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The cytotoxicity of a Grb2-SH3 inhibitor in Bcr-Abl positive K562 cells

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ARTICLE INFO

Article history:
Received 14 May 2007
Accepted 7 December 2007

Keywords:
Bcr-Abl
Chronic myelogenous leukemia
Grb2
SH3 domain
Cell cycle
Apoptosis

ABSTRACT

Chronic myelogenous leukemia (CML) is characterized by the presence of Bcr-Abl oncoprotein. Gleevec has been designed to treat many CML patients by specifically targeting Bcr-Abl, but resistance to it is already apparent in many cases. In CML cells, Bcr-Abl activates several signaling pathways, including the Ras-dependent pathway, in which growth factor receptor binding 2 (Grb2) acts as an adaptor protein. A specific Grb2-SH3 inhibitor (denoted as peptidimer-c) that disrupts Grb2–Sos complex was designed and synthesized in our laboratory.

In this study, we investigated the effect and the molecular mechanism of this inhibitor. Peptidimer-c was shown to bind to Grb2 in K562 cells, a cell line over-expressing Bcr-Abl oncoprotein. It caused cytotoxicity in the cells, and inhibited their ability of colony formation in the semi-solid medium. It was shown to induce apoptosis of K562 cells in a dose-dependent mode, the apoptotic effect of peptidimer-c being associated with caspase-3 activation. The effect of peptidimer-c on growth inhibition was also shown to be accompanied by S-phase arrest of cell cycle mediated by down-regulation of cyclin A and Cdk2, as well as phospho-Cdk2. The above results indicated that peptidimer-c may be another potential therapeutic agent for CML, which can induce S-phase arrest in the Bcr-Abl positive K562.

1. Introduction

Chronic myelogenous leukemia (CML) is a malignancy of pluripotent stem cells, and is characterized by the genomic reciprocal translocation t(9; 22)(q34; q11), which results in the formation of the Philadelphia (Ph) chromosome where the *bcr* gene on the chromosome 22 is fused to the *abl* gene on the chromosome 9. The chimeric gene encodes a 210-kDa protein, named Bcr-Abl, which is a constitutively activated tyrosine kinase [1,2]. The pathology of CML depends on the presence of Bcr-Abl, which induces cell transformation, triggering several signaling pathways. Among these Bcr-Abl-dependent signals, the MAPK cascade activated by Ras is essential. This transduction is initiated by the binding of growth factor receptor binding 2 (Grb2) adaptor on Bcr-Abl, involving the recruitment of Sos, the nucleotidic exchange factor of Ras.

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0006-2952/$ – see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.bcp.2007.12.021

The advent of tyrosine kinase inhibitors (TKIs) has ushered in a new area in the management of chronic myelogenous leukemia. Imatinib (Gleevec®) [3], the first TKI to be approved for the treatment of CML and the current standard first-line therapy, has significantly improved the prognosis of patients with this pathology. Nevertheless, still a minority of patients with chronic-phase CML and a large portion of patients in advanced-phase disease demonstrate resistance to imatinib or develop resistance during treatment [4]. In 40–50% of cases, the resistance is attributed to the development of mutations that impair the ability of imatinib to bind to and inhibit the constitutively active Bcr-Abl kinase [5]. Consequently, attempts to search for other kinds of drugs are currently ongoing.

One area of research of our laboratory focuses on the inhibition of protein–protein interactions, and particularly those involving the Grb2 protein. Grb2 is constituted by one Src homology 2 (SH2) domain surrounded by two SH3 domains [6,7]. Grb2 binds to the tyrosine-phosphorylated motif of Bcr-Abl by its SH2 domain, and interacts with proline-rich motives of Sos through its SH3 domains. Direct binding of Grb2 is required for the efficient induction of CML-like myeloproliferative disease by oncogenic Abl protein [8] and in other cancers [9]. Interestingly, Grb2 mutant proteins lacking N- or C-terminal SH3 domain could suppress Bcr-Abl induced Ras activation and revert the oncogenic phenotype [10]. Therefore, inhibition of Grb2 may contribute to target the Bcr-Abl-expressing cancer cells.

Grb2 is an adaptor protein (not an enzyme) and its functions are exclusively due to the presence of its binding SH2 and SH3 domains. On this basis, and since SH2 or SH3 domains might constitute targets for anti-proliferative agents [11], we have designed a peptide dimer (peptidimer) able to simultaneously bind to the two SH3 domains of Grb2 with high affinity (K_d = 40 nM), and it specifically recognizes Grb2 and does not interact with PI3K or Nck, two SH3 domain-containing adaptors [12]. This peptidimer was conjugated with penetratin, a cell-permeable peptide sequence and the resulting molecule, $(VPPPVPPRRR)_2$-K-Aha-RQIKIWFQNRRMKWKK, denoted as peptidimer-c in this paper, is able to inhibit cancer cell growth in vitro [12] but also exhibits an anti-tumor effect on mice xenografted with HER2-expressing human tumor [13].

In this study, we have investigated the mechanisms underlying the inhibitory effect of the peptidimer-c on K562 Bcr-Abl-positive cell growth. We have tested the effects of peptidimer-c on K562 cell proliferation and apoptosis and analyzed how this inhibitor produced its effect on cell proliferation and survival. We demonstrated that peptidimer-c, which binds to Grb2 protein, inhibits proliferation of K562 by arresting the cells in S phase and inducing cell apoptosis.

2. Materials and methods

2.1. Reagents and antibodies

Grb2-SH3 inhibitor conjugated to penetratin (peptidimer-c) and penetratin were synthesized by solid-phase peptide synthesis using Fmoc chemistry as described by Cussac et al. [12]. Gleevec® was product from Novartis, Switzerland.

Phospho-ERK1/2 (p44/42 MAP kinase) (Thr202/Tyr204) antibody, phospho-AKT (Ser473) antibody and AKT antibody were purchased from Cell Signaling Technology Inc. (Beverly, MA). cyclin A(C-19), cyclin B1(H20), cyclin D1(M20), cyclin E(C-19), Cdk2(M2), phospho-Cdk2(Thr160), Cdk1(C-19), phospho-Cdk1(Thr14/Thr15), actin and Grb2 antibodies were obtained from Santa Cruz Biotechnology (CA).

2.2. Cell culture and lysis

K562 [14] a human cell line derived from a patient with CML blastic crisis, was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI 1640 (Gibco Co., USA) containing 10% fetal bovine serum (Gibco Co.), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco Co.) in 5% CO_2 atmosphere at 37 °C.

For lysis, K562 cells were collected and washed with cold PBS buffer. K562 cell lysate was prepared by homogenization in modified RIPA buffer (150 mM sodium chloride, 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate, 50 mM NaF, 1% of protease cocktail from Roche) and incubated at 4 °C for 30 min. Cell lysate was centrifuged at 13,200 rpm at 4 °C for 10 min, and the supernatant was stored at −20 °C. Protein concentration was determined with Bio-Rad protein assay.

Before electrophoresis, K562 cell lysate was boiled for 5 min in 1× SDS sample buffer (50 mM Tris–HCl pH 6.8, 12.5% glycerol, 1% sodium dodecylsulfate, 0.01% bromophenol blue) containing 5% β-mercaptoethanol.

2.3. Pull-down experiment

Peptidimer-c and penetratin were coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) as already described by Cussac et al. [12]. Thirty microliters of peptide-coupled beads were then incubated with 50 μg of K562 cell extracts. Affinity-precipitated proteins were eluted by boiling sodium dodecyl sulfate (SDS) sample buffer for 5 min, and western blot assay was performed with antibody directed against Grb2.

2.4. Trypan blue exclusion assay

K562 cells were treated with drugs at different doses for various times. After the cells had been harvested, routine trypan blue staining was performed and viable cells were counted under microscope. For each concentration, the cell count was triplicated and the average value was obtained. Results are presented with S.D. values.

