Drug-fatty acids conjugates in Diabetes treatment: Role of chain length and degree of unsaturation of fatty acids

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INTRODUCTION

Diabetes mellitus (DM) is a widespread chronic metabolic disorder classified into autoimmune insulin-dependent type 1 DM (T1DM) and non-insulin dependent type 2 DM (T2DM) caused by insulin resistance resulting in high blood glucose level. T1DM develops owing to proinflammatory cytokine-mediated β -cells dysfunction and inflammation in the islet of Langerhans of the pancreas leading to activation of macrophages and release of inflammatory Th1 lymphocytes and cytotoxic T cells which lead to chronic complications after few years[1]. There is now an understanding that has developed regarding natural T1D which comes from prospective birth cohort studies following at-risk children, those with a first degree relative with T1D or high-risk human leukocyte antigen (HLA) genes, to the development of islet autoimmunity and eventual clinical T1D onset. Also, the presence of two or more autoantibodies directed against insulin confers ~85% risk of developing diabetes within 15 years and nearly 100% over the lifetime of an at-risk individual. With the known fact of development of T1D diseases and early diagnostic systems, none have been successful in preventing T1D and the only reliable source left is insulin. Here, we have tried to enhance the efficiency of anti-diabetic drug, Lisofylline (LSF) which acts on specific molecular targets involved in disease pathogenesis are needed to improve prevention efforts including preserving residual beta cell function in new-onset T1D patients.

In the present study, the drug of choice is LSF which is a broad-spectrum drug bearing significant clinical utility in preventing both T1DM and T2DM. It regulates the immune cell function to suppress autoimmunity and also retains the insulin secretory function of β-cells in the presence of inflammatory cytokines. It is, however, known to be non-bioavailable by oral route of administration because it has an extremely short half-life. Also, in pre-clinical studies, it has been administered parenterally, twice daily in T1D animals at a high dose of 25 mg/ kg because of its high metabolism and conversion into pentoxifylline (PTX). LSF has a free hydroxyl group in its structure which is responsible for its high solubility and fast metabolism. Considering this, our group has reported a hydrophobic conjugate of LSF

with a fatty acid (FA), linoleic acid (LA), LSF-LA; it exhibited self-assembly to form micelles in nanosize range and displayed improved anti-diabetic efficacy at a reduced dose of 15 mg/ kg once daily compared to that of free LSF (25 mg/ kg, twice daily).[2] Choice of fatty acid in liposomes is reported to influence the encapsulation efficiency and cytotoxicity of the payload depending upon the carbon chain length of the fatty acid selected. The major focus of our study was to explore different fatty acids that could be conjugated to LSF to obtain LSF-fatty acid prodrugs and to investigate the impact of carbon chain length and degree of unsaturation of the fatty acids on self-assembling nature of the prodrug, pharmacokinetic behaviour of LSF prodrugs and their anti-diabetic activity.

Numerous studies have been reported wherein, a drug is covalently bonded to a lipid moiety, i.e. phospholipids, glycerides or FAs, particularly for oral and intravenous (i.v.) administration for the treatment of cancer or other diseases and these drug-FA conjugates have shown increased efficacy and permeability, improved oral bioavailability, reduced toxicity, active targeting along with enhancing the stability of the compound in biological fluids. Fatty acids generally contain a linear chain of carbon atoms with a reactive carboxyl end that can form a strong ester or amide bond with any drug having α-hydroxyl or amine group.

FAs are classified as saturated and unsaturated fatty acids (SFA and USFA), among these, USFA are further designated according to the positions of double bonds like n-3, 6 and 9 (indicates the number of carbon atoms after double bond). Long chain polyunsaturated fatty acids (PUFA; containing two or more double bonds) play an important role in cholesterol metabolism, blood clotting, immune system regulation and also constitute an important component of most of the biological phospholipidic membranes. The hydrophobicity of these molecules' favours translocation of unionized fatty acid through the phospholipid bilayer of the gastro-intestinal membrane. Membrane fluidity and transportation across the membrane are reported to be significantly higher as their degree of unsaturation and chain length increases. It has earlier been reported that higher the number of double bonds in a FA, greater are the chances of interference in the physiological activities of cells. This has been exemplified in a study on understanding the alteration in structure and function cardiolipin (CL), a dimeric phospholipid present in the inner membrane of mitochondria. Different 18-carbon FAs were administrated orally and it was observed that among these FAs, oleic acid (OA; C18:1, 18 carbons with 1 double bond) remained stable whereas, linoleic acid (LA; C18:2) and α-linoleic acid (C18:3) exhibited instability and underwent chain length elongation (up to C20) along with an increase in the number of double bonds before getting incorporated into mitochondrial CL thus imparting a significant change in the structure and function of CL[3].

Among the different fatty acids selected for the study, linoleic acid (LA) is an omega-6 FA with an 18 carbon long chain and two double bonds (ω-6 FA). LA is mainly present in vegetable and fish oil. Oleic acid (OA) is also an 18-carbon chain length FA and liquid in nature but has a single double bond (ω-9) which imparts the fluidic nature to cell membrane. SFA like palmitic acid contains 16 carbon chain without any double bond making cell membrane structure straight and rigid. A functional FA, α-lipoic acid was also chosen for the study; it is also known as thioctic acid as it comprises of pentanoic acid with 1,2-dithiolan-3-yl at 5^{th} position. It is a heterocyclic fatty acid derived from an octanoic acid.

In the present study, different fatty acids prodrugs, LSF- α-lipoic acid (LSF-ALA), LSF-palmitic acid (LSF-PA), LSF-oleic acid (LSF-OA) and LSF-LA were synthesized and analysed for self-assembling behaviour by determination of critical micellar concentration (CMC) and micelle aggregation number. The stability and release of free LSF from these prodrugs in plasma was also studied. The non-toxicity of the fatty acids and synthesized prodrugs was initially confirmed in MIN-6 cells. These cells were then exposed to inflammatory conditions similar to those observed in diabetes to study the protective effect of micelles of LSF prodrugs followed by cell uptake study. All the prodrugs were studied for their pharmacokinetic behaviour in Wistar rats at a single dose of ~15 mg/kg of free LSF. Anti-diabetic activity was monitored by administering the prodrugs by i.p. route daily for 35 days in streptozotocin (STZ) induced T1DM rat model. Post-treatment, evaluation of efficacy was carried out by measuring blood glucose level, plasma insulin level and determining biochemical parameters like SGPT, SGOT, cholesterol, triglyceride, uric acid and total protein. The excised pancreatic tissues of the experimental animals were also studied for histopathological changes by haematoxylin/ eosin (H&E) staining and expression of CD4+ and CD8+ T-cells.{Italiya, 2019 #1}

OBJECTIVES

1. To characterize and evaluate synthesized LSF and LSF-fatty acid prodrugs.
2. To develop an HPLC analytical method for newly synthesized prodrugs.
3. To analyse and estimate the self-assembling nature of prodrugs.
4. To analyse the influence of carbon chain length and degree of unsaturation present in each prodrug

    i) To perform in vitro studies- stability, hemocompatibility, cell internalisation, cytotoxicity study.

    ii) To perform pharmacokinetic and anti-diabetic efficiency study.

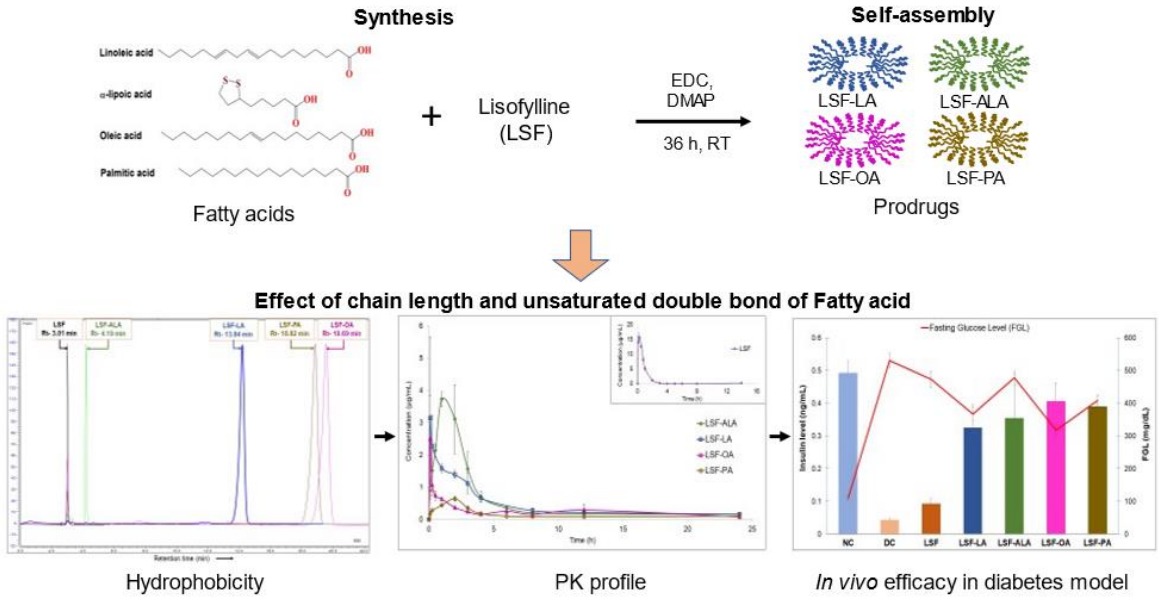

Figure 1- Overall graphical representation

MATERIAL AND REAGENTS

LSF (purity > 98%, HPLC) was synthesized in house. As a reference standard, LSF was also procured from Cayman Chemical (Michigan, USA). 3-Isobutyl-1-methylxanthine (IBMX as internal standard; HPLC ≥ 99%), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and STZ were bought from Sigma Aldrich (St. Louis, MO). 4-dimethylaminopyridine (DMAP) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC.HCl) were procured from Spectrochem Ltd. Dulbecco's modified eagle medium (DMEM), TrypLE, fetal bovine serum (FBS) and recombinant proteins (TNF-α, IL-1β and IFN-γ) were obtained from Invitrogen (USA); bovine serum albumin (BSA), dimethyl sulfoxide (DMSO, molecular biology grade) and phosphate buffered saline (PBS) pH 7.4 were purchased from Hi-media laboratories (Mumbai, INDIA). Fatty acids, LA and OA (99%) were purchased from Acme synthetic chemicals (Mumbai, INDIA) and PA (98%) from Suvidhinath laboratories (Vadodara, Gujarat). ALA (>99%) was purchased from TCI (Tokyo, Japan). Accucheck active glucometer was purchased from Roche diabetes care India Pvt. Ltd. (Mumbai, INDIA). Biochemical parameters were estimated using kits purchased from Coral clinical systems (INDIA). All other chemicals and reagents were of analytical grade and used as obtained. MIN-6 cells were procured from NCCS, Pune (INDIA). Wistar rats (male; 8–10 weeks, 200–220 g) were procured from Central Animal Facility, BITS-PILANI (Pilani, India). All animal experiments were performed in accordance

