit the growth of 786-O, 769-P, and ACHN cells in a dose-dependent manner. After cells were treated with the indicated doses of ADEP 1 (1, 10, 20, 50, and 100 μM) for 48 and 72 h, cell viabilities were determined by MTT assay. (B and C) ADEP 1 and ADEP 2 inhibit the colony formation of renal cancer cells. 100 cells were planted onto 24-well plates and treated with of ADEP 1 (50 μM) for 48 h, and then cells were stained by crystal violet after 5 days. The representative photographs were shown. *$p < 0.05$ versus control.

cell cycle, were measured by Western blotting. As shown in Fig. 4, cyclin D1 levels were markedly down-regulated in 786-O and 769-P cells by ADEP1. Meanwhile, decreased expression of CDK4 was also observed (Fig. 4). In addition, expression of PCNA, which is a marker of cell growth, was significantly reduced by ADEP1 treatment. These results confirmed that ADEP1 inhibited the growth of renal cancer cells through G1 phase arrest.

The expression level of cyclin D1 is regulated by the ERK1/2 MAPK pathway, which is mainly through the activities of c-Fos and Jun related proteins [10]. Inhibition of the ERK1/2 MAPK pathway leads to the decrease of cyclin D1 expression and inhibition of cell cycle progression [11]. To investigate whether ADEP1 inhibited cyclin D1 through suppressing the activity of ERK1/2 MAPK pathway, 786-O and 769-P cells were treated with ADEP1 (50 μM) for 48 h and Western blotting analysis showed an obvious decrease of phosphorylated ERK1/2. Furthermore, c-Fos, a downstream molecule and effector of ERK1/2, was also inhibited by ADEP1. These results suggest that ADEP1 down-regulated phosphorylation of ERK1/2 and expression of c-Fos, which may lead to the reduced expression of cyclin D1 and G1 arrest in renal cancer cells.

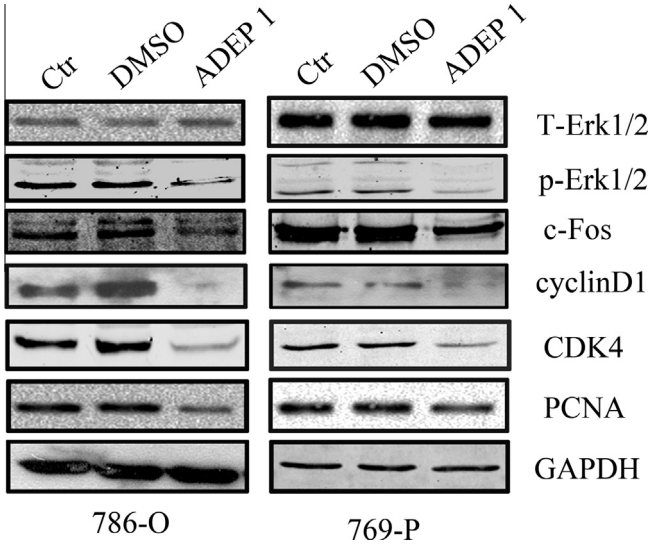

Fig. 4. ADEP 1 decreases the activity or expression of proteins promoting cell cycle progress in renal cancer cells. Cells were treated with 50 μM of ADEP 1 for 48 h, and then cell lysates was subjected to SDS–PAGE followed by immunoblotting. Membranes were probed for T-ERK, p-ERK, c-Fos, cyclin D1, CDK4, and PCNA protein levels, and GAPDH served as a loading control.

4. Discussion

Acyldepsipeptides (ADEPs) are a group of important secondary metabolites of microorganisms with the antimicrobial activities. ADEP1 and 2, the major components in the fermentation broth of *S. hawaiiensis* NRRL 15010, together with other low yield ADEPs were discovered as potent antibiotics [7]. Recently, the study of antibacterial mechanism showed that these ADEPs prevented cell division by switching the bacterial ClpP peptidase from a regulated to an uncontrolled protease [12]. And transition of the ClpP N-terminal segments upon activation as well as conformational changes was markedly different from those of other ATP-dependent proteases [13]. The research on these compounds mainly focused on their antimicrobial activities. However, we found in this study that ADEP1 and 2 could inhibit the growth of RCC cells through suppressing cyclin D1 and preventing cell cycle progress from G1 to S phase. This is the first report on the antitumor activities of ADEPs isolated from *S. hawaiiensis* NRRL 15010.

We fermented the strain of *S. hawaiiensis* NRRL 15010 and isolated ADEP1 and ADEP2, and then utilizing well-characterized renal cancer cell lines, firstly demonstrated the novel anticancer effect of ADEP1 and provided mechanisms that are responsible for its inhibitory effect on cell growth*in vitro*.

