Chapter 2
Literature Review

2.1 Cancer

Cancer is a leading cause of death worldwide viz. characterized by uncontrolled growth of abnormal cells in body. Worldwide deaths, going to rise from 7.6 million deaths in 2008 to 13.2 million by 2030 as per world health organization (WHO) (1). As the normal body cells grow, divide into new cells, and die in well-ordered manner but instead of dying, cancer cells continue to grow and multiply in an uncontrolled manner to form tumors. Cancer cells often travel to other parts of the body, where they begin to grow in uncontrolled manner and form new tumors that replace normal tissue viz. referred to as metastasis. No matter where a cancer may spread, it is always named for the place where it started. For example, breast cancer that has spread to the liver is still called breast cancer, not liver cancer. Extremely slow progress in cancer diagnosis and treatment options owing to dose related side effects, lack of tumor specificity and effective intracellular delivery, poorly predictive preclinical models, development of drug resistance etc. leads to poor prognosis in patients with common metastatic tumors such as breast, prostate, lung and gastrointestinal cancers (2).

2.2 Breast Cancer

Breast cancer is a malignant tumor that starts in the tissues of the breast that can grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body. Most breast cancers begin in the cells that line the ducts called as ductal carcinoma. Some begin in the cells that line the lobules called as lobular carcinoma while a small number start in other tissues (3).

2.2.1 Normal Breast

To understand breast cancer it's crucial to have some basic knowledge about the anatomy of the breasts which is shown in **Figure 2.1.** The breast is made up primarily of lobules (milk-producing glands), ducts (tiny tubes that carry the milk from the lobules to the nipple), and stroma (fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels).

2.2.2 Lymphatic System of the Breast

The lymph system has several parts that are vital to know because it is one way through which breast cancers can spread. Breast cancer cells can enter lymphatic vessels and begin to grow in lymph nodes. Most lymphatic vessels in the breast connect to lymph

nodes under the arm (axillary nodes).Some lymphatic vessels connect to lymph nodes inside the chest (internal mammary nodes) and those either above or below the collarbone (supraclavicular or infraclavicular nodes) shown in **Figure 2.2.** If the cancer cells have spread to lymph nodes, there is a higher chance that the cells could have also gotten into the bloodstream and spread (metastasized) to other sites in the body (3).

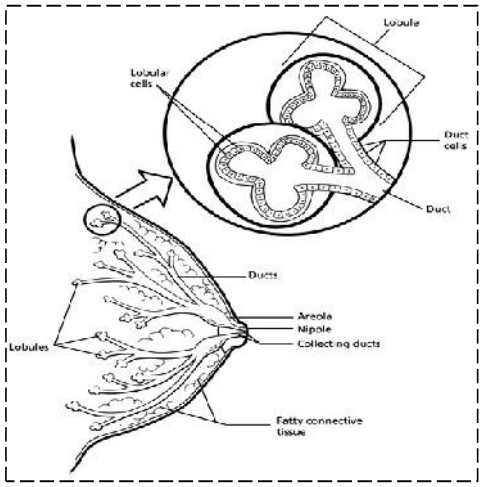


Figure 2. 1 Normal Structure of the Breast

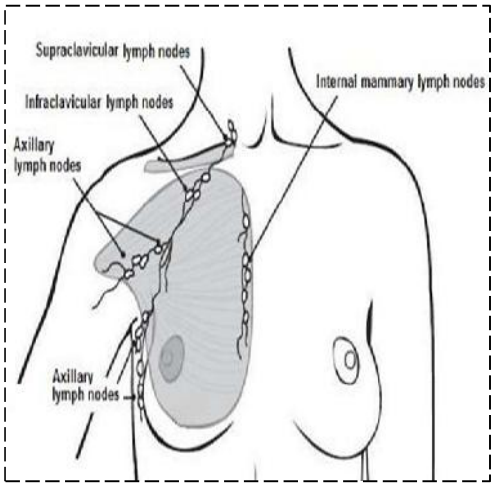


Figure 2. 2 Lymphatic System of the Breast

2.2.3 Breast Cancer Pathophysiology

Although normal cells are protected by several protein clusters and pathway, they commit suicide (apoptosis) when they are no longer needed. PI3K/AKT and RAS/MEK/ERK are the protective pathways and sometimes the genes along these protective pathways are mutated in a way that turns them permanently "on", rendering the cells incapable of committing suicide when they are no longer needed. This is one of the causes of cancer in combination with other mutations. Normally, the PTEN protein turns off the PI3K/AKT pathway when the cell is ready for suicide and mutation in gene of PTEN protein leads to PI3K/AKT pathway stuck in the "on" position, and the cancer cell does not commit suicide. Overview of signal transduction pathways involved in apoptosis shown in **Figure 2.3** and mutations leading to loss of apoptosis can lead to tumorigenesis.

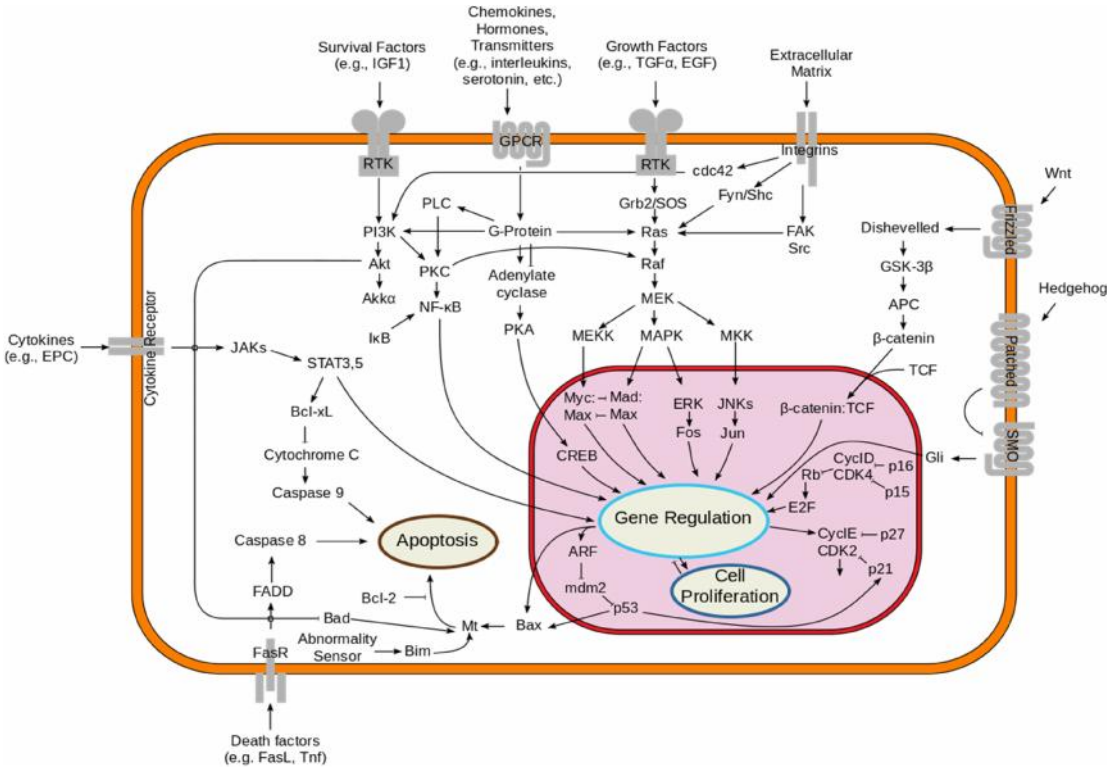


Figure 2. 3 Overview of signal transduction pathways involved in apoptosis

2.2.4 Breast Cancer Terminologies

- **Carcinoma:** Cancer that begins in a tissue that lines the inner or outer surfaces of the body i.e. epithelial cells of organs like breast. Nearly all breast cancers are carcinomas (either ductal carcinomas or lobular carcinomas).
- **Adenocarcinoma:** Cancer that develops in the glandular tissues (tissue that makes and secretes a substance) of the body. The ducts and lobules of the breast are glandular tissues (they make breast milk), so cancers starting in these areas are often called adenocarcinomas.
- **Carcinoma in situ (non-invasive or pre-invasive):** Cancerous growth or tumor is still confined to the site from which it started, and has not spread to surrounding tissue or other organs in the body. In breast cancer, in situ means that the cancer cells remain confined to ducts (ductal carcinoma in situ). The cells have not grown into (invaded) deeper tissues in the breast or spread to other organs in the body. When cancer cells are confined to the lobules it is called lobular carcinoma in situ.
- **Invasive (infiltrating) carcinoma:** Grown beyond the layer of cells where it started (as opposed to carcinoma in situ). Most breast cancers are invasive carcinomas either invasive ductal carcinoma or invasive lobular carcinoma.

- **Sarcoma:** Sarcomas are cancers that start in connective tissues such as muscle tissue, fat tissue, or blood vessels. Sarcomas of the breast are rare.

2.2.5 Types of breast cancers

There are several types of breast cancer as mentioned in **Figure 2.4**, but some of them are quite rare. In some cases a single breast tumor can be a combination of these types or be a mixture of invasive and in situ cancer (3).

