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ADVANCES IN TUMOR-TARGETED THERAPY USING NANOMEDICINE

By

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M.S., Pharmaceutical sciences

A Thesis Submitted to the Faculty of the School of Medicine of the
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in Pharmacology and Toxicology

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University of Louisville

Louisville, KY

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A Thesis Approved on

July 31st, 2017

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DEDICATION

This thesis is dedicated to my beloved husband, my sweet son and to my parents for their continuous support, encouragement and love.

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It is my pleasure to express my gratitude to my mentor Dr. Ramesh Gupta for his unwavering support, patience, guidance, and encouragement. His true passion for science and unflinching work ethic commitment has inspired me in developing as a person going forward. I would also like to thank my committee members Dr. Jun Yan, Dr. Carolyn Klinge and Dr. Radha Munagala for their comments and assistance. I express my special thanks to Dr. Radha Munagala and Dr. Ashish Agarwal for their valuable suggestions and assistance during the preparation of this thesis. I would also like to thank my lab members – Dr. Farrukh Aqil, Jeyaprakash Jeyabalan, Al Hassan Kyakulaga and Ashley Marie Mudd for their continuous support during the course of my laboratory work.

ABSTRACT

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Divya Karukonda

July 31st, 2017

Despite continuous improvement and significant progress made in diagnostic and therapeutic approaches for cancer, it is still the leading cause of death worldwide. Although conventional chemotherapy has made significant advances in improving patient survival the indiscriminate destruction of normal cells leads to severe side effects and poor clinical outcomes. Thus, there is a need for effective delivery of drugs to the tumor site avoiding normal tissues to reduce toxicity in the rest of the body. For this reason, a novel multidisciplinary field called Nanotechnology has evolved in recent years and advances in this field have contributed to the development of nanoscale materials to overcome the lack of specificity of conventional chemotherapeutic agents for optimized cancer therapy. Nanoparticles can be designed to preferentially target the tumor site and deliver high drug payloads by either passive or active targeting. Passive targeting exploits the preferential drug accumulation in tumor cells through enhanced permeability and retention (EPR) effect. On the other hand, active

targeting uses functionalized nanoparticles to carry a drug to the specific site. This targeting strategy is becoming a new standard in cancer treatment. A selective and tumor site-specific treatment can be achieved by using various ligands such as aptamers, antibodies, peptides, and small molecules. Targeting nanocarriers serve as a highly promising strategy for effective cancer treatment, as shown by encouraging results in many recent studies. This thesis highlights the diversity of nanoparticle types, targeting mechanisms and active targeting strategies. I will also discuss an emerging field of nano drug delivery using biological nanovesicles called exosomes. Finally, I will discuss the current clinical status of nanoparticle formulations.

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CHAPTER I

INTRODUCTION

Cancer and therapy

Cancer is the leading cause of death worldwide despite continuous improvement in the therapeutic strategies and early detection. Cancer incidence has been increasing in recent decades and American Cancer Society estimates that the number of new cases are projected to increase by 50% worldwide from 14 million in 2012 to 22 million by 2030. In 2017, 1,688,780 new cancer cases and 600,920 cancer deaths are estimated to occur in the United States (1). Cancer is a complex disease caused by uncontrolled growth and division of abnormal cells due to gene mutations. As a result of mutational changes, cancer cells exhibit certain characteristics including proliferation, resistance to signals that inhibit their growth and resistance to apoptotic signals that cause cell death, which make it difficult to treat the disease (2). Cancer cells interact with the microenvironment to acquire different capabilities during the multistep development of a tumor particularly, overcome immune response for survival, activate stromal cells to inappropriately promote angiogenesis, and invade through tissues, and metastasize to distant organs induced by tumor microenvironment components (3).

Standard treatment for cancer includes combinations of surgery, radiation and chemotherapy (chemo). Other treatment options include hormonal therapy and targeted therapy (including immunotherapy such as monoclonal antibody therapy). The choice of therapy depends upon the location, grade of the tumor and the stage of the disease. Over the last decade a number of potent anticancer drugs have been developed with various mechanisms of action such as blocking nucleic acid biosynthesis, interfering with gene transcription, causing cell cycle arrest, inducing apoptosis, and inhibiting angiogenesis (4).

Limitations of conventional therapy

Conventional chemotherapy that targets DNA is very effective and has resulted in improved survival rates of cancer patients. However, it has several limitations such as poor solubility, poor selectivity, non-specific drug distribution, fast clearance rate, systemic toxicity, multi-drug resistance, cancer reoccurrence, off- target effects along with severe side effects (5). These limitations pose a significant challenge in the effective treatment of cancer. Most of the chemotherapeutics in the market at present such as doxorubicin, paclitaxel, and vincristine have less selectivity toward the target and are systemically distributed without selective localization to site of tumor. Thus, higher doses are required to achieve pharmacological levels at the target site and this leads to increased toxicity to the normal tissues causing severe side effects. An example of this is anthracycline drug causing cardiotoxicity, severe in some cases (6). In order to avoid toxic side effects, chemo drugs are often given at lower doses, which are

less than the required doses resulting in subsequent failure of therapy accompanied by development of drug resistance and metastatic disease.

Hence, eradication of cancer still remains a major problem due to its heterogeneous nature and inability of chemotherapeutics to reach the tumor site without damaging the normal healthy tissues.

Effective solution

Delivering drug to the disease site is a major hurdle for many of the diseases including cancer. Because of the limitations noted above there has been enormous interest in developing an innovative technologies that can deliver drug at the target site. Over a century ago Paul Ehrlich introduced a concept of “Magic Bullet” for targeted drug delivery (7). It has two entities: the first one is that the drug should recognize the target and the second is that the drug should provide therapeutic action at the targeted site. Cell- or tissue-specific targeting is achieved by encapsulating a drug and targeting moiety in an appropriate pharmaceutical carrier which is the revised version of Ehrlich magic bullet (8). Nanoparticles can be designed including all three entities and could be used as both therapeutics and diagnostics. A number of studies show that most of the limitations of conventional drugs can be overcome by nanotechnology and that nanoparticles as carriers have huge potential to overcome the limitations of chemotherapeutics (9, 10).

Nanotechnology in cancer

Nanotechnology involves use of nanometer scale materials and systems by controlling the matter on a level of atomic, molecular and supramolecular scale. (11-14). The size of nanomaterials is around 10-100 nm and their unique size is ideal for increased solubility, oral bioavailability, rate of dissolution, surface area, high therapeutic loading and rapid onset of therapeutic action upon intracellular uptake (15). In contrast, conventional drugs are rapidly cleared from the body, reducing the amount of drug at the tumor site (16). Nanocarriers with drugs incorporated increase the half-life of drugs in circulation, allowing a greater amount of drug to reach the target site (11). Anticancer drugs in nano formulations exhibit enhanced therapeutic index due to improved pharmacokinetics, tissue distribution, and enhanced accumulation or release of the drug at the tumor site (17). The nano-sized particles exhibit more extravasation and permeability into tumor tissues with leaky vasculature in contrast to neo-vasculature of normal tissues, minimizing off-target toxicities, and enhancing delivery to site of action. Their small size also facilitates oral, nasal, ocular, and parenteral routes of drug administration. Thus, nanoparticle drug delivery systems can serve as the successful tools to anticancer therapy.