2.5. WST-1 test

The cytotoxicity of peptidimer-c on K562 cells was determined using WST-1 cell proliferation assay [15]. Cells were inoculated in RPMI 1640 with 10% FBS and antibiotics (100 U/mL penicillin and l00 μg/mL streptomycin), plated into 96-well flat-bottom microplates (Costar) with $0.4 \times l0^4$ cells per well for 24 h, and treated with peptidimer-c or penetratin at required concentration. After 72-h incubation, 10 μL of WST-1 (4-[3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene

disulfonate) was added to each well, and plates were further incubated at 37 °C for 2 h. After being shaken thoroughly for 1 min on an adapted shaker, plates were then read on a microplate reader (Bio-Rad, model 550) at 450 nm with a reference wavelength at 630 nm.

2.6. Clonogenic assay

Clonogenic assay for K562 cells were performed as described [16]. Briefly, in 96-well plates, 800 cells per well were treated with drugs and plated in each well in triplicate in a culture medium consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.8% methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada). The colonies (>50 cells) were counted after 7 days incubation at 37 °C in 5% CO_2. Cells were treated by peptidimer-c during the 7 days.

2.7. Cell cycle analysis by FACS

The cell cycle distribution was analyzed using a CycleTESTy PLUS DNA reagent kit (Becton Dickinson Immunocytometry Systems) according to the manufacturer's instructions. Cells were collected to a Falcon tube after being treated with drugs at different doses for 6 h, and were adjusted to an optimal concentration of $1.0 \times l0^6$ cells/mL in buffer solution. The cells were treated in 250 μL solution A (trypsin buffer) for 10 min, 200 μL solution B (trypsin inhibitor and RNase buffer) for 10 min, and 200 μL cold solution C (propidium iodide stain solution) for 10 min. The samples were examined on the flow cytometer (FACScalibur, BD Company, USA), and analyzed with CELL Quest software and ModiFit software.

2.8. Western blot analysis

After drug treatment, the nuclear proteins of the cells was extracted with Norvagen NucBuster protein extraction kit. Briefly, cell pellets were suspended in 150 μL of NucBuster extraction reagent I for 5 min on ice to release nuclei. The nuclei were harvested by centrifugation (16,000 × *g* for 5 min at 4 °C) and washed with ice-cold PBS to remove cytoplasmic proteins. The nuclei were resuspended in 50 μL of NucBuster extraction reagent II for 5 min on ice, and nuclear extracts were separated by centrifugation (16,000 × *g* for 5 min at 4 °C). The protein concentration in nuclear extracts was determined by Bradford assay with bovine serum albumin (BSA) (Sigma) as the standard. The nuclear proteins (25 μg) from each sample were separated on 7.5% or 10% polyacrylamide gels by SDS-PAGE, and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech., UK) using standard procedures. Membranes were blocked for 1 h at room temperature with 5% BSA in Tris-buffered saline (50 mM Tris–HCl (pH 7.6) and 150 mM NaCl) containing 0.1% Tween-20 (TBS-T) and then incubated overnight at 4 °C with the appropriate antibody diluted 1:1000 or 1:500 in 5% BSA in TBS-T. Membranes were washed several times in TBS-T and incubated at room temperature for another 1 h with 1:10,000 diluted anti-rabbit (or anti-mouse) IgG coupled to horseradish peroxidase. Proteins were detected by using the enhanced chemiluminescence reagent (ECL Western Blotting Detection System, Amersham Pharmacia Biotech., UK). Membranes were then stripped in Tris–HCl buffer with 100 mM β-mercaptoethanol and 2% SDS for 30 min at 50 °C. The membranes were washed three or four times with water and another two times with TBS and incubated in a new blocking buffer before incubation with anti-actin antibody as a protein loading control.

2.9. Terminal deoxynucleotidyltransferase-mediated biotin-dUTP nick end labeling (TUNEL) experiment

TUNEL assays were performed with an In Situ Cell Death Detection Kit (Roche Molecular Biochemicals). Briefly, after treatment with drugs for 6 h, cells were fixed with a freshly prepared 4% Paraformaldehyde in PBS for 1 h at 15–25 °C, rinsed with PBS and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice (2–8 °C). After washed with PBS, cells were resuspended in TUNEL reaction mixture containing terminal deoxynucleotidyltransferase enzyme and digoxigenin-nucleotide for 1 h at 37 °C. An alkaline phosphatase staining system was used to detect the incorporation of nucleotides into 3′-DNA. The apoptotic cells were observed under microscope.

2.10. Apoptosis assay by FACS

Analysis of phosphatidyl serine (PS) exposure was performed as described by the introduction of Annexin V apoptosis detection kit (BD Biosciences Pharmingen). Briefly, K562 cells treated with drugs at different concentrations were harvested, stained with Annexin V and propidium iodide, and analyzed with a FACS calibur cytometer. Simultaneously, K562 cells were treated with permeabilizing solution, incubated with caspase-3 antibody. Fas expression was detected by a direct staining with anti-Fas antibody.

To confirm whether caspase-3 was activated after treatment of cells with peptidimer-c, a blocking test was carried out in which a 10 μM concentration of Z-VAD-fmk (a specific inhibitor of caspase-3, product from R&D Co.) was applied to K562 cells for 2 h, and then various concentrations of peptidimer-c were added to the cells and incubated for another 6 h. Flow cytometric assays were performed as described above.

2.11. Statistical analysis

Data are expressed as means ±S.D. The significance of differences between control and treated groups was evaluated using Student's *t*-test. Differences were considered as significant if $p < 0.05$.

3. Results

3.1. Grb2 is correctly expressed in K562 cells and can be pulled-down by peptidimer-c beads

In order to explore if Grb2 was correctly expressed in K562 cells and to control the ability of peptidimer-c to bind Grb2, CNBr-activated Sepharose beads linked with either peptidimer-c or penetratin were used to precipitate Grb2 from K562 cell lysate. Linked proteins were analyzed by western blot and the result

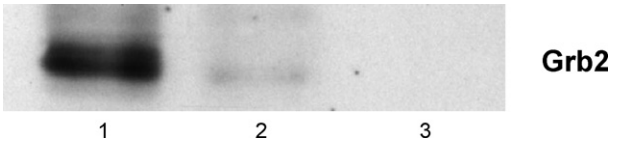

Fig. 1 – Peptidimer-c, and not penetratin, specifically binds to Grb2 protein from the K562 cells. CNBr Sepharose beads coupled with either peptidimer-c (lane 1) or penetratin (lane 2) or beads alone (lane 3) were incubated with K562 cell extract. Grb2 was revealed by specific antibody western blot. Only beads coupled with peptidimer-c were able to pull-down Grb2.

is shown in Fig. 1. Grb2 was correctly expressed by K562 cells and specifically bound peptidimer-c beads (lane 1) but did not bind beads coupled with penetratin alone (lane 2) or control beads without any coupled peptide (lane 3).

3.2. *Peptidimer-c effects on K562 cells proliferation*

Grb2 is a key protein in cellular signaling and is essential in the Ras–Raf–MAPK pathway that induces cell proliferation. Consequently, blocking the interaction of Grb2 with either Sos or tyrosine kinase receptor inhibits Ras pathway and cell proliferation. K562 cells, which express Bcr-Abl oncoprotein were treated with either peptidimer-c (Fig. 2A) at 0, 4.5, 9, 18, 27, and 36 μM or penetratin as control (Fig. 2B) for 3, 6, 24, 48, and 72 h.

Cell growth was quantitated by trypan blue exclusion as described in Section 2. As compared to the control, peptidimer-c inhibited the proliferation of K562 cells in a dose-dependent manner (Fig. 2A), and the penetratin vector did not influence cell growth at the same concentrations (Fig. 2B). Gleevec, a specific bcr-abl targeted inhibitor, obviously inhibited K562 cell growth after 24 h (Fig. 2C).