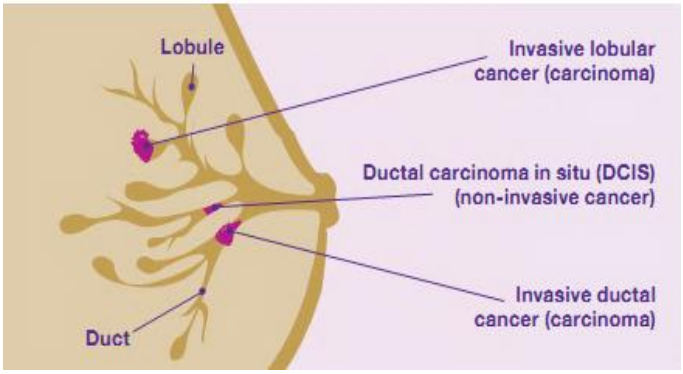


Figure 2. 4 Types of Breast Cancer

- **Ductal carcinoma in situ (DCIS) or Intraductal carcinoma:** Most common type of non-invasive breast cancer.
- **Lobular carcinoma in situ (LICS):** Least common type of non-invasive breast cancer.
- **Invasive (or infiltrating) ductal carcinoma (IDC):** Most common type of breast cancer. Invasive (or infiltrating) ductal carcinoma starts in a milk duct of the breast, breaks through the wall of the duct, and grows into the fatty tissue of the breast. At this point, it may be able to spread (metastasize) to other parts of the body through the lymphatic system and bloodstream. About 8 of 10 invasive breast cancers are infiltrating ductal carcinomas.
- **Invasive (or infiltrating) lobular carcinoma:** Invasive lobular carcinoma (ILC) starts in the milk-producing glands (lobules). Like IDC, it can spread (metastasize) to other parts of the body. About 1 invasive breast cancer in 10 is an ILC. *Invasive lobular carcinoma may be harder to detect by a mammogram than invasive ductal carcinoma.*
- **Less common types of breast cancer:** Inflammatory breast cancer is uncommon type of invasive breast cancer accounts for about 1-3% of all breast cancers. Triple-negative breast cancer term is used to describe breast cancers (usually invasive ductal

carcinomas) whose cells lack estrogen receptors, progesterone receptors, and do not have an excess of the HER2 protein on their surfaces. Paget disease of the nipple is type of breast cancer starts in the breast ducts and spreads to the skin of the nipple and then to the areola (the dark circle around the nipple). It is rare, accounting for only about 1% of all cases of breast cancer. Phyllodes tumor is very rare breast tumor develops in the stroma (connective tissue) of the breast, in contrast to carcinomas, which develop in the ducts or lobules. Angiosarcoma is form of cancer starts in cells that line blood vessels or lymph vessels. It rarely occurs in the breasts.

- **Special types of invasive breast carcinoma (sub-types of invasive carcinoma):** These are often named after features seen when they are viewed under the microscope, like the ways the cells are arranged. Some of these may have a better prognosis than standard infiltrating ductal carcinoma. These include adenoid cystic (or adenocystic) carcinoma, low-grade adenosquamous carcinoma (this is a type of metaplastic carcinoma), medullary carcinoma, mucinous (or colloid) carcinoma, papillary carcinoma, tubular carcinoma. Some sub-types have the same or maybe worse prognosis than standard infiltrating ductal carcinoma. These include metaplastic carcinoma (most types, including spindle cell and squamous), micropapillary carcinoma and mixed carcinoma (has features of both invasive ductal and lobular) (3).

2.2.6 Breast cancer chemoprevention

Chemoprevention is the use of drugs to reduce the risk of cancer. Several drugs have been studied for lowering breast cancer risk including tamoxifen and raloxifene that blocks the effect of estrogen on breast tissue. Aromatase inhibitors such as anastrozole and exemestane blocking the production of small amounts of estrogen that post-menopausal women normally make. Other drugs include aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen seem to have a lower risk of breast cancer. Studies have also looked to see if drugs called bisphosphonates may lower the risk of breast cancer. Bisphosphonates are mainly used to treat osteoporosis, but they are also used to treat breast cancer that has spread to the bone (4).

2.3 Treatment for Breast Cancer

Main types of treatment for breast cancer include surgery, radiation, chemotherapy, hormonal, targeted and bone-directed therapies (bisphosphonates and

denosumab) (5, 6). Treatments can be classified into broad groups, based on how they work and when they are used such as local versus systemic therapy and adjuvant versus neoadjuvant therapy.

2.3.1 Surgery for breast cancer

Most women with breast cancer have some type of surgery. Surgery is often needed to remove a breast tumor. Options for this include breast-conserving surgery (lumpectomy and quadrantectomy) and mastectomy. Breast reconstruction can be done at the same time as surgery or later on (7).

2.3.2 Radiotherapy

Radiotherapy uses high energy x-rays to destroy any cancer cells left behind in the breast area after surgery and is given to reduce the risk of the cancer returning in the breast. Radiotherapy also affects healthy cells, but they are generally able to recover and repair themselves. Damage to healthy cells can be kept to a minimum by giving small doses of radiotherapy regularly (8).

2.3.3 Chemotherapy

Chemotherapy is treatment with cancer-killing drugs given by oral and intravenous route. The drugs travel through the bloodstream to reach cancer cells in most parts of the body. Chemotherapy is given in cycles, with each period of treatment followed by a recovery period. Treatment usually lasts for several months. It is most effective when combinations of more than one drug are used. Many combinations are being used, and it's not clear that any single combination is clearly the best (9). Clinical studies continue to compare today's most effective treatments against something that may be better. Some of the most commonly used drug combinations are:

- **CMF:** Cyclophosphamide (Cytoxan®), Methotrexate, and 5-Fluorouracil (fluorouracil, 5-FU)
- **CAF (FAC):** Cyclophosphamide, Doxorubicin (Adriamycin®), and 5-Fluorouracil
- **AC:** Doxorubicin (Adriamycin®) and Cyclophosphamide
- **EC:** Epirubicin (Ellence®) and Cyclophosphamide
- **TAC:** Docetaxel (Taxotere®), Doxorubicin (Adriamycin®) and Cyclophosphamide
- **AC → T:** Doxorubicin (Adriamycin®) And Cyclophosphamide followed by Paclitaxel (Taxol®) or Docetaxel (Taxotere®). Trastuzumab (Herceptin®) may be given with the Paclitaxel or Docetaxel for HER2/neu positive tumors.

- **A → CMF:** Doxorubicin (Adriamycin®), followed by CMF
- **CEF (FEC):** Cyclophosphamide, Epirubicin and 5-Fluorouracil (this may be followed by docetaxel)
- **TC:** Docetaxel (Taxotere®) and Cyclophosphamide
- **TCH:** Docetaxel, Carboplatin and Trastuzumab (Herceptin®) for HER2/neu positive tumors

Other chemotherapeutic drugs used to treat breast cancer include cisplatin, Vinorelbine (Navelbine®), Capecitabine (Xeloda®), Liposomal Doxorubicin (Doxil®), Gemcitabine (Gemzar®), Mitoxantrone, Ixabepilone (Ixempra®), Albumin-Bound Paclitaxel (Abraxane®) and Eribulin (Halaven®). The targeted therapy drugs Trastuzumab and Lapatinib (Tykerb®) may be used with these chemotherapeutic drugs for tumors that are HER2/neu-positive.

2.3.4 Hormonal Therapy

Hormone therapy is another form of systemic therapy. It is most often used as an adjuvant therapy to help reduce the risk of the cancer coming back after surgery, but it can be used as neoadjuvant treatment, as well (10). Hormonal agents are Tamoxifen, Toremifene (Fareston®), Letrozole (Femara®), Anastrozole (Arimidex®), and Exemestane (Aromasin®), Fulvestrant (Faslodex®), Megestrol Acetate (Megace®) and Androgens (Male Hormones).

2.3.5 Targeted and Biological Therapy

As researchers have learned more about the gene changes in cells that cause cancer, they have been able to develop newer drugs that specifically target these changes (11). These targeted drugs work differently from standard chemotherapy drugs (12). They often have different (and less severe) side effects. They are most often used along with chemotherapeutic agents at this time. Trastuzumab (Herceptin®), Pertuzumab (Perjeta™), Lapatinib (Tykerb®), Everolimus (Affinitor®) and Bevacizumab (Avastin®).

2.3.6 Other agents

These agents are not used for breast cancer treatment but used to treat bone metastasis associated with breast cancer. Bisphosphonates {Pamidronate (Aredia®) and Zoledronic acid (Zometa®)(13)}, are drugs that are used to help strengthen and reduce the risk of fractures in bones that have been weakened by metastatic breast cancer.

Denosumab (Xgeva®, Prolia®) may help other systemic therapies, like hormonal and chemotherapies to work better.

