THERANOSTIC APPLICATION OF CURCUMIN LOADED MESOPOROUS SILICA NANOPARTICLE ON BREAST ADENOCARCINOMA (MCF-7) CELLS

THESIS
Submitted by
L. HARINI
(Reg. No: 200901114)
in partial fulfillment for the award of the degree of
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOTECHNOLOGY
KALASALINGAM UNIVERSITY
ANAND NAGAR
KRISHNANKOIL–626 126
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DECEMBER 2016
Minutes of the Ph.D. Viva-Voce Examination of Mrs. L. Harini (Reg. No. 200901114) of the Department of Biotechnology, Kalasalingam University, Anand Nagar, Krishnankoil - 626126.

The Ph.D. Viva-Voce Examination of Mrs. L. Harini (Reg. No. 200901114) on his Ph.D. thesis entitled “Therapeutic application of curcumin loaded mesoporous silica nanoparticle on breast adenocarcinoma (MCF-7) cell” was conducted on 24th April, 2017 at 11:00 a.m. in the IIC Seminar Hall at Kalasalingam University, Anand Nagar, Krishnankoil-626126.

The following members of the Oral Examination Board were present:

3. Dr. T. Kathiresan
   Professor
   Department of Biotechnology
   Kalasalingam University
   Anand Nagar, Krishnankoil - 626 126
   Supervisor & Convener

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   Chennai 600 036
   Indian Examiner

4. Dr. K. Sundar
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   Kalasalingam University
   Anand Nagar, Krishnankoil - 626 126
   Chairman/DIC

The candidate, Mrs. L. Harini, presented the salient features of his Ph.D. work.

This was followed by questions from the board members. The queries and clarifications raised by the Foreign and Indian Examiners were also put to the candidate. The candidate answered the questions to the full satisfaction of the board members.

The corrections and suggestions pointed out by the Indian/Foreign examiners have been carried out and duly incorporated in the thesis.

Based on the candidate’s research work, her presentation and also the clarifications and answers by the candidate to the questions raised by the examiners, the board recommends that Mrs. L. Harini be awarded the Ph.D. degree in the FACULTY OF BIOTECHNOLOGY.

Dr. T. Kathiresan
(Supervisor & Convener)

Dr. S. Mahalingam
(Indian Examiner)

Dr. K. Sundar
(Chairman/DIC)
CERTIFICATE

This is to certify that no corrections/suggestions were pointed out by the Indian/Foreign Examiner(s) in the Thesis titled “THERANOSTIC APPLICATION OF CURCUMIN LOADED MESOPOROUS SILICA NANOPARTICLES ON BREAST ADENOCARCINOMA (MCF-7) CELLS” submitted by Mrs. L. HARINI.

Place: Krishnankoil

Date: 26/04/2017.

SUPERVISOR
Dr. T. KATHiresan
DECLARATION

I hereby declare that the thesis entitled “THERANOSTIC APPLICATION OF CURCUMIN LOADED MESOPOROUS SILICA NANOPARTICLE ON BREAST ADENOCARCINOMA (MCF-7) CELLS” submitted by me for the Degree of Doctor of Philosophy in Department of BIOTECHNOLOGY is the result of my original and independent research work carried out under the guidance of Dr. T. KATHiresan, Professor, Department of Biotechnology, Kalasalingam University, Anand Nagar, Krishnankoil and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University or Institution.

L. Harini
KALASALINGAM UNIVERSITY
KRISHNANKOIL 626 126

BONAFIDE CERTIFICATE

Certified that this Thesis title “THERANOSTIC APPLICATION OF CURCUMIN LOADED MESOPOROUS SILICA NANOPARTICLE ON BREAST ADENOCARCINOMA (MCF-7) CELLS” is the bonafide work of Mrs. L. HARINI who carried out the research under my supervision. Certified further, that to the best of my knowledge the work reported herein does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion on this or any other scholar.

Dr. T. KATHIRESAN
SUPERVISOR
Professor
Department of Biotechnology
Kalasalingam University
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Krishnankoil–626 126
ABSTRACT

Breast cancer accounts for the leading cause of mortality among the female in the world. Nearly, 1.7 million population was diagnosed with breast cancer in 2012. Change in life style and family history was considered as the major risk factor in breast cancer development. Of the breast cancer patients, India holds 17% of breast cancer cases in the world. However, the mortality rate of breast cancer patients of 54% was reported in India. Detection at the late stage was considered as the main reason for high mortality rate in India. Drawbacks with the current cancer treatment include the cardiotoxicity of therapeutic drugs and the development of resistance to chemotherapy and radiotherapy.

Nutraceuticals with anticancer activity and less toxicity would overcome the present chemotherapeutic challenges. Curcumin was one of the potent molecule with pleiotropic effect, including its anti-cancer activity. Curcumin was effective against many cancer cells in vitro. But the major limitation with curcumin was its hydrophobicity which results in their faster excretion and low bioavailability in vivo. The solubility of curcumin was enhanced by conjugating it with nano carriers. Few nano formulation of curcumin lack target specificity and drug leakage. The characteristics of an efficient nano drug delivery system had the ability to load high quantity of drug, target
specificity, sustained release and lack toxicity. One such nano delivery system which gratifies these properties was mesoporous silica nanoparticles. These particles were porous in nature with size ranging from 2-50 nm for drug loading. Another advantage with these mesoporous silica nanoparticles attributes for targeted and sustained release by its surface modification. This work focus on the MSN loaded curcumin’s anticancer properties against MCF-7 breast adenocarcinoma cells.

The drug delivery potential of MSN was studied with the well-known MSN, MCM-41 with curcumin loaded on it. MCM-41 was decorated with PEI for better adsorption of curcumin. Further, spherical shape of MCM-41, the parallelly arranged pores and curcumin loading on MCM-41 coated with PEI were confirmed by characterisation with SEM and TEM. PEI coating of MCM-41 enhanced curcumin loading to 70% compared to uncoated MCM-41. Another advantage of PEI coating on MCM-41 assist the effective release of curcumin at the acidic pH. The accumulation time point of MCM-41 inside MCF-7 cells is an hour and the cells remain saturate with MCM-41 at 3 h. As the maximum accumulation was obtained in 1 h, MCM-41 with curcumin loaded was incubated with MCF-7 cells for 1 h and the viability was assessed till 48 h. Curcumin released inside MCF-7 cells by the MCM-41P reduced cell viability to 50% at the 48 h. In addition, the cell death was confirmed by DAPI
nuclear staining and FACS revealed DNA fragmentation and the cell membrane leakage which are the hallmarks of apoptosis.

Shape of the MSN was one of the factor which determines its biological activity. Non-spherical MSN had the capacity to escape from the immunogenic response better than the spherical particles. Non-spherical MSNA had a better drug delivery potential than the spherical MCM-41 which was proved by comparing the properties of MCM-41 and MSNA. Non-spherical MSNA was taken up by cells faster (30 min.), had a better curcumin release at neutral pH and had reduced toxicity compared to spherical particles. Toxicity of MCM-41 particle was confirmed by its accumulation inside the autophagosomes, whereas no such autophagosomes were formed on MSNA treatment. As a better NDDS, MSNA loaded with curcumin was further assessed for the mechanism of apoptosis. CUR-MSNAP was able release 15 µM of curcumin inside the cell at 72 h with 30 µM of curcumin loaded on it. The apoptosis of MCF-7 cells was induced by intracellularly released curcumin from MSNA activates the caspases and CHOP but downregulates the PERK, IRE1α and GRP78 proteins which are the markers of ER. Also the mechanism underneath confirmed with TEM indicates that CUR-MSNA disrupts the mitochondria and nuclear membrane leading to apoptosis.
Development of resistance against drugs was one of the major challenge in anti-cancer drug development. Increased drug efflux, alteration of drug targets, modification of drug’s functional group was reasonable for treatment resistance. Hence a nano-targeted drug delivery approach was used against drug resistance cell lines. MCF-7R cells was treated with CUR and DOX co-loaded MSNA for 72 h. CUR and DOX loaded MSNA exhibit a synergistic relation on inducing cell death in MCF-7R. Further, effective cell death of MCF-7 and MCF-7R was obtained with lower CUR and DOX loaded on MSNA compared to the individual drug. Thus, non-toxic and non-spherical MSNA targets nucleus and mitochondria of cancer cells leading to apoptosis and also MSNA targeted with drug could effectively overcome drug resistance in MCF-7R cells.
Dedicated
to
My beloved Parents
I would like to thank my mentor Dr. T. Kathiresan, Professor, Department of Biotechnology, Kalasalingam University, for his guidance throughout this research study. I am grateful to Dr. Sweta Srivastava, Associate Professor, St. Johns National Academy of Health science and Dr. Gnanakumar, Assistant Professor, Madurai Kamaraj University for extending their valuable ideas for the progress of my work. I am also grateful to Dr. Cecil Ross, Professor, department of Medicine and Hematology, St. Johns National Academy of Health science and Dr. Rajagopalan, Emeritus Professor, Madurai Kamaraj University for their support. I would like to gratefully thank Dr. K. Sundar, Professor, Department of Biotechnology, Dr .K. Palanichelvam, Head of our Department, and Dr. A. Muthukumaran, Asst. Professor, Department of Biotechnology all the other faculty and non-teaching staff members of the Department of Biotechnology, Kalasalingam University, for their help throughout the course of this study. I sincerely thank our late Chairman, “Kalvivallal” Thiru. T. Kalasalingam, Honourable Chancellor Dr. K. Sridharan, Director Dr. S. Sashianand and Vice Chancellor, Dr. S. Saravanasankar, and for extending me all facilities throughout my research. I’m extending my gratitude to Dr. B. Karthikeyan and Dr. Muthumareeswaran, Mr. Anand, Ms. Mugdha Sharma, Ms. Pavana Thomas, and all my lab members for their support. Finally, I would like to thank my parents, my husband and my in-laws and my family members for their prayers and encouragement over the years.
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<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>Caspases</td>
<td>Cysteine-aspartic proteases</td>
</tr>
<tr>
<td>c-caspase</td>
<td>cleaved caspase</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>Cur</td>
<td>Curcumin</td>
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<td>CUR- MCM-41P</td>
<td>Curcumin loaded MCM-41 coated with PEI</td>
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<td>Curcumin loaded with PEI coated MSNA</td>
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<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenyindole</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy ribo nucleic acid</td>
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<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive X-rays</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retension</td>
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<tr>
<td>FACS</td>
<td>Fluorescence assisted cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GRP78</td>
<td>Glucose regulated protein 78</td>
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<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>IMDM</td>
<td>Iscove's Modified Dulbecco's Medium</td>
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<td>IRE 1α</td>
<td>inositol-requiring enzyme alpha</td>
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<td>MDR</td>
<td>Multidrug resistance</td>
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<td>MSN</td>
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<td>3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide</td>
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<td>NDDS</td>
<td>nano drug delivery system</td>
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<td>NP</td>
<td>nanoparticles</td>
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<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylene imine</td>
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<tr>
<td>PERK</td>
<td>protein kinase RNA-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate – Polyacrylamide Gel</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
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<tr>
<td>TEMED</td>
<td>N, N, N’, N’ tetra methyl ethylene diamine</td>
</tr>
<tr>
<td>TEOS</td>
<td>Tetraethyl orthosilicate</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolding protein response</td>
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<td>XRD</td>
<td>X-ray diffraction</td>
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<td>WST</td>
<td>Water soluble tetrazolium salt</td>
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<table>
<thead>
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<tr>
<td>%</td>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>µM</td>
<td>Micro molar</td>
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<tr>
<td>Fig.</td>
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</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g/L</td>
<td>Gram per litre</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>N</td>
<td>Normality</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomole</td>
</tr>
<tr>
<td>P&lt;0.05</td>
<td>Probability value less than 0.05</td>
</tr>
<tr>
<td>pH</td>
<td>Acidity or alkalinity of a solution</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromole</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolts</td>
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CHAPTER 1

1. INTRODUCTION

Breast cancer incidences are the leading cause of woman death due to malignancies in the world. In spite of the recent advances in diagnostics and treatment, mortality due to cancer prevails till date. ICMR consensus, for the management of breast cancer, documented 1, 44,000 new cases of breast cancers was reported in India every year. India was listed third in cancer mortality with ratio of 68.4% death every year and nearly 30% of the population survive after 5 years of cancer diagnosis [77]. India holds 17% of world breast cancer population. 50% of breast cancers were diagnosed in India at late stage (III and IV) however, in developed nations, 12% were detected in their late stage [99]. Delayed diagnosis and poor cancer awareness were considered as the main criteria for low cancer survivors in India. An age-standardized incidence rates of cancer varies from region, ethnicity, and religion [16].

Markers for breast cancer were used for diagnosis and treatment. Breast cancers are classified based on place of origin like luminal, basal, ductal and also on the expression of receptor on cell surface. Classification of breast cancer based on receptors include Herceptin receptor (HER2), Estrogen receptor (ER), Progesterone receptor (PR) either alone or in their combination. Treatment for ER positive, HER2 positive or PR positive breast cancers include inhibitors to their receptors or their signalling cascade. Triple-negative breast cancer (TNBC) is absence of all three receptors and they are difficult to do chemothrapeutic treatment [141]. Recently, immunogenic molecules like
CD44, CD24, CD29, CD49f, matrix proteins and integrin were used as markers for cancer identification and prognosis [76].

Genetics of breast cancer pivot to the germinal mutation of genes involved in DNA repair mechanism BRCA1, BRCA2, p53, PTEN and in cell cycle checkpoint. Genomic instability like telomere dysfunction, increased copy number of gene variation, high DNA amplification is complicating the breast cancer [66, 67]. Another striving factor is the ineffectiveness or resistance of drug against cancer cells. Inactivation of the drug, alteration of drug targets and the enhancement of drug efflux by pumps were the primary mechanism which activated by tumour cells against anti-cancer drugs. The heterogeneity of breast cancer cells and development of resistant against drug increases the difficulty in targeting a drug towards breast cancers [49].

Chemotherapy for breast cancers includes doxorubicin, tamoxifen, paclitaxel, 5-fluorouracil, cisplatin, trastuzumab, bevacizumab. But the major side effects like cardiac dysfunction in clinics limited their therapeutic approach. Other side effects include nausea, diarrhea, infection, memory issues, hair loss, and eye problems. Hence, the lacuna for an effective anti-cancer drug with non-toxic nature and the minimal side effect is essential to develop in treatment of cancer. Natural compounds with least toxicity and potential activity against cancer were considered to treat breast cancer. One such natural compound with pleiotropic activity is curcumin. In addition to that, safety administration and non-development of resistance made curcumin as the first choice of drug against cancer.

Curcumin is the polyphenol from *curcuma longa*, known for its activity against cancer, inflammation, diabetes, bacterial, viral, helminths and parasitic infections. Curcumin also acts as an antioxidant by preventing reactive oxygen
species. Curcumin is known to induce cell death in many cancer cell lines like breast, colon, lung, head and neck, cervical, gliomas and retinoblastomas of both in vitro, and in vivo conditions. Curcumin induces apoptosis and cell cycle arrest which inhibit the progression of cancer cell [134]. Curcumin affects the signalling molecules like NF-kB, Bcl2, STAT, TNF, IL-1, growth factors, growth factor receptors, enzymes, adhesion molecules, apoptosis-related proteins, and cell cycle proteins [107]. In addition to this, curcumin act as an epigenetic modulator by interacting with histone through acetylation and methylation of DNA. Curcumin is used in combination with chemotherapy for sensitising resistant pool of cells and to increase the chemotherapeutic efficiency.

Though curcumin exhibits significant activity against many cancers, its therapeutic potential is limited to its low solubility in water. Curcumin has low bioavailability, bio distribution, tissue absorption, faster excretion, and degradation. Even though curcumin of 9 g was administered orally only the tracer amount of curcumin (2 mg) was detected in human serum. Similarly the mouse model with orally administered 2g/kg curcumin had retained only 1.35 µg/mL of curcumin in serum [91]. The therapeutic efficiency of curcumin is increased by modifying its functional group or combining with other drugs. Formulating curcumin in nano forms increases its bio absorption and prolonged circulation time. Conjugating curcumin with nano drug delivery vehicles like liposomes, dendrimers, magnetic nanoparticles, silica particles, PLGA enhances targeting of curcumin to tissues.

Therapeutic efficiency of a drug attributes to its bio distribution, bioavailability, specific targeting and potency. Conventional delivery methods were limited to lack of controlled release, nonspecific binding, and low availability at target site. So an increased dosage form administered results
fluctuation in their plasma concentration. Increasing the efficacy of the conventional drugs is the focus of current research rather than synthesising new drugs. Formulating the available potential anticancer drugs and natural compounds to nano form for targeted release at specific site is the present challenge for drug delivery systems.

