A SYNOPSIS OF THE THESIS ENTITLED

"SURFACE FUNCTIONALISED MESOPOROUS SILICA NANOPARTICLES AS A DELIVERY SYSTEM FOR ANTICANCER DRUGS"

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1. INTRODUCTION:

Cancer is the leading cause of death amongst various lethal diseases prevalent today. There is no sure shot treatment of cancer available, despite large amount of research going on throughout the world. A critical barrier in treating cancer in general is to discover a technology for targeted drug delivery to eliminate tumor cells while reprieving normal cells. The human circulatory system can distribute a drug to almost every cell in the body; however, delivering the anticancer drug specifically into the affected cancer cell past its membrane and thereafter releasing the drug into the tumor cells as required without affecting the healthy cells remains a daunting task(1). Until recently, there has been a shotgun approach to killing cancer cells – administering drug that reaches everywhere in the body with the hope that most of it reaches malignant cells to kill them. The flip side of this approach are often dangerous side effects and dissatisfactory therapeutic response. Therefore, in order to develop more effective chemotherapeutics, there is a need to improve strategy—switching to a sniper's rifle to deliver drugs more accurately to tumors. Nanoparticles, with the capacity to store large payloads within their cores and "targeting" molecules on their surfaces, would seem ideally suited to the task(2). Contemporary research attempts to tackle this fundamental challenge by utilizing nanoparticles as carriers for anticancer drugs.

Prostate cancer has become one of the paramount causes of deaths occurring due to cancer. As per the recent statistics, in the United States itself, 29,430 patients suffering from prostate cancer, died and about 1,64,690 new cases were reported (3). It is also reported that tumors of lung, colon and prostate account for about 42% (percent) of fresh cases in men, with prostate cancer alone having an occurrence rate of 1 among each 5 new diagnosis. Owing to such an alarming increase in the number of patients suffering prostate malignancy it becomes imperative to find a sure shot treatment to increase the life expectancy and for the abatement of the mortality rate in cancer.

Most of the anticancer drugs currently available fall into the biopharmaceutical classification system (BCS) category II and IV, possessing solubility limitations. About more than half of marketed products are classified as having low solubility (4-6) and about 70 percent (%) of new chemical entities (NCEs) discovered face same issue of low solubility (7, 8). This in turn limits their therapeutic value. Low solubility leads to a low oral bioavailability, limited by their low dissolution rate in the gastrointestinal tract (8). Poor aqueous solubility still is the foremost

challenge faced today in biopharmaceutics, which the scientists need to tackle. Due to lack of specification and solubility of drug molecules, patients have to take high doses of the drug to achieve the desired therapeutic effects for the treatment of cancer.

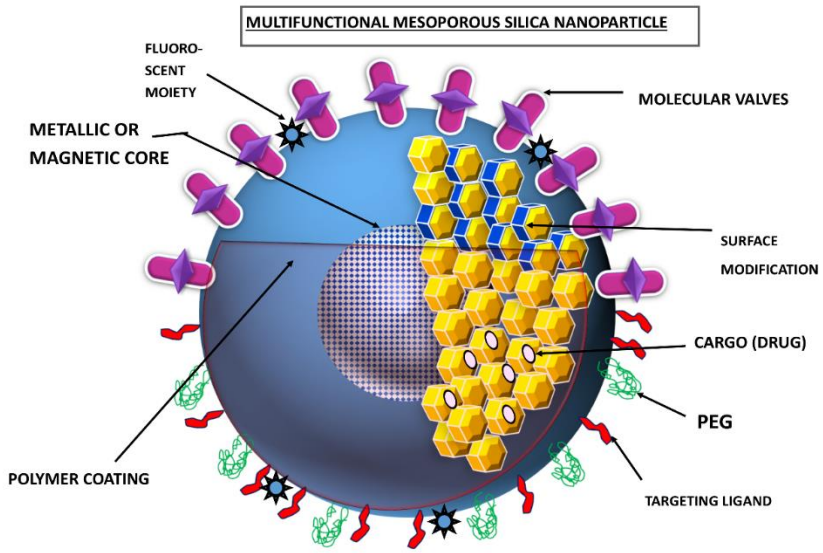
Nanotechnology is the branch of science dealing with engineering materials and systems on a molecular scale. Its application to therapeutic field, nanomedicine, has enabled the development of various nanoparticle drug-delivery vehicles. These have the ability to deliver therapeutics to cancer affected sites (9, 10). Conventional chemotherapeutics disseminate throughout the body; hence they affect both cancer cells as well as normal cells. Nanoparticles, possess enhanced permeability and retention (EPR) effect, therefore favourably accumulate in tumours. This divergent bio distribution allows nanoparticle-based chemotherapeutics to achieve enhanced intratumoral drug concentration and lower concentrations in normal tissue than their conventional small- molecule counterparts. It can also lead to higher therapeutic efficacy and lower toxicity for nanoparticle therapeutics (11). Various nanocarriers include Polymeric nanoparticles, dendrimers, polymeric micelle, liposome, inorganic (iron, silica, quantum dot core), biological nanoparticles, hybrid nanoparticles amongst many others. Over last couple of decades many nano drug delivery systems have been developed using organic as well as inorganic materials. Numerous nanodevices have been reported like carbon nanotubes, quantum dots, and polymeric micelles, etc., in the field of nanotechnology. Nanocarriers can encapsulate a variety of therapeutic agents and small molecules, which leads to increase in solubility and controlled release to maintain drug concentration within therapeutic window. Due to strong Si-O bond, silica-based mesoporous nanoparticles are also more stable to external response such as degradation and mechanical stress Hence, potential drugs which were earlier ignored because of poor pharmacokinetics, can be re-evaluated(12).