2.4 Current Breast Cancer Research and Treatment

Research on the causes, prevention, and treatment of breast cancer is being done in many medical centers worldwide. Current status of research in treatment of breast cancer is given below:

2.4.1 Surgery

Presently more focus towards oncoplastic surgery (14), breast reconstruction surgery and mastectomy. Recently concept of skin, aerola and nipple sparing mastectomy has evolved in which the only the tumor is removed sparing the above mentioned structures which can further help in breast reconstitution surgery.

2.4.2 Radiation therapy

For women who need radiation after breast-conserving surgery, newer techniques such as hypofractionated radiation or accelerated partial breast irradiation may be as effective and offering a more convenient way to receive.

2.4.3 Chemotherapy

Advanced breast cancers are often hard to treat, so researchers are always looking for newer drugs. A drug class has been developed that targets cancers caused by BRCA mutations called as PARP inhibitors (15) and they have shown promise in clinical trials treating breast, ovarian, and prostate cancers that had spread and were resistant to other treatments. Recently, new drug everolimus included in class of mTOR inhibitor was developed which could prove effective in treatment of estrogen positive breast cancer.

2.4.4 Targeted and Biological therapies

Targeted therapies are a group of newer drugs that specifically take advantage of gene changes in cells that cause cancer. Recently, a new drug for patients whose cancer cells have too much HER2 receptors has been approved by the FDA. This drug, ado-trastuzumab emtansine (Kadcyla™) was formerly called TDM-1 (16). It is made up of the same monoclonal antibody found in trastuzumab attached to a chemotherapy drug known as emtansine (DM-1). In this type of antibody drug conjugate, the antibody acts as a homing device, taking the chemotherapeutic agent directly to the cancer cells.

Bevacizumab and several other anti-angiogenesis drugs are being tested in clinical trials. Everolimus (Afinitor) is a targeted therapy drug that seems to help hormonal therapy drugs. It is approved to be given with exemestane (Aromasin) to treat advanced hormone receptor positive breast cancer in post-menopausal women. The epidermal growth factor receptor (EGFR) is another protein which is expressed on the surfaces of many cancer cells. Drugs that targets EGFR mainly comprises monoclonal antibodies (cetuximab and panitumumab) and tyrosine kinase inhibitors (gefitinib, erlotinib, lapatinib, canertinib) are already in use to treat other cancers, while other anti-EGFR drugs are still considered experimental (17, 18).

2.5 Challenges in Development of Therapies for Cancer

Several challenges are involved in the development of therapies for cancer and some of the major ones include narrow therapeutic index of cytotoxic drugs and their nonspecific distribution in the body. To overcome these challenges, researchers are working on two diverse approaches which are enlightening the path in development of effective therapy for cancer. First approach is genomics and proteomics research which is assisting in the identification of new tumor specific molecular targets (19) that will aid in the synthesis of 'perfect fit' drug molecule with delicate therapeutic activity and minimum side effects. Second approach involves developing innovative drug delivery systems which include nanoparticles, liposomes, polymersomes, micelles, dendrimers, microemulsion etc. These systems can provide tumor specificity, maintain therapeutic concentration of drug for long periods of time and reduce drug related toxicities. However, these novel drug delivery systems also suffer from one or more drawbacks; specifically, low encapsulation efficiency and poor storage stability of liposomes; limited drug loading capacity and amenability for only small molecules in case of polymersomes and micelles; and small size of dendrimer causing their diffusion into unwanted regions. Proteins nanoparticulate systems have advantages over other novel drug delivery system in treatment of cancer. So, the development of protein nanoparticulate system can be a good option.

2.6 Protein Carriers for Delivery of Cancer Therapeutics

Protein carriers are emerging as good alternative to overcome the challenges resulting from unfavorable properties of cytotoxic drugs and shortcomings of novel drug delivery system in the development of chemotherapeutics. Approaches in protein carrier

based delivery of cancer therapeutics include either conjugation of drug directly to the protein or formulation of drug-protein nanoparticles. Recent understandings on identification and modification of protein structures and their physicochemical properties have significantly extended the application of proteins for the production and utilization of drug-protein conjugates in anticancer therapy. Such conjugation increases the solubility and molecular weight of drug resulting in reduce renal clearance and prolonged circulation half-life. Furthermore, multifunctional protein carriers can be designed by the introduction of different functional groups to the protein in which each group is attached at a distinct position on protein surface and a defined conjugate is gained. Protein conjugates have numerous biological applications which include (i) use as reagents for immunoassay (enzyme-labeled antibodies or antigens) (20); (ii) in immunohistochemistry to enhance or suppress the immunogenicity of an antigen (coupling of a protein antigen to different protein carriers) (21); (iii) for elucidating hormone-receptor interactions (conjugates of peptide hormone with a protein carrier) and (iv) for treatment of various diseases like cancer, viral infections and diabetes (22) etc.

Another approach comprises the preparation of colloidal systems, such as nanoparticles using proteins like albumin, gelatin, gliadin and legumin. Protein based nanoparticle system has derived its special importance due to unique features of proteins and their potential applications in biological and material fields. Protein nanoparticles are biodegradable, non-antigenic and amenable for surface modification. Such nanoparticulate systems help avoid the undesirable toxic effects of drugs by modifying their body distribution and improving their cellular uptake. Toxic effects of commercially available surfactant (i.e., Tween 80 and Cremophor EL) based formulations (Taxotere and Taxol) are also avoided along with injection site reactions of vinorelbine and anticancer drugs.

Not only the demerits associated with the use of free drug but also the biological drawbacks of the carrier mediated drug delivery systems can be eliminated by developing a delivery system with targeting potential. These in turn include (i) finding a specific target for a particular disease; (ii) finding the drug that is effective in treatment of particular disease and (iii) finding how to carry the drug to a particular site. Proteins, being composed of various amino acids, not only allow various binding sites for drugs but also for various targeting ligands. In addition, proteins themselves act as passive as well as active targeting moieties.

2.6.1 Drug-Protein Conjugates

The concept of drug-protein conjugates arose from the finding that various plasma proteins including albumin, globulin etc. can bind to various drug molecules. This in turn evolved as a development and use of these proteins as drug carriers. Initially drug-protein conjugates were synthesized by direct coupling of drug with protein i.e., azide coupling method, carbodiimide reaction etc. Afterwards, elucidation of the structural features of various proteins and identification of various specific sites on protein (cysteine-34 position of albumin, lysine side-chain amines or active sulfhydryl groups generated by inter-chain disulfide bonds of antibodies) led to the use of various peptides and synthetically modified drugs that can selectively bind to protein for development of drug-protein conjugates. Advancement of recombinant DNA technology has also made it easier to synthesize therapeutically active proteins (e.g., interferon, interleukins, etc.) and other bioactive peptides directly fused with albumin or antibodies as well as allow to insert a binding site in the antibodies that can be used for conjugation (23).

Use of such drug-protein conjugates in cancer therapy follows the fact that tumors are able to capture plasma proteins and utilize them as a source of amino acids for their growth. One study reported uptake of different proteins having molecular weight of 12-160 kDa by tumors and proteins with longer circulation half-life provide enhanced uptake in tumor. The study also showed similar tumor accumulation characteristics of albumin (66.5 kDa) and immunoglobulin (160 kDa) (24).Additionally, the tumor vasculature has the pore size ranging from 100-1200 nm and macromolecules with molecular weight exceeding 40 kDa show reduced clearance therefore proteins that hold these two criteria can be used for developing drug-protein conjugates and nanoparticulate system that will show increased accumulation in tumor tissue through EPR effect. Albumin (MW 66.5 kDa, hydrodynamic diameter of 5-7 nm), Gelatin (hydrodynamic diameter of 25-200 nm, MW ~300 kDa Daltons) and immunoglobulin (IgG) (MW 150 kDa, hydrodynamic diameter of 7-10 nm) fit into this criteria allowing themselves to be good candidates for preparing anticancer drug conjugates. Protein drug conjugates evaluated till date for anticancer therapy is given in **Table 2.1** and clinical status of antibody drug conjugates (ADCs) is given in **Table 2.2.**

Table 2. 1 Protein Drug Conjugates for Anticancer Therapy

Drug	Conjugated to	Evaluation	Remarks	Ref.
Methotexate	Albumin	*In vivo* in Walker-256 carcinoma bearing rats	• Loading (equivalents of MTX per albumin molecule) affected the tumor targeting potential (1 equivalent MTX per albumin molecule provided optimal response). • Conjugate not stoichiometrically defined.	(25)
		Preclinical in Human xenograft models SXF 1301, PRXF PC3M, SXF 1410, LXFE 409, LXFE 529, MAXF 449, BXF 1258	• Better response in soft tissue sarcoma, prostate cancer and osteosarcoma models as compared to free drug • No response of either MTX or conjugate in other cancer models	(26)
		Renal cell carcinoma, mesotheliom a (Phase I and II)	• Response to therapy was observed, but not confirmed in phase II study • Dose limiting (above 50 mg/m^2) stomatitis was observed	(27)
		Advanced bladder cancer (Phase II)	• In combination with cisplatin • One complete and one partial remission obtained	(28)

