Bioorganic & Medicinal Chemistry Letters 18 (2008) 4633–4637

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

A novel peptide from α5 helix of *Asterina pectinifera* cyclin B conjugated to HIV-Tat$^{49-57}$ with cytotoxic and apoptotic effects against human cancer cells

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ARTICLE INFO

Article history:
Received 8 April 2008
Revised 30 June 2008
Accepted 7 July 2008
Available online 10 July 2008

Keywords:
Anticancer
Apoptosis
Cyclin B
Peptide
Asterina pectinifera

ABSTRACT

A series of novel peptides from various motifs of *Asterina pectinifera* cyclin B and their derivatives conjugated to HIV-Tat$^{49-57}$ were designed and synthesized. Their bioactivities on two human cancer cell lines were determined. Among them, **Tat-a5** (KAQIRAMECNILGRKKRRQRRR) exhibited significant cytotoxic effects on cancer cell lines EC-9706 and HCT-116. **Tat-a5** could arrest cancer cells at G_2/M phase and make them apoptotic. Our results suggested that **Tat-a5** could be a novel leading peptide with anticancer activity.

Cyclin-dependent protein kinases (CDKs) play important roles in the pathways which control the cycle of proliferation and differentiation of eukaryotic cells.[1–3] There are about twelve known members of the CDK family. Among these, CDK1, CDK2, CDK3, CDK4, and CDK6 are considered more important in controlling the cell cycle.[4–6] Recent studies showed that CDK3/cyclin C complex could help the cells to exit the G_0 state and enter the G_1 phase efficiently,[7] but it did not really participate in the cell cycle. Recent genetic and RNA interference studies in mammalian cells revealed that CDK2, CDK4, and CDK6 were not essential for cell cycle progression, thus leaving CDK1 as the only non-redundant cell cycle driver.[8–10] RNAi experiments also showed that cyclin B conferred M phase-like properties on CDK2 when CDK1 was knocked down, but mouse cells containing a temperature sensitive lesion in CDK1 were unable to enter mitosis and were arrested at G_2 phase.[11] These studies indicated that CDK2 could not completely compensate for CDK1.[12] Therefore, CDK1 is indispensable for the cell cycle progression. It has been established that CDK1/cyclin B complex regulated the transition between late G_2 and mitosis phases.[13] Recent investigations in human cells also showed that gradually increasing CDK1/cyclin B1 activity after centrosome separation was critical to coordinate mitotic progression.[14] In some cases, aberrant expression of CDK1 and cyclin B1 in primary tumors seemed to correlate with the survival rates of the patients.[15–17] CDK1 was required in tumor-derived cell lines, and it was the only mammalian CDK member which was present and active during late G_2 and mitosis phases.[18] So the design of CDK1-specific inhibitors was very important. Previous studies suggested that the dysfunction of cell cycle could lead to the malignant cellular proliferation. Therefore, peptides/compounds designed to inhibit CDK1 could have potential anticancer activity.

The most used strategy to inhibit CDKs was to design small molecules targeting the ATP-binding sites,[19] but the high degree of sequence conservation among the catalytic clefts of protein kinase family members caused non-specific effects. Because of the specificity of individual protein–protein interactions, modulation of these protein complexes was viewed as a putative source of future new highly selective drugs.[20–22] Accordingly, a series of peptides derived from CDK/cyclin complexes or their substrates were developed as novel specific CDK inhibitors. These peptide inhibitors were mainly designed according to the binding properties of the complexes CDK2/cyclin A, CDK2/cyclin E, and CDK4/cyclin D1 from human. They mainly came from endogenous CKIs,[23–25] E2F,[26,27] and combinatorial library of peptides.[21,28] Recently, a peptide inhibitor from human cyclin A was reported. It was designed according to the crystal structure of human CDK2/cyclin A complex and was derived from the α5 helix of cyclin A.[29] These peptides targeted CDK/cyclin complexes interfaces or conformational changes involved in the activation of CDKs. They displayed growth inhibitory activities when coupled with the protein transduction peptides.[21,23,24,27,28,30] Peptide inhibitors of CDKs designed from CDK1/cyclin B complex and/or from other species have not been reported yet.