Failure of conventional cancer therapy was due to the (a) bio distribution of drug: cancer drug effectively inhibits the actively proliferating cells, and induce cell death which occurs on the periphery of tumour mass but not in core of solid tumour. (b) rapid clearance of drug by the reticuloendothelial system, (c) evolution of multi-drug resistant cancer cells [119, 59]. Nano drug delivery system (NDDS) has influenced cancer therapeutics by taking advantage of enhanced permeability and retention (EPR) effect in cancer microvasculature. Other than this NDDSs, longer circulation time, increased bio distribution, effective release of drug at the target site, high drug loading capacity created their niche in drug delivery against cancer [87].

Mesoporous silica nanoparticle (MSN) is one of NDDS, which has hexagonally arranged pores of 2-50 nm with size ranging from 20-200 nm. Application of MSN extends to the field like catalysis, adsorption chemistry, polymers, bio imaging, sensors, optical devices, drug delivery, tissue engineering, artificial implants etc. The porous nature, unique architecture, modifiable structures and easy synthesis methods gained importance of MSN in various field. Inertness, non-toxicity of silica particles, their high surface area for increased drug loading, and tunable pore size for drug loading discloses the potential of MSN in drug delivery system (DDS). Additionally, MSN increases the solubility of drug by the modification of the surface functionality which possess an advantage to cargo both hydrophobic and hydrophilic drugs. MSN increases the dissolution of drugs which are in their crystalline form. Recently,
MSN is conjugated with ‘gate keepers’ for stimuli response release of drug at the target site. Efficacy of MSN depends on its size, shape and functional groups on its surface. Loading the drug to MSN increases the drug bioavailability, solubility, surface affinity and also aid in the slow and sustained release of the drug. Polymer coating of MSN also enhances the sustained release of drug intracellularly at the target site. So far, drugs like doxorubicin, tamoxifen, nucleic acid like DNA and siRNA, peptides, enzymes, antibodies and nutraceuticals loaded on MSN showed significant in vitro and in vivo delivery at target site.

Though MSN gratifies the properties an efficient drug delivery vehicle, its usage is limited to its toxicity and faster in vivo excretion. It was also reported the shape of the nanoparticle determines the nanoparticle bio distribution efficiency. The spherical shaped particle has low circulation time and also undergo phagocytosis than the non-spherical particles. Another major drawback of MSN was uptake by cell which was mostly mediated through an endosomal pathway, and was consequently thrown out by cells through exocytosis. However, MSN with sharp surface was able to escape from the endosomal pathway and retain inside the cell. Similarly, MSN coated with cationic polymers exhibits ‘endosomal escape’ and retain MSN intracellularly compromising its toxicity. Hence, MSN which is non-toxic, non-spherical, with multiple drug loading efficiency was required to overcome the present drawbacks with MSN.

Another therapeutic challenge in breast cancers is the development of resistance to chemotherapeutics. The main factors which govern resistance are the epigenetics, drug efflux, drug inactivation, and target alteration, mutation in DNA repair mechanism, apoptosis evasion and epithelial to mesenchymal transition where the tumours attain metastasis [49]. Intracellular targeting of
drug to cancer cells would overcome resistance developed by the drug or target alteration and drug efflux. The nanomedicine provides an advantage by depositing drug on plasma membrane which saturates the Pgp with excess drug subsequently blocking ABC transporter function [140].

The combinatorial effect is used to prevent the development of resistant in cancers and also to avoid toxicity. The combination of drugs reduces their dosage concentration resulting increases their anti-cancer effect. Combining two drugs has another advantage of minimising the side effects because of lesser dosage administration. There is also possibility of an additive adverse effect when both the drug distribute and excrete independently. There are also reports of adverse effects when administering the combination of molecular targets to patients. Another drawback with combination of two drugs was its different formulations and different route of administration, which further complicate to monitor its pharmacokinetic and pharmacodynamics profile. Also, the uniform temporal and spatial coherency in co-delivery was not easily achievable. Hence a carrier-mediated delivery of combination drugs could efficiently overcome the hindrance in combinatorial drug delivery. Primary advantages of a carrier-mediated dual drug delivery its uptake mediated by endocytosis, which overcomes the multidrug resistance and also ratio metric drug dosage could be formulated. So far curcumin and doxorubicin synergism against resistance cells were not known.

As described earlier, though MSN is a better DDS, the toxicity elicited decreases it’s in vitro vehicle potential and fast excretion in vivo. Hence a better non-spherical MSN with less toxicity was obligatory. Also, the MSN should possess a better drug delivery property and should be flexible to multiple cargos. In addition to this, MSN loaded with the drug should be able to
overcome the resistance of the cancer cells to the therapeutics. These were the primary demands in the contemporary DDS which was focused in this work.

**OBJECTIVE**

With the above mentioned concepts the objective was focused to improve the drug delivery potential of MSN. The current study might provide insights on synthesis of a non-spherical MSN and its drug delivery properties. These concepts afford the objectives as described below

1. To find the curcumin-loaded MCM-41 could induce apoptosis in MCF-7 cells.
   - MCM-41 characterisation and the indication of curcumin loading.
   - MCM-41 time point accumulation inside MCF-7 cells.
   - Curcumin-loaded MCM-41 induces dose and time-dependent cell death of MCF-7 cells.
   - MCM-41 with curcumin inducing apoptosis confirmation with DNA fragmentation.

2. To prove non-spherical MSNAP was better DDS than MCM-41P and understand the mechanism of curcumin-loaded MSNAP inducing apoptosis.
   - Comparison of characteristics of MCM-41P and the non-spherical MSNAP.
   - Comparison of drug delivery properties of MCM-41P and MSNAP.
   - An efficiency of MSNAP in drug delivery studied with curcumin.
3. To elucidate the combinatorial effect of curcumin and doxorubicin loaded MSNAP in overcoming the resistance in DOX-resistant MCF-7 cells.
   - Characterisation of curcumin and doxorubicin loaded MSNAP
   - Dose-dependent effect of MSNAP conjugated with curcumin and doxorubicin on MCF-7 and MCF-7R cells.
   - Curcumin and doxorubicin loaded MSNAP induced cell death in MCF-7 and MCF-7R cells.
   - Synergistic effect of curcumin and doxorubicin loaded MSNAP induced cell death in MCF-7 and MCF-7R cells.
CHAPTER 2

2. REVIEW OF LITERATURE

2.1. Breast cancer scenario in India

Breast cancer is one of the most common tumours diagnosed among women in the world. In India, an increasing trend in cancer incidence is reported in recent years [80] with doubling pattern of incidence from 1985-2005. The age-standardized incidence rate varies from 6.2 to 39.5 among Indians, which depends on region, ethnicity, and religion [64]. Also, 100,000 new cases of cancers was detected every year [4]. Of these breast cancers, 20% has reported with familial incidence. Almost 50% of breast cancer cases in India are in advanced stages, 16% were reported to be in stage II and 5% in stage I [1]. Within Breast cancer population in India, 50% belong to triple negative (TNBC), 20-45% belong to Estrogen and Progesterone receptor positive category [64]. The ignorance of breast cancer awareness, delayed diagnosis, and demanding diagnostic pathway limits the early detection of breast cancer. Therefore, the higher mortality rate of Indian population is found to compare with the western world. Though initiatives for breast cancer awareness has been implemented, low level of awareness on breast cancer still prevails [44].

2.1.1 Classification of Breast cancers

Primarily classification of breast cancer based on their aggressiveness as non-invasive and invasive breast cancer. Non-invasive cancers is further classified based on the origin such as lobular carcinoma (lobules) and ductal carcinoma (Ducts). Infiltrating lobular (ILC) and ductal carcinoma (IDC) signifies the metastasis to other tissues. ILC constitutes to 10-
15% and IDC constitutes 80% of the diagnosed breast cancer. Other less commonly occurring breast cancers belong to medullary carcinoma, mucinous carcinoma, tubular carcinoma, inflammatory breast cancer and phyllodes tumour which accounts for 1-5% [104]. Based on prognosis, cancer was staged with TNM system clinically with size of tumour (T), state of the regional lymph node (N) and presence or absence of distant metastasis (M) [107]. The gene expression profiling of breast cancer is classified as luminal, HER2-enriched, basal-like and normal-like forms [131]. Breast cancer is the heterogeneous disease with broader ranges of molecular, histological varieties. Hence WHO released “Classification of Tumors of the Breast” in 2012 which include all wide subtypes of cancers [112].

2.1.2 Metastasis

Metastasis or secondary cancer is the late attained incident in tumorigenesis which involves two steps intra vasation, dissemination, and extravasation, colonization. Metastasis depends on the lymph-node, size of the primary tumour and undifferentiated histopathology. It is also reported that 0.02% of circulating tumour cells initiates micro metastasis which rapidly spread over from its original site to different sites in the body by using different proteins [67]. Recently, biomarkers used to identify the breast cancer metastasis include epidermal growth factor receptor 2 (ERBB2), detection of disseminated tumour cells (CTC), urokinase plasminogen activator (uPA) and its plasminogen activator inhibitor (PAI1) [134]. Till now chemotherapy, anti-angiogenesis therapy, hormonal therapy, and antibody-based therapy were implemented against metastatic breast cancer [101]. Interestingly, six metastasis suppressor genes were identified as NME1, KiSS1, KAI1, CAD1, BRMS1, and M KK4 but their suppression mechanisms was not yet fully understood [135].
2.2. Morden breast cancer therapy and limitation:

2.2.1. Surgery

Surgery was preferred when the breast cancer size is more than 3 cm diameter and initial surgical removal is known as lumpectomy or mastectomy which was proceeded with both chemotherapy and radiation therapy. In breast-conserving therapy, lumpectomy, wide excision, quadrantectomy was followed where a small portion of tissues along with the tumour tissues. Surgery was followed by radiation therapy with X-rays and gamma rays in breast conserving therapy [104]. In addition, lymphatic mapping and sentinel lymph node biopsy was also performed to reduce the morbidity.

2.2.2. Chemotherapy

Neoadjuvant (surgery after chemotherapy) therapy for locally advanced and operable breast cancer includes in the combination of i) doxorubicin with cyclophosphamide, ii) fluorouracil, with doxorubicin, and cyclophosphamide, iii) axanes, with vinorelbine and other alkaloids, iv) gemcitabine, capecitabine, ixabepilone and eribulin to reduce or eliminate tumour. Adjuvant therapy is based treatment reduce tumour size by surgery followed by chemotherapy. The targeted therapies include 1) Anti-estrogen therapies, tamoxifen or aromatase inhibitors were widely administered for ER-expressing breast cancers which reduce or inhibit breast cancer in women. 2) Adjuvant trastuzumab is used against HER2-positive cancer which increases overall survival. 3) Small molecule tyrosine kinase inhibitor like lapatinib, pertuzumab, gefitinib, or erlotinib, canertinib or neratinib [112]. 4) Epothilones are macrolides which compete with paclitaxel for binding with β-tubulin. 5) Pure EGFR inhibitors Gefitinib, anilinoquinazoline Erlotinib, quinazolinamine
6) Trastuzumab–DM1, ado-trastuzumab emtansine the antibody drug conjugate was also used as chemotherapeutics, 7) Ertumaxomab is a trifunctional bispecific antibody which targets HER2 on tumor cells and CD3 on T cells, 8) Sunitinib malate, Motesanib, Vandetanib, Vatalanib mainly target VEGF and PDGF receptors, 9) Farnesyltransferase inhibitors inhibits downstream of Ras downstream effectors, tipifarnib and lorafarnib (Ricardo H Álvarez), 10) Epigenetic modifiers i) DNMT inhibitors are nucleoside analogues 5-azacytidine (5-aza) and 5-aza-2'-deoxycytidine (decitabine), zebularine and 5-fluoro-2'-deoxycytine, ii) HDACi include vorinostat (suberoylanilide hydroxamic acid)[74] 11) Temozolomide is an alkylating agent [60]. 12) The triple-negative breast cancer and BRCA1 mutated tumours were targeted with PARP inhibitors. Though many drug are available, the exact mechanism behind the drugs anti-cancer activity and their interaction the proteins of the cell remains unclear.

2.3 Challenges in modern therapy

The major drawback of the anti-cancer drugs was severe side effects such as nausea, diarrhoea, fatigue, neutropenia, elevation of liver enzymes, epistaxis and conjunctival disorder [19]. Therefore, there is a need for developing the drug which overcomes above side effects.

2.3.1 Limitation in detection

Mammography was used as the initial screening process and the usage of radiation in this process leads to side effects. Other early detection was with circulating biomarkers. Low sensitivity, low specificity, and undefined markers were considered as the limiting factor in early detection of circulating tumour biomarkers [93].
2.3.2 Disadvantages with radiation therapy

Radiation therapy was used as the primary management of breast cancer. The major serious side effects of radiation include ischemic heart disease and pneumonitis [97].

2.3.3 Disadvantages with chemotherapy

Development of resistance, heterogeneity of cancer tissue and drug toxicity was considered as the disadvantages of chemotherapy. Epigenetic modifications induced by DNA methyltransferase (DNMTs) and Histone deacetylase inhibitor (HDACi) was reversible and transitory. Hence targeting histone alterations was remains a challenge [74]. Antibodies therapy was limited to toxicity, high cost, and limited duration of action, non-specific immunogenicity. Large size, shape, affinity, and valence of mAb influence its therapeutic efficiency. It was reported that only 20% of administered mAb reaches the tumour site. Other adverse effects with mAb therapy include cardiotoxicity, degradation of the peptide, low bioavailability [81].

2.3.4 Disadvantages with conventional doxorubicin treatment

Doxorubicin intercalates DNA and inhibits topoisomerase II activity. Side effects of doxorubicin are bone marrow depression, Nausea and vomiting, oral ulceration, alopecia, skin pigmentation, Acute and chronic cardiotoxicity, tachycardia, hypotension arrhythmia, cardiomyopathy leading to cardiac failure, glomerular atrophy, and increased permeability. These side effects with the conventional therapy were minimized by formulating doxorubicin with liposomes and pegylation. Though liposomal doxorubicin overcome these side effects, phospholipid functional group of liposomes are removed by the reticuloendothelial system (RES) which results in short circulating time. Similarly, PEGylated doxorubicin has dose-limiting HFS palmar-plantar
erythrodysesthesia (HFS). High cost of Myocet®, a non-pegylated liposomal doxorubicin (NPLD) limits its application in therapy [93].

2.3.5 Strategies for developing better therapy

For the effective treatment of breast cancer, horizons of research should be expanded to i) characterize the stroma, cell adhesion and the extracellular matrix, ii) determine the development of metastatic disease or drug-resistant against cancers, iii) optimal duration of therapy for drugs, iv) targeted cells for breast cancer prevention, v) mechanism of breast cancer prevention [122].

2.4. Role of natural compounds in breast cancer therapy

Nutraceuticals are bioactive molecules derived from natural sources which aid in prevention or treatment of malady other than diet supplements. Usage of nutraceuticals is the measure to reduce the toxicity with therapeutic effectiveness [61]. Dietary factors influence chronic disease appearance, disease progression, morbidity, and mortality. Deficiency of these dietary factors hallmark cardiovascular disorders, osteoporosis [47] and cancers [17]. Natural products with bioactivity can be classified based on their functional groups as carotenoids (isoprenoids) like β-carotene, lutein, lycopene, tocotrienol (isoprenoids), saponins, polyphenolic compounds like flavonones, flavonols, flavones and anthocyanins, phenolic acids include resveratrol, curcumin and glucosinolates, phytoestrogens include isoflavones (genistein, daidzein), lignans, dietary fibre varieties include soluble fibre (prebiotics) and insoluble fibre, sulfides/thiols like dithiolthiones, fatty acids like omega 3 fatty acids (polyunsaturated fatty acids) and mono saturated fatty acids, probiotics/prebiotics are lactobacilli, bifidobacteria, minerals like (calcium, selenium, zinc) and polyols sugar alcohols (xylitol, sorbitol) [122].
Some of the natural compounds with anticancer activity are tabulated

<table>
<thead>
<tr>
<th>Anticancer compounds</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>Turmeric</td>
</tr>
<tr>
<td>Cyanidins</td>
<td>Grapes</td>
</tr>
<tr>
<td>Diindolylmethane/Indole-3-carbinol</td>
<td>Brassica</td>
</tr>
<tr>
<td>EGCG</td>
<td>Green tea</td>
</tr>
<tr>
<td>Fisetin</td>
<td>Strawberries, Apples</td>
</tr>
<tr>
<td>Genistein</td>
<td>Soybean</td>
</tr>
<tr>
<td>Gingerol</td>
<td>Gingers</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>Tea, Broccoli, grapefruit</td>
</tr>
<tr>
<td>Lycopene</td>
<td>Tomato</td>
</tr>
<tr>
<td>Phenethylisothiocyanate</td>
<td>Cruciferous vegetable</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Grapes</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>Rosemary</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>Cruciferous vegetable</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>Wax-like coating of fruits and herbs</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Mushroom</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>vegetable oils</td>
</tr>
</tbody>
</table>

**Table 1.1:** Natural compounds with anti-cancer activity and their source.
Natural compounds with anti-carcinogenic effect which are in clinical trial are curcumin, DIM (Diindolylmethane), genistein, I3C (indole-3-carbinol), lutein, PEITC, quercetin, and sulforaphane [131].

