Contents

1. Introduction

Major depressive disorders (MDD), commonly known as depression is a serious disorder of enormous sociological and clinical relevance (1). The lifetime prevalence of depression is as high as 20%. In the general population, worldwide with a female to male ratio of about 5:2. Heritability based on twin studies show 40% to 50% chances and perhaps higher when measurement error is modeled based on repeated assessments. Adoption studies provide some support for a role for genetic factors, although these studies have methodological limitations. The relative risk (RR) (ratio of risks to first-degree relatives of MDD probands vs. the general population) is around 2 to 3. Depressive disorders can be classified as melancholia, psychotic depression, antenatal or postnatal depression, bipolar depression, cyclothymic disorder, dysthymic disorder, seasonal affective disorder according to symptoms present in person (2). Symptoms commonly present in depressed person includes:

- depressed mood;
- loss of interest or pleasure;
- significant weight loss or appetite alteration;
- insomnia or hyposomnia;
- psychomotor agitation or retardation;
- fatigue or loss of energy;
- feelings of worthlessness;
- diminished ability to think or concentrate or indecisiveness;
- suicidal ideation.

1.1 Medications

Drugs that are used clinically for depression, mostly based on monoamine theory. They can be classified as mentioned below:

- MonoAmine Oxidase inhibitors (MAOIs);
  Generic and brand names: Phenelzine (Nardil), Tranylcypromine (parnate), Isocarboxazid (marplan), Selegiline (emsam)
- Tricyclic antidepressants
  Generic and brand names: Amitrypriline (tryptizol), Clomipramine (anafranil), Imipramine (tofranil), Nortriptyline (allegron)
- Selective serotonin reuptake inhibiotors (SSRIs);
  Generic and brand name: Fluoxetine (Prozac), Sertraline (Zoloft), Citalopram (celexa), Escitlopram (lexapro)

- Atypical Anti Depressants:

  Generic and brand name: Bupropion (wellbutrin), Duloxetine (cymbalta), Mirtazepine (remeron), Trazodone (desyrel)

1.2 Limitations of current therapy

- Low remission rates

  It is shown that by current medication treatment remission rate around only 40-60 %, it is indicating that there is need of new medications for the treatment of disease.

- High rate of recurrence

  In patients who do recover, there is a high rate of recurrence and it has been found that approximately 75% of patients experience more than one episode of major depression within 10 years.

- Non-adherence to therapy

  It is widely believed that antidepressants generally show only a slow onset of efficacy, often taking up to 6 weeks for maximum effect, in spite of rapid pharmacological actions. Such a delay may promote non-adherence to therapy and increase the risk of suicide early in treatment.

- Side effects

  Side effects profile is also high as the treatment continues for a long time. SSRI class of drugs having side effects related to sexual dysfunctioning, insomnia and feeling of sickness. Tricyclic antidepressants and MAOIs are relatively more toxic than SSRI class of drugs. They sometime cause problem related to blood pressure, chest pain, severe migraine, abnormal fast heart beats.

- Drug – drug interaction is common due to long term therapy

  Most of the antidepressants especially SSRI class of drugs inhibits the enzyme CYP450. This CYP450 is important in metabolizing many drugs due to long treatment of depression it is possible for a patient to take some other medication for any other complications and these drugs may alter the pharmacokinetic profile of other drugs.

1.3 Gene delivery for depression: current status or genes involved in depression

Gene	Protein encoded	Role
SLC6A4	SERT	Decreases serotonin neurotransmitter level by increasing presynaptic serotonin transporters

p11 (S100A10)	**P11**	**Affects serotonnin signal by fetch out serotonin receptor on cell surface**
FKBP5	FKBP51	Bound to glucocorticoid receptor (GR), so corticoids can't bound to GR, affecting GR translocation and its trafficking
NR3C1	GR	Increases number of GR
CRHR1	CRHR1	important in negative feedback mechanism of glucocorticoid
BDNF	BDNF	Play important role in neuroplasticity
NTRK2	TrkB	Important in BDNF signaling

Cationic liposomes for gene delivery.

Due to their safety and versatility, cationic lipoosmes have emerged as promoting alternative to viral vectors for the development of gene therapeutics (3). Numerous in vivo applications have been reported in literar]ture, focusing not only aspects related to their pharmacokinetics and distribution but also on their toxicity and immunogenicity. However, it is generally recognized that the efficacy of lipid based gene delivery system is still far from that observed for viral vectors. Aiming at circumventing these limitations, much work has been devoted to the synthesis of new cationic lipids and the design of new plasmid constructs with more promoters (4).

BBB targeting by IGF-1 monoclonal antibody (mAb) or nasal spray

CNS disorders, such as Alzheimer's and Parkinson's disease, depression are an increasing burden on our ageing society because there are currently no effective treatments for these disabling conditions. Treatment as well as early diagnosis of these and other diseases that originate in the brain remain challenging because the majority of suitable therapeutic molecules and diagnostics cannot penetrate the tight and highly restrictive blood-brain barrier. The blood-brain barrier (BBB) prevents transport of molecules larger than 500 Daltons from blood to brain. Receptor-mediated transcytosis (RMT) facilitates transport across the BBB of specific

molecules that bind receptors on brain endothelial cells that form the BBB. An insulin-like growth factor 1 receptor (IGFlR)-binding antibody or fragment thereof is identified that transmigrates the BBB by RMT (5). The antibody or fragment is used to deliver a cargo molecule across the BBB, wherein here the cargo molecule is therapeutic p11 gene entrapped cationic liposomal nano carrier.

