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Research article

Anti-tumor Property of Plant Protease Inhibitor on Human Chronic Myeloid Leukemia Cells

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ABSTRACT

Many plant protease inhibitors are found to have effects of tumor suppression in certain cell lines. In this work attempt has been made to determine anti-tumor/apoptotic property of trypsin inhibitor protein (WbTI2) from plant *Psophocarpus tetragonolobus* (L.)D.C. (winged bean) on human chronic myeloid leukemia cells (K562). Pure winged bean trypsin inhibitor protein was purified by affinity chromatography from recombinant molecule and human chronic myeloid leukemia cells were treated by it. Then viability of the treated cells were determined by simple microscopic study, MTT assay and flow cytometric analysis. All results revealed significant effect of WbTI2 on the viability of the human chronic myeloid leukemia cells. The IC_{50} value was determined to be 5 µg/mL in MTT assay. The data was found to be statistically significant.

Introduction

Chronic Myeloid Leukemia (CML) is a malignant disease of myeloid tissue where bone marrow, lymph nodes and spleen are involved. Association of BCR-ABL oncogene, generated by Philadelphia chromosome is the principal feature of this disease. The balanced reciprocal translocation between chromosome number 9 and 22, t (9; 22) (q34.1; q11.2) (Philadelphia Chromosome) causes constitutive synthesis of BCR-ABL protein tyrosine kinase which triggers a number of proliferative pathways in CML, cells. [1] The incidence of occurrence of CML in India has been reported annually to be 0.8–2.2/100,000 in adult males and 0.6–1.6/100,000 in adult females. Although CML occurs in children and adolescents, less than 10% of all cases occur in subjects between 1 and 20 years old.[2] Before advent of tyrosine kinase inhibitors CML was treated with cytarabine, busulfan, hydroxyurea or IFNα. These are still potential curatives for patients unresponsive to newer therapies. But the landmark development regarding treatment of CML is introduction of tyrosine kinase inhibitors (TKIs) like imatinib mesylate.[3] After getting resistance to imatinib mesylate in increasing numbers,[4] two "second-generation" TKIs,i.e., dasatinib and nilotinib have been approved for the first-line treatment of CML. Dasatinib was initially approved in 2007 and then nilotinib was subsequently approved for the patients having indication of Imatinib resistance or intolerance. Both dasatinib and nilotinib were approved as first-line treatment options in 2010 following demonstration of high cytogenetic response and molecular response rates.[2] Mechanisms

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Table 1: Purification of rWbTI-2 from induced *E. coli**

Steps	*Total proteina (mg)*	*Recombinantb WbTI-2 (mg)*	*Specific protein contentc (mg/mg)*	*Yield (%)*
Bacterial lysate	60	4.0	0.067	100
Anti-WbTI-2 column eluent	3.2	3.14	0.981	78.5

* starting from 2.25 gm cells
a: Total protein was estimated by Bradford assay
b: rWbTI-2 was estimated by radial immune diffusion assay
c: Specific content is defined as rWbTI-2/ total protein

Table 2: Simple microscopic study on effect of WbTI-2 on k562 cells

Samples	*Set I (Cell count x 105/mL)*	*Set II (Cell count x 105/mL)*	*Average cell count (Cell count x 105/m:)*
1. +ve C (no protein)	Living cell 32 Dead cell 0	Living cell 30 Dead cell 0	Living 31 Dead 0
2. –ve C (with Imatinib)	Living 3 Dead 10	Living 2 Dead 13	Living 2.5 Dead 11.5
3. 5 µL/mL WbTI2	Living cell 13 Dead cell 4	Living cell 11 Dead cell 5	Living 12 Dead 4.5
4. 10 µL/mL WbTI2	Living 1 Dead 4	Living 4 Dead 3	Living 2.5 Dead 3.5

No. of initial K562 cells: 2.7 x 10^5/mL (before 24 hours incubation)
Imatinib (10 µg/mL) was taken as control
Stock solution of WbTI-2: 6 mg/mL (estimated by Bradford assay)

Table 3: Flow cytometric data showing the effect of treatment of k562 cells (106 cells/mL for 24 hourss) with WbTI-2 stained with annexinv and pi.