2.4.1 Druggability of phytochemicals

Phytochemicals crystal structures, polymorphism, amorphous nature, its salt form and excipient compatibility were considered for their drug formulation. The phytochemicals properties determine their physiological characteristics like absorption, pharmacokinetic efficiency, large-scale manufacturing, drug consistency, drug stability and its degradation product [133].

Most of the natural bioactive compound’s molecular structures possess a greater number of chiral centres, and steric hindrance. Additionally, their more number of oxygen and hydrogen atoms, low ratio of aromatic rings, higher molecular rigidity and molecular mass hinder their biological activity. Non-reproducibility of these phytochemicals in vitro efficacy and toxicity elucidation in vivo at high concentration of these molecules declines its therapeutic potential. A better drug formulation is achieved when the natural bioactive compound have less than five hydrogen bond donors, less than 10 hydrogen bond acceptors, molecular weight less than 0.5 kDa and lesser partition coefficient [133, 61].

2.4.2 Polyphenols and anticancer activity

Polyphenols are the plants secondary metabolites with hydroxyl group attached to the aromatic ring. Polyphenols were classified as flavanoids and non-flavanoids. Flavonoids are further sub-divided as flavonols, flavones,
flavan-3-ols, anthocyanidins, flavanones and isoflavones. Non-flavanoids includes phenolic acids (benzoic acids and cinnamic acid), stilbenes, lignans, tannins and other polyphenols like curcumin, rosemarinic acid and gingerol. Flavonoids and have 15 carbon atoms (C6-C3-C6) with two aromatic rings bound through a 3 carbon chain. Flavonols, have 3-hydroxyl group in the pyrone ring and they exert anticancer properties through the inhibition of kinase activity. The phenolic acids subgrouped into seven carbon atoms (C6–C1) benzoic acids, containing and nine carbon atoms (C6–C3) cinnamic acids. Stilbenes have two aromatic rings linked by an ethene bridge (C6-C2-C6). These are secondary metabolites synthesized by plant due to infection or injury [84, 35, 122].

Polyphenol is double-edged sword having both antioxidant and pro-oxidant activity. The catechol (1,2-dihydroxibenzen), 2,3-double bond conjugated with the 4-oxo-function (oxygen atom double bonded to carbon) of the carbonyl group and hydroxyl groups at positions 3 and 5 determines the free radical scavenging activity of the polyphenols. In addition to this, another mechanism of anticancer activities includes metal chelation, inhibition of oxidases and stimulates enzymes with antioxidant properties. On the other hand, oxidative induction of polyphenols is by reactive oxygen species (ROS) generation which consequently affects the mitochondrial-mediated apoptosis and oxidative DNA damage. Anticancer activity of the polyphenols was mediated by inhibiting the aromatase, reversing the glycolytic metabolism, and regulating cell cycle and apoptosis [84, 35, 122].

2.5 Curcumin structure and function

Curcumin is the active polyphenol found in rhizomes of *Curcuma longa*, chemically known as 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-
heptadiene-3,5-dione and also called diferuloylmethane. Demethoxycurcumin and bis-demethoxycurcumin also constitute the curcuminoids group of C. longa. Curcumin belongs to diarylheptanoid group of compounds, with two aromatic rings by seven carbon bonds. The aryl groups have methoxy substitution and ortho hydroxyl substitution on both the rings. The seven carbon links have a two enone groups conjugated to 1, 3-diketone groups. Curcumin exhibit nine different keto-enol and cis-trans configuration. The methoxy phenolic groups, enone groups and 1, 3- keto-enol groups were the active functional groups of curcumin. Metabolism of curcumin starts with the transformation of curcumin to dihydrocurcumin and tetrahydrocurcumin by reduction, then β-glucuronidase conjugate them to monoglucuronide as dihydrocurcumin-glucuronide and tetrahydrocurcumin-glucuronide. Curcumin is converted to curcumin glucuronides and sulfate and also reduced to hexahydrocurcumin in gut and liver [87]. Antioxidant property of curcumin depends on its functional groups. The phenolic –OH groups involves single or double electron transfers and also hydrogen electron transfer for reactive oxygen species quenching. Methoxy group of curcumin involves in the antioxidative reactions and the molecule with least number of methoxy group has decreased antioxidant activity. Tetrahydro curcumin is the potent antioxidative molecule and bismethoxy curcumin has the lowest antioxidant potential [57].

Biological properties of curcumin was influenced by interactions with biomolecules through covalent or non-covalent interactions. Curcumin’s functional groups like phenolic group, keto-enol group, and the seven carbon linker groups interact with biomolecules depending on the biomolecules nature. Non-covalent interaction of curcumin is mediated through the phenolic group π- π interactions with aromatic group of the biomolecule, keto-enol group hydrogen bonding and also the linker carbon chains induce flexibility for
hydrophobic interactions. Curcumin’s covalent interaction is exhibited by Ketone-enol group which act as Michael acceptor or electron donor. Non-covalently curcumin binds to DNA (minor groove), Proteins (hydrophobic pocket) and Lipids (phosphate group of choline). Covalent bonding of curcumin is to metals (β-diketogroup) and Protein thiols (keto-enol group through Micheal addition). In addition to this, ROS neutralizing activity of curcumin is by the (O-methoxy phenolic OH group) [53, 54].

Curcumin molecular targets are serum albumin, xanthine oxidase, 5-LOX (lipooxygenase), COX-2 (cyclooxygenase 2), thioredoxin reductase, IKK (IkB kinase), iron, p-glycoprotein, PKA (protein kinase A), GST (glutathione-S-transferase), PKC (protein kinase C), PhK, cPK, autophosphorylation-activated protein kinase, Ca^{2+} -dependent protein kinase (CDPK), inositol 1,4,5-triphosphate receptor, pp60c-src tyrosine kinase, Ca^{2+} -ATPase of sarcoplasmic reticulum, rat liver cytochrome p450s, aryl hydrocarbon receptor, Topo II isomerase and glutathione [92].

Curcumin’s biological activity extends to anti-cancer effects in colorectal, pancreatic, breast, prostate, multiple myeloma, lung cancer, cancer lesions, head and neck squamous cells. Additionally, curcumin was effective against inflammation and infection related ailments like inflammatory bowel disease, irritable bowel syndrome, arthritis, uveitis, post-operative inflammation, peptic ulcer, H. pylori infection, idiopathic orbital inflammatory pseudotumor vitiligo, psoriasis, Dejerine-Sottas Disease, alzheimer’s disease, acute coronary syndrome, atherosclerosis, diabetes, type 2 diabetic nephropathy, diabetic microangiopathy, lupus nephritis, Acquired Immundeficiency Syndrome (AIDS), β-Thalassemia, biliary dyskinesia, gallbladder contraction, recurrent respiratory tract infections, ATT-induced hepatotoxicity, and chronic bacterial prostatitis [45].
2.5.1 Difficulties in delivery of curcumin

The major therapeutic limitation of curcumin is low aqueous solubility and bioavailability. Curcumin administered at 2g/kg in rat resulted in serum concentration of 1.35 ± 0.23 µg/mL. Though bioactive concentration of curcumin is micromolar, the water solubility is at nanomolar concentration. Another disadvantage of curcumin is instability at pH 7.4 and half-life time for 20 min. The curcumin uptake by cells is limited and lesser concentration of cytoplasm. Adverse effects of curcumin on clinical trials include diarrhoea, headaches, rashes, yellow stool and nausea. Elevation of serum alkaline phosphatase, lactate dehydrogenase and intractable abdominal pain was also observed in patients administered with curcumin at 48 h [88, 89, 45].

2.6. Anticancer drug targeting

2.6.1 Barriers for tumour targeting

Tumour microenvironment favours the progression of the tumour and prevents the drug to target. The abnormal vasculature which surrounds the tumour prevents the drug delivery to tumour site. The absence of lymph vessels increases the interstitial fluid pressure leading to lesser distribution of drug also compresses the blood vessels. In addition, the impaired blood flow and the development of hypoxic regions are limit exposure of drugs to tumour site. One more factor which influences the drug and tumour interaction is the stromal cells which reduce the pressure of arteriole and venule reducing the drug efficacy [122]. Further, the drug-tumour targeting is inhibited by anatomical, physiological, chemical and clinical barriers. The vascular endothelium, perivascular space, cell membranes, nucleus, blood-brain barrier, high
interstitial fluid pressure, hepatic degradation, renal filtration, drug efflux, and density of the tumour microenvironment which prevents drug targets. Even the large volume of drug distribution, low stability and solubility, charge and low molecular weight limits the therapeutic potential to target cancer [58].

### 2.6.2. Enhanced permeability and retention effect

Characteristics of tumour microenvironment depends on its heterogeneity, larger size, high and improper vasculature with more permeability and leakage compared to the normal blood vessels. Elevation of vascular mediators along with defective lymphatic system in these vasculature leads to enhanced permeability and retention effect (EPR).

Figure. 1.1 Representation of EPR effect in tumour cells. [144].
Molecules like NO, CO, bradykinin, VEGF enhances the EPR effect. NOS synthase produce NO from L-Arginine, the main second messenger required for tumour growth and nutrients and oxygen supplement. Bradykinin (BK) is accumulated in malignant ascetic and pleural fluids in cancer patients. Angiotensin converting enzyme breakdowns BK which also enhances EPR effect. CO is released from heme breakdown by hemeoxygenase, has vaso modulation properties which also enhance EPR effect [144, 41]. EPR effect was advantageous in targeting tumours.

2.7. Cancer – drug targeting strategies

2.7.1. Active drug targeting

Conjugating target molecule to surface of the drug carriers like nanoparticle is to cancer site is known as active targeting. These targeting molecules binds to the tumour tissue and delivering the drug intracellularly overcoming the efflux [58].

2.7.2. Passive drug targeting

Delivery of drugs directly to the tumour tissues is passive targeting. Properties of the drug and the tumour characteristics were considered for efficient drug delivery [58]. In passive targeting, the drug carrier complex is stable in metabolism, excretion, opsonisation, and phagocytosis for longer circulation in blood. Leaky vasculature, pH, temperature play vital role in drug delivery [62]. Passive targeting of the nanoparticles to cancers depends on EPR effect. Particles with 10-100 nm and charged showed enhanced accumulation. Polymer coating of these drug carriers enhance systemic circulation by hindering protein adsorption and recognition by the phagocytic system. But also
the accumulation of the drug with the nano carrier varies on the cancer type [122].

2.8. Novel drug delivery system (NDDS)

Most of the anti-cancer drugs has higher molecular weight, which increases their lipophilicity and decreased solubility. Increasing the solubility of these drugs influence their pharmacological properties [144]. Solubility of these drugs are enhanced by conjugating with nanoparticles. Antibody targeting to tumour has wide distribution and high dose is administered leading to functional impairment in patients. Similarly, nucleic acid delivery exhibit innate immune responses in patients also the high molecular weight, hydrophilic property, and negative charge of the nucleic acid and its endosomal entrapment limit the nucleic acid therapeutic potential [122].

Nanotechnology extents its horizons to cancer research in drug delivery and diagnosis. The main aspects of nanoparticle in tumour diagnosis with localization, margin detection, knowing adjacent structures, mapping the lymph nodes, and detection of metastases. Nano delivery enhance opportunities for variable targets and also overcoming drug resistance [122]. Poor ribonuclease (RNase) resistance, short half-life (2–6 min), inability to bind plasma proteins limits tissue accumulation, and rapid clearance due to their small size [2]. Targeted systemic administration with drug carrier is would overcome the drawbacks. Nanoparticles were used in chemotherapy, radiotherapy, phototherapy, immunotherapy against cancer. Delivery systems like liposomes, polymeric micelles, dendrimers, nano cantilevers, carbon nanotubes, quantum dots, magnetic nanoparticles, AuNP, PLGA, multimodal nanoparticles were widely researched on cancer targeting [2, 3].
The major drawback with nano drug delivery systems is its toxicity. The detailed study with the interaction between the nano carriers and host organs and organelles was still not clear [144]. The study for the nanoparticles physicochemical properties, biological properties and their dosage concentration would provide a better understanding of the nanotoxicity. Also development of specific standard testing and characterisation procedures would support the fabrication of better nano therapeutics [11]. Knowledge on nanoparticle dispersiveness, agglomeration, aggregative nature and concentration would provide better understanding its behaviour in vivo and in vivo. Nanoparticles are reported to cause oxidative damage leading oxidative stress and genotoxicity. Lung inflammation, macrophage activation, alteration in liver clotting factors and systemic thrombosis. Nanoparticles also crosses the blood brain barrier through the intravenous route induces neurotoxicity [2, 3]. Other drawbacks in nano drug delivery system like polymer micelles and liposomes is its non-specific uptake by the RES, long term stability, smaller size (10-100nm) limits the drug loading quantity, drug leakage and also high production cost [58].

2.9. Mesoporous silica nanoparticles (MSN)

Mesoporous silica nanoparticle has attracted researchers with its high drug loading capacity and high surface area and tunable pore size. Tetraalkoxysilane is the main precursors used in the synthesis of MSN, silanol group provide the surface hydroxyl group. Surface modification was performed by additional hydrolysis and condensation with the functional groups. The pore size, ordering, particle size and its structures are tunable with usage of different templating molecules. Alteration in the synthesis condition results in various shape of MSN from spherical to rad shape. MSN has wide application in almost all fields. MSN was used in improving dissolution, chemical and pharmaceutical
purification, purification and decolorization of food, improving bioavailability, improving solubility, improving surface affinity, sustained/controlled release. The porous nature of MSN has applications as catalyst supports, filters for molten metals and hot gases, refractory linings for furnaces and porous implants in the area of biomaterials. Crystalline microporous materials generally have a narrow pore size distribution. The first commercial albumin-bound paclitaxel (PTX) nano carrier (Abraxane) was approved as the first new drug for pancreatic ductal adenocarcinoma [56].

Surface properties of MSN determines its bio distribution and surface charge, surface functionalization, particle size and shape determines the pharmacokinetic behaviour of MSN. Solvation of MSN with larger size is better compared to smaller sized MSN. Charge of the MSN play vital role in its excretion, negatively charged particles accumulate in the Kupffer cells, positively charged are eliminated by hepatobiliary action [96]. Toxicity of MSN depends in vivo on its administrational route, intraperitoneal and intravenous routes is toxic, while the subcutaneous route had less toxicity. Cationic particles have better cellular uptake also elicit immune response and cytotoxicity, whereas neutral particles have longer circulation time and negatively charged particles reside in endosomes. Entrapment in endosomes of negatively charged particle was rectified by cationic polymer coating [102].

2.9.1 Controlled drug delivery system

Another advantage of MSN is its adaptability to stimulus induced drug delivery. Non-specific uptake of anti-cancer drugs by normal cells leads to severe side effects. So ‘Gate keepers’ were used to seal the drugs and on stimulus release drug effectively at the target site.
2.9.2. pH dependent systems

Compared to the normal cells, tumor cells and inflammatory cells have lower pH. MSNs are loaded with drug and functionalized with molecules active in acidic pH. Polyamines, acid-labile bonds like acetals were used for sensitisation with pH. Other reactions like the polyalcohols and boronic acids interact in a reversible way to form boronate esters, trisammonium with one anilinium and two – CH2NH2+CH2 – centers [142] and pseudorotaxanes, rotaxanes rings which remains closed at neutral pH and on protonation in acidic condition opens the pores which releases the drug. Similarly, polyamines remain saturated at normal pH and under low pH amines involve in protonation resulting in drug release [149].

2.9.3. GSH mediated release/ Redox

Intracellular concentration of GSH is high compared to extracellular environment [149]. Hence, thiol linked drugs were targeted for intracellular release. Yanes et. al reported that rotaxanes with disulfide bonds get reduced inside the cell. Disulfide bonds are stable outside the cells, and the intracellular environment with glutathione reduce the disulphide bond [142].