Multiple studies performed in animal models demonstrated evidence for intranasal absorption of drug molecules as a potential direct route of transport to the CNS. However, many controversies still remain. Direct intranasal transport is not always evident, and ambiguous results were observed even when testing similar molecules. It was noted that several factors can influence drug delivery such as head position of animals, drug administration technique, volume, and assessment of CNS distribution and drug formulation (6).

2. Hypothesis

Preparation of therapeutic p11 gene entrapped cationic liposomal nano carrier formulated from alkali amino acid modified lipid, will provide a better and safe delivery vector for gene delivery as compared to viral delivery vectors. In addition, this will provide better stability to therapeutic gene from nuclease mediated degradation. Also surface modification with a targeting ligand via intravenous route and local targeting of liposomes in form of nasal spray via intranasal route will provide brain targeted delivery of gene providing higher concentration of gene at the target site.

3. Objectives

The aim of the current project is to develop a novel treatment approach for depression to achieve the goals of

- developing a safe and effective p11 gene delivery vector for depression treatment
- targeting the same for brain delivery using Insulin like growth factor- I (IGF- I) mAb
- local targeting by nose to brain delivery through nasal spray

4. Isolation of p11 plasmid by alkaline lysis method

Alkaline lysis was first described by Birnboim and Doly in and has, with a few modifications, been the preferred method for plasmid DNA extraction from bacteria ever since (7).

1. Inoculate 3 ml of LB containing G418 antibiotic with a single colony of transformed bacteria and grow it overnight at 37°C with shaking.

2. Pour culture into a microfuge tube. Centrifuge at 8000 rpm for 2 minutes at 4°C in a microfuge. Remove the medium and leave the bacterial pellet at bottom of the tube.

3. Resuspend the bacterial pellet in 1ml of ice-cold solution I by vortexing.

4. Centrifuge at 8000 rmp for 2 minutes and discard the supernatant.

5. Resuspend the bacterial pellet in 100 µl of ice-cold solution I by vortexing and incubate on ice for 5 minutes.

6. Add 200 µl of freshly prepared solution II to each bacterial suspension. Close the tube tightly, and mix the contents well by inverting the tube.Store the tube in ice for 5 minutes.

7. Add 150 µl of ice-cold solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube in ice for 15 minutes.

8. Centrifuge the bacterial lysate for 15 minutes at 12000 rpm at 4°C in a microfuge. Collect the supernatant to a fresh tube.

9. Add equal volume of phenol: chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at 10,000 rpm for 5 minutes at 4°C in a microfuge. Transfer the aqueous upper layer to a fresh tube.

10. Add equal volume of isopropanol and 1/10 th volume of 3M sodium acetate. Mix thoroughly by repeated gentle inversion.

11. Centrifuge at 12000 rpm for 15 minutes at 4°C.

12. Discard the supernatant leaving pellet in the tube which is DNA.

13. Add 400 µl of 80% ethanol to the pellet and centrifuge at 12000 rpm for 10 minutes at 4°C.

14. Remove all of the supernatant by aspiration. Take care with this step, as the pellet sometimes does not adhere tightly to the tube.

15. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube.

16. Dissolve the pellet in 20 µl of sterile water containing 20 µg/ml RNase A. Vortex the solution gently for a few seconds and store the DNA at -20°C.

Recipes for solutions

Solution I:*	**Solution I:****	**Solution III:*****
50 mM glucose. 25 mM Tris-Cl (pH 8.0). 10 mM EDTA (pH 8.0).	0.2 N NaOH (freshly diluted from a 10 N stock). 1% (w/v) SDS	5 M potassium acetate, 60.0 ml. Glacial acetic acid, 11.5 ml. H2O, 28.5 ml.

* Prepare Solution I from standard stocks in batches of approx. 100 ml, sterilize by autoclaving and store at 4°C.

** Prepare Solution II fresh and use at room temperature.

***The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.

5. **Analytical methods used for quantification of pDNA**

a. UV spectroscopy

b. Gel electrophoresis

5.1 UV spectroscopy

pDNA was quantified using a UV spectrophotometric method (8). Absorbance of the solution of the pDNA was checked by taking the absorbance values at four wavelengths i.e. 230 nm, 260 nm, 280 nm using NanoDrop 2000 instrument (NanoDrop,Germany). Absorbance values at all the wavelengths were corrected for scattering from sample by substracting the absorbance value at 320 nm (correction performed automatically by software). Purity of the pDNA was determined by evaluating the ratio of A260/A280 and A260/A230. Once DNA was confirmed for its purity, calibration curve was constructed. pDNA stock solution of 1 µg/µL was prepared in DFW and by appropriate dilutions pDNA solutions of various concentrations between 1 ng/µL to 100 ng/µL were prepared. Absorbance values of these solutions were recorded at 260 nm NanoDrop UV spectrophotometer. Content of pDNA was calculated by corrected absorbance at 260 nm i.e. A260-A320 and multiplying the reading by dilution factor and using the relationship that A260 of 1.0 = 50 µg of dsDNA. Whole experiment was performed in triplicate with regression co-efficient of 0.999 was found and %RSD was less than 2 % meeting the acceptance criteria as per ICH guidelines.

