RESEARCH ARTICLE

Selective Inhibition of Bicyclic Tetrapeptide Histone Deacetylase Inhibitor on HDAC4 and K562 Leukemia Cell

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Abstract

Histone deacetylase (HDAC) inhibitors of cyclic peptide have been proved to be the most complex but the most stable and relative efficient inhibitors because of their large cap region. In this paper, a series of studies were carried out to evaluate the efficacy of synthetic bicyclic tetrapeptide inhibitors 1-5 containing hydroxamic acid referring molecular docking, anti-proliferation, morphology and apoptosis. Docking analysis, together with enzyme inhibitory results, verified the selective capability of inhibitor 4 to HDAC4, which might closely related to haematological tumorigenesis, with Phe227, Asp115, Pro32, His198 and Ser114 participating into hydrophobic interactions and Van der Waals force which was familiar with former study. Moreover, inhibitor 4 inhibited K562 cell line at the IC_{50} value of 1.22 μM which was 51-67 times more efficient than that for U937 and HL60 cell lines. Inhibitor 4 exhibited the cell cycle-arrested capability to leukemia at S phase or G2/M phase as well as apoptosis-induced ability in different degrees. Finally, we considered that bicyclic tetrapeptide inhibitors were promising inhibitors used in cancer treatment and inhibitor 4 could prevent K562 cell line well from proliferation, arrest cell cycle and induce K562 towards apoptosis to achieve the goals of reversing cancer cells which could become a potential leukemia therapeutic agent in the future.

Keywords: Histone deacetylase inhibitor - bicyclic tetrapeptide - leukemia

Asian Pac J Cancer Prev, **14 (12)**, 7095-7100

Introduction

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are two opposing classes of enzymes, which finely regulate the balance of histone acetylation affecting chromatin packaging and gene expression (Aversanaa et al., 2012). An increasing number of clinical researches in cancer patients have illustrated that the most prevalent alteration of HDAC function is overexpression. Eighteen mammalian HDACs have been identified so far, which can be classified into four different families according to the homology with yeast. Classes I, II and IV consist of 11 members, whereas the 7 class III members are called sirtuins. Recently, HDAC inhibitors are in advanced clinical research with considerable potential as cancer therapeutic agents which could impede HDAC substrate recognition, interfering with pathways such as gene expression, differentiation, apoptosis, cell cycle progression, autophagy, ROS (reactive oxygen species) production and DNA damage repair (Frandy et al., 2005; Humeniuk et al., 2009).

It is well known that the basic composition of an inhibitor includes the zinc binding group (ZBG), the cap group and the cavity-binding linker region according to chemical structure obtained through the X-ray crystal diffraction techniques. There exist several classes of HDAC inhibitors characterized: short-chain fatty acids, benzamides, hydroxamic acids, cyclic tetrapeptides, electrophilic ketones and sulfer-containing inhibitors. Among these inhibitors, hydroxamic acids were regarded as the most effective Zn^{2+} binding group such as natural products Trichostatin (TSA) and selected SAHA in synthesized hybrid (Figure 1). SAHA (Zolinza) was the first one approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma (CTCL) (Grant et al., 2007). Meanwhile, cyclic peptide inhibitors were proved to be the most complex inhibitors because of their large cap region with the most stable structure and relative better efficacy. FK228 (Istodax) (Figure 1) was a cyclic peptide containing

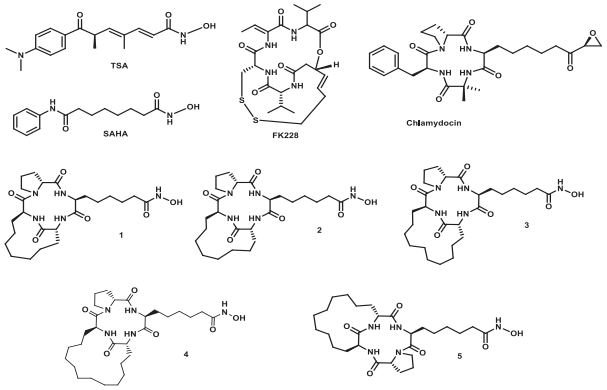

Figure 1. The Structure of Histone Deacetylases Inhibitors

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sulfur moiety, isolated from Chromobacterium violaceum and has received FDA approval in 2009 to treat CTCL in patients who have received at least one prior systemic therapy (Grant et al., 2010). And FK228 exhibited better selectivity to HDAC1 and HDAC2 (class I) respectively than HDAC4 (class IIa) and HDAC6 (class IIb) (Furumai et al., 2002). Nevertheless, researches about this kind of cyclic inhibitors were limited and the exploration of them with good efficacy and subtype selectivity was still a research focus.

