

## *In-vivo* Inhibition of Glutathione S-Transferase P1 by Bovine Lactoferricin in Swiss Albino Tumor Bearing Mice

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### ABSTRACT

Many cancers overexpress GSTP1 which contributes to multidrug resistance via conjugation of antineoplastic drugs. The aim of this paper is to investigate the in-vivo interaction of GSTP1 and Lfcin B in Swiss albino female mice induced with skin cancer using 7, 12 dimethylbenz (a) anthracene. Group B and C received topical application of DMBA, whereas group C was subcutaneously injected with 200 $\mu$ l (20 $\mu$ g/ $\mu$ l) Lfcin B while keeping group A as a control. The activity of GSTP1 in all the groups was then estimated spectrophotometrically at 340nm by taking the increase in absorbance of GS-DNB conjugate. Administration of 200 $\mu$ l (20 $\mu$ g/ $\mu$ l) Lfcin B three times per week was found to significantly ( $p < 0.01$ ) inhibit GSTP1. GSTP1 was observed to be overexpressed in group B (treated with DMBA only as skin carcinogen). Significant differences ( $P < 0.01$ ) were observed between group A (control) and C (DMBA+ Lfcin B group) indicating in vivo inhibition of GSTP1 by Lfcin B. We concluded that Lfcin B competitively inhibits GSTP1 in-vivo. Accordingly, Lfcin B may emerge as a suitable pharmacological agent that can selectively target GSTP1 thereby inducing cancer cells to apoptosis.

### INTRODUCTION

Glutathione S-transferases (GST) consists of a family of enzymes with various functions i.e. xenobiotic detoxification. GSTP1 which comes in the Pi class of GST is found to be overexpressed as well as contributes to multidrug resistance by the conjugation of therapeutic drugs. Furthermore, GSTP1 have antiapoptotic properties as it interacts with JNKs (c-jun NH2-terminal kinase) which is a key regulator of programmed cell death. This marks GSTP1 a promising chemotherapeutic agent. (Federici *et al*; 2009). Glutathione S-transferase pi (GSTP1) is an essential enzyme which is categorized in Glutathione S-Transferase family. Their main function is to perform

detoxification reaction of xenobiotic compounds thus it inactivates various electrophilic carcinogens through conjugating glutathione reduced (Toffoli *et al*; 1992; Jeronimo *et al*; 2001). GSTP1 takes part in various detoxification reactions by the catalysis of conjugation of many electrophilic and hydrophobic compounds with glutathione reduced (Jeronimo *et al*; 2001). GSTP1 is found to be overexpressed in variety of cancers such as breast, bladder, pancreas, colon, stomach, lung, neck, head, cervix, ovary and various soft tissues such as sarcoma, carcinoma, of testicles, glioma and meningioma (Niitsu *et al*; 1989; Randall *et al*; 1990; Kantor *et al*; 1991; Satta *et al*; 1992; Green *et al*. 1993; Inoue *et al*; 1995; Bentz *et al*; 2000; Trachte *et al*; 2002; Simic *et al*; 2005; Arai *et al*; 2006). The function of GSTP1 is to metabolize various xenobiotics and to involve with susceptibility to cancer and other diseases. Various reports show that overexpression of GSTP1 is linked to acquired resistance to various chemotherapeutic drugs (Wang *et al*; 2008).

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GSTP1 has a variety of substrate including endogenous compounds as well as antineoplastic drugs such as cisplatin, chlorambucil interact with difference and control the signaling pathways such as proliferation and apoptosis and differentiation. GSTP1 interact and inhibit c-Jun-N-terminal kinase (JNK), a transcription factor which is having a specific role in apoptosis and cell signaling. Protein kinase C (PKC) and epidermal growth receptor (EGFR) phosphorylates GSTP1 which results in increased catalytic activity of the GSTP1 and subsequently responsible for drug resistance (Rabindran *et al*; 2004; Singh *et al*; 2010; Okamura *et al*; 2009). Recently we have demonstrated the interaction of Lactoferricin B (Lfcin B) with GSTP1 from human placental and breast cancer cell line MDA-MB-231. The findings suggest that Lfcin B inhibits 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 the GSTP1 enzyme (Khan and Taneja, 2015).

In this current research, we have investigated the in vivo interaction of GSTP1 and Lfcin B in Swiss albino female mice induced with skin cancer using 7, 12 dimethylbenz (a) anthracene.

## MATERIALS AND METHODS

### Animals

Female Swiss Albino mice 5-8 weeks old, weighing 20-35g were purchased from Luvas, Haryana, India. The animals were maintained in the Central Animal House, Sharda University. The animals were housed in polypropylene cages and provided standard pellet diet and water ad libitum and maintained under controlled conditions of temperature and humidity, with a 12 h light/ dark cycle.