with the guidelines laid down by CPCSEA and the protocols were approved by the institutional animal ethics committee (IAEC), BITS-Pilani.

METHODS

Synthesis of LSF and LSF- fatty acid prodrugs

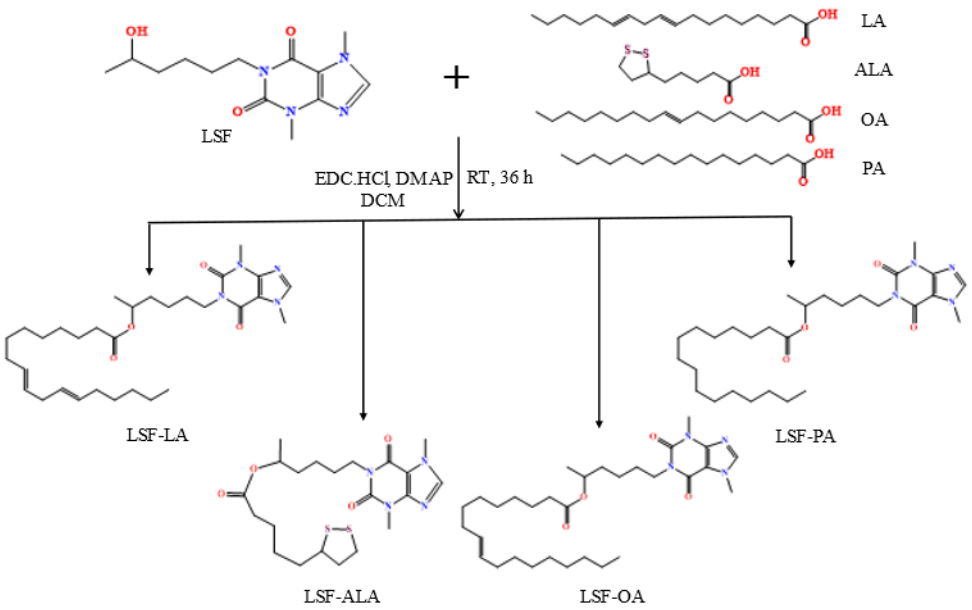

Figure 2- Reaction Scheme of the Synthesized Prodrugs with Different Fatty Acids and LSF

LSF was synthesized in house as reported earlier [5]. Briefly, in the presence of $NaBH_4$ (reducing agent) and protic solvent methanol, the carbonyl group present in the PTX was reduced to a secondary alcohol. The reaction mixture was stirred at room temperature wherein, the reaction progress was monitored by thin layer chromatography (TLC, toluene: acetone in 1:1 ratio). The resultant product that is, LSF was characterized by high-performance liquid chromatography (HPLC), high resolution mass spectroscopy (HR-MS), ^1H proton nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infra-red spectroscopy (FT-IR) and it was compared to the commercially available LSF (Cayman Chemical).

LSF was chemically conjugated to various fatty acids namely ALA, LA, PA and OA to obtain prodrugs- LSF-ALA, LSF-LA, LSF-PA and LSF-OA (**Figure 2**), using our previously reported method [2]. In brief, the coupling reaction of carbodiimide/ DMAP was used to conjugate fatty acids to the LSF hydroxyl pendant group. At RT and under N_2, different fatty acids including ALA, LA, OA and PA (21.6, 22, 19.2

and 21.1 mmol) were mixed with DMAP (1.2 eq., 21.6 mmol) and EDC.HCl (1.5 eq., 27 mmol) to form a uniform mixture in anhydrous DCM (250 mL). To the reaction mixture, LSF (19.8 mmol) was added and left on stirring in dark for next 36 h. After completion of the reaction (as monitored by TLC using ethyl acetate: hexane in 90:10 ratio), the reaction mixture was washed twice with water and saturated solution of salt, dried over Na_2SO_4 and then DCM was evaporated under vacuum. The crude prodrugs so obtained from the above reaction were semi-solid in texture. These crude prodrugs were then purified using flash chromatography (Bonna-Agela Technologies, USA) using ethyl acetate: hexane as mobile phase in different ratios ranging from 45:55 to 95:5 depending upon the lipophilicity of the prodrug (**Table 1**). The instrumental parameters for all the prodrugs were set constant; λ_{max} 273 nm and 20 ml/min flow rate using standard previously packed column (40 g; dimensions 3.1*14 cm) with silica gel (40-60 µm). The purified prodrugs LSF-ALA, LSF-LA, LSF-PA and LSF-OA were characterized for their physical appearance and by HPLC, HR-MS, ^{1}H NMR and FTIR.

Table 1- Mobile phase ratios optimized for elution of purified prodrugs using flash chromatography.

S. No.	Prodrugs	Mobile Phase		Elution time (min)
		Hexane	Ethyl acetate	
1	LSF-ALA	45 %	55 %	71
2	LSF-LA	22 %	78 %	102
3	LSF-PA	5 %	95 %	140
4	LSF-OA	8 %	92 %	125

Stability of prodrugs in plasma

Each prodrug was dissolved in DMSO (20 mg/ mL) and 10 µL of aliquot was spiked in rat plasma (1 mL) and maintained at 37 °C. At predetermined time points, 100 µL aliquots were withdrawn from each prodrug sample. To each of the aliquots, 50 µL IBMX (IS, 2 µg/mL) and 1.8 mL DCM were added and mixed, which was then centrifuged at 3500 rpm, 4°C for 15 min. The organic phase was then transferred into another tube and DCM was evaporated under nitrogen stream. Using 200 µL mobile phase, the dried samples were reconstituted and analysed using our previously reported HPLC based method [6].

Self-assembly of prodrugs

Hydrophilic nature of LSF and hydrophobic nature of fatty acids, imparted an amphiphilic nature to all the prodrugs leading to their self-assembly into micelles. To study this, solution of prodrugs in DCM was evaporated under vacuum to obtain a thin film which was allowed to undergo self-assembly in aqueous

media. The micelles so formed were sonicated for 3 min to enable size reduction. The size and zeta-potential were then recorded for all the prodrug micelles with a scattering angle of 173° using Zetasizer Nano-ZS (Malvern, UK).

CMC of prodrugs

The self-assembling ability of the prodrugs was confirmed by fluorescence spectroscopy (RF-5301 Shimadzu, Japan). The CMC value of each prodrug was estimated by using pyrene in aqueous solution. Pyrene dissolved in acetone (6×10^{-7} M) was incubated with different concentrations of prodrugs (1.0×10^{-5} mg/ mL to 1.0 mg/ mL) and kept on stirring at RT under dark conditions for 24 h. The fluorescence intensity of each sample was measured at an excitation range of 300–360 nm and emission at 390 nm with slit width of 5 nm. The ratio of intensities of peaks at I_1 and I_3 corresponding to wavelength 333 nm and 337 nm were recorded and a graph was plotted between I_3/I_1 versus log concentration of prodrugs.

Micelle aggregation number (N_{agg}) of the prodrugs

Determination of micellar aggregation number (N_{agg}) of prodrugs was performed by fluorescence steady state method at an excitation wavelength of 318 nm assuring that no Raman scattering will occur near to the emission peaks projected from 320 nm to 450 nm at 5 nm bandwidth. The probe and quencher (pyrene and CPC, respectively) were used here, wherein, the sample preparation was done by dissolving pyrene (10^{-4} M) in 2 mL ethanol which was then evaporated under nitrogen leaving out a thin film of pyrene. The film was reconstituted into 100 mL aqueous solution of prodrugs' micelles at 30 times CMC value of the respective prodrug, with overnight stirring at room temperature (*solution 1*, 2 x 10^{-6} M pyrene in 100 mL of micellar solution). Solution 2 was prepared by taking 110.05 mg of CPC in 10 mL of solution 1 (CPC concentration 2.8 x 10^{-3} M). The molar concentration of quencher was further varied from 0 to 1.12 x 10^{-3} M by diluting it with solution 1 and probe intensities were thereafter recorded.

$$\ln \frac{F_0}{F_Q} = \frac{Q}{M} \qquad \text{... Equation 1}$$

where, $$M = \frac{C - CMC}{N_{agg}} \qquad \text{… Equation 2}$$

Here, M= micellar concentration, F_Q and F_0 = fluorescence intensities of probe with and without quencher, Q = concentration of quencher in micelle solution, C= concentration of prodrug, CMC = critical micelle concentration of prodrug and N_{agg} = aggregation number.