Leukemia is a treatable malignant tumor of the blood cells started in the bone marrow which commonly occurs in children. The most common leukemia seen in childhood is acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML) and other myeloid leukemias, such as chronic myelogenous leukemia (CML). HDAC inhibitors have been performed in several in vivo or in vitro studies to test their roles in leukemia treatment (Göttlicher et al., 2001; Rosato et al., 2003; Editorial, 2009). For instance, FK228 has been approved with some clinical activity in chronic lymphocytic leukemia and acute myeloid leukemia (Byrd et al., 2005). However, there is no related research of bicyclic tetrapeptide HDAC inhibitors on leukemia cells so far.

In this paper, studies were carried out to research the efficacy of several more complex and relatively novel synthetic bicyclic tetrapeptide HDAC inhibitors 1-5 (Figure 1) (Islam et al., 2010). All these inhibitors were composed of hydroxamic acid as metal-binding group, the optimized 5-carbon hydrophobic spacer (Huang et al., 2011) and bicyclic tetrapeptide surface recognition region. We had researched primarily that position 2 and position 3 in the large loop of cyclic skeleton were closely associated with inhibitory selectivity of inhibitors, so these bicyclic tetrapeptide inhibitors might possess different selective characteristics. Docking methods was used to confirm the binding mode as well as the selectivity of inhibitors to HDAC4, in comparison with the results of enzyme inhibitory activity. Subsequently, cytological experiments were applied to further assess bicyclic tetrapeptide inhibitors using three different kinds of leukemic cells HL60 (Acute promyelocytic leukemia, AML), K562 (Chronic myelocytic leukemia, CML), U937 (Diffuse histiocytic lymphoma, monocytic characteristics) (Sundström et al., 1976; Andersson et al., 1979; Collins, 1987) to detect their anti-proliferation, cell cycle-arrest and apoptosis-induction capabilities.

Materials and Methods

Synthesis of bicyclic tetrapeptide inhibitors

Bicyclic tetrapeptides containing hydroxamic acid used in this paper were synthesized by Nishino et.al involving cascade catalytic chemistry (Islam et al., 2010). And they were dissolved into dimethyl sulfoxide (DMSO) for experimental usage.

Docking study

Docking study could analyze their binding conditions in the aspects of energy. Molecular docking was conducted by AutoDock4.2 program whose parameters were defaults values during test to know the binding energy and mode between them. Before inhibitors were put into the active pocket of enzyme which was covered by 70×70×70 Grid box with 0.375 Å spacing, both ligand and receptor were saved as PDB files using Chimera. AutoDock tool was applied to delete waters and add hydrogen to enzyme. Meanwhile, inhibitors were also needed to be treated by AntoDock tool and saved as PDBQT files, torsion happened in all single bond except amino bond and cyclic bonds. Docking began when enzyme and inhibitors were prepared well, the Lamarckian genetic algorithm (LGA) was employed with a maximum number of 2.5×10^7 energy evaluations and a maximum number of 5×10^3 generations during the overall procedure. 150 docking runs were performed during all the calculations. Results were clustered on the basis of energy from low to high according to Root-Mean-Square Deviation (RMSD) which should be under 2.0 Å. Upon finished, AutoDock tool could be employed to know the free energy and residues participating in hydrophobic interactions. Pymol software was then used to analyze hydrogen bond and mark hydrophobic interact amino acids (Goodsell et al., 1996; Morris et al., 1998; Morris et al., 2008; Morris et al., 2009). Chimera software was used to indicate hydrophobic interactions further together with Ligplot procedure.

Cell line and cell culture

HL60 (acute promyelocytic leukemia, AML), U937 (diffuse histiocytic lymphoma, monocytic characteristics) cell lines were maintained in RPMI 1640 medium, supplemented with 15% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO2 at 37°C. K562 (chronic myelocytic leukemia, CML) cell line was cultured in Dulbecco,s Modified Esgle,s Medium (DMEM) containing 15% FBS at 37°C in a 5% CO2 incubator and routinely screened for Mycoplasma contamination.

