A SYNOPSIS ON

"DEVELOPMENT OF NANO DRUG DELIVERY SYSTEMS FOR BIOAVAILABILITY ENHANCEMENT OF SOME ANTI-CANCER DRUGS"

By

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Research guide

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1. INTRODUCTION:

Cancer is one of the leading causes of death amongst all the lethal diseases prevalent. Around 30% of the world's population is suffering from cancer, which puts a tremendous pressure on the researchers to find a cure for the same. Everyday new advances are being made towards cancer treatment, by synthesizing new moieties or by researching new molecular mechanisms. Currently drug molecules available for cancer treatment include peptides, steroid molecules and oligonucleotides. Most of the anti-cancer drugs are hydrophobic in nature and hence, having low solubility and bioavailability. Therefore, the desired therapeutic dose will not reach to the target, hence, requiring a higher dose for therapeutic efficacy. This may damage healthy cells and tissues, leading to severe side effects, *e.g.* severe hair falls resulting in baldness, acute vomiting and nausea, low blood cell counts making patients more susceptible towards developing infection or anaemia [1].

An ideal drug delivery system should encourage the intracellular accumulation of drug in the targeted cells and maintain drug concentration to its effective level. This is possible by two ways *viz.*, (a) by designing a formulation that gives controlled or sustained release *i.e.* modified release or (b) by targeting the drug to its target *i.e.* tumour cell. The research thrust these days is on designing a targeted drug delivery system that will deliver a therapeutic dose to the cancerous cell and ultimately reduce its reach to healthy cells thereby decreasing their side effects. Targeting concept is now in focus and vast researches are involved to formulate a nano system, including liposomes [2-5], solid lipid nanoparticles [6], self-micro emulsifying drug delivery system (SMEDDs) [7] having tumour-targeting efficiency. Mesoporous silica nanoparticle (MSNs) are one of the nano carriers, which are in focus for targeting the tumour cells [8, 9].

Mesoporous silica nanoparticles (MSNs) are emerging as an efficient and prominent drug delivery carriers due to their excellent properties like high surface areas and pore volumes, pore size, high drug loading capacity, zero premature release, biocompatibility, tailored drug release capability by tuning the pore size and surface of MSNs *etc*. MSNs can be used to satisfy various purposes like enhanced dissolution rate, high bioavailability, targeting the desired cells, diagnosis of disease, *etc*. Though MSNs have high therapeutic potential, it still remains to be untapped and yet to be explored. Different MSN carriers like MCM-41, MCM-48, MCM-50, SBA-15, SBA-16, HMS, TUD, MCF, FSM, *etc*. are available, each having a unique morphology and pore size. Amongst these, MCM and SBA are gaining attention because of their unique internal structure, surface properties and pore size [10-14]. MCM-41 is in the

limelight in the pharma field having 2D hexagonal structure, whereas MCM-48 is gaining attention now a days because of its exclusive 3D cubic structure and having a similar or higher capacity of drug loading, drug release compared to former [15, 16].

2. SELECTION OF ACTIVE PHARMACEUTICAL INGREDIENTS:

2.1. RALOXIFENE (RLX):

RLX is a potent anti-cancer drug used in the treatment of breast cancer. Being a BCS class II drug (biopharmaceutical classification system), it faces a solubility issue which becomes a major obstacle to its efficacy. Many research groups have explored RLX to satisfy different purposes. E.g. Shah *et al* have synthesized nanostructured lipid carrier for bioavailability enhancement of RLX [17]. Bikiaris *et al* have formulated biodegradable polyesters drug carrier to enhance bioavailability and solubility of RLX hydrochloride [18]. Similarly, Jay Prakash *et al* have constructed Gellan gum nanoparticles for bioavailability enhancement of the same [19]. Fontana *et al* had formulated Eudragit coated RLX nanocapsules to control release to improve the anti-proliferative effect [20]. But no research work has yet been carried out to formulate mesoporous silica nanoparticles for RLX.

2.2. BEXAROTENE (BXR):

BXR is another API being used in the treatment of breast cancer. As has been BCS class IV drug, it faces solubility and permeability problems to show its efficacy. Being recently discovered molecule, it is not much explored, only a few literature involving formulation of BXR nanoparticle are available. e.g. Li *et al* had prepared chitosan folate conjugated nanoparticles as a targeted drug delivery system [21]. Another research group had formulated nanocrystal to show its efficacy in lung cancer [22]. Lisi Qi *et al* had incorporated BXR into bovine serum albumin nanoparticles [23]. However, the field showing application of mesoporous silica nanoparticle for encapsulation of BXR was not explored.