To verify the cytotoxicity of peptidimer-c on K562 cell, cells were treated with increasing peptidimer-c or penetratin concentrations for 72 h and cell survival was assessed by WST-1 assay [15]. Its effect was compared to imatinib (Gleevec®), an active molecule which targets the kinase domain of Bcr-Abl and which is largely used in therapeutics. K562 cells were treated at the same doses compared to previous experiment with peptidimer-c or imatinib at 0, 0.045, 0.09, 0.18, 0.27, and 0.36 μM. Peptidimer-c exhibited IC_{50} value of 18 μM, and the IC_{50} of Gleevec was 0.3 μM (data not shown). This result shows an effect of peptidimer-c on Bcr-Abl-expressing cells proliferation is less important than that of imatinib.

Subsequently, in order to evaluate the anti-tumor effect of peptidimer-c on K562 cells, we performed a clonogenic assay in RPMI 1640/methylcellulose medium (Fig. 3A). While peptidimer-c decreased the colony formation of K562 cells

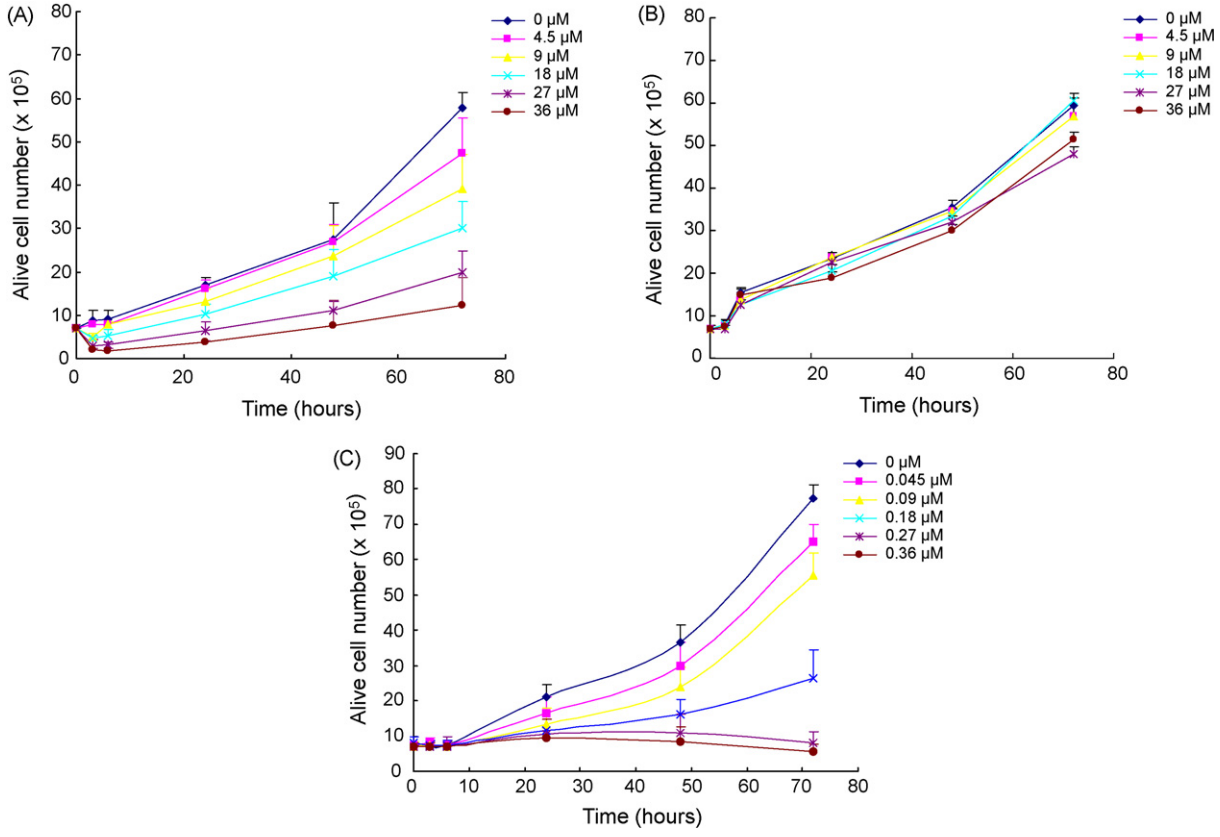

Fig. 2 – Effects of peptidimer-c on the cell growth by trypan blue exclusion test. (A) K562 cells were treated with peptidimer-c of various concentrations for 3, 6, 24, 48, and 72 h ($n = 3$). (B) K562 cells were treated with penetratin of various concentrations for 3, 6, 24, 48, and 72 h ($n = 3$). (C) K562 cells were treated with Gleevec of various concentrations for 3, 6, 24, 48, and 72 h ($n = 3$).

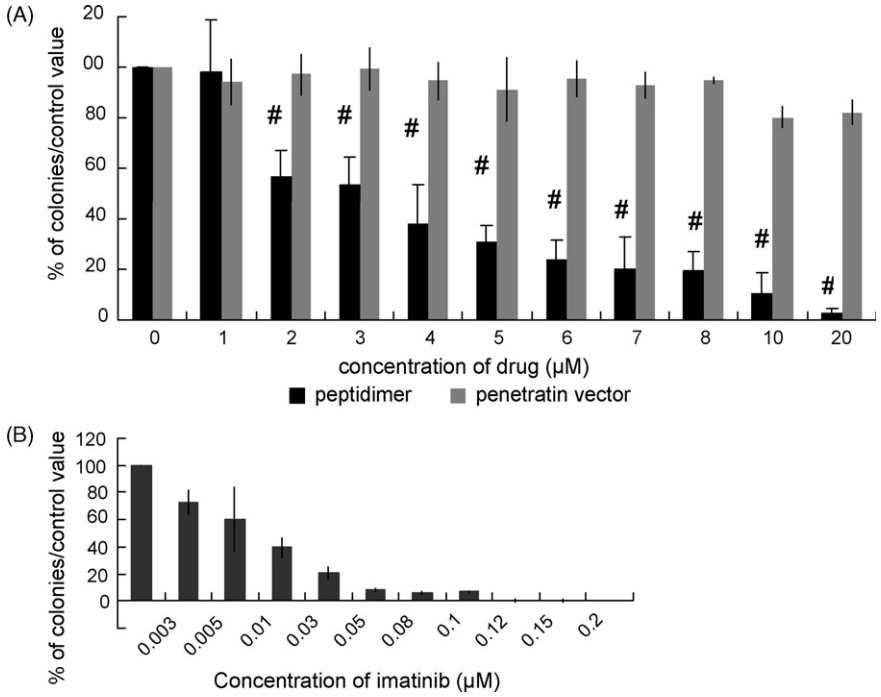

Fig. 3 – Effects of peptidimer-c and Gleevec on the colony formation of K562 cells. Eight hundred cells per well in 96-well plate were treated with drugs in a culture medium consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum, and 0.8% methylcellulose. The colonies (>50 cells) were scored after 7 days of incubation at 37 °C in 5% CO_2. (A) The relative colony formation of K562 cells treated with peptidimer-c, compared to the penetratin ($n = 4$). (B) The relative colony formation of K562 cells treated with Gleevec ($n = 3$).

with an IC_{50} around 3–4 μM, penetratin vector did not exhibit any activity at these doses. On the same assay, imatinib exhibited an IC_{50} value around 0.005–0.01 μM (Fig. 3B). Even if its active dose is not of the same order of magnitude than that observed with imatinib, these results demonstrate an inhibitory effect of peptidimer-c on proliferation of Bcr-Abl overexpressed K562 cells. The active dose range of peptidimer-c is in the same order of magnitude as those published by Feller et al. with a peptide inhibiting Grb2–Sos interaction [17].