A variety of nanostructures have been investigated such as synthetic biodegradable polymers, lipids (liposomes), mesoporous silica nanoparticles (MSNs), micelles, quantum dots, carbon nanotubes, and gold nanoparticles for the treatment of cancer (18-22). A summary of their properties is presented in Table (1) (23)

Liposomes, first discovered by Dr. Alec Bangham in 1961 (24), and are extensively explored as the nanocarriers for the targeted drug delivery. A separate field of liposomal technology research was started by the approval of first nano drug - Doxil^R which is a big hit in the market. The field of liposomal literature is only focused on liposomes without the term nano until 2000. Liposomes are closed vesicles surrounded by a lipid bi-layer membrane composed of phospholipids. Their hydrophilic core can be used for the entrapment and delivery of water-soluble drugs. These vesicles are uni- or multi-lamellar and have a potential to carry both hydrophilic and lipophilic molecules entrapped within the lipid bilayer. Availability of liposomes with diverse properties makes them the most intelligent drug carrier systems available (25)

Polymeric micelles are nano-sized vectors that contain amphiphilic block copolymers which assemble to form nanoscopic core-shelled colloidal structures termed micelles. Their advantage is in trapping drugs physically within the hydrophobic cores or linking drugs covalently to component molecules of the micelle. Additionally, they proved to be an excellent novel drug delivery system due to their high stability in physiological conditions, high loading capacity, and high accumulation of drug at target site (26).

Dendrimers are a class of polymeric materials. First discovered in the early 1980's by Donald Tomalia and colleagues (27), these hyper-branched, tree-like, structured polymeric molecules originate from the Greek word *dendron*, meaning a tree. As the chains growing from the core molecule become longer and more branched, they adopt a globular structure. Dendrimers become

densely packed as they extend out to the periphery, forming a closed membrane-like structure. Their sizes range between 1.9 nm and 4.4 nm, the smallest nanocarriers so far developed. Dendrimer-drug interactions or drug loading in dendrimers may be achieved by various approaches: simple encapsulation in the interior of dendrimers (illustrated in Fig. 1) involves electrostatic interactions and covalent conjugations to the surface of the dendrimers. They serve as an ideal carrier for drug delivery due to several advantages, for example, they can be modulated for target-specific drug delivery, have a defined molecular weight, are of a small size, and have good entrapment efficiency, thus offering a good surface for functionalization (28).

The general term nanoparticles (NPs), describes a wide range of nano systems including organic polymeric NPs, composed of synthetic or natural polymers or proteins (i.e, albumin), solid lipid nanoparticles comprising of physiological lipids, as well as inorganic NPs such as semiconductor NPs, iron oxide NPs, quantum dots and gold NPs (29)

Polymeric nanoparticles are widely investigated nanoparticles in clinical trials, and received much attention after the initial work of Langer and Folkman in 1976 (30). Because of their biodegradability, biocompatibility, high drug loading, stability and flexibility, polymeric nanoparticles are used for controlled release of drug. They usually consist of a general core-shell structure and are also subdivided into various categories according to their basic chemical and core shell composition and their morphology, including nanocapsules (NCs) and nanospheres (NSs). Nanocapsules are hollow spherically-shaped vesicular

particles, where the drug is confined to a hollow core, usually composed of oil droplets, which is surrounded by a polymeric shell or membrane (31). Nanospheres are solid colloidal matrix systems, ideally uniform in their core-shell polymer partition, where a drug is dispersed or dissolved in the polymer matrix (32) (Fig. 1). Various synthetic and natural polymers currently being investigated for the design and potential applications of nanoparticles are polyethylene glycol (PEG), poly lactic acid (PLA) and poly D,L-lactide co-glycolide) (PLGA) and their copolymers PEG-PLA, PEG-PGA, PLGA and PEG-PLGA; these nanocarriers are the most widely investigated synthetic polymers for drug and gene delivery (33-38).