5.2 Gel electrophoresis

Gel electrophoresis tank was filled with tank buffer and electrodes were placed in tank. Electrodes were connected with voltage supplier. 1.2% agarose gel in gel tray was placed in the tank buffer immersing the gel 2-3 mm below the level of buffer with gel-end having wells towards the negative electrode and other end of gel towards the positive electrode. Each DNA sample was mixed with 3µL of 6X loading dye and then loaded on to gel. Electrophoresis run was carried out at 5 V/cm depending on the distance between the electrodes for 30 min to 45 min. Gel was removed after run and pDNA migrated on the gel was visualized under UV light at 254 nm on GelDoc TM Imaging System. The gel was removed and the pDNA in the agarose gel was visualized under UV light using GelDoc™ XR + Imaging System. Gel images were

taken on ImageLab Software. Gel images were analyzed by ImageJ software for quantification which showed regression co-efficient of 0.998 .

6. Vector synthesis for formulation

6.1 Alkali amino acid based non-viral vector synthesis

Alkaline amino acids are positively charged at neutral pH; subsequently they could be good hydrophilic headgroups in cationic lipids. Three cationic lipids with alkaline amino acids (lysine, histidine, arginine) headgroups and a DOPE hydrophobic moiety were prepared. N α,N ß -di-Boc-L-lysine (L), N α ,N im -di-Boc-L-Histidine (H) , and Boc- Arg(Mtr)-OH (A) were three Boc protected amino acids used in synthesis of the same.

Step I:

Protected amino acids, DOPE and 4-dimethylaminopyridine (DMAP) were placed in a three-necked flask (100 mL) equipped with a constant pressure dropping funnel and an inlet. The mole ratio of protected amino acid, DOPE (148.8 mg, 0.2 mmol) and DMAP was 1:2:02. Anhydrous dichloromethane (10 mL) was added in an atmosphere of nitrogen. A solution of dicyclohexylcarbodiimide (DCC, 2 equivalent of DOPE) in dichloromethane (10 mL) was added dropwise in the mixture over 1 h. After stirring for 1 h at 0º C, the mixture was stirred at room temperature for another 22 h. The white solid dicyclohexylurea (DCU) precipitate was removed by filtration (9). Filtrate was washed twice with 50 mL saturated sodium hydrogen sulfate, 50 mL saturated Sodium bicarbonate solution, and 50 mL saturated sodium chloride solution. Organic solution was dried over anhydrous potassium sulfate and concentrated.

Step II:

Deprotection of protected lipids. Protected lipid (0.2 mmol) was dissolved in 2 mL of anhydrous dichloromethane. Trifluoroacetic acid (2 mL) was added in the mixture and stirred for 2 h at 0º C in an atmosphere of nitrogen. Mixture was stirred at room temperature for another 2 h. Mixture was concentrated and recrystallized in anhydrous diethyl ether.

Conjugation of protected amino acids to DOPE was confirmed using IR spectroscopy and conjugation efficiency was measured using TNBS assay. Amount of residual solvent i.e. DCM, in synthesised lipids was calculated using gas chromatography.

6.2 TNBS assay

TNBS reacts with primary amino group of DOPE lipid making it possible to estimate the percentage conjugation efficiency of DOPE to protected amino acids. Briefly, 50 µl of TNBS (20 mg/mL) solution was added to 1 mL of standard DOPE solution and amino acid conjugated DOPE solution. After addition of 200 µl of sodium bicarbonate (0.8 M, pH 8.5), the solutions were incubated at room temperature for 30 min. The absorbance was measured at 410 nm using

UV spectrophotometer and primary amine modification of DOPE lipid is depicted in table below (10).

Modified lipid	Conjugation efficiency (%)
DOPE- H	37.26 ± 4.95
DOPE- A	52.68 ± 5.64
DOPE - L	41.72 ± 5.09

7. Formulation and optimization of lipoplex

Liposomes were prepared by ethanol injection method (11). Synthesised cationic lipids, HSPC and cholesterol were taken in the molar ratios of (4:4.5:1.5) and solubilized into 0.8 ml of ethanol (minimum quantity needed to dissolve lipids) and injected into the preheated (60-65°C) aqueous solution. The lipid portion was added to preheated aqueous phase with continuous stirring for 15 -20 min till liposomal dispersion was formed. The formed liposomal dispersion was sonicated at 60 amplitudes and 0.6 second cycle time for one minute in probe sonicator.

Lipoplexes were prepared by incubating preformed cationic liposomes with pDNA based on the ratio of moles of cationic lipid (DOPE-amino acid conjugate) to moles of phosphate of pDNA. Briefly, sufficient quantity of liposomes (diluted with DFW if necessary) was taken and incubated with pDNA at 25°C ± 2°C temperature for a period of 30 min to 1.5 h (12).

Various process and formulation parameters involved in the preparation of liposomes and of lipoplexes were optimized to arrive at the best suitable formulation of pDNA lipoplexes. Process parameters characterized for the development of liposomes were ratio of organic to aqueous phase, rotation speed and stirring time while those for preparation of lipoplexes involved incubation time, pH and temperature for maximum complexation of pDNA. Formulation parameters i.e. lipid types, content and L/P ratio (lipid:pDNA molar ratio) were optimized based on the desired particle size distribution characteristics of liposomes and lipoplexes.

7.1 Characterisation of lipoplex

7.1.1 Complexation efficiency

After incubating liposomes with cDNA, formed lipoplexes were subjected to gel electrophoresis to assess complexation efficiency of liposomes. Lipoplexes were mixed with 2 µL of 6X gel loading buffer and loaded onto a 1.2 % agarose gel containing 0.5 µg /mL ethidium bromide and electrophoresed for 30 min at 100 V in TAE buffer. Afterwards, cDNA was visualized by UV trans-illumination and gel photography using a Gel Doc System.

