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ORIGINAL ARTICLE

Antitumor activity of novel chimeric peptides derived from cyclinD/CDK4 and the protein transduction domain 4

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Abstract CyclinD1/CDK4 and cyclinD3/CDK4 complexes are key regulators of the cell progression and therefore constitute promising targets for the design of anticancer agents. In the present study, the key peptide motifs were selected from these two complexes. Chimeric peptides with these peptides conjugated to the protein transduction domain 4 (PTD4) were designed and synthesized. The chimeric peptides, PTD4-D1, PTD4-D3, PTD4-K4 exhibited significant anti-proliferation effects on cancer cell lines. These peptides could compete with the cyclinD/CDK4 complex and induce the G1/S phase arrest and apoptosis of cancer cells. In the tumor challenge experiment, these peptides showed potent antitumor effects with no significant side effects. Our results suggested that these peptides could be served as novel leading compounds with potent antitumor activity.

Keywords CyclinD · CDK4 · Peptide · Antitumor · Apoptosis

Haili Wang and Xi Chen contributed equally to this work.

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Introduction

In higher eukaryotes, cell cycle progression is coordinated by several closely related Ser/Thr protein kinases resulting from the association of a catalytic cyclin-dependent kinase (CDK) subunit with a regulatory cyclin subunit (Swanton 2004; De Falco and Luca 2010; Gerard and Goldbeter 2009). The transition of cells through the early G1 stage of the cell cycle is coordinated by the activities of CDK4 and CDK6 complexes that are formed following the mitogen-dependent expression of D-type cyclins (Moeller and Sheaff 2006; Emmerich et al. 2004). CDK4 and CDK6 associate with the D-type cyclins (D1, D2, D3) phosphorylate and inactivate the retinoblastoma (Rb) protein family members (p107, p130, pRb) (Kudo et al. 2005; Cooper 2006). Phosphorylation of pRb by CDK4/6 then leads to the derepression and activation of E2F target genes, including the E-type cyclins, which facilitate progression through the G1 phase of the cell cycle. Because of their critical role it is perhaps not surprising that D type cyclins and CDK4 are frequent targets of mutagenesis in various types of cancer (Liu et al. 2010). So the design of cyclinD/CDK4 specific inhibitors was very important to the development of antitumor drugs.

Previous studies suggested that the dysfunction of cell cycle could lead to the malignant cellular proliferation (Lange and Yee Killing 2011). Therefore, peptides/compounds designed to inhibit CDK4 could have potential

anticancer activity (Gondeau et al. 2005; Toogood 2001). One of the currently most developed strategies to block malignant cellular proliferation involves the design of peptide-based inhibitors that target the substrate recruitment binding groove identified on cyclin partners and on a number of cell cycle regulatory proteins (McInnes et al. 2003; Honma et al. 2001; Andrews et al. 2006). Recognition of this groove is mediated by a specific sequence termed the cyclin binding motif (CBM). Several CBM-derived peptides have been reported to induce either cell cycle arrest or apoptosis in tumor cells in vitro and in vivo when conjugated to cellular delivery vectors (Lou et al. 2008).

Determination of the crystal structure of several CDK and CDK-cyclin complexes has provided essential information on the mechanism of formation and activation of these protein kinases (Takaki et al. 2009; Day et al. 2009; Echalier et al. 2010). In most CDK/cyclin complexes, the CDK C-helix packs against helix 5 of the cyclin partner and the packing surface are characterized by extensive hydrophobic interactions. Upon binding to CDK4, cyclin D induces a marked structural reorganization of the catalytic kinase subunit. On the basis of some data and structure, the observation that deletion of the N-terminal region of cyclin D1 resulted in a large reduction in catalytic activity of the complex perhaps suggests that this region may play a role in this remodeling process. By analogy, the flexibility of the cyclin D3 C-terminal helix, evidenced by its ability to unfold from the cyclin C-terminal cyclin box fold, may reflect a protein binding or regulatory role (Echalier et al. 2010).

In the present study, the key peptide motifs from cyclinD1, cyclinD3, and CDK4 were selected and conjugated to the protein transduction domain 4 (derived from HIV-Tat$^{49-57}$). Their antitumor effects were studied in vitro and in vivo.

Materials and methods

Peptide synthesis and purification

All peptides were synthesized using standard solid phase Fmoc strategy (Yamaguchi et al. 2005; Yamaguchi and Kiick 2005). DIC/HOBt/DIPEA were used as coupling reagents. Final cleavage from the resin was carried out with trifluoroacetic acid/phenol/H_2O/thioanisol/ethanedithiol (82.5:5:5:5:2.5) for 3 h. The crude peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a C_{18} column, monitored at 228 nm, and eluted at a flow rate of 5 ml/min. After being lyophilized, their molecular weights were confirmed by electrospray ionization-mass spectrometry (ESI–MS, Bruker-Esquire 3000, Germany). The purity of all the peptides was more than 95 %. They were stored at −20 °C until required.

Animals

BALB/c nude mice, aged 5–6 weeks (18 ± 2 g), male, were purchased from Beijing HFK Bioscience (Beijing, China). The animals had free access to food and water in animal cages that were maintained in a pathogen-free environment (24 ± 0.8 °C, humidity of 55 ± 5 %) with a 12 h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the Ethical Regulations on the Care and Use of Laboratory Animals of Zhengzhou University and were approved by the university committee for animal experiments.

Cell lines

The esophageal carcinoma cell line EC-9706 and the breast cancer cell line MDA-MB-231 were maintained in our laboratory. Murine sarcoma S180 and hepatoma H22 cell lines were obtained from Henan Medical and Pharmaceutical Sciences Institute (Zhengzhou, China) and subcultured in our laboratory. All cells were cultured at 37 °C under humidified air with 5 % CO_2, in RPMI 1640 medium (Invitrogen, CA, USA) containing 10 % FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Suojanen et al. 2011).

Measurement of cell proliferation

The synthesized peptides were assayed at a dose range of 10–100 μM for their anti-proliferation activity in vitro by the MTT assay. Briefly, EC-9706, MDA-MB-231, S180 and H22 cells were plated at a density of 5×10^4 cells/well in 96-well plates. Twenty-four hours after plating, the medium was removed, and cells were treated with various concentrations of peptides for 3 h at 37 °C in serum-free RPMI 1640 medium, and then supplemented RPMI 1640 with FCS. After the cells were incubated for 48 h, 20 μl MTT reagent (5 mg/ml in phosphate-buffered saline) (Sigma-Aldrich, MO, USA) was added to each well for another 4 h at 37 °C. The formazan crystals formed were dissolved in DMSO (200 μl/well) by constant shaking for 10 min. The plate was then read on a microplate reader at 570 nm.