There are no direct structural data about human CDK1/cyclin B1 complex, but McGrath et al. described the binding model of CDK1/cyclin B complex of *Asterina pectinifera* in detail.[31] In view of the similarity of the tertiary features between the structures of

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doi:10.1016/j.bmcl.2008.07.017

A. pectinifera CDK1/cyclin B and human CDK2/cyclin A complexes, we selected peptides from the sequence of cyclin B of *A. pectinifera* based on the sequence homology of cyclin B (*A. pectinifera*) and cyclin A (human) and based on the binding properties of CDK2/cyclin A. Divita et al. have demonstrated that the molecular mechanism of CDK2/cyclin A complex formation was a two-step process. The first step involved the rapid association between the PSTAIRE helix of CDK2 and helices of α3 and α5 of cyclin A to yield a non-processive intermediate complex. The second step involved additional contacts between the C-lobe of CDK2 and the N-terminal helix of cyclin A, which induced subsequent isomerization of the CDK into a fully mature form by promoting exposure of the T-loop for phosphorylation by the CDK activating kinase (CAK) as well as formation of the substrate-binding site.[29] This made the α3, α5, and N-terminal motifs of cyclins very important for the activation of CDKs. So we selected three motifs from cyclin B of *A. pectinifera* which contacted with CDK1. They were encompassed in the α5 helix, α3 helix, and N-terminal regions of cyclin B (*A. pectinifera*) and termed as **a5**, **a3**, and **N**, respectively. Investigations have suggested that the protein transduction domain of the HIV-1 transactivator of transcription, **Tat**, could successfully facilitate peptides or proteins entering into cells.[24,25] To facilitate peptides **a5**, **a3**, and **N** entering into cells and contacting with the CDK1/cyclin B complex, their derivates were conjugated with HIV-Tat (49–57) (**Tat**, RKKRRQRRR).[28] The bioactivities of each peptide were evaluated on two human cancer cell lines, HCT-116 (human colon adenocarcinoma cells) and EC-9706 (esophageal carcinoma cells). Up to now, CDK/cyclin complexes from other organisms have not been used as the source of anticancer peptides.

Table 1
The sequence and source of each peptide

Peptide	Sequence	Source
a5	KAQIRAMECNIL	Cyclin B (285–296)
a3	LIAAYEEM	Cyclin B (261–268)
N	YVNDIYLY	Cyclin B (178–185)
Tat	RKKRRQRRR	HIV-Tat (49–57)
Tat-a5	KAQIRAMECNILGRKKRRQRRR	
Tat-a3	LIAAYEEMGRKKRRQRRR	
Tat-N	YVNDIYLYGRKKRRQRRR	

All peptides were synthesized using the standard Fmoc-chemistry-based strategy on Wang resin. PyBOP/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. Crude peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a C_{18} column, lyophilized, and then identified by electrospray ionization mass spectrometry (ESI-MS). The purity of all the peptides was greater than 95%. They were dissolved with serum-free RPMI 1640 to make a stock solution of 1 mM and stored at −80 °C until required.

The synthesized peptides are listed in Table 1. **Tat** was the previously reported protein transduction domain of the HIV-1 Tat