3.3. *Peptidimer-c-induced apoptosis in K562 cells*

To confirm that peptidimer-c was able to inhibit cell proliferation and to reduce cell viability, we further investigated whether peptidimer-c was able to induce K562 cells apoptosis. According to the results of the anti-proliferation test, where peptidimer-c showed already significant inhibitory effect after 6 h, and since apoptosis phenomenon is an important cell death event, its induction was quantitized after 6-h treatment. Cells were treated with various doses of drugs for 6 h, and stained with DNA reagent (BD Company). The percentage of cells in sub-G1 was counted by flow cytometry (FCM). Results, in which percentage of hypodiploid cells were quantitated in a dose-dependent manner, are shown on Fig. 4. Peptidimer-c significantly increased hypodiploid percentage of K562 cells (30%), while the penetratin vector alone had no effect on the cells. This is a dose-dependent effect and the difference between penetratin control and peptidimer-c is clearly significant ($p > 0.05$).

During apoptotic phenomenon, one of the most important characteristics is DNA fragmentation and degradation, which occurs in early stages and is selective for the inter-nucleosomal DNA linker regions. This DNA cleavage leads to strand breaks. Thus we used TUNEL assay to detect both types of breaks in the K562 cells treated with peptidimer-c. The results showed that peptidimer-c induced 29.9% apoptosis of K562 cells when treated at 18 μM and that there was a significant difference between the peptidimer-c treatment and the penetratin one ($p < 0.01$) at high concentrations (Table 1).

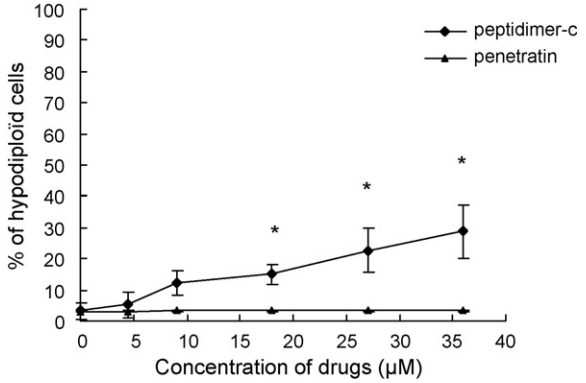

Fig. 4 – Peptidimer-c increased the percentage of hypodiploid K562 cells and penetratin had no effect ($p < 0.05$ at 18, 27, and 36 μM). Peptidimer-c-induced DNA degradation in K562 cells in a dose-dependent manner.

Table 1 – Apoptosis of K562 cells induced with peptidimer-c (measured by TUNEL assay)

Concentration of drugs (μM)	Percentage of apoptotic cells (%)	
	Peptidimer-c treatment	Penetratin treatment
0	0.67 ± 0.35	1.43 ± 1.02
4.5	3.43 ± 0.98	2.30 ± 0.89
9	20.20 ± 1.31*	3.63 ± 1.46
18	29.87 ± 1.69*	3.87 ± 2.38
27	34.53 ± 1.29*	4.30 ± 1.48
36	41.73 ± 3.10*	5.33 ± 2.41

* $p < 0.01$ compared to the penetratin treatment group.

In the FACS two-dimensional scatter diagram of Annexin V/PI test, Annexin V(+)/PI(−)cells is characteristic from apoptotic cells and Annexin V(+)/PI(+) from necrotic cells. Fig. 5 shows the result of non-treated K562 cells (5A), or cells treated by 9 μM (5B), 18 μM (5C) or 27 μM (5D) of peptidimer-c for 6 h. The percentage of both necrotic and apoptotic K562 cells clearly increased when peptidimer-c dose increased. Necrosis clearly increased for higher peptidimer-c doses (18 and 27 μM with respectively 9.66 and 36.67%).

As a control, K562 cells were treated with the same doses of penetratin vector. No significant difference was observed between control cells without any treatment (5A′) and cells

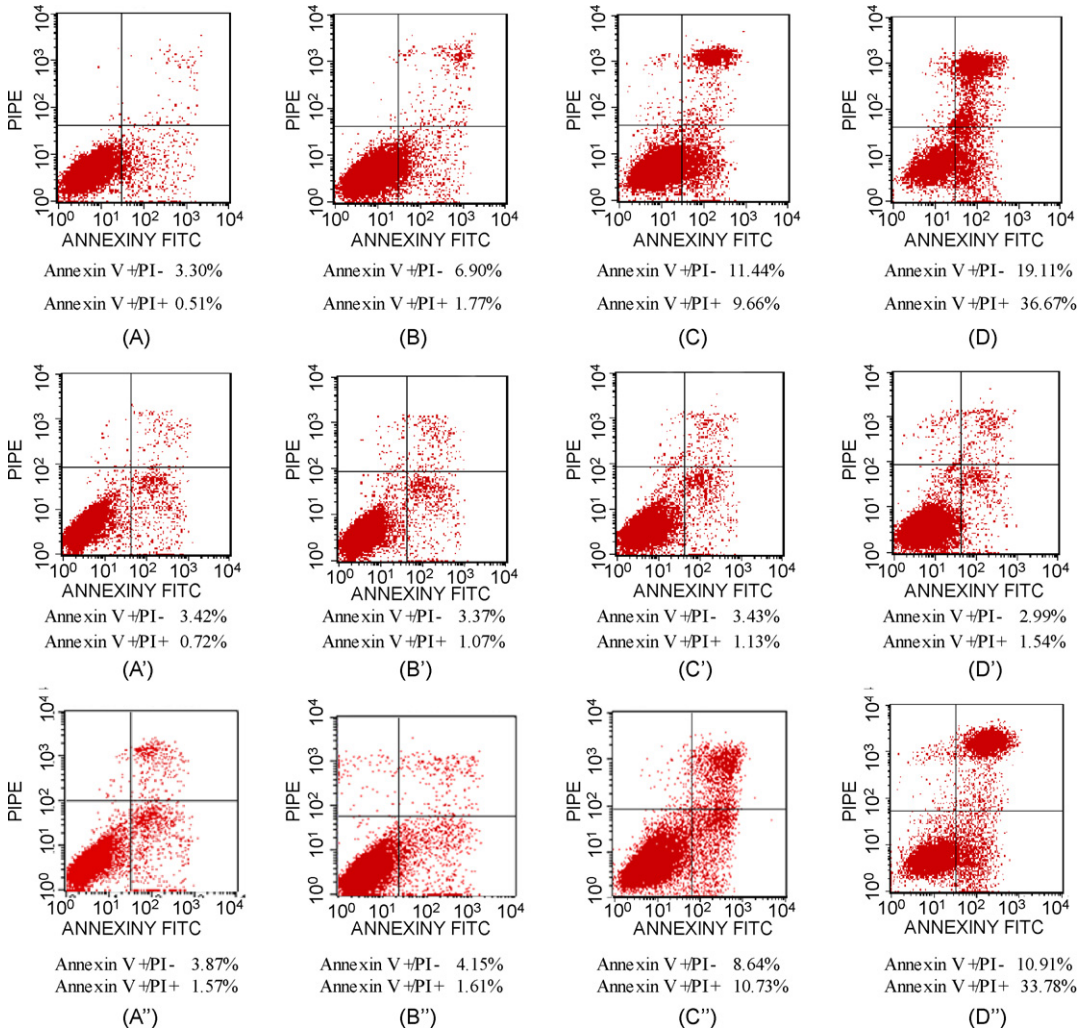

Fig. 5 – The effect of peptidimer-c on the expression of Annexin V/PI of K562 cells. K562 cells were treated with peptidimer-c in 0 μM (A), 9 μM (B), 18 μM (C), and 27 μM (D), for 6 h, or treated with penetratin in 0 μM (A′), 9 μM (B′), 18 μM (C′), and 27 μM (D′) for 6 h. K562 cells were treated with 20 μM of Z-VAD-fmk for 2 h and then with peptidimer-c in 0 μM (A″), 9 μM (B″), 18 μM (C″), and 27 μM (D″) for another 6 h. The results showed the percentages of apoptosis (Annexin V+/PI−) and necrosis (Annexin V+/PI+).

