A SYNOPSIS ON

INVESTIGATION ON SPHERULITES AS NOVEL CARRIERS FOR DRUG TARGETING TO NON-SMALL CELL LUNG CANCER

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1. Introduction:

Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumours and neoplasms. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. This process is referred to as metastasis. Metastasis is the major cause of death from cancer.[1]

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The burden of cancer is increasing in economically developing countries as a result of population ageing and growth as well as, numerous factors enlisted below.[2]

Key Facts of Cancer: [1]

- Cancer is a leading cause of death worldwide, accounting for 14 million new cases and 8.2 million deaths in 2012.
- Lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year.
- The most frequent types of cancer differ between men and women.
- About 30% of cancer deaths are due to the some behavioural and dietary risks.
- Tobacco use is the most important risk factor for cancer causing 22% of global cancer deaths and 71% of global lung cancer deaths.

Causes of Cancer: [3]

- Smoking
- Alcohol
- Overweight
- Lack of Physical Activity
- Infection
- Sunlight (increased UV radiation)
- Occupational Exposure to cancerous agents
- Environmental Exposure.

Pathophysiology of Lung Cancer:

Lung cancer is a disease characterized by uncontrolled cell growth in tissues of the lung. If left untreated, this growth can spread beyond the lung in a process called metastasis into nearby tissue and, eventually, into other parts of the body. Most cancers that start in lung, known as primary lung cancers, are carcinomas that derive from epithelial cells. The main types of lung cancer are *small cell lung carcinoma* (SCLC), also called oat cell cancer, and *non-small cell lung carcinoma* (NSCLC).[4]

Symptoms of Lung Cancer: [5]

The symptoms of lung cancer are persistent cough, coughing up blood, breathlessness, wheezing, hoarseness, chest or shoulder pain, tiredness and weight loss.

Currently available treatment options of Lung Cancer:

The class of drugs used for treatment of lung cancer are alkylating agents eg. Ifosfamide, antimetsbolites eg. Gemcitabine, antibiotic eg. Mitomycin, plant derived products eg. Vinblastine, miscellaneous agents eg. Cisplatin.[6]

Gemcitabine and Vinorelbine are first line agents used in the treatment of lung cancer and both show good cytotoxicity against lung tumour.

Gemcitabine, a nucleoside analog related to cytarabine, is a novel pyrimidine antimetabolite that shows significant cytotoxicity against lung tumours. [7]

As with fluorouracil and other analogues of pyrimidines, the triphosphate analogue of Gemcitabine replaces one of the building blocks of nucleic acids, in this case cytidine, during DNA replication. The process arrests tumour growth, as only one additional nucleoside can be attached to the "faulty" nucleoside, resulting in apoptosis.

Another target of Gemcitabine is the enzyme ribonucleotide reductase (RNR). The diphosphate analogue binds to RNR active site and inactivates the enzyme irreversibly. Once RNR is inhibited, the cell cannot produce the deoxyribonucleotides required for DNA replication and repair, and cell apoptosis is induced. [8]

Gemcitabine is marketed as “Gemzar” by Eli Lilly and Company. “Gemzar” is a white to off white solid lyophilized powder and it is supplied in vials of either 200 mg or 1 gm administered by i.v. infusion over 30 minutes. It is formulated with mannitol and sodium

acetate. Hydrochloric acid or /or sodium hydroxide may have been added for pH adjustment.[9]

Our another drug of interest is Vinorelbine. Vinorelbine (5'nor-anhydro-vinblastin) is a semisynthetic vinca alkaloid that is manufactured from alkaloids extracted from the rosy periwinkle, Catharanthus roseus. Vinorelbine induces cytotoxicity by inhibiting the polymerisation of tubulin dimers into microtubules, which in turn disrupts mitotic spindle formation and prevent cell division. This promotes apoptosis in cancer cells. [10]

Vinorelbine is marketed by Abbott Healthcare under the name Navelbine. Navelbine is available in vial containing vinorelbine tartarate equivalent to 10 mg (1 mal vial) or 50 mg (5 ml vial) and formulated in water for injection administered by i.v. route over 6-10 minutes.[11]

Both Gemcitabine hydrochloride (Vd: i.v. 50 L/m^2) and Vinorelbine tartarate (Vd: i.v. 25.4-40.1 L/kg) due to their non-specific distribution throughout the body, are associated with serious side effects like anaemia, thrombocytopenia, cardiac arrhythmia, alopecia etc. [12, 13.] Development of a targeted drug delivery systems containing these drugs reduces its distribution to the other organs and tissues resulting into decrease in the side effects. Various nanocarrier based delivery systems for targeting these drugs to the lung tumor have been reported. However, Gemcitabine loaded nanocarriers are found to have limitations like failure to achieve desired particle size and also low drug entrapment efficiency in case of liposomes [14], low drug loading capacity and low stability of the formulation in aqueous media in case of polymeric micelles [15], in the dendrimers based formulation the selection of suitable vector is tedious process [16] and for carbon nanotubes it may show toxicity by bioaccumulation [17]. Vinorelbine has also been entrapped in nanocarriers but they are having some of the disadvantages such as from the liposomes the drug gets rapidly released thus it eliminates the benefits of liposomal encapsulation [18], the polymeric nanoparticles show slight decline in drug entrapment efficiency [19], dendrimers are having the risk of bioaccumulation [20]. Thus a tumor specific targeted drug carrier is required which eliminates these limitations of the presently available systems.

Types of Targeted Drug Delivery Systems to Lung Tumour: [21]

- Passive Tumour Targeting: Passive targeting is achieved via generalised Enhance Permeation and Retention (EPR) effect. Anticancer drugs used in chemotherapy have no tumour selectivity in passive targeting. Polymer carriers bearing physically entrapped or chemically conjugated drugs are strategy for passive tumour targeting.
- Active Tumour Targeting: Cancer cells often display increased cell surface expression of proteins that may be found at low levels on normal cells (tumour-associated antigens), as well as proteins that are found exclusively on cancer cell surfaces (tumour-specific antigens). Active drug targeting is usually achieved by chemical attachment to a targeting component that strongly interacts with antigens (or receptors) displayed on the target tissue, leading to preferential accumulation of the drug in the targeted organ, tissue, or cells.

Types of Nanocarriers for drug delivery: [22]

- Polymeric Nanoparticles: Drugs are conjugated to side chain of a linear polymer with a linker.
- Polymeric micelles: Amphiphillic block copolymers assemble and form a micelle with hydrophobic core and hydrophilic shell.
- Dendrimers: Radially emerging hyperbranched synthetic polymer with regular pattern and repeated units.
- Liposomes: Self assembling close colloidal structures composed of lipid bilayers.
- Viral Nanoparticles: molecular cages derived from the assembly of viral structural proteins.
- Carbon Nanotubes: Carbon cylinders composed of benzene ring.

Advantages of Nanocarriers: [23]

- Increased drug localization at tumour site.
- Protect drug from degradation and from premature clearance.
- Retain drug at target site for desired period of time.
- Facilitate cellular uptake and intracellular trafficking.
- Biocompatible and biodegradable.

Disadvantages of Nanocarriers: [24]

- Nanocarriers taken up by reticuloendothelial system (RES).
- Short circulation time.
- Failure to achieve desired particle size.
- Drug leakage during reaching targeted site.