The surface of the nanocarriers can be modified to increase blood circulation half-life and enhanced biodistribution, while attachment of targeting ligand to the surface of nanocarrier can lead to increase in their cellular uptake(13). Among a variety of nano drug-delivery systems, mesoporous silica materials have several attractive features for use in the delivery of water-insoluble drugs (14). These particles have porous interiors and large surface areas which can be used as reservoirs for storing hydrophobic anticancer drugs. Textural properties of MSNs provide the possibility to load high amount of drugs within MSNs carriers. The pore size is tunable can be tailored to selectively store different molecules of interest, (15, 16) while the size and shape of the particles can be altered to maximize cellular uptake. Unlike polymer-based nanoparticles, these sturdy inorganic materials can withstand many organic solvents.(17)

Silica-based materials have been successfully used as drug-delivery agents(18, 19), gene transfection reagents,(19) cell markers,(20) and carriers of molecules(21).

Amorphous silica was proposed as a drug delivery carrier as early as 1983 (22). Amongst these, ordered mesoporous silica materials (OMMs) have been recognized as one promising class due to the controllable structural and morphological features on the nanometer and micrometer scale. Silica is an endogenous substance, especially abundant in bone, cartilage and other supporting tissue. Silica is 'generally recognized as safe' by the US FDA. Recently, silica nanoparticles in the form of 'C-dots' (Cornell dots) (23)were US FDA approved for stage I human clinical trial (24)

The functionalization of Mesoporous Silica Nanoparticles (MSNPs) with molecular, supramolecular or polymer moieties, gives them great versatility while performing drug delivery tasks, which makes the delivery process highly targeted and controllable (25). On the other hand, there are abundant silanol groups on the surfaces of mesoporous channels and the outer surfaces of MSNs, which facilitate the surface functionalization to allow for a better control over the drug diffusion kinetics (26). Surface modification and attachment of various moieties on MSNPs as per requirement of drug release is depicted in Fig 1. The surface functionalization is generally needed to load proper type of drug molecules (hydrophobic/hydrophilic or positive/negative charged), specific actions can also have a natural quality or characteristics by the functionalization through chemical links with other materials such as stimuli-responsive, luminescent or capping materials, leading to smart, and multifunctional properties(27). In addition to the attractive structural features of OMMs, the flexible and straightforward surface functionalization means allow for control of OMM surface charge, optimization of drug–carrier interactions, as well as enhanced OMM dispersion stability. The platform can easily be further functionalized, for instance, by attachment of fluorophores for imaging and ligands for cell-specific targeting. Active surface enables functionalization to changed surface properties and link therapeutic molecules. The biggest challenge in cancer treatment is not able to achieve targeted or selective delivery of drug molecule. Various targeting moieties like folate (28), aptamers, antibodies (29) and others can be attached on surface functionalized MSNs. They are used as widely in the field of diagnosis, target drug delivery, bio-sensing, cellular uptake, etc., in the bio-medical field. They are also suitable for multi drug delivery.