Reagents

RPMI Medium 1640 and Dulbecco's Modified Eagle Medium were purchased from Invitrogen Corporation and Life Technologies Corporation respectively. Standard fetal bovine serum was bought from Beijing Solarbio Science & Technology. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), bisBenzimideH33342 (Hoechst 33342), Trochostatin A were purchased from Amersco company while Propidium Iodide and RNase were bought from Sigma. Annexin v-fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) staining kit was purchased from Nan Jing KeyGen Biotechnology.

In vitro cytotoxicity assay

In vitro cytotoxicity of leukemia cell lines were assessed by MTT assays. 100 μl cells all at the normal growth state were seeded in a 96-well plate at about 3000 cells and then treated with 100 μl different synthetic inhibitors 1-5 for 48 h at 37°C. Following the suitable treatment period, 20 μl of a 5 mg/ml solution of MTT was added to each well. After 4 h incubation, cells were lysed using 100 μl of a 30% sodium dodecyl sulfonate

Table 1. HDAC Inhibitory Activity (nM) and p21 Promoter Assay (nM) Data for Bicyclic Tetrapeptide HDAC Inhibitors

Inhibitors	HDAC1	HDAC4	HDAC6	P21 promotor assay	
				EC_{1000}	EC_{10000}
1	9.1	5.4	330	92	130
2	9.1	5.5	410	7.2	21
3	25	26	230	510	>1000
4	13	5	240	2	5
5	25	31	490	58	310
TSA	23	34	65	20	31

(SDS) formamide solution. Plates were left to incubate overnight and read the following day using a 570/630 nm double wavelength which could improve the accuracy of measured date. IC_{50} values were calculated as drug concentrations necessary to inhibit 50% proliferation compared with untreated control cells.

Hoechst33342 staining

In order to detect possible drug-induced apoptotic changes in leukemic cell nucleus, HL60, K562, U937 cells (1×10^6) were seeded on a 6-well plate and then treated with drugs or dimethyl sulfoxide (DMSO) for 24 h. Cells were collected in 5 ml tubes, washed by phosphate buffer (PBS) and then fixed in a 1% paraformaldehyde solution for 10 min, after washed by PBS, cells in each group were incubated with Hoechst33342 (10 μg/ml) for 15 min. Nuclear morphological changes in each treatment were identified by fluorescence microscope.

Effects of inhibitor 4 on cell cycle progression

Cancer cell lines were treated for 24 h on 6-well plate and then cells were harvested and fixed with cold 70% (v/v) ethanol for 24 h. After collected by centrifugation at 1000 r for 5 min, the cells were washed twice by PBS and suspended in PBS containing RNase (50 μg/ml) for 30 minutes at 37°C water bath, the function of RNase was stopped by ice bath and then 50 μg/ml Propidium RnaseA (PI) was added to treat for at least 20 min. The cell cycle profiles were determined by flow cytometry (FAC) followed by filtration of 300 mesh nylon membrane. Cell cycle analysis was done with the Modfit software.

Apoptosis assessment

AML or CML leukemic cells were treated with the modest drug for 24 h culture, the doses for the cell lines were IC_{50} values determined by MTT assays. Then, at least 1×10^5 untreated or drug-treated leukemic cells were collected at 1000 r for 5 min, and washed twice with cold PBS, each at 1000 r for 5 min. Apoptosis was measured by flow cytometry (FAC) after a stain with Annexin v-FITC or Propidium Iodide (PI) or both for 15 min in the dark at room temperature following the instruction of Annexin v-FITC apoptosis detection kit.

Statistical analysis

All the data were expressed as the mean of triplicate independent experiments performed in a parallel manner unless otherwise indicated and the quantitative data were expressed as a mean of ± standard deviation (SD).

Table 2. The Binding Free Energy Between HDAC4 and Inhibitors 1-5 (kcal/mol)

Inhibitors	Estimated Free Energy of Binding	vdW + Hbond + desolv Energy	Electrostatic Energy	Final Total Internal Energy	Torsional Free Energy
1	-10.2	-10.7	-1.61	-0.2	2.09
2	-9.4	-10.9	-0.5	-0.85	2.09
3	-9.57	-10.1	-1.51	-0.3	2.09
4	-10.5	-10.6	-1.92	-0.4	2.09
5	-9.09	-9.87	-1.31	-0.49	2.09
TSA	-8.04	-9.45	-0.98	-0.52	2.39