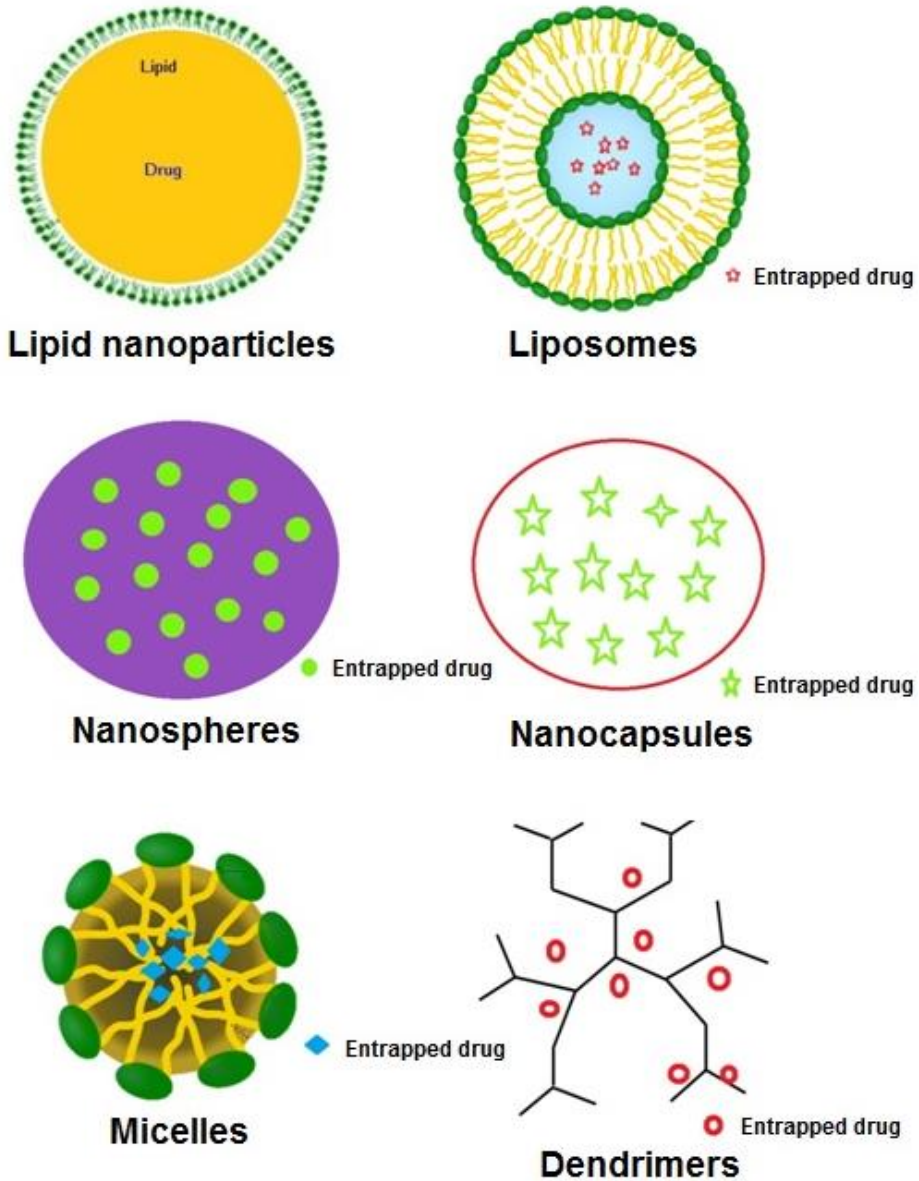


Figure 1. Basic structure of nanoparticles used for cancer therapy entrapped with drug (Source : With permission from Katayoun *et al.*, 2015 Active-targeted Nanotherapy as Smart Cancer Treatment) (39).

Table 1

Summary of characteristics and representative applications of various nano systems

Nano particle	Size(nm)	Characteristic properties	Applications	Ref
Carbon-nanotubes	0.5-3	Cylindrical tube of crystal carbon sheets, Biocompatible	Drug, gene and peptide delivery	(23)
Dendrimers	<10	Highly branched synthetic polymeric structures, low polydispersity, Biocompatible	Controlled drug delivery	(28)
Liposomes	50-100	Phospholipid bilayered vesicles, Biocompatible and good entrapment efficiency	Passive and active drug delivery of drugs, gene, peptides and others	(25)
Polymeric micelles	10-100	Hydrophobic core wrapped by single layer of hydrophilic polymers, high drug entrapment, payload, biostability	Active and passive drug delivery, cell specific targeting	(26)
Polymeric NPs	10-1000	Biodegradable, Biocompatible, complete protection of drug	Site selective delivery, excellent carrier for controlled and sustained drug delivery	(23)
Gold	<100	Small size with large surface area, biocompatible	Hyperthermia, drug delivery, diagnostic assay, radiotherapy enhancement	(40)

Despite the variety of nanomaterials designed for tumor targeting, only a limited number of formulations are clinically approved (Table 4). Treatment efficacy is often impeded by nonspecific drug distribution and lack of specificity to the target tissue site. Ideally, enhancing drug accumulation at the site of tumor will lower the systemic exposure and result in a more efficient and patient-friendly treatment. Several drug-targeting strategies can be engaged to reach target tissues. These include active and passive targeted drug delivery which are described in Chapter II. Targeted delivery of anticancer agents is a rapidly evolving and is a highly promising field of research. Indeed, targeted drug delivery potentially increases the local concentration of the fraction of the systemically administered dose reaching the tumor site, minimizing toxicity to the adjacent healthy cells. A particular focus has been the active targeting of nano drug delivery systems for the treatment of cancer because of the discovery of new molecular targets, a deep understanding of biology of cancer, and the failure of conventional treatment. Together, these lead to the enormous interest in developing tumor targeted nanomedicine for the development of novel drug delivery systems.

CHAPTER II

DRUG TARGETING MECHANISMS

The key to success in cancer treatment is the therapeutic concentration at the tumor site. The concentration of therapeutic agent reaching the tumor tissue should be precise after crossing and penetrating all the biological barriers in the body. Once the drug is at the active site, it should selectively destroy the cancer cells, avoiding healthy tissues to reduce adverse effects and toxicity. To achieve these goals, nanoparticle drug delivery systems use the characteristics of the disease tissue to target their payloads. The drug-loaded nanoparticles reach the tumor site by two of the principal mechanisms: passive and active drug targeting.

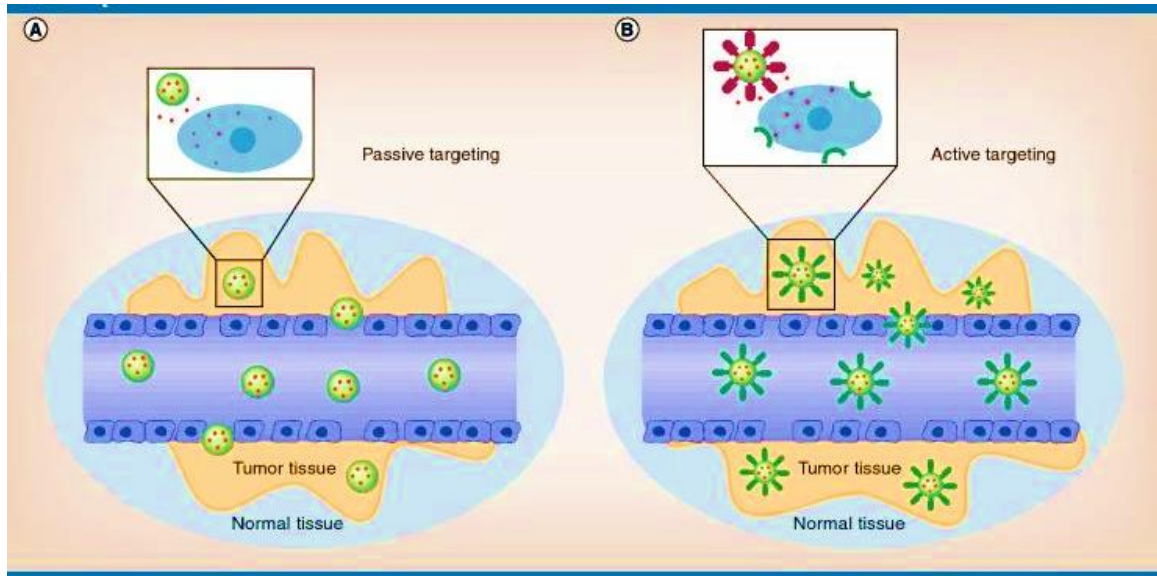


Figure 2. Passive and active targeting to enhance permeability and retention. , Nanoparticles (NPs) can be passively extravasated through leaky vascularization, allowing their accumulation at the tumor region (A). In this case, drugs may be released in the extracellular matrix and then diffuse throughout the tissue or tumor. Active targeting (B) can enhance the therapeutic efficacy of drugs by increasing accumulation and cellular uptake of NPs through receptor-mediated endocytosis. (Source: With permission from Suwassa *et al.*, A focus on nanoparticles as drug delivery system) (41).