Paclitaxel	Albumin, Albumin-PEG_{2000} and Albumin-PEG_{5000}	*In vitro* (HT-29,A431, MeWo) Pharmacokinetics and acute toxicity studies in mice	• Reduced toxicity of paclitaxel after conjugation • Higher cytotoxicity as compared to native paclitaxel in all cell-lines • Improved pharmacokinetics with clearance rate in order paclitaxel>Albumin-Paclitaxel>2kDa PEG conjugate>5kDa PEG conjugate	(29)
	Albumin and Albumin-PEG_{3400}-Folate	*In vitro* (KB Fr-α +ve cells, HT-29 Fr-α -ve cells)	• Folate modified conjugates showed better activity in KB cells • Nonmodified conjugates showed better activity in HT-29 than pegylated and targeted conjugates	(30)
Doxorubicin	Albumin	*In vitro* (AH66P, and AH66DR cells)	• Improved cytotoxicity in parental cell-line • Effectiveness of conjugated doxorubicin in resistant cell-line, thus providing possibility to overcome multidrug resistance using such conjugates	(31)
	Albumin (via glutaraldehyde or dextran bridge)	*In vitro* (K562 and K562/DXR cells)	• Possibility to treat multidrug resistant cancer	(31, 32)

	Transferrin (via glytaraldehyde or dextran bridge)	*In vitro* (K562 and K562/DXR cells)	• Possibility to treat multidrug resistant cancer	(31)
	Transferrin (benzoyl hydrazone or phenyl acetyl hydrazone linker)	*In vitro* (MDA-MB-468, U937, and LXFL 529 cells)	• Benzoyl hydrazone containing conjugate showed highest activity • Acid sensitive conjugates showed tumor specificity	(33)
	Transferrin	*In vitro* (Daudi and HL-60 cells)	• The cytotoxicity of conjugate correlates with conjugate concentration and time of exposure	(34)
	Transferrin	*In vitro* (K562 cells)	• Cell killing action without intercalating into DNA • May provide activity in resistant cells, and reduce side effects	(35)
	Transferrin	*In vitro* (K562 cells and normal peripheral blood mononuclear cells)	• Higher cytotoxicity as compared to free drug	(35)
	Transferrin	*In vitro* (Lovo, HL-60, H-meso	• Conjugate showed more potency against resistant cell lines than free drug	(36)

		and Hep2 cells) *In vivo* in nude mice bearing human mesotheliom a tumors		
	Gelatin (mPEG bridge)	*In vitro* (LLC cells)	• Matrix metalloproteinase mediated release of active drug in cancer cells	(37)
Artemisinin	Transferrin	*In vitro* (Molt-4 cells) *In vivo* in breast cancer rat model	• Selective uptake by tumor cells through transferrin receptors • Conjugate releases iron and artemisinin in cells which allows reaction of both to form free radicals and provide high cytotoxicity	(38, 39)
N-alkyl isatin derivatives	Transferrin	*In vitro* (MCF-7 cells)	• Para-phenylpropionic acid linker allowed acid based hydrolysis releasing free isatin derivative • Selective uptake through transferrin receptors • *In vivo* efficacy at $(1/10)^{th}$ dose of free drug	(40)
	plasminoge n activator inhibitor type 2 (PAI-2)	*In vitro* in cancer cell lines *In vivo* in breast cancer rat model	• Receptor mediated uptake • *In vivo* efficacy at (1/20)th dose of free drug	(40)

Tumor necrosis factor-α	Transferrin (via PEG chains)	*In vitro* (K562 and KB cells) *In vivo* sarcoma mice model	• Dual advantage of PEGylation and targeting through transferrin receptors	(41)
HSV-TK gene	Transferrin (Via biotin-streptavidin bridge)	*In vitro* (K562, M7609, TMK-1) *In vivo* in highly metastasized leukemia mice model	• High transfection as compared tolipofectin and retroviral vector • Biotin-steptavidin bridge avoids aggregation during preparation as well as allows easy release of DNA from conjugate	(42)
Mitomycin-C	Transferrin	*In vitro* (S-180 cells)	• Mitomycin-C content below 10 mol/mol of trnasferrin retained a binding activity of more than half that of TF • Increase in mitomycin-C content of conjugate decreased binding capacity of transferrin to its receptors	(43)
Cisplatin	Transferrin (Complex known as MPTC-63)	*In vitro* (feline lymphoma cells, HeLa cells) *In vivo* in tumor model in Fischer rats and in 5 human	• Conjugate decreased rate of growth of lymphoma cells • Conjugate killed the HeLa cells in 7 days • Treated rats never showed systemic disease • Marked response in two human patients • Conjugate may work synergistically with tamoxifen	(44)

		patients with advanced breast cancer		
	Transferrin	*In vitro* (A431 cells) *In vivo* in B16-bearing mice	• 3:1, 7:1, 15:1 cisplatin:transferrin (mol/mol) conjugations • Cytotoxicity of conjugates reduced in order of 3:1>7:1>15:1 ratio • Prolonged systemic circulation complex *in vivo* with order of 15:1>7:1>3:1>free cisplatin	(45, 46)
	Albumin	*In vitro* (A431 cells)	• 7:1 cisplatin:albumin (mol/mol) conjugation • Extremely reduced activity as compared to transferrin comjugates	(46)
Chlorambucil	Transferrin	*In vitro* (MCF7 mammary carcinoma and MOLT4 leukemia cell)	• Conjugates with acetaldehyde carboxylic hydrazine bond exhibited 3-18 times lower cytotoxicity as compared to free drug	(47)
CRM107 (a genetic mutant of diphtheria toxin)	Transferrin	*In vivo* clinical studies in humans	• 50% tumor volume reduction in 60% patients with 2 complete responses • Responses in refractory tumor patients • Regional perfusion showed no systemic toxicity	(48)

Gallic acid	Gelatin	*In vitro* (DU145, PC-3, and A498 cells)	• Conjugate maintained the anticancer activity of gallic acid	(49)
Doxorubicin	Gelatin (PEGylated and PEGylated)	*In vivo* in murine squamous cell carcinoma mice model	• Rapid clearance from blood of non-PEGylated conjugates • Poor tumor selectivity	(50)
pDNA for NK4 (an angiogenesis inhibitor)	Cationized gelatin	*In vivo* in Lewis lung carcinoma bearing mice	• Microsphere formulation • Suppression of metastases • Prolonged release over 28 days	(51)
pDNA for NK4	Cationized gelatin	*In vivo* in pancreatic cancer cells bearing mice	• Microsphere formulation • Better activity than free pDNA • Prolonged release over 28 days	(52)
pDNA expressing siRNA for VEGF	Cationized gelatin	*In vivo* in NRS1 model	• Microsphere formulation • Microspheres were present around tumor even after 10 days of injection	(53)
pDNA for IL-12	Cationized gelatin	*In vitro* (immature dendritic cells)	• Nanoparticulate formulation • Developed for cytokine based immunostimulatory cancer therapy • Efficient gene transfer into cells having phagocytic capacity	(54)
PTEN gene	Cationized gelatin	In vivo (bcl-2 over expressing PC-3 cells	• Microsphere formulation tumors that exhibit radiation resistance associated with expression of phosphorylated	(55)

		bearing mice)	Akt and Bcl-2 can be effectively treated	

SXF 1301 – soft tissue sarcoma xenograft model, PRXF PC3M – Prostate cancer xenograft model, SXF 1410 – osteosarcoma model, LXFE 409 – lung cancer model, LXFE 529 – lung cancer model, MAXF 449 – breast cancer model, BXF 1258 – bladder cancer model, HT-29 – colorectal adenocarcinoma cell line, A431 – epithelial adenocarcinoma cell line, MeWo – Melanoma cell line, KB Fr-α +ve cells – nasopharyngeal epidermal carcinoma cells expressing folate receptors, HT-29 Fr-α -ve cells – colorectal carcinoma cells lacking Folate receptors, lacking FR-, AH66P – parental hepatoma cell line, AH66DR – daunorubicin-resistant mutant hepatoma cell line, K562 –myelogenous leukemia cell line, K562/DXR – multidrug resistant myelogenous leukemia cell line, MDA-MB-468 – breast cancer cell line, U937 – leukaemic cell line, LXFL 529 – lung carcinoma cell line, Daudi – human Burkitt's lymphoma cell line, LOVO – human colon adenocarcinoma cell line, HL-60 – Human promyelocytic leukemia cells, H-meso – human lung mesothelia cell lline, HEP-2 – human epithelioma type-2 cell line, Molt-4 – human leukemia cell line, MCF-7 – breast cancer cell line, KB – HeLa derivative cell line, HSV-TK – herpes simplex virus-thymidine kinase, M7609 – human colonic cancer cell line, TMK-1 – human gastric cancer cell line, S-180 – mouse sarcoma 180 cell line, HeLa – Hernietta Lacks cervical cancer cell line, A431 – human epidermoid carcinoma cell line, B-16 – mouse melanoma cell line, PC-3 – human prostate cancer cell line, LLC – Lewis lung carcinoma cell line, DU145 – prostate cancer cell line, A498 – renal cell carcinoma cell line NRS1 – murine squamous cell carcinoma xenograft model, NK4 – NH_2-terminal hairpin and subsequent four-kringle domains of hepatocyte growth factor (HGF) (an HGF-antagonist and angiogenesis inhibitor), VEGF – vascular endothelial growth factor, IL-12 – interleukin 12, PTEN – phosphatase and tensin homolog)