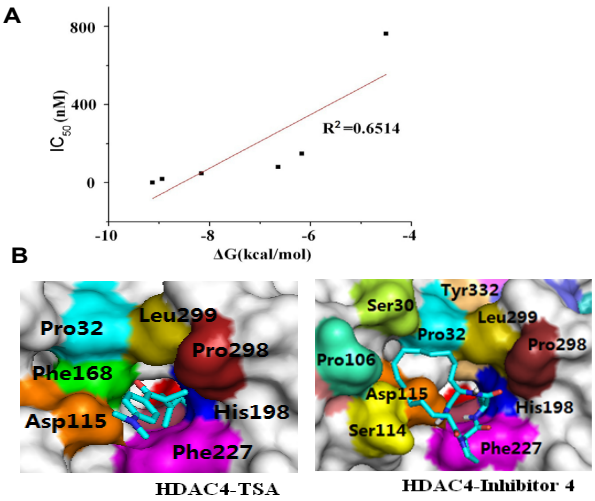

Figure 2. Docking Results of Inhibitors to HDAC4. (A) Correlation between the predicted binding energies (ΔG) and the experimental IC_{50} values. (B)The hydrophobic interactions of HDAC4 with TSA and inhibitor 4 together with the amino acids participating in surface interactions

Results

Docking results

The enzyme inhibitory activities of inhibitors were showed in Table 1 (Islam et al., 2010). All inhibitors were more efficient than TSA against HDAC4. However, inhibitors 1, 2 and 4 showed better selective inhibition than TSA against either HDAC1 or HDAC4 at nanomolar concentrations. Moreover, inhibitors 1, 2 and 4 possessed a better selectivity to HDAC4 and inhibitor 4 was the best one with the IC_{50} of 5 nM which was 6.8 times better than TSA to HDAC4 (IC_{50} = 34 nM). Energetic analysis in Table 2 demonstrated the lower binding energy of inhibitors 1-5 than TSA (-8.04 kcal/mol) with HDAC4 under the participation of Van der Waals force, hydrogen bonds and hydrophobic interaction during the process of enzyme-inhibitor interaction. Moreover, inhibitors 1 and 4 showed the lowest free binding energy of -10.27 kcal/mol and -10.47 kcal/mol respectively, which was absolutely consistent with the results of enzyme inhibitory activity. And we knew that ΔG had positive correlation with experimental IC_{50} values (Figure 2A), so we could roughly guess the better cytotoxicity of inhibitor 4.

The interac pattern between HDAC4 and inhibitor 4 was then studied that oxygen of L-Asu side chain in metal-binding area stretched deep into the active pocket participating into the formation of hydrogen bond with zinc ion (Figure 2B). Hydrophobic channel of HDAC4

Table 3. Anti-proliferative Activities (IC50 μM) of Bicyclic Tetrapeptide HDAC Inhibitors Towards U937, K562 and HL60 Leukemic Tumor Cell Lines

	1	2	3	4	5	TSA
HL60	47.6±0.2	56.6±0.5	53.6±1.4	51.3±0.6	71.2±1.8	53.9±2.6
K562	1.24±0.61	1.25±0.31	1.29±0.72	1.22±0.38	1.25±0.21	0.56±0.05
U937	80.3±2.1	95.0±10.2	83.2±0.8	68.1±1.2	>100	39.4±2.1

Table 4. Effects of Inhibitor 4 on Cell Cycle in K562, HL60 and U937 Cell Lines

	G0/G1 (%)	S (%)	G2/M (%)
HL60 control	20.5±2.7	12.1±0.6	67.5±1.9
HL60	26.4±2.1	56.0±0.1	17.6±1.5
U937 control	44.8±1.6	24.9±1.8	30.2±0.7
U937	30.9±0.3	7.70±0.8	61.4±2.3
K562 control	24.3±0.6	5.80±0.1	69.9±0.6
K562	6.20±1.3	28.5±1.8	65.3±3.1

was also occupied with linker region of inhibitor 4. It was clear to find in Figure 1 that inhibitor 4 possessed a 12-carbon loop and this large loop might increase the hydrophobic interactions in surface recognition region. Just like what we saw, Phe227, Asp115, His198, Ser30, Ser114 in HDAC4 interacted closely with the largr bicyclic structure and meanwhile, only Phe227, Asp115 and His198 mainly participated into the hydrophobic interactions with TSA. So we could speculated that Ser30 and Ser114 played remarkable roles in enhancing the combination of inhibitor 4 and HDAC4 and giving inhibitor 4 better inhibitory activity.