Protein interaction of prodrug micelles

BSA was selected here as a model protein which undergoes a change in its UV absorption spectra when incubated with different concentrations of prodrugs micelles. With the help of fluorescence quenching method, the change in fluorescence spectra of the protein was recorded after incubating it with micelles using Scatchard equation (equation 3). Binding site present per BSA molecule represents the prodrugs binding on the vacant sites of the protein.

$$\log\frac{F_0-F_Q}{F_Q} = \log K_b + n\log[Q] \quad \text{... Equation 3}$$

where, F_0 and F_Q as in the above formula represent the fluorescence intensities in the absence and presence of quencher at any particular concentration (Q), n denotes the number of binding sites per protein molecule and K_b is the apparent biding constant.

Preparation of samples was done by making a series of different concentrations of prodrug micelles (0 to 100 µM) and spiking with a constant amount of BSA (2 µM). Fluorescence intensities were recorded after 30 min of incubation of each sample at excitation and emission wavelength of 280 nm and 343 nm respectively. For the determination of number of binding site and binding constant, a graph was plotted between log $(F_0\text{-}F_Q)/F_Q$ vs. log of quencher concentration (LSF or prodrug). Further, the mechanism of quenching of BSA molecule by LSF or prodrug was studied by UV-visible spectrophotometer (JASCO V-650) using quartz cell having 1 cm path length.

***In vitro* Cell culture studies of self-assembled micelles of the LSF-fatty acids prodrugs**

Efficacy of LSF prodrug micelles was evaluated in MIN-6 cells (originated from insulinoma cells of mouse). These cells were cultured in DMEM media supplemented with 10% FBS and incubated in 5% CO_2 at 37 °C.

***In vitro* hemocompatibility study**

The interaction of prodrugs' micelles with blood was studied *in vitro* with modification in the previously reported method (88). Blood was collected from Wistar rats and mixed with EDTA solution (10% w/v) to prevent coagulation. It was washed 3 times with normal saline solution followed by centrifugation at 1500 rpm for 10 min at 4 °C. The red blood cells (RBCs) so obtained was then diluted in 1:5 ratio with normal saline solution. Free LSF (20 µM) and prodrugs' micelles (~ 20 µM LSF) were dispersed in 1 mL of RBC suspension with negative and positive control groups (normal saline and 0.1% triton X solution, respectively). After incubation for 1 h at 37 °C, each sample was centrifuged at 2000 rpm for 5 min and 200 µL of the supernatant was analysed for optical density (OD) at 540 nm using Epoch microplate

spectrophotometer (BioTek Instruments, VT, USA). The percentage hemolysis was determined by using equation 4.

$$\% \text{ Hemolysis} = \frac{OD_{Sample} - OD_{Negative\ control}}{OD_{Positive\ control} - OD_{Negative\ control}} \times 100 \quad \text{…. Equation 4}$$

Cell internalization of prodrugs micelles

The internalization of free drug and micelles of self-assembled LSF-prodrugs into the cells was studied. In this study, micelles of all the four types of prodrugs (~20 µM free LSF) and free LSF (20 µM) were added to the MIN6 cells for 6 h. The cells without treatment served as control. After incubation, media was collected from each well and centrifuged for 10 min at 1200 rpm to remove any cells / debris. Extraction of the prodrugs and LSF from the media (0.5 mL) was carried out using DCM (1.3 mL) followed by centrifugation at 3500 rpm. DCM was transferred in a fresh tube and dried under nitrogen. The residue was then redispersed into 100 µL of mobile phase and analysed for LSF/prodrugs by HPLC, this concentration when subtracted from the initial drug/conjugate added provided the amount of drug internalized by the cells.

Cell viability assay (under normal and inflammatory conditions)

MIN-6 cells (5000 cells/well) were seeded and allowed to adhere for 24 h in a 96-well culture plate under normal growth conditions. Different treatments were given to the cells including the prodrug micelles, free LSF and free fatty acids (LA, OA, PA and ALA) at 20 µM concentration and incubated at 37 °C. After 48 h, MTT assay was performed and the inhibition in cell growth was determined with respect to untreated cells (media only).

$$\% \text{ Cell viability} = \frac{Absorbance_{Sample\ wells}}{Absorbance_{Untreated\ wells}} \times 100 \quad \text{…. Equation 5}$$

Once the cell compatibility was confirmed, freshly seeded MIN6 cells (5000 cells/well) were further exposed to a mixture of 03 different proinflammatory cytokines (TNF-α; 10 ng/mL, IL-1β; 5 ng/mL and IFN-γ; 100 ng/mL) to induce inflammation [7] followed by treatment with free LSF and synthesized LSF prodrugs (~20 µM free LSF). Post 48 h, cell viability was analysed by MTT assay.

***In vivo* evaluation of Prodrug**

All the experiments were carried out in compliance with CPCSEA guidelines and protocols were approved by IAEC, BITS PILANI, Pilani. The rats were housed in well ventilated cages with periodic light/ dark periods for 12 h and fed within standard laboratory conditions with routine *ab libitum* diet.

Pharmacokinetics of prodrugs

The micelles of LSF prodrugs and free LSF were injected intravenously into the Wistar rats (200-220 g) at a dose of 30 mg/ kg (~15 mg/ kg of LSF) and 15 mg/ kg respectively. Blood samples were collected by retro-orbital route at pre-set time points till 24 h. Plasma was retrieved from the collected blood followed by sample extraction and sample analysis (previously described in stability section). Using non-compartmental model, plasma concentration-time profile was plotted for free LSF and its prodrugs employing Phoenix 2.1 WinNonlin (Pharsight Corporation, USA).

Efficacy studies in STZ induced T1DM model

STZ induced diabetes model was created in Wistar rats (200-220 g) with standard dose of STZ (55 mg/ kg) prepared in cold citrate buffer (0.01M, pH 4.5) using i.p. route, while control rats were administered only buffer. Fasting glucose level was measured after 72 h of STZ injection and animals exhibiting plasma glucose level of 250 mg/ dL or greater were labelled as diabetic. Rats were randomly grouped into normal control (NC), diabetic control (DC), free LSF treated and self-assembled prodrug micelles treated rats. Treatment was initiated on the 3rd day after the hyperglycaemic state was verified.

For the daily treatment regimen, one of the groups was treated with 15 mg/kg i.p. injection of free LSF solution in water. The self-assembled micelles of the respective four prodrugs were delivered at 30 mg/ kg dose (~15 mg/ kg of free LSF) once daily to each of the groups. The blood glucose levels were measured using Accu-Check active glucometer by tail bleeding method on every 3rd day till 5 weeks. Plasma was collected after 21 and 35 days and used for estimating the insulin levels and biochemical parameters for lipid and protein profiling of liver and kidney functions.

Histopathology and immunohistochemical analysis

After giving treatment to the animals for 5 weeks, animals were sacrificed for the assessment of pancreatic islet morphology with the help of H&E staining. Standard protocol was followed for immunohistochemical (IHC) analysis of expression of CD4+ and CD8+ T-cells in the pancreata samples. The primary antibodies used were CD4 Rabbit mAb (1:400) and CD8a Mouse mAb (1:20). As a

secondary antibody, signal stain IHC boost reagent and anti-mouse IgG antibody were used at 1:1 and 1:500 dilutions, respectively.

RESULTS

Synthesis and Characterization of LSF and LSF-fatty acid prodrugs

LSF was successfully synthesized with >98% purity as determined by HPLC (UHPLC, ThermoFisher Scientific, USA) and a complete reduction of the ketone group present in PTX was seen, giving a high % yield. LSF-fatty acid prodrugs were then successfully synthesized and purified by flash chromatography with approx. 50% yield except for LSF-LA (41.91 % yield) **(Table 2)**. The physical appearance of the purified prodrugs was quite different from each other wherein, LSF-LA and LSF-OA were colourless and semi-solid but LSF-PA was white coloured and LSF-ALA was light yellow in colour; both were solid in nature. LSF and its fatty acid prodrugs exhibited the following attributes when characterized by different spectroscopic and analytical techniques.

Table 2- HPLC and HR-MS analysis of synthesized LSF and LSF prodrugs

S. No.	Drug/ Prodrugs	Molecular Formula	% Yield	Retention time (R_t min)	Purity (%)	Expected mass (g/mol)	$(M+H)^+$ peak (g/mol)
1	Free LSF	$C_{13}H_{20}N_4O_3$	92%	6.21	99.7	280.1535	281.1551
2	LSF-ALA	$C_{21}H_{32}N_4O_4S_2$	52%	4.19	95.1	468.1865	469.1933
3	LSF-LA	$C_{31}H_{32}N_4O_4$	41.91%	13.84	98.4	542.3832	543.3994
4	LSF-PA	$C_{29}H_{50}N_4O_4$	55.75%	18.82	98	518.3832	519.3889
5	LSF-OA	$C_{31}H_{52}N_4O_4$	49.25%	19.69	95.3	544.3989	545.4044

Proton NMR (400 MHz, $CDCl_3$) of the synthesized LSF drug showed a peak at chemical shift value of 3.75 ppm (-CH-OH) corresponding to the hydroxyl group in the side chain of LSF formed by the reduction of ketone group of PTX. Further, the successful conjugation of fatty acid with LSF was confirmed wherein, all the prodrugs exhibited a peak at 4.9 ppm corresponding to the formation of an ester (-CH-COO^-) group and removal of peak for hydroxyl proton (–CH-OH) signifying a complete reaction between LSF and fatty acids and absence of any free drug in the purified products. In HPLC analysis, lab synthesized LSF exhibited retention time (Rt) of 6.21 min indicating its hydrophilic nature, no additional peak was detected in the chromatogram of LSF indicating absence of any impurity/ reaction by-products. The spectrum of the synthesized LSF was similar to that of the commercially available LSF