	LR Quadrant	*UR Quadrant*	*UL Quadrant*	*LL Quadrant*
Control cells (K562)	1.36% ± 0.12	2.18% ± 0.05	2.08% ± 0.04	94.38% ± 1.12
WbTI-2 treated cells (5 µg/mL)	16.18% ± 0.6	16.43% ± 0.07	7.28% ± 0.38	60.99% ± 1.16
Imatinib treated cells (10 µg/mL)	11.59% ± 0.55	22.37% ± 0.27	3.32% ± 0.08	62.71% ± 0.75

*LL quadrant= viable cells, LR quadrant = early apoptotic cells, UL quadrant = necrotic cells; UR quadrant = late apoptotic cells
*Data are expressed as percentage mean ± SEM for 4 independent

which cause resistance against imatinib are generally lack of dependence of CMC stem cells on BCR-ABL for survival.[5, 6] Mutations in the kinase domain of BCR-ABL and non-compliance of patient that causes emergence of resistant clones through suboptimal target inhibition.[7,8] Therefore studies are required to identify more potential curative products to overcome the limitations of such treatment.

Plant protease inhibitors are versatile proteins, which have been established to be influential in various biological processes like inflammation reaction, infection, extracellular matrix degradation, blood coagulation, programmed cell death, tumor invasion as well as cancer metastasis.[9,10] Soybean trypsin-chymotrypsin inhibitor has been found to inhibit ovarian sarcoma M5067 by increasing expression of Cx43 molecule.[11] It has also been found to induce apoptosis of LNCap cancer cell of colorectal cancer and HT-29 colon cancer cells.[11-14] Anti-proliferative effect has been shown from trypsin-chymotrypsin inhibitor (BWI-1) of buckwheat (*Freguagopyrum esculentum*) on acute T-lymphoblastic leukemia cells.[15] Cytotoxic activity could also be triggered by up regulation of proteins like Caspase-3, Caspase-9 in hepatoma like solid tumor cells, cervical carcinoma cells and esophageal squamosal carcinoma cells.[16]

Winged bean trypsin inhibitor is a kunitz type protease inhibitor with 182 amino acid residues. Cloning, functional expression and site directed mutagenesis study has been done and reported earlier by our group.[17] The single peptide protein has been found to have a blocked n-terminal end and is originated from intron less gene. This 20 kDa protein has no chymotrypsin inhibitory activity. The protein was fragmented by cyanogen bromide digestion and its largest fragment (about 65% of the original protein, which contained the RSL loop) has been found to retain about 50% of its trypsin inhibitory activity. Such retention of activity in a fragmented molecule implicates that it is an ideal candidate for consideration as a drug which by character should generally be small enough to ease targeted delivery. Moreover, winged bean is an eatable crop consumed as a vegetable in many parts of India. So, it is expected that a protein isolated from such a pod if is used as a drug would cause lesser lateral damage to patient. Thus the protein WbTI-2 appeared to have a great potential as a natural therapeutic agent in CML. Therefore the aim of this study is to assess the anti-tumor property of WbTI-2 on chronic myeloid leukemia cell line and determine its IC_{50} value.

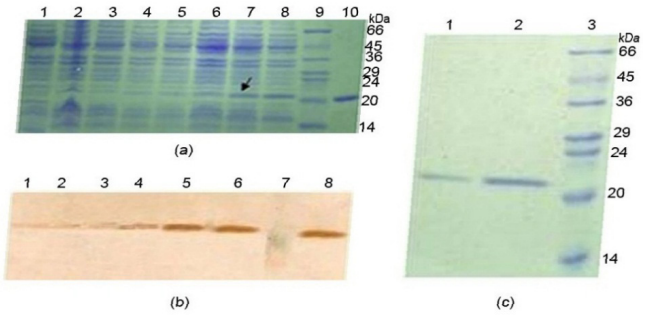

Fig. 1(a): showing expressed protein (WbTI-2) in 15% SDS-PAGE with varying amount of IPTG induction. [Lane 1: total protein profile of *E. coli* (BL21DE3) as negative control, Lane 2: expressed protein with no IPTG induction, Lane: 3 to Lane 8: IPTG induced expressed protein with 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM and 0.6 mM IPTG concentrations respectively, Lane: 9 marker, Lane 10: native protein purified from winged bean seeds]. **(b):** showing expressed protein (WbTI-2) in western blot analysis. **(c):** showing purified WbTI-2 after immune-affinity chromatography. Lane 1: WbTI-2 purified by immune-affinity Chromatography, Lane 2: Native protein, purified from winged bean plant, Lane 3: marker.

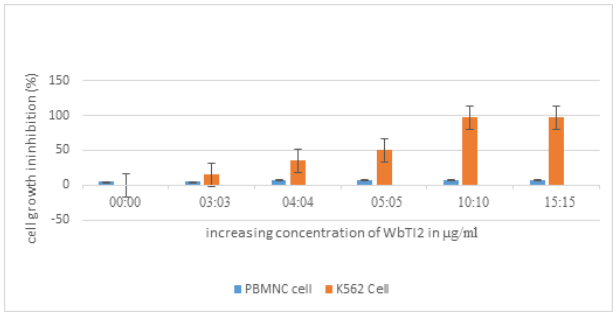

Fig. 2: Result of MTT Assay, showing growth inhibition of PBMNC cells and K562 cells on administration of WbTI-2.