Spherulites: A Novel vesicular drug Delivery System for Tumour Targeting:

Spherulites are concentric multilamellar vesicles obtained by shearing lamellar phase of lipids and surfactants. They consists of concentric bilayers of amphiphiles alternating with layers of aqueous medium in which hydrophilic drugs can be sequestered with high yield. To be useful for drug targeting applications, spherulites should be of desired size and long circulating.[25]

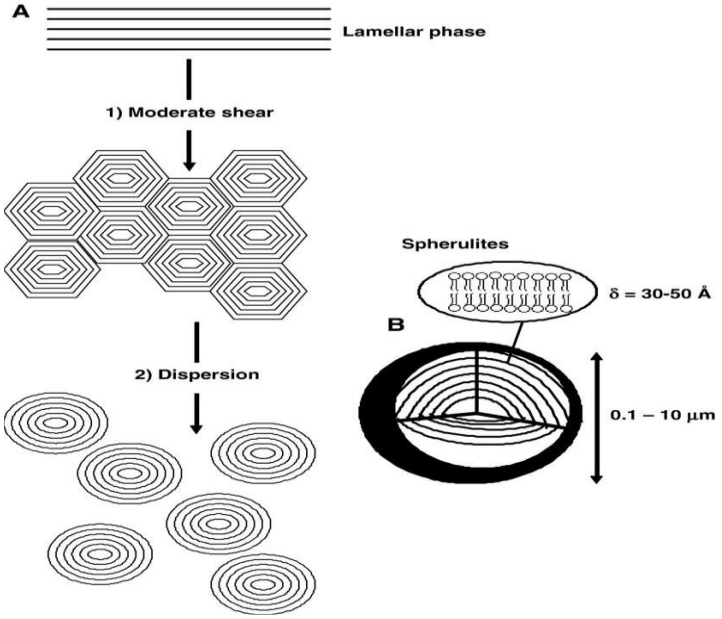

Fig 1: Graphical representation of Spherulites assembly

Spherulites are also called as Lyotropic Lamellar Phases or Onion phases. Spherulites are made of surfactants, water and sometime an additional hydrophobic component (cosurfactant).[26]

The important properties of Spherulites are as follows: [27]

- High stability and protection of the incorporated molecule against enzymatic degradation.

- Ability to incorporate both hydrophilic and lipophillic active molecules with high encapsulation yield.
- Manufacture without use of organic solvents and with little stress (pressure, shear, temperature) allowing the encapsulation of fragile molecules.
- Have desired size in nanometric range which is required for the Enhanced Permeation and retention effect for drug targeting to the tumour.

Hypothesis:

The drug loaded Spherulites are expected to overcome the limitations of existing nanocarrier formulations like instability, drug leakage during circulation and low entrapment efficiency. The drug loaded spherulites, because of their nano size will be targeted to the tumor due to the Enhanced Permeation and retention (EPR) effect. PEGylation will increase the circulation half-life of spherulites.

2. Aim and Objectives:

Our aim is to develop a spherulites formulation loaded with an anticancer drug Gemcitabine hydrochloride and Vinorelbine tartrate separately in order to target it to the lung tumour and decrease its distribution to other tissues and organs resulting into decreased side effects.

3. Plan of Work:

1. **First Year :** Literature survey
   Procurement of API and Excipients
   Preformulation Studies

2. **Second Year:** Preparation of Formulation
   Optimization of the formulation

3. **Third Year :** Evaluation of Formulation
   i) Particle size analysis
   ii) Zeta potential
   iii) Surface morphology
   iv) Drug entrapment efficiency

v) Drug release study

vi) Cell line studies

vii) In-vivo-Pharmacokinetic, Acute toxicity study, biodistribution and Comparative study with other existing formulation.

4. Materials

Gemcitabine HCl was obtained as a gift sample from Sun Pharmaceutical Industries Ltd., Vadodara, India. Vinorelbine tartrate was obtained as a gift sample from Cipla Ltd. Mumbai, India. The lipids Phospholipon® 90G and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] were obtained as a gift sample from Lipoids, Germany. Cholesterol, Potassium Oleate and Mannitol were purchased from Sigma Aldrich, Bangalore, India. Methanol and chloroform (A. R. grade) were purchased from Spectrochem, Mumbai, India. All other reagents were purchased from authentic source and were of analytical grade. Cellulose dialysis membrane (Molecular weight cut of 12KD) was purchased from Himedia Lab, Mumbai, India and membrane filter of pore size 0.2 µm was purchased from Pall Corporation, Mumbai, India. A549 (Human adenocarcinomic alveolar basal epithelial cells) cell line was procured from National Culture Collection Society (NCCS), Pune. 6-well plates, 96-well plates, tissue culture flask (25 and 75 cm^2), and other sterile material for cell culture were obtained from Thermo scientific, India. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and Propidium Iodide were purchased from Sigma Aldrich, India. Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), Antibiotic solution**,** Trypsin-EDTA solution were purchased from Himedia lab Pvt. Ltd., Mumbai, India. Distilled water was prepared with an in house assembly.

5. Methods

5.1 Preformulation studies: As a part of the pre-formulation studies following tests were performed:

a) Capillary melting point method was used to determine the melting point of the drug. A few crystals of the compound are placed in a thin walled capillary tube 10-15 cm long, about 1 mm in inside diameter, and closed at one end. The capillary, which contains the sample, and a thermometer was then suspended so they can be heated slowly and

evenly. The temperature range over which the sample was observed to melt is taken as the melting point.

b) Drug-excipient compatibility was assessed for any physico-chemical interaction. For this pure drug and mixture of drug along with excipients were taken and sample preparation was done by appropriate method and subjected to Fourier Transform Infra-Red (IR) spectroscopy (Bruker, Germany) and Differential Scanning Calorimetric (DSC) studies (Shimadzu, Japan).

5.2 Analytical Methods:

Analytical Method Development of Gemcitabine HCl:

Preparation of Stock solution:

Accurately weighed 10 mg of drug was taken in 10 ml volumetric flask. It was initially dissolved in 4-5 ml of distilled water. Then the volume was made up to 10 ml with distilled water with the help of calibrated pipette to get a stock solution of 1 mg/ml (1000µg/ml).

Determination of Absorbance maxima (λmax): From the stock solution, an aliquot of 0.1ml was withdrawn with the micropipette and was taken in 10ml volumetric flask. It was diluted with distilled water up to the mark to obtain 10 µg/ml solution of Gemcitabine Hydrochloride. UV spectrum of it was recorded on a UV-Vis spectrophotometer between the range of 200-400 nm. Maximum absorbance was obtained at 266 nm.

Preparation of calibration plot: From the stock solution of 1000 µg/ml, aliquots of 5, 10, 15, 20, 25, 30 µg/mL were withdrawn and further diluted up to 10 ml with distilled water to obtain a concentration range of 5-30 µg/ml. The absorbance of these solutions was measured at the λmax of 266 nm. A concentration vs. absorbance calibration plot was plotted.

Method validation was done according to ICH Q2B guideline to determine all the validation parameters of the analyte.

Analytical Method Development of Vinorelbine tartrate:

Calibration Plot of Vinorelbine tartrate in Distilled water

Preparation of stock solution: Accurately weighed 5 mg of drug was taken in 5 ml volumetric flask. It was initially dissolved in 2-3 ml of distilled water. Then the volume was made up to 5 ml with distilled water with the help of micropipette to get a stock solution of 1mg/ml (1000µg/ml).