Similarly, conjugation efficiency of prepared lipoplexes were determined using Nanodrop UV. The conjugated cDNA from the lipoplexes was released by phenol-chlorofrom extraction and the resulting aqueous phase was used to determine conjugation efficiency.

Modified lipid	Complexation efficiency (%)
DOPE- H lipoplex	98.72 ± 2.35
DOPE- A lipoplex	99.51 ± 3.48
DOPE-L lipoplex	96.43 ± 2.87

7.1.2 Particle size and zeta potential

The average particle size and zeta potential of lipoplexes wer determined using dynamic light scattering technique using Malver Zetasizer. Prior to measurements, lipoplexes were diluted with nuclease free water; measurements were carried out at 25 c in triplicates and size of all lipoplex formulations was found to be between 90 to 110 nm and zeta potential was found to be between 15 to 30 mV.

7.1.3 Liposome morphology and lamellarity

For imaging of liposome in their intact state Cryo-TEM is a preferred Technique. The sample was diluted two times with water and then 20 ml of sample was applied on suitable Carbon grid, surface of which was modified for proper adhesion of liposomal preparation to the grid surface. Later, a thin film of sample in amorphous ice was made using liquid ethane. Subsequently, the grid was transferred to cryo holder already maintained at cryo temperature and observed for morphology and lamellarity using Tecnai G2 cryo-TEM.

7.1.4 Heparin polyanion competition assay

This assay was used to evaluate the stability of cDNA lipoplexes in vivo and to confirm that cDNA forms stable complexes with cationic liposomes which are resistant to decomplexation by polyanions like sulphated glaycosaminoglycans found inside body (13). The formulations were exposed to varying amount of heparin sodium and resulting dispersions were incubated for 30 min at room temperature. After incubation, the amount of cDNA decomplexed from liposomes were evaluated using gel electrophoresis. Heparin:cDNA ratio at which dissociation of cDNA from different lipoplex formulations occurs is shown in table below.

Formulations	Heparin:cDNA (w/w)
DOPE- H lipoplex	1:1
DOPE- A lipoplex	4:1
DOPE-L lipoplex	3:1

7.1.5 Serum stability study

In order to evaluate the potential of prepared lipoplex to protect cDNA from DNAses, especially those present in serum when administration is meant for intravenous route; serum stability studies were carried out by incubating formulations under high serum conditions (50% v/v) and it was analyzed for stability by nanodrop uv spectrophotometer at different time points (14).

% cDNA retained				
Time	**Naked cDNA**	**DOPE- H**	**DOPE- A**	**DOPE- L**
0	100	100	100	100
1	81.37 ± 2.68	98.12 ± 3.62	99.65 ± 0.61	99.51 ± 2.85
2	58.68 ± 3.89	96.39 ± 2.71	98.32 ± 1.70	98.35 ± 2.63
4	24.53 ± 4.08	91.71 ± 3.18	96.55 ± 1.67	94.66 ± 1.94
8	-	83.36 ± 2.69	95.36 ± 2.09	90.93 ± 3.48
16	-	81.25 ± 4.23	89.64 ± 4.27	86.64 ± 4.30
20	-	74.28 ± 4.92	87.85 ± 1.65	83.17 ± 2.62
24	-	69.64 ± 3.56	86.94 ± 3.46	82.94 ± 1.53

7.1.6 Electrolyte induced flocculation study

Electrolyte flocculation study was done to evaluate steric stability of prepared lipoplex by estimating extent of steric barrier present around liposomes (15). For proposed study, lipoplex formulations were dispersed in 1 ml PBS and diluted such that final lipid concentration of 1 mg/ ml was obtained. From this dispersion 1 ml of aliquot was taken and 1 ml of sodium sulphate solution of various concentrations ranging from 0 to 5% prepared in 16.7% sucrose solution, were added to it. The resulting dispersions were mixed thoroughly and absorbance was measured for all the concentrations within 5 min at 400 nm on UV–vis spectrophotometer against respective blank. This was further confirmed by measuring the size of the liposomes by Zetasizer. From the results, it was found that all the lipoplex formulations was stable till exposed to external electrolytic concentration minimum equivalent to 2% Na_2SO_4 solution while exposure to certain higher concentration resulted in liposomal aggregation as the steric barrier, preventing aggregation, was lost.

7.1.7 Haemolysis assay

Hemolytic potential was determined to check compatibility of different formulations with red blood cells and thereby estimate its safety when used as a carrier for cDNA (16). Briefly, blood

sample was collected from Wistar rats in heparinized microcentrifuge tubes by retro-orbital sinus puncture. It was centrifuged at 3000 rpm for 5 min and settled erythrocyte pellet was re-dispersed in normal saline to achieve 2% erythrocyte dispersion. Subsequently, 1 ml of 2% erythrocyte dispersion was mixed with 1 ml test samples such that final concentration btained was from 1 to 1000 nM on lipid basis which was incubated for 1 h at 37 ° C. It was entrifuged at 3000 rpm for 5 min and supernatant was estimated for free hemoglobin content by determining absorbance at 545 nm using UV–Visible spectrometry. From results it was concluded that modified lipoplex formulations were negligibly toxic as hemolysis rate was less than 2 % at highest concentration.

In addition, solutions obtained by incubating 2% erythrocyte dispersion with formulations equivalent to 1000 nM on lipid basis were observed under microscope for heme-aggregation study using normal saline treatment as negative control.