2.9.4. ATP-mediated release

Another stimulated release of drug was done with ATP with the advantageous higher concentration of ATP inside cells which could trigger the drug release [149].

2.9.5. Enzyme mediated release

Substrate for the enzyme was functionalized with delivery system for triggered release at specific site. Tri (ethylene glycol) chains threaded by α-CD
tori with ester-linkage, intracellular esterase cleave the ester links opening the channels of MSN for drug release. Similarly, another system with polyethylene glycol diacrylate (PEGDA) and peptide macromer was cleaved by MMPs [142].

2.9.6. Light mediated release

Photo activation involves electron or proton transfer with exited photons. Activation of azobenzene switches between cis-trans configuration, releasing the drug inside the cell. Irradiation with \( \lambda=351 \) nm led to the trans-to-cis isomerization of azobenzene [142]. 9-anthracenecarboxylic acid or \([\text{Ru(bpy)}_2(\text{bpy(CH}_2\text{OH})_2)]^{2+}\) (bpy = 2,2'-bipyridine) photo induced electron transfer to the CBPQT\(^{4+}\) rings which aids in drug release [6].

2.9.7. Magnetism mediated release

Ferromagnetic substance were the first choice for stimulus with magnetism. MSNs with zinc-loaded iron oxide releases the drug on magnetic activation. Magnetic field generated induce local heating which disassemble the system. MSNs with iron oxide particles and DNA saturated delivery system on alternating magnetic field generate heat. The heat generated by this system results in melting of the DNA double strand and the iron oxide crystals [142].

2.9.8. Other stimulated models

Other than this Glucose-responsive and H2O2-responsive drug release from MSN was also reported by Chun-Ling Zhu et.al. [142].

MSN which were effective in drug delivery in vitro didn’t produce a significant effect in vivo. As size, shape and surface functionalisation of MSN governs the drug delivery potential, MSN with different shape could influence the biological
activity. Also, unavailability of a standard *in vitro* characterisation could be the possible reason for the *in vivo* failure. Also, MSN reported so far was prone to be toxic and the biological effect of the intracellular released drug was also not clearly understandable. The amount of drug released inside the cell was prejudiced by polymers coated on MSN surface. With these the work was focused on MSN cellular uptake, toxicity and the effect of intracellularly released drug.
CHAPTER III

3. CUR-MCM-41 INDUCED CELL DEATH IN MCF-7 CELLS.

3.1 INTRODUCTION

Breast cancer incidence in India is predicted to be 90,723 in year 2015 which is less when compared with World breast cancer occurrence. However, mortality of breast cancer patients in India is around 50% which is much higher compared to other countries [108]. Till now drugs developed for breast cancer are being targeted for its receptors on cell surface, angiogenesis blockade, HDACi (Histone deacetylase) inhibitors and inhibitors for survival pathway. Of these, drugs are being targeted for HER2 (human epidermal growth factor receptor) and EGFR (epidermal growth factor receptor) such as Trastuzumab, Pertuzumab, Lapatinib, Neratinib, and Afatinib are in clinical trials. These drugs are potential anticancer effect and they are leads to severe side effects such as nausea, diarrhea, fatigue, neutropenia, elevate liver enzymes, epitaxis and conjunctival disorder [19]. Therefore, there is a need for developing drug which overcomes above side effects.

Curcumin is an antioxidant and polyphenolic compound found in turmeric (curcuma longa) is known for its non-toxic nature. Curcumin pleotropic effects include anti-oxidant, anti-inflammatory, antibiotic, antidiabetic, anti-cancer anti-depressive and inhibit of reactive oxygen species (ROS) formation. Curcumin hamper the proliferation of various cell lines including MDA-MB-435 and HCT-116 and arrest these cells at G1 phase [13]. Curcumin inhibits the cell growth and induced apoptosis in vitro by increasing Bax/Bcl ratio [75] and blocks the nuclear factor kappa B (NF-kB) [103].
In spite of all these effects, curcumin’s low bioavailability, absorption and digestion decline its eligibility in therapy [91]. In phase I clinical trial, oral consumption of curcumin is found only in the gastrointestinal track but not in other parts of the body. Oral uptake of 8 g of curcumin showed peak plasma curcumin concentration of 2 nm after 1 h. The number of clinical trials is proved that curcumin as potential cancer preventive molecules. However, it is limited by its poor availability in different parts of the human body and inhibits therapeutic potential [25]. The encapsulation of curcumin with drug delivery vehicles like liposomes, nanoparticles, polymers, emulsion, colloidal suspension, and adjuvant were proven to increase curcumin retention in animal system and also increase its bioavailability [9, 129].

The present study focuses on mesoporous silica nanoparticles (MCM-41) as a carrier for delivering curcumin. MCM-41 has cylindrical channels, tunable pore size of 2-12 nm, high surface area, non-toxicity and inertness which make them a better carrier for delivering the curcumin molecules. The shape of these nanoparticles determines its biological activity and distribution in different organelles of the cells [80, 51]. The present study investigates the characterization of curcumin-loaded with PEI-coated MCM-41 (CUR-MCM-41P), efficiency of drug loading and release, drug delivery inside the cell, and apoptosis induction in MCF-7 cell. Improving the therapeutic potential of CUR-MCM-41P could be a better curation of breast cancer.
3.2. EXPERIMENTAL METHODOLOGY

3.2.1 Materials

Polyethylenimine (PEI), curcumin, tetraethyl orthosilicate (TEOS) were purchased from Alfa Aesar (Ward Hill, MA, USA). IMDM, FBS, Trypsin EDTA were purchased from Gibco BRL (Waltham, MA USA). All tissue wares are purchased from Greiner (Bahlingen, Germany). The chemicals such as cetyl trimethyl ammonium bromide (CTAB), tetra sodium salt WST-1, paraformaldehyde (PFA), and DAPI were purchased from Sigma (Bengaluru, India).

3.2.2 MCM-41 synthesis, curcumin loading and release

For MCM-41 synthesis, 48 ml of water containing 100 mg of CTAB, 350 µl of 2M NaOH was heated and stirred at 80 °C. 500 µl of 21.6 mM TEOS was added into the mixture and stirred for 2 h. The white precipitate obtained was refluxed for CTAB was removal by adding HCl and methanol for 18 h [12]. The precipitate was washed with ethanol, dried and coated with 0.3% of 10 kDa PEI [94]. The 10 mg of MCM-41 coated with PEI (MCM-41P) was kept in a shaker along with 50 µM curcumin in ethanol for 24 h. The unbounded curcumin was washed with ethanol and dried CUR-MCM-41P.

The drug release studies was performed with 4 mg/mL concentration of curcumin loaded with MCM-41P kept in three different pH ranges of 3, 6, and 7.4 in phosphate buffer saline (PBS). 200 µl of PBS was collected at every 6 h for up to 48 h and curcumin concentration was measured in fluorescent microplate reader excited at 420 nm and emission and excitation at 495/40 nm [Biotek, Model FLx800, Vermont, USA].
3.2.3 Characterization of MCM-41 and CUR-MCM-41P

The synthesized silica nanoparticles were dried and characterized by high resolution transmission electron microscopy (HR-TEM), X-ray diffraction (XRD), and Fourier transform infrared (FT-IR). To determine the structure of MCM-41 and CUR-MCM-41P by using HRTEM and these nanoparticles were dissolved in ethanol and loaded on copper grid. A Carl Zeiss microscope operated with HT650 ES1000 W at 100 kV was used for TEM analysis and pore size of MSN measured by using image J software. XRD analysis was done in X-ray tube 3 kW with copper target, Real time multiple strip solid state detectors was used, K alpha was maintained at 0.001°, and it was scanned fast by D8 Advance ECO XRD System (Bruker, Madison, WI, USA). FTIR measurements of MCM-41, MCM-41P, and CUR-MCM-41P were analyzed by Shimadzu spectrometer (Nishinokyo, Japan) in the ranges of 400-4000 cm\(^{-1}\) in transmission mode.

3.2.4 Culture condition

Breast adenocarcinoma cells line MCF-7 were grown in IMDM medium containing 10% FBS with 5% CO\(_2\) at 37 °C. MCM-41 and CUR-MCM-41P were treated separately with MCF-7 cells containing serum free media for all the experiments.
3.2.5 Toxicity analysis of MCM-41 and curcumin

The MCM-41 and curcumin toxicity was analysed with WST-1 [139], MTT [117] assay for MCF-7 cells in 96 well plate separately. Briefly, 10,000 cells were grown in 96 well plate for 36 h. followed by 500 ng/mL to 1 mg/mL of MCM-41P and 10 µM to 100 µM of curcumin added separately in each well. After 24 h 5 µL WST-1 and 5 µL MTT reagent was added to each well and incubated for 30 min. followed by read at 450 nm for WST-1 and 595 nm for MTT assay in a microplate reader.

3.2.6 Accumulation of nanoparticles in MCF-7 cells

The accumulation of MCM-41P in MCF-7 cell was analysed in confocal laser scanning microscopy (CLSM). MCF-7 cells were grown in petri dishes (MetTek, Ashland, MA, USA) and treated with doxorubicin-coated MCM-41P (DOX-MCM-41P) at different time intervals 20 min, 40 min, 1 h, 2 h, 3 h and 4 h. Then cells were fixed with 4% paraformaldehyde (PFA) followed by DAPI stain and analysed in CLSM (Zeiss LSM 500, Heidelberg, Germany) exited with 405 nm and emission from 580 to 620 nm.

3.2.7 Curcumin release and MCF-7 cell Viability

Approximately 10,000 MCF-7 cells were seeded on 96 well plate and incubated at 37 °C with 5% CO₂ for 36 h. CUR-MCM-41P was added to MCF-7 cells and incubated for 4 hours and the used media was replaced with fresh media. This was further incubated for different time intervals such as 3, 7, 15, 24, and 48 h. Followed by 5 µL WST-1 reagent added to each well and incubated for 30 min and the plate was read at 450 nm in a microplate reader.
3.2.8 Apoptosis assay

DAPI staining used to identify the apoptosis of MCF-7 cells. Initially, the cells were grown in chamber slides and treated with MCM-41 coated with PEI alone and in combination with 50µM of curcumin. After 48 h the slides were washed with cold PBS. Then the cells were fixed with 4% paraformaldehyde for 20 min. The fixed cells were again washed with PBS and stained with DAPI. Apoptotic MCF-7 nuclei were examined under a fluorescence microscope (Zeiss, Heidelberg, Germany) and condensed nuclei as visible morphological changes.

Fluorescence Assisted Cell Sorter was used to analyse the percentage of cell death. MCF-7 cells were grown in 24 well plate and treated with free curcumin, MCM-41P and CUR- MCM-41P. After 24h of treatment, the cells were washed, trypsinized to single cell suspension, incubated in 10µg/ml of propidium iodide (PI) for 15 min and cells were analysed for PI positive cells.

3.2.9 Statistical Analysis

All results were analyzed as the mean ± standard error of the mean (SEM) values. Statistical analysis was approved by using Graph pad prism 5 (Graph pad software, San Diego, CA, USA). A significance level of P value is less than 0.0001, 95% confidence interval was used and data with control and sample.
3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of MCM-41 and CUR-MCM-41P

MCM-41P was synthesized and different concentration of curcumin was loaded in this nanoparticles. These nanoparticles were characterized by HRTEM, FTIR and XRD. The MCM-41 and CUR-MCM-41P were analysed by HR-TEM (Fig. 3.1) revealed that carrier size ranges from 100-200 nm and measured pore size from the instrument software ranges from 4-12 nm. The MCM-41 pores are wide open however; CUR-MCM-41P pore closure occurred because of curcumin loaded on MCM-41. Amorphous nature of MCM-41 was analysed with XRD, (Fig. 3.3) addition of curcumin and PEI reduces the amorphous nature of MCM-41 at 20-30 two theta degree. FTIR analysis Fig. 3.2 revealed that peaks around 500 and 1055 cm\(^{-1}\) represented the presence of silica functional group Si-O-Si and Si-OH. C=O and phenolic -OH groups peaks were found in the ranges of 1750cm\(^{-1}\) and 3780cm\(^{-1}\) which indicated that curcumin loaded on MCM-41. Significant variation on the peak was found in CUR-MCM-41P when compared with MCM-41. The peak of 3050 cm\(^{-1}\) indicated the presence of imine group and -C-H group at peak of 2660cm\(^{-1}\) which are functional groups of PEI.
**Figure 3.1**: HR-TEM images of MCM-41P and MCM-41P+cur. 200nm image of (A) MCM-41 coated with PEI (C) MCM-41P loaded with cur, 50nm image of (B) MCM-41+PEI with parallelly arranged pores (D) MCM-41P+Cur with saturated pores.
**Figure 3.2**: FT-IR graph for MCM-41, MCM-41P, MCM-41P+Cur:

Imine group of PEI shifts the peak around $3100\text{cm}^{-1}$ and curcumin loading on MCM-41P indicates the functional phenolic group, ketonic group.
3.3.2 Curcumin loading and release in MCM-41

The MCM-41 and MCM-41P containing flasks were kept in a shaker separately and added 50 µM curcumin dissolved in 100% ethanol. The amount of curcumin loaded in MCM-41 and MCM-41P was calculated from unbound curcumin in a flask at 0 h and 24 h after incubation (Fig. 3.4 A). Curcumin loaded on MCM-41P was found to be 90%, while MCM-41 showed significantly reduced 10% of curcumin. Therefore, curcumin loading on MCM-41 was significantly enhanced 80% through PEI coating. The release of curcumin from MCM-41 in PBS with three different pH ranges 3.0, 6.0 and 7.4 as shown in Fig. 3.4 B. We found that curcumin is released effectively at pH 3.0 when compared with pH 6 and 7.4. The 65 nM curcumin released in pH 3.0 and 58 nM in pH 7.4 at 48 h. Amount of curcumin released depends on degradability of PEI coating with MCM-41. The secondary amines of PEI are easily protonated with acid, resulting in releasing curcumin effectively at acidic pH [32].

3.3.3 In vitro accumulation of DOX-MCM-41P in MCF-7 cells

The DOX-MCM-41P was treated with MCF-7 cells in petri dishes for different time intervals. The cells were fixed, stained and documented in CLSM. Fig. 3.5 showed that DOX-MCM-41P started accumulating in MCF-7 cells from 1 h incubation and after 3 h there is no significant accumulation of DOX compared with 1 h after incubation. Our result was consistent with previous report [130], that MSN accumulating DOX inside the cell from 30 min to 1 hour. The accumulation of MCM-41 start accumulates from 1 h and gets saturated at 3 h.
Figure 3.3: X-ray Diffraction of MCM-41: Amorphous nature of MCM-41 is confirmed by the broader peak.
Figure 3.4: Drug entrapment and release: (A) The curcumin was loaded on MCM-41 and PEI coated MCM-41 nanoparticles at 0 and 24 h after incubation at room temperature (B) The curcumin released from PEI coated MCM-41 at different pH 3, 6 and 7.4 for 48 h.
**Figure 3.5** Intracellular accumulation doxorubicin loaded MCM-41: Doxorubicin loaded MCM-41 in MCF-7 cells was observed in confocal microscope at consecutive intervals such as 0, 1 and 3 h.
3.3.4 Toxicity analysis of MCM-41 and Curcumin

The different concentrations of curcumin was treated with to MCF-7 cells and analysing the IC\textsubscript{50} by MTT assay (Fig. 3.6 A). 50 µM concentration of curcumin loaded could induce 50% cell death in MCF-7 cells. Therefore, these studies suggested that threshold levels of toxicity were depending on the concentration of curcumin in MCF-7 cells.

3.3.5 Curcumin released from MCM-41 and induction of Cell death in MCF-7 cells

The accumulation of curcumin released in MCF-7 cells from 50 µM CUR-MCM-41P particles is observed in different time intervals (Fig. 3.6B). We observed intracellular MCM-41 concentration increased from 1h and saturated within 3 h. Therefore, curcumin released from MCM-41 could effectively increase MCF-7 cell death. Curcumin released from MCM-41 inside MCF-7 cell reduced the viability of MCF-7 cells (Fig 3.6B). The 50 µM CUR-MCM-41P reduced cell viability around 50% at 48 h. The similar results are previously reported [33] that IC\textsubscript{50} of curcumin released from curcEmulsome.
Figure 3.6 Cell survival of MCF-7 with curcumin and MCM-41 loaded curcumin: (A) Variation in viability of MCF-7 cells treated with curcumin for 24 h. (B) Different time intervals of curcumin released from MCM-41 and its effect on MCF-7 survival.
3.3.6 Cell death induced by CUR-MCM-41P

DNA laddering or fragmentation assay indicated that curcumin induced apoptosis [55] of MCF-7 cells as shown in Fig.3.7. DAPI stained cells were imaged in fluorescence microscope. Fig. 3.7 showed that cells with extended DNA revealed that fragmentation of DNA in the cell. Cells which have undergone curcumin treatment showed increased DNA fragmentation. Whereas, cells treated with MCM-41 coated with PEI didn’t show presence of fragmented DNA. But MCM-41 coated with PEI and loaded with 50µM of curcumin induced DNA fragmentation in cells which has taken up MCM-41 particle. The cells which didn’t take up any MCM-41 particle showed intact DNA.