On a very general level, surface functionalized MSNs seem to reduce the observed harmful effects as compared with pristine mesoporous silica(30). Mesoporous silica nanoparticles are biodegradable and mostly eliminated through renal clearance. Blocking the access to the silanol groups by functionalization with organic groups, however, diminished the observed hemolysis. Remarkably enough, primary amine groups were also very (and almost equally as PEG) effective in preventing hemolysis in a dose-dependent manner (31). As nanocarriers, mesoporous silica nanoparticles with unique mesoporous structure have been explored as effective drug delivery systems for a variety of therapeutic agents to fight against various kinds of diseases including diabetes,(32) inflammation,(33) and cancer(34).

Present drug delivery systems, however, do not have the ability to guide themselves to a target. So, the active targeting is required to guide the drug/drug carriers to a target site. Hence, the current research is focused on developing a mesoporous silica nanoparticle to obtain effective treatment for cancer.

2. SELECTION OF ACTIVE PHARMACEUTICAL INGREDIENTS:

2.1. ETOPOSIDE (ETO):

Etoposide (ETO) is one of the most widely used anticancer drug and is used in the treatment of various cancers like lung cancer, prostate cancer, advanced gastric cancer, leukaemia and testicular cancer. It belongs to BCS Class IV and faces both solubility and permeability limitations. Several nanofomulations were researched upon for enhancing therapeutic efficacy

of ETO like PLGA NPs (35) , solid lipid nanoparticles(36), PLGA and PCL NPs (37) and surface modified poly(lactide-co-glycolide) NPs(38). The literature survey revealed that no mesoporous formulation of ETO has been reported till date.

2.2. BICALUTAMIDE (BIC):

Bicalutamide (BIC) is a standout amongst the most broadly utilized medication and considered among the principal first line treatment for prostate cancer with or without castration. It belongs to BCS class II possessing poor solubility issues which in turn limits its efficacy. Several formulations like PLGA NPs (39) and Magnetic NPs (40) have been researched upon for BIC. However, mesoporous formulation is not been reported for this drug.

3. AIMS AND OBJECTIVES:

- Synthesis, characterization and solid-state evaluation of mesoporous silica nanoparticles (MSN) i.e. MCM-41.
- Drug (ETO and BIC individually) loading and thorough characterization of formulated nanoparticles.
- To synthesize pH and receptor based mesoporous silica nanoparticles by Surface modification of MSN followed by further characterizations to confirm success of surface decoration
- Drug (ETO and BIC individually) loading into surface functionalized MSNs and thorough characterization of formulated nanoparticles.
- To perform *in vitro* release and kinetics study in different dissolution and diffusion media (at different pH) including simulated and biorelevant fast and fed state gastric and intestinal media.
- To perform cell permeability study on Caco-2 cells.
- To perform *in vitro* cell line study on androgen dependent and independent prostate cancer cell lines namely LNCaP and PC-3 respectively.
- To perform cellular uptake study
    - ✓ Qualitative cellular uptake study by confocal microscopy on LNCaP and PC-3 cells.
    - ✓ Quantitative cellular uptake study by flow cytometric analysis.
- To study cell death mechanisms by apoptosis study.
- To perform *in vivo* animal study

- ✓ Bioavailability enhancement study
- ✓ Bio distribution study

4. METHODOLOGY:

4.1. ETO:

4.1.1 Synthesis of MSN (MCM-41)

MCM-41 was synthesized as per the literature reported method based on template based synthesis with slight modifications (41). Concisely, around 9.84 g of CTAB was dispersed in 67 grams (g) deionized water at room temperature (RT) under continuous stirring. Thereafter, 6.52 g TMAOH and 6 g fumed silica was added to the mixture under continuous stirring for 5 hours (h) at RT. Subsequently, hydrothermal treatment was given for 48h. The thick slurry obtained was filtered and washed with de-ionized water and dried at RT yielding uncalcinated-MCM-41. The final step consisted of removal of the surfactant template. This was achieved by calcination of the product at 823 K for 6 h. Eventually, calcined MCM-41 type MSN was obtained.

4.1.2 Functionalization with Amine groups

The amine functionalization was done using a methodology adapted (42) with trivial modifications. Precisely weighed 500 mg MCM-41 was transferred to a round bottom flask (RBF). 50 mL of toluene was added to the RBF. Followed by addition of 6.87 ml of APTES. The reaction was kept at a higher temperature of 70 °C for 12 h. After completion of reaction, RBF was carefully removed and allowed to cool to room temperature. The reaction mixture obtained was filtered and washed with Methanol. The formation of aminated carrier was confirmed by FT-IR spectroscopy. They were tagged as MCM-41-AMN. The functionalization of MCM-41 with amine groups offered positive charged groups and imparted a base functional group to MSN for further decoration with PAA.