(Cayman chemical). For quantification of prodrugs, HPLC analysis was carried out as mentioned in our previous report [2]. Here, LSF showed Rt of 3.01 min whereas, the prodrugs showed an increased Rt as compared to the drug alone. Among all, the minimum Rt was observed for LSF-ALA (4.19 min) followed by other prodrugs as shown in **Table 2**, indicating enhanced hydrophobicity of the prodrugs. All the prodrugs were obtained with a purity >95% with negligible amount of free LSF present in final product. Peak corresponding to any other impurity was also not observed in any of the chromatograms. HR-MS showed $(M+H)^+$ ion peaks (expected mass of the drug/prodrug + Mass of ^1H) which were found to be closely matching with the molecular formula of the respective prodrug and exact calculated mass of LSF/ prodrugs **(Table 2)**. In FTIR, the LSF spectra showed peak at 3380 cm^{-1} corresponding to –CH-OH group that was absent in the spectra of all the prodrugs as it was consumed in ester bond (–C=O) formation as depicted by presence of the –COOH peak at 1700-1650 cm^{-1} in the FTIR spectra of the prodrugs**.**

Analytical method development for Prodrugs

FAs have short and long carbon chains with/without double bonds resulting in variation in their hydrophobicity and therefore, only the retention time of the different prodrugs was different in the HPLC **(Table 3)**. The polynomial regression for the calibration plots showed good linear relationship with correlation coefficient (R^2) for LSF-ALA and LSF-OA was found to be > 0.9995 over the concentration range of 1-100 µg/mL. The methods were also found to be selective for the analytes **(Table 2)**. The limit of detection (LOD) and limit of quantification (LOQ) were 13 ng/mL and 41 ng/mL for LSF-ALA and 52 ng/mL and 159 ng/mL for LSF-OA. Results of method validation have been provided in supporting information **(Table 4 and 5)**.

Table 3. Chromatographic condition for analytical method development of LSF-FA prodrugs.

Chromatographic conditions	
Instrument	Ultimate HPLC 3000, ThermoFisher Scientific
Column	Inertsil® ODS (C18), (250 × 4.6 mm, 5µ)
Mobile Phase (Isocratic mode)	Acetonitrile: Acetate buffer pH 3.5 (95:05 % v/v)
Flow rate	1 mL/min
Column Temperature	30 ± 0.5°C
Injection Volume	20 µL
Wavelength	273 nm

Table 4- Precision (% RSD) and accuracy (% Bias) of analytical methods of LSF prodrugs- LSF-ALA and LSF-OLA

Analyte	Nominal Conc. (µg/mL)	Measured Conc. (Mean ± SD, µg/mL)	% Precision (%RSD)	% Accuracy (%Bias)
LSF-ALA	100	99.40 ± 1.89	1.89	0.60
	50	51.42 ± 0.63	1.27	-2.83
	20	19.52 ± 0.38	1.89	2.39
	10	10.09 ± 0.27	2.73	-0.87
	1	0.99 ± 0.02	1.91	0.52
LSF-OA	100	99.84 ± 0.58	0.58	0.16
	50	50.34 ± 0.40	0.79	-0.67
	20	19.88 ± 0.12	0.60	0.58
	10	10.01 ± 0.37	3.67	-0.06
	1	0.95 ± 0.01	0.58	4.55

Table 5- Precision and accuracy of quality control (QC) samples of LSF prodrugs

Analyte	Level	Nominal Conc. (µg/mL)	Inter-Day			Intra-Day		
			Measured Conc. (Mean ± SD, µg/mL)	% Precision (%RSD)	% Accuracy (%Bias)	Measured Conc. (Mean ± SD, µg/mL)	% Precision (%RSD)	% Accuracy (%Bias)
LSF-ALA	LQC	5	5.04 ± 0.11	2.22	-0.83	4.91 ± 0.01	0.20	1.78
	MQC	30	30.62 ± 0.73	2.42	-2.08	29.69 ± 0.13	0.45	1.04
	HQC	80	83.18 ± 1.86	2.33	-3.98	80.87 ± 0.81	1.02	-1.09
LSF-OA	LQC	5	4.90 ± 0.16	2.08	3.35	4.86 ± 0.11	2.19	2.74
	MQC	30	29.97 ± 0.23	0.77	0.10	30.33 ± 0.21	0.69	-1.09
	HQC	80	77.33 ± 1.62	2.09	3.33	79.42 ± 0.80	1.00	0.72

Stability of prodrugs in plasma

Due to the conjugation of LSF to FAs, its stability in rat plasma increased and the prodrugs released LSF in a sustained manner for 72 h wherein, LSF-LA exhibited maximum release of 60% in 72 hours followed by LSF-OA and LSF-PA (**Figure 3**).

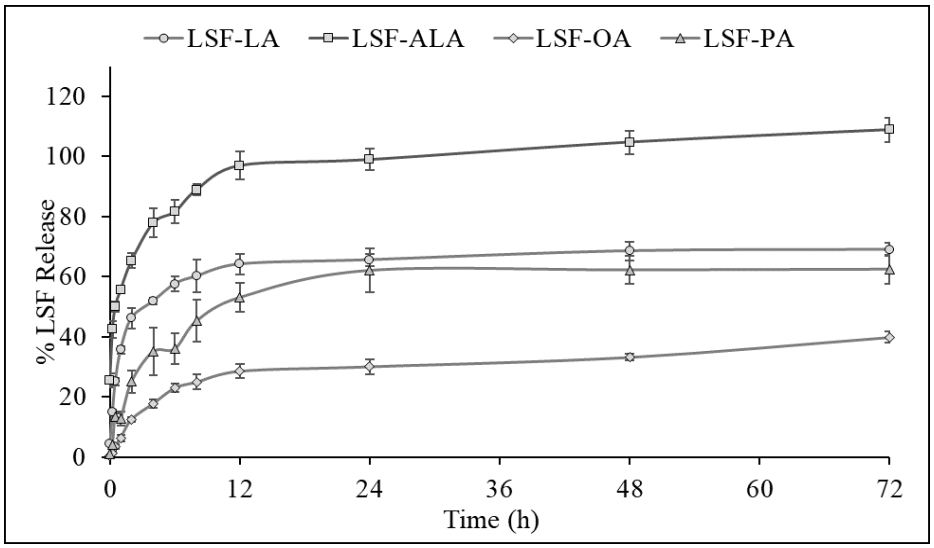

Figure 3- Stability of LSF prodrugs and the rate of release of free LSF from the prodrugs in the rat plasma. Data represents mean (n=3) ± SD.

Self-assembly of prodrugs

These prodrugs are amphiphilic in nature as these comprise of hydrophilic (LSF) and hydrophobic components (fatty acids) in 1:1 ratio (on mole basis) which imparts the self-assembling property to them enabling formation of micelles in the presence of water. The self-assembled micelles were characterized for particle size and zeta potential using Malvern dynamic light scattering system (**Table 6**). The micelles size ranged from 70.4 nm to 170.5 nm and zeta potential values -4.3 mV to -22.6 mV for different prodrugs. For confirming self-assembly of micelles, CMC value was obtained for each of the prodrugs and was found to be in range of 0.81 µg/mL to 7.58 µg/ mL (**Figure 4A**).

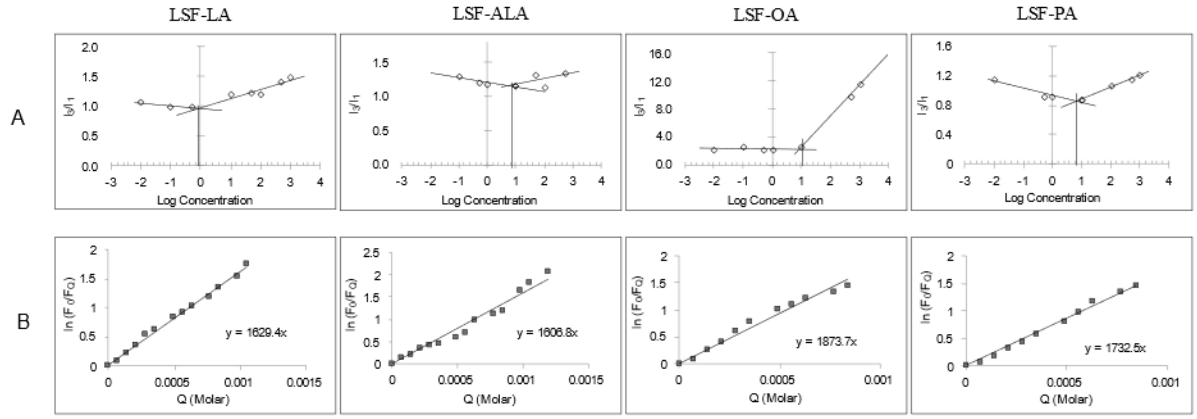

Figure 4- Study of self-assembling nature of LSF prodrugs by (A) critical micelle concentration (CMC) and, (B) micelles aggregation number.

Estimation of aggregation number

The aggregation number was determined after estimating the concentration of micelles (M) by the steady state fluorescence and the intensities were compared with and without quencher. So, the decrease in the intensity of pyrene with prodrugs micelles in the presence of various concentrations of CPC (quencher) was recorded. A straight line was obtained when a graph was plotted between $\ln(F_0/F_Q)$ and Q (concentration of quencher in micelles), M was determined by the slope of the graph using equation 1 and then aggregation number (N_{agg}) was calculated using equation 2 **(Figure 4B)**.