PI is used to stain the DNA of apoptotic cells which have damaged cell membrane. PI positive cells analysed with FACS suggest that curcumin (Fig. 3.8 B) induced 41.6 % of cell death, whereas CUR-MCM-41P (Fig. 3.8 D) induced 50.47 % of cell death. The sustained release of curcumin from CUR-MCM-41P was able to cause 50% cell death at 48 h. The amount of curcumin released from MCM-41P (58 nm) curcumin dissolved in ethanol. Low solubility, short half-life, large volume distribution, low absorption [33] could be the reason for high concentration of free curcumin required to cause 50% of cell death whereas the same is acquired from 58 nm curcumin released from MCM-41 inside the cell.
Figure 3.7: Alteration in nuclear morphology by CUR: Apoptosis is a process which involves DNA fragmentation of cells. Curcumin and MCM-41+Cur induced DNA fragment is shown in arrows.
Figure 3.8: Cell death percentage in flow cytometry: FACS data of (A) Control, (B) Curcumin alone, (C) MCM-41P alone, (D) MCM-41P with 50µM of curcumin loaded and (E) Representative graph of percentage cell death analysed by FACS.
3.4 CONCLUSION

Conjugating curcumin with MCM-41P increased its hydrophilicity and dispersity. Our results suggested that MCM-41 could enter the cell and release curcumin which results in reduced cell survival and induce apoptosis similar to exogenously administered curcumin.
CHAPTER IV

4. INDUCTION OF APOPTOSIS BY CUR-MSNA IN MCF-7 CELLS

4.1 INTRODUCTION

Recent research demands in drug delivery systems (DDS) focus on improving effectiveness of the conventional drug than investing on new drugs. Delivery of a drug in a controlled and slow mode at target site is required to overcome the disadvantages in the prevailing scenario [127, 109]. Mesoporous silica nanoparticles (MSNs) are the most widely researched drug carrier due to its chemical inertness and its high drug loading capacity. In addition, their high surface area, desired surface functionalisation and tunable pore size intensifies the potential of MSN as a drug carrier [115, 142, 14]. Efficacy of MSN was determined by its size, shape and surface functionalisation. The shape of the nanoparticle influences its biological activity, phagocytosis evasion and increased circulatory time in blood [120]. The rod shaped MSN with high aspect ratio has increased bio distribution and bioaccumulation compared to the spherical one with low aspect ratio [50, 148]. Non-spherical MSN have the greater ability to escape from the host immune response like cylindrical and filamentous bacteria and virus [128]. Surface functionalization of nano drug carriers plays a vital role in target specification and for efficient cellular uptake. Polymer coating of nanoparticle enhances drug encapsulation, efficient translocation inside the cell and sustained release at target sites [37].

Cationic polymers have an advantage in binding to the negatively charged plasma membrane than anionic counterparts. Polyethyleneimine (PEI),
a polycation was used as an efficient transfecting agent for DNA and siRNA. Transfection efficiency of PEI increases with its increased molecular weight and branching, but PEI with high molecular weight is toxic whereas the linear PEI has poor transfection efficiency. The major positive trait of PEI as a drug carrier is its endosomal escape through ‘proton sponge effect’ [37, 139, 43, 85, 34]. In order to limit the toxicity, PEI has been conjugated with polyethylene glycol (PEG) [73], polymethyl methacrylate (PMMA) [113] and cholic acid [8]. But still the usage of PEI was restricted in DDS. Hydrophobic nature of curcumin and its effective anticancer activity against many cancer cell lines made it the first drug of choice in nano formulation. Till now, curcumin has been conjugated with liposome, PLGA, cyclodextrin, micelles, dendrimers, polymers, and with nano forms of metal oxides, carbon nanotubes, magnet, and silica. Each formulations has its own disadvantages like accumulating in liver and spleen, low circulation time and non-specific binding [134, 91, 141]. In this study, we conjugated curcumin to MSN by its surface functionalisation through polyethylene imine.

The size, shape and surface charge of the nanoparticles not only determine the effectiveness of the nanoparticle but also governs the toxicity elicited by these particle. Platelet aggregation, physiological and reproductive mutilation were the toxic response to silica nanoparticle reported earlier [63]. Nanotoxicity at cellular level generate ROS which leads to DNA damage and apoptosis. In addition to this, complement activation by silica nanoparticles was also described previously. Further, nanoparticles also act as adjuvants and promote antigenicity [63,121]. Toxicity of nanoparticles were correlated with its bio distribution and excretion in vivo. Decrease in aspect ratio of MSN has also decreased its excretion and increased absorption in organs when administered orally [70] but rods with higher aspect ratio were up taken by cells
faster and easier [150]. Controlling the size, shape and surface functionalization could be possible approach to reduce the toxicity of the nanoparticle [71]. Toxicity of the nanoparticles were minimized by binding them with quercetin, ascorbic acid to reduce the ROS generation and by inhibiting cytokines signalling to reduce the inflammation [63]. Also surface modification with biocompatible polymers was also known to reduce the toxic effects of nanoparticles [145]. Spheres, rods and wires were the most reported nanostructures studied for their in vitro and in vivo toxicological effects. Rods and wire shaped nanoparticle had a better intracellular accumulation [150] but has increased toxicity and faster excretion compared to spherical particles [70]. However, a different shape nanoparticle with less toxicity and higher intracellular accumulation could be ultimatum.

The present study deals with drug delivery efficiency of a non-spherical, nose-coned cylindrical mesoporous nanoparticle with PEI coating against MCF-7 cell line. We observed nose-cone headed cylindrical nanoparticle (MSNAP) is less toxic, has fast intracellular uptake, release the drug curcumin faster than spherical MCM-41P. Further, non-toxic nature of MSNAP analysed with TEM indicates its cytoplasmic distribution and absence of auto phagosome in MCF-7 cells. MSNAP loaded with curcumin (CUR-MSNAP) induce cell death with lesser curcumin concentration compared to the curcumin administered in their soluble form. Intracellular curcumin released from CUR-MSNAP was able to induce apoptosis which was mediated by mitochondrial impairment and also by disturbing proteins involved in ER homeostasis. Our preliminary study indicate the competent drug carrier properties of MSNAP in MCF-7 cells which could be further characterized in vivo.
4.2. EXPERIMENTAL METHODOLOGY:

4.2.1 Synthesis of spherical (MCM-41) NP

The synthesis of MCM-41 was achieved as per the procedure described earlier [139]. Briefly, 100 mg of CTAB (Alfa Aesar, Ward Hill, MA, USA) and 2M NaOH (0.35 mL) was dissolved in 48 mL of deionized water and stirred at 800 rpm and 80 °C for 20 min. This mixture was allowed to react with 0.5 mL of 0.0216 M TEOS (Alfa Aesar), which was added dropwise. This mixture was stirred at 800 rpm for acceleration of TEOS hydration at 80 °C for another 2 h. Template was removed by refluxing with the mixture of HCl and MeOH (1:20) for 18 h.

4.2.2 Synthesis of Non-spherical (MSNA) NP

‘Origami’ method was adopted for the synthesis of non-spherical MSNA (MSNA – As synthesized in acid medium) with the slight modification [27]. In brief, H2O, HCl, formamide, CTAB, TEOS were used in a molar ratio of 100:7.8:10.2:0.11:0.13 and magnetically stirred. Initially, 2.4 mL of HCl, 4 mL of formamide and 0.4 g of CTAB (Cetyl trimethylammonium bromide) was mixed in a conical flask with deionized water and stirred at 600 rpm for 40 h at room temperature. 0.3 mL TEOS was added dropwise to this mixture and incubated for another 18 h. The obtained white precipitate was refluxed with HCl and MeOH (1:20) overnight for the removal of the template. Both these nanostructures were coated with 0.3% of 10 kDa PEI (Alfa Aesar) dissolved in EtOH [31].
4.2.3 MSN characterization

The synthesized nanoparticles were characterized by its morphology, size, and surface charge. Surface morphology of fabricated nanoparticles, MCM-41, MCM-41P, and curcumin-loaded MCM-41 (CUR-MCM-41P), MSNAP, curcumin-loaded MSNAP (CUR-MSNAP) was analyzed by scanning electron microscopy (SEM) (Evo18 Zeiss Munich, Germany) at 20 KV adjuncts with Energy Dispersive X-ray (EDX) (Bruker, Madison, WI, USA). The structure of MCM-41P, CUR-MCM-41P was observed in High-resolution transmission electron microscope (HRTEM, T12 tecnai, Hillsboro, Oregon USA) HT650 ES1000W at 120 kV. The pore size of these nanoparticles was determined by image J software of HRTEM. All these above-mentioned nanoparticles were dispersed in EtOH and placed on either carbon paper for SEM studies or on copper grid for TEM analysis and dried before analyzing. Diffraction patterns were obtained from X-ray diffractometer (Bruker Eco D8 Advance, Madison, WI, USA) with Cu Kα (40 kV, 25mA) scanned at 0.02/ 2.4 sec. The Nano structures dispersed in water were analyzed for their size and surface charge through dynamic light scattering (DLS) and zeta potential by using Nanoparticle analyzer SZ-100 (Horiba, Kyoto, Japan) respectively.

4.2.4 Drug loading and release

Each 10 mg of synthesized MCM-41P and MSNAP were suspended separately in 5 mM curcumin (Alfa Aesar) in EtOH for 24 h in an orbital shaker. The unbound free curcumin was removed at 24 h and its absorbance at 420 nm was compared with 0 h unbound curcumin in nano drop (Biospec Nano, Shimadzu). Percentage of curcumin loaded in both nanostructure was determined by the formula (Abs at 0 h- Abs at 24 h/ Abs at 0 h)*100.
The concentration of curcumin released from CUR-MCM-41P and CUR-MSNAP was determined in phosphate buffer saline (PBS) at pH 7.4. The 4 mg/mL of CUR-MCM-41P and CUR-MSNAP was immersed in PBS. The released curcumin in PBS was measured at different time intervals from 0 to 96 h at 420 nm in nano drop. The concentration of curcumin released was calculated by referring the standard curcumin graph.

4.2.5 Cell culture

The breast adenocarcinoma (MCF-7) cells were grown in IMDM (Gibco/Life Technologies, Gaithersburg, MD, USA) containing 10% FBS (Gibco BRL) and incubated with 5% CO₂ at 37 °C. Serum free media was used in all the experiments and cells were treated with different concentrations of MCM-41, MCM-41P, CUR-MCM-41P, MSNAP and CUR-MSNAP. Main stock of curcumin was prepared by solubilizing in EtOH and desired working concentration was obtained by reconstituting stock in IMDM medium.

4.2.6 MSNs induced Toxicity assays

Nanotoxicity of MCM-41P and MSNAP against MCF-7 cells was used to determine their optimal dosage. The nanotoxicity induced cell death was determined by Water soluble tetrazolium-1 (WST-1) assay (Roche, Switzerland) as per manufacture’s instruction. Briefly, 8,000 cells were grown in 96 well plate for 36 h. Both these nanoparticles were used to treat cell in concentration ranges from 500 ng/mL to 100 µg/mL for 24 h separately. 5 µL WST-1 was added to each well and incubated for 1 h. The plate was read at 450
IC$_{50}$ of curcumin was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reduction assay (Roche, Germany GmbH) as per the manufactures instruction. The variable concentrations of curcumin from 10-100 µM was added to MCF-7 cells grown in 96 well plate (Greiner, Bio-One, Ireland) for 24 h. followed by addition of 5 µL MTT to each well. Formazan crystal developed was dissolved in isopropanol and the plate was read at 590 nm in a microplate reader. The concentration of curcumin which induces 50% of cell death was also determined by the above-mentioned formula.

4.2.7 Accumulation of MSNs in MCF-7 cells

The accumulation of nanoparticles in MCF-7 cells was analyzed by confocal laser scanning microscopy (CLSM), (LSM 500, Zeiss, Munich, Germany). Cells were grown on coverslips till they attain 60% confluence. 5 nM Doxorubicin (DOX) (Doxotero, Khendeiwa Laboratories) loaded silica nanostructures (DOX-MCM-41P and DOX-MSNAP) were incubated with MCF-7 cells for different time intervals such as 0, 20, 40, 60, 120 and 180 min. The coverslips were washed with 1X PBS then fixed with 4% paraformaldehyde for 10 min. followed by DAPI staining. Cells were imaged with CLSM which was exited at 405 nm and emission was set from 580 to 620 nm.
4.2.8 Bio-TEM studies for subcellular localization of MSNs in MCF-7 cells

The non-toxic concentration of 500 ng/mL MCM-41P and 30 µg/mL MSNAP were treated with MCF-7 cells for 72 h. The efficacy of 30 µM CUR-MSNAP treated MCF-7 cells at 24h and 48 h. After incubation cells were harvested and fixed in fixative mixture of 2.5% glutaraldehyde, 2% sucrose and complete media for 12 h. These fixed cells were centrifuged and stained with 1% osmium tetroxide. The cells were infiltrated with graded series of 70 to 100% EtOH for 1 h each at room temperature. The fixed cells were added with 100% propylene oxide then gradually transferred to 100% epoxy resin (TAAB, England) by decreasing the percentage of propylene oxide. Initially, the cell pellet was kept in an equal mixture of epoxy resin and propylene oxide for overnight, followed by epoxy resin and propylene oxide (2:1) for 1 h. Finally, cells were kept in pure resin for 5 h and embedded in a freshly prepared resin at 50 °C for 48 h. Resin embedded samples were made to 80 -100 nm thin sections with ultra-microtome. These sections were imaged with transmission electron microscope (Tecnai G², Hillsboro, Oregon, USA) at 80 KV.

4.2.9 Evaluation of CUR-MSNAP induced apoptosis of MCF-7 cells

IC₅₀ value of CUR-MSNAP in MCF-7 cells was determined by WST-1 assay. 7000 cells were seeded in 96 well plate and grown for 36 h. Cells were treated with different concentration of curcumin (5 – 50 µM) loaded in non-toxic concentrations of 30 µg/mL MSNAP. After 72 h, the cell viability was measured with WST-1 reagent as described earlier. Percentage of live cells were determined by the formula (Absorbance of treated cells / Absorbance of control cells)*100.
FACS analysis was used to quantify live and dead cells on curcumin, MSNAP, CUR-MSNAP. Cells were grown in 12 well plate (Greiner) and treated independently with 30 µg/mL MSNAP, 50 µM curcumin, and 30 µg/mL CUR (30µM)-MSNAP for 72 h. Cells were harvested and stained with 5µL of 10 µg/ml propidium iodide (PI) for 10 min. The stained cells were analysed in FACS (FC500, Beckman Coulter, Brea, CA, USA) and PI positive cells were gated in FL3. The forward scatter (FSC) and side scatter (SSC) was also analysed simultaneously.

4.2.10 Measurement of intracellular curcumin released from nanoparticles

The concentration of curcumin released from CUR-MSNAP in MCF-7 cells was determined by nano drop. Briefly, 20,000 cells were grown in 12 well plate and incubated with 30 µg/mL CUR (30µM)-MSNAP (IC$_{50}$ value) and curcumin with concentrations 1, 5, 10, 25, 50 µM. After 72 h, the cells were harvested and lysed with lysis buffer (Tris pH 10, 150 mM NaCl, 10% DMSO) for 30 min. and the lysate was sheared with 25 gauge needle followed by centrifugation at 12,000 rpm for 10 min. at 4 ºC. The supernatant was measured at 420 nm in nano drop. The absorbance of curcumin was compared with standard curcumin graph and calculates the concentration of curcumin released from MSNs in MCF-7 cells.