Apoptosis effects of peptides on esophageal carcinoma and breast cancer cells

EC-9706 and MDA-MB-231 cells at logarithmic growth were seeded in 6-well plates by density of 1×10^6 cells/ml. Treatment groups (peptides or 5-FU, 100 μM) and control group were cultured for 48 h. Cells were harvested by centrifuging at 1,000 rpm for 5 min after trypsinization and washed twice with cold PBS. The supernatant was discarded and the pellet was resuspended in the binding buffer at a density of 1×10^5–1×10^6 cells/ml. Then,

100 μl of the sample solution was transferred to a 1.5 ml culture tube, and 400 μl of the binding buffer was added to each sample. After being incubated with 5 μl of FITC-conjugated Annexin V and 10 μl of propidium iodide (PI) for 30 min at room temperature in the dark, the samples were subsequently analyzed by a flow cytometer (FACSCalibur, Becton–Dickinson, CA, USA).

Cell cycle analysis

EC-9706 and MDA-MB-231 cells were treated and harvested as described above. After being fixed with cold ethanol/PBS (7:3, v/v) at 4 °C overnight, cells were then digested by 1,000 U RNase A, and stained with 1 % propidium iodide at 37 °C for 30 min in dark. The DNA profiles were determined within 4 h of the PI staining by the FACScalibur flow cytometer with a 488 nm argon laser. Events were evaluated for each sample and the cell cycle distribution was analyzed using ModFit software (Becton–Dickinson).

Western blot

EC-9706 and MDA-MB-231 cells were treated with peptides for 48 h, and then washed three times with ice-cold PBS before lysis. Cells were lysed with a RIPA buffer (Beijing Biosynthesis Biotechnology, Beijing, China) containing a protease inhibitor for 30 min on ice. The supernatants were collected after centrifugation at 12,000 rpm for 20 min at 4 °C. Equal amounts of protein were separated by 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), After electrophoresis, proteins were electroblotted to nitrocellulose membranes, and subsequently incubated in blocking buffer (in PBS, 5 % skim milk, and 0.1 % Tween 20) at room temperature for 1 h. After blocking, the membranes were incubated with the antibodies, rabbit anti-cyclinD1 (1:1,000), rabbit anti-cyclinD3 (1:1,000), rabbit anti-CDK4 (1:1,000), rabbit anti-actin (1:1,000), or rabbit anti-caspase-3 (1:1,000) (Beijing Biosynthesis Biotechnology, Beijing, China). Subsequently, membranes were washed five times in a wash buffer and incubated with appropriate secondary antibody, and the bands were visualized with the ECL plus system (Strasberg Rieber et al. 1996; Hui et al. 2011).

Table 1 The sequence and source of the peptides

Peptide	Sequence	Source
PTD4	YARAAARQARA	Analog of HIV-Tat (49–57)
D1	SIRPEELLQMELLLVNK	CyclinD1 α5 (131–147)
D3	AVSPRQLRDWEVLVLGK	CyclinD3 α5 (131–147)
K4	LPISTVREVALLRRLEAF	CDK4 αC (49–66)
PTD4-D1	YARAAARQARA GSIRPEELLQMELLLVNK	
PTD4-D3	YARAAARQARAGAVSP RQLRDWEVLVLGK	
PTD4-K4	YARAAARQARAGLPIS TVREVALLRRLEAF	

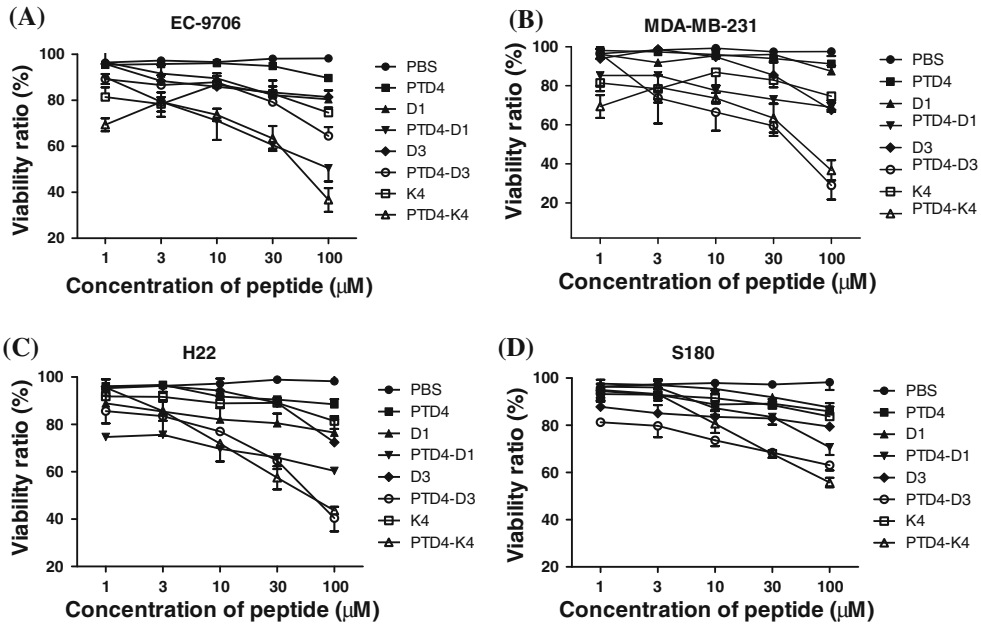

Fig. 1 In vitro anti-proliferation effects of the peptides. Viability ratios were measured by MTT assay. **a** Human esophageal carcinoma cells (EC-9706). **b** Human breast cancer cell (MDA-MB-231). **c** Murine hepatoma cells (H22). **d** Murine sarcoma cell (S180). All the cancer cells were treated with various concentrations of peptides for 48 h. The viability ratio of each dose of peptide was shown as mean ± SD ($n \geq 3$)