Protein interaction of prodrugs with BSA

This study was performed to check the interaction of protein with prodrugs by fluorescence quenching method wherein, the emission spectra of BSA was recorded before and after interaction of BSA with free LSF and prodrugs. A decrease in relative intensity of BSA with increase in concentration of prodrugs was observed revealing an interaction between BSA and prodrugs. A bathochromic peak shift was observed only in case of LSF-PA which itself showed fluorescence (**Figure 5**). After plotting the graph of log $(F_0\text{-}F_Q)/F_Q$ and log Q, the binding constant K_b and number of binding sites (n) present on BSA were determined (**Table 6**). The K_b and n for free LSF were estimated to be 6.32×10^4 and 1.16.

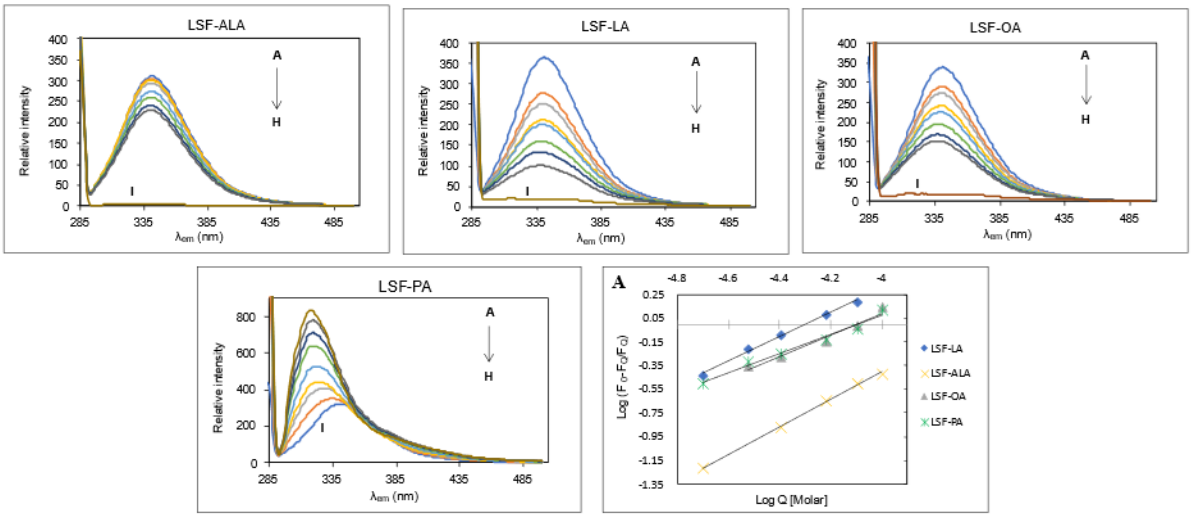

Figure 5. Protein interaction study between LSF prodrugs and BSA. Emission spectra of BSA (λ_{ex} = 280 nm) at 2.0 µM concentration in the presence of different concentrations of prodrug micelles that is 0, 10, 20, 30, 40, 60, 80 and 100 µM, corresponding to curves A–H. Plot I correspond to the emission spectrum of respective prodrug micelle only (500 µM). (A) Plot of Log (F_0-F_Q/F_Q) against log (Q) at different concentrations of the prodrugs at room temperature.

Table 6- Characterization of self-assembled LSF- fatty acid micelles

S. No.	Prodrugs	Particle size (nm)	PDI	Zeta potential	CMC (µg/mL)	N_{agg}	K_b ($L.mol^{-1}$)	N
1	LSF-ALA	170.5	0.257	-13.0	6.31	47	2.05×10^4	1.18
2	LSF-LA	92.67	0.326	-22.6	0.81	52	2.70×10^4	1.03
3	LSF-PA	71.74	0.268	-4.3	7.4	69	5.55×10^4	0.85
4	LSF-OA	70.4	0.232	-15.6	7.58	61	6.34×10^4	0.92

In vitro studies of prodrugs

Hemocompatibility study

To understand if the micelles can cause hemolysis during systemic circulation, % hemolysis was calculated. Hemolysis exhibited by the different prodrugs was very less as compared to the hemolysis caused by the positive control (Triton X) (**Figure 6A and 6B**).

Cell internalisation of prodrug micelles

As micelles are nano-sized systems, the prodrugs micelles exhibited higher cell uptake into MIN-6 cells as compared to the free drug after 6 h wherein, among all the prodrugs, LSF-LA showed maximum internalization of 72.9% while, only 16.48 % of free drug was found inside the cells (**Figure 6C**).

Cell viability under normal and inflammatory conditions

Under normal cell culture conditions, all the prodrugs and free drug exhibited no cell death after 48 h, confirming that the synthesized prodrugs of LSF are non-toxic to the cells (**Figure 6D**). As seen in **Figure 6E**, upon exposure to the cytokine mediated inflammatory conditions, a drastic reduction in cell viability was observed in all the samples. However, presence of LSF-prodrugs with the cells under these conditions significantly preserved their viability in comparison to the control cells (in media alone).

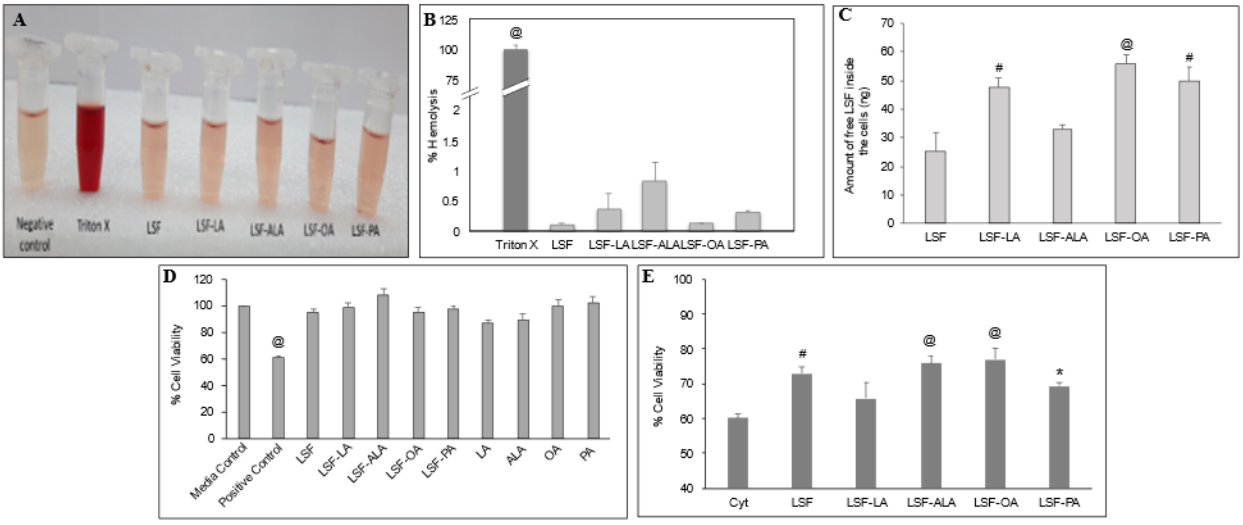

Figure 6- *In vitro* evaluation of the LSF prodrugs. (A) and (B) *In vitro* hemocompatibility study $^{@}$Triton X vs. all ($^{@}$P<0.0001), (C) cell uptake study in MIN-6 cells after 6h of incubation $^{@}$LSF vs. LSF-OA ($^{@}$P<0.0001); $^{\#}$LSF vs. LSF-LA and LSF-PA ($^{\#}$P<0.001), (D) cytotoxicity study of FAs, LSF (20 µM) and prodrugs (~20 µM free LSF) under normal conditions and, (E) cell viability in presence of cytokine induced inflammation $^{@}$Cyt vs. LSF-ALA and LSF-OA ($^{@}$P<0.0001); $^{\#}$Cyt vs. LSF (P<0.001); *Cyt vs. LSF-PA (*P<0.05).

***In vivo* studies of prodrugs**

Pharmacokinetic study

The non-compartmental estimation of pharmacokinetic parameters showed that the conjugation of LSF increased the half-life of LSF by 2 to 6 folds (from 0.752±0.03 h to 4.44±0.47 h) and the apparent volume of distribution of prodrugs also increased from 3 to 15 folds. The mean residence time (MRT) of prodrug elevated to 9 h as compared to 0.8 h for free LSF. The clearance values of LSF-LA and LSF-ALA prodrugs were found to be lower than free LSF but found much higher in LSF-OA and LSF-PA as shown in **Table 7** (**Figure 7**).

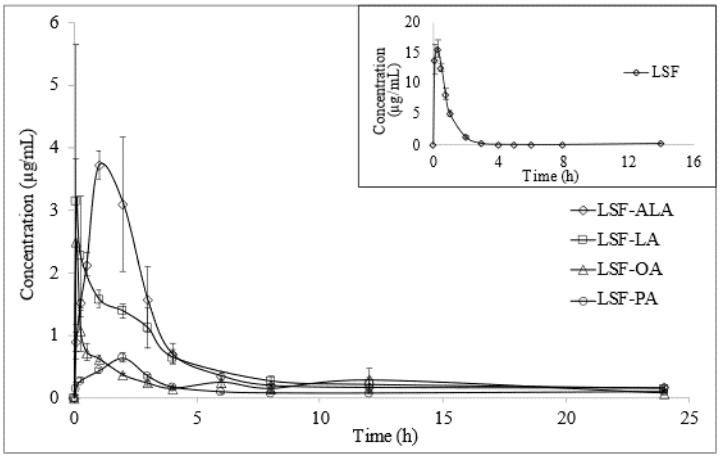

Figure 7- Systemic Pharmacokinetic study of LSF and LSF prodrugs in Wistar rats, IV administration. Each point represents mean (N = 4) ± SD at ~15 mg/ kg dose of free LSF.