4.2.11 Western blot analysis

The qualitative differences of CUR-MSNAP influenced protein expression in MCF-7 cells were analysed using Western blot. The cells grown in 100 mm dishes (Greiner) were treated with 50 µM curcumin, 30 µg/mL
MSNAP, 30 µg/mL CUR (30µM)-MSNAP and 20 µM DTT for 72 h. DTT was used as a positive control for UPR induction [Dicks, N]. After incubation, MCF-7 cells were lysed with RIPA (Radio immuno precipitation assay) buffer (pH 7.4) containing protease and phosphatase cocktail inhibitors (Roche, Switzerland) on ice for 20 min. and the lysates were sheared with 25 gauge needle, followed by centrifugation at 12,000 rpm for 25 min. at 4 °C. The protein was quantified with BCA reagent (Sigma-Aldrich, St. Louis, MO). Each 50 µg of proteins were loaded onto SDS-PAGE and proteins were run at 110V for 2 h. The proteins were transferred to nitrocellulose membrane (Amersham bioscience, Piscataway, NJ, USA). Anti-PERK, anti-IRE1α, anti-GRP78, anti-calnexin, anti-phospho-Akt (Ser473), anti-total Akt, anti-PTEN, anti-PARP, anti-caspase 12, anti-caspases 9, 6, anti-CHOP, and anti-GAPDH were obtained from Cell Signalling Technology (Danvers, MA, USA). The membrane was incubated overnight with primary antibodies at 4 °C followed by either anti-rabbit IgG or anti-mouse IgG HRP-linked secondary antibodies (Santa Cruz, CA, USA) for 1 h. The presence of the protein was detected with addition of lumiglo (Thermo scientific, Rockford, IL, USA) reagent and viewed in gel documentation system (Bio-Rad, Hercules, CA, USA) with Image lab 5 software. Densitometry of respective bands was analysed by Image J software. Expression of proteins was represented as fold change with a ratio of each protein to its loading control GAPDH.

4. 2.12 Statistical analysis

All results were analysed as the mean ± standard error of the mean values. Statistical analysis was done by Graph pad prism 5 (Graph pad software, San Diego, CA, USA). A significance level of P value, less than 0.005 with 95% confidence interval was used for control and treatment.
4.3 RESULTS AND DISCUSSION:

4.3.1 Comparison of Synthesis, Fabrication and Biophysical characterisation of MSNAP and MCM-41:

Both the synthesized MCM-41 and MSNA was coated with PEI and was characterised for their structure and function. Synthesis of MSNs is usually achieved by two methods (a) simultaneous grafting of surfactant micelles along with silica precursor, and (b) the silica precursor is allowed to accumulate over the pre-formed surfactant micelle [120, 15, 96]. MCM-41 was synthesised by former method [26] whereas MSNA was prepared by the later method in midst of acid hydrolysis and condensation.

SEM images of MSNAP revealed that they are formed in different shapes and sizes. Nose cone headed cylindrical form is the predominant form, few gyroid, discoid, cylindrical and a few particles with spherical shapes were also observed (Fig. 4.1). Silica particle obtained from this method vary from micro to nano sized structures [27]. CTAB removal from MSNA and its PEI coating resulted in a unique shaped (nose cone headed cylindrical particles) and smaller sized particles compared to an earlier reported form [27,139]. PEI was coated on these silica particles through the electrostatic interaction between the NH4+, NH2+ and NH3+ ions of PEI and Si-OH, Si-O-Si, O-Si-O anions of silica nanoparticles. Further to analyse the drug was efficiently loaded onto MSNAP, curcumin loaded MSNAP (CUR-MSNAP) was also considered for its structural and functional characteristics. Curcumin loading on MSNAP was mediated by electrostatic interaction between the PEI cationic groups and –OH and -C=O anionic group of curcumin. Rough surface of curcumin loaded MSNAP indicated by SEM images, confirmed the presence of crystalized curcumin on MSNAP surface (Fig. 4.1 and Fig 4.2). But MCM-41, MCM-41P (MCM-41
coated with PEI), CUR-MCM-41P (Curcumin loaded MCM-41P) was also characterisation with SEM analysis of reveals that most of these nanoparticles are homogenous and spherical in shape (Fig. 4.2). Further, surface of MCM-41 and MCM-41P are smoother than CUR-MCM-41P. The CUR-MCM-41P showed that crystallinity and increased roughness on the surface of the nanoparticles.

TEM analysis of MSNAP (Fig. 4.4c), revealed it parallelly arranged pores and also indicated the variation in particle shape. TEM image of CUR-MSNAP appeared darker compared to MSNAP without a clear distinction of the pores. Curcumin saturated the pores of MSNAP resulting in a darker image. Particle size analysed with DLS indicated MSNAP has micro particles with average diameter of 679.38 nm and nanoparticles with average diameter of 23.58 nm (Fig. 4.4 A & B). Particle size analysed with DLS indicate, curcumin loading on MSNAP resulted in micro particle with average of 604.92 nm and nanoparticle of 25.83 nm (Fig. 4.4). However MCM-41 had uniform size and shape distribution. Particle size analysed with DLS indicated MCM-41, MCM-41P, CUR-MCM-41P nanoparticles had an average diameter of 104 nm (Fig. 4.3A), 100 nm (Fig. 4.3B), and 102 nm (Fig. 4.3C) respectively.

Functional groups of MSNA was determined with energy dispersive X-rays. EDX data of MSNAP suggested that major energy peak of silica was at 1.75 KeV, oxygen at 0.5 KeV and nitrogen at 0.4 KeV. EDX study showed an additional peak of carbon around 0.3 KeV which corresponds to carbon of curcumin on MSNAP along with the silica and nitrogen peaks of MSNAP (Fig. 4.5). EDX pattern (Fig. 4.6) of MCM-41 has predominant peak of silica and oxygen at 1.75 and 0.5 KeV with atomic percentage 19.59 and 64.34 respectively. PEI coating of MCM-41 was confirmed by nitrogen peak at 0.4 KeV (12.36%), along with silica of 10.94% (1.8 KeV), oxygen 45.27% (0.5
KeV) and carbon 30.87% (0.3 KeV). CUR-MCM-41P was confirmed by increased carbon atomic percentage to 41.65%.

XRD (Fig. 4.7 A) analysis of MSNAP with curcumin showed an increased two theta peak around 20-30 compared to MSNAP. This confirms the amorphous nature of MSNAP was further decreased upon curcumin loading. MSNAP has a positive zeta potential with net positive charge of 115.2 mV (Fig. 4.7B).

Zeta potential value confirmed curcumin-loaded MSNAP surface charge was 102.2 mV. The reduction in charge was due to the curcumin addition on its surface. Zeta potential examination of these nanoparticles indicated that net surface charges are exhibited. The zeta potential of MCM-41 is -22.6 mV, which is due to the silanol group of silica. MCM-41P and CUR-MCM-41P have positive zeta potential of +74 and +93 mV respectively. Positive charges from cations of PEI contribute to higher positive zeta potential of MCM-41P and CUR-MCM-41P. Higher positive zeta potential indicated the particles stability.
Figure 4.1: SEM analysis of MSNA with PEI and MSNA+PEI loaded with curcumin: 10 µM, 2 µM, 300 nm images of MSNA with PEI coating indicating its non-spherical cylindrical cone headed particle. Curcumin loading on MSNA+PEI indicates the curcumin as crystal on MSNA surface.
Figure 4.2: Comparison of SEM images of MCM-41 and MSNAP: SEM images of MCM-41 (a) 200nm and (b) 100nm images of MCM-41, MCM-41P (C) 200nm and (d) 100nm, MCM-41P with loaded cur image (e) 200nm and (f) 100nm. Various non-spherical shape of MSNAP compared to spherical shape of MCM-41.
Figure 4.3: Particle size analysed with DLS indicate the diameter (A) MCM-41 is 104.58nm (B) MCM-41 decorated with PEI is of 100nm (C) Cur loading on MCM-41+PEI is of 102 nm
Figure 4.4: Particle size analysed with Dynamic light scattering: Diameter of MSNAP (A) 679nm and (B) 23.58nm. Diameter of MSNAP with curcumin incubated (A) 604.92 and (B) 25.83. (C) TEM images of MSNAP with parallel pores and Cur-MSNAP with pore closure.
Figure 4.5: EDX of MCM-41: Elemental analysis with Energy dispersive X-ray of (A) MCM-41 (B) MCM-41 with PEI (C) MCM-41 with PEI and loaded curcumin.
**Figure 4.6:** EDX prediction of MSNA and MSNAP+Cur: (A) Graph representing the energy peaks of atoms with their atomic percentage of MSNAP. (B) Atomic percentage and elemental energy peaks of MSNAP with curcumin.
Figure 4.7: Particle charge and amorphous nature of MSN: (A) X-ray diffraction of MSNA, MSNA+PEI with and without curcumin loading. (B) Zeta potential analysis of the nanostructures.
4.3.2 In-solution analysis of drug uptake and release by MSNAP

Drug loading capacity of nano carriers plays a vital role in theranostic application which depends on the composition of both drug and its carrier [18]. Drug adsorption studies were performed to determine the drug loading capacity of these nanostructures. Comparison of curcumin loading on MCM-41 and MSNA in ethanol revealed, loading percentage of curcumin on MSNA was 1.7% higher than MCM-41. Surface modification of these nanostructures with PEI enhanced curcumin loading on MCM-41 to 86.7% and MSNA to 82.8% (Fig. 4.8A).

The analysis of curcumin-released from CUR-MCM-41P and CUR-MSNA was monitored in PBS at pH 7.4 at different time intervals from 0 to 96 h (Fig. 4.8B). Though, both nanoparticles have a sustained release of curcumin; CUR-MSNAP has enhanced drug releasing capacity as compared with CUR-MCM-41P. As bigger pore sized nanostructures release drug efficiently [48], MSNAP release curcumin better due to its bigger pore size compared to MCM-41P.

Electrostatic interaction between the functional groups of curcumin and PEI forms a zwitterionic complex. This zwitterionic complex is converted to protonated amines and free isomer of curcumin in water or buffer at neutral pH [72]. The gradual PEI protonation is directly proportional to the amount of drug released from PEI coated MSN. 20% of PEI was protonated at neutral pH and 45% of PEI was protonated at pH 5.0 [7]. Our drug release studies at different pH indicate 65 nM of curcumin was released from CUR-MCM-41P at pH 3.0, and 58nM was released at pH 7.4 [46]. Thus, neutral pH plays a vital role for sustained release of curcumin from CUR-MSNAP.
Figure 4.8. Comparison of drug entrapment and release from MSNAP and MCM-41P: (A) Bar diagram denoting curcumin loading percentage on MCM-41, MCM-41P, MSNA and MSNAP. (B) Graph signifying concentration of curcumin released from MCM-41P and MSNAP in different time intervals from 0 to 96 h in PBS (pH 7.4) at 37 °C.
4.3.3 Biological activity of MSNAP in MCF7 cells

The biological activity of MSNAP was assessed with its biocompatibility and its efficient uptake by cells. Biocompatibility of these nanostructures was determined by its toxicity against MCF-7 cells which was assessed by quantifying the viable cell with WST assay. When the cells were incubated with MCM-41P and MSNAP, we observed that the LD$_{50}$ of MCM-41P was 10µg/mL (Fig. 4.10A) however the LD$_{50}$ of MSNAP was 80 µg/mL (Fig. 4.10B) after 24 hours. MSNAP with nose cone-headed cylindrical shape is less toxic compared to spherical MCM-41P. It has been earlier reported that the shape of the nanoparticle influences the toxicity [70].

Another drug that was used to assess the cellular uptake of these nanostructures was doxorubicin. MCF-7 cells was treated with doxorubicin loaded MCM-41P (Dox-MCM-41P) or MSNAP (Dox-MSNAP) for 20 min. to 180 min. Time point accumulation study (Fig. 4.9), suggests MCF-7 start Dox-MCM-41 uptake from 60 minutes and attain saturation as 180 min. Whereas Dox-MSNAP accumulation in MCF-7 cells starts from 20 minutes with a maximum accumulation observed at 180 min. This indicate the rapid cellular accumulation of MSNAP compared to MCM-41P. Non-spherical particles have an advantage of better intracellular accumulation [91] and also evade immune response [141] of the host. The non-spherical nanoparticles exhibit low hydrodynamic shear stress than spherical nanostructures [134] which may account for MSNAP’s easy and faster uptake by cell.
Figure 4.9: Accumulation time point of nanostructures in cells: Confocal images of MCF-7 cells with MCM-41P and MSNAP coated with DOX (red) with its corresponding DAPI (blue) staining at 0, 20, 40, 60, 180 min in MCF-7 cells.
Figure 4.10 Toxicity study of MCM-41P and MSNAP against MCF-7 cells. (a) Graph representing cell viability percentage of MCF-7 cells in presence of increasing concentration of MCM-41P from 500 ng/ml to 50 µg/mL. (b) Bar diagram representing MCF-7 viability by treating with MSNAP from 20-100 µg/mL.
4.3.4 TEM based understanding of reduced toxicity exhibited by MSNAP

Intracellular localisation of MCM-41P and MSNAP was analysed using transmission electron microscope in MCF-7 cells. MCF-7 cells treated with MCM-41P and MSNAP showed an increased cellular vacuolisation compared to control cells (Fig. 4.11). We observed that a significant number of MCM-41P particles were localised in vacuoles (Fig. 4.11f &g) but MSNAP was localized in endosomes (Fig. 4.11j). MCM-41P primarily localized in mitochondria (Fig. 4.11h) and also in autophagosomes along with the mitochondria Fig. 4f. Whereas MSNAP was not accumulated (Fig. 4.11 i-k) in any organelle and it was mostly distributed in cytoplasm and cytoplasmic vesicles.

Autophagy is the cellular process to eliminate the toxins and pathogens from the mammalian cells [78]. Earlier reports with silica particles has indicated the formation of autophagosome in MRC-5 cells [132]. The possibility of MCF-7 cells undergoing autophagocytosis could be attributed to the toxic nature of MCM-41P. Toxicity of MCM-41P was also confirmed by the formation of dilated ER in MCF-7 cells (Fig. 4.11g), which was not observed in control cells. However, in MSNAP treated cells no autophagosomes were observed and MSNAP was mostly found in cytoplasm (Fig. 4.11 l) and vesicles (Fig. 4.11j). This further endorses the lesser toxic nature of MSNAP. In addition to this, different shapes of MSNAP enable increased accumulation of nanoparticle inside the cells than the spherical MCM-41P of the same size.
Figure 4.11 Subcellular localization of MCM-41 and MSNAP in MCF-7 cells: (a, b, c, d) 5 µm, 500 nm and 200 nm TEM images of control MCF-7 cells with nucleus (N), endoplasmic reticulum (ER) Mitochondria (M), Golgi (G), Plasma membrane (PM). Images of MCM-41P treated MCF-7 cells (e) 2µm image of cell, (f) 500nm image indicating MCM-41P in autophagosome (g) 200nm image indicating MCM-41P treated MCF-7 cells with bulged ER, (h) MCM-41P localized in mitochondria. Images of MSNAP treated MCF-7 cell (i) whole cell, (j) MSNAP in cytoplasmic vesicles (k) 500nm image of MSNAP localised in mitochondria and (l) 200nm image with MSNAP present in cytoplasm. Black arrows indicate the presence of nanoparticle.
4.3.5 MSNAP efficiently delivers curcumin to the MCF-7 resultanty inducing apoptosis.

MSNAPs drug delivery capacity was assessed with CUR-MSNAP induced cell death. MCF-7 cells treated with CUR-MSNAP were subjected to viability assay and flow cytometry to analyse cell death. Further to confirm MSNAP release curcumin inside the MCF-7 cells, intracellular curcumin concentration was determined using nano drop.

IC\textsubscript{50} concentration of curcumin and CUR-MSNAP was determined using cell viability assay. Free curcumin induced 50% cell death at 50\textmu M concentration (Fig.4.12A) but CUR-MSNAP was able to induce a similar cell death 30 \textmu M (loaded concentration) as shown in Fig. 4.10A. Intracellular curcumin concentration was estimated using cellular extracts from cells treated with curcumin and CUR-MSNAP. The absorption spectra of curcumin in the cellular extract was measured at 420nm and showed an effective concentration of 14 \textmu M (Fig. 4.12B). This is interesting because the free curcumin induces cell death at 50 mM while curcumin released by MSNAP achieves a similar cell death at a lower intracellular concentration. Similarly, analysis of cell death using propidium iodide followed by flow cytometric analysis of DNA content showed that the cells treated with free curcumin had 45% cell death while CUR-MSNAP treated cells exhibited 43% cell death (Fig.4.13).