Table 2. 2 Clinical Status of Antibody Drug Conjugates (56)

Antibody Drug Conjugate	Drug Class	Target	Disease	Company	Clinical Status	Remark
Trastuzumab Emtansine	Maytansine	HER2	HER2-positive metastatic breast cancer	Genetech/ Roche/ Immunogen	Approved	1st FDA approved antibody-drug conjugate.
Gemtuzumab Ozogamicin; Gemtuzumab -hydrazone-calicheamicin	Calicheamicin	CD33	Acute Myeloid Leukemia	Wyeth	Post Approval Withdrawn	Approved in May 2000 under the FDA's accelerated approval program. Wyeth in 2004 started the confirmatory, post approval clinical trials.
Brentuximab Vedotin; Brentuximab-MC-VC-MMAE	Auristatin MMAE	CD30	Hodgkin's Lymphoma; Haematologic Maliganancies	Seattle Genetics	Phase III	Randomized, double-blind, placebo-controlled, multicenter phase III trial to evaluate the efficacy and safety of brentuximab vedotin (SGN-35).
Inotuzumab ozogamicin; Inotuzumab-hydrazone calichaemicin	Calicheamicin	CD22	Lymphocytic leukemia; non-Hodgkin's lymphoma	Wyeth	Phase II	The purpose of this study is to assess the safety, tolerability and efficacy at increasing dose levels of inotuzumab ozogamicin in subjects.
Glembatumumab Vedotin CDX-011-MC-VC-MMAE	Auristatin	GPNMB	Melanoma, Breast Cancer	Celldex Therapeutics/ Seattle Genetics	Phase II	The randomised, multi-center, controlled EMERGE study was performed on 124 patients to receive CDX-011 or "Investigator's

						Choice" single agent, approved chemotherapy.
Lorvotuzumab Mertanaine HuN901-SPP-DM1 (IMGN 901)	Maytansine	CD56	Merkel Cell Cancer, Small Cell Lung Cancer	Immunogen	Phase I	Lorvotuzumab mertansine was generally well tolerated in this heavily pretreated population of patients.
SAR3419	Maytansine	CD19	B-cell malignancies	Sanofi/ Immunogen	Phase II	Recently, the phase II program began with 2 trials in patients with DLBCL - a single-agent study and a study of SAR3419 in combination with rituximab.
IMGN388	Maytansine	Integrin	Solid Tumor	Immunogen	Phase I	IMGN388 has been well tolerated at the doses tested, demonstrating initial evidence of safety in early clinical phase I testing.
BT062	Maytansine	CD138	Multiple myeloma	Biotest AG	Phase II	Acceptable toxicity profile for BT-062 is seen in phase I trial.
AGS-16M8F	Auristatin	AGS -16	Renal cell carcinoma	Astellas Pharma/ Agensys	Phase I	--
ASG-22ME	Auristatin	Nectin-4	Solid tumors	Astellas Pharma/ Agensys	Phase I	--
ASG-5ME	Auristatin	SLC44A4	Prostate, pancreatic Cancer	Astellas Pharma/ Agensys	Phase I	Trial identified maximum tolerated dose (MTD) for weekly administration, demonstrated

						tolerability and provided preliminary evidence for antitumor activity of ADC.
BAY 79–4620	Auristatin	CA-IX	Solid tumors	Bayer Health care	Phase I	--
BAY 94–9343	Maytansine	Mesothelin	Solid tumors	Bayer Health care	Phase I	--
BIIB-015	Maytansinoid	Cripto	Solid tumors	Biogen Idec	Phase I	--
IMGN529	maytansinoid	CD37	Non-Hodgkin's lymphoma, Chronic lymphocytic leukemia	ImmunoGen	Phase I	Study evaluates the safety, tolerability, pharmacokinetic profile and anticancer activity of increasing doses of IMGN529.
IMMU-130 (labetuzumab -SN-38)	Camptothecin analog (SN-38)	CEACAM5	Colorectal	Immunomedics Inc.	Phase I	Phase I study evaluated the safety of the IMMU-130.
IMGN 242	--	--	Gastric Cancer, Small cell lung cancer	ImmunoGen	Phase I, II	Marked response has been found in one of six patients who were subsequently treated with chemotherapy and radiation therapy.
MDX-1203	Duocarmycin	CD70	Non-Hodgkin's lymphoma, Renal cell carcinoma	Medarex	Phase I	--
PSMA ADC	Auristatin	PSMA	Prostate Cancer	Progenics	Phase I	Results identified that ADC was well tolerated upto MTD of 2.5 mg/kg.
RG7593	Auristatin MMAE	CD22	Hematologic malignancies	Roche/ Genetech	Phase I	Among 17 patients treated at clinically relevant doses, 7 had

						an objective response to RG7593 monotherapy with encouraging evidence of durable responses.
SAR566658	Maytansine	CA6	Solid tumors	Sanofi Aventis	Phase I	--
SAR 3419	Maytansine DM4	CD19	Non-Hodgkin's lymphoma	Sanofi Aventis	Phase II	Phase I trial demonstrated promising clinical activity, including patient's refractory to rituximab.
SGN-75	Auristatin	CD70	Non-Hodgkin's lymphoma	Seattle Genetics	Phase I	--
SGN-25	Auristatin	CD30	Hodgkin's lymphoma and CD 30+ T Cell Lymphomas	Seattle Genetics	Phase I	--

2.6.2 Protein Nanoparticles

With the advancement of novel drug delivery systems, protein nanoparticles have drawn very much attention of formulation development scientists. In recent times nanoparticles have been developed for the delivery of therapeutics using various proteins including albumin, gelatin, casein, silk proteins, elastin and lectins. Various protein carriers used for nanoparticulate delivery of therapeutics along with their advantages have been briefly summarized in **Table 2.3.**

Table 2. 3 List of Different Protein Carriers used for Nanoparticulate Delivery of Cancer Therapeutics (56)

Carrier	Advantages	Nanoparticle Formulation
Albumin	• High binding capacity to various drugs due to	Introduction of nab-technology (nanoparticle albumin-bound technology) by American Bioscience, Inc. made it possible

	presence of multiple drug binding sites • Promising targeting capability to tumor cells • Enhanced uptake in solid tumors is mediated by binding of albumin to albumin-binding proteins gp60 (albondin) and secreted protein, acidic and rich in cysteine (SPARC) • Transcytosis of albumin across endothelium of tumor vessels occur through albondin receptors while overexpressed SPARC help tumor tissue accumulation of albumin	for the first protein based nanoparticulate system, Abraxane® (nab-paclitaxel, ABI-007), to get the US FDA approval for metastatic breast cancer after combination therapy failure or relapse within 6 months of adjuvant chemotherapy. This nab-technology involves drug dispersed with HSA in aqueous solvent passed through a jet under pressure to form drug loaded nanoparticles of nanometer size range (100-200 nm). The advantage of this method lies in the fact that this method allows efficient encapsulation of lipophilic drug into the hydrophilic matrix of protein. Advancement in this technology can be quoted by development of nab-paclitaxel for non-small cell lung cancer (NSCLC), melanoma and pancreatic cancer, as well as development of three new nab-based products, namely nab-docetaxel (ABI-008) which is under phase I/II clinical investigation for hormone refractory prostate cancer and metastatic breast cancer; nab-rapamycin (ABI-009, rapamycin mTOR inhibitor) and nab-17AAG (ABI-010, 17AAG-Hsp-90 inhibitor) which are under phase I clinical investigation for advanced non-hematological malignancies.
Gelatin	• Low antigenicity • Functional groups are accessible for various	Paclitaxel-loaded gelatin nanoparticles prepared by coacervation-phase separation showed longer retention and higher accumulation compared to paclitaxel

	chemical modifications, which may be especially useful in developing targeted drug delivery vehicles	solution which uses cremophor/ethanol as solubilizers.
		Cisplatin-loaded gelatin/poly acrylicacid nanoparticles in which complex were formed between platinum of cisplatin and carboxylic groups in the nanoparticles have shown superior anticancer activity than free cisplatin in murine hepatic H22 tumor-bearing mice model.
Casein	• Surface-activity • Stabilizing property • Gelation and emulsification • Self-assembly and water binding capability	Chemotherapeutic drugs such as mitoxantrone, vinblastine, irinotecan, docetaxel and paclitaxel were successfully entrapped within β-casein (β-CN)-based nanoparticles. Paclitaxel-loaded β-CN nanoparticles have shown better cytotoxic activity in human N-87 gastric carcinoma cells.
Silk proteins	• Biocompatible and slow biodegradability • Self-assembly and excellent mechanical properties • Controllable structure and morphology	Silk sericin/poloxamer nanoparticles loaded with paclitaxel have shown significant apoptosis in breast cancer cells comparable to free paclitaxel.
Elastin	• Elastic protein located in connective tissues • Elastin retains all the advantages of polymeric drug delivery systems	Doxorubicin nanoparticle prepared using thermally responsive chimeric polypeptide, derived from an elastin-like polypeptide, were found to be stable upon dilution to low micromolar concentrations, cytotoxic at both 37 and 42°C and found useful for targeting of solid tumors.