8. *In vitro* cell line studies

8.1 *In vitro* cell cytotoxicity study

SHSY5Y cells were seeded onto a 96- well plate at a cell density of 5000 cells/well and allowed to grow in MEM media with sodium pyruvate + 10 % FCS + 1 % antibiotic-antimycotic solution) for 24 h. After 24 h of incubation, cells were separately treated with developed lipoplexes formulations. Such treated cells were incubated in incubator for 24 h, after which the transfecting medium was replaced with fresh media. The cells were incubated for 48 h and then treated with 20 µL of 5 mg/mL solution of MTT. After 4 h of incubation with MTT solution, the culture media was removed and 200 µL of DMSO was added and solubilised formazan crystals in DMSO was measured by colorimetry at 570 nm using ELISA plate reader. Cell viability of each treated group was expressed as a relative percentage against that of negative control (PBS treated cells); lipoplex formulation of commercially available cationic vector (positive control). From the results, it was concluded that all the lipoplex formulations formulated from modified lipids are relatively nontoxic.

8.2 Cell uptake study

Confocal microscopy

Media preparation, subculturing of cells, cell counting and preparation of formulations were followed as described in the MTT assay earlier. Cells were seeded onto 6-well plates with a glass cover slip at the bottom. Cells were seeded at a density of 50,000 cells/well on flame sterilized 0.17 mm square glass cover slips in a 6 well plates and allowed to grow for 24 h in complete media. After 24 h, cells were transfected with lipoplexes prepared using optimized liposomal formulations using eGFP . Treatement was carried out for 6 h and post-treatment,

cells were washed with phosphate buffered saline. Fresh media was replaced and cells were incubated for 24 h for expression of eGFP. Cells were washed with PBS followed by treatment with nucleus stain DAPI for 10 minutes followed by washing again with sterile phosphate buffered saline to remove excess DAPI from milieu. Coverslips were mounted on sterile glass slides and confocal microscopy was performed on confocal laser scanning microscope. Cells treated with naked eGFP cDNA and lipoplexes prepared with Lipofectamine-2000 were used as controls.

8.3 *In vitro* permeation study

The adherent cells were trypsinised and detached from tissue culture flask and seeded in each transwell at density of 1 x 10^{6} cells/insert nd then 1.5 ml of complete media was added in basolateral chamber. This was incubated in CO_2 incubator and media was changed every 24 h. The monolayer integrity of cells was checked regularly by voltohmeter and permeabilty assay was commenced after 9-12 days when TEER (Transepithelial electrical resistance) value above 170 Ω.cm^2 was achieved (17). Before starting media was replaced by transport media (HBSS + 25mM HEPES + 0.35 g/l $NaHCO_3$) was added to both chambers and plate was allowed to equilibrate for 1 h. Subsequanlty, flurocscent labelled formulations diluted in 0.5 ml transport media were added and plate was incubated at 500 rpm in shaker. 20 µl sample was withdrawn at 15, 30, 45, 60, 90 and 120 min and replaced with equal amount of fresh transport media. The sampels were analysed by fluorimetry to determine formulation transported to receptor compartment. The results from study showed that lipoplex formulations improved permeation of cDNA and that would ultimately help in improving its therapeutic efficacy.

8.4 *In vitro* gene expression studies by Real Time-qPCR

In vitro mRNA expression efficiency of different cDNA formulations was evaluated in order to quantify gene expressing potential of lipoplex formulations. RT-PCR was used to quantify mRNA expressed in SHSY5Y cells transfected with different lipoplex formulations. SHSY5Y cells seeded on a 24 well plate at a density of 1,00,000 cells/well were incubated for 24 h to get 80 % confluency. After incubation cells were treated with INF-α-2b (interferon), to reduce the p11 mRNA level. After 48 h of incubation, cells were treated with different lipoplex formulations (18). Basal gene expression level was evaluated using PBS control i.e. negative control. After another 48 h of incubation, total RNA was isolated using TRIzol reagent and reverse transcriptase into cDNA was carried out using RNA to cdna was carried out using RNA to cDNA conversion kit. mRNA level was quantified using Step One real time PCR using SYBR Green Master mix, forward and reverse primers and lipoplex formulations in a total

volume of 10 µL. The mRNA expression level was normalised against housekeeping gene GAPDH.

The results of gene expression studies showed that INF-α-2b considerably reduced the mRNA level of p11, which was again elevated by cDNA complexed lipoplex formulation.

Groups	**Normal control**	**Disease control**	**DOPE- H**	**DOPE- A**	**DOPE- L**
% gene expression	100	42.6 ± 2.3	48.3 ± 1.39	89.5 ± 2.71	72.9 ± 2.84

"From above depicted studies, lipoplex formulated from modified DOPE-A, showed promising outcomes, consequently for further studies i.e. targeting approach, nasal spray formulation and in vivo studies, lipoplex formulated from DOPE- A modified lipid was used. "

9. <u>CNS delivery of DOPE- A lipoplex by nasal spray</u>

(nose to brain delivery)

9.1 Formulation and characterization of nasal spray

9.2 Formulation of nasal spray

Glycerine was mixed with water in beaker I and stirred well for 30 min. simultaneously, in another beaker, beaker II, Di sodium EDTA was dissolved in water; phosphate buffers and sodium chloride were added to the solution once the solution became clear and mixed with the solution of beaker I. Finally, benzalkonium chloride was added as preservative and volume was made up with nuclease free water. The optimised formula for the preparation of nasal spray is represented in table below.