3. AIMS AND OBJECTIVES:

- Synthesis, characterisation and solid state evaluation of mesoporous silica nanoparticles (MSN) i.e. MCM-41 and MCM-48
- Drug (RLX and BXR individually) loading and thorough characterisation of formulated nanoparticles
- To synthesise pH and target based mesoporous silica nanoparticles by Surface modification of MSN followed by further characterisation to confirm success of surface modification
- To perform *in vitro* release study in different dissolution and diffusion media

- To perform *in vitro* cell line study
- To perform cellular uptake study
    - ✓ Qualitative cellular uptake study by confocal microscopy
    - ✓ Quantitative cellular uptake study by flow cytometric analysis
- To perform *in vivo* animal study
    - ✓ Bioavailability enhancement study
    - ✓ Bio distribution study

4. METHODOLOGY:

4.1. RLX:

4.1.1. MCM-41 synthesis:

MCM-41 synthesis was carried out according to procedure reported in the literature [24]. Briefly accurately weighed 9.84 gram (gm) of CTAB was dissolved in 67 gm of deionized water and subjected to vigorous stirring for 5 minute (min) followed by dropwise addition of 6.92 gm of TMAOH under constant stirring for 10 min. Reaction mixture was subjected to continuous stirring for 1.5 hour (h) subsequent to incorporation of 6 gm fumed silica which resulted in formation of thick slurry carrying a composition of 1 SiO_2: 0.27 CTAB: 0.19 TMAOH: 40 H_2O. This slurry was given hydrothermal crystallization treatment for 48 h in Teflon lined autoclave for at 373K. The mixture was then filtered, washed with deionised water, and dried at 353K overnight. Dried MSN was incinerated in a muffle furnace for 5.5 h at 823K for removal of surfactant.

4.1.2. MCM-48 synthesis:

MCM-48 nanoparticles were synthesised as per the procedure given in the literature [25]. Precisely weighed 4.4 gm CTAB was dissolved in 40 mL moderately warm water (35 °C) under gentle and continuous stirring followed by addition of 2M 5 mL aqueous NaOH. Following to this 5 mL TEOS, a silica source was incorporated in a dropwise manner with uninterrupted stirring. The gel mixture having molar composition of 1 SiO_2: 0.23 NaOH: 0.55 CTAB: 11 H_2O was stirred for 1.5 h and hydrothermal treatment was given by transferring this homogeneous blend to Teflon lined autoclave at 283K for 72 h. Eventually, the product was recovered by filtration, washed thoroughly with plenty of water and dried overnight at 353K. Dried product was ignited at 573K initially for 2 h and at 823K for subsequent 8 h for total surfactant elimination.

4.1.3. Synthesis of amine decorated MSN (MCM-41 and MCM-48):

Amine modified MSN was prepared by following post synthesis grafting approach. Wherein, 0.1 gram (gm) MCM-41 was dispersed in 25mL of dried toluene, which was followed by subsequent addition of 1.381mL of APTES and the reaction mixture was refluxed at 120 ºC for 24 hour (h).

4.1.4. Synthesis of chitosan coated MSN (MCM-41 and MCM-48):

Chitosan grafting above MSN-NH_2 was carried out according to the reported method [26]. 25 mg chitosan was dissolved in 5mL 3% acetic acid and the suspension was stirred at 600rpm for 24 h to form a chitosan solution (0.5%v/v). On the other side, 10mg bare nanoparticles were ultrasonicated in 5mL methanol for 10 min. Dispersion was adjusted to pH 3.5-4.5 by adding acetic acid. Subsequently, 200µL APTES was added into the dispersion of bare mesoporous silica nanoparticles and was stirred for 4h at room temperature (RT). Finally 5mL chitosan solution was added into the mixture of the bare MSN and was subsequently stirred at RT for 24 hr. The chitosan-coated MSN were collected by centrifugation at 10000 rpm, following washing with excessive distilled water and methanol before freeze drying.

4.1.5. Synthesis of folate-chitosan conjugated MSN (MCM-41 and MCM-48):

4.1.5.1. Synthesis of folate chitosan conjugates:

STEP-1: *Solution 1*: 500mg chitosan was dissolved in 100mL 1 %v/v acetic acid to get concentration of 5mg/mL. The solution was vacuum filtered after 24 h. pH of the above solution was made to 5.5 by 0.1M sodium hydroxide. The complete solution was diluted to 500mL to get concentration of 1mg/mL with MES buffer (10mM, pH:5.5).

STEP-2: *Solution 2*: 0.88mg folic acid and 0.3094mg EDC were mixed in 20 mL methanol and stirred at RT for 24 h. *Solution 3*: 19.5mg MES and 11.5mg NHS were added into 10 mL water in separate beaker and mixed well for 15 min. Sol-3 was added to sol-2, and kept for stirring for 2-3 h. Above mixture was added to sol-1 and stirred for 4h at RT. Final mixture contains chitosan : folic acid in 1 : 0.16 ratio. pH of the solution was made from 5.5 to 9 with 1M NaOH. White precipitates were obtained, thoroughly washed and collected by centrifugation.