Table 7- Pharmacokinetic study of LSF (15 mg/ kg; i.v. dose) and LSF prodrug (30 mg/ kg; i.v. dose) administered in rat and analysed by non-compartmental method (Mean ± SEM)

	LSF	**LSF-LA**	**LSF-ALA**	**LSF-OA**	**LSF-PA**
C_0 (ng/mL)	16395.65± 1630.78	4902.86±3223.37	4117.86±588.65	4747.3±3028.56	266.75±110.19
$t_{1/2}$ (h)	0.752±0.03	3.12±0.29	1.26±0.13	4.44±0.47	3.59±0.51
Ke (1/h)	0.924±0.04	0.23±0.02	0.51±0.06	0.16±0.02	0.17±0.01
AUC_{0-t} (ng.h/mL)	14692.73±603.55	16157.94±5437.84	13467.82±1538.58	6107.96±1322.07	3491.96±145.55
$AUC_{0-\infty}$ (ng.h/mL)	14738.52±590.77	16984.11±5365.74	13838.8±1439.5	6635.59±1270.95	3887.79±268.31
$AUMC_{0-t}$ (ng.h/mL)	12068.34±1247.91	77115.13±13161.58	67512.96±3282.77	30549.55±7409.28	29926.06±1339.38

$AUMC_{0-\infty}$ (ng.h/mL)	12797.01±1278.92	98737.84±18277.97	77295.34±5430.21	43158.66±14430.4	24806.43±3905.12
MRT (h)	0.818±0.06	6.55±0.55	5.16±0.67	7.91±0.93	9.07±0.33
V_d (mL/kg)	961.55±79.16	2587.82±558.86	1769.5±395	14974.4±1588.02	13774.62±2501.16
CL (mL/h/kg)	1020.9±39.46	634.05±191.99	951.84±107.22	2415.3±407.29	3894.14±260.65

***In vivo* efficacy studies in STZ induced T1D model**

The LSF- fatty acid self-assembled micelles were administered once daily intra-peritoneally at a dose of 30 mg/ kg (~15 mg/ kg of LSF) over a period of 5 weeks and compared with free LSF dosed at 15 mg/ kg, once daily. In this study, fasting glucose levels (FGL) of animals (mg/dL) were recorded every week. An overall FGL level at 3rd and 5th week, exhibited decreased FGL levels. Diabetic control group (DC) showed significant increase in FGL level (555.5 ± 22.73 mg/ dL), when compared to normal animals (106.25 ± 4.77 mg/ dL) as no treatment was given to this group except for saline. Free LSF also showed significant increase in FGL in 3rd and 5th week. LSF-LA and LSF-PA demonstrated slight increase in FGL in 5th week compared to their 3rd week level. LSF-OA showed a better control of FGL than other prodrugs by maintaining a constant level throughout the study. In LSF-ALA group, FGL level exhibited significant increase in 5th week as compared to 3rd week **(Figure 8A).** All the prodrugs showed significantly increased insulin levels as compared to DC and free LSF group (i.p.), among these LSF-OA and LSF-PA prodrugs showed higher insulin levels than LSF-LA and LSF-ALA which indicates increased protection of the residual beta cells owing to controlled blood glucose levels (**Figure 8B)**.

In T1DM, progressive liver injury occurs due to an increase in oxidative stress which is further associated with higher levels of SGOT (ASAT) and SGPT (ALAT) as seen in DC group. Treatment with the prodrugs lowered the levels of ALAT and ASAT, with minimum levels of ASAT being observed in LSF-OA and LSF-LA showed the lowest ALAT level. All the groups exhibited similar total protein level. Due to persistent hyperglycaemia in the body, the renal functions get compromised which increases the urea and uric acid levels in plasma. Here, all the prodrugs showed significant reduction in uric acid level as compared to DC and free LSF with the highest reduction being observed in LSF-OA. It has also been reported that cholesterol and triglyceride levels are significantly increased in patients with diabetes due to

insulin resistance and dyslipidaemia. All the prodrugs demonstrated significant reduction in cholesterol and triglycerides levels as compared to DC group **(Figure 8C)**.

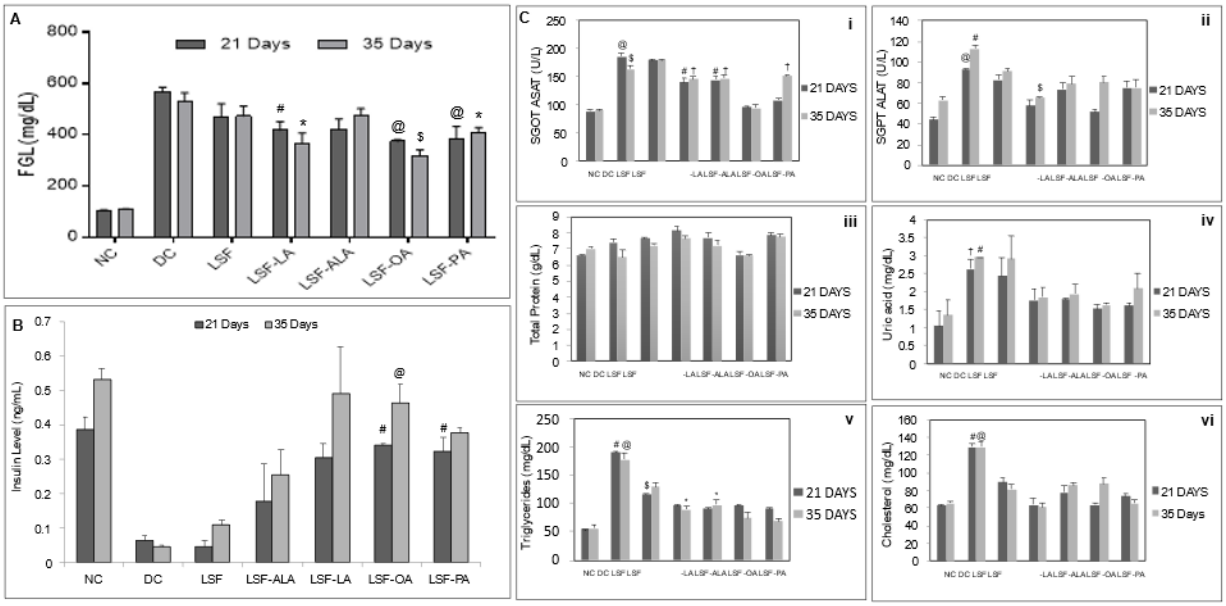

Figure 8- *In vivo* anti-diabetic activity of the LSF prodrugs evaluated in a STZ induced diabetic model. A) Fasting blood glucose levels after 35 days $^{\$}$DC *vs.* LSF-OA ($^{\$}P$<0.005); $^{@}$DC *vs*. LSF-OA and LSF-PA; *DC *vs.* LSF-LA and LSF-PA ($^{@}$*P<0.01); $^{\#}$DC *vs*. LSF-LA ($^{\#}P$<0.05), B) serum insulin level $^{\#}$DC *vs*. LSF-OA and LSF-PA; $^{@}$DC *vs*. LSF-OA ($^{\#@}P$<0.05), C) Biochemical analysis of plasma i) SGOT $^{@}$DC *vs*. LSF-OA and LSF-PA; $^{\$}$DC *vs*. LSF-OA ($^{@\$}P$<0.005); $^{\#}$DC *vs*. LSF-LA and LSF-ALA ($^{\#}P$<0.01); †DC *vs*. LSF-LA, LSF-ALA and LSF-PA ($^{\dagger}P$<0.05), ii) SGOT $^{@}$DC *vs*. LSF-LA and LSF-OA, $^{\$}$DC *vs*. LSF-LA ($^{@\$}P$<0.005); $^{\#}$DC *vs*. LSF-ALA, LSF-OA and LSF-PA ($^{\#}P$<0.05), iii) Total protein (non-significant), iv) Uric acid †DC *vs*. LSF-LA, LSF-ALA, LSF-OA and LSF-PA ($^{\dagger}P$<0.01); $^{\#}$DC *vs*. LSF-LA, LSF-ALA and LSF-OA ($^{\#}P$<0.005), v) Triglycerides $^{@}$DC vs. LSF-LA, LSF-ALA, LSF-OA and LSF-PA; $^{\#}$DC vs. LSF-OA and LSF-PA ($^{@\#}P$<0.001); $^{\$}$DC *vs.* LSF; *DC vs. LSF-LA and LSF-ALA ($^{\$*}P$<0.05), vi) Cholesterol $^{\#}$DC vs. LSF-LA and LSF-OA ($^{\#}P$<0.005); $^{@}$DC vs. LSF-LA and LSF-OA ($^{@}$P<0.01).

Histopathology and Biochemical assay

The pancreatic islets isolated after sacrificing the experimental animals at terminal time point were analysed for the morphology and immunohistochemical expression. The β-cells in DC were distorted and very few of these were visible in islets after H&E staining compared to NC and other prodrugs. Although,

LSF, LSF-ALA and LSF-PA–exhibited proper arrangement of pancreatic islets however, number of β cells were lesser as compared to LSF-LA and LSF-OA treated groups. A higher population of CD4+ and CD8+ inflammatory cells was seen in DC, along with depletion of other cells in the vicinity. The presence of these inflammatory cells was significantly diminished in prodrug treated diabetic rat's pancreas (**Figure 9**).