Ingredients	**Role**	**Quantity in percentage**
Sodium Phosphate, monobasic	Buffer component	0.5525 %
Sodium Phosphate, dibasic	Buffer component	0.0975 %
Di Sodium EDTA	Chelating agent	0.03%
Glycerine	Humectant	0.5 %
Sodium Chloride	Osmotic agent	0.8 %
Benzalkonium chloride	Preservative	0.1 %

9.3 Characterization of nasal spray

9.3.1 pH

pH of prepared nasal spray was found to be between 6 to 6.2 determined using Labindia pH meter. Briefly, fixed sample volume was taken in beaker and electrode was dipped in it. This was allowed to stabilize for a while and then stable pH was recorded.

9.3.2 Osmolality

Osmolality was determined using osmometer which was found to be around 290mOsm. The instrument was operated as per manufacturer's guidelines. Briefly, empty sample tube was placed inside sample well and test was started; result displayed was recorded.

9.3.4 Viscosity

The viscosity of nasal spray formulation was measured using Brookfield viscometer which was around 46-47 cP. The test formulation was loaded in sample holder with a constant volume and rested for 40 to 45 min. an appropriate spindle was immersed in the test liquid and rotated at different speeds (10 to 100 rpm). The reading was taken after five full spindle rotations. Three replicate measurements per formulations were carried out at temperature of 25 ±2 º C.

9.3.5 Shot weight

Shot weight was found to be 78.6 mg/actuation with % RSD 0.1. It was assessed by weighing the spray pump filled with 10 gm nasal spray prior to and after each actuation using an analytical balance with maximum weighing capacity of 200 gm and readability of 0.1 mg.

9.3.6 plume geometry and spray pattern

Plume geometry and spray pattern were measured using a SprayVIEW NSP system and analysed by sprayVIEW software. The SprayVIEW NSP combines laser sheet illumination and high speed digital imaging; is designed specifically to characterise pharmaceutical nasal spray. The spray pump was filled with test fluid and placed in the mouth of the actuator, which was precalibrated for compression force and duration as per testing guidelines. The plume geometry was characterised by spray angle, spray width which was found to be 26.5 and 27.2 mm respectively while spray pattern was characterised by its shape and ovality value which was around 1.3 to 1.4. Spray pattern was calculated at 3 cm distance and plume width 6 cm from nozzle orifice (18) (19).

9.3.7 Droplet size distribution

Droplet size distribution was determined by laser diffraction technique employing HELOS BR instrument with SPRAYER module and force actuator (18). Various instrument parameters like spraying angle (30, 60, 90), actuation force (35, 45, 55), stroke length was set as 3 cm and

hold time was kept 2 sec. time resolved measurement was performed and data were analysed by Fraunhofer theory. From data analysis value of D10, D50, D90 was found to be 28.42 µm, 77.16 µm, 151.85 µm respectively.

9.3.8 Ex- vivo nasal permeation study

Freshly excised sheep nasal mucosa was obtained and dipped immediately in nuclease free water to maintain hydration. Superior nasal conche was recognised and detached from the nasal mucosa and washed properly with nuclease free water to remove debris and other contaminants. The membrane was mounted on franz diffusion cell. The tissue was stabilised using phosphate buffer (pH 7.4) in donor as well as receptor compartments for 15 min. later both compartments were emptied and receptor compartment was filled with fresh PBS (pH 7.4) such that nasal membrane touches the buffer and assembly was maintained at 37 ± 2 º C with continuous stirring. Subsequently, desired concentration of suitably diluted lipoplex dispersion was added to the donor compartment. 0.5 ml sample was withdrawn from the receptor compartment at predefined time intervals and fresh PBS (pH 7.4) was added to receptor compartment. The withdrawn samples were suitably diluted and analysed by fluorimetry (20). Results from this study depicted permeation of significant fraction of cDNA from lipoplex formulation (86.29 %) in comparison to naked cDNA (26.14 %), to receptor chamber of franz diffusion cell through sheep mucosa after 8 h.

10. Intravenous CNS delivery of IGF-I conjugated lipoplex

10.1 Formulation and characterisation of immunoliposomes

10.1.1 Preparation of maleimide functionalised liposomes

Functionalized liposomes were prepared by the post-insertion method (21). In contrast to the pre-insertion method, post-insertion comprises two consecutive reactions. First, micelles are formed with DSPE-PEG-Mal in the presence of aqueous phase and subsequently insertion of micellar solution to preformed liposomes containing 1 mole % of DSPE-mPEG. Briefly, a micellar solution of 1 mole % of DSPE-mPEG2000 maleimide was prepared and pre-equilibrated at 55 º C. Optimised batch of liposomes was taken in one beaker and temperature around 60 º C was maintained (> tg of HSPC), the micellar solution was then added drop by drop to the liposomal dispersion and was incubated for 2 to 3 h at 60 º C. The micellar solution then was from liposomal fraction using sepahrose CL-4B column.

10.1.2 Confirmation of functionalization

Ellman's assay was used to determine the functionalization of liposomes. For that, a known amount of thiols which was provided by cysteine , were reacted with maleimide in excess, after

which the unreacted fraction of thiol present in solution was determined using ellman's reagent (22). For the above procedure, solution of cysteine HCl (1.5 mM) and sodium phosphate (pH 8.0, 0.1 M) solution containing 1mM EDTA were prepared and mixed well with functionalised liposomes with overnight stirring in cold room. After overnight stirring, 50 µl of ellmans reagent was mixed and stirred for 15 min at room temperature. The absorbance of above solution was determined using UV visible spectrophotometer at 412 nm to determine unreacted cysteine.