STEP-3: Residues obtained after centrifugation were purified using PBS solution (pH:7.4) for 3 days and water for 4 days in dialysis membrane (10000 Da). After 7days purification procedure, the spongy material was recovered and subjected to freeze drying.

4.1.5.2. Synthesis of folate chitosan conjugated MSN (*MCM-41 and MCM-48)*:

100 mg of RLX-MCM-41-NH_2 was mixed with 150 mg freeze dried CHITO-FA conjugates in MES buffer. This mixture was stirred for 2 days at RT. The solution was filtered and collected

residues were labelled as RLX-MCM-41-CHITO-FA. A similar procedure was followed to synthesise MCM-48-NH_2-NP by taking RLX-MCM-48-NH_2 initially.

4.1.2. Drug loading into silica nanoparticles:

A novel immersion-solvent evaporation method was employed for incorporation of RLX into prepared nanoparticles. Concentrated RLX solution was prepared by dissolving 50 mg RLX into 10 mL of MeOH and 75 mg of different mesoporous silica nanoparticles were added to maintain drug: silica nanoparticle ratio 1:1.5. This mixture was stirred for 1.5 h for efficient loading followed by rota evaporation treatment. Subsequently the blend was vacuum dried for overnight and %Entrapment efficiency and %loading efficiency of RLX and silica nanoparticles respectively, were calculated using following formula.

$$\%Entrapment\ Efficiency = \frac{Total\ weight\ of\ RLX\ present\ in\ nanoparticles}{Weight\ of\ RLX\ added\ initially} \times 100$$

$$\%Loading\ Efficiency = \frac{Total\ weight\ of\ RLX\ present\ in\ nanoparticles}{Total\ weight\ of\ drug\ loaded\ nanoparticles} \times 100$$

4.1.3. Solid state evaluation of drug loaded MSNs:

Drug loaded nanoparticles were evaluated performing SEM, TEM, nitrogen sorption analysis, small and wide angle XRD, DSC, FT-IR and particle size and zeta potential-DLS analysis.

4.1.4. Formulation of tablet and evaluation:

RLX loaded plain and amine modified nanoparticles were formulated in tablet performing direct compression method. Mixture of different excipients and tablets equivalent to 60 mg RLX 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 (IP) methods.

4.1.5. *In vitro* dissolution study

%Release was checked in simulated gastric fluid (SGF) having pH 1.8, acetate buffer with pH 4.5 and simulated intestinal fluid (SIF) of pH 6.8 were prepared. In Addition to stimulated media, biorelevant media like fasted state simulated gastric fluid (FaSSGF), fed state simulated gastric fluid (FeSSGF), fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) were prepared to mimic human physiology. Dissolution study was performed using USP apparatus II at 50 rpm maintaining temperature of dissolution medium at 37 ± 0.5 ºC. The study was carried out in aforementioned dissolution media of different pH for plain drug (RLX), Ralista®, a marketed formulation (MF) and for RLX filled MSN tablets

equivalent to 60mg RLX. 5mL of aliquots were withdrawn periodically and subsequently replenished by fresh media every time. 0.45µ nylon filter was utilized to filter the aliquots followed by UV spectrophotometric analysis at 287 nm λ_{max}.

4.1.6. *In vitro* diffusion study

The pH responsive behaviour of chitosan and folate coated MSNs were studied by performing *in vitro* release study in PBS buffer having three different pH (PBS: 5.5, 6.5, and 7.4). 10 mg Plain drug RLX was dispersed in 5mL diffusion release media and obtained dispersion was filled in dialysis bag (cut off molecular weight 5000Da). Subsequently, sealed bag was immersed into 100mL diffusion media. The entire assembly was kept at 37 ºC temperature and rotated at the speed of 75 rpm. Aliquots were withdrawn at predefined time interval and it was replenished by an equivalent volume of fresh diffusion medium upto 48 h. Eventually, the %cumulative drug release (%CDR) was calculated and the release pattern for RLX at different pH was constructed.

4.1.7. Estimation of cell viability assay by MTT

Cell viability assay or cytotoxicity of the formulated nanoparticles was determined by MTT assay against human breast carcinoma cell line i.e. MCF-7 cell line. The first step of the MTT assay is seeding. Where, the cells were seeded in the 96 well plates using DMEM containing sodium pyruvate, L-glutamate, sodium bicarbonate along with high glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen/strep) solution which resist bacterial growth. MTT assay initiated with cell seeding step. Where, 100µL cell suspension in each well was poured (10000 cells/well) followed by incubation at 37 ºC for 24h in the incubator. Seeding step was followed by the prime important step of incorporation of the formulation i.e. drug treatment. Wherein, formulation containing 0.1 to 10 ppm equivalent RLX was added and eventually well plate was incubated for a desired period time (24h and 48h). Lastly cells were given MTT dye treatment which was followed by plate reading at 570nm. To nullify the false negative results, negative control i.e. cells treated exclusively with solvent (DMSO) was taken into consideration.

