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Original Article

CATIONIC PEPTIDE LACTOFERRICIN B INHIBITS GLUTATHIONE S-TRANSFERASE P1 FROM HUMAN PLACENTA AND BREAST CANCER CELL LINE MDA-MB-231 PREVENTING ANTICANCER DRUG METABOLISM

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ABSTRACT

Objective: To investigate the interaction of Lfcin B (Lactoferricin B) with GSTP1 (Glutathione S-Transferase P1) from human placental and breast cancer cell line MDA-MB-231.

Methods: We examined the interaction of Lfcin B with human placental GSTP1 and breast adenocarcinoma MD-MB-231 cell line. Enzyme activity of GSTP1 was measured with and without pre-incubation with Lfcin B. Kinetic variables were determined by incubating the enzyme reaction mixture with fixed GSH (reduced glutathione) concentration and varying CDNB (1-chloro-2, 4-dinitrobenzene) concentrations or fixed CDNB concentration and varying GSH concentrations.

Results: Lfcin B is a competitive inhibitor with respect to GSH binding site (G site) and noncompetitive inhibitor with respect to hydrophobic substrate unit (H site) of human placental GSTP1 enzyme. Lfcin B was also incubated with GSTP1 from breast adenocarcinoma MDA-MB-231 cell line. The activity of GSTP1 was much higher (0.2665 µ mol/ml/min) in Lfcin B untreated MDA-MB-231 cell line, whereas MDA-MB-231 with Lfcin B treatment showed a very low activity (0.0254 µ mol/ml/min).

Conclusion: Our Findings suggest that Lfcin B can inhibit the GSTP1 activity in human placental and MDA-MB-231 breast cancer cell lines, which may induce synergistic effects when used in combination with antineoplastic drugs that are substrates of GSTP1 enzyme. This combination will exert a double attack on cancers over expressing GSTP1, first sensitizing them to anticancer drugs by preventing their metabolism.

Keywords: Cationic peptide, Lactoferricin B, Glutathione S Transferase P1, Enzyme activity, Inhibitor.

INTRODUCTION

Glutathione *S*-transferases (GSTs) belong to a family of phase II drug metabolizing enzymes that catalyzes the conjugation of tripeptide glutathione (GSH) with electrophilic compounds resulting in the formation of corresponding conjugates [1]. The active site of GSTs exists as dimer with two functional regions; a hydrophobic site (H-site) where, electrophilic substrates bind and a hydrophilic GSH binding expression site (G-site) [2]. GSTs, especially Glutathione S-transferase P1 (GSTP1) isoform have been reported to play an important role in the development of tumoral drug resistance [3]. GSTP1 isoform has been reported to in a variety of cancer including, breast, lungs, ovary, pancreas, melanoma, brain, kidney, colon and leukemia [4-12] Beside exogenous and endogenous toxic compounds, several chemotherapeutic agents including, cisplatin, chloroambucil, doxorubicin, melphalan, cyclophosphamide, ifosphamide and thiotepa have been demonstrated to be the substrates of GSTP1 [13]. During the last decade, non-detoxifying functions of GSTP1 have emerged, providing the enzyme with significant biological importance. GSTP1 has been shown to interact with different proteins and modulate signaling pathways controlling proliferation, apoptosis and differentiation. For example, GSTP1 binds to and inhibit c-Jun-N-terminal kinase (JNK) thus, playing a significant role in apoptosis and cell signaling [14]. These regulatory functions of GSTP1 suggest why cancers over expressing GSTP1 show drug resistance towards drugs that are not its substrates. Recently GSTP1 has been demonstrated to undergo phosphorylation by protein kinase C (PKC) and epidermal growth factor receptor (EGFR), leading to increase in catalytic efficiency of GSTP1 which further contributes to drug resistance. [15-17]. Though consequences of non-enzymatic functions of GSTP1 are still being elucidated, there is enough evidence to suggest that pharmacological inhibition of GSTP1 may be useful for the treatment of cancer. GSTP1 inhibitors presently under pre-clinical and clinical trials include ethacrynic acid, TLK-286, TLK 199, 6-(7-nitro-1,2,3-benzoxadiazol-4-ylthio) hexanol (NBDHEX), 4-aminobenzoic acid/nitric oxide (PABA/NO) and bromosulfophthalein [18]. TLK-286 and NBDHEX have also have been demonstrated to block the interaction of GSTP1 with JNK leading to sensitization of cancer cells to alkylating agents [19, 20]. In spite of various inhibitors there is a need of new inhibitors since toxicity of the reported compounds limit their use in clinic.