10.1.3 Thiolation of monoclonal antibody

mAb was dissolved in 0.15 M Na-borate buffer/0.1 mM EDTA, pH 8.5 followed by the addition of Traut's reagent. After incubation for 60 min at room temperature, mAb solutions were concentrated and the buffer exchanged with 0.1 M Na-phosphate (pH 8.0, 0.1 M) using a Centriprep-30 concentrator (Amicon). Thiolated mAb was immediately used for conjugation with liposomes and was determined for size, zeta potential and complexation efficiency. Ellmann's reagent was used to determine the number of sulfhydryl groups added by thiolation to mAb (23).

10.1.4 Preparation of immunolipsomes

Monoclonal antibody was incubated with maleimide functionalised liposomes in weight ratio of 1: 50. The incubation was kept overnight in cool condition under inert atmosphere of nitrogen. Then after, formed immunoliposomes were incubated in same condtion with excess amount of cysteine so that leftover (unconjugated) maleimide groups were occupied by cysteine. The extra unreacted cysteine was removed using ultracel membrane (50 KDa) and was characterised for size, zeta potential, complexation efficiency and permeation ability. mAb conjugation to liposomes were confirmed by bradford's method SDS-PAGE using coomassie staining. Size of lipoplex found to be around 130-150 nm but there was not ample change in the zeta potential and complexation ability was observed (24).

10.1.5 Bradford's method for estimation of mAb conjugation

To the 0.5 ml of immunoliposomal fraction, 2.5 ml of methanol was added to dissolve all lipids. Methanol was then evaporated at 50 ° C to precipitate all the lipids. These precipitated lipids were than separated by centrifugation and the supernatant containing mAb was estimated by Bradford's method. For this assay, 10 µl of prepared mAb solution and extracted mAb solutions from supernatant were added to 96 well plate (25). To each well 100 µl of Bradford's reagent was added and absorbance was taken at 595 nm using ELISA plate reader and amount of conjugated mAb was found to be 86.98 % ± 2.39 %.

11. *In vivo* studies

11.1 Nasal ciliotoxicity studies

Freshly excised sheep nasal mucosa, except for the septum, was collected from the slaughter house in saline phosphate buffer pH 6.4. Three sheep nasal mucosa pieces (S1, S2, and S3) with uniform thickness were selected and mounted on Franz diffusion cells. S1 was treated with 0.5 mL of PBS pH 6.4 (negative control), S2 with 0.5 mL of isopropyl alcohol (positive control), and S3 was treated with lipoplex formulation for 1 h (26). After 1 h, the mucosa was rinsed with PBS at pH 6.4 and subjected to histological studies to evaluate the toxicities of lipoplex nasal spray formulation photographed by microscope to check effect of formulation on nasal epithelia and the result showed no adverse effect on nasal epithelia compared to IPA which damaged nasal mucosa and also had adverse effect on nasal cilia.

11.2 Acute toxicity studies

The swiss albino mice were randomly divided into respective groups for administration of lipoplexes such that 3 mice were allocated to each group. Lipolexes were administered by intravenous and intranasal routes in separate groups and animals were observed for 14 days for any signs of toxicity and mortality but there was no sign of any kind of toxicity was observed for both the routes even after escalating dose. All the animals used in the studies were weighed before and after the completion of studies (27).

11.3 Brain distribution study

For brain distribution study animals were divided in 4 groups (3 rats per group), each for intravenously administirred lipoplex and IGF-I targeted lipoplex, intranasal lipoplex and intransal flouroscent naked cDNA. All rats were anaesthetised using ketamine (100 mg/kg) and xylazine (20mg/kg); then liposomal formulation containing eGFP was administered. To determine the distribution in brain tissue after intranasal and intravenous routes, the brains were removed after 4 h of liposomal administration for cryo sectioning. The tissue was embedded in OCT media and cut into 7 µm thickness which was stained for nucleus visibility by DAPI dye (28). Confocal images of cryo sectioned tissue of group administered with intravenousIGF-I targeted lipoplex exhibited more accumulation of in brain contrast to other groups .

11.4 Interferon α 2B (INF α 2B) induced animal models

11.4.1 Selection of dose

Mice were divided into 5 groups, (6 mice per group), one of which was control and remaining groups were treated intraperitonially with different doses of INF α 2B (100, 400, 800 and 1200 IU/ day) for 15 days for induction of depression like behavioural despair detected by TST and

FST assays. Results showed that 800 IU/day was sufficient to induce depression like behaviour which was confirmed by TST and FST assays.

11.4.2 TST and FST assay

Male swiss albino mice were divided into 4 groups. For TST studies, two swim sessions were conducted: a baseline 10-min free exposure session, 1 day before the start of the study, to acclimatize the mice to the FST; and an end point 6-min session to assess behavioural despair, 24 h after the last IFN-α2b dose (29). The dose for depression induction was selected as per the results of studies depicted above. The FST apparatus was filled with fresh water at ambient temperature. Mice were judged to be immobile when they floated in an upright position and made only small movements to keep the head above water. Mice were tested individually, and the time of immobility was recorded during the last 4 min of the 6-min period of exposure, leaving the first 2 min for habituation (30) (31).