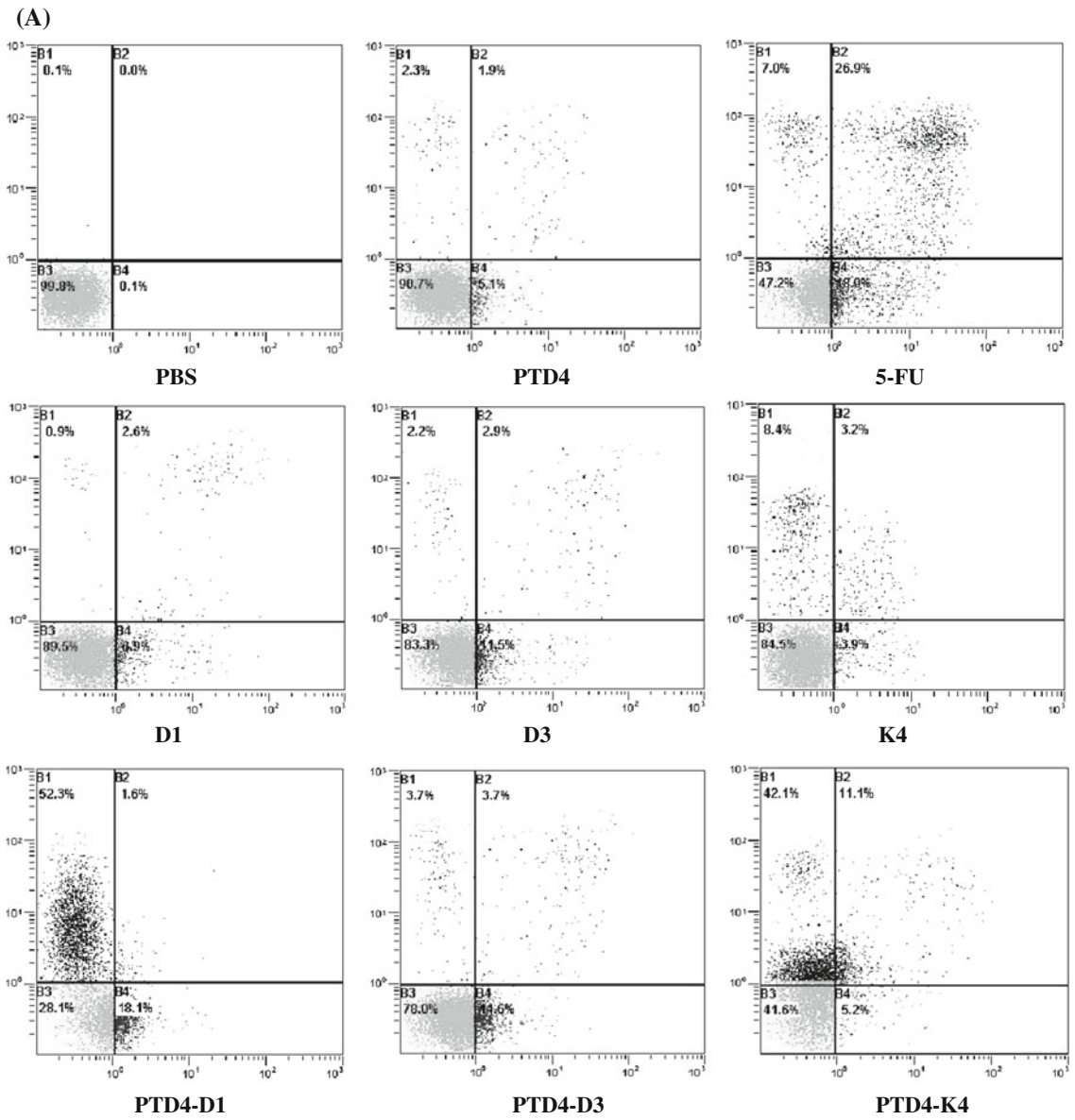

Fig. 2 Flow cytometry analysis of the early apoptosis induced in human cancer cells. EC-9706 (**a**) and MDA-MB-231 (**b**) cells were treated with peptides (100 μM) for 48 h. Apoptosis was measured by Annexin V-FITC and PI (propidium iodide) double staining. Cells with early apoptosis and late apoptosis (or death) were stained as annexinV-FITC positive (*X* axis) and PI positive (*Y* axis), respectively

Co-immunoprecipitation assay

Cell lysates (EC-9706) were prepared and supplemented with phosphatase inhibitor for 30 min at 4 °C, followed by centrifugation at 12,000×*g* for 15 min at 4 °C (Guo et al. 2011; Quintanilla-Martinez et al. 2003). The protein concentrations of the lysates were determined using the BCA protein assay kit. Lysates were precleared with protein A plus-agarose beads (Santa Cruz Biotechnology, CA, USA) overnight at 4 °C, and then cell lysates were immunoprecipitated with the antibodies, rabbit anti-CyclinD1 (1:1,000), rabbit anti-CyclinD3 (1:1,000), rabbit anti-CDK4 (1:1,000), or normal rabbit IgG (1:10,000, as control) overnight at 4 °C. Secondary antibodies included an anti-rabbit IgG horseradish peroxidase. Aliquots of cell lysates obtained before immunoprecipitation were used as input control. Immune complexes were precipitated by protein A plus-agarose beads (10 μl packed beads). After incubation for 12 h, beads were washed five times with the lysis buffer. Precipitated proteins were eluted from the beads by re-suspending the beads in NuPAGE sample buffer (Invitrogen) and heating them at 75 °C for 10 min. The resulting protein from immunoprecipitation or total cell lysates (input) were analyzed as described above.