During the last decade, peptide has been established as an effective therapeutic option for the treatment of cancer [21]. Bovine lactoferricin (Lfcin B) is a cationic amphipathic peptide produced by acid pepsin hydrolysis of bovine lactoferrin (bLF) and reported to exhibit anticancer, antimicrobial and antifungal properties [22]. Burrow *et al.* showed interaction of selenium saturated bovine lactoferrin (Se-bLF) with [(GSH)/GPx (Glutathione Peroxidase/Glutathione Reductase) GR/GST (Glutathione S-Transferase) [23]. However, there is no report examing the interaction between Lfcin B and GSTP1. In this study, we reported that Lfcin B is a competitive inhibitor of human placental GSTP1 also we had shown Lfcin B decreases the GSTP1 activity in MDA-MB-231 breast cancer cell lines and could be developed as an alternate to existing inhibitors. Finding suggest that Lfcin B may exert a double-barrel attack on cancer cells; first by acting as an anticancer agent itself and secondly by inhibiting GSTP1, thereby, sensitizing cells to chemotherapeutic drugs that are either substrates of GSTP1 or induce apoptosis by activating JNK.

MATERIALS AND METHODS

Materials

Reduced glutathione (GSH), human placental glutathione *S*-transferase P1 (hp-GSTP1) and 1-chlro-2, 4-dinitro benzene (CDNB) were purchased from Sigma Aldrich (New Delhi, India). Lfcin B (with sequence RRWEWRMKKLG), was custom designed at BioConcept Lab Pvt. Ltd. (IMT Manesar, Gurgaon, India.) MDA-MB-231 cell,

culture media, fetal calf serum and Hank's basal salt solution was brought from Institute of Nuclear Medicine and Allied Sciences (New Delhi, India). All other reagents were purchased from Sigma-Aldrich unless otherwise stated.

Methods

GSTP1 enzymatic assay

GSTP1 activity assay was determined following the method of Habig *et al.* 1974 by measuring GSH conjugation with CDNB [24]. Briefly, the reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.8), 1 unit/ml hp-GSTP1 or 25µg protein from cell lysates, GSH and CDNB. The rate of product formation was monitored by measuring the change in absorbance at 340 nm using a Shimadzu UV 1800 Spectrophotometer. The enzyme activity was calculated after correction for non enzymatic reaction. The molar absorption coefficient for CDNB was $\Delta\epsilon 340 = 9.6\ mM^{-1}\ cm^{-1}$.

Determination of steady-state kinetic parameters

Steady-state kinetics of GSTP1 was carried out at a constant GSH concentration of 2.5 mM, by varying CDNB concentration (0.5-4.0 mM) keeping CDNB concentration at 1.0 mM and varying GSH concentration (0.125-2.5 mM). All assays were performed in 0.1 mM potassium phosphate buffer (pH 6.8) containing 1 unit/ml hp-GSTP1 in the presence or absence of Lfcin B. Experimental data were plotted with Line weaver-Burk plot and kinetic parameters determined using the Michaleis-Menten equation.

Kinetics of enzymatic inhibition

A reaction mixture containing 0.1 M potassium phosphate buffer (pH 6.8), 1.0 unit/ml hp-GSTP1 and 1 µM Lfcin B was prepared and incubated in a water bath set at 37 ºC for enzyme activity. Aliquots (350 µl) were withdrawn for enzyme activity assay and the remaining solution transferred back to the water bath. Aliquots were withdrawn at 1,5,10,15,30,45 and 60 min mixed with 125 µl of 10 mM GSH and 50 µl of 10 mM CDNB and GSTP1 activity determined spectrophotometrical as described above.

Inhibition of GSTP1 activity in cells

MDA-MB231 cells were cultured in serum free DMEM/F12 (Dulbecco's Modified Eagle's medium) media in 100-cm2 dishes. They were treated with 100 µM Lfcin B for 6 h. After treatment cells were harvested using a cell scraper, lysed in 1 ml of 50 mM Tris-Hcl buffer (pH 7.4) containing 1 mM DTT (Dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride), 100 µM Na3VO4 and 2 µg/ml aprotinin and centrifuged at 20,000 x g for 20 min at 4 ºC. Supernatants were collected and protein concentration determined by Lowry's method. Another set of cultures dishes were similarly set but without Lfcin B treatment, which served as a control. GSTP1 activity in the cell lysates was measured using CDNB and GSH as substrates, as described above and expressed as n mol/min/mg protein.