### Chemicals

Reduced glutathione (GSH) was purchased from calbiochem (India), 1-chloro-2, 4-dinitro benzene (CDNB) were procured from Sigma Aldrich (India). Lfcin B (with sequence RRWEWRMKKLG), was designed at Bioconcept Lab Pvt Ltd. IMT Manesar, Gurgaon, Haryana, India.

### Experimental protocol and treatment

A total number of 30 animals were divided equally into 3 groups i.e. A, B and C. Each group consists of 10 animals. Group A was used as a control and was given 200µl of acetone as a topical application on shaved skin. Group B and C animals were applied with 200µl of DMBA (0.025µg/µl) per animal three times per week as a topical application on the shaved skin. Group C animals were also injected subcutaneously with 200µl of Lfcin B (20µg/µl) after a gap of 30 minutes after topical application of DMBA.

Group A (10 animals) (Control) - Treated with acetone only

Group B (10 animals) - Treated with DMBA alone.

Group C (10 animals) -Treated with DMBA+ Lfcin B

### Preparation of Tissue Homogenate

Liver and kidneys were carefully excised from the animals, washed in a phosphate buffer saline (PBS) and weighed. A homogenate of the tissues was made in 100mM potassium phosphate buffer (pH 6.8) and was used for the estimation of glutathione S-transferase P1 (GSTP1).

### Determination of enzymatic activity

The activity of GST enzyme was estimated by the protocol described by Habig *et. al*; 1974. The reaction mixture consists of 1000µl of 100mM potassium phosphate buffer (pH 6.8) , 100µl of 1-chloro-2, 4-dinitrobenzene (CDNB), 100µl of homogenate and 1700µl of D.W. was incubated at 37°C for 5 min. The reaction was then started by the addition of 1000µl of glutathione. The increase in absorbance was followed for 3 minutes at 340 nm.

### Total protein

Total protein concentration was calculated according to the protocol described by Lowry *et al*; 1951 using Folin-Ciocalteu reagent (FCR). Bovine serum albumin was used as a standard. Absorbance was recorded at 660nm and the amount of protein was calculated using the standard curve and expressed as µg/ml.

### Statistical Analysis

The investigational results acquired are expressed as mean  $\pm$  standard deviation (SD). The data was imperiled to one-way analysis of variance (ANOVA) and differences between samples were determined by Tukey multiple comparison tests using the SPSS 22. The level of significance was set at  $p < 0.01$ .

## RESULTS AND DISCUSSION

The outcomes of the study show that Lfcin B possesses chemoprotective effect. Administration of DMBA has produced 100% tumor incidence in Group B (DMBA alone treated group) at the end of the 16<sup>th</sup> week while only 20% developed tumors in the Group C (DMBA+ Lfcin B) treated group. The size of the tumors in the group C was found to be significantly ( $P < 0.01$ ) reduced than those treated by Group B. The tumor volume of DMBA alone was found to significantly ( $P < 0.01$ ) higher than those group C. The tumor burden and total number of tumor were also found to be higher in group B in comparison to group C. (Table 1). The results of the analysis of GSTP1 are presented in table 2. Lowest level of GSTP1 was observed in group C (DMBA + Lfcin B treated group). A significant difference was observed between all the group at  $P < 0.01$ . This is due to the fact that the GSTP1 is inhibited in vivo by Lfcin B in group B and C (DMBA alone and DMBA + Lfcin B treated group respectively) and GSTP1 was overexpressed in the group B (treated with DMBA only). In the liver of the experimental mice, GSTP1 in group C was found to be lower than the other groups (group A and B) presented in table 2.

**Table 1:** The impact of Lfcin B on various tumor parameters in DMBA treated mice.

Group	Tumor Incidence	Tumor size (cm)	Tumor volume (cm <sup>3</sup> )	Tumor burden (cm <sup>3</sup> )	Total number of tumors
Group B	100% (10/10)	0.60±0.	0.36±0.40 <sup>a</sup>	3.43±4.16 <sup>a</sup>	7.70±3.89 <sup>a</sup>
Group C	20% (2/10)	0.12±0.15 <sup>b</sup>	0.004±0.005 <sup>b</sup>	0.005±0.007 <sup>b</sup>	0.50±0.71 <sup>b</sup>

Values are expressed as mean±SD (n=10). The control Group (Group A) was compared against treated groups: DMBA alone (Group B) and DMBA + Lfcin B (Group C) "a" and "b" are the superscript denoting statistical significance.