Our viability assay and FACS data suggest that 30 \textmu M curcumin loaded on MSNAP (14\textmu M effective concentration) was able to induce similar percentage of cell death as that of 50 \textmu M of free curcumin. Higher intracellular accumulation and sustained drug release from MSNAP induced cell death at lower curcumin concentration compared to extracellularly administered curcumin.
Figure 4.12: Effects of curcumin released from MSNAP: (A) Bar diagram of MCF-7 cells viability on treatment with 0-50 µM curcumin and MSNAP (30 µg/mL) loaded with 0-50 µM of curcumin. (B) Graph indicating the intracellular curcumin concentration which was released from MSNAP at 72 h.
Figure 4.13: Cell death assessed with FACS: Images of live, dead MCF-7 cell quantification which are treated with curcumin, MSNAP and CUR-MSNAP by FACS.
4.3.6 CUR-MSNAP induce apoptosis by targeting mitochondria:

Further, in order to understand the mechanism of MSNAP released curcumin mediated apoptosis, we studied the changes in expression and activation of several signalling pathways regulating apoptosis in MCF-7 cells. Curcumin has been reported to induce apoptosis by modulating proteins of ER and mitochondria in numerous cancer cells [65, 83, 137]. We thus analysed the change in expression of protein which are involved in ER homeostasis, apoptosis and cell survival on curcumin and CUR-MSNAP treatment.

CUR-MSNAP and curcumin increased the expression of CHOP, C-PARP, C9, C-C9, C12, calnexin and PTEN (as shown in Fig. 4.13 and Fig. 4.14). Additionally, expression of pAkt, IRE1α, PERK, and GRP 78 proteins were markedly downregulated on treatment with curcumin and CUR-MSNAP (Fig. 4.14). Calnexin, an ER protein, expression was upregulated to two folds on curcumin and CUR-MSNAP treatment [Fig.4.15]. GRP78 the HSP chaperone, is the main stress sensor of ER which controls the activity of PERK, IRE1α and ATF6. PERK, ATF6 and IRE1α dissociate from GRP78 under stress condition and activate the downstream signalling molecules to restore the ER homeostasis [98]. Downregulation of these proteins by curcumin and CUR-MSNAP indicate an altered ER homeostasis.

Curcumin and CUR-MSNAP downregulated the Akt phosphorylation at Ser 473 however total Akt level was not altered on their treatment. Treatment of cells with curcumin and CUR-MSNAP enhanced PTEN expression by 1.75 fold compared to the untreated cells [Fig. 4.16]. Reports indicate that phosphorylation of Akt at ser473 enhances the cell survival whereas increased PTEN activity decreases the cell survival [40]. Our data thus suggests that free
curcumin and CUR-MSNAP may regulate the cell survival by modulating Akt phosphorylation and PTEN upregulation in MCF-7 cells.

We also studied that changes in cleaved PARP (C-PARP) and caspase 12 in these cells. Immunoblot analysis showed no significant variation in their expression upon treatment with free-curcumin compared to untreated cells (Fig. 4.16). However C-PARP and caspase 12 expression were increased two folds upon CUR-MSNAP treatment as compared to the control (Fig. 4.16). Caspase9 and cleaved caspase9 expressions were also elevated on CUR-MSNAP treatment with cleaved caspase9 showing a fourfold increase. Studying the changes in CHOP expression showed that both free curcumin and CUR-MSNAP treatment elevated expression of CHOP to 1.5 fold and 1.3 fold respectively compared to the control cells. Caspases are the link between regulations of cell death and inflammation [79]. Proteolytic cleavage of caspases amplify the signal to induce apoptosis [30]. Caspase12 aid in cleaving procaspase 9 which cascadically cleave caspase 3 [21]. Our result suggests curcumin and CUR-MSNAP apoptosis activation may be implemented through caspase12, caspase9 and PARP.

Induction of apoptosis was also studied by understanding the ultrastructural changes associated with treatment. MCF-7 cells were treated with 30 µM curcumin loaded MSNAP for 24 h and 48 h. Bio-TEM images of these samples indicated that CUR-MSNAP treated cells at 24 h and 48 h have distinct morphological changes in mitochondria and nucleus as compared to MSNAP treated and control cells. MSNAP (Fig. 4.17) localizes primarily in the cytoplasm whereas curcumin loaded MSNAPs are mostly distributed in nucleus and mitochondria. The CUR- MSNAP treated cells at 24 h (Fig. 4.17c, d) showed swollen mitochondria with cristae and disrupted nuclear membrane. Additionally, cells treated with CUR-MSNAP for 48h (Fig. 4.17g, h) showed
cells with swollen mitochondria, damaged plasma membrane and apoptotic bodies.

Apoptosis mediated through mitochondria induces alteration in the inner mitochondrial membrane convoyed with cisternae degradation. Vesicular structure of mitochondrial inner membrane was altered during apoptosis which eventually lead to loss of cisternae [52, 36, 117, 106]. Fig. 4.8 c, suggests CUR-MSNAP treated cells at 24 h. exhibit a swollen mitochondria with vesicular inner membrane, but at 48h. (Fig. 4.15g) vesicular inner membrane was also lost and a swollen outer mitochondrial membrane was observed. Ultrastructural images suggest that CUR-MSNAP treated cells undergo apoptosis by remodelling inner mitochondrial membrane from normal vesicular structure to swollen vesicular at 24 h and a completely swollen mitochondria at 48 h. Interestingly, it has been reported that free curcumin induces apoptosis by damaging chromosome and the plasma membrane in MCF-7 cells, however there is no report to suggest that free curcumin cause mitochondrial insult [24].

Caspases are involved in mitochondria mediated apoptosis [90, 42]. CUR-MSNAP treatment resulting in mitochondrial disruption might be the cause for increased expression of cleaved caspase9 and caspase12 as compared to free curcumin treatment.
Figure 4.14: Signalling induced by intracellular released curcumin from MSNAP. (a) Images of blot probed for PERK, IRE1a, GRP78 and their corresponding graph of fold change with respect to GAPDH.
Figure 4.15: Modification of survival proteins determined by western blot: Panel indicating western blots of pAkt, TAkt, PTEN and calnexin and their graphical representation ratio of individual proteins to loading control.
**Figure 4.16:** Induction of apoptotic proteins by CUR and CUR-MSNA: Blots of proteins involved in apoptosis and their representative fold change graph compared to GAPDH.
Figure 4.17: Mechanism of apoptosis induced by CUR-MSNAP: (a) TEM images indicating MCF-7 cells treated with CUR-MSNAP for 24 h (a) whole cell with (N) nucleus, (Nu) Nucleolus, (M) Swollen Mitochondria (MS), (NE) Nuclear envelop, black arrows indicating MSNAP, (b) (c) (d) 200nm TEM image with swollen mitochondria. TEM images of CUR-MSNAP treated MCF-7 cells for 48 h (e) whole cell, (f), (g) 200 nm image with fully swollen mitochondria (h) 200 nm image with disrupted nuclear membrane. Black arrows indicate the presence of nanoparticle.
4.4 CONCLUSION

To summarize, a novel nose cone headed cylindrical mesoporous silica nanoparticle coated with PEI was characterized for its drug delivery efficiency in MCF-7. The non-spherical shape of MSNAP had reduced toxicity compared to spherical MCM-41P. Our uptake result indicate, MSNAP accumulates intracellularly within 20 min whereas the spherical nanostructure uptake was observed from 1h. Novel shape of the nanoparticle MSNAP and its amine modified surface aided a faster MSNAP uptake and increased bio-distribution in MCF-7 cells. Electron microscopy study of MCF-7 cells treated with MCM-41P and MSNAP showed difference in their subcellular localisation. MCM-41P induce autophagosome formation and dilated the ER which signifies its toxicity but non-toxic MSNAP mostly localized in cytoplasm and cytoplasmic vesicles (Fig. 4.18).

Efficiency of MSNAP released drug inside the cell was studied using curcumin. MSNAP loaded with 30µM of curcumin induced percentage of cell death similar to 50µM free curcumin. An effective intracellular curcumin concentration measured with nano drop which released from MSNAP was 14µM. Further, immunoblotting of CUR-MSNAP treated cells showed elevated levels of caspase12, cleaved caspase9, C-PARP, PTEN and also decreased levels of pAkt, PERK, GRP78 and IRE1α were observed. Mechanism of CUR-MSNAP induced cell death analysed with TEM suggests an increased mitochondrial insult. Intracellularly released curcumin of 14µM concentration released from MSNAP disrupts mitochondria and induce apoptosis better than free curcumin. A further detailed in vivo study with MSNAP vehicle could help to develop MSNAP as a potential DDS against cancers. We thus report the synthesis and characterisation of a new modified nanoparticle with a better biological activity.
Figure 4.18: Schematic overview of MCM-41P, MSNAP and CUR-MSNAP effects on MCF-7 cells. MCM-41P triggers toxic response in cells but shape modification of MSNAP has reduced toxicity. Intracellular curcumin released from MSNAP disrupts mitochondria and nucleus eventually leads to apoptosis.
CHAPTER V

5. CUR-DOX-MSNAP SYNERGISTICALLY INDUCED APOPTOSIS IN DOX RESISTANT (MCF-7R) CELLS.

5.1 INTRODUCTION

Breast cancer is the second most common neoplastic and chromosomal instability is the major role of the bearer of breast cancer. Based on the heterogeneity of tumour tissues, drugs were encouraged to approach more than one target. Targets of breast cancer include (i) hormonal receptors like epidermal growth factor receptor and nuclear receptor, (ii) the non-hormonal targets like the modulators of signal transduction which involved in cell-cycle, uncontrolled cell proliferation, angiogenesis, invasion, and metastasis [29]. However, the main disadvantages of chemotherapy are inducing cardiotoxicity at an older age and infertility in younger age breast cancer patients [10]. Anthracyclines and trastuzumab drugs enhances the chronic heart failure from cardiac dysfunction in cancer patients [22, 69]. Another significant challenge in anticancer therapy is the acquired resistance against chemotherapy in tumour cells and the also initiation of tumour growth through cancer stem cells [118].

Drug resistance mechanisms adapted by cancer cells implicate changes in the drug uptake, drug metabolism, efflux, and detoxification. Mutation in the intracellular drug transporter or mutation in the surface receptor of cancer cells also leads to decreased uptake of the drug. Drug transporter like ATP-binding cassette (ABC) protein is responsible for increased efflux of drug on cellular
surface. In addition, cancer cells inactivate the drug by mutation induction of metabolic targets downstream molecules or modifying their functional groups. Acquisition of resistance to one drug may also lead resistance to other drugs. Hence, a single drug target is inefficient to treat the development of resistance in cancer [146].

In order to overcome the resistance acquired by cancer cells, the present drawbacks in chemotherapy was strategized to used include a choice of drug which are non-substrate of ABC, sensitisation of MDR-ABC transporters by employing inhibitors to these pumps, targeting transcriptional regulation of ABC transporters, microRNA, signalling pathways of ABC transporters, combinational targeting with CSC targeting agents and transporter modulating drugs or dual targeting with a single agent, multiple drug targets, nanoparticle mediated drug delivery of single or combination of drugs [99, 86, 28]. Combinatorial therapy possesses the better anti-cancer effect compared to the administration of a single drug. Combining two or more anti-cancer agents increases the therapeutic efficiency of both drugs and tumour size reduction. Chemotherapeutics with synergistic effect was preferred rather than an additive effect which shows effective therapy against cancer with least toxicity [118]. Simultaneous dual, triple or multiple targeting of anti-cancer therapeutics was trailed clinically yet the resistance development by primary and secondary tumours was not resolved [39].

Nanoparticle-mediated delivery of anti-cancer drugs would target intracellularly rather than efflux by MDR-ABC. The mesoporous silica nanoparticle (MSN) loaded doxorubicin reverse the chemotherapeutic resistant in MCF-7R cells [105]. Even though the co-delivery effect of doxorubicin with curcumin on MSNAP in MCF-7 and MCF-7R was remain unanswered. Therefore, the proposed work focused on effect of dual drug CUR-DOX-
MSNAP on MCF-7 and doxorubicin-resistant MCF-7 (MCF-7R) cells. Curcumin and doxorubicin loaded MSNAP synergistically induce apoptosis in breast adenocarcinoma cells (MCF-7 and MCF-7R). The mechanism of both the drug-induced apoptosis would be the future study.

5.2 METHODOLOGY

5.2.1 Materials

Polyethylenimine (PEI), curcumin, tetraethyl orthosilicate (TEOS) were purchased from Alfa Aesar (Ward Hill, MA, USA). IMDM (Iscove's Modified Dulbecco's Medium), FBS (Fetal bovine serum), Trypsin-EDTA were purchased from Gibco BRL (Waltham, MA USA). All tissue wares are purchased from Greiner (Bahlingen, Germany). The chemicals such as cetyl trimethyl ammonium bromide (CTAB), water soluble tetrasodium salt WST-1, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide), paraformaldehyde (PFA), and PI (Propidium Iodide) were purchased from Sigma (Bengaluru, India).

5.2.2 Synthesis of mesoporous silica nanoparticle (MSNAP)

MSNAP was synthesized as described previously in section 4.2.2. Briefly, H₂O, HCl, formamide, CTAB, TEOS was used in a molar ratio of 100:7.8: 10.2: 0.11: 0.13 and magnetically stirred. Initially, 2.4 ml of concentrated HCl, 4 mL formamide and 0.4 g CTAB was mixed in conical flask with deionized water and stirred at 600 rpm for 40 h. 0.3 ml TEOS was added dropwise to this mixture and incubated for another 18 h. The obtained white
precipitate was refluxed in concentrated HCl and MeOH (1:20) overnight for the removal of the template. MSNA was coated with 0.3% of 10 kDa PEI dissolved in EtOH (MSNAP) [139].

### 5.2.3 Dual drug loading on MSNAP

Curcumin and doxorubicin alone and in combination were loaded on MSNAP. The 5 mM concentration of doxorubicin and 10 mM curcumin was mixed in ethanol and incubated in a shaker along with 10 mg/mL MSNAP for 12 h. 10 mM curcumin was added to 10 mg/mL MSNAP along with 5 mM doxorubicin in a shaker for 12 h. Drug loading was confirmed by its absorbance at 420 nm and 595 nm in microplate reader (Biotek, Model FLx800, Vermont, USA). Dual drug loaded MSNAP was further subjected for their characterisation.

### 5.2.4 Characterisation of MSNAP

Curcumin and doxorubicin loaded MSNAP was analysed with SEM and EDAX. Dual drug loaded MSNAP was dispersed in ethanol and placed on a carbon paper and dried. A zeiss scanning electron microscope (SEM) with 20 KV with an adjacent with Energy Dispersive X-ray (EDX) was used to characterize MSNAP structure and its elemental content.
5.2.5 Cell culture

MCF-7 cells were cultured in T25 flask with IMDM growth medium and supplemented with 10% FBS. Cultures were maintained in 5% CO$_2$ at 37 °C till they attain 70% confluence and then these cells were split with 0.5% trypsin-EDTA. The stock of curcumin and doxorubicin was dissolved in ethanol and the required concentrations were obtained by dissolving the stock in IMDM media.

5.2.6 Development of doxorubicin resistant (MCF-7R) cell lines

MCF-7 cells were treated with an initial doxorubicin concentration of 1 µM for 48 h. Treated cells were replaced with fresh media and were incubated further till the plate reached confluence. Similarly, the doxorubicin resistant MCF-7 cells were acquired by treating with an increasing concentration of 2.5, 5, 7.5, 10, 15, 20, 25 and 30 µM of doxorubicin in MCF-7 cells.

5.2.7 Cell viability

Concentration of curcumin and doxorubicin which induces 50% of cell death was determined by cell viability assay with MTT assay. Briefly, 7,000 cells of MCF-7 and Dox-resistant MCF-7 cells were grown separately on 96 well plate for 48 h. Curcumin with concentrations ranging from 20-100 µM and doxorubicin concentration ranging from 10-100 µM and 50-300 µM was treated to MCF-7 and MCF-7R for 24 h. 5 µL of MTT was added to these plates and was further incubated for 2 hours and the plate was read at 595nm.
Similarly, MSNAP with curcumin and MSNAP with doxorubicin were incubated with MCF-7 and MCF-7R for 72 hours followed by addition of 5 µL of WST. The plate was read at 450 nm. Concentration of curcumin and doxorubicin loaded on MSNAP was from 20-100 µM and 50-300 µM respectively. IC\textsubscript{50} value was determined from formula \((\text{absorbance of treated sample} / \text{absorbance of the sample}) \times 100\).

### 5.2.8 Flow cytometry

MCF-7 and MCF-7R cells were grown on 12 well plate and treated with IC\textsubscript{50} concentrations of curcumin, doxorubicin, and CUR-MSNAP, DOX-MSNAP for 24 h and 72 h respectively. After incubation, cells were trypsinized and made to single cell suspension. These cells were incubated with 5 µg/mL of PI for 10 min. before analysing in flow cytometry. PI positive cells were gated with band pass FL3.