Cytotoxicity of HDAC inhibitors to leukemic cell lines

In order to detect the efficacy and specificity of inhibitors towards haematological malignancies, we screened the effects on leukemic cancer cell lines. The anti-proliferative activity of these analogues were listed in Table 3, three leukemic cancer cell lines from different sources were selected as candidates for MTT assays, they were HL60, K562 and U937. It was obvious that bicyclic tetrapeptide inhibitors showed the best anti-proliferative activity against K562 cell line and appeared significant selective difference between three leukemic cancer cell lines with 51-67 folds more efficient against K562 than another two leukemic cell lines. Meanwhile, we could also see that inhibitor 4 was still the most valid one among these five inhibitors with the IC_{50} of 1.22 μM (SD=0.38) which was consistent with the result of HDAC inhibitory efficacy in Table 1. Hence, we hoped to further explore their function to leukemic cell lines in consideration of genetic differences.

Hoechst staining

The morphological features of apoptosis were tested using Hoechst33342 staining methods in Figure 3, the average diameters of HL60, K562, U937 cells were at the range of 20 μm to 30 μm and K562 possessed a larger cell with diameter of 25 μm. There existed visible apoptosis in treated HL60, U937 and K562 cells with inhibitor 4, including nuclear condensation, cell shrinkage and cell cavities which could be clearly seen. However, except for the morphological detection, the intracellular changes were also needed to check and prove.

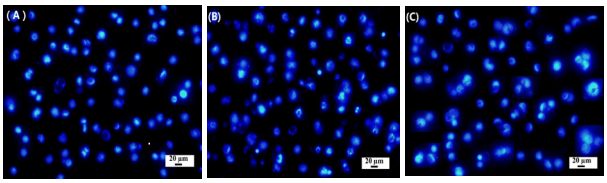

Figure 3. Morphological Studies with Hoechst33342 Staining under Fluorescence Microscope. (A) Hoechst staining for HL60 cell line (B) Hoechst staining for K562 cell line (C) Hoechst staining for U937 cell line

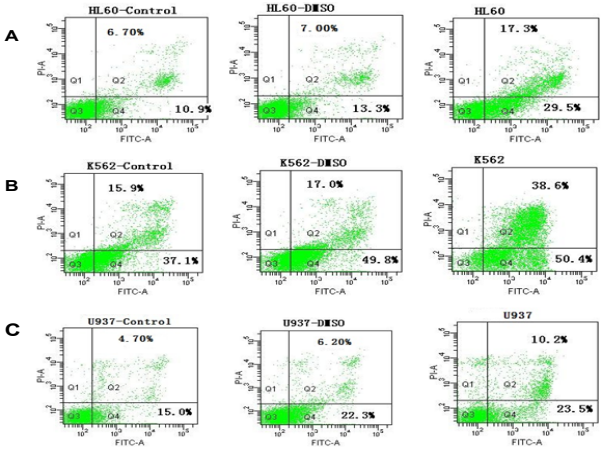

Figure 4. Effects on Cell Apoptosis of Bicyclic Tetrapeptide Inhibitor 4. (A) Apoptosis in HL60 induced by inhibitor 4. (B) Apoptosis in K562 induced by inhibitor 4. (C) Apoptosis in U937 induced by inhibitor 4. Q1 represented necrotic cells, Q2 represented the cells in terminal apoptosis, cells in Q3 were normal cells and cells in Q4 are primary apoptotic cell

Effects of bicyclic tetrapeptide HDAC inhibitor 4 on cell cycle progression

The effect of inhibitor 4 on K562, HL60, U937 cell cycle were examined. As shown in Table 4, inhibitor 4 affected the cell cycle progression of three cell lines and accumulated cell in different phases compared with untreated control cells treated with DMSO. HL60 cells were prevented from passing through the S phase of cell cycle whose proportion of cells in the S phases (56%) was much higher than phases G0/G1 (26.4%) and G2/M (17.6%). In U937 cell line, most of cells were arrested evident in the G2/M phase relevant to DNA damage checkpoint. However, K562 cells were less affected than other cell lines but with a little fluctuation on S phase.