Total protein determination

Total protein contents for each samples in the particle-free supernatants were determined spectrophotometrically according to the Folin-Ciocalteu reagent (FCR) method described by Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. Absorbance at 660 nm against the blank measured. A standard graph drawn, the amount of protein in the sample calculated and results expressed as µg protein/g sample.

Statistical analysis

Enzyme activity data curves were fitted using a non-linear regression program (GraphPad Prism, San Deigo, CA, USA) to calculate the maximum velocity of substrate formation (*Vmax)* and the Michaelis constant (K_M) for substrate affinity determined using the following equation:

$$Y = (Vmax)\ (S)/(Km+S)$$

Where; Y represents enzyme activity (µmol/min/mg) and S represents substrate concentration (mM).

RESULTS

Kinetic profiling of human placental GSTP1 in response to Lfcin B

The Km values for GSH (Varied) and CDNB (varied) were found to be 0.75±1.0 and 0.63±0.59 mM, respectively. The Vm values were varying according to the fixed substrate. When CDNB was fixed substrate (1 mM), Vm value obtained was 0.75±0.44 U/mg protein but when GSH is the fixed substrate (1 mM), a lower value for Vm was obtained: 0.38±0.14 U/mg protein (table 1).

Table 1: The kinetic parameters of substrates and Lfcin B inhibition of GSTP1

Substrates	Vm (U/mg protein)	Km, mM
Varying GSH	0.75±0.44	0.75±1. 0
Varying CDNB	0.38±0.14	0.63±0.59

The total protein content of MDA-MB-231 cell line of treated and untreated with Lfcin B determined spectrophotometrically by Lowry's method were 9.3 µg and 7.3 µg respectively. The GSTP1 enzyme activity in MDA-MB231 treated with Lfcin B and without any treatment showed a difference. The activity of GSTP1 was much higher (0.2665 µ mol/ml/min) in control (Lfcin B untreated) cell line, whereas MDA-MB231 with Lfcin B treatment showed a very low activity (0.0254 µ mol/ml/min). (table 2).

Table 2: Total protein and GSTP1 activity in MDA-MB231 cells in untreated and Lfcin B treated groups

Cancer Cell line groups	MDA-MB231 (Untreated)	MDA-MB231 (Lfcin B treated)
Total Protein	7.3 µg	9.3µg
GSTP1 Activity	0.2665 µ mol/ml/min	0.0254 µ mol/ml/min

DISCUSSION

In recent years, studies of GSTs have revealed new roles for some of the members of this family. It has been demonstrated that GSTs of classes' alpha, mu, and pi are involved in cell proliferation, differentiation and control of cell death via interactions with special signaling proteins [22, 23, 30]. In particular, GSTP1 is over expressed in some cancer cells and it has been reported to be responsible for tumoral drug resistance [25] Therefore, the use of inhibitors to suppress the GSTP1 activity in cancer cells is a promising method to overcome such drug resistance. New GSTP1 inhibitors with better efficacy are required. Recently peptides have been reported to exert anticancer effects [26]. Examples Lfcin B and Lactoferrin have various anticancer properties [27, 28]. Here we report Lfcin B is the competitive inhibitor (with respect to G site) and non-competitive inhibitor (with respect to H site) of GSTP1 enzyme (fig. 1), (fig. 2) and (fig. 3). In Lactoferricin interaction with human placental GSTP1 the differences in Vm values could be explained by the controversial effects of the substrates on GSTP1; high GSH converts GSTP1 to a more active conformation but high CDNB to a less active conformation. In addition the treatment of Lfcin B with breast cancer MDAMB231 cells, we noted a difference