Determination of Absorbance maxima (λmax): From the stock solution, an aliquot of 0.1ml was withdrawn with the micropipette and was taken in 10ml volumetric flask. It was diluted with distilled water up to the mark to obtain 10 µg/ml solution of Vinorelbine tartrate .UV spectrum of it was recorded on a UV-Vis spectrophotometer between the range of 200-400 nm. Maximum absorbance was obtained at 271 nm.

Preparation of calibration plot: From the stock solution of 1000 µg/ml, aliquots of 0.15, 0.2, 0.25, 0.3, 0.35, and 0.4 were withdrawn and further diluted up to 10 ml with distilled water to obtain a concentration range of 15-40 µg/ml. The absorbance of these solutions was measured at the λmax of 271 nm. A concentration vs. absorbance calibration plot was plotted.

Method validation was done according to ICH Q2B guideline to determine all the validation parameters of the analyte.

5.3 Preparation of Non-PEGylated Gemcitabine HCl loaded Spherulites[28]

For optimization of Gemcitabine HCl loaded Spherulites, a 3^3 full factorial design was applied by identifying the independent variables as (A) Phospholipid conc. (% w/w), (B) Hydration time (Hrs), (C)Probe-cylinder distance (mm) and dependent variables as % drug entrapment and size of the spherulites.

The spherulites were composed of Soyabean Phosphatidylcholine (SPC)/Cholesterol (Chol). Lipid phase comprising of SPC: Chol (1:1 molar) along with potassium oleate, were dissolved in chloroform: methanol mixture (9:1 V/V). The organic solvent was removed under pressure and the resulting film was dried in a rotary evaporator (IKA RV10, Karnataka, India). 20 mL aqueous phase comprised of mannitol and Gemcitabine HCl was used to hydrate the film by hand shaking of the round bottom flask. The resulting dispersion was kept for overnight hydration and then it was homogenized

(Eurostar power control-visc, IKA, Bangalore, Karnataka, India) using a customized assembly of teflon probe and glass cylinder having gap of 0.5 mm for 1 hour at shear rate of 65 min-1, followed by 5 times extrusion of the sheared dispersion through stainless steel syringe filter assembly fitted with 0.22 μm nylon filter.

5.4 Preparation of PEGylated Gemcitabine HCl Loaded Spherulites:

For the preparation of PEGylated Spherulites, SPC/DSPE- PEG 2000/Cholesterol in 51:2:47 mole % were taken and rest of the method was same as above.

5.5 Preparation of Non-PEGylated Vinorelbine tartrate loaded Spherulites

For optimization of Vinorelbine tartrate loaded Spherulites Box-Behnken design was applied by identifying the independent variables as (A) Phospholipid conc. (% w/w), (B) Hydration time (Hrs), (C)Probe-cylinder distance (mm) and dependent variables as % drug entrapment and size of the spherulites.

The spherulites were composed of Soyabean Phosphatidylcholine (SPC)/Cholesterol (Chol). Lipid phase comprising of SPC: Chol (1:1 molar) along with potassium oleate, were dissolved in chloroform: methanol mixture (9:1 V/V). The organic solvent was removed under pressure and the resulting film was dried in a rotary evaporator (IKA RV10, Karnataka, India). 20 mL aqueous phase comprising of mannitol and Vinorelbine tartrate was used to hydrate the film by hand shaking of the round bottom flask. The resulting dispersion was kept for overnight hydration and then it was homogenized (Eurostar power control-visc, IKA, Bangalore, Karnataka, India) using a customized assembly of teflon probe and glass cylinder having gap of 0.5 mm for 1 hour at shear rate of 65 min-1, followed by 5 times extrusion of the sheared dispersion through stainless steel syringe filter assembly fitted with 0.22 μm nylon filter.

5.6 Preparation of PEGylated Vinorelbine tartrate Loaded Spherulites:

For the preparation of PEGylated Spherulites, SPC/DSPE- PEG 2000/Cholesterol in 51:2:47 mole % were taken and rest of the method was same as above.

6. Characterization

The drug loaded spherulites were characterized in order to check the following parameters:

6.1 Particle Size and Zeta Potential:

Spherulites size, its polydispersity index and zeta potential were measured by Dynamic light scattering (DLS) using Malvern Zetasizer (NanoZS, Malvern Instruments, UK). 50 µL spherulites dispersion was diluted to 2 mL distilled water, it was then taken in polystyrene disposable cuvette and used for analyzing the Particle Size and Zeta Potential

6.2 Determination of Drug Entrapment efficiency (%) and Drug Loading (%):

Non-PEGylated and PEGylated spherulites were taken in centrifuge tubes separately and centrifuged at 20000 rpm at 4 ºC for 30 minutes (REMI Laboratory Instruments, Mumbai, India). Spherulites get settled down in the centrifuge tube, forming a pellet. This pellet was air dried and weighed for total weight comprised of the solid content of the formulation. The pellet was lysed using 2% solution of Triton X100, suitably diluted and the amount of drug was estimated at 266 nm for Gemcitabine HCl and 271 nm for Vinorelbine tartrate by UV spectrophotometer (Shimadzu 1800, Kyoto, Japan).

To calculate the % Entrapment Efficiency (EE) and loading (% w/w), formula used was:

$$\% \text{ EE} = \frac{\text{Estimated Entrapped drug in Spherulites}}{\text{Total drug added to formulation}} \times 100$$

$$\text{Loading (\%w/w)} = \frac{\text{Estimated Entrapped drug in Spherulites}}{\text{Total weight of formulation}} \times 100$$

While the unentrapped drug that remains in the supernatant was analyzed to determine the mass balance. 0.1 ml of the supernatant was diluted sufficiently and the amount of drug was quantified at 266 nm for Gemcitabine HCl and 271 nm for Vinorelbine tartrate by UV spectrophotometer (Shimadzu 1800, Kyoto, Japan).

6.3 In-Vitro Drug Release study:

2 mL each of Non-PEGylated Gemcitabine HCl loaded Spherulites, PEGylated Gemcitabine HCl loaded Spherulites and Gemcitabine HCl plain drug solution was filled individually in activated dialysis bag (Molecular cut off 12KD) with both the ends closed using closure clips. It was immersed in 100 ml Phosphate Buffer pH 7.4. The medium was maintained at 37±0.5 ºC and 100 rpm speed was kept constant. 5 ml sample was withdrawn at predetermined time intervals of 0, 1, 2, 4, 8, 12, 24, 36, 48 hrs. Equal volume of fresh medium was replaced after each sample withdrawn. The concentration of drug was estimated using UV spectrophotometer (Shimadzu 1800, Japan).

Similar method was followed for Non-PEGylated Vinorelbine tartrate loaded Spherulites, PEGylated Vinorelbine tartrate loaded Spherulites and Vinorelbine tartrate plain drug solution.

6.4 Morphological Study by Scanning Electron microscope (SEM) and Transmission Electron microscope (TEM):

Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) were used to characterize the surface and structure of the spherulites, respectively.

To observe the liquid sample by SEM, a drop of sample dispersion was taken on a glass slide and direct air-drying of the sample was performed using an air blower before viewing under SEM (Phillips XL30 ESEM TMP+EDAX) operated at 30 KV.