4.1.3 Preparation of MCM-41-PAA: Grafting of PAA groups

The grafting was performed as per literature reported method (43) with various modifications. To a 100 mL round-bottom flask covered with aluminum foil, MCM-41-AM (392 mg) dispersed in 30 mL DMF was added followed by addition of 400 mg PAA to the aforementioned mixture. After 2 h of vigorous stirring at 140 °C, the final product was then

recovered by filtration. The final product was washed with anhydrous methanol and dried for 4 h at 45 °C. The success of grafting was ascertained using FT-IR spectroscopy. Obtained NPs were labelled as PAA-MSN.

4.1.4 Surface functionalization: FA grafting on Bare MSNs

A facile post-synthetic grafting strategy was adopted for attachment of FA molecules onto the surface of MCM-41 NPs as reported earlier with minor moderation (44). 50 mL Round bottom flask (RBF) containing reaction mixture comprising of 10mL DMSO, FA (300 milligrams (mg)), NHS (90 mg), EDC.HCl (150 mg), and APTS 200µL was taken. After vigorous stirring for 7 h, 12 mL toluene and 150 mg of MCM-41MSNs were added. The aluminum foil covered RBF was kept under continuous stirring for 48 h at room temperature (RT). Further, the reaction mixture obtained was filtered and washed properly with various solvents in the order toluene (30mL), DMSO (30 mL), water (50mL) and acetone (40mL). Final product was dried and named FA-MSNs.

4.2. Drug loading into silica nanoparticles:

For the purpose of ETO encapsulation into MSN, different drug carrier ratio and loading methods were tried, striving for maximum loading of the drug. Loading and entrapment were determined by Ultraviolet (UV) and TGA. Drug: carrier ratio (D: C) of 0.5:1, 1:1 and 1:1.5were tried. The different loading methods tried for ETO included modified incipient impregnation centrifugation method, incipient impregnation filtration method and novel immersion solvent rotary evaporation method. For modified incipient impregnation centrifugation method, different concentrations of ETO in methanol were prepared and added to the vial containing MCM-41 nanoparticles (NPs). Thereafter, the residue was collected and supernatant assayed for non-encapsulated ETO by UV spectrophotometer at a wavelength maximum of 285 nm. Further, in second method, fixed amount of ETO was dissolved in methanol and specific amount of MCM-41 was added. The mixture was kept under magnetic stirring for 4 h at RT and then filtered. The recovered solid was dried further and filtrate checked for unloaded drug. ETO was loaded into mesoporous materials by immersion solvent rotary evaporation method. A 10 mL solution of the drug in methanol was prepared and MCM-41 NPs were added to it. The mixture was stirred continuously for 2 h at RT. The solvent was evaporated by rotary evaporator under reduced pressure at 50℃ for 10 minutes (min). The obtained ETO loaded

MCM-41 nanoparticles (ETO-MCM-41) were dried at RT. The loading of ETO into MCM-41 was confirmed by Differential scanning calorimetry (DSC).

However, it was found that former two methods were unsuitable for loading, as they were showing poor results with respect to loading and entrapment. Therefore, immersion solvent rotary evaporation method was finalized for ETO loading in D: C ratio 1:1.5.

Additionally, ETO loading and entrapment in MCM-41 was calculated by the formulae (1) and (2) respectively.

$$Drug\ loading\ (\%) = \frac{Wt\ of\ ETO\ (mg)\ in\ MSNPs}{Initial\ wt\ of\ ETO\ (mg)} \times 100 \ldots\ldots\ldots\ldots\ldots (1)$$

$$Entrapment\ efficiency\ (\%) = \frac{Wt\ of\ ETO\ (mg)\ in\ MSNPs}{Total\ wt\ of\ ETO\ loaded\ MSNPs\ (mg)} \times 100 \ldots\ldots\ldots\ldots (2)$$

4.1.5. Solid state evaluation of drug loaded MSNs:

Drug loaded nanoparticles were evaluated by SEM, TEM, nitrogen sorption analysis, small and wide angle XRD, DSC, FT-IR and particle size and zeta potential analysis. Elemental composition was determined by EDX analysis.