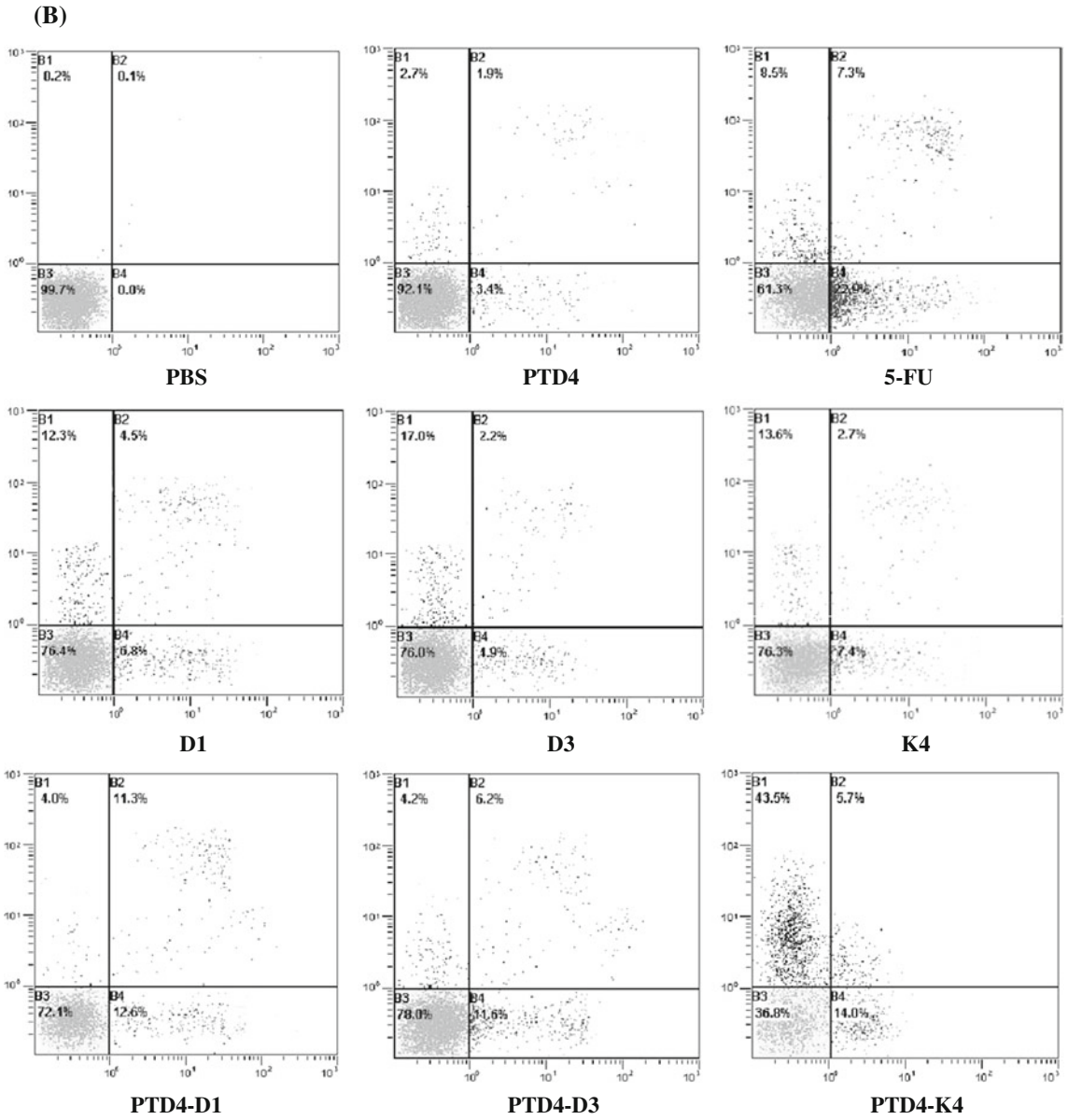

Fig. 2 continued

Evaluation of antitumor effects in nude mice

S180 and H22 cells were harvested and washed three times with normal saline (NS). The cells were pelleted by brief centrifugation at $300\times g$, and then resuspended in NS at a density of 1×10^7 cells/ml. BALB/c nude mice were subcutaneously implanted with 2×10^6 cells/mouse on the right flank (day 0). Twenty-four hours after inoculation, forty-eight mice with S180 cells and H22 cells were randomly divided into eight groups. Peptides (dissolved in NS) were continuously administrated subcutaneously (near the implanted tumor) for 7 days (0.1 ml/10 g, once a day): group 1 with NS (negative control), group 2 with 5-FU (positive control), group 3 and 4 were injected with peptide PTD4-D1 (1 and 2 mg/kg, respectively), groups 5 and 6 were injected with peptide PTD4-D3 (1 and 2 mg/kg, respectively), group 7 and 8 were injected with peptide PTD4-K4 (1 and 2 mg/kg, respectively). The tumor volumes were monitored every day, and primary tumor volumes were calculated by the formula $V = a \times b^2/2$(Yuan et al. 2008), where a is the longest dimension parallel to the skin surface and b is the dimension perpendicular to a and parallel to the surface, 24 h after the last administration, all mice were euthanized with ether, and the tumors, liver and kidney tissues were excised and weighed.