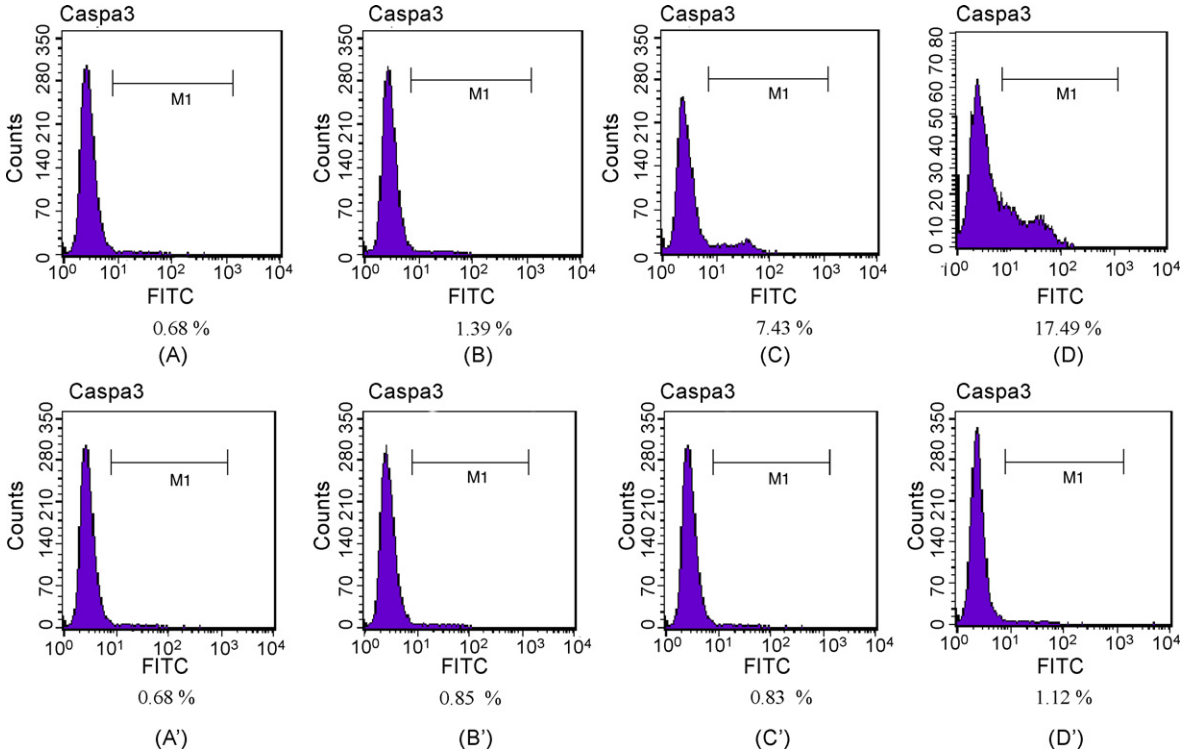

Fig. 6 – The effect of peptidimer-c on the expression of caspase-3. K562 cells were treated with peptidimer-c in 0 μM (A), 9 μM (B), 18 μM (C), and 27 μM (D) for 6 h, or treated with penetratin in 0 μM (A′), 9 μM (B′), 18 μM (C′), and 27 μM (D′) for 6 h. The results showed the percentages of caspase-3.

treated by 9 μM (5B′), 18 μM (5C′) or 27 μM (5D′) of penetratin for 6 h and the percentage of apoptotic cells was in the 3–3.5% range while necrotic cells represented 1–1.5%.

In order to reveal which death pathway was induced in the peptidimer-c apoptosis process observed in K562 cells, we assessed caspase-3 (Fig. 6) and Fas expression (Fig. 7) by FACS. K562 cells were treated with 9 μM (Fig. 6–7B), 18 μM (Figs. 6–7C) or 27 μM (Fig. 6–7D) of peptidimer-c (Figs. 6 and 7) or 9 μM (Fig. 6B′), 18 μM (Fig. 6C′) or 27 μM (Fig. 6D′) of penetratin (Fig. 6) and compared with untreated cells (Fig. 6–7A and Fig. 6A′). The results indicated that caspase-3 (Fig. 6A–D) expression was clearly up-regulated (0.68, 1.39, 7.43, and 17.49%) when cells were respectively treated by peptidimer-c, while treatment with penetratin vector as a control had no effect (Fig. 6A′–D′). In contrast, Fas expression (Fig. 7) was not modified when cells were treated by peptidimer-c.

Furthermore, to evaluate whether caspase-3 activation is involved in the apoptosis induced by peptidimer-c in K562 cells, K562 cells were treated with 10 μM caspase inhibitor (Z-VAD-fmk) for 2 h followed by 0, 9, 18, and 27 μM of peptidimer-c for another 6 h, and assessed caspase-3 expression by FACS. The results showed that the percentage of caspase-3 was significantly decreased, compared to those treated only with peptidimer-c (Fig. 5A″–D″). These findings suggested that peptidimer-c might induce the apoptosis of K562 by activating the caspase-3 signaling.

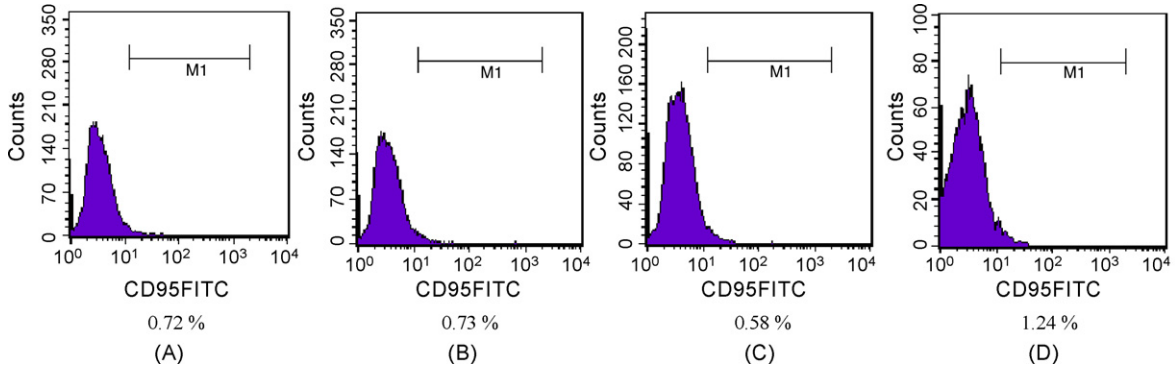

Fig. 7 – The effect of peptidimer-c on the expression of Fas. K562 cells were treated with peptidimer-c at 0 μM (A) 9 μM (B) 18 μM (C), and 27 μM (D) for 6 h. The results showed the percentages of Fas expression.