The samples were prepared for TEM, by applying a drop of spherulites dispersion on to a copper grid, and left for one minute to allow some of the particles stick on the grid. After removing the excess of dispersion the grid was left to air-dry. The samples were then mounted on single tilt holder and viewed under TEM (Tecnai 20, Philips, Holland) operated at 200 KV.

Also, the effect of PEG concentration on the morphology of spherulites was also studied using TEM.

7. In vitro cytotoxicity study:

Culturing of A549 cells:

The cells were maintained as monolayer culture in T-25 cell culture flasks. Cell were sub-cultured at 37°C in a humidified atmosphere (95% air and 5% CO2) using DMEM

medium supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% FBS. Below mentioned procedure was followed for the sub culturing.

1. Culture medium from the cell culture flask was removed.
2. Trypsin-EDTA solution (2 ml) was added to flask and was shaken to allow the detachment of the cells.
3. Cells were observed under the inverted microscope until cell layer was dispersed (usually within 5 min).
4. Complete medium (2 ml) was added to cell dispersion to neutralize trypsin and then centrifuged at 2000 rpm for 3 min.
5. Pellet of cells was resuspended in minimum volume of complete growth medium.
6. Cell culture was (10^5 Cells) then added to cell culture flask and 10 ml of complete growth medium was added to it.
7. Culture was incubated at 37 °C, 5% CO^2.

Preparation of stock solution:

Stock solution of Gemcitabine HCl, Gemcitabine HCl Spherulites, Vinorelbine tartrate and Vinorelbine tartrate Spherulites was prepared. For standard drug stock solutions 10 mg each Gemcitabine HCl and Vinorelbine tartrate were dissolved in 10 mL sterile PBS pH 7.4 separately to get the final concentration of 1000 µg/mL. From this, serial dilutions were made to get 0.001µg, 0.01µg, 0.1µg, 1µg and 10µg of Gemcitabine HCl and Vinorelbine tartrate.

The Spherulite formulations of both the drugs were also diluted to get the same concentrations.

7.1 MTT assay:

A549 cells were seeded onto 96-well plates at a density of 3×10^3 cells/well. After 24 h, cells were treated separately with Standard Gemcitabine HCl, Gemcitabine HCl Spherulites, Standard Vinorelbine tartrate and Vinorelbine tartrate Spherulites (at concentrations of 0.001µg , 0.01µg, 0.1µg, 1µg and 10µg were added to wells). In all wells, after 96 hrs. transfection media was replaced by fresh DMEM containing 10% of FBS and antibiotics and 20 µL of 5 mg/mL MTT solution was added to each well. After incubating for 4 hr with MTT solution, the culture medium was removed and 200 µL of a

dimethyl sulfoxide (DMSO) (Sigma, USA) was added. The reduction of viable cells was measured by calorimetry at 570 nm wavelength using an enzyme-linked immunosorbent assay (ELISA) plate reader (Fluostar, Germany). Percentage inhibitions were calculated and plotted with the concentrations and IC_{50} values were calculated.

7.2 Cell Cycle and Apoptosis study by *Flow cytometry*:

$1x10^5$ A549 (Human adenocarcinomic alveolar basal epithelial cells) were seeded on 6 well plates and allowed to grow for 24 hours. Cells were treated with 0.1μM of test sample Plain drug solution and Spherulites formulation of Gemcitabine HCl and Vinorelbine tartrate for 24h. Cells were gently trypsinized and washed with PBS pH 7.4 twice. Cells were fixed in methanol and stained with 2 μL of 0.1% Propidium Iodide and incubated at 4 ^oC for 10-15 minutes. After incubation suspension was analyzed for cell cycle and apoptosis on flow cytometer (BD FACSCalibur, BD Biosciences, India).

8. In Vivo Biodistribution Studies by Gamma Scintigraphy

Radiolabelling of Gemcitabine HCl with ^{99m}Tc

The radiolabelling of Gemcitabine HCl with ^{99m}Tc was done by standard protocol developed at Institute of Nuclear Medicine and Allied Sciences (INMAS), New Delhi. Briefly, 2 mg Gemcitabine HCl was dissolved in 1 ml Phosphate Buffer Saline (PBS) pH 7.4 followed by the addition of 75 μg of $SnCl_2.2H_2O$. The pH of the solution was adjusted to 6.5. The contents were filtered through 0.22 μm membrane filter (Millipore Corporation, Bedford, MA USA). Freshly prepared 99mTcO-4 (74 Mbq) was added to the contents, mixed and incubated for 20 minutes at room temperature.

All the necessary quality control tests of ^{99m}Tc-Gemcitabine HCl complex viz. ^{99m}Tc-Gemcitabine HCl complex labelling efficiency, Radiocomplex stability in serum and saline and Transchelation Study (DTPA Challenge) were carried out.

Radiolabelling of Vinorelbine tartrate with ^{99m}Tc

The radiolabelling of Vinorelbine tartrate with ^{99m}Tc was done by standard protocol developed at Institute of Nuclear Medicine and Allied Sciences (INMAS), New Delhi. Briefly, 2 mg Vinorelbine tartrate was dissolved in 1 ml double distilled water followed by the addition of 50 μg of $SnCl_2.2H_2O$ in 0.1 N HCl. The pH of the solution was

adjusted to 6.5-7.0. The contents were filtered through 0.22 µm membrane filter (Millipore Corporation, Bedford, MA USA). Freshly prepared ^{99m}TcO-4 (74 Mbq) was added to the contents, mixed and incubated for 20 minutes at room temperature.

All the necessary quality control tests of ^{99m}Tc- Vinorelbine tartrate complex viz. ^{99m}Tc-Vinorelbine tartrate complex labelling efficiency, Radiocomplex stability in serum and saline and Transchelation Study (DTPA Challenge) were carried out.

Biodistribution studies in Rats

All animal experiments conducted were approved by the Institutional Animals Ethics Committee of the Institute of Nuclear Medicine and Allied Sciences (INMAS), DRDO, New Delhi, India. Experimental animals used were healthy female Sprague Dawley rats weighing 200±4 g (14 weeks), kept on normal diet with free access to water. Animals prior to the experiment were housed in Experimental Animal House facility of DRDO.

^{99m}Tc-Gemcitabine HCl/^{99m}Tc-Vinorelbine tartrate loaded PEGylated and non-PEGylated spherulites were prepared by procedure mentioned in preparation of Spherulites section except the overnight hydration step. Dose of the drug to be administered in rat was calculated using the formula:

Human Equivalent Dose (mg/kg) (HED) = Animal Dose (mg/kg) x Animal Km/Human Km; where Km is body weight (kg) divided by body surface area (m2); the factor that varies for all animals.

0.3 ml of ^{99m}Tc-Gemcitabine HCl/^{99m}Tc-Vinorelbine tartrate (1.48 MBq) loaded PEGylated and non-PEGylated spherulites and Plain drug suspension, each containing 0.3 mg of Gemcitabine HCl/Vinorelbine tartrate, was injected in tail vein of rat (n=3). Rats were anaesthetized by injecting ketamine (100 mg/kg) through intraperitoneal route and were fixed on a board. Imaging was carried out at predetermined time point's viz. 0.25, 0.5, 1, 2, 3, 4 hours using a Single Photon Emission Computerized Tomography (SPECT, LC 75–005, Diacam, Siemens AG, Earlangan, Germany) gamma camera.