**Table 2:** Hepatic and Renal GSTP1 and total protein values after Lfcin B inhibition.

	Group A (μmol/ml/min)	Group B (μmol/ml/min)	Group C (μmol/ml/min)
Hepatic GSTP1 inhibition by Lfcin B	174.69 ± 2.19*	207.95 ± 1.48**	135.17 ± 2.61***
Renal GSTP1 inhibition by Lfcin B	215.68 ± 1.40*	352.28 ± 1.46**	141.65 ± 1.68***
	Group A (g/ml)	Group B (g/ml)	Group C (g/ml)
Total hepatic protein content.	4.65*	4.38***	4.59***
Total renal protein content.	3.37*	2.97**	3.33***

Values are articulated as mean ± SD (n=5). Values with dissimilar asterisk differ significantly at p<0.01. Group A (control) was compared against treated groups: Group B (DMBA alone) and Group C (DMBA+ Lfcin B)

Normal expression was seen in the control group (group A) whereas high expression was observed in the group B (DMBA alone). A similar result was observed in the kidneys of experimental animals. In the kidneys, overexpression of GSTP1 was observed in group B (DMBA alone treated group) whereas lowest expression of GSTP1 was in group C (DMBA + Lfcin B treated group). A significant difference was observed between group A, B, and C (P<0.01). Administration of 200μl (25μg/μl) Lfcin B three times per week was found to significantly inhibit GSTP1 in both liver and kidneys of group C indicating the in vivo inhibition of GSTP1 by Lfcin B.

Estimation of protein content has revealed that total protein content in group B (DMBA alone) treated animals was found to be depleted compared to other groups as presented in table 2. This indicated the toxicity of DMBA to the genetic materials thereby disrupting the protein synthesis in both kidneys and liver. A significant difference was observed between group A, B, and C and topical application of 200μl of DMBA at concentration of 0.025μg/μl per animal for three weeks was found to significantly inhibits protein synthesis in both group B and C. Compared to group B, group C was to be closer in the protein contents than the group B. This also indicates the reversal effect of 200μl (25μg/μl) of Lfcin B as a result of the assault induced by the topical application of DMBA. In both the liver and kidneys protein was found to be depleted following topical application of DMBA probably due to stress, however, subcutaneous injection of Lfcin B significantly restored by the protein contents. It was described that the carcinogen, DMBA upsets intracellular calcium balance thereby damaging endoplasmic reticulum which results in depletion of protein (Sharma *et al*; 2010).

A similar result to this research in our previous finding was reported in vitro by observing the interaction of Lfcin B with human placental GSTP1 and breast adenocarcinoma MD-MB-231 cell line. The result of the research suggested that Lfcin B is a competitive inhibitor with respect to GSH binding site (G-site) and noncompetitive inhibitor with respect to the hydrophobic substrate unit (H-site) of the human placental GSTP1 enzyme. Following the observation that GSTP1 was overexpressed in most cancer

cells (Khan and Taneja, 2015), inhibitors of GSTP1 could play important role in suppression of GSTP1 activity thereby overcoming and sensitizing the cancers cells to a chemotherapeutic agent (Tsuchida and Sato, 1992). Lfcin B was proposed to modulate the activity of GSTP1 by three different ways (Khan and Taneja, 2015) i.e. by directly interacting with GSTP1 and inhibiting its active site, by inhibiting GSTP1 binding with JNK and c-Jun or by inhibiting GSTP1 complex formation with TRAF2 (TNF receptor-associated factor 2) and ASK (apoptosis signal-regulating kinase). Chemotherapeutic sensitization can be initiated following JNK activation and phosphorylation of C-jun as a result of ROS and other stress responses (Khan and Taneja, 2015). Topical application of DMBA induced oxidative stress to genetic materials thereby initiating the process of carcinogenesis. Accordingly, JNK associated with stress response, apoptosis, inflammation, cellular differentiated and proliferations (Karin and Gallagher, 2005). Peptides have become apparent as assuring remedial agents in the cure of cancer, diabetes, and cardiovascular diseases, and presentation of peptides in a variety of other therapeutic sections is growing swiftly (Thundimadadathil, 2012). In spite of the progress that was recorded in the development of anticancer drugs, challenges continue to rise particularly due to the resistance of the cancer chemotherapies and low sensitivity of the commercially available anticancer drugs (Loza *et al* ; 2013). Peptides for eg. Lfcin B which are host resistance peptides also acting as effector molecules of innate immunity as well may signify an innovative approach for the development of substitute anticancer drug molecules. Cationic peptides are unaffected by multidrug resistance mechanisms (Riedl *et al*; 2011; Fadnes *et al*; 2011) and have been revealed to provoke a defending immune response in contrast to solid tumors, thus making them noteworthy candidates for emerging novel prime substitutes for anticancer treatment. Further studies will be required to elucidate the more clinical significance of the above findings.

## CONCLUSION

Subcutaneous administration of 200μl of Lfcin B inhibits GSTP1 in both liver and kidneys of DMBA induced tumor-bearing

mice. Accordingly, Lfcin B may emerge as a suitable pharmacological agent that can selectively target GSTP1 thereby inducing cancer cells to apoptosis. Further studies will be required to elucidate its mechanism of chemoprevention.

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