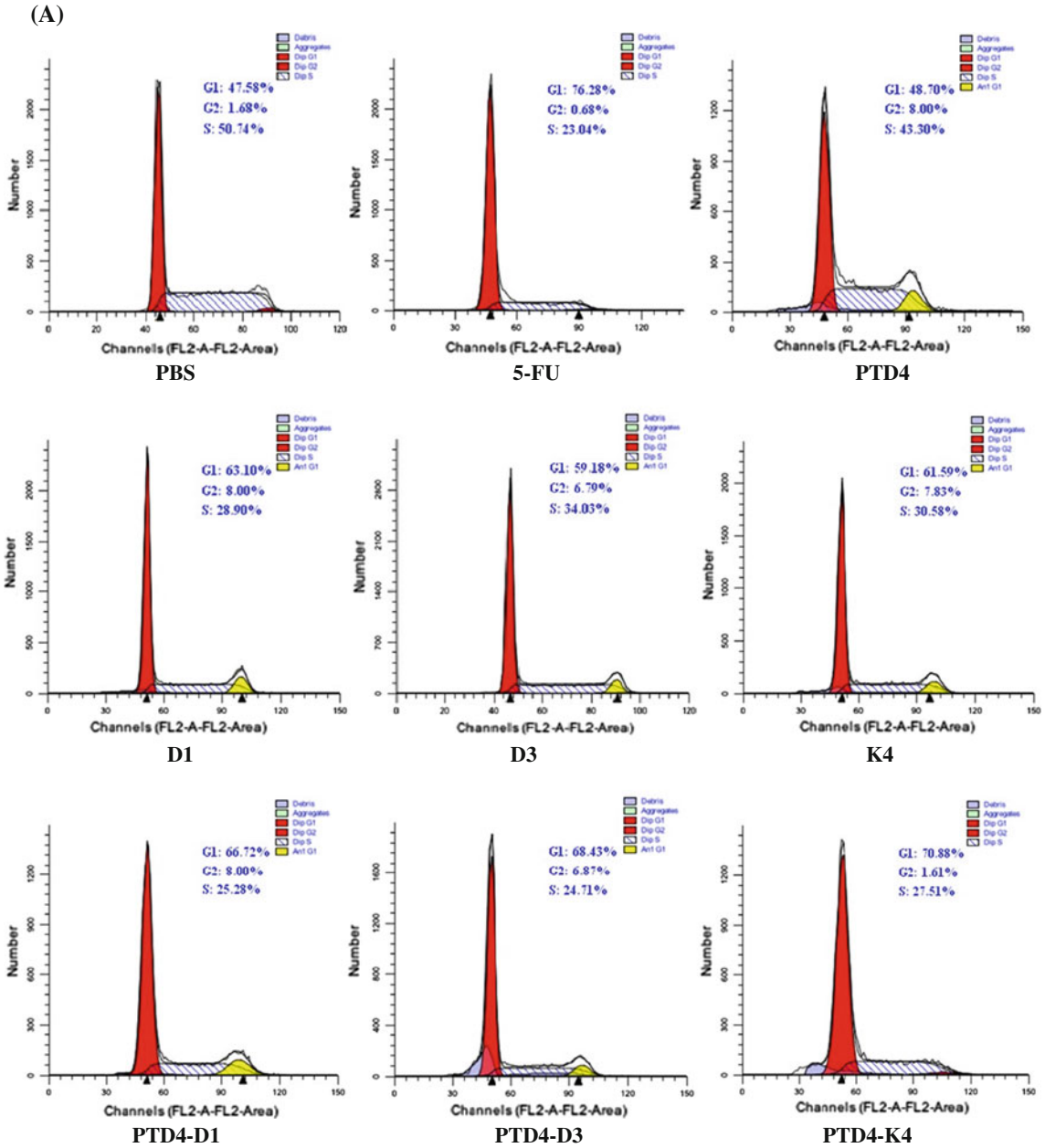

Fig. 3 Flow cytometry analysis of the percentages of the cells in the G1, S, and G2/M phases. EC-9706 (**a**) and MDA-MB-231 (**b**) cancer cells were treated with peptides (100 μM) for 48 h, stained with propidium iodide (PI) and analyzed on a FACSCalibur flow cytometry

HE staining

Samples of the tumors, livers, and kidneys were fixed in 10 % neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and processed according to the hematoxylin and eosin (HE) staining protocol. The stained tissues were observed under a light microscope (200×).

Statistical analysis

All data were presented as mean ± SD ($n \geq 3$). The statistical significances of difference between each group were analyzed by one-way analysis of variance (ANOVA). Statistical differences were presented at probability levels of $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results

Design of peptides targeting CDK4/cyclinD1 and CDK4/cyclinD3 complexes

Both the determination of the crystallographic structure of several CDKs and cyclin/CDK complexes in their inactive

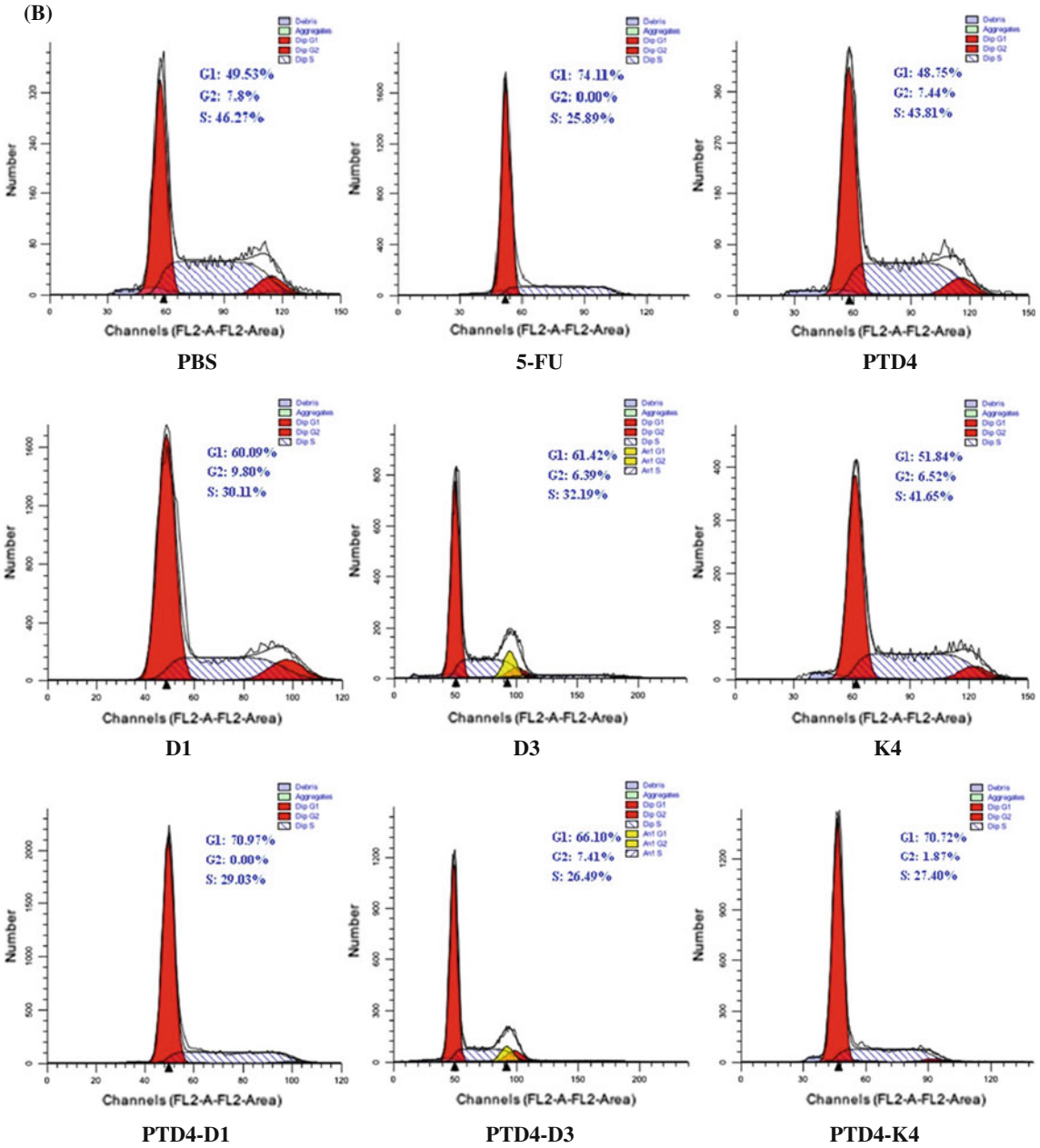

Fig. 3 continued

and active conformation and the characterization of their dynamics of association and activation have enabled identification of the critical features of these complexes (Reynaud et al. 2000; Echalier et al. 2010; Liu et al. 2009). The crystal structures of cyclinD/CDK4 complexes revealed that the regulatory cyclin subunit could bind to one side of the catalytic cleft, interacting with both the N- and the C-lobes of the CDK to form a large, continuous protein–protein interface (Day et al. 2009). Based on these structural and biochemical characterizations, we proposed to specifically inhibit the activity of cyclinD/CDK4 complexes by targeting the protein–protein interface.