### 5.2.9 Combinatorial effect

Similar to viability assay and flow cytometry assay for curcumin and doxorubicin, MCF-7 and MCF-7R cells were treated with combination of curcumin and doxorubicin at its predicted IC\textsubscript{50} values and also sub IC\textsubscript{50} values for 24 hours. Percentage of viable cells were determined with MTT assay. Also, MSNAP loaded with combination of curcumin and doxorubicin at its IC\textsubscript{50} and sub IC\textsubscript{50} values was determined for its anti-cancer effect against MCF-7 and MCF-7R for 72 hours.
Combination of curcumin and doxorubicin loaded MSNAP (CUR-DOX-MSNAP) treated cells were subjected to flow cytometry analysis. Concentration of curcumin and doxorubicin was loaded on MSNAP at its IC$_{50}$ values and sub IC$_{50}$ values. Cells which taken up PI were gated. Auto fluorescence exhibited by cells were neutralized by incubating the cells with 0.5% trypan blue for 20 min before the PI staining. FL3 band pass was used to quantify the fluorescence of PI. Combinatorial effect drug was calculated from the formula AB/A*B. AB – effect of drug combination, A- effect of drug A compared to control, B- effect of drug B compared to control. The resulting value less than 1 indicate its synergistic effect, value equal to 1 indicate additive effect and value greater than 1 represents antagonist effect.

**5.2.10 Statistical Analysis**

All results were analysed as the mean ± standard error of the mean (SEM) values. Statistical analysis was approved by using Graph pad prism 5 (Graph pad software, San Deigo, CA, USA). A significance level of P value is less than 0.001, 95% confidence interval was used to compare data with control and sample.
5.3 RESULTS

5.3.1 MSNAP characterisation

The synthesised CUR-DOX-MSNAP was characterized for its structural and functional properties with SEM and EDAX. SEM images (Fig. 5.1A) revealed that MSNAP has the unique cone headed cylindrical shape. MSNAP synthesized has both micro and nanoparticle of 2 µm and 20 nm respectively. Fig. 5.1B indicates unique shape of MSNAP and Fig. 1c showed the nano-sized MSNAP. CTAB removal from MSNAP with methanol and HCl by refluxing aided an increased the pore size of MSNAP for better loading compared to CTAB removal with water [139]. Fig. 1F and G revealed the nose cone cylindrical structure of MSNAP with doxorubicin and curcumin. The rough surface in Fig. 5.1F and G, indicated the loading of curcumin and doxorubicin on MSNAP. Unloaded MSNAP had a smooth surface compared to drug loaded MSNAPs (Fig. 5.1B).

Elemental analysis with EDAX of MSNA (Fig. 5.2A) revealed that oxygen peak at 0.5 KeV with an atomic percentage of 55.10%, Silica peak at 1.75 KeV with atomic percentage of 16.34, Nitrogen peak at 0.4 KeV with atomic percentage 20.41. EDAX data of MSNAP also indicated that presence of silica and oxygen from SiO₂, nitrogen from PEI. EDAX graph of MSNAP loaded with curcumin and doxorubicin (Fig. 5.2B) revealed oxygen atomic percentage was 33.67 with peak at 0.5 KeV, Nitrogen atomic percentage was 6.56 with peak at 0.4 KeV, Silicon atomic percentage was 8.84 with peak at 1.75 KeV and carbon atomic percentage was 50.28 with peak at 0.27 KeV. And an additional peak of ruthenium at 2.6 KeV with atomic percentage of 0.17 indicated the presence of doxorubicin. Increased carbon atomic percentage in Fig. 5.2B, revealed the contribution of carbon atoms from curcumin and
doxorubicin. Presence of ruthenium ion and increased carbon atomic percentage confirms the presence of curcumin and doxorubicin loaded on MSNAP. Similarly decrease in atomic percentage of nitrogen; silica and oxygen in CUR-DOX-MSNAP compared to MSNAP indicate these molecules were involved in interaction with curcumin and doxorubicin on its surface.

**Figure 5.1:** SEM images of MSNAP and Cur-Dox-MSNAP: A – C SEM MSNAP images of 10 µm, 1 µm, 200 nm. D-I. SEM images of MSNAP with curcumin and doxorubicin with cone headed cylindrical structure.
Figure 5.2. EDAX of MSNAP and Cur-Dox-MSNAP: A. EDAX graph of MSNAP with N, C and Si peaks. B. EDAX graph of MSNAP with doxorubicin and curcumin with N, C, Si and an additional Ru representing doxorubicin.
5.3.2 Dual drug loading

Dual drug loaded on MSNAP was confirmed by analysing the absorbance at 420 nm and 595 nm in nanodrop. The MSNAP, CUR-MSNAP, DOX-MSNAP and CUR-DOX-MSNAP nanoparticles were dispersed in ethanol before measuring its absorbance. MSNAP with curcumin cargo showed the maximum absorbance of 0.8 at 420 nm (Fig. 5.3) and a minimum absorbance (0.1) at 495 nm. MSNAP with doxorubicin showed maximum absorbance (0.802) in the red region corresponding to the ruthenium molecule and has a minimum absorbance (0.09) at 420 nm. The absorbance of curcumin and doxorubicin in dual drug loaded MSNAP was around 0.683 and 0.693 respectively. There was a decrease in absorbance (0.78) in MSNAP with curcumin and doxorubicin compared to MSNAP with single loaded drug was due to shift in wavelength on dual drug loading. However, an increased absorbance of 0.09 was observed in MSNAP with curcumin at 495 nm and MSNAP with doxorubicin at 420 nm due to the overlap of absorbance range of both these drugs.

5.3.3 Curcumin and doxorubicin-induced cell death in MCF-7 and MCF-7R cells

The concentration of curcumin and doxorubicin which induces 50% of cell death was determined by MTT assay. IC$_{50}$ of 50 µM curcumin and 100 µM doxorubicin were solubilized in media containing MCF-7 cells (Fig. 5.4). The MCF-7R cells was treated with different concentrations of curcumin (25-100 µM) and doxorubicin (50 – 350 µM) The IC$_{50}$ value concentration of curcumin which induces of cell death was 75 µM (Fig. 5.5A) and doxorubicin induced
50% of cell death was 250 μM in MCF-7R (Fig. 5.5B). These results clearly depicted MCF-7R cells required 1.5 fold of curcumin and 2.5 fold of doxorubicin for induction of 50% cell death when compared with normal MCF-7 cells. Therefore, drug resistant cell lines require more drug to induce apoptosis of cells and simultaneously increased side effects of breast cancer patients.

**Figure 5.3:** Absorbance of MSNAP with dual drug loaded at 420nm and 485nm. Absorbance at 420nm indicates CUR loading and absorbance at 495nm indicates DOX loading.
**Figure 5.4:** Effect of curcumin and doxorubicin on MCF-7. Curcumin at 50 µM concentration inhibits 50% of cell viability, Doxorubicin at 100 µM concentration induces 50% of cell death, combination of doxorubicin (100 µM) and curcumin (50 µM) reduces 70% of cell viability.

### 5.3.4 MSNAP cargo with curcumin and doxorubicin induce cell death in MCF-7 and MCF-7R cells

Both MCF-7 cells and the DOX resistance MCF-7 (MCF-7R) cells were treated with MSNAP, Curcumin loaded-MSNAP (CUR-MSNAP) and DOX loaded-MSNAP (DOX-MSNAP) for determining their efficacy on induction of apoptosis at 72 h by WST assay. The 50µM and 75 µM CUR-MSNAP induced 50% of cell death in MCF-7 and MCF-7R cells respectively (Fig. 5.6A&B). 150 µM DOX-MSNAP induce 50% of cell death in MCF-7 250 µM DOX-MSNAP induces 50% of cell death in MCF-7R cells. The DOX-MSNAP induces 1.6 fold more cell death than DOX-treated in MCF-7 cells. Nanoparticle formulated
doxorubicin was able to sensitize MCF-7R at a lower concentration compared to its soluble formulation.

5.3.5 Determination of cell death by flow cytometry

Percentage of cell death was analysed with fluorescence assisted cell sorter. 30 µg/mL of Cur-MSNAP and 30 µg/mL of DOX-MSNAP at its IC$_{50}$ concentrations was incubated with MCF-7 and MCF-7R cells for 72 h. The 30 µg/mL CUR-MSNAP induced apoptosis nearly 34% of cells and 30 µg/mL DOX-MSNAP induced cell death nearly 30% of MCF-7 cells (Fig. 5.7). Similarly, CUR-MSNAP induced 25% of cell death whereas DOX-MSNAP induced 49% of cell death in the MCF-7R cells (Fig. 5.8).
**Figure 5.5:** IC$_{50}$ concentration determination of resistant MCF-7R cells: Graph of IC$_{50}$ concentration of curcumin and doxorubicin on MCF-7R cells. (A) IC$_{50}$ of curcumin against MCF-7R 75 µM. (B) IC$_{50}$ of doxorubicin against MCF-7R 250 µM.
Figure 5.6: IC$_{50}$ of Cur-MSNAP and Dox-MSNP: (A) Cur-MSNAP IC$_{50}$ was found to be 75 µM against MCF-7R and (B) Dox-MSNAP IC$_{50}$ was 150 µM.
**Figure 5.7:** Dead cell induced by Cur-MSNAP and Dox-MSNP percentage determination by FACS: Flow cytometry graphs of MSNAP, MSNAP+CUR and MSNAP+DOX. PI stained percentage of MCF-7 cells were 30% for MSNAP with DOX and 34% for MSNA with CUR.
Figure 5.8: MCF-7R dead cell percentage determination by FACS: Flow cytometry graphs of MSNAP, MSNAP+CUR with 25% of dead cells, MSNAP+DOX with 48% of dead cells.
5.3.6 Combinatorial effect of curcumin and doxorubicin co-loaded on MSNAP:

Avoidance of toxicity and side effects of anticancer agents was achieved by combining two drugs with lesser dosage. The combined effect of curcumin and doxorubicin interaction with cancer and assess their cell death in MCF-7 and MCF-7R through cell viability assay and flow cytometry analysis. The IC$_{50}$ concentration of 25 µM curcumin and 50 µM doxorubicin were co-loaded on MSNAP and determine their synergistic effect on MCF-7 and MCF-7R cells. The CUR-DOX-MSNAP treated cells showed decreased cell death from 50% to 40% when compared with curcumin and doxorubicin alone treated MCF-7 cells (Fig. 5.9). The 75 µM CUR-MSNAP had 52% of live cells and 150 µM DOX-MSNAP had 54% of live cells on treatment with MCF-7R (Fig. 5.10). Also, sub IC$_{50}$ concentration of curcumin (37 µM) and doxorubicin (75 µM) loaded MSNAP has the cell survival to 50%.

Flow cytometry analysis of MCF-7 cells treated with MSNAP co-loaded with 50 µM curcumin and 100 µM doxorubicin showed 80% percentage of cell death (Fig. 5.11). MCF-7 cells treated with CUR-MSNAP showed 30% and DOX-MSNAP showed 34% of cell death. But dual drug loaded MSNAP treated MCF-7 cells showed 80% of cell death and the sub IC$_{50}$ concentration of these both drugs loaded on MSNAP induced 50% of cell death. Similarly, FACS determination of cell death in MCF-7R cells revealed that 75 µM CUR-MSNAP and 150 µM DOX-MSNAP induced cell death of 82%, and MSNAP with sub IC$_{50}$ loading of both the drugs induced 78% of cell death (Fig. 5.12).
**Figure 5.9:** Combinatorial effect of MSNA loaded cur and dox on MCF-7 viability: Combination of cur and Dox on MCF-7 reduced the cell survival compared to cur and dox alone on MCF-7 cells.

**Figure 5.10:** Combinatorial effect of MSNA loaded cur and dox on MCF-7R viability: Enhanced reduction of cell survival in MSNA loaded with CUR and DOX.
Figure 5.11: Synergistic effect of CUR-DOX-MSNAP on MCF-7 dead cell percentage: MSNAP+CUR (50)+DOX (100) had 80% of cell death. (B) Sub IC$_{50}$ conc of CUR (25) and DOX(50) loaded on MSNAP induce 50% of cell death on MCF-7 cells.
Figure 5.12: Synergistic effect of CUR-DOX-MSNAP on MCF-7R dead cell percentage: MSNAP+CUR (50)+DOX (100) had 82% of cell death. (B) Sub IC₅₀ concentration of CUR (25) and DOX (50) loaded on MSNAP induce 78% of cell death on MCF-7 cells.
5.4 DISCUSSION

Development of resistance to chemotherapy was one of the major challenges in anti-cancer therapy. Resistant cells were used as a model to study the mechanism of drug resistance in cancer both \textit{in vitro} and \textit{in vivo} system. Nowadays combining drugs and nanoparticles mediated delivery of anti-cancer drugs were the widely used strategies to overcome drug resistance. In the present study, we developed DOX-resistant MCF-7 at their clinically relevant concentration of 25 µM doxorubicin. The cells were initially treated with lower concentration of doxorubicin and the resistant pool of cells were selected and administered with a higher concentration of the doxorubicin. MCF-7 cells IC$_{50}$ with DOX is 100 µM but the IC$_{50}$ value of acquired resistance of MCF-7R is 250 µM. Fold of resistance was calculated from the formula (IC$_{50}$ of resistance cells/ IC$_{50}$ of normal cell). Resistant cells have acquired 2.5 fold resistance compared to the normal cells.

A difference in the percentage of cell death was observed in MTT and flow cytometry. The concentration which induces 50% of cell death was 100µM with MTT assay but the same cells on FACS quantification yield only 31%. Viability assay with MTT quantifies all the live cells including the cells which are quotient, but PI stains only the cells which have disrupted plasma membrane resulting in an increased percentage of live cells by MTT compared to flow cytometry.

Combination of two drugs against cancer would result in increased anticancer effect with lower concentration of drug thus minimizing the toxicity [126]. Combining two anticancer drugs may result in synergistic, additive or antagonistic effect. When the combinatorial effect of the drugs was higher compared to the sum of the individual drugs the combination of drug results in
synergism, when the outcome of drug combination was equal to the sum of the individual drugs the results in additive effect. On contrary, antagonism is the effect of drug combination resulting in lesser effect compared to the sum of individual drugs [23]. More than additive effect, synergism implemented by anticancer drugs has gained significant importance [68].

Calculated combinatorial effect of curcumin and doxorubicin loaded on MSNAP indicates the synergistic effect with value of 0.38 for its IC$_{50}$ concentration loaded on MSNAP and sub IC$_{50}$ concentration resulted in 0.93 for MCF-7 cells. A potential synergism was observed between curcumin and doxorubicin in their soluble form and also in their nanoformulation with MSNAP loaded with both curcumin and DOX.

5.5 CONCLUSION

Acquiring resistance of cancer cells against drugs is one of the major drawbacks of chemotherapy in cancer. Changing the formulation of drug was one the adapted methods to overcome the drug resistance. Anticancer drug doxorubicin was conjugated with the nanoparticle MSNAP along with curcumin and its efficacy was analysed against MCF-7 and MCF-7R cells. MSNAP mediated delivery of doxorubicin sensitize the acquired resistance of MCF-7R. In addition to this, combining curcumin and doxorubicin on MSNAP executed synergistic effect against MCF-7 and MCF-7R. CUR-DOX-MSNAP effectively overcomes the resistance of MCF-7R with lesser drug loading yet with increased cell death.
6. SUMMARY

Mesoporous silica nanoparticle are efficient drug carriers with adjustable pore size, shape and surface functionality. But toxicity exhibited by these particle limit their usage in drug delivery system. Characteristics of nanoparticle like their size, shape influence its biological activity. Variation in the shape of the nanoparticle from spherical to non-spherical particle reduce the toxicity of the nanoparticle. Non-spherical MSN was synthesized and drug delivery potential was compared with the spherical particles in MCF-7 with curcumin. Though the spherical molecule (MCM-41) loaded with curcumin induce cell death in MCF-7 cells, non-spherical particles was able to induce cell death with lesser curcumin loaded on it.

Further, the non-spherical MSN enters the cell faster, release drug intracellularly in a sustained manner, and was less toxic compared with spherical particle. Non-spherical MSNs were localized in cytoplasm but the spherical molecule accumulates in auto-phagosome which reveals its cytotoxic effect. Intracellularly released curcumin in MCF-7 cells induces the activation of caspase 3, 12, 9, 6 and PARP and downregulated the proteins involved in cell survival. Further, mechanism of apoptosis was mediated by degradation of mitochondria.

Resistance of cancer cells complicates the anti-cancer treatment options. Non-spherical MSN loaded with curcumin and doxorubicin induce synergistic effect on cell death in the doxorubicin resistant MCF-7. Thus the mechanism of drug resistance like drug modification, drug efflux was efficiently nullified by intracellular targeting drug.
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