Lectins (Wheat germ agglutinin)	• Lectin belongs to the group of glycoproteins. • High stability and low toxicity • Binding to glycosylated membrane components. • This makes it a good candidate for surface modification rather than a delivery vehicle.	Paclitaxel-loaded PLGA nanoparticles conjugated with wheat germ agglutinin exhibited superior anti-proliferation activity against the malignant pulmonary and colon cancer cells compared with conventional paclitaxel formulations due to wheat germ agglutinin receptor-mediated endocytosis

2.6.3 Preparation Techniques

Research in the area of nanoparticulate anticancer therapeutics can be represented by plenty of protein nanoparticulate formulations prepared for doxorubicin, methotrexate, cisplatin, paclitaxel, docetaxel, etc. Various methods have been used for the preparation of protein nanoparticles including desolvation, nab-technology (nano-particle albumin bound), emulsification, thermal gelation, nano spray drying, and self-assembly technique.

2.6.3.1 Desolvation (coacervation)

Nanoparticles are obtained in this technique by dropwise addition of desolvating agent (ethanol/methanol/acetone) in aqueous solution of albumin under continuous stirring until the solution became turbid. During the addition of desolvating agent into the albumin solution, albumin get separated due to its diminished water-solubility (57). The morphologically formed albumin particles being not sufficiently stabilized could consequently re-dissolve again after dispersion with water (58). Therefore, co-acervates were hardened by crosslinking with glutaraldehyde where the amino moieties in lysine residues and arginine moieties in guanidino side chains of albumin are solidified by a condensation reaction with the aldehyde-group of glutaraldehyde (59). After removal of desolvating agent by evaporation under reduced pressure, nanoparticles were purified by centrifugation to eliminate the free albumin and the excess of cross-linking agent.

Weber *et al*. optimized desolvation method for the preparation HSA nanoparticles and it was observed that particle size mainly depends on the amount of desolvating agent and not the cross-linker (60). The pH of HSA solution was another major factor identified determining the particle size of nanoparticles and it was observed higher pH values led to smaller nanoparticles and the mean particle size could be adjusted between 150 and 280 nm. Langer *et al.* established a pump-controlled desolvation method to enable a controllable particle size in combination with a narrow size distribution (57). Washing the particles by differential centrifugation led to significantly narrower size distributions (61). As an alternative to blood derived albumin, recombinant HSA (rHSA), a genetically engineered protein expressed in yeast cells, has shown comparable safety, tolerability, pharmacokinetics and dynamics to native HSA. Monodisperse rHSA nanoparticles could be prepared and enzymatic degradation of HSA and rHSA nanoparticles was possible over 24 h with different enzymes such as trypsin, proteinase K and protease. Furthermore, particle degradation in the presence of the intracellular enzyme cathepsin B confirms the biodegradability of the nanoparticles as a prerequisite of drug release after cellular uptake (62).

2.6.3.2 Nanoparticle albumin-bound technology (nab-technology)

American Bioscience, Inc. has developed a unique albumin-based nanoparticle technology (nab-technology) that is ideal for encapsulating lipophilic drugs into nanoparticles. The drug is mixed with HSA in an aqueous solvent and passed under high pressure through a jet to form drug albumin nanoparticles in the size range of 100–200 nm (63, 64). Abraxane® (nab-paclitaxel; paclitaxel-albumin nanoparticle) with an approximate diameter of 130 nm is the first FDA approved nanotechnology based chemotherapeutic that has shown significant benefit in treatment of metastatic breast cancer. The market approval of Abraxane® can be viewed as a landmark not just for albumin-based drug delivery technology but also for nanomedicine (65-68).

2.6.3.3 Emulsification

Emulsification technique has been extensively used for preparation of polymeric nanoparticles. Two main methods are used for stabilization of albumin nanospheres prepared by emulsification; thermal or chemical treatment (69). Albumin nanospheres (0.3–1 μm) were formed by homogenizing the oil phase (e.g. cotton seed oil) containing the albumin droplets at a high speed then thermally stabilized by heating at 175 to 180

°C for 10 min (70). This mixture was cooled and diluted with ethyl ether to reduce the oil viscosity to facilitate separation by centrifugation. Alternatively, in chemical stabilization, albumin aqueous solution was emulsified in cottonseed oil at 25 °C then denatured by re-suspension in ether containing the cross-linking agent 2, 3-butadiene or formaldehyde.

2.6.3.4 Thermal gelation

Thermal gelation is a sequential process that involves heat-induced unfolding followed by protein–protein interactions including hydrogen bonding, electrostatic, hydrophobic interactions and disulfide–sulfhydryl interchange reaction (71-73). In a study performed by Yu *et al*., spherical core–shell structure nanogels (about 100 nm) were manufactured using thermal gelation method where ovalbumin and lysozyme solutions were mixed at pH 5.3, the pH of the mixture solution was adjusted to 10.3 and the solution was subsequently stirred and heated (73). The gelation property of BSA on heating has been also reported (74). By heating a mixture of chitosan and BSA–dextran conjugates, biocompatible BSA–dextran–chitosan nanoparticles were formed (72). BSA molecules gelate forming the core of the nanoparticles whereas chitosan chains are partly trapped in the nanoparticle core because of the electrostatic attraction between chitosan and BSA.

2.6.3.5 Nano spray drying

Spray drying is a well-established method commonly used in the pharmaceutical industry for producing a dry powder from a liquid phase. Unlike conventional spray dryers that use rotary atomizers and pressure nozzles for forming the spray droplets, the new Nano Spray Dryer utilizes a vibrating mesh technology for fine droplets generation. Basically, the piezoelectric crystal driven spray head is incorporated with a small spray cap that contains a thin perforated membrane (spray mesh) having an array of precise micron-sized holes. When the piezoelectric actuator is driven at an ultrasonic frequency (i.e. 60 kHz), the mesh will vibrate upwards and downwards, injecting millions of precisely sized droplets from the holes and generating the aerosols. In contrast to the common cyclone technology where particles smaller than 2 μm are typically not captured, particle separation in the Nano Spray Dryer involves the use of the electrostatic precipitator whereby the collection mechanism is independent of particle mass. Collection of fine particles with high efficiency is achieved with the novel electrostatic

particle collector consisting of a grounded star electrode (cathode) and cylindrical particle collecting electrode (anode). The presence of a high voltage around the particle collector creates an electrostatic field that accelerates the deposition of negatively charged particles onto the inner wall of particle collecting electrode. This is followed by a discharging process (75).

2.6.3.6 Self-assembly

Increasing the hydrophobicity of albumin by addition of a lipophilic drug and diminishment of primary amine groups on protein surface, could drive the self-assembly of HSA and formation of polymeric micelles. Xu *et al.* prepared nanoscale HSA micelles for targeted delivery of doxorubicin. The inner core was formed by albumin conjugated with doxorubicin via disulfide bonds. Additional doxorubicin was physically adsorbed into this core to attain a high drug loading capacity. This process gave rise to multimeric albumin aggregates that contain about 50 doxorubicin molecules per albumin with a mean diameter of about 30 nm (76).

2.6.4 Surface Modification of Protein Nanoparticles

Surface modification of protein nanoparticles alters surface properties and enhances performance of delivery system in biological environment. Conjugation of protein nanocarrier's surface with specific ligand is usually achieved through covalent bond formation between the functional groups on protein surface and the ligands. Electrostatic adsorption and surface coating techniques are also utilized for surface modification. Surface modification of protein based delivery systems provides various possibilities owing to the presence various functional groups on the surface i.e., carboxylic and amino group on the surface of albumin nanoparticles. Ligand used for various purposes ranging from pharmacokinetic modification to drug release modification and targeting are as follow:

2.6.4.1 Surfactants

Surfactants are used to modify the pharmacokinetic parameters of formulation. Such as modification with Polysorbate 80 effectively reduced various toxicities of drug i.e., cardiac, testicular, and hematological toxicities of doxorubicin (77).

2.6.4.2 PEG

PEGylation of delivery system is performed in order to reduce their immunogenicity and prolong their circulation half-life and therefore to increase the passive tumor targeting capability of nanoparticles (78).

2.6.4.3 Cationic polymers

Cationic polymers are mainly used for retardation of drug release. The superior way for protection of protein nanoparticles against enzymatic degradation is coating of nanoparticles with biocompatible materials which eliminate the utilization of hazardous crosslinkers for stabilization of protein nanoparticles i.e., glutaraldehyde crosslinking which is used for stabilization of albumin and gelatin nanoparticles prepared by desolvation or coacervation method. Cationic polymers such as polyethylenimine (PEI) can be used for surface coating of anionic albumin nanoparticles which controls the rate of drug release and it depends on the concentrations of PEI used for coating (79).