Investigations have suggested that the protein transduction domain of the HIV-1 transactivator of transcription (Gros et al. 2006; Eiriksdottir et al.2010), Tat, could successfully facilitate peptides or proteins to enter into cells (Park et al. 2010; Hidema et al. 2012). PTD4 is the analog of the previously reported protein transduction domain, HIV-Tat$^{49-57}$ (Ho et al. 2001); D1, D3 are encompassed in the α5 helix regions of human cyclinD1 and cyclinD3, respectively. K4 is encompassed in the αC helix region of human CDK4. Using glycine as the linker, PTD4-D1, PTD4-D3, and PTD4-K4 were designed as the tandem chimeric peptides by conjugating PTD4 to the N-terminal

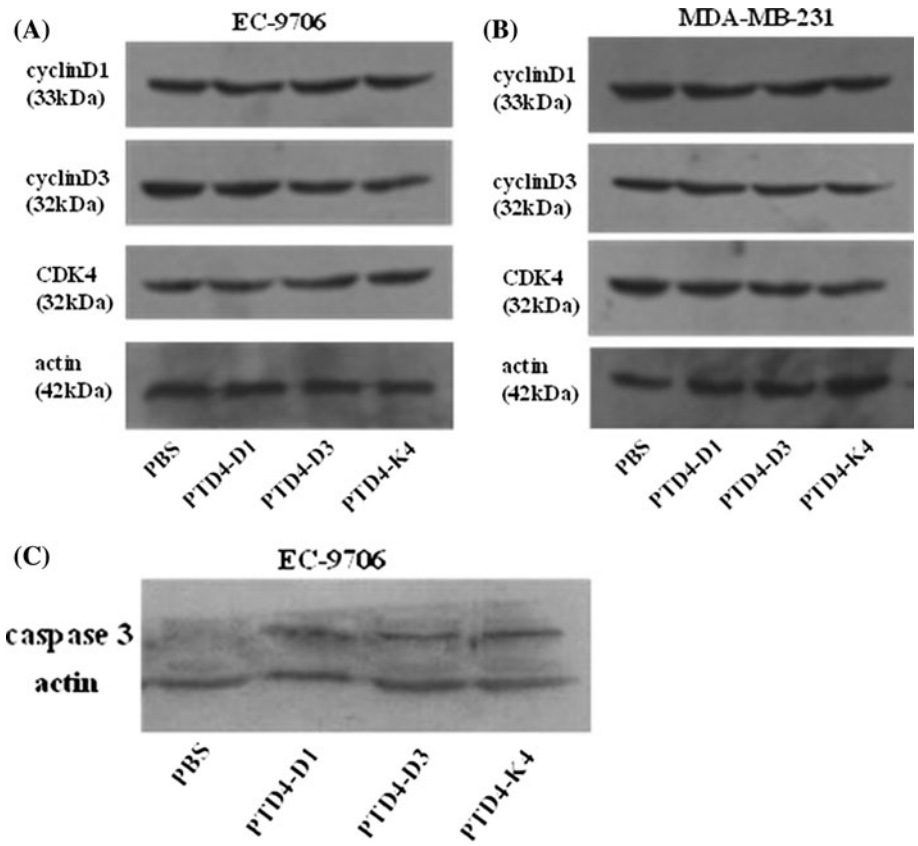

Fig. 4 Western blot analysis of the expression of cyclinD1, cyclinD3, CDK4 and caspase-3. **a** EC-9706 and **b** MDA-MB-231 cells were treated with 100 μM PTD4-D1, PTD4-D3 or PTD4-K4 for 48 h. **c** EC-9706 cells were treated with each peptide at dose of 100 μM for 48 h. The cell lysates were prepared and analyzed for caspase-3 expression by Western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing for β-actin. Each lane contains 30 μg protein extract from the cancer cells

of peptides D1, D3, and K4, respectively. The synthesized peptides were listed in Table 1.

MTT assay

MTT assay was carried out to evaluate whether these peptides could inhibit the proliferation of cancer cells. As shown in Fig. 1, all these three chimeric peptides could not only inhibit the proliferation of human esophageal carcinoma cells (EC-9706, Fig. 1a) and breast cancer cells (MDA-MB-231, Fig. 1b), but also inhibit the proliferation of murine heptoma cells (H22, Fig. 1c) and sarcoma cells cell (S180, Fig. 1d). When the cancer cells were treated for 48 h with increasing doses of PTD4-D1, PTD4-D3, and PTD4-K4, the viability ratios of the cells were markedly decreased. PTD4, D1, D3, and K4 could hardly inhibit the proliferation of these cancer cells at all the tested doses. We speculated that the peptides D1, D3, and K4 could be transduced into cells when conjugated to the membrane-permeable peptide, PTD4.

Peptides could induce the apoptosis of cancer cells

Phosphatidylserine is normally situated on the inner layer of the plasma membrane (Rekdal et al. 2012). In the course of cell death, phosphatidyl serine is translocated to the outer layer of the membrane, this occurs in the early stage of apoptosis, while the cell membrane itself remains intact (Xie et al. 2009; Thapa et al. 2008). To detect the early apoptotic changes, staining with fluorescein isothiocyanate (FITC) labeled annexin V was used, because of its known high affinity to phosphatidylserine (Sawai and Domae 2011; Shang et al. 2011; Roy Choudhury et al. 2011). Based on the MTT assay, we further studied if PTD4-D1, PTD4-D3, and PTD4-K4 could induce apoptosis in cancer cells, EC-9706 and MDA-MB-231. To confirm that the apoptosis observed was induced by these peptides, cancer cells were subjected to annexin V-FITC/PI doubling staining after incubation with 100 μM of peptides for 48 h. As shown in Fig. 2, cancer cells underwent early stage apoptosis after exposure to these three chimeric peptides.