9. Stability Studies:

All four formulations were subjected to short term accelerated stability studies at two different storage conditions. Stability protocol was designed as per ICH guidelines for countries falling under Zone III and IV. Three batches were stored under refrigerated

condition (2–8 ºC) and three at room temperature (25±2 ºC) for a period of three months. Samples from each were characterized for spherulites particle size, zeta potential, % drug entrapment, % loading, % drug release after 1, 2 and 3 months.

10. Results

Preformulation studies

Preformulation studies were performed in order to confirm the authenticity of the drug product, so as to confirm that the product meets the standard specification. Melting point determination of Gemcitabine HCl was performed by capillary method and it was found to be in the range of 166 °C- 170 °C (168.64 °C reported), while the melting point of Vinorelbine tartrate was found to be 184-187 °C (181-183 °C reported). The melting point of both the drugs were found in agreement with the standard reported values.

Drug-excipient compatibility was studied by Fourier transform infrared (FTIR) spectroscopy. FTIR spectra of Gemcitabine HCl and Vinorelbine tartrate showed no change in their characteristic peaks, indicating there was no chemical interaction between the drug and the excipients. Hence, drug and excipients were found to be compatible with each other. These results were further confirmed by DSC study, which showed there was no change in the characteristic endothermic peak of drug.

Analytical methods

a) Gemcitabine HCl

The λmax was found to be 266 nm in distilled water and the plot was found to obey Beer's law in the concentration range of 5 to 30 µg/ml with correlation coefficient (R^2) 0.999.

b) Vinorelbine tartrate

The λmax of Vinorelbine tartrate in distilled water was found to be 271 nm, and the plot was found to obey the Beer's law in the concentration range of 15 to 40 µg/ml with correlation coefficient (R^2) 0.983.

Validation of the analytical method was carried out and the results obtained were found to meed the specification according to ICH Q2B guidelines.

Formulation Development

Preliminary trails of the formulation were carried out to confirm the major formulation parameters as well as process parameters affecting the size of spherulites and entrapment efficiency of drug. Hence based on these results following parameter were chosen for the optimization of drug loaded spherulites:

- Phospholipid concentration: Phospholipid concentration is a critical parameter, since, very less phospholipid in lamellar phase leads to non-arrangement of vesicles, similarly very high concentration leads to aggregation of vesicles.
- Hydration Time: The mixture of phospholipids along with water leads to the formation of homogeneous lamellar phase. Greater the hydration time better the vesicle formation.
- Type of homogenizer: The lamellar phase gets grinded in between the teflon rod and glass tube, so maintaining the fixed distance between Teflon rod and glass tube is an important factor, since it will affect the vesicle size.

Based on the results obtained from preliminary trails, a 3^3 full factorial design was applied for the optimization of Gemcitabine HCl loaded spherulites using a software tool (Design Expert 7.0). 27 batches were run to identify the optimized formulation.

PEGylation of Gemcitabine HCl loaded spherulites was done in order to increase the circulation time of the spherulites by incorporation of DSPE-PEG 2000 in the optimized formula of the formulation in the ratio of SPC/DSPE-PEG 2000/Cholesterol in 51:2:47 mole %.

Table 1: Optimized Formula For preparation of Non-PEGylated Gemcitabine HCl loaded Spherulites:

Thin Film on Rota evaporator			**Hydration**		
Potassium Oleate	**Cholesterol**	**SPC**	**Mannitol**	**Water**	**Gemcitabine HCl**
5 mg	**20.9 mg**	**42 mg**	**3.5 mg**	**20 mL**	**10 mg**

Table 2: Optimized Formula For preparation of PEGylated Gemcitabine HCl loaded Spherulites:

Thin Film on Rota evaporator				Hydration		
Potassium Oleate	**Cholesterol**	**SPC**	**DSPE-PEG 2000**	**Mannitol**	**Water**	**Gemcitabine HCl**
5 mg	**20.9 mg**	**42 mg**	**4 mg**	**3.5 mg**	**20 mL**	**10 mg**

The check point analysis of the optimized batch was performed:

Table 3: Predicted:

Phospholipid conc. (% w/w)	**Hydration time (Hrs)**	**Probe-cylinder distance (mm)**	**PDE (%)**	**Particle size (nm)**	**Desirability**
42	19.68	0.57	77.50	206.3	0.915

Table 4: Characterization of optimized batch of Gemcitabine HCl loaded Spherulites

Characterization	**Non-PEGylated Spherulites**	**PEGylated Spherulites**
Size (PDI)	204.9±1.2 nm (0.43)	209.2±1.4 nm (0.43)
Zeta Potential	-26.5±1.2 mV	-33.3±1.8 mV
%Drug entrapment	76.28±1.1 %	77.42±1.5 %
Drug loading (%w/w)	9.38±0.94	9.07±0.89

Vinorelbine tartrate loaded spherulites were optimized by applying Box-Behenken design using a software tool (Design Expert 7.0). 17 batches were run to know the optimized formulation.

PEGylation of Vinorelbine tartrate loaded spherulites was done in order to increase the circulation time of the spherulites by incorporation of DSPE-PEG 2000 in the optimized formula of the formulation in the ratio of SPC/DSPE-PEG 2000/Cholesterol in 51:2:47 mole %.

Table 5: Optimized Formula For preparation of Non-PEGylated Vinorelbine tartrate loaded Spherulites:

Thin Film on Rota evaporator			Hydration		
Potassium Oleate	**Cholesterol**	**SPC**	**Mannitol**	**Water**	**Vinorelbine tartrate**
5 mg	20.9 mg	42 mg	3.5 mg	20 mL	10 mg

Table 6: Optimized Formula For preparation of PEGylated Vinorelbine tartrate loaded Spherulites:

Thin Film on Rota evaporator				Hydration		
Potassium Oleate	**Cholesterol**	**SPC**	**DSPE-PEG 2000**	**Mannitol**	**Water**	**Vinorelbine tartrate**
5 mg	20.9 mg	42 mg	4 mg	3.5 mg	20 mL	10 mg

Table 7: Predicted:

Phospholipid conc. (% w/w)	Hydration time (Hrs)	Probe-cylinder distance (mm)	PDE (%)	Particle size (nm)	Desirability
42	12	0.53	96.06	120.1	0.992

Table 8: Characterization of optimized batch of Vinorelbine tartrate loaded Spherulites

Characterization	Non-PEGylated Spherulites	PEGylated Spherulites
Size (PDI)	122.4±1.6 nm (0.24)	131.6±1.9 nm (0.32)
Zeta Potential	-26.9±2.4 mV	-37.8±2.1 mV
%Drug entrapment	95.65±0.86 %	94.2±0.74 %
Drug loading (%w/w)	11.76±0.59	11.04±0.76

In vitro drug release from Gemcitabine plain drug solution, Gemcitabine HCl loaded Spherulites and PEGylated Gemcitabine HCl loaded spherulites was studied. Plain drug solution showed 78.76% release after 1 hour and within 2 hours entire drug was diffused through the dialysis bag. This was followed by non-PEGylated, which showed 93.09% drug release and PEGylated Spherulites 89.49% after 48 hours. PEGylation of Spherulites relatively retarded the drug release compared to non-PEGylated formulation.