Passive drug Targeting

Nanoparticles drug delivery systems use pathophysiological characteristics of the tumor vasculature through the enhanced permeability and retention (EPR) effect. The EPR concept was originally described by Maeda *et al.*, 1986 and this theory is based on the characteristics of tumor vasculature of leaky blood vessels and lack of lymphatic drainage (42). This allows the diffusion of longer circulating nanoparticles to the tumor site, avoiding health tissues, and thereby being selectively site-specific (43, 44). Most passive-targeting nanoparticles are surface-coated with PEG for biocompatibility, *e.g.*, SP1049C, Genexol- PM, NK911 (45, 46) in early clinical trials for treating various types of cancer.

However, high heterogeneity of the EPR effect in tumors which varies from patient to patient and within same subject is a significant limitation to this strategy (47). The determination of precise impact of the EPR effect on nanoparticle accumulation in tumor tissues becomes difficult since a variety of parameters including size, shape, and zeta potential of nanoparticles are involved in this process. Only a small part of injected dose is accumulated in target cancer tissues which becomes a significant restriction in passive drug strategy (42). In view of these limitations of passive targeting a considerable amount of work is done and focused on developing active drug-targeting strategies.

Active drug Targeting:

Active drug targeting is aimed at delivery of active drug selectively to the tumor site. Active drug-delivery strategies comprise use of a targeting ligand or moiety attached on the surface of nanocarrier, which recognizes and enables the nanoparticle to bind to receptors (tumor-specific epitope) overexpressed on tumor cells. These receptors serve as tumor markers which are either expressed at lower levels or essentially not expressed on normal cells. The interaction between ligand and receptor is affected by binding affinity and selectivity of the targeting unit and by the targeted receptor's capacity (48). Receptor levels depend not only on synthesis and stability, but also on recycling rate after receptor activation and internalization (49). Hence the binding affinity of the targeting ligand and number of ligand units conjugated and decorated on the surface are the important factors affecting targeting efficiency. To target the nanoparticles effectively to the desired site it is essential to have sufficient quantity of ligands/targeting moieties along with high affinity binding to cell surface receptors (50). Therefore, the most important feature of the targeting ligand is to induce receptor-mediated endocytosis causing the internalization of the drug carrier into the desired tumor tissue specific intracellular site (51, 52).

Currently there are many approaches available for active targeting to tumor cells. All the ligands that can be attached to the nanoparticles can serve as targeting moieties. There are wide variety of tumor-targeting moieties including small molecules, peptides, monoclonal antibodies, aptamers and

nucleic acids which specifically recognize receptors overexpressed on tumor cell surface which will be discussed in Chapter III.

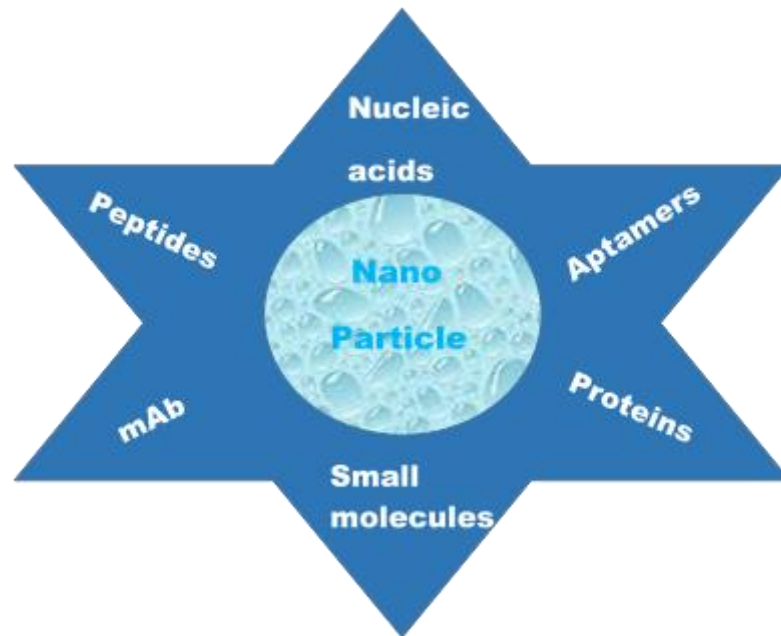


Figure 3. Types of ligands decorated on surface of nanoparticles for tumor targeting.

CHAPTER III

TYPES OF LIGANDS FOR ACTIVE TARGETING

The identity and characteristics of the targeting ligands are extremely important for circulation time, cellular uptake, affinity, and extravasation. Targeting ligands can be broadly classified as proteins (mainly antibodies and their fragments), nucleic acids (aptamers), or other small molecules (peptides, vitamins, and carbohydrates).

Monoclonal Antibodies (mAb)

Targeting cancer with a mAb was described by Milstein in 1981 (53). mAbs bind to a receptor on the cell surface to induce several antibody-based anticancer mechanisms including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cellular toxicity (CDC) (54, 55). The feasibility of antibody-based tissue-targeting has been clinically demonstrated with 17 different mAbs approved by the US Food and Drug Administration (FDA) (56). The mAb rituximab (Rituxan) was approved in 1997 for treatment of patients with non-Hodgkin's lymphoma — a type of cancer that originates in lymphocytes (57). A year later, Trastuzumab (Herceptin), an anti-HER2 mAb that binds to ErbB2 receptors, was approved for the treatment of HER2+ breast cancer (58). The first angiogenesis inhibitor for treating colorectal cancer, Bevacizumab (Avastin), an anti-VEGF mAb that inhibits the growth of new blood

vessels, was approved in 2004 (59). Today, over 200 delivery systems based on antibodies or their fragments are in preclinical and clinical trials (60). Recent developments in the field of antibody engineering have resulted in the production of antibodies that contain animal and human origins such as chimeric mAbs, humanized mAbs (those with a greater human contribution), and antibody fragments. However, there are several limitations to this approach including immunogenicity, large size, and cost of mAb synthesis, rapid clearance, and environmental factors. The use of antibody fragments like Fab and single chain variable fragments reduce the immunogenicity by keeping high antigen binding specificity (52).