Peptides could induce the cell cycle arrest at G1/S phase

Cell proliferation and apoptosis correlated with cell cycle progression (Caldon et al. 2010). So, we examined the cell cycle determined by propidium iodide staining and flow cytometry analysis. As shown in Fig. 3, changes of the cell cycle profile induced by peptides were studied. When EC-9706 and MDA-MB-231 cells were treated with these chimeric peptides at the concentration of 100 μM for 48 h,

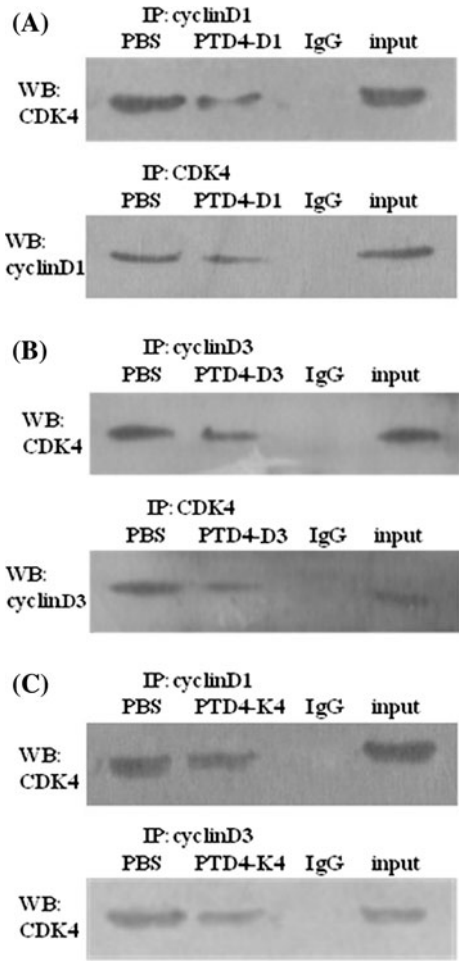

Fig. 5 Co-immunoprecipitation of CDK4 with cyclinD1 or cyclinD3 after the treatment of peptides. After the treatment of the chimeric peptides (100 μM) for 48 h, protein extracts from cancer cells EC-9706 were immunoprecipitated with anti-cyclinD1, anti-cyclinD3 or anti-CDK4. Lanes contain positive control (input) and negative control (IgG)

the proportion of cells in the G1 phase was significantly increased.

The effects of the peptides on the expression of cyclinD1, cyclinD3, CDK4, and caspase-3

To study the action mechanism of these peptides, western blotting and co-immunoprecipitation assays were performed in EC-9706 and MDA-MB-231 cells. As shown in Fig. 4a and b, the treatment of these chimeric peptides could have no significant effects on the expression of cyclinD1, cyclinD3, and CDK4 as compared to the PBS control group.

To confirm the apoptic effects of the peptides, the expression of caspase-3 was tested in EC-9706 cells after peptide treatment. As shown in Fig. 4c, the caspase-3 protein levels were all increased after the three chimeric peptides treatment. The results indicated that the chimeric peptides could induce the apoptosis of cancer cells by activation of caspase-3.

The effects of the peptides on the binding of the cyclinD1/CDK4 or cyclinD3/CDK4 complexes

As shown in Fig. 5, PTD4-D1 treatment could significantly inhibit the binding of the cyclinD1/CDK4 complex. When the cell lysates (EC-9706) were co-immunoprecipitated with anti-cyclinD1 antibody or anti-CDK4 antibody, the amounts of CDK4 and cyclinD1 were decreased, respectively. Similar results were observed for PTD4-D3 and PTD4-K4. These results indicated that the antitumor mechanism of these chimeric peptides might be that they could be transduced into cancer cells and inhibit the binding of the cyclinD1/CDK4 or cyclinD3/CDK4 complexes, which induced the cell cycle arrest and apoptosis.

Antitumor activity of the peptides in S180 and H22 xenograft models

As the antitumor effects of the chimeric peptides in vitro, we further investigated their antitumor effects on the H22 hepatoma and S180 sarcoma xenografts in nude mice. After the tumor cells were inoculated, all the nude mice were inoculated successfully (tumorigenicity was 100 %). Experimental results showed that there was no significant difference in the tumor volume between each group before administration. As shown in Fig. 6a and b, the growth of the tumor volume was slowed down when treated by the 1 or 2 mg/kg/day of the chimeric peptides, PTD4-D1, PTD4-D3, and PTD4-K4. As shown in Fig. 6c–f, the tumor weight of the animal groups treated by these chimeric peptides was significantly decreased as compared to the negative control. As the HE staining results shown in Fig. S1(A), the chimeric peptides could induce the necrosis of the tumor cells. The results indicated that all these three chimeric peptides had potent in vivo antitumor effects on both H22 hepatoma and S180 sarcoma xenografts models.

The side effects of these peptides were also evaluated. As shown in Fig. 7 and Fig. S1(B–C), there were no significant differences in body weight changes, liver and kidney morphological changes, between each peptide-treated group and the PBS control. The results indicated that these peptides might have no significant side effects.

Discussion

Protein kinases constitute important targets in cancer chemotherapy. In the last few years, several CDK inhibitors, essentially ATP antagonists, have been developed and