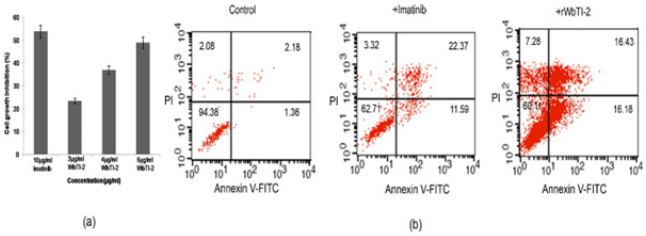

Fig. 3: analysis of cell viability by flow-cytometry **(a):** showing the cell growth inhibition of WbTI-2 treated K562 cell which increases with increasing quantity of protein and maximum inhibition is shown at 5 µg/mL concentration (K562 cells treated with 10 µg/mL of imatinib was taken as positive control). **(b):** The K562 cells treated with 5 µg/mL WbTI2 shows maximum number of dead cells compared to imatinib treated cells (10 µg/mL) and untreated control cells.

Methodology

Cell Culture of Human Chronic Myeloid Leukaemia K562 Cells

Human chronic myeloid leukaemia K562 cells are cultured in RPMI 1640 medium with 8 U/mL gentamycin sulphate, 0.0044 U/mL Pen-Step solution and 10% FCS in a 10% humidified CO_2 atmosphere at 37°C. PBMCs are grown in RPMI 1640 medium containing 8 U/mL gentamycin sulphate, 0.0044 U/mL Pen-Step solution under same temperature, humidity and CO_2 atmosphere. The culture are incubated for 24 hours under same temperature, humidity, and CO_2 conditions and the apoptotic behaviour of cells are observed by different methods.

Expression and Purification of Recombinant WbTI-2

WbTI-2 gene was cloned and was preserved previously by our group[17] which was cloned and expressed in expression vector. The expressed protein was purified by affinity chromatography.

The recombinant WbTI-2 gene was sub cloned into high expression vector pTrC99A and over expressed under varying concentrations of IPTG e.g., 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mM for 5 hours. Cells were boiled with SDS gel loading buffer and the amount of expression was analyzed on Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The maximum amount of expression of rWbTI-2 was obtained at 0.5m M IPTG (lane 7). Western blot analysis of expressed rWbTI-2 using rabbit anti-WbTI-2 antibody also indicated very low expression of rWbTI-2 in un-induced *E. coli* (Fig. 1b, lane 1) but gradual increase of expression as the IPTG concentration increased (lane 2-6).

Single step immune-affinity chromatography was done using rabbit anti-WbTI-2-sepharose column to purify WbTI2 from bacterial cell lysate. The yield of the purified recombinant protein was around 3.14 mg from 2.25 g wet cells (Table 1). Homogeneity of the purified WbTI-2 (lane 2) was ensured by SDS-PAGE (Fig. 1c) against native protein (molecular mass ~20 kDa, lane 1).

Trypsin Inhibition Assay by Purified WbTI-2

Trypsin assay was performed from increase in absorbance at 247 nm by using N-α-Tosyl-L-Arginine-Methyl Ester (TAME) as substrate.[18] For determining the inhibitory activity of WbTI-2, the molar ratio enzyme: inhibitor was varied from 1:0 to 1:5 (Fig. 2). At 1:2 enzyme: inhibitor molar ratio trypsin was completely inhibited by WbTI-2, which is also true for the native protein.[17]

Study for Reduction of Cell Viability after Treatment with WbTI-2 on K562 Cell Line

Simple Microscopic Study

2.7×10^5/mL of cells were treated separately with various amount of WbTI-2 (Table 2), 10 mg/mL imatinib (negative control), and incubated for 24 hours. The viable cells of all cultures were stained with trypan blue dye the cells were counted under simple microscope (40x objective lens) using hemo-cytometer. Positive control was set using only cell line Table 2.

MTT Assay

MTT assay was used to examine the effect of WbTI-2 on proliferation of K562 cells.[19] K562 cells and normal human PBMCs were treated with WbTI-2 for 24 hours. For treatment with WbTI-2, 100 µL and 1 mL the cell cultures (10^5 cells/mL) are taken in sterile 96 well microtiter plates and 24 well microtiter plates respectively, and various amount of protein (WbTI-2) are added to each of those wells. Colour intensity was measured at 590 nm. As shown in Fig. 3a, WbTI-2 inhibited the proliferation of K562 cells in a dose dependent manner (3, 4, 5 µg/mL) but there was no effect on the proliferation of PBMCs. The IC_{50} value was estimated to be 5 µg/mL for WbTI-2 by way of MTT assay.