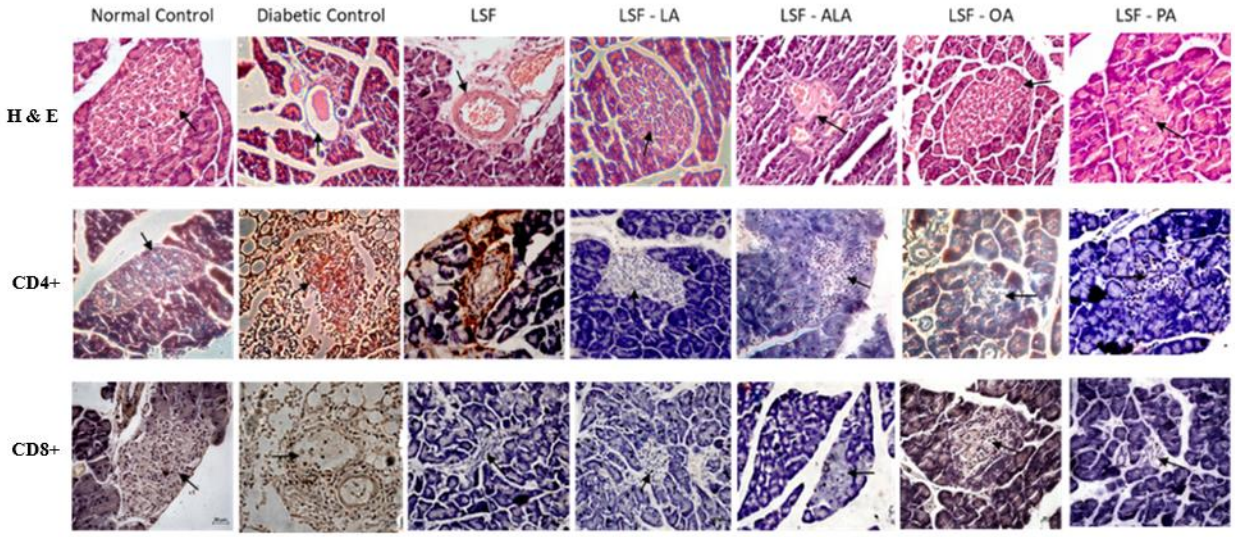

Figure 9- Immunohistochemical (IHC) study of pancreatic islets of control and treated groups after 35 days of treatment by Haematoxylin-eosin (H&E) staining and expression of CD4+ and CD8+ T-cells (indicated by brown colour and black arrows) (*under 400 × magnification; 20 µm scale bar).

Statistical Analysis

All the data has been expressed as mean ± standard deviation and analysed using GraphPad prism 7.0 software (GraphPad software, San Diego, CA, USA). Statistical analysis was performed using one-way ANOVA by Tukey's multiple comparison test with 95% confidence interval for confirming the difference between the groups. A p-value < 0.05 was considered statistically significant.

Discussion

LSF is a synthetic drug molecule that shows potent anti-diabetic activity along with its well-known immunomodulatory and anti-inflammatory activities. To improve its physicochemical and pharmacokinetic attributes, we conjugated it with different types of fatty acids which are also

endogenously present in the human body and are synthesized by de novo lipogenesis of triglycerides and lipids. These are also extracted from plant and animal sources and are being consumed in diet and food supplements extensively. To reduce the solubility of hydrophilic LSF and to enhance its efficacy and bioavailability, we conjugated it with hydrophobic FAs belonging to different classes like – LA (omega 6), OA (omega 9), PA (conjugated fatty acid) and ALA (functional fatty acid) to form prodrugs with LSF with increased lipophilicity. Here, the objective was to elucidate the effect of length of carbon chain in terms of short and long chain, presence or absence of double bonds and degree of unsaturation on the LSF-FA prodrug properties and associated pharmacological activity. Inspite of same carbon-chain length; FAs can demonstrate huge difference in their physiological behaviour owing to their geometrical and conformational differences. As reported earlier by our group, LA conjugation to LSF also reduced the rapid conversion of LSF to PTX and increased the drug stability by offering protection to the hydroxyl group present in the side chain of LSF. The nano-sized micellar system generated by amphiphilic nature of prodrugs helps in bypassing the first-pass metabolism of LSF, increasing its bioavailability which prolongs its mean residence time in the body, RES escape and reducing clearance and ease of handling formulation without the use of any other surfactant or excipients.

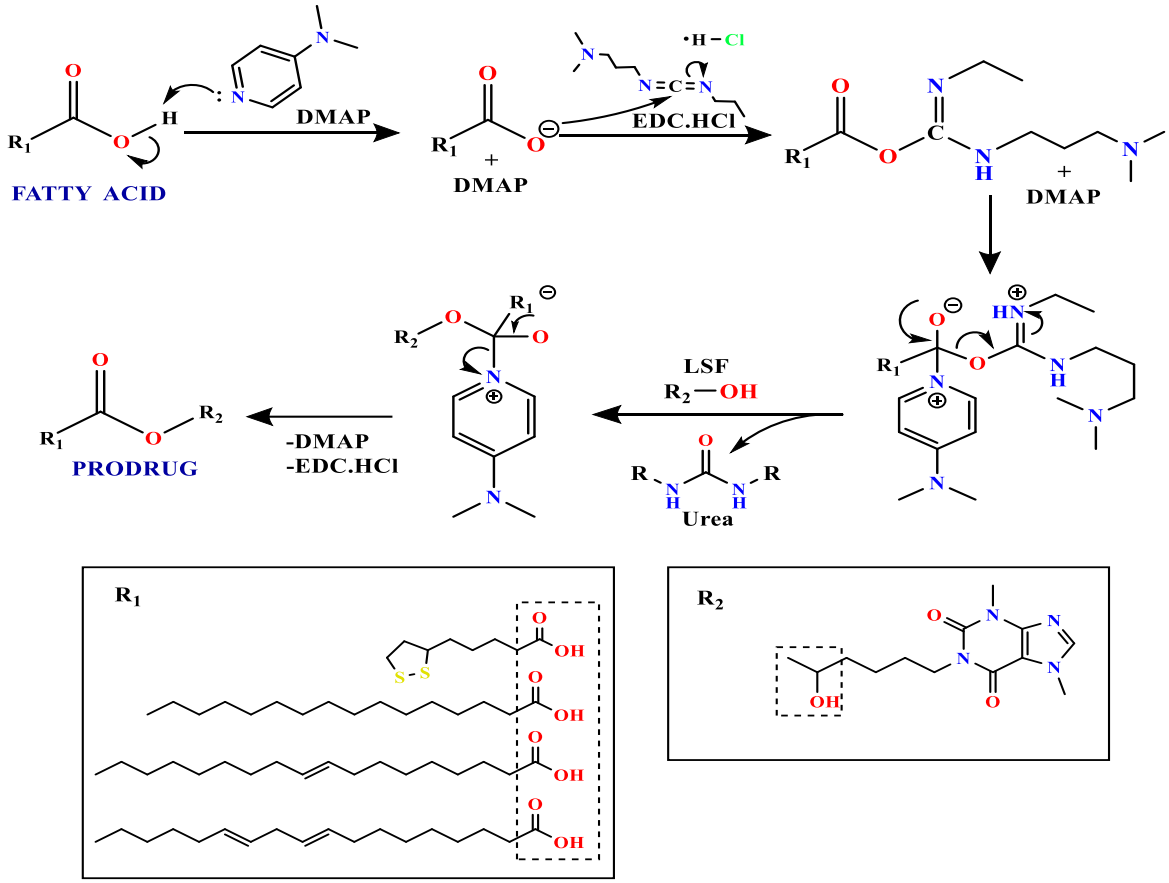

Figure 10- Mechanism involved in the formation of LSF-fatty acid prodrugs

Firstly, LSF was synthesized in-house by reduction of keto group of PTX using simple green chemistry followed by its purification. The synthesized drug was characterized by HPLC, FT-IR, HR-MS and DSC and compared with LSF procured from Cayman chemical, USA. The prodrugs were synthesized by conjugation of LSF with different categories of fatty acids LA, OA, PA and ALA, all having the same structural chemistry (–COOH) at one end of carbon facilitating a similar procedure for the synthesis for all the prodrugs. There was a progressive increase in the hydrophobicity of the prodrugs as the alkyl chain length of the associated fatty acid increased, this was reflected during the purification of the prodrugs in terms of the mobile phase ratio required and the elution time observed in flash chromatography; the same was further confirmed by the retention time of the prodrugs in HPLC (**Table 2**). The purified prodrugs also exhibited different physical states, LSF-LA and LSF-OA were in liquid form while LSF-PA and LSF-ALA were solid in nature, this might be attributed to the presence of *cis* double bond in the long alkyl carbon chain of LA and OA that generates kinks and bents and does not allow the resultant molecules to attain a tightly packed structure [8]. The calculated molecular weight of all the prodrugs matched with the molecular weight as obtained by HRMS. The proton NMR and FTIR of prodrugs, confirmed the removal of –CH-OH group from 3360 cm^{-1} in FTIR and 3.9 ppm in NMR of LSF and the formation of –CH-COO^{-} ester bond as revealed by signals at 2917-2933 cm^{-1} (FTIR) and 4.85-5 ppm (NMR). The CMC value is important to determine the minimum media volume required to form micelles wherein, it depends on the hydrophobic chain length and varying structural properties of the compounds. It has been reported that with increase in the carbon chain length, the CMC value decreases along with low aggregation number [9, 10]. Aggregation number represents the average number of monomers involved in formation of spherical micelles at or beyond CMC [11]. In our study, the nano-sized self-assembled prodrugs showed CMC values ranging from 0.8 to 7.6 µg/ mL wherein, LSF-LA exhibits CMC lower than LSF-ALA and LSF-PA owing to the longer chain length of LA and, LSF-OA has a higher CMC value due to a different degree of unsaturation as compared to LSF-LA. LSF-ALA showed least aggregation number which might be attributed to the short carbon chain of ALA resulting in higher relative aqueous solubility in comparison to micelles of other prodrugs. A higher aggregation number results in a low diffusion coefficient and reduced rate of transport from the interface to the bulk causing hindrance in the solubilization of the micelles. The self-assembled prodrugs when evaluated for their stability and rate of release of free LSF in fresh rat plasma at 37ºC, exhibited sustained release of the drug up to 72 h (**Figure 3**) reconfirming that conjugation of the fatty acids with small/large molecules enhances their *in vivo* stability as reported in literature also.