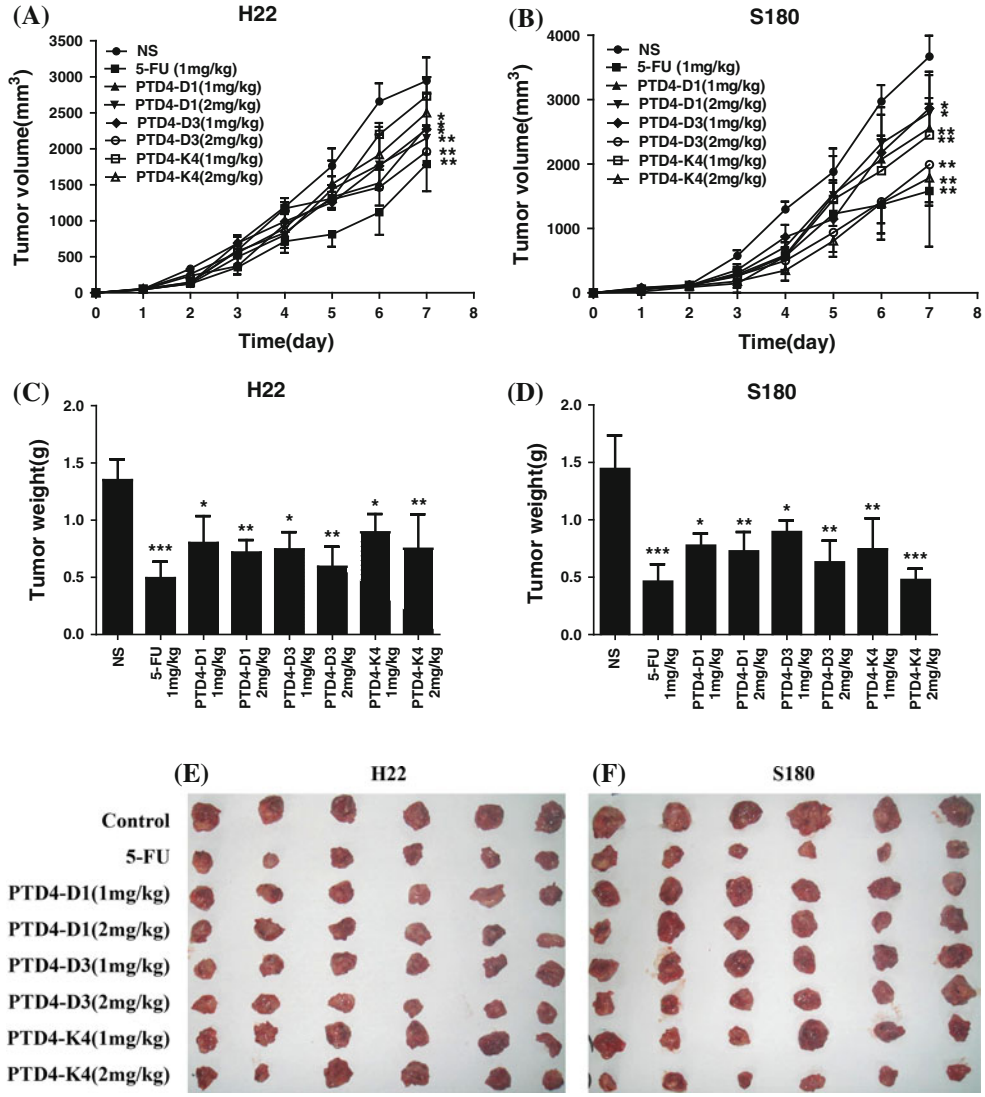

Fig. 6 Effects of peptides PTD4-D1, PTD4-D3, and PTD4-K4 on the growth of H22 hepatoma and S180 sarcoma xenografts in BALB/c nude mice. S180 and H22 cells (2×10^6cells/200 μl) were implanted subcutaneously at the right axilla of the mice. **a**, **b** The in vivo tumor volumes were messured with a caliper every day and calculated according to the equation: tumor volume = length × width^2/2. **c**–**f** At the end of the experiment (day 7), the mice were sacrificed, and the tumors were removed, weighed, and photographed. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group. All data were expressed as mean ± SD ($n = 6$)

studied at preclinical or clinical level (Gerard and Goldbeter 2009). One of the major drawbacks of the inhibitors that target the ATP-binding site of the kinase is their poor selectivity among CDKs and other ATP-based kinases (McInnes et al. 2003; Honma et al. 2001; Andrews et al. 2006). In the present work, we proposed a strategy to design CDK inhibitors targeting to the interface of the cyclin/CDK binding complexes (Chen et al. 2004). Although the relevance of the targets and the specificity of the cell cycle regulators remain issues, the design of inhibitors with unique selectivity offers the potential to treat a wide range of tumor types (Singh et al. 2011).

As both the structure and the mechanism of cyclinD1/CDK4 and cyclinD3/CDK4 have been extensively characterized, and they are major complexes which are very important to the proliferation and cell cycle progression of the cancer cells, we chose them as the targets to develop antitumor leading peptides. Crystal structures of cyclinD in its free and CDK4-bound state revealed that the cyclin box (56-141) might constitute the main interface with CDK4 through interactions with the PSTAIRE helix and contacts with the T-loop as well as the N-terminal β-sheet of CDK4 (Takaki et al. 2009; Day et al. 2009; Echalier et al. 2010). The major contacts involve the α3, α4, and α5 helices of cyclinD1 and cyclinD3, which are critical for the first step of the complex formation. With the aim of specifically targeting the protein–protein interface between CDK4 and cyclinD1 or cyclinD3, we therefore designed and

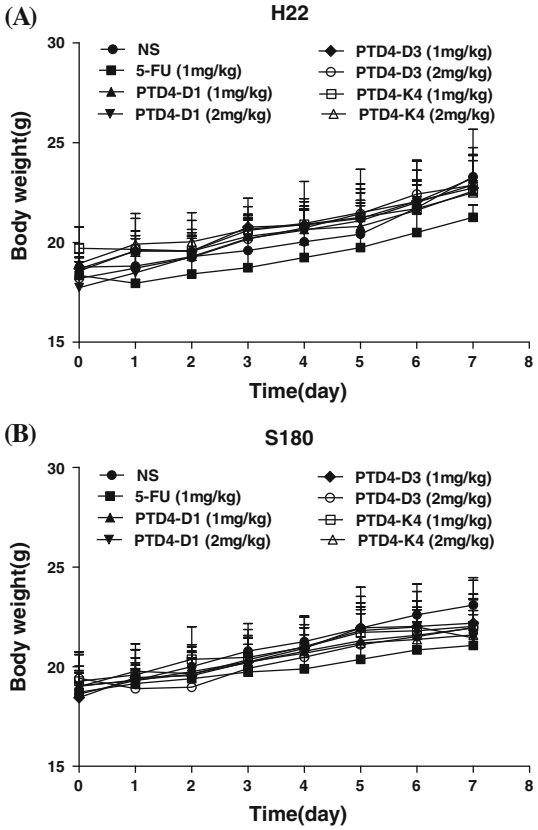

Fig. 7 Body weight changes of the nude mice. The body weight changes curve of each group of BALB/c nude mice during the subcutaneous administration of peptides for 7 days ($n = 6$)

characterized a series of peptides derived from the α5 helix of cyclin D1 and cyclinD3, and αC helix of CDK4.

Because that the cyclinD/CDK4 complexes are existed in cells, it is necessary to conjugate a carrier to carry the peptides into cells. Our results also indicated that the cell-penetrating peptide analog PTD4 could carry the conjugated peptides into cells. It contains less positive charge arginine, and could be a good membrane-permeable carrier similar to HIV-Tat$^{49-57}$.

In this study, when conjugated to a membrane-permeable peptide PTD4, the novel chimeric peptides, PTD4-D1, PTD4-D3, and PTD4-K4 exhibited significant antitumor effects. They could compete with the cyclin/CDK4 complex, and then induce the cell cycle arrest and apoptosis of the cancer cells. In the in vivo experiment, they showed potent antitumor effects and fewer side effects. All of these results provided further evidence to support the binding model of cyclinD/CDK4 and suggested that the α5 helix of cyclinD might be the most important binding region in contact with CDK4. Our results provided valuable information for the design of CDKs peptide inhibitors. In contrast to most kinase inhibitors described in the last few years, the prototype peptides of this study might compete with the cyclinD/CDK4 binding interface.

In conclusion, we have described a novel class of peptide inhibitors of cell cycle progression that target the cyclinD/CDK4 complex. These peptides could serve as novel and important leading compounds with antitumor activities.

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Conflict of interest The authors declare that there are no conflicts of interest.

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