RESEARCH ARTICLE

Antiproliferative Activity of *Lavatera cashmeriana*- Protease Inhibitors towards Human Cancer Cells

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Abstract

Background: Proteases play a regulatory role in a variety of pathologies including cancer, pancreatitis, thromboembolic disorders, viral infections and many others. One of the possible strategies to combat these pathologies seems to be the use of protease inhibitors. LC-pi I, II, III and IV (*Lavatera cashmerian*-protease inhibitors) have been found in vitro to strongly inhibit trypsin, chymotrypsin and elastase, proteases contributing to tumour invasion and metastasis, indicated possible anticancer effects. The purpose of this study was to check in vitro anticancer activity of these four inhibitors on human lung cancer cell lines. Material and Methods: In order to assess whether these inhibitors induced in vitro cytoxicity, SRB assay was conducted with THP-1 (leukemia), NCIH322 (lung) and Colo205, HCT-116 (colon) lines. Results: LC-pi I significantly inhibited the cell proliferation of all cells tested and also LC-pi II was active in all except HCT-116. Inhibition of cell growth by LC-pi III and IV was negligible. IC_{50} values of LC-pi I and II for NCIH322, were less compared to other cell lines suggesting that lung cancer cells are more inhibited. Conclusion: These investigations might point to future preventive as well as curative solutions using plant protease inhibitors for various cancers, especially in the lung, hence warranting their further investigation.

Keywords: Proteases - *Lavatera cashmeriana* - protease inhibitors - anticancer activity

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Introduction

Proteases, also known as proteolytic enzymes, are enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds. Until fairly recently, proteases were considered primarily to be protein-degrading enzymes. However, this view has dramatically changed and proteases are now seen as extremely important signaling molecules that are involved in numerous vital processes. Protease signaling pathways are strictly regulated, and the dysregulation of protease activity can lead to pathologies, important being cancer. The dysregulated proteases cause the dysregulation of cell cycle, apoptosis, cell growth and activation, cell-cell adhesion, cellular interactions and signal transduction. This all suggest that proteases are Hallmarks of cancer. Mammalian proteolytic enzymes are divided into five classes (aspartic, cysteine, metallo, serine and threonine), and the serine protease (SP) family is the largest (Rawlings et al., 2006). It is widely accepted that SPs degrade extracellular matrix and facilitate neoplastic progression. The trypsin, one of the representative member of SPs, is involved in colorectal carcinogenesis and promotes proliferation, invasion, and metastasis (Uchima et al., 2003; Yamamoto et al., 2003; Soreide et al., 2006; Matěj et al., 2007). Trypsin activates, and is co-expressed with matrix metalloproteases (MMPs), which are known to facilitate invasion and metastasis (Nyberg et al., 2002; 2006). Polymorphonuclear leukocyte elastase (referred to as elastase) disintegrates matrix proteins (Barrett, 1981), implicating this enzyme in cancer cell invasion and metastasis. Increased levels of elastase have been shown to be strongly associated with recurrence and death in breast cancer patients (Foekens et al., 2003) suggesting that elastase could have a role in tumor progression leading to metastasis in breast cancer (Queen, 2005; Akizuki et al., 2007; Mittendorf, 2012). Thus one possibility of cancer treatment is to suppress the activity of proteases (SPs and MMPs) that play an important role in tumour invasion and metastasis (Jedina and Maliar, 2005; Hunt et al., 2013). Therefore, proteases present a promising strategy for anticancer and antimetastatic therapy.