Aptamers

Aptamers are another emerging class of targeting ligands which are short single-stranded RNA or DNA sequences of oligonucleotides that can be designed as targeting ligand capable of binding to target receptors on the surface of cancer cells with high selectivity and affinity (61). They form unique three-dimensional structures with high ligand binding specificity needed for target affinity. They are small size (~15 KDa), less immunogenic when compared to antibodies, and can be chemically synthesized. Several aptamers have been developed to bind specifically to receptors on cancer cells, and can be considered suitable for nanoparticle-aptamer conjugate therapy (62). Docetaxel (Dtxl)-encapsulated nanoparticles with aptamer (targets the antigen on the surface of prostate cancer cells) functionalized surface were delivered with high selectivity and efficacy *in vivo* (63). Similarly, doxorubicin encapsulated

DOTAP nanoparticles functionalized with DNA-based aptamer demonstrated a significant reduction in tumor growth in a tumor xenograft model (64). RNA-based aptamers have also been developed which can selectively bind to the T cell factor 1 and beta catenin in colon cancer cells (65-67). Locked nucleic acid-modified aptamers (LNA) used in iron oxide saturated lactoferrin nanocarriers demonstrated improved survival rate in colon cancer xenograft (68). RNA-A10 aptamer PMSA (prostate specific membrane antigen) has also been reported for better therapeutic efficacy (69-71).

Protein/peptides

A variety of proteins/peptides have been investigated for tumor targeting. Several endogenous proteins which bind specifically to cell surface receptors have been used for targeting purposes (72). For example, transferrin, a protein involved in transport of iron, binds specifically to transferrin receptors which are overexpressed in variety of malignancies. Choi *et al.* showed that transferrin decorated PEGylated gold nanoparticles accumulated specifically in cancer cells avoiding nonspecific transport of nanoparticles to the healthy cells (73, 74). Muthu *et al.* demonstrated the enhanced efficacy of transferrin-functionalized vitamin E-based micellar nanosystems in MDA-MB 231 breast cancer cells (18). Jain *et al.* showed that transferrin-based nanosystems improved the antitumor activity against breast cancer cells (21). Krishna *et al.* developed a unique transferrin receptor targeting using apotransferrin protein as drug carrier for nanoparticles (75).

In addition to proteins, various peptides have also been used as targeting ligands, which are specific to the receptors overexpress on tumor cells. In order to find the best suitable peptide for targeting ligand, several peptide phage display libraries are available for identification of specific targeting ligands (76). A tumor homing penta-peptide CREKA that recognizes fibrin-associated plasma protein has been used as a targeting ligand on iron oxide nanoparticles and liposomes (77). Also penta-peptide LFC-131, an antagonist for CXCR4, a chemokine responsible for majority of inflammatory related cancers, has been used as a targeting ligand on polymeric nanoparticles for targeting CXCR4 overexpressed in cancer cells (78). Peptides have also been reported for the receptor proteins *viz.* interleukin 11 receptor α (IL-11R α) and 78 KDa glucose-regulated protein (GRP78) in prostate and breast tumors (79-81). Among the different peptides, RGD peptide is a commonly used targeting ligand, which selectively binds to $\alpha v\beta 3$, $\alpha v\beta 5$ integrin (angiogenesis markers) overexpressed in endothelial and smooth muscle cells of tumor blood vessels. In an earlier investigation by Danhier *et al.* (2009) RGD-decorated paclitaxel-loaded nanoparticles demonstrated significantly enhanced tumor growth inhibition and prolonged animal survival (82). RGD-conjugated PLGA NPs have also shown enhanced antitumor efficacy *in vivo* (83).

Small molecules

Small molecules with molecular weight less than 500 Da are a promising class of targeting ligands because of their small size, low cost of synthesis, and high stability. Pomper *et al.* identified small hydrophilic molecules from urea-based PMSA inhibitors which specifically target PMSA receptor overexpressed on the surface of prostate cancer cells (84). Chandran *et al.* developed docetaxel-encapsulated PLA/PCL-based targeted nanoparticles using PMSA as a targeting moiety (85). This moiety proved to be an efficient targeting ligand for the uptake of nanoparticles by PMSA-overexpressing cells. This small molecule is also used as a targeting ligand for the development of a novel polymeric nanoparticle BIND-014, composed of biodegradable hydrophobic PLA polymeric core and hydrophilic PEG. This is the first targeted- and controlled-release polymeric nanoparticle to reach clinical phase I trials for cancer chemotherapy (86).

Among the different targeting strategies, vitamins are another class of molecules widely investigated for tumor targeting. The vitamins employed for targeting include folate, vitamin B12, thiamine, and biotin. The principal advantages associated with vitamins, particularly folic acid, include stability (both on the shelf and in the body), relative cost (low), lack of toxicity and immunogenicity, and wide flexibility for diverse chemical reactions (87). Folic acid has been widely investigated as a ligand in targeted drug delivery (88-90). Folic acid has high affinity for folate receptors which are over expressed in many types of solid tumors such as ovarian, lung, uterine, breast, head and neck cancers

(91). Besides the different tumors, folate has also been used as targeting ligand for delivery of many drug conjugates and delivery systems including liposomes, polymeric NPS through folate receptor mediated endocytosis (92). Folic acid-functionalized PLGA nanoparticles and deoxycholic acid-o-carboxymethylated chitosan-folic acid micelles have shown enhanced efficacy of doxorubicin and paclitaxel, respectively (93, 94).

Table 2

Examples of targeting ligands used in anticancer nanoformulations

Targeting ligand	Receptor	Nanoformulation	Indication	Ref
Folate	Folate receptor	PLGA polymeric NPs- Doxorubicin	Breast cancer	(93)
Folate	Folate receptor	Deoxy cholic acid-o-carboxymethylated chitosan-folic acid micelles- Paclitaxel	Breast cancer	(94)
Folate	Folate receptor	Chitosan polyplex liposomes- Nucleic acid	Melanoma	(95)
RGD	$\alpha v\beta 3, \alpha v\beta 5$ integrin receptors	RGD modified liposomes-paclitaxel	Hepatocellular carcinoma	(96)
Transferrin	Transferrin receptor	Lipid coated PLGA Nps- Aromatase inhibitor	Breast Cancer	(97)
Transferrin	Transferrin receptor	Vit E TPGS micelles- Doxirubicin	Breast Cancer	(18)
Transferrin	Transferrin receptor	PLGA polymeric NPs- Methotrexate	Brian cancer	(21)
EGFR	EGFR receptor	DSPE-PEG lipid polymeric complex	Hepatocellular Carcinoma	(98)
EGFR	EGFR receptor	Poly(lactic acid-co-l-lysine) nanoparticles	Hepatocellular Carcinoma	(99)
Fibrin associated plasma proteins	Clotted Plasma proteins of tumor vessels	CREKA conjugated liposomes-Doxirubicin	Breast Cancer	(77)
Aptamers	Tumor DNA	DOTAP Liposomes- Doxirubicin	Breast Cancer	(100)
Aptamers	CD133	PEGYLATED PLGA NPs	Hepatocellular Carcinoma	(101)
Aptamers	EGFR receptor	Triple function RNA NPs	Triple-negative Breast Cancer	(78)

Bioconjugation for surface-functionalization of nanocarriers

In spite of the availability of a wide variety of targeting ligands, surface functionalization remains a challenge. The major requirement for surface functionalization is the presence of a targeting ligand on the NPs surface until the active load is delivered to the target site. To make the functionalization stable over the NP surface, a conjugation strategy that covalently links the ligands over the surface of the NPs by using simple chemistry was established (102, 103). The selection of the appropriate conjugation strategy is an important step, as the chemicals used for the conjugation may affect the targeting ligand during the process of conjugation (104). The most commonly used covalent conjugation approaches are through amide linkages, which link carboxyl group to amine using carbodiimide chemistry. It occurs by activation of carboxylic group present on the NP surface by EDC (1-ethyl-3-(3-dimethyl amino propyl) carbodiimide and NHS (N-hydroxysuccinimide) forming reactive intermediate which couples with amine groups present in targeting ligand.