2.6.4.4 Thermosensitive polymers

Shen *et al.* developed new thermal targeting drug carriers by conjugating the thermo-responsive poly (N-isopropylacrylamide-coacrylamide)-block-polyallylamine (PNIPAM-AAm-b-PAA) to the carboxylic group on the surface of albumin nanospheres through carbodiimide (EDC) coupling reaction and active targeting was observed with these nanoparticles (80).

2.6.4.5 Folic acid

Among various receptors overexpressed in cancer cells are folic acid receptors. Folic acid is a low molecular weight vitamin. Various advantages of folic acid as targeting agent are its stable, inexpensive, non-immunogenic nature, its compatibility with organic solvents used during processing, and being primary substrate for folate receptors; it binds to the folate receptors at cell surfaces with very high affinity and is internalized by receptor mediated endocytosis (81). Carbodiimide (EDC) coupling technique was used for conjugation of carboxylic group of folic acid to the amino groups on the surface of albumin nanoparticles (82).

2.6.4.6 Monoclonal antibodies (mAbs)

One of the interesting group of ligands emerged for tumor targeting are mAbs. Surface over expression of the epidermal growth factor receptor (EGFR) was observed

in many malignancies like breast, ovarian, colorectal, non-small cell lung, head, neck, and prostate cancers, as well as in glioma. In treatment of patients with metastatic breast cancer, EGFR-2 (HER2) serves as a tumor targeting marker. Humanized anti-HER2 specific antibody, trastuzumab (Herceptin®), was used for the surface modification through avidin–biotin complex formation between the biotin-binding protein (NeutrAvidin) attached to the nanoparticles and the biotinylated antibody for Human serum albumin (HSA) nanoparticle. Wartlick *et al.* conjugated the HSA nanoparticles with trastuzumab and evaluated in HER2-overexpressing cells (cell lines BT474, MCF7 and SK-BR-3) and it was observed that effective internalization of the nanoparticles via receptor-mediated endocytosis is time and dose dependent (83).

2.6.4.7 Peptides and proteins

Expression of high levels of αvβ3 integrin (a membrane receptor for extracellular matrix ligands such as vitronectin and fibronectin) in cancer cells from various entities is reported in literature (84). The αvβ3 integrin has shown high binding affinity with the cyclic arginine–glycine–aspartic acid (RGD) peptide ligand. *Dubey et. al* prepared RGD peptide-anchored sterically stabilized BSA nanospheres (RGD-SN) bearing 5-fluorouracil for targeting tumor vasculature and it was observed that RGD-SN were significantly effective in the prevention of lung metastasis, angiogenesis and in effective regression of tumors compared with free fluorouracil (85).

2.6.5 Preclinical and Clinical Investigations of Protein Nanoparticles

Albumin-paclitaxel nanoparticles prepared by using novel nab technology has an approximate diameter of 130 nm and was approved in 2005 for the treatment of metastatic breast cancer. Nab-paclitaxel was examined for the toxicity profile, pharmacokinetics and mean therapeutic dose (MTD) in phase I study (68). Dose from 135 to 375 mg/m^2 was administered as infusion in nineteen patients with advanced solid tumors without any premedication. Only mild hematologic toxicity was observed. Grade 3 superficial keratopathy was observed in two patients at the highest dose studied (375 mg/m^2). The MTD was determined to be 300 mg/m^2.Ibrahim *et al.* concluded that nab-paclitaxel can be administered rapidly and safely without premedication (68). In a multicenter phase II trial, 63 women with metastatic breast cancer received 300 mg/m^2 nab-paclitaxel by intravenous infusion without premedication (86). No severe hypersensitivity reactions were reported in the phase II study despite the absence of

premedication. Routine ophthalmological examinations yielded no severe ocular events such as superficial keratopathy, suggesting that this toxicity occurred by chance in the phase I study or occurs only at doses more than the MTD. Open-label multicenter study of phase III trial was conducted on 210 Chinese patients suffered with metastatic breast cancer. Preliminary results of the study suggested that nab-paclitaxel provides higher response rates and longer time to tumor progression without increased toxicity compared with solvent-based paclitaxel (87).

Preclinically, nab-paclitaxel has shown superior antitumor efficacy as compared to paclitaxel in a number of human tumor xenograft models. However, dramatic improvement in antitumor response for mice treated with nab-paclitaxel was observed at the MTDs. Antitumor response and tumor uptake data for nab-paclitaxel was comparable to albumin-binding prodrugs such as DOXO-EMCH. The overall increase in drug tumor accumulation can be estimated to be 3 to 6 fold at an equitoxic comparison was the result of shift of the MTD of nab-paclitaxel and of DOXO-EMCH over the respective free drug in mice. Pathophysiology of tumor tissue mediates the enhanced uptake of albumin-based drug delivery systems in solid tumors and characterized by angiogenesis, hypervasculature, and an impaired lymphatic drainage. Transcytosis initiated by binding of albumin to a 60-kDa glycoprotein receptor of cell as well as due to albumin binding to SPARC (secreted protein acid and rich in cysteine) enhance the accumulation of nab-paclitaxel in tumor cells (88).After the approval of Abraxane from FDA, it was further evaluated for adjuvant, neoadjuvant, and first-line treatment in breast cancer as well as for other indications such as non-small cell lung cancer, ovarian cancer and pancreas cancer. Various other nab-technology based products under clinical evaluation include those with docetaxel, rapamycin etc.

Targeted delivery to cancerous cells was achieved by coupling of transferrin protein to different nanoparticles. Doxorubicin loaded apotransferrin nanoparticles have delivered the drug more efficiently against cell-mediated ascetic liver cancer upon intraperitoneal administration as compared to free doxorubicin. Hepatic uptake of transferrin-bound iron occurs through receptor mediated endocytosis into a low-density vesicle compartment of hepatocytes followed by the release of iron and recycling of transferrin. However, transferrin receptor-1, transmembrane protein divalent metal transporter 1 (DMT1), divalent metal transporter ZIP14 were also reported to be involved in transferrin mediated iron transport.

Systemic delivery of pDNA encoding VEGF receptor-1 was done by using the gelatin-based nanoparticles. pDNA was encapsulated with gelatin (Gel), thiolated gelatin (SHGel), and polyethylene glycol-modified gelatin (PEG-Gel), as well as with polyethylene glycol-modified thiolated gelatin (PEG-SHGel). *In vivo* studies showed that several anticancer genes can be delivered by number of gelatin-based delivery systems. Gelatin based delivery system demonstrated that almost 13%-15% of the recovered dose of and PEG-SHGel nanoparticles, respectively, accumulated in the tumor for up to 12 hr following intravenous administration. On the whole, PEG-Gel nanoparticles may offer a safe and efficient strategy for systemic administration of therapeutic plasmid to solid tumors. Furthermore, the cationic gelatin microspheres incorporating NK4 pDNA, noticeably inhibited angiogenesis in Lewis lung carcinoma tumor and also the suppression of disseminated pancreatic cancer cells *in vivo* studies

2.7 Epidermal Growth Factor Receptor Targeting in Cancer

The epidermal growth factor receptors (EGFR)/Her1/ErbB1 are the cell-surface receptors belonging to ErbB family of tyrosine kinase and they have been of much attention for the molecular targeting of cancer therapeutics owing to their abnormal expression in many epithelial tumors and their influence on the growth and survival in malignant states (89). Advances in genetic engineering and understanding of the EGFR signaling pathways in cancer have led to the development of many therapeutic agents including monoclonal antibodies (mAbs), small molecule tyrosine kinase inhibitors (TKIs), antisense oligonucleotides, antibody based immuno-conjugates and other agents like FR-18, peptides, affibodies, nanobodies etc.

mAbs bind to the extracellular domain of EGFR and compete with endogenous ligands to inhibit the ligand-induced EGFR tyrosine kinase activation by blocking the ligand-binding region (90, 91). Cetuximab and panitumumab are the two most advanced mAbs targeting the extracellular domain of the EGFR. Cetuximab (Erbitux), approved in February 2004 by United States Food and Drug Administration (USFDA), is a chimeric (mouse/human) monoclonal antibody for intravenous infusion for the treatment of metastatic colorectal and head/neck cancer (92). On the other hand Panitumumab (Vectibix), manufactured by Amgen, is a fully human mAb specific to EGFR approved in September 2006 by USFDA for metastatic colorectal cancer. Panitumumab is the first mAb to demonstrate the use of KRAS (Kristen RAS) as a predictive biomarker and it was further approved by the European Medicines Agency in 2007 and by Health

Canada in 2008 for the treatment of refractory EGFR-expressing metastatic colorectal cancer in patients with wild-type KRAS. Later in July 2009 USFDA approved Erbitux for treatment of KRAS wild type (non-mutated) colon cancer.