4.1.5. In vitro dissolution study

Dissolution study was performed using Veego USP type II dissolution apparatus in 900 mL dissolution media at 50 rpm maintaining temperature of dissolution medium at 37 ± 0.5°C. The *in vitro* release study was performed for plain ETO (API), MF, ETO-MCM-41 and ETO-MCM-41-A in acetate media (pH 4.6), simulated gastric fluid (SGF) (pH 1.2) and simulated intestinal fluid (SIF) (pH 6.8) containing pepsin and pancreatin respectively. Enzyme free SGF (pH 1.2) and SIF (pH 6.8) were also taken to study the presence of any interaction between hard gelatin capsule shell and amine group of MCM-41-A and whether it has any effect on the release of ETO. The drug release pattern was also studied in the presence and absence of food as well. Hence, the fed and fasted state simulated gastric and intestinal media were prepared (FaSSGF, FeSSGF. FaSSIF, FeSSIF) for this purpose. The composition of all media is summarized in Table 1 (45, 46).The powder was filled in the hard gelatin capsule shell prior to dissolution study. 5 mL aliquots were withdrawn at regular intervals. The withdrawn samples were filtered through 0.45µ PVDF filter membrane and analysed by UV spectrophotometer at 285 nm. Sink conditions were maintained throughout the study by adding an equivalent amount

of fresh medium as that of withdrawn sample. A comprehensive dissolution kinetics study was carried out for ETO release in biorelevant and simulated dissolution media.

4.1.5.1 Kinetics study

For the purpose of quantifying the differences already observed in the release profiles of ETO. The drug release data from both MCM-41 and MCM-41-A nanoparticles was fitted to various kinetic models and the best fit was determined amongst them. Different parameters by fitting the experimental data to different release models was calculated. Criteria for judgement on best model included lowest AIC (Akaike information criterion), highest MSC (Model selection criterion) and Regression values (R^2) (47). The various release models to which dissolution data were fitted include zero order, first order, Higuchi, Weibull, Hixon-Crowell and Korsmeyer peppas model (48).

4.1.6 Caco-2 permeability study

Caco-2 cell line was procured from NCCS-Pune and maintained at 5% Carbon dioxide, 37℃ and complete RPMI-1640 with 20% FBS and 1% antibiotics (Pen-Strep solution). Lucifer yellow was used to check the membrane integrity. The Caco-2 cells were grown on trans well inserts having 0.4µ pore diameter with 1.13 cm^2 area. The inserts were thoroughly washed with 25mM HBSS Hank's balanced salt solution and 7.4 pH. The integrity of the monolayer formed was tested by monitoring Lucifer yellow dye permeability across the layer. Time dependent transport of ETO loaded MSNs was studied in unidirectional apical to basal manner. The donor compartment (apical) was treated with 0.5 mL of transport solution i.e. HBSS containing 0.1 mg/mL ETO and basal side was treated with 1.5 mL of HBSS solution. The samples were analysed by HPLC equipped with fluorescence detector with excitation wavelength of 247 and emission measured at 323nm.

After incubation of 30, 60, 90, 120, 180, 240 and 300 min, 100µL aliquots were withdrawn from the receiver and replenished with same volume of fresh HBSS. The collected samples were further analysed by HPLC equipped with fluorescence detector. The apparent permeability coefficient (P_{app}) was measured using the following equation. $P_{app}=dQdt/A\times C_0*60$

Where,

P_{app}: Apparent permeability coefficient (cm/h)

dQ/dt: drug permeation rate (mg/min)

A: cross-sectional area *i.e.* 1.13 cm_2

C_0: Initial drug concentration in the donor compartment (mg/mL)

4.1.7. In vitro diffusion study

In the *in vitro* drug release study, a suspension of ETO, MCM-41-AMN and PAA-MSN loaded with ETO was filled into a dialysis tube (cutoff Molecular weight (Mw) = 7000 g/mol). For ETO loaded FA-MSNs, dialysis tube of (cut-off Mw=3500) was used. Further, the bag containing filled dispersion was then immersed into 100 mL (Phosphate buffer saline) PBS solution of different pH (5.5, 6.8 and 7.4) with continuous magnetic stirring at room temperature (RT). Sink conditions were maintained by immediate replenishment of the withdrawn samples with fresh PBS. The drug release at particular time intervals was calculated by measuring fluorescence intensity by Spectrofluorophotometer with excitation wavelength fixed at 247 nm and emission measured at 323 nm.

4.1.8. Estimation of cell viability assay by MTT

Human prostate cancer cell lines PC-3 and LNCaP (NCCS, Pune) were used to evaluate the cytotoxicity of MCM-41-PAA, ETO-AMN-MSN, ETO-PAA-MSN, ETO-FA-MSN and free ETO by colorimetry based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were cultured at low passage. The media used was RPMI-1640 supplemented with 10 % (Fetal Bovine serum) FBS and 1 % antibiotics (Pen-Strep). The cell line was regularly screened and confirmed for absence of any sort of contamination. Cells were maintained in a humified, 5% CO_2 tissue culture incubator at 37 °C.