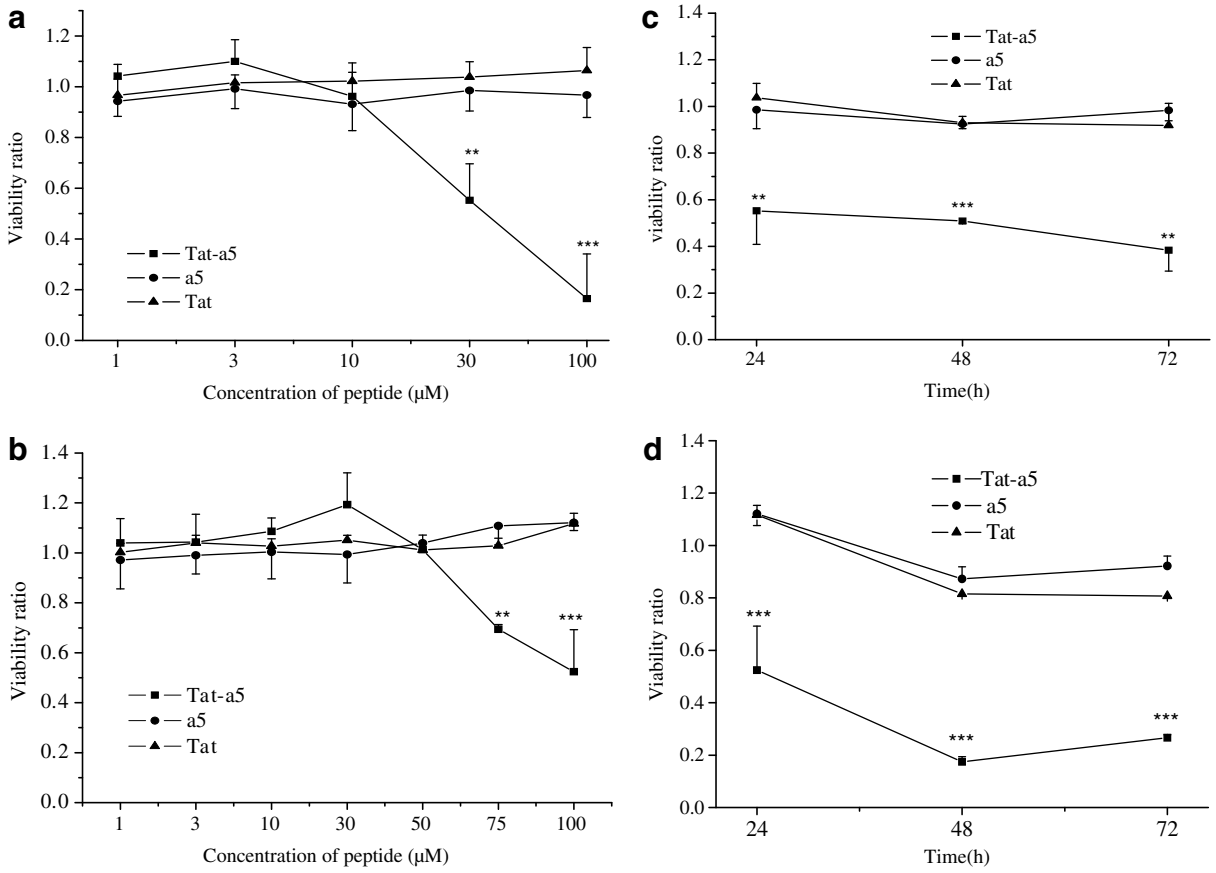

Figure 1. Dose and time curve of in vitro cytotoxic effects of **Tat-a5**. Viability ratios were measured by MTT assay. (a) Human colon adenocarcinoma cells (HCT-116) were treated with various concentrations of **Tat-a5**, **a5**, or **Tat** for 24 h. (b) Human esophageal carcinoma cells (EC-9706) were treated with various concentrations of **Tat-a5**, **a5**, or **Tat** for 24 h. (c) HCT-116 cells were treated with **Tat-a5** (30 μM), **a5** (30 μM), and **Tat** (30 μM) for 24, 48, and 72 h. (d) EC-9706 cells were treated with **Tat-a5** (100 μM), **a5** (100 μM), and **Tat** (100 μM) for 24, 48, and 72 h. The viability ratio of each peptide is shown as mean ± SD ($n \geqslant 3$). The statistical significance of differences between groups of each dose of peptides and **Tat** was assessed with a one-way ANOVA followed by the Dunnett's post-hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$.

protein (49–57)[28]; **a5**, **a3**, and **N** are encompassed in the α5 helix, α3 helix, and N-terminal regions of cyclin B of *A. pectinifera*, respectively. By using glycine as the linker, **Tat-a5**, **Tat-a3**, and **Tat-N** were designed as the tandem chimeric peptides conjugating **Tat** to the C-terminal of **a5**, **a3**, and **N**, respectively.

The synthesized peptides **Tat-a5**, **a5**, **Tat-a3**, **a3**, **Tat-N**, **N**, and **Tat** were assayed at a dose range of 0–100 μM for their antiproliferative activity in vitro against two cancer cell lines by MTT assay.[25,32,33] In the preliminary study, **Tat-a3**, **Tat-N**, and their control peptide (**a3** and **N**) did not show strong cytotoxic activity (data not shown). Only **Tat-a5** peptide exhibited the potential to kill the cancer cells. The data are shown in Figure 1.