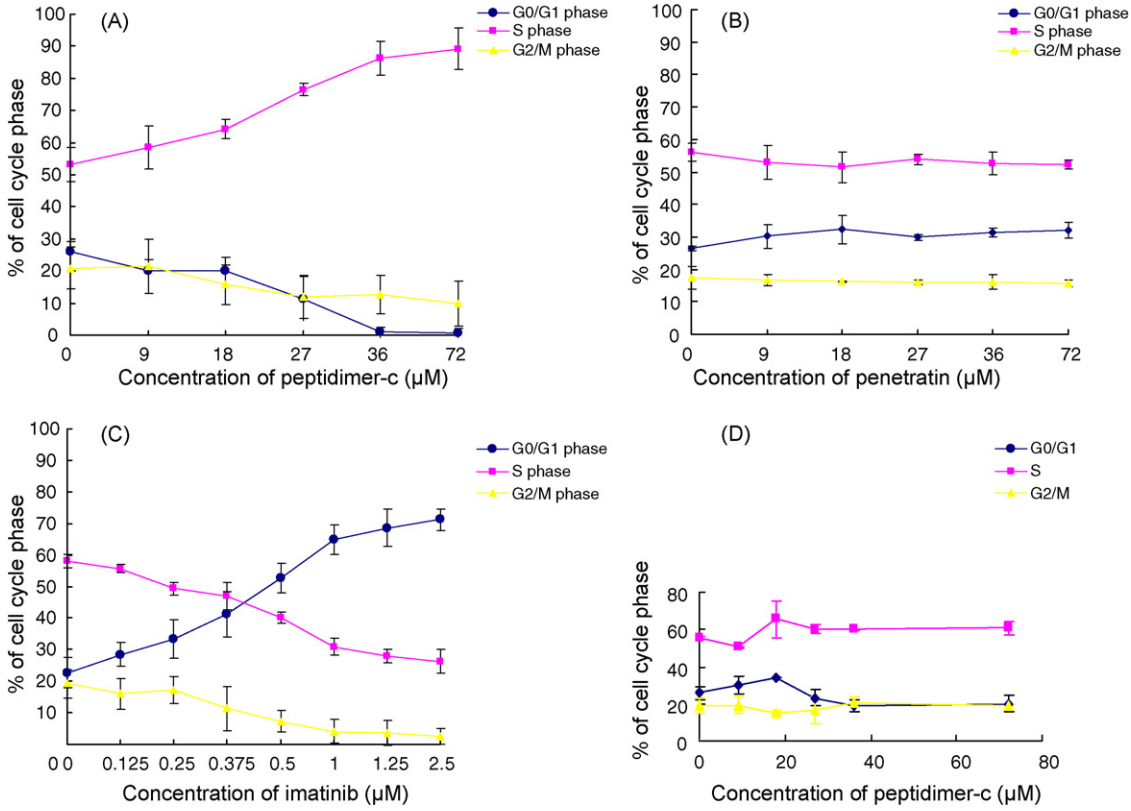

Fig. 8 – Cell cycle analysis on K562 cells. (A) Peptidimer-c-induced K562 cells being arrested at S phase. K562 cells were treated with peptidimer-c in an increasing dose for 6 h. (B) Penetratin had no effect on K562 cell cycle. K562 cell treated with penetratin for 6 h. (C) Gleevec caused G1 phase arrest of K562 cells. Cells were treated with varying doses of Gleevec for 24 h. (D) Cell cycle distribution of K562 cells treated with peptidimer-c in various concentration for 24 h. All the statistical values were based on three respective experiments.

3.4. *Peptidimer-c inhibition of K562 cells proliferation is mediated in part by S-phase arrest*

To elucidate the mechanism by which peptidimer-c inhibits K562 cell proliferation and determine if cell growth inhibition involved cell cycle changes, flow cytometry analysis was carried out to determine the modifications of cell cycle of K562 cells after treatment with various doses of peptidimer-c (Fig. 8A) or penetratin vector (Fig. 8B) for 6 h.

When cells were treated with peptidimer-c (Fig. 8A), while the percentage of cells in S phase (red curve) was $53.09 \pm 5.36\%$ before treatment, it clearly increased to $89.21 \pm 6.54\%$ after 6-h treatment with 72 μM peptidimer-c. Concomitantly, the percentage of cells in G0/G1 phase (blue curve) decreased from $25.99 \pm 3.16\%$ in the case of untreated cells to $0.79 \pm 1.37\%$ for cells treated with 72 μM peptidimer-c. Thus, peptidimer-c treatment for 6 h led to a significant increase of S-phase cells clearly correlated with a decrease of G0/G1 phase cells in a concentration-dependent manner. At the same time, the cell proportion in G2/M phase slightly decreased, while the penetratin vector treatment (Fig. 8B) did not induce any change in G0/G1, S, and G2/M phases of cell cycle.

These results demonstrate that the changes in cell cycle progression are specifically due to peptidimer-c and that the inhibition of K562 cells proliferation proceeds via an S-phase arrest.

In order to compare these results with the effect of Gleevec® on cell cycle, FCM analysis was performed to test the cell cycle progression of K562 cells treated with various doses of imatinib. After 6-h treatment by imatinib at 0, 0.125, 0.25, 0.375, 0.5, 1, 1.25, and 2.5 μM, no effect on G0/G1, S, and G2/M phases was observed (data not shown). However, after 24-h treatment, imatinib obviously induced a G0/G1 arrest (blue curve in Fig. 8C) in K562 cells. Concomitantly, a decrease of cells either in S (red curve) or G2/M (yellow curve) phases was observed, indicating that imatinib-induced K562 cell growth was mediated by G0/G1-phase arrest.

As described above, peptidimer-c showed inhibition of K562 cells in a mechanism different from that of Gleevec. To confirm this point, cell cycle distribution of K562 cells treated with peptidimer-c in various concentrations for 24 h was observed by flow cytometry, as well as the cell cycle distribution of K562 cells treated with 27 μM peptidimer-c or 0.375 μM Gleevec in various time. The results showed that peptidimer-c still arrested K562 cells in S phase, but some cells seemed to grow again(Fig. 8D). Peptidimer-c seemed to have the most strong inhibition on K562 cells at 6 h (Fig. 9A), while Gleevec at 24 h (Fig. 9B).

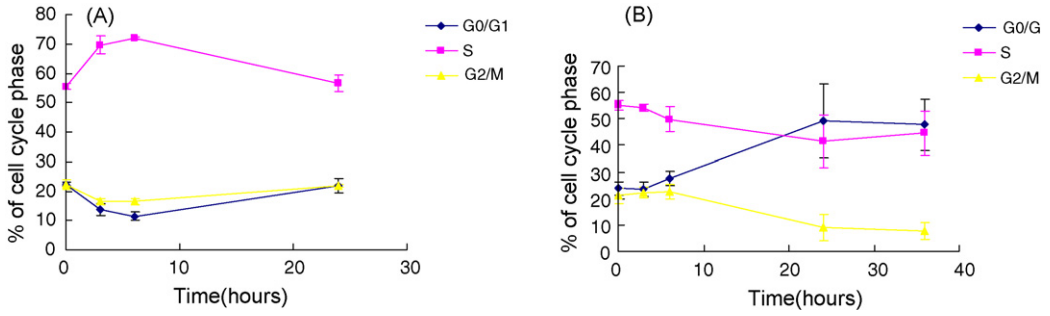

Fig. 9 – The cell cycle distribution of K562 cells in various time. (A) K562 cells treated with 27 μM peptidimer-c (n = 3). (B) K562 cells treated with 0.375 μM Gleevec (n = 3).