Adsorption and binding of the endogenous proteins on the surface of nanocarriers is a predominant factor affecting their behaviour in the systemic circulation. To understand protein binding with the prodrugs, BSA was selected for the study owing to its structural similarity with human serum albumin (HSA) that is endogenously present and is majorly found in the circulatory system. It is well reported that the free FAs can easily bind to the HSA which makes FAs and its conjugates potent and stable in blood stream. Under normal physiological conditions, FA molecules bind to HSA in 1:1 or 1:2 ratio; this however, increases by 4-5 folds under diabetic conditions [12] further prolonging the mean residence time of the molecules in the circulation and thus supporting the choice of conjugating FAs with LSF to enhance its anti-diabetic potential. The binding site per BSA molecule was found to be slightly higher in free LSF and LSF-ALA as compared to other micelles indicating lesser affinity of these micelles towards endogenous proteins in contrast to LSF-PA and LSF-OA micelles which show higher binding affinity possibly due to the fact that FAs like OA and PA are abundantly present in the tissues and are known to bind firmly to albumin and use it as a carrier in the blood. The *in vitro* study showed a lower binding constant of LSF-ALA followed by LSF-LA however, LSF-OA and LSF-PA exhibited a stronger interaction with the protein (revealed by a higher value of binding constant and lower aggregation number) indicating a probable increase in half life and mean residence time and hence controlled release of the drug *in vivo*.

A proof of non-toxicity was established by the *in vitro* hemocompatibility study wherein, negligible hemolysis of RBCs in the presence of prodrugs' micelles was observed **(Figure 6A & B)**. PUFAs, especially are advantageous to the system because the presence of long carbon chain increases the fluidity and flexibility of the membrane while maintaining the curvature of the membranes [8, 13]. This was reflected in the higher cell uptake and internalization of the micelles of LSF-LA as compared to other prodrugs and free LSF, signifying its optimum lipophilicity attributed due to presence of a longer carbon chain and higher degree of unsaturation. This further indicates that use of these FAs based prodrugs would enable higher uptake of LSF into the cells as supported by the cell uptake data (**Figure 6C**) and hence greater therapeutic efficacy. The cell viability study of prodrugs micelles in MIN-6 cells demonstrated the non-toxic nature of the FAs as well as the prodrugs with almost 100% cell viability **(Figure 6D)**. Although some toxic and inflammatory effects of FAs have been reported earlier like PA exhibits increased apoptosis and cytotoxicity in HepG2 cells at 300 µM or higher concentrations [14, 15] however, these were found to be safe within the concentrations used in the present study. LSF prodrugs restored the cell viability in presence of cytokines to a significant extent.

Conjugation of hydrophobic moieties like FAs to the drugs has been reported to improve the *in vivo* performance significantly by us as well as by other groups [16-19]. Conjugation of palmitic acid to the peptide, GLP-1 increased its $t_{1/2}$ from 5 min to 13 h providing it a sustained release. Likewise, in this

study also the synthesized prodrugs exhibited 1.5 to 6 folds higher $t_{1/2}$ than free LSF with a simultaneous increase of 7 to 11 folds in MRT due to the increased hydrophobicity of the prodrugs attributed to the carbon chain present in FAs; MRT increases linearly with increase in the chain length of the FA as shown in **Table 7**. The volume of distribution (V_d) of prodrugs was found to be higher than that of free LSF wherein, LSF-PA and LSF-OA demonstrated 15 times higher V_d indicating distribution to peripheral organs attributed to their longer chain length and hence greater lipophilicity.

In vivo efficacy of the prodrugs was evaluated in STZ induced T1D model. The micelles used for treatment were formulated by film hydration method and exhibited a particle size range of 78 – 112 nm with a narrow PDI. The FGL level was maintained at a significantly lower level than the diabetic control group in all the treatment groups representing the anti-diabetic potential of LSF but among all the prodrugs, LSF-PA and LSF-OA showed the best control of glucose level with decreased mortality as compared to other prodrugs (**Figure 8A**). Among all the prodrugs, LSF-ALA exhibited comparatively higher FGL which might be due to the inadequate hydrophobicity of shorter carbon chain length of ALA effecting the half-life and MRT of LSF as seen in the PK data [2, 20]. A remarkable increase in the insulin level was found on 21st day of the study which further improved on 35th day in all the prodrugs as compared to the diabetic control and free LSF treated group with LSF-OA treatment showing the highest insulin levels in comparison to the diabetic control group.

Persistent hyperglycaemia induces certain adverse effects in kidney, pancreas and other organs also. Study of different biochemical parameters known to undergo a drastic change under diabetic conditions, revealed that the prodrugs (particularly LSF-LA and LSF-OA) showed reduced oxidative stress as seen by the reduced plasma ASAT and ALAT levels compared to DC. Renal function also gets compromised in T1D as seen by the increased uric acid level in DC; LSF-OA showed significantly reduced level of uric acid than other prodrugs. Diabetes can cause hepatic dyslipidaemia wherein, low density lipoproteins, total cholesterol and triglycerides increase with a significant decrease in high density lipoproteins; these stress induced lipid metabolism changes are adequately addressed by the anti-inflammatory property of LSF. LSF prodrugs' treatment also reduced the total cholesterol and triglyceride levels rapidly after 21 days that was maintained till 35th day of the study. There was no significance difference seen in total protein content in both DC and treatment groups.

Presence of pro-inflammatory cytokines induces the infiltration of CD4+ and CD8+ T lymphocytes in the pancreas causing destruction and depletion of β-cells in the islets in diabetic model. The effectiveness of LSF released from the prodrugs in protecting the β-cells from inflammation and hence T cell infiltration was shown by H&E and IHC staining of pancreatic tissues collected after 5 weeks of the treatment. H&E

staining confirmed the intactness of β-cells in islets after treatment with the micelles of prodrugs as compared to the diabetic control, most prominently visible in LSF-LA and LSF-OA prodrug treated groups. Daily administration of LSF prodrugs also reduced the entry of T cells (CD4+ and CD8+) into the islets which otherwise cause β-cell destruction in comparison with DC group (**Figure 9)**.

The present study confirms the successful conjugation of LSF with different types of fatty acids and self-assembly into nano-sized micelles along with improved *in vitro* and *in vivo* performance than the free drug itself. Among the different drug-FA prodrugs studied, LSF-OA showed comparatively better pharmacokinetic and pharmacodynamic profile in diabetes. OA, the fatty acid *per se* has also been proven to be significantly better than PA in terms of its anti-inflammatory action, improved beta cell survival and increased insulin sensitivity. LSF-OA exhibits increased drug bioavailability and efficiently treats diabetic condition with decreased mortality and maintaining FGL in diabetic Wistar rats. Overall, this research study presents oleic acid (OA) as the most suitable fatty acid among ALA, LA, PA and OA for enhancing the stability and efficacy of LSF in diabetes treatment.

IMPACT OF THE RESEARCH

Diabetes mellitus has resulted into an epidemic proportion of a chronic metabolic noncommunicable disease where more than 463 million adults were reported to be suffering as of 2019 (IDF 2019), which was said to increase to 700 million by 2045 worldwide. Although 95% of the population suffers from T2DM, there is a significant increase in the T1DM population. T1DM is an autoimmune disorder where the body's immune system destroys the insulin producing beta cells in the pancreas. Hence, as a result the body is not able to produce insulin, or the production is very less. There are various hypothesis proposed as to why this condition happens where a most likely explanation is that there might be a combination of genetic susceptibility and environmental factors. The number of deaths due to type 1 diabetes mellitus were reported to be 128,900 out of 1,110,100 patients suffering from it. It is reported that the cases of T1DM are increasing by 3-5% every year, and in India there are 3 new cases of T1DM per 100,000 children aged 0-14 years. In the presence of two or more autoantibodies directed against insulin confers ~85% risk of developing diabetes within 15 years and nearly 100% over the lifetime of an at-risk individual. With the known fact of development of T1D diseases and early diagnostic systems, none have been successful in preventing T1D and the only reliable source left is insulin. Here, we have tried to enhance the efficiency of anti-diabetic drug, Lisofylline (LSF) which acts on specific molecular targets involved in disease pathogenesis are needed to improve prevention efforts including preserving residual beta cell function in new-onset T1D patients. The discovery of small molecules that can protect β-cells

against proinflammatory cytokines and preserve functional β-cell mass could potentially prevent life-long insulin therapy and complications in diabetic patients. LSF is one such small synthetic molecule with anti-diabetic activity,6 majorly attributed to its ability to a) inhibit the proinflammatory cytokine (IL-1β, TNF-α and IFN-γ) production and, b) effective suppression of T-cell activation and differentiation *via* inhibition of the STAT4-mediated IL-12 signaling. Most importantly, LSF can also maintain β-cell insulin secretory function in the presence of inflammatory cytokines and regulate immune cellular function to suppress autoimmunity.

With this conjugation of fatty acid, hydrophobicity of whole molecule increases, which makes system more appropriate for the drug delivery by also converting and making it into a micellar formulation which itself is promising technology. This prodrug system can further given by oral delivery by encapsulating into a polymeric system enhances the further adaptability of LSF prodrug technology. These prodrugs can replace or reduces the burden of exogenous insulin patients and also helps in faster recovery or maintenance of insulin level in the body. The future therapy has many advantages and needed to explore further for preventing the diabetes complications and increase the patient compliance.

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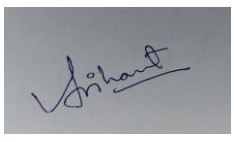

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