The chemical conjugation approach has been reported by Kocbek *et al.* to functionalize the PLGA NPs by using Mb as targeting ligand and by Acharya *et al.* who developed the nanoparticle bioconjugate by using epidermal growth factor (EGF) as A targeting ligand (105, 106). In addition to the use of carboxylic and amino groups, thiol functional groups have also been reported to form disulfide bonds for surface functionalization (107). Thiol group can react with other thiol group to form disulfide bond and also react with maleimide group to form thioether groups. Shaik *et al.* used the similar concept of forming disulfide

bond to conjugate anti-My9 antibody to stealth liposomes (107). Similarly, several other reports also demonstrated disulfide bond formation as a conjugation strategy between maleimide processing NPs and thiol-bearing ligands and *vice versa* (83, 104, 108). The highly specific, non-covalent reaction between avidin and biotin has been used to functionalize avidin containing liposomes with biotinylated antibodies (109). Other reaction complexes with streptavidin and neutravidin are also in public domain for conjugation (110). Although these non-covalent binding techniques are available, the immunogenic reactions due to the source of avidin make this approach the second choice after covalent conjugation (111).

'Click' chemistry is another interesting technique to conjugate targeting moieties to NPs (112). The use of click reactions became prevalent because of their high efficiency, specificity, ease of availability of reagents, low nonspecific binding, and physiological stability as compared to traditional crosslinking carbodiimide chemistry. Click chemistry is a single step reaction carried out under mild conditions in aqueous solutions producing high yield of product. It involves reaction between azide and alkyne under various conditions and the major classes of reactions involved are copper-catalyzed azide-alkyne cycloaddition (CuAAC), Strain-promoted azide-alkyne click chemistry (SPAAC) and Tetrazine-trans-cyclooctene (TCO) ligation (113, 114). Koo *et al.* have reported the biorthogonal copper free click chemistry for tumor targeted delivery of nanoparticles (115).

Exosome-mediated drug delivery as an emerging nanomedicine approach

Exosomes are lipid bilayer biological nanoparticles secreted by all the cells in the body, present in almost all the body fluids, and play an important role in cell-to-cell communication (116-119). Exosomes are emerging as potential drug delivery nano vehicle (Figure 4). Exosomes have the advantages of being less immunogenic and showing better biological tolerability and cellular internalization compared with synthetic NPs (118, 120-122). There is a growing interest in exploiting these biological NPs for delivering chemotherapeutics and genetic material to the tumor site. With this growing interest, surface functionalization of exosomes for selective delivery of chemotherapeutics and siRNAs to cancer cells have been reported (123-125). Exosomes isolated from different sources and the different small molecules delivered are summarized in Table 3.

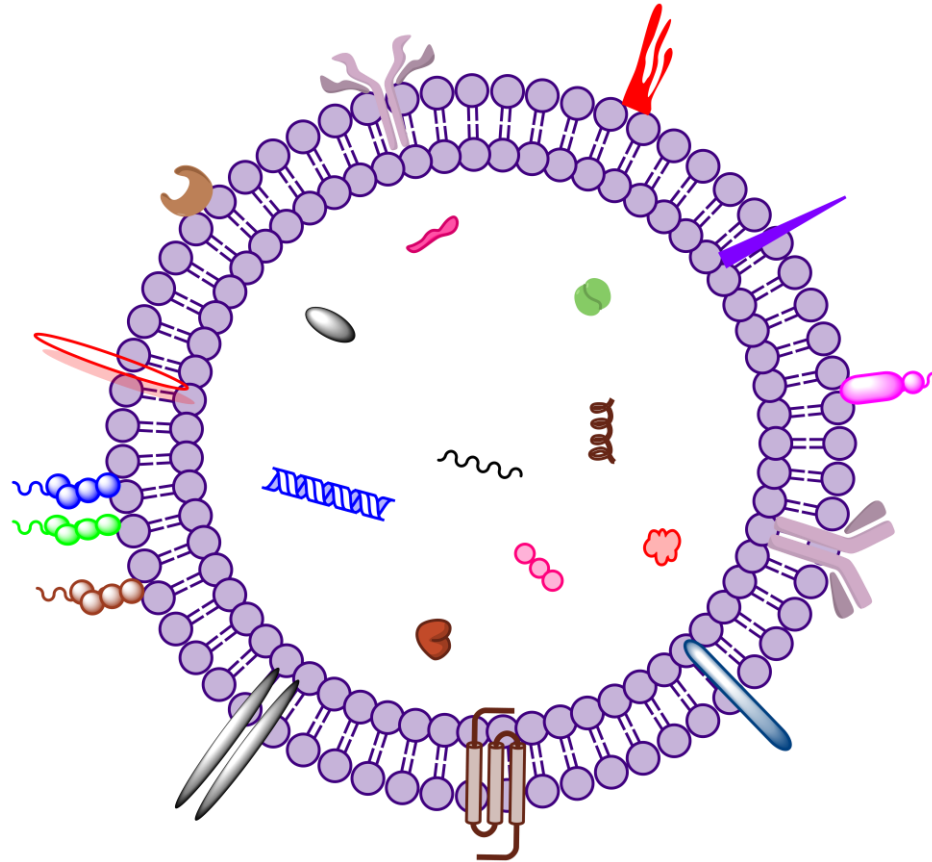


Figure 4. Diagram depicting the structure of exosome carrying lipid, DNA, RNA and protein (Source: With permission from Munagala *et al.*, 2016 Bovine milk derived exosomes for drug delivery) (126).