PEG serves as a barrier for release of hydrophilic drug from Spherulites. The drug release data were fitted to various mathematical models to evaluate the kinetics of release. First order model fitted the best with highest R^2 value. Release from vesicular system is generally first order i.e. dependent on the concentration of drug in Spherulites.

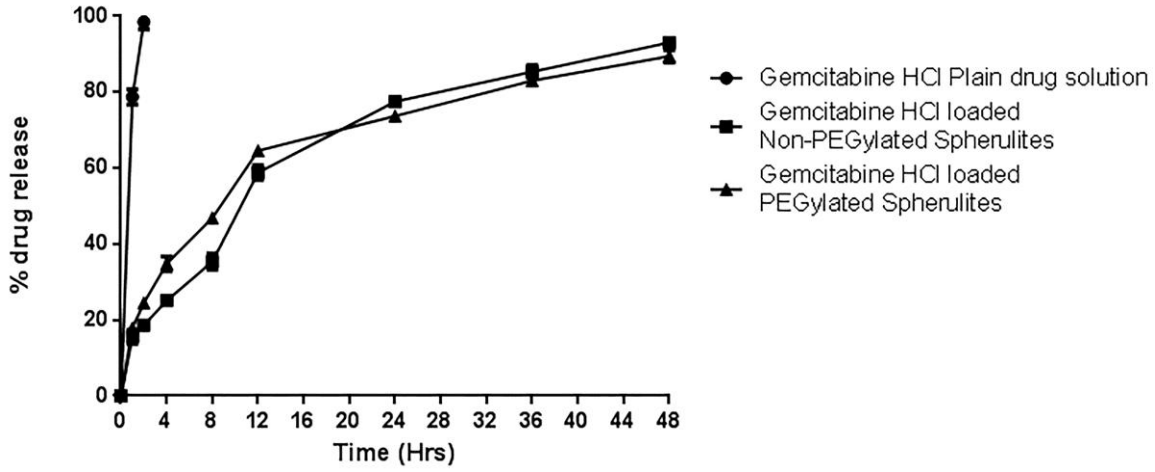

Fig 1: In vitro drug release of Gemcitabine HCl plain drug solution, Gemcitabine HCl loaded Non-PEGylated Spherulites, Gemcitabine HCl loaded PEGylated Spherulites.

Results obtained from in vitro drug release study of Vinorelbine tartrate plain drug solution, Vinorelbine tartrate loaded Spherulites and PEGylated Vinorelbine tartrate loaded Spherulites, showed that 92.3% of Vinorelbine tartrate plain drug solution was diffused within 12 hours, followed by Vinorelbine tartrate loaded Spherulites and PEGylated Vinorelbine tartrate loaded Spherulites 90.84% and 86.85% in 48 hours respectively. The drug release data were fitted to various mathematical models to evaluate the kinetics of release. First order model fitted the best with highest R^2 value.

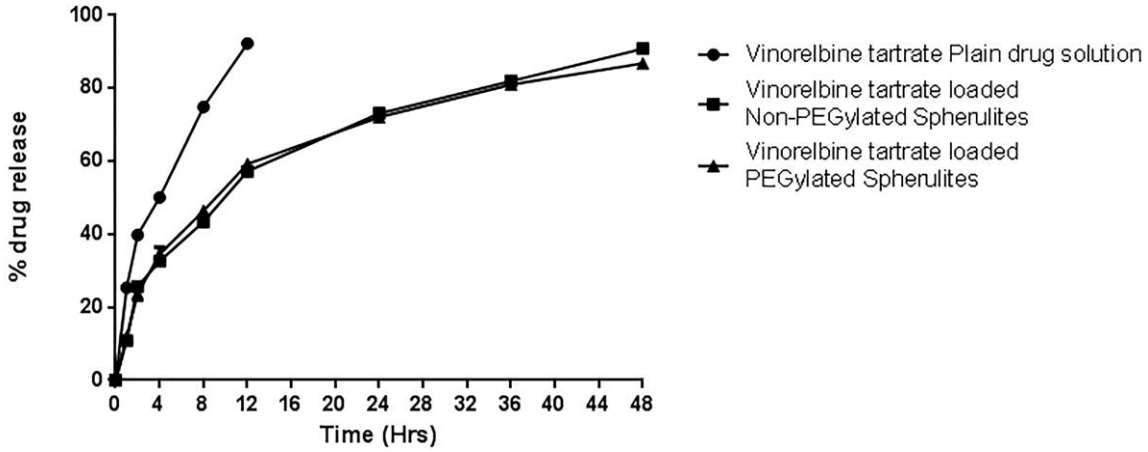

Fig. 2: In vitro drug release of Vinorelbine tartrate plain drug solution, Vinorelbine tartrate loaded Non-PEGylated Spherulites, Vinorelbine tartrate loaded PEGylated Spherulites.

SEM of optimized batch of Gemcitabine loaded Spherulites, Vinorelbine tartrate loaded Spherulites, PEGylated Gemcitabine loaded Spherulites and PEGylated Vinorelbine tartrate loaded Spherulites was done. SEM results indicated that the surface morphology of the spherulites was even and showed spherical structure.

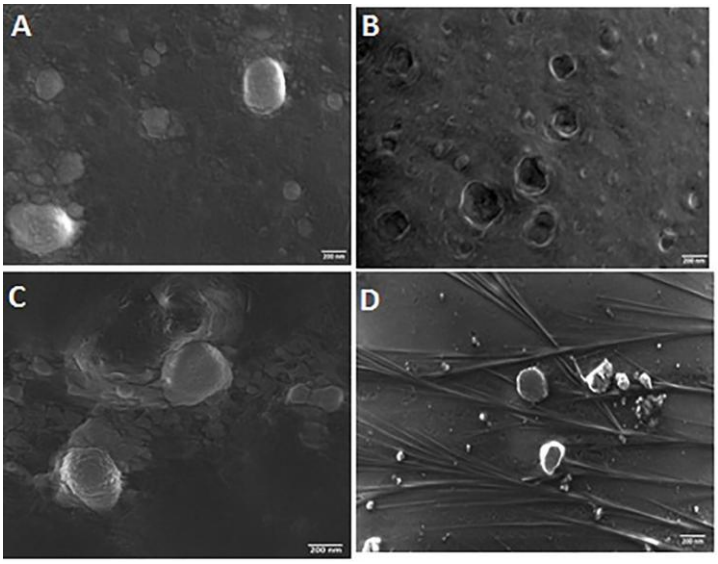

Fig 3: SEM images showing the surface morphology of **A:** Gemcitabine HCl loaded Non-PEGylated Spherulites, **B:** Gemcitabine HCl loaded PEGylated Spherulites, **C:** Vinorelbine tartrate loaded Non-PEGylated Spherulites, **D:** Vinorelbine tartrate loaded PEGylated Spherulites

TEM results showed the internal multilamellar structure of the spherulites, PEGylated spherulites showed a dark region around the outer periphery of the spherulites, as it confirms successful PEG attachment on the surface of spherulites. Also the effect of increased concentration of PEG on the morphology on the spherulites was observed as the spherulites were seen in disk shape.