Cancer cells were seeded in 96-well microtiter plates at a density of 9,000 cells per well in complete RPMI-1640 media supplemented with 10% FBS. After 24, 48 and 72 h cells were incubated with fresh RPMI-1640 containing ETO, (0.1-20 and 2-60 µg/mL); known amount of ETO loaded NPs corresponding to the aforementioned range. For adjudging the carrier toxicity; MCM-41-PAA NPs were incubated with cells for 72h in the concentration range of 0.1 to 100 µg/mL. All the dilutions were done in RPMI-1640 media. Furthermore, on next day MTT treatment was given to cells with 100µL dye added to each well and again incubated for 4 h. This was followed by addition of 100 µL DMSO to solubilize the formazan formed. The difference in intensity of purple color obtained in wells was analyzed by Plate reader at 590nm. Finally, cell viability was determined by the following equation

$$Cell\ viability\ (\%) = \frac{I\ (sample) - I\ (blank)}{I\ (control) - I\ (blank)} \ldots\ldots (3)$$

Where I sample, I control and I blank represents absorbance intensity at 590 nm, for cells treated with different samples (Positive control), for Negative control cells (untreated cells+ MTT+ DMSO), and blank well devoid of cells (Media + MTT + DMSO) respectively.

4.1.9 Intracellular Uptake and intracellular distribution of MSNs

4.1.9.1 FITC Labelling of MSNs

The NPs were labelled as per the procedures adopted earlier with slight modifications(49, 50). For FITC labelling, a methanolic solution of FITC (0.3 mg/mL) was used. In which, the 10 mg A@MSNs and FA@MSNs were kept for 12h, under continuous stirring in dark. Furthermore, the solution was centrifuged and washed with methanol for complete removal of unconjugated FITC until the supernatants obtained were rendered colorless. The final product was tagged as FITC#A@MSNs, FITC#PAA-MSNs and FITC#FA@MSNs.

4.1.9.2 Fluorescence microscopic images

LNCaP and PC-3 cells were seeded on a coverslip in 6 well plates and grown in a complete RPMI-1640 medium. Thereafter, the cells were exposed to FITC#A@MSN, FITC#PAA-MSNs ,FITC#FA@MSN and Free FA+FITC#FA@MSN for 12 and 48 h. The medium was removed and then washed thrice with PBS. After completion of the predefined time, the coverslips were washed thoroughly with the PBS and fixed with 3% paraformaldehyde (51). Lastly, LNCaP and PC-3 cells on coverslips were counter stained with DAPI (nuclear stain) in PBS (1ug/ml) at RT for 30 min and images were taken on a confocal microscope.

4.1.9.3 Flow Cytometry analysis (FCM)

The cellular uptake was determined by FCM. PC-3 and LNCaP cells were seeded in a 12-well plate at density of 1×10^6 cells per well and incubated for 24h. Untreated cells were taken as control. Both the cells were treated with FITC#A@MSN, FITC#PAA-MSNs, FITC#FA@MSN and Free FA+FITC#FA@MSN. After appropriate incubation time of 48 h medium was completely removed and cells were washed with PBS two times followed by trypsinization. Here, all the free FITC MSNs and dead cells were removed. All the cells were collected and centrifuged at a speed of 1200rpm and washed with PBS. This was again followed by resuspending in PBS. The cell suspensions were filtered through cell strainer and further subjected to flow cytometric analysis (FACS Caliber).

4.1.10 Evaluation of cell death mechanisms by apoptosis assay

Annexin V-FITC double stain apoptosis detection kit from BD Biosciences was used for determining percentage of apoptotic and necrotic cells by standard Fluorescence activated cells sorting (FACS) assay. LNCaP and PC-3 cells were seeded each at a density of 10^6 cells per well and incubated for 24 h. The cells were treated with ETO solution, ETO-AMN-MSN, ETO-PAA-MSNs and ETO-FA-MSN and incubated for 24 h followed by cold PBS (4℃) wash. Untreated cells were taken as control. The washed cells were then stained using FITC-Annexin V apoptosis detection kit. Concisely, the cells were suspended in 1 mL of 1 × binding buffer at a concentration of 1×10^6 cells/mL. Further, 5µL of FITC-Annexin V and 5µL of PI were added per 100µL of the suspension (1×10^5). After mild vertexing, the cells were incubated for 15 min in dark. Finally, 400 µL of 1 × binding buffer was added to each tube and analyzed by FCM (52).