Table 3

Therapeutic applications of Exosomes as nanocarriers

Source	Cargo	Target cancer type	Outcome	Ref
Bovine milk	Wittheferin- A	Breast and Lung cancer	Tumor growth inhibition	(126)
Bovine milk	Celastrol	Lung cancer	Tumor growth inhibition	(127)
Bovine milk	Paclitaxel	Lung cancer	Tumor growth inhibition	(128)
Mouse Dendritic cells	Doxorubicin	Breast cancer	Tumor growth inhibition	(129)
Breast cancer cells	Doxorubicin	Breast and Ovarian cancer	Tumor growth inhibition	(130)
Prostate cancer cells	Paclitaxel	Prostate cancer	Increased cytotoxicity	(131)
Machrophage	Paclitaxel	Lung cancer	Tumor growth inhibition	(132)
Mesenchymal stem cells	mir-122	Hepatocellular carcinoma	Tumor growth inhibition	(133)
Mesenchymal stem cells	mir-143	Osteosarcoma	Inhibition of migration	(134)
Monocytic cell	c-Myc siRNA	Lymphoma	Induction of apoptosis	(135)
HEK 293 cell	PIK-1 siRNA	Bladder cancer	Induction of apoptosis	(136)

The field of exosomes as NPs is rather young and its use as a nanocarrier to deliver cytotoxic drugs and phytochemical compounds has recently been explored with encouraging results (126, 127, 129, 130, 137-139). For example, Tian *et al.*, 2014 and Srivastava *et al.*, 2016 reported enhanced efficacy of doxorubicin exosomal formulations (129, 138). Moreover, some studies have shown enhanced therapeutic efficacy of cytotoxic drugs and natural compounds while encapsulating these compounds in exosomes (127, 130, 131, 137, 139). Saari *et al.* showed that cytotoxic effect of paclitaxel to prostate cancer cells increased when encapsulated in exosomes (131). Milk derived exosomes have been reported to enhance therapeutic response of withaferin A (126) and celestrol (127), as well as to enhance the stability and therapeutic response of anthocyanidins (139) in various cancers. Zhuang *et al.* showed that the exosomal formulations of curcumin inhibited LPS-induced inflammation in a mouse model compared with free curcumin and reported their positive effects against brain tumor when given through intranasal route (137).

Due to their high complexity and variable composition, the cell specificity of these exosomes is not predictable, leading to non-specific targeting. Such off-target effects can be minimized through active targeting by functionalizing extracellular vesicles with targeting ligands. Exosomes are functionalized in several ways by decorating with specific ligands to improve the targeting ability (129, 140, 141). Folic acid-functionalized milk-derived exosomes were shown to enhance the therapeutic efficacy of withaferin A against lung cancer *in vitro* and *in vivo* (126). The peptide i-RGD, which is specific to the $\alpha\beta 3$ integrin receptor

that overexpressed in cancer cells, has been reported to fuse to the exosomal membrane proteins and lipids, *i.e.*, Lamp2b and glycosylphosphatidylinositol (142). Alvarez *et al.* showed siRNA delivery to mouse brain by functionalizing exosomes with RVG peptide by fusing with Lamp2b exosomal membrane protein. These RVG exosomes could bind to specific receptor overexpressed in brain tumors. Tian *et al.* used the same mechanism to deliver doxorubicin to breast cancer cells (129). Chemical conjugation techniques, similar to those used for NPs, have also been used for the functionalization of exosomes (143). Kooijmans *et al.* reported a post insertion technique for exosome functionalization. In this technique, EGFR nanobody-conjugated PEG phospholipid micelles were mixed with extracellular vesicles derived from Neuro 2A cells (143). Click chemistry has also been used for making functionalized exosomes (144).

Clinical status

To date only a handful of nanoformulations are FDA approved and available for clinical use. Liposomal formulations like Doxil[®], Myocet, DaunoXome, Depocyt, polymeric nanoparticles such as Abraxane[®], and polymeric micelles like Glenexol-PM are FDA approved. The majority of the FDA-approved nanomedicines were developed based on passive targeting which utilizes the EPR effect, due to the leaky vasculature of the tumor. There are certain functionalized nanoformulations which have been specifically designed to undergo ligand-mediated targeting selective to tumor site. The clinical status of novel nanoformulations has been summarized in Table 4.

MCC-465 is a novel PEGylated liposomal formulation encapsulating doxorubicin tagged with human monoclonal antibody fragment F(ab') in Phase I clinical trial against metastatic stomach cancer (145). Recently, a PEGylated liposomal formulation of doxorubicin functionalized with F (ab') fragment of antibody cetuximab (C225) was approved for clinical use (146). Liposomal formulation of oxaliplatin (SGT53) functionalized with single chain antibody fragment (TfRscFv) as targeting ligand is in a Phase I of clinical trial (147). Cyclodextrin-based nanoparticles (CALAA-01) is the first nanoformulation in clinical trial for the siRNA delivery to tumor site (148). Heat-activated PEGylated liposomes containing doxorubicin (Thermodox) is in Phase III clinical trial for treating liver cancer (149). Similar to liposomal formulations, some polymeric nanoparticles are at different stages of clinical trials. PEG-poly(aspartic acid) polymeric nanoparticles like NK 105 and NK 911, and PEG-cyclodextrin nanoparticles like CRLX101 are in phase I and phase II clinical trials (150). Targeted-polymeric nanomedicines like BIND-014, PEGylated PL(G)A docetaxel formulation has completed the phase I and is now in Phase II clinical trials (86).

Table 4

Summary of nanoformulations in market and clinical development

Nano Carrier type	Product Name	Formulation	Drug	Indication	Clinical Status
Liposomes	Doxil® (Caelyx® in EU)	PEGylated liposome	Doxorubicin	Breast cancer, ovarian cancer, multiple myeloma, Kaposi's sarcoma	Approved
	Myocet®	Non-PEGylated liposome	Doxorubicin	Breast cancer	Approved
	DaunoXome®	Non-PEGylated liposome	Daunorubicin	Kaposi's sarcoma	Approved
	DepoCyt®	Non-PEGylated liposome	Cytarabine	Lymphomatous meningitis, leukaemia, glioblastoma	Approved
	Lipoplatin	PEGylated liposome	Cisplatin	Various malignancies	Phase III
	S-CKD602	PEGylated liposome	CKD-602	Various malignancies	Phase I/II
	NL CPT-11	PEGylated liposome	Irinotecan (CPT-11)	Glioma	Phase I
	CPX-1	Liposome	Irinotecan	Colorectal cancer	Phase II
	LE-SN38	Liposome	SN-38	Colorectal cancer	Phase II
	MBP-426	Tf-NGPE-liposome	Oxaliplatin	Various malignancies	Phase II