4.2. BXR:

4.2.1. Large pore MCM-41 synthesis:

Accurately weighed CTAB was dissolved in deionised water and stirred for 5 min. Followed by dropwise addition of TMAOH under constant stirring for 10 min. Followed by incorporation of fumed silica and NH_4OH which resulted in formation of thick slurry carrying a composition of 1 H_2O: 0.06 SIO_2: 0.02 TMAOH: 0.01 CTAB:0.01 NH_4OH. Resultant gel was treated at 70

ºC for the required time (3days). Following this, the samples were aged in their mother liquor for the required time (1 days, 2days and 3 days). The mixture was then filtered, washed with deionised water and dried at 353 K overnight. Dried MSN was incinerated in a muffle furnace for 5.5 h at 823 K for removal of surfactant.

4.2.2. Large pore MCM-48 synthesis:

Precisely weighed 3.85gm TMAOH (25%) was diluted with 37.1 gm water before adding 5.89 gm of CTAB under vigorous stirring. After 15 min, 2gm fumed silica was added followed by stirring for 30 min. The gel mixture having molar composition of 1 SiO_2: 0.317 TMAOH: 0.45 CTAB: 67H_2O was stirred for 1.5 h. Hydrothermal treatment was given by transferring this homogeneous blend to Teflon lined autoclave at 130 ºC for 72 h. The product was recovered by filtration, washed thoroughly with plenty of water and dried overnight at 353 K. Dried product was ignited at 573 K initially for 2 h and at 823 K for subsequent 8 h for total surfactant elimination and labelled as MCM-48 nanoparticles.

4.2.3. Synthesis of hyaluronic acid (HA) coated MSNs:

First, 1.5 g calcined MSNs was added to 60 mL toluene and stirred for 6 h before adding 1.0 mL APTES. After stirring at RT for 24 h, the particles were extensively washed with toluene and dried in a fume-hood at RT. Next, in order to graft HA, the carboxyl group of HA was activated with the NHS, using EDC as a coupling agent. The activated carboxyl group of HA then combined with the amine end of theNH_2-MSNs. Specifically, 1 g of the prepared NH_2-MSNs powder was dispersed in 100 mL deionized water. In another reaction vessel, 20 mL of an aqueous solution containing NHS (0.37 g) and EDC (0.2 g) was mixed with 60mL HA(113 mg) deionized water solution. Finally the two solutions were mixed and the pH was adjusted to 9.0 using triethylamine as a catalyst. The mixture was stirred at 38 ºC overnight. The HA modified MSNs (HA-MSNs) were obtained by centrifugation, washing three times with deionized water and freeze-drying.

4.2.4. Drug loading, solid state evaluation and release study:

Identical methodology was adopted for BXR loading into MSNs as applied for RLX. 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.

5. RESULTS AND DISCUSSION:

5.1. RLX:

- ✓ RLX loaded nanoparticles were successfully synthesised 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 RLX 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.
- ✓ Confirmation of formation of surface coated 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 data showed superior release behaviour 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 RLX release in cancer cells.
- ✓ In vitro cell viability MTT assay on human breast carcinoma cell line result favoured pH responsive drug release from chitosan coated nanoparticles. Furthermore, folate conjugated MSNs showed selective folate receptor targeting to MCF-7 cancer cells which confirmed by incubating the cells for 24 and 72hrs.

5.2. BXR:

- ✓ Similar to RLX, BXR loaded nanoparticles were successfully synthesised and characterized thoroughly. FT-IR, DSC, XRD results confirm complete drug loading in final formulation.
- ✓ 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.
- ✓ Confirmation of formation of surface coated MSN was done through DLS, FT-IR, BET analysis.
- ✓ For all formulations exhibited considerable a %loading capacity and %entrapment efficiency
- ✓ In vitro drug release data showed superior release behaviour of prepared formulation with respect to prepared synthetic mixture.

- ✓ In vitro diffusion study exhibited controlled and pH responsive release pattern for hyaluronic acid 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.

6. FUTURE WORK:

- ✓ To perform cellular uptake study by confocal microscopy and flow cytometry analysis
- ✓ To perform in vivo pharmacokinetic study and biodistribution for formulated nano drug delivery systems.
- ✓ Data compilation and submission.

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