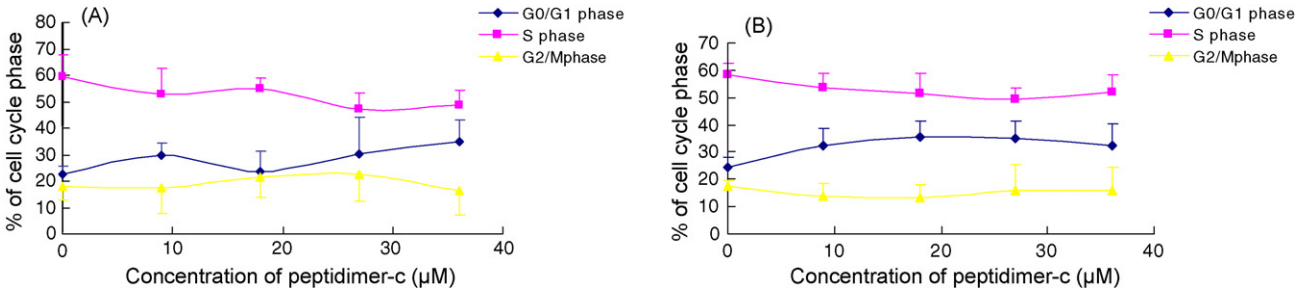

Fig. 10 – The effect of caspase inhibitor on the cell cycle of K562 cells. K562 cells were treated with 20 μM of Z-VAD-fmk for 2 h and then with peptidimer-c in an increasing dose for 6 h (A) and 24 h (B). All the statistical values were based on the three respective experiments.

In the last part, we showed that peptidimer-c activated caspase-3 and the apoptosis in K562 cells. In order to further clarify the effect of caspase inhibitor on the cells treated with peptidimer-c, FCM assay was performed to analyze the effect ofn K562 cell cycle of K562 successively treated with 20 μM of Z-VAD-fmk for 2 h and then with increasing doses of peptidimer-c for 6 h (Fig. 10A) and 24 h (Fig. 10B). These results indicate that caspase-3 inhibitor (Z-VAD-fmk) influenced the distribution of K562 cell cycle phases treated with peptidimer-c. These results also support that apoptosis is mediated by peptidimer-c associated with caspase-3 activation.

3.5. *Peptidimer-c down-regulated the expression of cyclin A*

Since cell cycle progression requires the co-ordinated interaction and activation of cyclins and cyclin-dependent kinases (Cdk), the expression levels of cyclin A, Cdk2, phospho-Cdk2, cyclin B, Cdk1, and phospho-Cdk1 was studied by western blot analysis after K562 cells treatment for 6 h with different doses of either peptidimer-c (Fig. 11A) or penetratin vector alone (Fig. 11B) as a control. Cyclin A expression was clearly decreased after peptidimer-c treatment (lane 1 in Fig. 11A). While total Cdk2 level (lane 3 in Fig. 11A) was constant during treatment with low concentrations of peptidimer-c, it slightly decreased for a peptidimer-c concentration of 27 μM. Phospho-Cdk2 clearly decreased after peptidimer-c treatment (lane 2 in Fig. 11A), most of all for 27 μM of peptidimer-c.

No effect of peptidimer-c treatment was detected neither in Cdk1 (lane 4 in Fig. 11A) nor in its phosphorylated form (lane 5 in Fig. 11A). No effect was observed in cyclin B and cyclin D levels in the same conditions. In all experiments, actin level was verified to be constant (lane 8 in Fig. 11A). When cells were treated by penetratin vector, no significant difference was observed in the expression of any of the studied proteins (Fig. 11B), proving the specificity of peptidimer-c.

Fig. 11C showed the expression levels of cell cycle associated molecules in K562 cells treated with varying concentrations of imatinib for 24 h. It was found by western blot assay that the level of cyclin D (lane 7 in Fig. 11C), cyclin B (lane 6 in Fig. 11C) got obviously decrease in a dose-dependent mode. There seemed not any changes for the cyclin A, Cdk1, and Cdk2. But the significant decrease of p-Cdk2 (lane 2 in Fig. 11C) and p-Cdk1 (lane 5 in Fig. 11C) was observed.

These results support different effect on K562 cell cycle of peptidimer-c and imatinib.

4. Discussion

Despite the efficacy of imatinib, some patients in chronic phase and more in advanced phases of CML develop resistance, frequently as a result of Bcr-Abl tyrosine kinase domain mutations that impair imatinib binding and retain enzymatic activity [4,5]. It is therefore important to propose alternative therapeutics. New tyrosine kinase inhibitors that inhibit Bcr-Abl more potently than imatinib have been

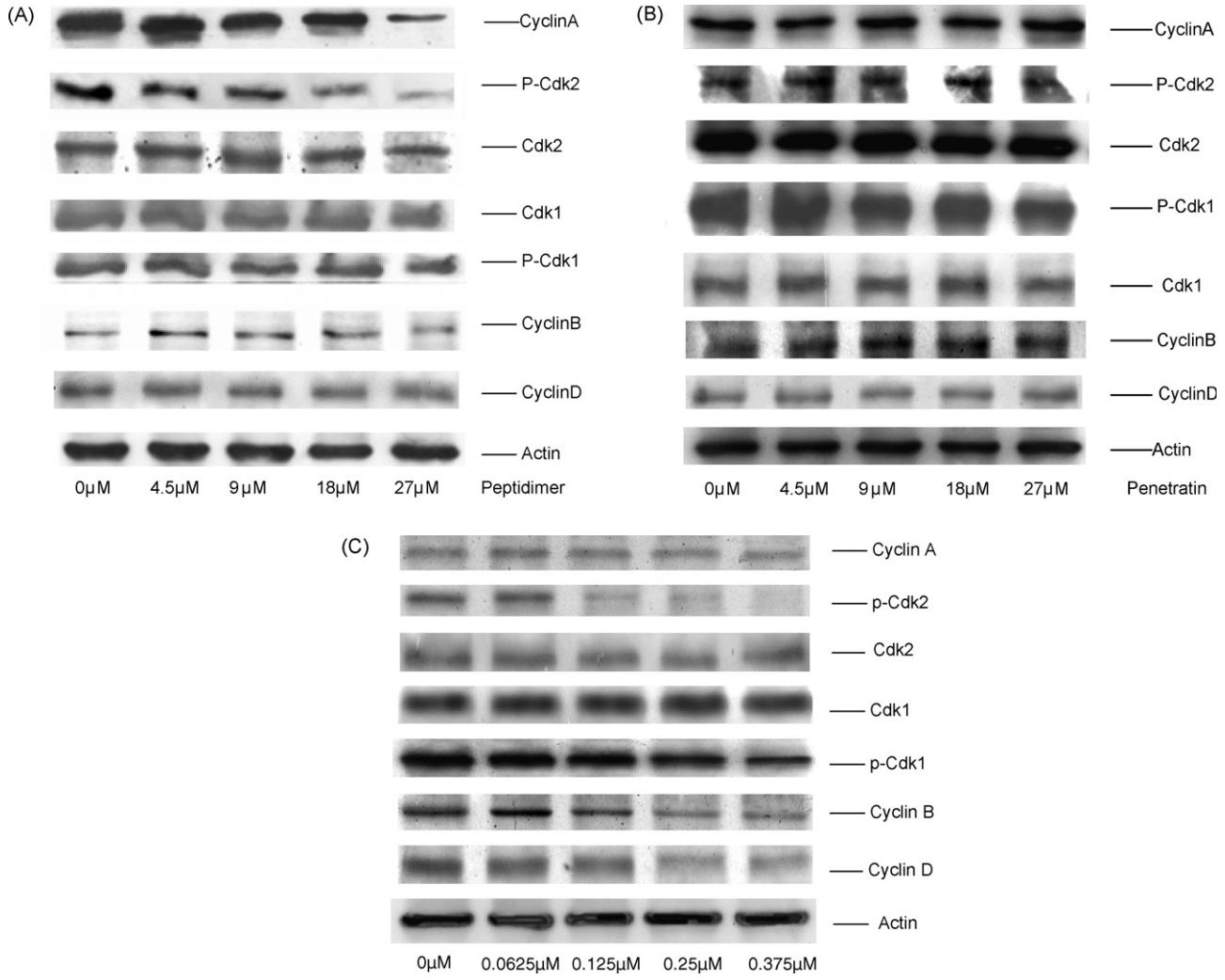

Fig. 11 – Expression of cell cycle related proteins of K562 cells. Cells were treated with various doses of peptidimer-c (A) or penetratin (B) for 6 h, and Gleevec (C) for 24 h. The nuclear extracts were prepared for western blot analysis. (A) Peptidimer-c obviously decreased the expression of cyclin A and phospho-Cdk2. It had a slight effect on the Cdk2 level, but no effect on the level of cyclin B, Cdk1, and phospho-Cdk1. (B) Penetratin had no effect on the level of all the proteins detected. (C) Gleevec significantly decreased the level of cyclin D, cyclin B, phospho-Cdk2, and phospho-Cdk1, but did not affect the level of cyclin A, Cdk2, and Cdk1. β-Actin was used as an internal control.