	MCC-465	Human antibody fragment (GAH)-PEG-liposome	Doxorubicin	Gastric cancer	Phase I
	Thermodox™	Heat-activated PEGylated liposome	Doxorubicin	Liver cancer, breast cancer	Phase III
	CPX-351	Liposome	Cytarabine+daunorubicin	Acute myeloid leukaemia	Phase II
	SGT-53	Transferrin-targeted DNA plasmid liposome	P53 gene	Various solid malignancies	Phase I
Polymeric nanoparticles	Abraxane® (ABI-007)	Albumin-bound nanoparticle (nab)	Paclitaxel	Breast cancer	Approved
	BIND-014	PEG-PLGA nanoparticle	Docetaxel	Various solid malignancies	Phase I
	Docetaxel-PNP	Polymeric nanoparticle	Docetaxel	Various solid malignancies	Phase I
	CRLX101	Cyclodextrin-PEG nanoparticle	Camptothecin	Various malignancies	Phase II
	CALAA-01	Cyclodextrin-PEG-transferrin-nanoparticle	Anti-RRM2 siRNA	Various solid malignancies	Phase I
Polymeric micelles	Genexol-PM®	PEG-PLA micelle	Paclitaxel	Breast cancer, lung cancer, ovarian cancer	Approved
	NK911	PEG-PAA micelle	Doxorubicin	Various solid malignancies	Phase III
	NK105	PEG-PAA micelle	Paclitaxel	Gastric cancer	Phase II

	NC-6004 (Nanoplain™)	PEG-polyglutamic acid micelle	Cisplatin	Pancreatic cancer	Phase II
	NK012	PEG-PGA micelle	SN-38	Various solid malignancies	Phase II
	SP1049C	P-glycoprotein micelle	Doxorubicin	Various malignancies	Phase II
	Paical®	Polymeric micelle	Paclitaxel	Ovarian cancer	Phase III
	NC-4016	Polymeric micelle	Oxaliplatin	Various solid malignancies	Phase I
Polymer-drug conjugate nanoparticles	Oncaspar®	PEG-drug	L-asparaginase	Leukaemia	Market
	PK1 (FCE28068)	HPMA-drug	Doxorubicin	Breast cancer, lung cancer, colorectal cancer	Phase II
	DOX-OXD (AD-70)	Dextran	Doxorubicin	Various malignancies	Phase I

CHAPTER IV

CONCLUSION

The rapid advances in the field of nanomedicine have created a new trend and opened the doors for the development of different tools and strategies for anticancer therapy. Nanoparticle drug formulations have the potential to overcome the limitations of conventional chemotherapy by their ability to selectively target cancer cells over healthy tissue. Properly designed nanoparticles have the ability to accumulate in tumors either by passive or active targeting and enhance the cytotoxic effects of antitumor agents. Several nanoformulations of anticancer drugs are being evaluated in phase II/III clinical trials while relatively few have been approved for clinical use. Nanotechnology provides an opportunity to revisit and reformulate the drugs that have been shelved due to poor oral bioavailability, lack of selectivity to the desired target, or extreme toxicity. Biological nanoparticles, *i.e.*, exosomes, provide another promising avenue for delivery of small and macromolecules. Nevertheless, the field of nanotechnology has the potential to shift the paradigm of treatment for cancer with an ever-growing arsenal of non-targeted and targeted nanomedicines.

Future perspective

In spite of various advantages of NPs, efficient delivery of drugs has never been completely achieved due to lack of ideal drug delivery system. The major limitations being low biocompatibility and toxicity. Their characterization, cost, scalability, inability to evade host immune system, limited circulation time and safety issues still remain as a challenge (151). Exosomes seem to overcome several of the limitations associated with the conventional nanoparticles. They have ability to target tissues by utilizing the intrinsic mechanisms of extracellular vesicles. Exosomes are biocompatible, potentially nontoxic, less immunogenic, and provide desired long-term safety for therapeutic use. They have the natural ability to carry nucleic acids and other therapeutic molecules cross membranes that are difficult to cross such as the blood brain barrier (BBB). There are several recent studies published which isolated exosomes from different sources like biological fluids and cell culture media using variety of strategies (129, 142, 152). However, they still suffer from biocompatibility and scalability issues. Particularly cost-effective, mass-scale production, drug loading and targeting strategies are limitations, which lessen the throughput of this field. More recent development of milk exosomes seem to overcome many of these limitations (126-128, 139). However, immune-compromised subcutaneous xenograft mouse models used in these studies have some limitations as the formulations are not being tested in tumor microenvironment.

Tumor growth is complex and heterogeneous microenvironment consist of different immune cells. So it is crucial to develop nanoformulations that can

adapt to the microenvironment and improve the selective targeting to tumors. Apart from few, most of the formulations have not yet been in clinic considering this aspect. The studies need to be performed with more sophisticated humanized mouse models (i.e., patient-derived tumor xenograft models) and also in different immunocompetent animal models (such as spontaneous tumor models, carcinogen-mediated tumor models) which take tumor microenvironment into consideration. This will better match the system of human disease and create a wide scope for clinical translatability.

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Education

Graduate Student

2015 - Present

Department of Pharmacology and Toxicology

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Masters in Pharmaceutical Sciences

2012 - 2014

Jawaharlal Nehru Technological University, India

Bachelor of Science in Pharmacy

2008 - 2012

Rajiv Gandhi University of Health sciences, India

Awards and Honors

IPIBS Integrated Programs in Biomedical Sciences (IPIBS); 2015-2017

Fellowship award, University of Louisville, Kentucky

Best intern student - Dr.Reddy's Pharmaceutical Industry, Hyderabad, India
Summer- 2013

Best player of the chess tournament

National level round 3- 2007

Award from Ministry of State of Andhra Pradesh as player of the tournament

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Abstracts

Milk-derived exosomes – a platform nanocarrier to enhance anti-proliferative, anti-inflammatory and anti-cancer activities of small drug molecules against multiple human cancers Ramesh C. Gupta, Farrukh Aqil, Manicka V. Vadhanam, , Jeyaprakash Jeyabalan, Ashish K. Agrawal, Ashley M. Mudd, Alhassan Kyakulaga, Divya Karukonda, Wendy Spencer and Radha Munagala

Conferences and Workshops

Attended National Poster Symposium on Organic/ Medicinal Chemistry as a delegate, conducted by Royal Society of Chemistry (London)-Deccan section (India) at IICT, Hyderabad, India.

Workshop on innovation in life sciences (Sept 2011) Bangalore, India.

3rd World Congress on Biotechnology (Sept 2012) Hyderabad, India.

Paper Presentations

Presented a paper on “Drug Discovery and Development Process” in DRAVYAKA’11, a National level seminar held at Geethanjali College of Pharmacy, Hyderabad

Presented a paper on “Pharmaceutical Engineering” in j-TALENT’10, a National contest on “Emerging Technologies” held at Jyothishmathi College of Pharmacy, Hyderabad

Motto of Life:

“Everyone in this world is born to accomplish a specific task. Forget about the failures, instead search for the true purpose of your existence”

- Dr. A.P.J. Abdul Kalam