between the GSTP1 levels supporting our hypothesis that Lfcin B inhibits the GSTP1 activity as shown invitro human placental GSTP1 and in cell line MDA-MB-231. Lfcin B is formed by acid pepsin hydrolysis of Lactoferrin naturally occurring in stomach. Lactoferrin have not been known for any major side effects. In very high doses diarrhea, skin rash, loss of appetite, fatigue, chills, and constipation have been reported [29]. We hypothesize that Lfcin B can modulate GSTP1 by three proposed pathways (1) by directly interacting with GSTP1 and inhibiting its active site, (2) by inhibiting GSTP1 binding with JNK and cJun, (3) by inhibiting GSTP1 complex formation with TNF receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase (ASK) (fig. 4). Activation of GSTP1 has been shown to be involved in anticancer drug resistance [30]. JNK has been shown to be associated with stress response, apoptosis, inflammation, and cellular differentiation and proliferation [31]. Moreover ROS and other stress response lead to JNK activation and phosphorylation of cJun which is involved in development of chemo sensitization (fig. 4). It has been found that GSTP1 inhibits (TRAF2) activation of JNK and p38 Mitogen associated protein kinase (p38-MAPK) [32]. Furthermore GSTP1 attenuates TRAF2-ASK induced apoptosis [32]. We think that Lfcin B might inhibit GSTP1 interaction with TRAF2 and ASK (fig. 4). Further studies are required to study the detailed mechanisms of this proposed hypothesis.

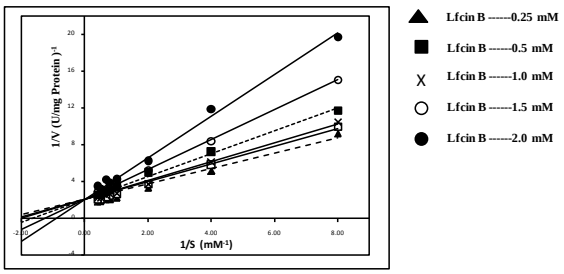

Fig. 1: GSTP1 inhibiting by Lfcin B at 1.0 mM CDNB and varying GSH concentration (0.125-2.5 mM)

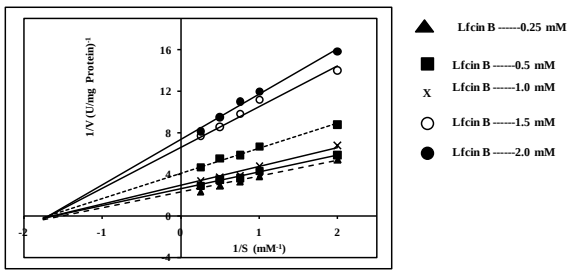

Fig. 2: GSTP1 inhibiting by Lfcin B at 2.5 mM GSH and varying CDNB concentration (0.5-0.4 mM)

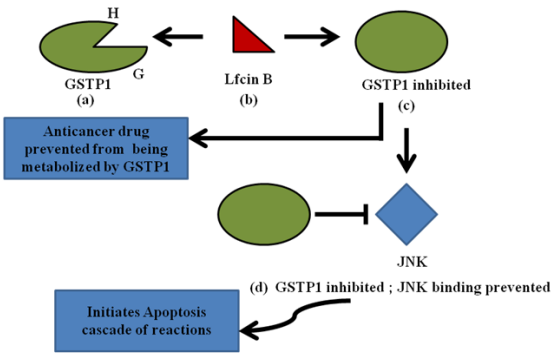

Fig. 3: (a) GSTP1 molecule showing H and G: (b) Lfcin B peptide: (c) Lfcin B is a competitive inhibitor (with respect to G site) and non competitive inhibitor (with respect to H site) of GSTP1 enzyme; (d) Inhibited GSTP1 prevented from binding with JNK

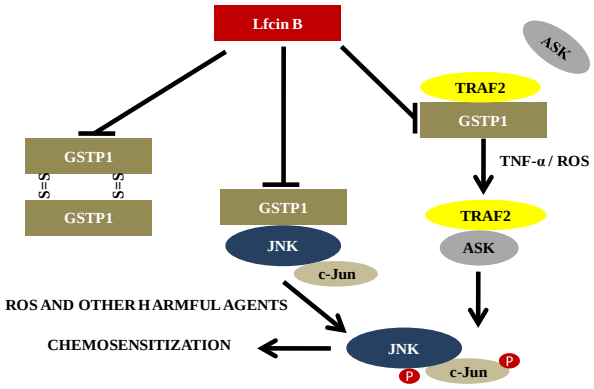

Fig. 4: Proposed role of Lfcin B in inhibiting GSTP1 signaling pathways

CONCLUSION

Our findings suggest that Lfcin B can be used in combination with drugs known to be substrates of GSTP1 enzyme. This combination will exert a double attack on cancers overexpressing GSTP1, first sensitizing them to anticancer drugs by preventing there metabolism and secondary Lfcin B themselves will act as an anticancer peptide.

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CONFLICT OF INTERESTS

Declared None

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