Flow Cytometric Study

AppoAlert Annexin V-FITC Apoptosis kit (Clontech) was used for flow cytometric analysis.[20] The K562 cells (10^6 cells) in complete RPMI medium were treated with and without rWbTI-2 (5 µg/mL) for 24 hours. The cells were washed in 50 mM cold PBS at pH 7.4 and collected by centrifugation at 1000 rpm for 10 min at 4°C. After rinsing and re-suspending in binding buffer K562 cells were double labelled with Annexin V-FITC (0.1 mg) and Propidium Iodide (0.5 mg) for 15 minutes in dark at room temperature. Negative control was set by treating K562 cells with Imatinib (10 µg/mL). The relative percentage of live/apoptotic/necrotic cells were analysed in Becton Dickinson FACS Calibur single laser flow cytometer (NJ, USA) (Fig. 3a and b).

Statistical Analysis

Four independent sets of flow-cytometric experiments and three independent sets of MTT assay were performed. Data were expressed as percentage mean ± SEM for all independent experiments. Student's t-test was performed comparing test samples to respective controls. Statistical analysis was done using graph pad prism software (San Diego, USA). '*p*-value' of 0.05 was considered statistically significant.

Result

In this study recombinant winged bean trypsin inhibitor-2 was expressed from recombinant WbTI-2 molecule which was cloned earlier by our group. In SDS-PAGE analysis of cloned and expressed protein (Fig. 1a) the maximum amount of expression of rWbTI2 was obtained at 0.5 mM IPTG (Fig. 1a, lane 7). Western blot analysis of expressed rWbTI-2 also indicated very low expression of rWbTI-2 in un-induced *E. coli* (Fig. 1b, lane 1) but with gradual increase of IPTG, the concentration of expressed protein increased (Fig. 1b, lane 2-6). Single banded pure protein was obtained using affinity chromatography (Fig. 1 b, c). The purified protein was found to retain its complete trypsin inhibitory activity. The yield of the purified recombinant protein was around 3.14 mg from 2.25 g wet cells (Table 1). Homogeneity of the purified WbTI-2 was ensured by SDS-PAGE (Fig. 1c, lane 1) against native protein with molecular mass ~20 kDa, (Fig. 1c, lane 2). WbTI-2 was tested to have a significant effect on reducing the viability of K562 CML cells by MTT assay (Fig. 2) and also it was found to inhibit the proliferation of K562 cells in a dose dependent manner (3, 4, 5,10 and 15 µg/mL) but there was no effect on the proliferation of PBMC cells. IC_{50} value of the protein determined by MTT assay was 5 µg/mL. Simple microscopic study also revealed significant increase in number of dead cells with increasing concentration of WbTI-2 (Table 2). Flow-cytometric analysis revealed approximately 16.18% of WbTI-2 treated and 11.59% of Imatinib treated cells were undergoing apoptosis whereas the percentage of apoptosis in untreated cell group was only 1.36% (Fig. 3).

Discussion

Here three independent sets of MTT assay was carried out and the data were expressed as percentage mean ± SEM. Student's t-test was performed with every set of PBMNC and K562 cells (n=3) where significant inhibition of growth of K562 cells was found ($p < 0.05$) compared to PBMNC cells with increasing concentration of WbTI-2. Moreover the IC_{50} value determined by MTT assay (5µg/mL) implied that the protein is potent enough to kill CML cells even in quite low concentration (Fig. 2, table 2). In comparison to the untreated K562 cells, cells treated with both WbTI-2 as well as imatinib showed shift of distribution from viable (LL quadrant) to early apoptotic (LR quadrant) and then to late apoptotic stage (UR quadrant) in flow-cytometric analysis. This establishes the apoptosis inducing ability of WbTI-2 protein (Fig. 3b, Table 3). Student's t-test was performed with the percentage means obtained (n=4) where the reduction of number of viable cells on administration of both imatinib as well as WbTI-2 was found to be significant ($p < 0.05$) with respect to the un-treated control cells (Table 3). Thus the data reveals that the protein WbTI2 has significant therapeutic potential and could be a non-toxic and safer option for cancer therapy as the protein has been naturally sourced (winged bean plant). However, further attempt should be made to study the collateral effects of WbTI-2 on various other normal and cancerous cell types so that the mechanism of action on such effect can be thoroughly understood.

Conclusion

This study thus gives a clear indication that the trypsin inhibitory protein WbTI-2 from winged bean plant has an anti-tumor property on human CML cells with an IC_{50} value of 5 µg/mL.

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Conflict of Interest

The author declares that there is no conflict of interest related to this study.

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