4.1.11 In vivo pharmacokinetic study

For pharmacokinetic study mice were randomly divided into required groups (n=6) and intravenously injected from the tail vein with a 0.5 CC U40 insulin syringe fitted with a 28-$g^{1/2}$ needle with 200µL of sterile suspension of free ETO, ETO-AMN-MSN, ETO-PAA-MSNs and ETO-FA-MSN (10 mg/kg) body weight. The blood samples (0.3 ml) were collected at specific time intervals and stored in EDTA containing centrifuge vials. Further, plasma was separated by centrifugation at 4000 rpm for 10 minutes at 4℃. 100µL plasma was mixed with internal standard Tapentadol (TAP). The samples were precipitated by adding Methanol (MeOH) and again centrifuged at 10000 rpm for 10 min. The supernatants were collected and ETO was quantified using a HPLC equipped with a fluorescence detector. The parameters of HPLC method developed were ammonium formate (20mM) and methanol in the ratio 49:51 on a waters symmetry 300 C-18 column (250mm× 4.6mm×5µ) at pH 3.9 with a flow rate of 1mL/min. Pharmacokinetic parameters viz C_{max}, T_{max} and AUC were calculated using excel add-in. Oral pharmacokinetic study was carried out for ETO, ETO-AMN-MSN, ETO-MCM-41 and Marketed formulation.

4.1.12 Biodistribution study

For studying Biodistribution of ETO and MSNs, in major organs, mice were assigned into 3 groups (n=3) including control, free ETO, ETO-AMN-MSN, ETO-PAA-MSN and ETO-FA-MSNs. The experimental groups were administered sterile ETO suspensions and NP suspensions at dose of 10 mg/kg. Sterile saline injections (0.9% NaCl) at equivalent volumes

were given to mice as control. Mice were sacrificed at 24 h after injection and major tissues like heart, liver, lung, kidney, brain and spleen were collected and weighed. Furthermore, PBS solution was added to each tissue sample by an equal volume to its weight and subjected to high speed homogenization. The mixtures obtained were centrifuged at 10000 rpm for 10 min. The supernatant was collected and extracted with Methanol. The obtained solution was transferred to centrifuge vials and subjected to evaporation. The dried residues were reconstituted in 100µL methanol. The amount of ETO in each tissue was quantified by HPLC equipped with a fluorescence detector with excitation at 247 and emission at 323 nm.

4.1.13 Histological examination

The major organs collected were fixed in 10 % formalin solution. The organs were embedded in paraffin and sectioned to 4um sections and placed onto the glass slides. The histological sections were stained with Hematoxylin and Eosin stain (H&E) and observed under microscope. This was performed to determine the *in vivo* toxicity of various ETO formulations.

4.2 BIC

4.2.1. Synthesis of carrier, Drug loading, solid state evaluation and release study:

Identical methodology was adopted for carrier synthesis and BIC loading into MSNs as applied for ETO. Same way remaining studies, including solid state evaluation, in vitro drug release and in vitro cell viability assay were performed in the same way as accomplished earlier.

4.1.2. Formulation of tablet and evaluation:

BIC loaded plain and amine modified nanoparticles were formulated in tablet performing direct compression method. Mixture of different excipients and tablets equivalent to 50 mg BIC was punched using single punch tablet machine equipped with punches of 9 mm diameter with flat faces. Tablets were characterized for several parameters like hardness, friability, weight variation, disintegration time *etc*. as per the official methods.

4.1.3. Pharmacokinetic study:

All the mice were given free access to food and water and acclimatized to the animal care facility for at least 7 days before starting the experiment. To study the oral bioavailability, mice were assigned into 5 groups including control. The other groups were administered oral dose equivalent to 10mg/kg BIC solution, BIC-MCM-41, BIC-PAA-MSN, BIC-MCM-41-AMN and Marketed Formulation by direct introduction into stomach using oral gavage. I.V. Pharmacokinetic study was carried out as per procedure described earlier for ETO.