Protease inhibitors being found to be as special agents in anticancer therapy, have been isolated from plants (Khan et al., 2008; Tochi et al., 2008) or bacteria (Hossain et al., 2007) as their natural source. Its main feature includes their ability to form strong protease-PI complex, inhibiting the protelytic activity. PIs are mostly isolated from plants suggesting plant PIs may open a new anticancer strategy

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in future. In this communication we have tried to explore the anticancer activity of potease inhibitors isolated from *Lavatera cashmeriana* seeds. *Lavatera cashmeriana* is a medicinal plant widely used in traditional folk medicine (Kaul, 1997). Four protease inhibitors viz LC-pi I, II, III and IV were purified from seeds of *Lavatera cashmeriana*. These four inhibitors inhibited trypsin, chymotrypsin and elastase in vitro (Rakashanda et al., 2013) and antibacterial activity against *Klebsiella pnuemoniae* and *Pseudomonas aeruginosa* (Rakashanda et al., 2012). To explore the application of all LC-pis as antitumor agents, we examined their suppressive effects on various cell lines. In this article, we report on their capacity to suppress the growth of cells, such as THP-1, NCIH322, Colo205 and HCT-116

Materials and Methods

Inhibitors and chemicals

LC-pis were purified from the seeds of *Lavatera cashmeriana*, as described previously (Rakashanda et al., 2012). RPMI-1640 medium (#N 3520), rhodamine-123 (Rh-123) (#R 8004), penicillin (#P 3032), streptomycin (#S 9137), fetal bovine serum (# 7524), mitomycin (#M 4287), sodium bicarbonate (#S 5761), phosphate buffer saline (PBS) (#P3831), sulphorhodamine (SRB) (#1402), trypsin (#T 4799), paclitaxel (#T 7402), 5 fluorouraci l (5-FU) (#F 6627), gentamycin sulfate (#G 1264) were procured from SigmaeAldrich. Trichloroacetic acid was purchased from Merck Specialties Private Ltd.

Cells and cell culture

Human acute monocytic leukemia cell line (THP-1) was procured from National Centre for Cell Sciences (NCCS), Pune, India. Human lung carcinoma cell line NCIH322 and human colon cancer cell lines (HCT-116 and colo-205) were obtained from National Cancer Institute, Frederick, U.S.A. All the cells that were used were grown in RPMI-1640/MEM medium containing 10% FCS, 100 unit Penicillin/100 μg Streptomycin per ml medium. Cells were allowed to grown in CO_2 incubator (Thermo Scientific USA) at 37°C with 98% humidity and 5% CO_2 gas environment. The cells used for the assay were in logarithmic phase.

Determination of suppressive activity of protease inhibitors

The suppressive activity of inhibitors was evaluated according to the soluble SRB assay (Hoghton et al., 2007). Briefly, required μl of a cell suspension containing required cells/ml was added to each well in a 96-well microtiter plate and was cultured in RPMI-1640 mediums previously stipulated. The cells were allowed to adhere overnight, and then media containing purified inhibitors at different concentrations were added. Originally 30, 50, 70 and 100μg/ml of all four test samples were used. Also peclitexal and 5- Fluorouracil were used as positive controls. The plates were assayed after 48hrs. The cells were fixed by adding 50μl of ice-cold 50% TCA to each well for 60min in case of adherent cells. The plates were washed five times in running tap water and stained with 100ml per well SRB reagent (0.4% w/v SRB in 1% acetic acid for 30min). The plates were washed five times in 1% acetic acid to remove unbound SRB and allowed to dry overnight. SRB was solublized with 100μl Tris-base (10mM) per well, shaken for 5min and the OD was measured at 570 nm with reference wavelength of 620nm. Further, the IC_{50} values in the presence of purified inhibitors on the cancer cells of different tissue origin used for screening were determined by non-linear regression analysis using Graph Pad Software (2236 Avenida de la Playa La Jolla, CA 92037,USA).

Statistical analysis

The experiments were performed at least three times, independently. A logistic non-linear regression model was fit to the data using using Graph Pad Software to calculate the IC_{50}. The data obtained were expressed as 'mean±standard deviation'. A value of $p<0.05$ was considered as significant.