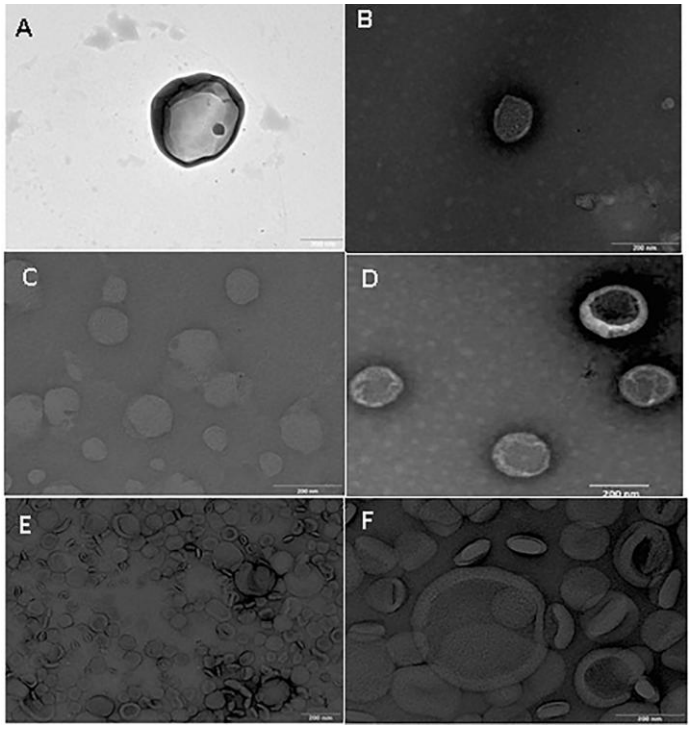

Fig 4: TEM micrographs **A:** Gemcitabine HCl loaded Non-PEGylated Spherulites, **B:** Gemcitabine HCl loaded PEGylated Spherulites, **C:** Vinorelbine tartrate loaded Non-PEGylated Spherulites, **D:** Vinorelbine tartrate loaded PEGylated Spherulites**, E:** Gemcitabine HCl loaded PEGylated Spherulites with 5% PEG, **F:** Vinorelbine tartrate loaded PEGylated Spherulites with 5% PEG.

In vitro cytotoxicity by MTT assay:

MTT assay was performed on A549 cells. Gemcitabine HCl loaded spherulites showed more cytotoxic effect at all concentrations than standard Gemcitabine HCl. This can be attributed to higher uptake of spherulites into the cell as compared to the plain drug solution. Similarly, Vinorelbine tartrate loaded spherulites were found to be more cytotoxic than the plain drug solution

IC50 values of formulation were found to be significantly lower than the plain drug solution. This means that lesser dose of formulation is required to cause cell inhibition than the plain drug.

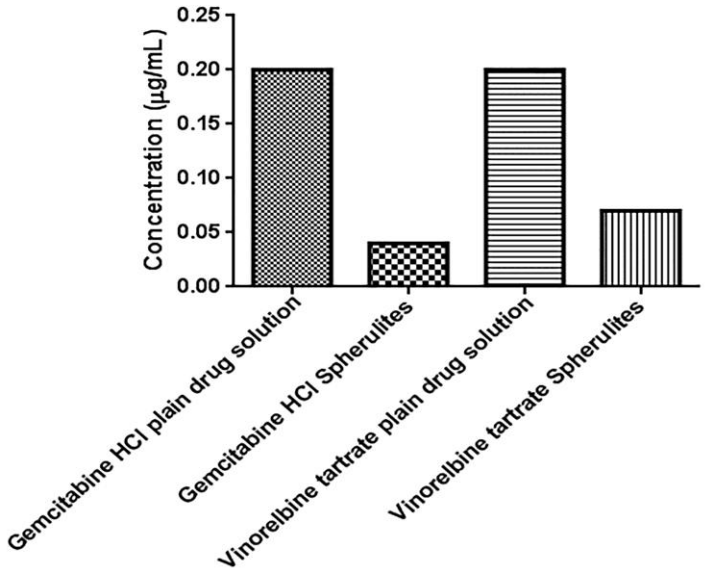

Fig 5: IC50 values of Plain drug solution and Spherulites formulation of Gemcitabine HCl and Vinorelbine tartrate.

Cell Cycle and Apoptosis study by *Flow cytometry*:

Results indicated that as Gemcitabine HCl is S-phase cell cycle specific, formulation showed more cells were arrested in this phase as compared with plain drug solution, which was correlated by increased number of cells seen in fragmented DNA region. Vinorelbine tartrate is M-phase cell cycle specific, formulation showed more apoptotic cells than plain drug solution, confirming that the enhanced uptake of spherulites is exerting its effect on the cell cycle.

In Vivo Biodistribution studies of ^{99m}Tc-Gemcitabine HCl

The mean labeling efficiency of Gemcitabine HCl was >98% at pH 6.5 (adjusted using 0.5M $NaHCO_3$ solution). Less than 1% radioactivity was dissociated after 24 hours incubation. Incubation of ^{99m}Tc- Gemcitabine HCl in human serum and 0.9% saline at 37^{o}C revealed that radiolabelled ^{99m}Tc-Gemcitabine HCl was extremely stable.

DTPA challenge study was performed to measure strength of binding of technetium with Gemcitabine HCl. This study demonstrated that the labeling efficiency of the complexes

did not alter much in the presence of DTPA. Even at 7.5 mM concentration of DTPA, the transchelation was found to be less than 3.5%. This indicates the stability of radiolabelled complexes.

The initial distribution, within 1 hour of administration, of PEGylated Spherulites to lungs was significantly higher (18.73%) than that of Non PEGylated (12.17%) and plain drug solution (14.62%) as seen in Figures 6, 8, and 10. Even after 4 hours of administration, lung retention of PEGylated Spherulites (9.11%) exceeded that of non-PEGylated (4.31%) and plain drug solution (1.20%).

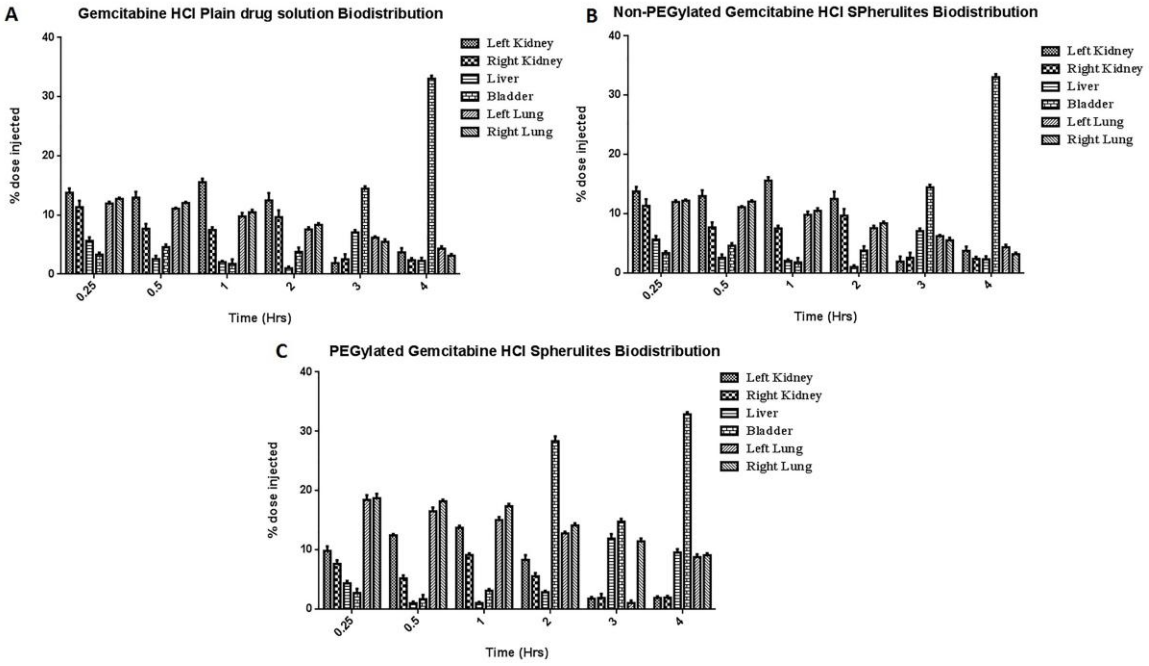

Fig 6: Biodistribution of (A) Plain ^{99m}Tc-Gemcitabine HCl solution, (B) ^{99m}Tc-Gemcitabine HCl loaded Non-PEGylated Spherulites, (C) ^{99m}Tc-Gemcitabine HCl loaded PEGylated Spherulites.