As shown in Figure 1, when the cancer cells were treated for 24 h with increasing doses of **Tat-a5**, viability ratios of the cells were markedly decreased. HCT-116 cells treated with 30 and 100 μM **Tat-a5** exhibited viabilities of 55% and 16%, respectively. EC-9706 cells treated with 75 and 100 μM **Tat-a5** exhibited viabilities of 69% and 52%, respectively. Significant differences were seen between **Tat-a5** and **Tat** at these doses. **Tat** or **a5** showed no effects on the viability ratios of these cells at all the tested doses. We speculated that the peptide **a5** could be transduced into cells when conjugated to the membrane-permeable peptide, **Tat**.

The cell cytotoxic effects of **Tat-a5** (30 μM for HCT-116 and 100 μM for EC-9706, Fig. 1) treated for 48 and 72 h were also determined. Combined with the results mentioned above, we found that the viability ratios of the cancer cells were decreased significantly when treated with **Tat-a5** for 24, 48, and 72 h. Therefore, **Tat-a5** showed potent cytotoxic effects in both the cell lines, HCT-116 and EC-9706.

To examine whether the cytotoxic effects of **Tat-a5** were related to apoptosis, an acridine orange stain assay was used to observe the morphological changes in the cancer cells.[34,35] Treatment of the cells (HCT-116 and EC-9706) with **Tat-a5** caused chromatin condensation in the nucleus, shrinkage of nucleus, loss of integrity of nuclear envelope, and induced apoptotic bodies. No morphologic evidence of apoptosis was seen in both the cell lines after exposure to **Tat** alone (Fig. 2).

To further explore whether the apoptosis-promoting effects of **Tat-a5** were related to G_2/M arrest in cancer cells, we treated the two cancer cell lines with 30 μM **Tat-a5** or **Tat** for 12 and 18 h. DNA analysis was then performed by flow cytometry to determine the percentages of cells in the G_1, S, and G_2/M phase of the cell cycle.[36–38] The DNA contents of EC-9706 and HCT-116 cells treated with **Tat-a5** were summarized as histograms, which are shown in Figure 3. As shown in the figure, compared with control cells (0 h), in the EC-9706 group, cells exposed to **Tat-a5** were significantly arrested at the G_2/M phase of the cell cycle at 12 and 18 h. In the HCT-116 group, cells exposed to **Tat-a5** were significantly arrested at the G_2/M phase of the cell cycle at 12 h, but after 18 h treatment, the proportion of cells in the G_2/M phase was significantly decreased and the ratio of cell apoptosis and debris increased (data not shown). We speculated that **Tat-a5** could arrest the cell cycle at the early G_2/M transition state, but thereafter the cells arrested at the G_2/M phase began to come into apoptosis and died. In contrast, the two cell lines treated with **Tat** showed no G_2/M arrest.

In this study, we selected another organism, *A. pectinifera*, and designed peptides, which targeted the CDK1/cyclin B interfaces. A