About 0.3 mL of blood was collected from retro orbital vein of mice in EDTA containing centrifuge vials. Protein precipitation method was used in sample processing. Plasma was separated from the blood sample by centrifugation at 4000 rpm for10 min at 4℃. Processed sample was mixed with internal standard Tadalafil (TAD). Acetonitrile (ACN) was used as a precipitating agent. Supernatant was collected post centrifugation and quantified by HPLC equipped with a fluorescence detector. The developed HPLC method consisted of ammonium Formate (20mM) (pH 4) and ACN in the ratio 46:54 run on a waters symmetry 300 C-18 (250mm× 4.6mm×5µ) column with a flow rate of 1 mL/min. Various Pharmacokinetic parameters were determined using an excel add-in PK-Solver.

4.1.4 Cytotoxicity, cellular uptake, biodistribution and apoptosis study

The concentration range for MTT assay was 0.1-70 µg/mL. Cellular uptake studies by confocal microscopy and flowcytometry were conducted as per the procedure descried earlier for ETO. Also, biodistribution and apoptotic study to determine the cell death mechanism were carried out in a similar way as per the aforementioned procedure.

5. RESULTS AND DISCUSSION:

5.1. ETO:

- ✓ ETO loaded nanoparticles were successfully synthesized and characterized thoroughly. FT-IR results confirm the functional group before surface modification and after surface modification. Furthermore, absence of drug peak in the final formulation showed complete drug loading. This was further confirmed by DSC and wide angle XRD analysis where drug loaded nanoparticles were free from characteristic ETO peak.
- ✓ Success of mesoporous structure was confirmed by TEM microscopy and small angle XRD. Furthermore, pore size, pore volume and surface area were calculated from nitrogen sorption technique (BET analysis).
- ✓ Confirmation of formation of surface coated (AMN, PAA and FA coating) MSN was done through DLS, FT-IR, BET analysis.
- ✓ For all formulations, obtained %loading capacity and %entrapment efficiency was more than 30 and 80 respectively.
- ✓ In vitro drug release and permeability data showed superior release behavior of prepared formulation with respect to marketed formulation.

- ✓ In vitro diffusion study exhibited controlled and pH responsive release pattern for surface modified nanoparticles which will help in selective ETO release in cancer cells.
- ✓ In vitro cell viability MTT assay on human prostate carcinoma cell lines (PC-3 and LNCaP) result favored pH responsive drug release from PAA coated nanoparticles. Furthermore, folate coated MSNs showed selective folate receptor targeting to prostate cancer cells which confirmed by incubating the cells for 24 and 72hrs.
- ✓ Further, confirmation of targeting efficiency could be ascertained from confocal microscopy images the cellular uptake efficiency of FITC labelled NPs which was more than 85%.
- ✓ Investigation of cell death mechanism revealed a programmed apoptotic cell death.
- ✓ In vivo pharmacokinetic data revealed enhanced bioavailability of the synthesized mesoporous formulation and prolonged blood circulation time.
- ✓ Lastly, the histological examination of tissues revealed no significant damage to the major organs.

5.2. BIC:

- ✓ Similar to ETO, BIC loaded nanoparticles were successfully synthesized and characterized thoroughly by FT-IR, DSC, XRD results confirm complete drug loading in final formulation.
- ✓ The mesoporous structural integrity was maintained and confirmed by SEM, TEM microscopy and small angle XRD. Furthermore, pore size, pore volume and surface area were calculated from nitrogen sorption technique.
- ✓ Confirmation of formation of surface coated MSN was done through DLS, FT-IR, BET analysis.
- ✓ EDX data confirmed the success of amination, PAA and FA coating onto bare MCM-41 MSNs.
- ✓ For all formulations exhibited considerable a %loading capacity and %entrapment efficiency
- ✓ In vitro drug release data showed superior release behavior of prepared tablet mesoporous formulation with respect to marketed formulation.
- ✓ In vitro diffusion study exhibited controlled and pH responsive release pattern for PAA and to some extent FA modified nanoparticles which will help in selective drug release in cancer cells.

- ✓ In vitro cell viability MTT assay showed promising cell death with respect to plain API and revealed superiority in cell killing efficiency.
- ✓ Confocal microscopic images and cellular uptake data revealed high intracellular uptake into both PC-3 and LNCaP cells.
- ✓ Investigation of cell death mechanism revealed a programmed apoptotic cell death.
- ✓ In vivo study carried out in male swiss albino mice gave encouraging results with significant increase in bioavailability.
- ✓ Safety of synthesized MSNs could be established by the histological examination of major organs. Which indicated no significant abnormality when compared with control.

6. FUTURE WORK:

- ✓ Data compilation and submission.
- ✓ Publication of research work
- ✓ Thesis writing.

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