In Vivo Biodistribution studies of 99mTc-Vinorelbine Tartrate

The mean labeling efficiency of Vinorelbine Tartrate was >98% at pH 6.5 (adjusted using 0.5M $NaHCO_3$ solution). Less than 1% radioactivity was dissociated after 24 hours incubation. Incubation of ^{99m}Tc- Vinorelbine Tartrate in human serum and 0.9% saline at 37^{o}C revealed that the labeling of the Vinorelbine Tartrate was extremely stable.

DTPA challenge study was performed to measure strength of binding of technetium with Vinorelbine Tartrate. This study demonstrated that the labeling efficiency of the complexes did not alter much in the presence of DTPA. Even at 7.5 mM concentration of DTPA, the transchelation was found to be less than 2.53%. This indicates the stability of radiolabelled complexes.

The initial distribution, within 1 hour of administration, of PEGylated Spherulites to lungs was significantly higher (18.10%) than that of Non PEGylated (10.90%) and plain drug solution (13.85%). Even after 4 hours of administration, lung retention of PEGylated Spherulites (8.39%) exceeded that of non-PEGylated (3.30%) and plain drug solution (7.85%).

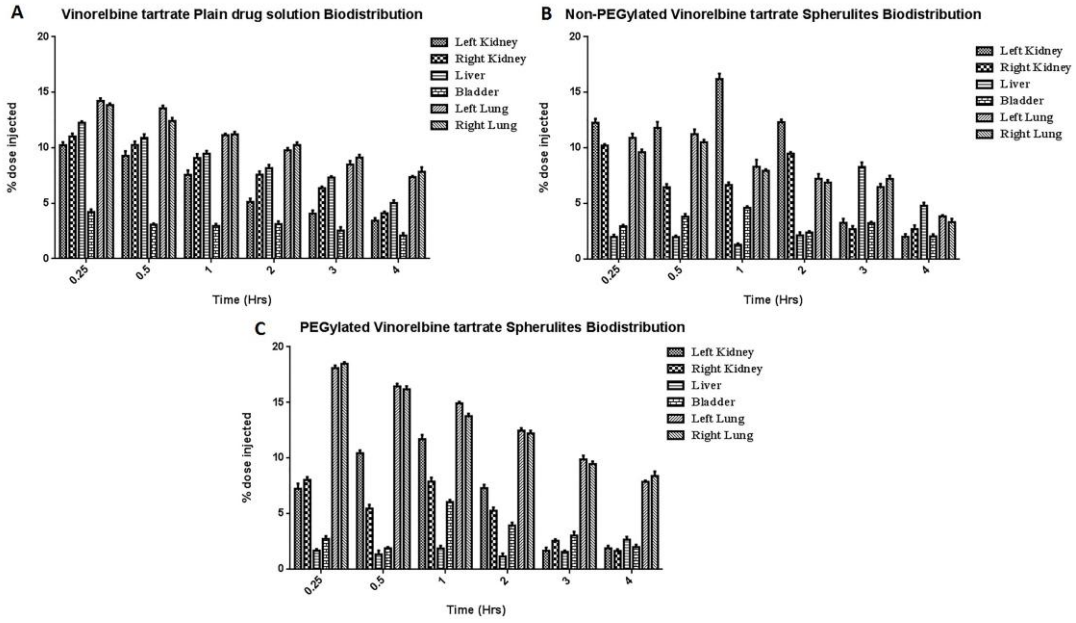

Fig 7: Biodistribution of (A) Plain ^{99m}Tc-Vinorelbine tartrate solution, (B) ^{99m}Tc-Vinorelbine tartrate loaded Non-PEGylated Spherulites, (C) ^{99m}Tc-Vinorelbine tartrate loaded PEGylated Spherulites.

Stability Study:

Stability study of Non-PEGylated and PEGylated formulations of Gemcitabine HCl and Vinorelbine tartrate was performed as per ICH guidelines for countries falling under Zone III and IV. The formulations were stored under refrigerated condition (2–8 ºC) and at room temperature (25±2 ºC) for a period of three months. Samples were subjected to

evaluation parameters like physical appearance, particle size, PDI, zeta potential, % drug entrapment, % drug loading and % drug release. There was no significant effect of storage conditions was found and all formulations showed good stability at every sampling time.

11. Ongoing work

i) Pharmacokinetic study

ii) Biodistribution study

iii) Acute toxicity study

iv) Spherulites-Haemo compatibility study.

v) Tumour induction and regression study (if possible)

12. Conclusion

In the present investigation, novel multilamellar Spherulites were formulated incorporating two anticancer drugs Gemcitabine HCl and Vinorelbine tartrate separately, which are used as first line drug in the treatment of Non-Small Cell Lung Cancer (NSCLC). Spherulites of both the drugs were PEGylated in order to increase their circulation time in vivo. Preformulation studies such as FTIR and DSC was performed in order to detect any incompatibility between drug and excipient. Formulation was developed by taking numerous preliminary trials and evaluated on the basis of particle size and drug entrapment efficiency. The Spherulites of both the drugs was optimized using Design of Experiment (DoE) tool with the help of Design-Expert 7.0.0 and JMP® 12.2.0, SAS Institute Inc. software. Gemcitabine loaded Spherulites were optimized by applying 3^3 full factorial design, whereas, Vinorelbine tartrate loaded Spherulites were optimized by applying Box-Behnken design. The optimized batch was evaluated for particle size, PDI, zeta potential, drug entrapment (%), drug loading (%). In vitro release study was performed and results indicated that PEGylated and Non-PEGylated formulation of both the drugs were obeying first order release kinetics. The morphology of the Spherulites was studied by SEM and TEM, where SEM images showed uniform surface of the particles with spherical structure. TEM images showed presence of multilamellar structure of Spherulites. Formulations were evaluated for their cytotoxicity on A459 cell line, results indicated that formulation was more cytotoxic than plain drug

solution. Also, cell cycle analysis and Apoptosis study revealed that the formulation was inducing apoptosis, as more number of cells were found in the fragmented DNA region. In vivo biodistribution studies depicted the results that PEGylated formulation of both the drugs had remained in the circulation and also it was able to reach the lungs relatively compared with Non-PEGylated formulation and plain drug solution. Short term stability study showed that the formulation was stable in both the storage conditions and possessed all its characters at the end of the study.

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