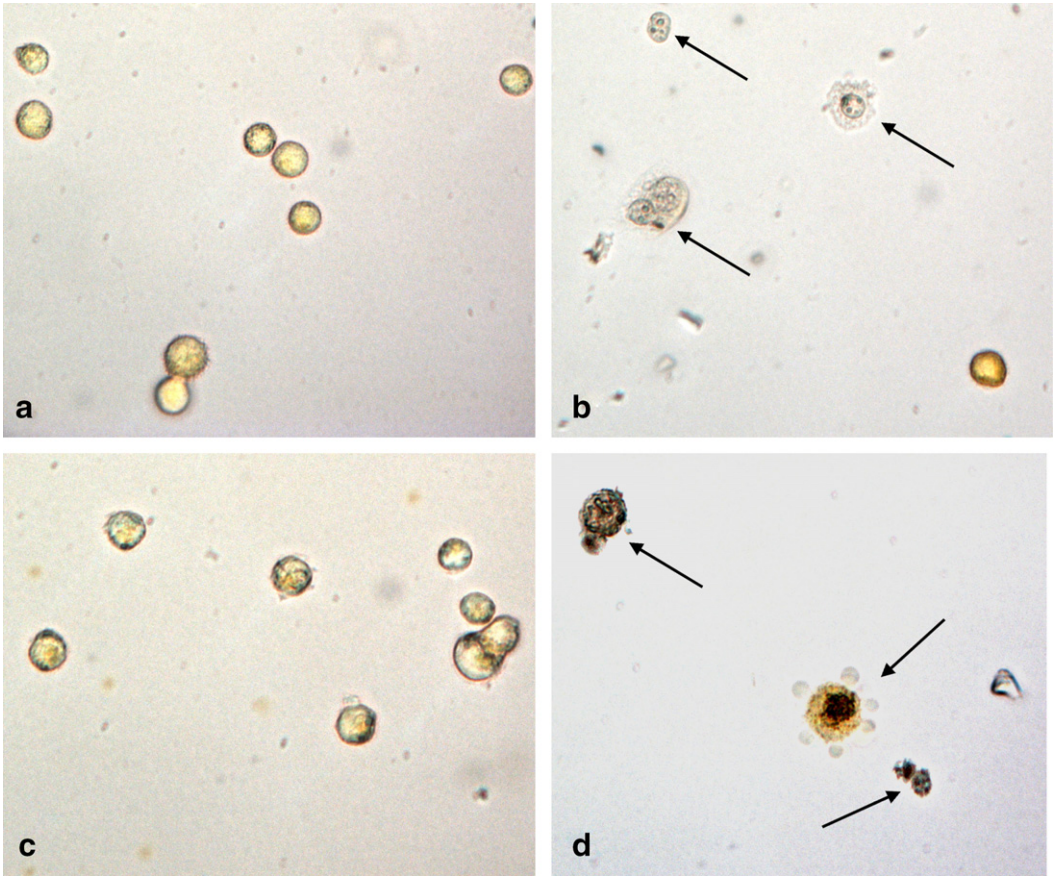

Figure 2. The effects of **Tat-a5** and control peptide, **Tat**, on the morphology of cancer cell lines, HCT-116 and EC-9706 (images at 400× magnifications). (a) EC-9706 cells treated with 100 μM **Tat** for 24 h. (b) EC-9706 cells treated with 100 μM **Tat-a5** for 24 h. (c) HCT-116 cells treated with 100 μM **Tat** for 24 h. (d) HCT-116 cells treated with 100 μM **Tat-a5** for 24 h. In images (b) and (d), chromatin condensation, loss of integrity of nuclear envelope, and visualization of apoptotic bodies were observed.