Cyclins, which interact with and activate specific CDKs, are key regulators for cell cycle progression. Cyclin D1, partnered with either CDK4 or CDK6, phosphorylates the retinoblastoma tumor suppressor Rb to generate hypophosphorylated Rb, which is critical for cell cycle progression into S phase [14]. Expression of both cyclin D1 and CDK4 were decreased by ADEP1 treatment in renal cancer cells (Fig. 4), indicating that ADEP1 is a potent inhibitor of the cyclin D1-CDK4/6 complex. This result is consistent with our finding that ADEP1 resulted in G1 phase cycle arrest in renal cancer cells (Fig. 3). Given that ADEPs suppress cell cycle in bacteria [9], the ability of ADEPs to inhibit the protein level of components of cell cycle molecular machine could be critical for ADEPs to function as either antibiotic or anti-tumor drug.

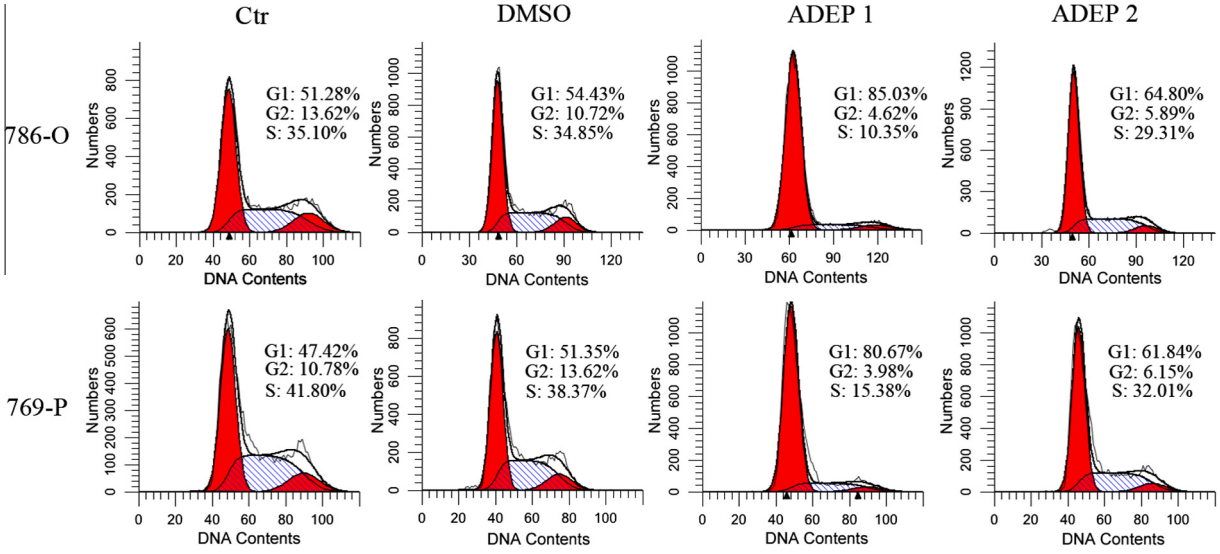

Fig. 3. ADEP 1 and ADEP 2 arrest renal cancer cells on cell cycle G1 phase. The cells were treated with DMSO or 50 μM of ADEP 1 and ADEP 2 for 48 h, stained with propidium iodide and analyzed by flow cytometry. Percentage of cells in G0–G1, S and G2–M phases were calculated using cell fit computer software and values plotted.

Cyclin D1 also functions as a transcriptional modulator by regulating the activity of several transcription factors and histone deacetylase (HDAC3) [15], and this activity is independent of CDK4 activity. Due to its multiple oncogenic functions, cyclin D1 is commonly up-regulated in cancers, including renal cancer [16–18], and it has been identified as a therapeutic target in cancer for a long time. Since ADEP1 could dramatically inhibit the cyclin D1 level, it could be an efficient antitumor seed compound in cyclin D1 overexpression cancer. On the other hand, a recent genetics study revealed that a SNP at 11q13.3, which is located in the enhancer of cyclin D1, highly correlated with the susceptibility of renal cancer [19], which further confirms the especial importance of cyclin D1 in renal cancer. These findings suggest that ADEPs maybe have antitumor function in cyclin D1-overexpressed cancer, which might explain the reason why ADEP1 suppresses the cell growth of renal cancer cells but not T24 bladder cells and PC-3 prostate cancer cells (data not shown).

Except the possible pivotal role of cyclin D1 as a target of ADEP1, activity of MAPK–ERK, and expression of CDK4 and PCNA were also inhibited by ADEP1 (Fig. 4). These results further support the inhibitory effect of ADEP1 on the growth of RCC cells; moreover, these findings also suggest that ADEP1 may target upstream molecules which promote cell growth though the unknown mechanism.

In conclusion, ADEP1, a purified secondary metabolite from the strain of *S. hawaiiensis* NRRL 15010, inhibits the cell growth of renal cancer cells through suppression on cyclin D1 and prevention of cell cycle progress from G1 to S phase. ADEP1 may become a promising lead compound for developing a novel chemotherapeutic agent for renal carcinoma.

Acknowledgments

This study was supported by the Fundamental Research Funds for the Central Universities and National Natural Science Foundation of China (No. 81171953 to P. Guo).

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