Results

LC-pi I and II were found to significantly inhibit the in vitro growth of THP-1, NCIH322, Colo205, whereas only LC-pi I inhibited growth of HCT-116 cells but not LC-pi II in a dose dependent manner. Further LC-pi III and IV could not inhibit the growth of these cells (Table 1). The calculated IC_{50} values are given in Table 2. IC_{50} values suggest that NCIH322 lung cancer cells are strongly inhibited by both inhibitors at lower concentrations in comparison to other cell lines. Their pharmacological effects may be mediated through inhibition of the protease activity and the subsequent modulation of the protease pro-survival signaling as LC-pi I and II strongly inhibited the in vitro activity of trypsin, elastase and chymotrypsin

Table 1. Cytotoxic Activity (%age growth inhibition) of LC-pi I, II, III and IV at Different Concentration Against Various Human Cancer Cell Lines

	Tissue Type		Leukemia	Lung	Colon	
	Cell Type		THP-1	NCIH322	Colo205	HCT-116
S.NO	Code	Conc(μg/ml)				
1	LC-pi I	100	**74±2**	**81±3**	**70±3**	**70±2**
		70	**65±2**	**73±3**	**60±2**	**65±3**
		50	**52±3**	**63±2**	**40±2**	**55±2**
		30	**46±2**	**60±1**	**30±1**	**41±4**
2	LC-pi II	100	**60±1**	**70±2**	**52±2**	25±2
		70	**50±2**	**65±1**	**50±1**	14±1
		50	37±2	**50±1**	49±1	11±1
		30	1±0	**35±2**	27±2	2±1
3	LC-pii III	100	36±2	28±2	23±2	39±3
		70	16±2	19±3	13±2	33±2
		50	6±3	15±3	8±2	24±3
		30	1±0	5±2	6±2	7±3
4	LC-pi IV	100	27±1	36±3	37±2	25±1
		70	25±1	32±3	30±2	18±1
		50	25±1	15±3	27±2	11±2
		30	22±1	18±2	38±2	4±2
5	5-FU	20µM	**84±3**	**72±3**	**71±1**	**50±1**
6	Paclitaxel	1µM	**71±2**	**50±1**	**98±2**	**72±3**

*The bold values are shown for those compounds which have proved to be active and those in normal font represent least significant. Results are mean±SD of three separate experiments, conducted in triplicate

Table 2. IC_{50} Values of LC-pi I, II, III and IV for Growth Inhibition of Various Human Cancer Cell Lines

Cell lines	LC-pi I	LC-pi II
THP-1	46±2	70±3
NCIH322	36±2	51±3
Colo205	60±3	68±2
HCT-116	43±1	NI[a]

*Results are mean ±SD of three separate experiments, conducted in triplicate. [a]NOT INHIBITED, [b]IC_{50} values are in µg/ml

(Rakashnada et al., 2013)

Interestingly several plant protease inhibitors have been reported as anti-proliferative, apoptosis inducing and chemopreventive agents against human cancer cell lines. The present study has been intended to explore the suppressive activity of LC-pis on various cancer cells. LC-pi I and II having anticancer activity may be further supported by anticancer activity of plant protease inhibitors viz., *Cicer arietinum* inhibitors and other Plant derived inhibitors inhibiting proliferation of breast and prostate cancer cell lines (Mageea et al., 2012), IBB1 and IBBD2, inhibitors from soybean (*Glycine max*) inhibiting proliferation of HT29 human colorectal cancer cells (Clemente et al., 2010), also TI1B, a major Bowman-Birk isoinhibitor from pea (Pisum sativum L.) on HT29 colon cancer cells (Clemente et al., 2012) and antitumor Protease Inhibitor from Mini-Black Soybean (Ye and Ng, 2011). These evidences have made the plant protease inhibitors to be considered as neutraceutical proteins, owing to prevention and treatment of cancer (de Mejia and Dia, 2010).

Discussion

In conclusion, LC-pi I and II may have a potential application as anticancer agents. Nevertheless, a better understanding on the detailed mechanism of how LC-pi I and II exert its suppressive activity would be valuable, thus warrants further investigations.

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