The total duration of immobility induced by tail suspension was measured according to the method described by steru et al. as a facile means of evaluating depressing behaviour and antidepressant potential of formulation. Mice were judged to be immobile when they floated in an upright position and made only small movements to keep the head above water. Mice were tested individually, and the time of immobility was recorded during the last 4 min of the 6-min period of exposure, leaving the first 2 min for habituation. Results of FST and TST assays showed that treatment with INF α-2B at 800 IU/day dose, significantly increased TST and FST immobility time.

Reversal of antidepressant behaviour

For this study, swiss albino mice were divided in 4 groups and they were administered with 800 IU /day of INF α 2B for 15 days, to induce depression type of behaviours. After inducing disease, each group was treated with different formulations comprising of control (physiological saline), targeted and non targeted i.v. formulations, intranasal lipoplex formulation and were assessed for their behavioural changes by TST and FST studies after a week. Treatment with lipoplex formulation containing therapeutic gene reduced immobility time when administered through both intranasal and intravenous routes separately showing signs of antidepressant behaviour but at different rates, targeted liposomal formulation showed early recovery from depression like behaviour.

Efficacy studies

11.5 Western blot studies

Young swiss albino mice were divided in 5 groups. Three groups of them received INF α-2B (disease control) and the remaining one received physiological saline pH 7.5 (control). After inducing depression, two groups received treatment of targeted and non-targeted liposomal formulation by i.v. route and one group treated with intranasal liposomes. The leftover group was used as disease control group. Hippocampi of mice treated with saline solution (control), INF α-2B (disease control), liposomal formulation (3 treatment groups) were collected and stored in −80°C until further process. The isolated hippoampi was homogenised in lysis buffer and agitated for 2h. This homogenate was centrifuged at 12000 rmp for 20 min at 4 c and the supernant was collected on ice and total protein conten was determined by Lowery method. Then required volume of sample was mixed with equivalent volume of 2x Laemmli buffer nd heated 95 c for 5 min. 30 µL samples were loaded in SDS-PAGE gel made up of stacking gel (4 %) and resolving gel (12%). 15-20 µl protein ladder was loaded in 1 well to estimate molecular weight. SDS-PAGE was operated at 100 V to allow migration of proteins. After completion, the bands were transferred on nitrocellulose membrane using transfer cassette at 100 V for 2h usinfg ice cold 2 liter transfer buffer and gel packs to maintain temperature. Complete transfer of bands were verified with Ponceau staining. The membrane was blocked using blocking buffer (5-10 ml) for 1 h at 37 c on gel rocker. This blocked membrane was incubated overnight with primary antibody on gel rocker at 4 c. the membrane was washed 4 times with PBST for 15 min. later, it was incubated with antibody for 1 h at 37 c. this was washed 3 times with PBST and PBS for 15 min on gel rocker. The blot was incubated with ECL substrate for 2 min in dark nd later observed in chemiluminescence detector (32). The membrane was washed 4 times with PBST for 15 min and reported with beta-actin antibody which was used as loading control. The results showed diminishing of p11 protein expression in mice treated with INF α-2B in comparison to normal control group but after treatment there was upsurge in p11 protein level through each and individual intranasal and intravenous routes nevertheless intravenous targeted liposomal formulation exhibited substantial improvement in p11 level than that of other groups.

11.6 Gene expression study

p11 mRNA level was examined in mice hippocampi of normal control, disease control and from treatment groups (intravenously and intranasally). Mice were divided in groups as per studies depicted above. After disease induction and its treatments, mice hippocampi were homogenized in cold PBS. RNA was extracted from tissue samples and were determined using spectrophotometric techniques. Then further process to quantify mRNA level by RT-PCR was

done. The results of gene expression study showed INF α-2B reduced p11 mRNA level which was yet again amplified by p11 lipoplex formulations individually by intravenous and intranasal routes however intravenous targeted liposomal formulation showed better efficacy than other groups.

12. Conclusion

In this work, novel tactic for the treatment of major depressive disorder was applied by CNS delivery of p11 gene therapeutic by intranasal administration; targeted and non-targeted intravenous administration; and their efficacy to treat depression was compared. For this, three different alkali amino acids (BOC protected arginine, histidine and lysine) having positive charge at physiological pH was conjugated to DOPE by amide bond formation between amine group of DOPE and -COOH group of amino acids, to increase its endosomal escape property. The modifications of lipids were confirmed by IR spectroscopy and residual solvent level was measured using gas chromatography. The modified lipids were then encorporated in liposomes with HSPC (bilayer forming lipid), cholesterol (stability imparting lipid) and were complexed with cDNA. Synthesized three different lipoplexes were characterized for size, zeta potential, complexation efficiency, heparin conjugation assay, serum stability and *in vitro* cell line studies like RT-PCR, Cellular uptake study and MTT assay to chose the best among them for further studies. The chosen lipoplex formulation was conjugated to IGF-I mAb for active targeting via intravenous route and same lipoplex formulation was delivered through intranasl route (without targeting) as nasal spray. IGF-I targeted lipolex were again evaluated for size, zeta potential, complexation and conjugation efficiencies while intranasal lipoplex formulation as nasal spray was evaluated for several parameters including pH, osmolality, plume geometry, droplet size distribution, shot weight, spray pattern and ex vivo permeation efficiency. This optimized formulation was later evaluated by *in vivo* studies like toxicity studies, brain distribution study and efficacy study by western blot and RT-PCR for both routes individually. All the parameters supported the envisioned goal of the study and of the two routes, intravenous and intranasal, intravenous IGF-I conjugated lipoplex provided better efficacy.

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