designed and maintain activity against an array of imatinib-resistant Bcr-Abl mutants [18]. Such kinase inhibitors are under investigation or already commercialized (dasatinib, Sprycel® Bristol-Myers Squibb Co.), and exhibit efficacy on the treatment of either CML or Ph+ ALL. Agents that target proteins downstream of Bcr-Abl (e.g. Ras/Raf and phosphatidylinositol 3-kinase) are also under investigation. Among these, Grb2 inhibitors appeared to constitute a potential new class of pharmacological agents. Indeed, since all imatinib resistances are clearly due to mutations in the tyrosine kinase active site of Bcr-Abl and since peptidimer-c acts downstream the protein, its effect on imatinib-resistant clones might be similar to that on imatinib-sensitive ones.

In this paper, we provide evidence for several aspects that demonstrate the anti-cancer activity of peptidimer-c, a Grb2-SH3 inhibitor, on Bcr-Abl positive K562 cells. Peptidimer-c, which acts as a protein–protein interaction inhibitor, is able to inhibit cell proliferation and to induce apoptosis in K562 cells in a dose-dependent manner. As described by Cussac et al. [19] and Gril et al. [20], purified Grb2 was tested by fluorescence for its ability to interact through its SH3 domains with the VPPPVPPRRR peptide or peptidimer. Moreover, Gril et al. [20] have shown that the VPPPVPPRRR sequence is specific for Grb2 when it is highly bound to Sepharose beads. So, in our pull-down assay, it was shown that the peptidimer-c (dimer of the VPPPVPPRRR peptide) could coherently bind to the Grb2 from K562 cells lysate.

As shown in the result section, the IC_{50} of peptidimer-c was approximate 18 μM in the WST-1 assay on K562 cells, and 3–4 μM on a colony formation assay, which both demonstrated the cytotoxic effect of peptidimer-c on K562 cells. Nevertheless, these effects are not as efficient as we expected considering the magnitude of the cytotoxic and anti-tumor effects that were obtained with peptidimer-c on HER2-

expressing cells and mice xenografted with HER2 positive human tumor [13]. The response of SKBr3 cells that over-express HER2, to this inhibitor was as low as in sub-micromolar range for IC_{50}. This difference can probably be explained by the fact that transduction pathways involved in HER2 or Bcr-Abl signaling are rather different. It is now believed that HER2 pathway is essentially triggered by MAPK activation, through Grb2/Ras pathway, and several reports suggest a major role of the MAP kinase cascade in HER2-induced cell transformation [21,22]. This was confirmed by the use of peptidimer-c in HER2 positive cells, which exhibited sub-micromolar IC_{50}. In the case of Bcr-Abl, MAPK activation is also observed. This activation also needs the recruitment of Grb2, but a recent paper clearly showed that Bcr-Abl-induced activation of Rap1 plays an important role in regulation of cell proliferation and survival [23]. Interestingly, Rap1 is a small G protein, whose activation in hematopoietic cells is not Grb2-dependent and which is able to activate MAPK through B-Raf signaling [24]. Therefore, if Grb2 is not the main signaling factor involved in ERK-activated cell division, it is logical that peptidimer-c exhibits lower activity on Bcr-Abl over-expressing cells as compared to those over-expressing HER2.

The effect of peptidimer-c was also tested on the cell cycle. To the best of our knowledge, only few papers have described the effect of Grb2 inhibitors on cell cycle. In 2005, Kim et al. described the effect of actinomycin, an inhibitor of Grb2 SH2 domain on cell cycle [25]. In this study, they have shown, by proteomic analysis, that this molecule is able to up-regulate MEKK3 and to down-regulate Hsp70 expression, which was correlated with G1 arrest of cell cycle. In our case, peptidimer-c, which is an inhibitor of Grb2-SH3 domains, induces S-phase arrest, concomitantly with down-regulation of cyclin A. In 2001, Shen and Guan [26] showed that targeting of Grb2 to focal contacts increased cell cycle progression, and biochemical analyses correlated ERK activation by means of Grb2, with its stimulation of cell cycle progression. This observation supported the important role of Grb2 in cell cycle progression.

The cell cycle is the process by which cells duplicate themselves, grow, and prepare to divide. Many studies demonstrated that ERK activation is associated with either stimulation or inhibition of cell proliferation [27]. Activation of ERK pathway induced by growth factors and cytokines resulted into over-expression of cyclin D and cyclin E which are G1 associated cyclins [28]. In many cases, blocking this signal arrested the cells in G1 phase, but some other data reported that ERK pathway activation also regulated the progression of G2/M phase [29]. In our experiments, Gleevec caused G1 arrest of K562 cells after treatment for 24 h, while peptidimer-c arrested cell cycle progression in S phase. This result clearly demonstrated that the two drugs affect the cell cycle of K562 cells by different mechanisms. Pytel et al. [30] also showed that the treatment with Gleevec reduced fraction of K562 cells in G2/M checkpoint and recovered regular cell cycle process. Furthermore, the inhibition of Bcr-Abl tyrosine kinase by Gleevec (imatinib) caused both cell cycle arrest in the G0/G1 phase and increased the portion of apoptotic cells, and the suppression of cyclin D2 may contribute to the G0/G1-phase arrest [31]. Cell cycle progression requires the co-ordinated interaction and activation of cyclins and cyclin-dependent kinases (CDKs) [32]. Cyclin A is required for both the initiation of cell DNA synthesis in the S phase and the entry in G2/M phase, while cyclin D is the key regulator for G0/G1 to S phase progression, and cyclin B is associated with G2/M phase. Castanedo et al. [33] analyzed a series of small peptides for blocking the recruitment site on cyclin A, and found that Cdk2/cyclin A inhibition affected E2F phosphorylation and blocked S-phase exit, thus sensitizing cancer cells to apoptosis. Here we found, by western blot assay, that peptidimer-c decreased the expression of cyclin A and phospho-Cdk2, and influenced as well the distribution of Cdk2 in the nucleus of K562 cells (data not shown). In addition to Cdk2, cyclin A also binds to Cdk1 (also called cdc2) and functions in mitosis before cyclin B/Cdk1, the classic M phase-promoting factor [34,35]. Peptidimer-c appears to have no effects on G2/M phase related proteins, such as cyclin B, Cdk1, and phosphorylated Cdk1. On the contrast, Gleevec may arrest the G0/G1 phase by down-regulating the expression of cyclin D, p-Cdk2, and cyclin B. It does not affect cyclin A and Cdk1.

These observations, correlated with the cytotoxic effect of peptidimer-c, suggest that Grb2 inhibitors might work as a new class of cytotoxic agents for the treatment of CML. In conclusion, peptidimer-c might act as an anti-proliferative agent on the K562 cells by causing S-phase arrest and inducing cell death, both by caspase-3-dependent apoptosis and by necrosis of K562 cells.

Acknowledgments

This work benefited from financial support from La Ligue Nationale contre le Cancer, Equipe Labellisée 2006, and from Ministère de la Technologie et de la Recherche, ACI 2002 Molécules et cibles thérapeutiques.

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