Assessment of bicyclic tetrapeptide inhibitors 4-induced apoptosis in leukemic cell lines

To examine whether bicyclic tetrapeptide inhibitors 4 could induce the apoptosis of human hematological malignancy, we carried out apoptotic detection through flow cytometry (Figure 4). It was clear that apoptosis occurred in each cell line after 24 h treatment, obtained from the proportion changes of Q2 area which represented the cells in terminal apoptosis and Q4 area which reflected primary apoptosis. The percentage of cells in terminal apoptosis increased from 15.9% to 38.6% in K562 cell line and in HL60 cell, the percentage increased from 6.7% to 17.3% whereas from 4.7% to 10.2% in U937 cell line. When it referred to primary apoptosis, K562 changed from 37.1% to 50.4%, HL60 increased from 10.9% to 29.5%

and U937 rose from 15.0% to 23.5%. So we could easily get to a conclusion that bicyclic tetrapeptide inhibitors possessed the ability to induce hematological malignancy towards apoptosis and inhibitor 4 induced apoptosis of K562 cell line at the greatest degree.

Discussion

HDAC inhibitors have been represented as a new generation of anti-cancer agents, as one class of them, a variety of natural cyclic peptide HDAC inhibitors and synthetic analogues have been found and explored their HDAC isoform-selectivity (Josea et al., 2004; Norikazu et al., 2004; Norikazu et al., 2004; Walton, 2006; Bhuiyan et al., 2006; Gu et al., 2007; Matsuoka et al., 2007; Wang et al., 2007; Im et al., 2008; Norikazu et al., 2008; Ahn et al., 2012) owing to their stable conformation and considerable efficacy. Here, we reported several studies about synthetic bicyclic tetrapeptide compounds containing hydroxamic-acid involving binding mode with HDAC4, anti-proliferation cell cycle arrest and apoptosis inducement with three hematological cancer cell lines K562, HL60 and U937 as objects.

Docking analysis exhibited the lower energy of bicyclic tetrapeptide inhibitors than TSA , together with a better selectivity of inhibitor 4 to HDAC4 in contrast with the selectivity of cyclic inhibitor FK228 (Ryohei et al., 2002) but in accordance with enzyme inhibitory results. Van der Waals force and hydrophobic interaction were more likely to enhance the inhibitory activity to HDACs under the promotion of Ser30 and Ser114. It has been known that HDAC4 controlled chondrocyte hypertrophy during skeletogenesis (Vega et al., 2004), so the good selectivity of compounds to HDAC4 might give them a better application in leukemia therapy.

We next proceeded to examine their anti-proliferative activity to three representative leukemic cell lines by MTT assay. Inhibitor 4 observably better than the others, inhibited the proliferation of K562 at the IC_{50} value of 1.22 μM which possibly resulted from the modest combination with HDACs. Moreover, another interesting phenomenon was found that they appeared significant selectivity to K562 with 51-67 times more efficient than U937 and HL60 cell lines. The cytological studies were performed referring to nuclear morphological changes, cell cycle arrests and cell apoptosis to further exploration. Morphological features of nuclear were assessed through Hoechst33342 staining which revealed visible morphological changes of apoptotic nuclear. Flow cytometry results showed that inhibitor 4 induced HL60 and K562 cancer cells at S phases while at G2/M phase in U937. Except for cell cycle test, apoptotic detection could also be applied to determine apoptotic condition inside cancer cell by flow cytometry and the results made perspicuous illustration of the best apoptosis-promoting capability of inhibitor 4 to K562 leukemic cell line. We have known that U937 had several parameters similar with HL60 like the lack of EBV (Epstein-Barr virus) -associated antigens and the expression of surface receptors for Fc fragment. However, the surface glycoprotein profile (GP) of K562 was completely different from those malignant cells like HL60 and human natural killer cell expresses strong lytic activity against K562 cell lines (Sundström et al., 1976; Andersson et al., 1979; Collins, 1987). It was possibly the sensibility of K562 determining the optimistic performance of bicyclic compounds.

Consequently, we arrived at a conclusion that bicyclic tetrapeptide inhibitor 4 did have a selective anti-proliferative ability to HDAC4 and leukemia cell K562 in the respects of anti-proliferation and apoptosis induction. Inhibitor 4 also possessed the cell cycle-arrest capabilities to leukemia in various degrees. When it referred to the reason why inhibitor 4 inhibited K562 cell line better than U937 and HL60, the surface glycoprotein profile and sensibility of K562 maybe play a role to some extent and we could make further exploration of this assumption. We hoped that bicyclic tetrapeptide inhibitors could be introduced into clinical trials as a fire new drug for the treatment of leukemia especially in childhood.

Acknowledgements

We thank Nishino Norikazu for the kindly support of synthetic compounds from the Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology.

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