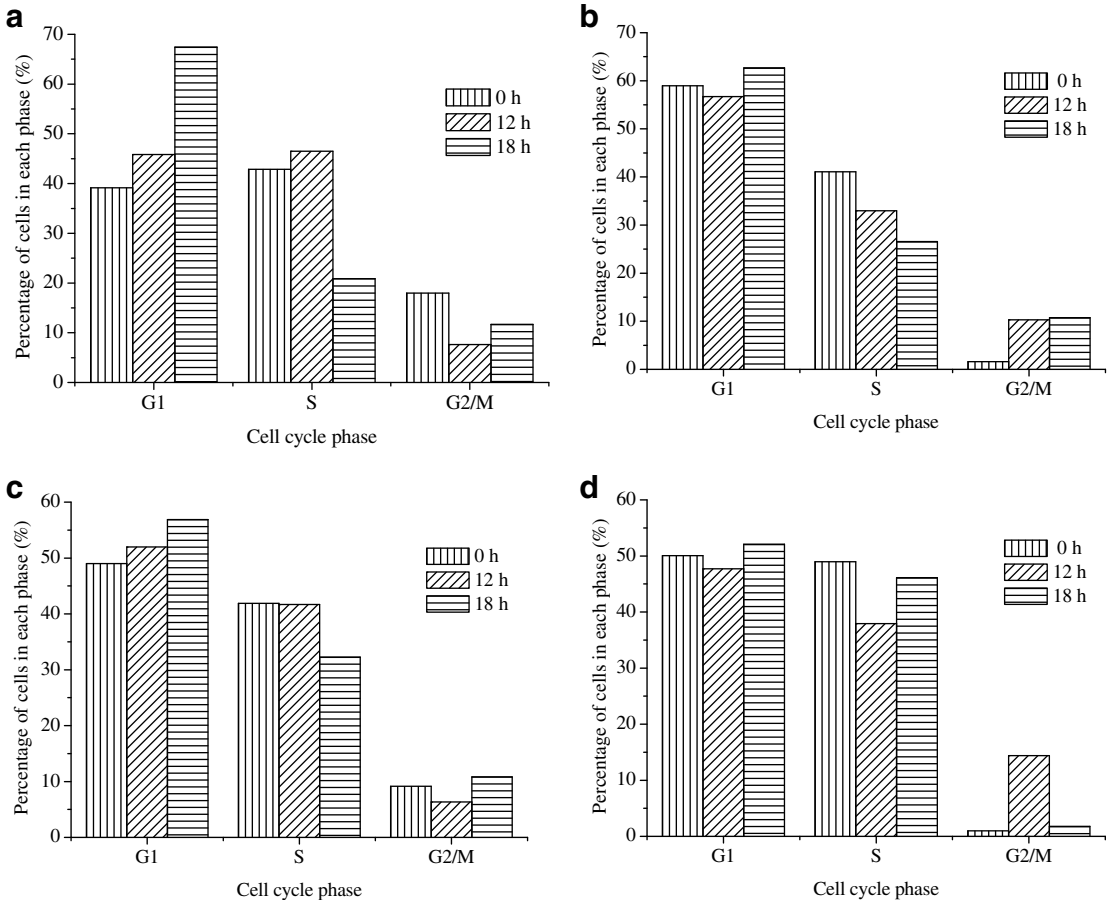

Figure 3. The percentages of the cells in the G_1, S, and G_2/M phases. (a) EC-9706 cells were treated with 30 μM **Tat**. (b) EC-9706 cells were treated with 30 μM **Tat-a5**. (c) HCT-116 cells were treated with 30 μM **Tat**. (d) HCT-116 cells were treated with 30 μM **Tat-a5**.

novel peptide, **a5**, was identified which had the significant anticancer effects when conjugated to a membrane-permeable peptide, HIV-Tat$^{49-57}$. From the bioassay results, we speculated that **Tat-a5** competed with cyclin B1, contacted with CDK1 prior to cyclin B1, and prevented the excessive activation of CDK1, which made the cancer cells get arrested at G_2/M phase and come into apoptosis. Compared with the previously reported peptide inhibitor of CDK2/cyclin A, C4-Tat (TYTKKQVLRMAHLVLKVLTFDLCRRRQRRKKR G),[29] **Tat-a5** is a much shorter leading peptide which showed potent cytotoxic and apoptotic activities, which makes it more convenient to synthesize and study further in the future. Since C4-Tat was the inhibitor of CDK2/cyclin A, while **Tat-a5** targeted the CDK1/cyclin B complex, and CDK1 was the only mammalian CDK member which was present and active during late G_2 and mitosis phases,[18] **Tat-a5** might be more valuable in anticancer drug research. Recent work in our group showed that **Tat-a5** would have the potential to inhibit tumor angiogenesis, which made it more attractive.

The structure of human cyclin B1 has been recently published together with a structural model of the human CDK2/cyclin B.[39,40] We aligned and analyzed the human cyclin B1 and *A. pectinifera* cyclin B by NCBI blast. There were segmental-same sequences, while obvious differences existed between the two α5 helices. We speculated that the differences might be the special point of this peptide from *A. pectinifera*.

Our results provided further evidence to support the binding model of CDK1/cyclin B and suggested that the α5 helix of cyclin B might be the most important binding region in contact with CDK1. Our results provided valuable information for the design of CDKs peptide inhibitors with anticancer activities from other organisms. The peptide **Tat-a5** could serve as a novel and important compound with anticancer and tumor angiogenesis inhibitive activities. The detailed mechanisms of this leading peptide as well as its selectivity toward CDK1 will be investigated in the future. Studies of the structure–activity relationships for a series of analogs are being undertaken in our laboratory to identify the key residues in the sequence of **Tat-a5**.

Acknowledgments

This work was supported by grants from Science and Technology Project (0624410024, 072102330038) and Outstanding Talent Program of Henan Province (2008), China.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.017.

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