Second class of agents targeting EGFR are TKIs which have a partially different activity profile than mAbs as they act in intracellular domain to inhibit enzyme tyrosine kinase which is responsible for signal transduction cascade and downstream activation of many proteins (91). USFDA approved TKIs include gefitinib (Iressa) in 2003 for the treatment of patients with advanced or metastatic non-small cell lung cancer (NSCLC); erlotinib (Tarceva) in 2004 for locally advanced or metastatic NSCLC and in combination with gemcitabine for locally advanced or metastatic pancreatic cancer. Lapatinib (Tykerb) in 2007 in combination with capecitabine for the treatment of patients with advanced or metastatic Her2-overexpressing breast cancer patients who have received prior treatment with an anthracycline, a taxane, and trastuzumab. Remaining EGFR inhibitors like antisense oligonucleotides, antibody based immunoconjugates and other agents like FR-18, peptides, affibodies and nanobodies are under preclinical and clinical investigations.

Recently, mutations and amplification of the EGFR gene have been identified and are implicated in development of resistance against mAbs and TKIs (93). To address these problems, broader acting inhibitors like dual EGFR HER-2 inhibitors, combined anti-pan-ErbB and vascular endothelial growth factor receptor inhibitors are under development. Current research focus is directed towards the selection of optimal dose regimen for particular cancer therapy, to identify molecular markers that can predict patients more likely to respond to anti-EGFR therapy, to find out combinatorial approaches with anti-EGFR agents and to bring new therapeutic agents with better clinical efficacy.

2.7.1 Biochemical and Structural Characterization of EGFR

EGFR is a 170 kDa protein containing approximately 20% of carbohydrate of its molecular mass and is heavily N-glycosylated (94). Glycosylation is important in case of protein-protein interactions that occur between protein ligand and their cognate receptors, because it plays a role in determining protein structure and known to affect the three dimensional configuration of proteins. Ligand binding brings two receptor monomers together and allows for the dimerization and subsequent activation of the kinase domain.

2.7.2 EGFR Signaling and Trafficking

EGFR plays a vital role in the regulation of cell proliferation, survival and differentiation. Various ligands are responsible for the activation of EGFR which include epidermal growth factor (EGF) (95), transforming growth factor alpha (TGF-α) (96), amphiregulin (97), betacellulin (98), epigen (99), epiregulin (100), heparin-binding EGF (101), neuregulin 2β (102), etc. These ligands are expressed as integral membrane proteins and are cleaved by metalloproteinases (typically members of a disintegrin and metalloproteinase family of membraneous proteases) to release soluble mature ligand and it represents a crucial point in regulation of EGFR signaling (103-105).

EGFR consists of an extracellular domain, a hydrophobic transmembrane domain, an intracellular catalytic tyrosine kinase domain and several intracellular tyrosine residues. Binding of ligand to the extracellular domain stabilizes the EGFR in an extended conformation and makes it capable of receptor dimerization (106). Dimerization facilitates the cytoplasmic domain of the regulatory monomer to stabilize the tyrosine kinase domain of the catalytic monomer in the active conformation and presents the tyrosine residues of the regulatory monomer to the catalytic site of the catalytic monomer. Different ligands cause the phosphorylation of distinct sets of EGFR tyrosine residues but mechanism is still unclear.

Activation of EGFR leads to multiple cell responses, inducing cellular growth, differentiation and migration. Factors contributing to these responses include the presence of other ErbB family receptors, specifically ErbB2, which is able to stabilize EGFR in a conformation required for dimerization and tyrosine phosphorylation even in the absence of ligand, resulting in ligand-independent EGFR signaling and increased ligand affinity for the EGFR (107-109). The heterodimerization of ErbB2 with EGFR alters EGFR endocytosis and intracellular trafficking (110-112). The agonist-induced heterodimerization of EGFR with a partner ErbB receptor changes the post effects of stimulation with a given EGFR ligand by coupling to different signaling pathways and biological responses than EGFR homodimers.

2.7.3 EGFR in Cancer

EGFR activation normally leads to cellular growth; its signaling can provide substantial advantage in tumor cells survival and it was the first receptor which was directly associated to human cancer (113). Dysregulation of EGFR has been observed in a wide variety of carcinomas, including head and neck, breast, bladder, ovarian, renal,

colon, NSCLC etc (114) and excessive EGFR waving can arise from receptor overexpression, autocrine signaling or mutation. Generally 4 x 10^4 to 1 x 10^5 EGF receptors per cell are expressed by normal cells, but tumor cells can express more than 2 x 10^6 receptors per cell (115). Generally observed percentages of tumors overexpressing EGFR in various types of cancer are listed in **Table 2.4**.

Table 2. 4 Observed Percentages of Tumors Overexpressing EGFR in Various Types of Cancer

Sr. No	Tumor type	Tumor overexpressing EGFR (%)
1.	Head and Neck	80-100
2.	Breast	14-91
3.	Renal	50-90
4.	Non-small cell Lung	40-80
5.	Colon	22-75
6.	Ovarian	35-70
7.	Glioma	40-63
8.	Pancreatic	30-50
9.	Bladder	31-48

2.8 EGFR Targeting Strategies

Great efforts have been made in the last 20 years to design therapeutic agents to target EGFR and these new treatment options have produced remarkable results in several human malignancies. mAbs and small-molecule TKIs are the most promising and widely used agents to target EGFR. They share the same target but display different mechanisms of action and different specificity for EGFR. mAbs bind to the extracellular domain of EGFR and compete with endogenous ligands to block the ligand-induced EGFR tyrosine kinase activation by blocking the ligand-binding region (116, 117). Small-molecule TKIs compete reversibly with adenosine 5′ triphosphate to bind to the intracellular catalytic domain of EGFR tyrosine kinase and inhibit the EGFR autophosphorylation and downstream signalling. Immunotoxin conjugates to deliver toxins (118); antisense oligonucleotides (119) or iRNA that decrease the expression of EGFR (120).

2.8.1 Monoclonal Antibodies

EGFR is the first molecular target against which mAbs have been developed for cancer therapy. Anti-EGFR mAbs are highly selective to receptor because they recognize EGFR exclusively. Presently, two anti-EGFR mAbs, cetuximab and panitumumab are widely used in treatment of metastatic colorectal and head/neck cancer. Newer mAbs are now being tested in clinical trials including murine and humanized mAbs. List of antibodies against EGFR which are clinically approved and/or under clinical trials are briefed in **Table 2.5.**

Table 2. 5 Anti EGFR Antibodies (18)

Sr. No.	Antibody	Class	Effective Against
1.	Cetuximab	Chimeric human-murine mAb	CRC, NSCLC, SCCHN, Pancreatic Cancer
2.	Panitumumab	Humanized mAb	CRC, Renal Cancer
3.	h-R3 (Nimotuzumab)	Humanized mAb	SCCHN (gliomas)
4.	EMD-72000 (Matuzumab)	Humanized mAb	SCCHN, ovarian, cervical, esophageal, CRC
5.	Zalutumab	Humanized mAb	Head and neck cancers
6.	MDX-447	Humanized mAb	SCCHN
7.	mAb-806	mAb	U87MG2–7 (EGFRvIII positive) glioma and epithelioid carcinoma (A431) cells and xenografts

2.8.2 TKIs

Tyrosine kinases play a vital role in the regulation of growth factor signaling. Activation of these enzymes can lead to tumor cell proliferation, anti-apoptosis, angiogenesis and metastasis. Owing to this key role played by receptor tyrosine kinases, they are the key targets for inhibition. Tyrosine kinases are activated by autophosphorylation of cytoplasmic domains as a result of ligand binding induced dimerization of receptor tyrosine kinases. Several TKIs have been found to have effective antitumor activity and have been approved or are in clinical trials. TKIs act by different

mechanisms: they can compete with adenosine triphosphate, the phosphorylating entity, the substrate or both or can act in an allosteric fashion. The further sections discuss different TKIs such as gefitinib (Iressa), erlotinib (OSI-1774; Tarceva), lapatinib (GW-572016), and canertinib (CI-1033).

2.8.3 Antibody Based Immunoconjugates

The unconjugated antibodies, with their ability to disrupt cellular functions and survival, are attractive therapeutics, but overall, are not very potent which makes it mandatory to use drugs in combination with them. The hypothesis states that one can improve the therapeutic window of chemotherapeutic agents or render the drug inactive by altering their *in vivo* distribution by conjugation to tumor-targeting monoclonal antibodies (56). Thus antibody–drug conjugates act as prodrug by releasing the drug into tumor cells after internalization. Although immunoconjugates are not currently established chemotherapeutic agents, several of them have demonstrated evidence of biologic activity in cancer patients. The current objectives are aimed at improving the efficacy and therapeutic index of immunoconjugates by optimizing selectivity and potency. With various research approaches to identify antigens and conjugation strategies with appropriate selectivity being pursued, the development of mAb therapies directed against the EGFR is current area of considerable interest.

2.8.4 Antisense oligodeoxynucleotides (AS ODNs)

AS ODNs as inhibitors of EGFR expression to regulate cell proliferation for potential anti-cancer therapy have been examined by several researchers. The latest disappointment of ZD1839 AS ODN, in clinical trials has directed search for unconventional approaches for the development of AS ODNs.

2.8.5 Other Newer Agents

Current research also focused on newer agents such as FR18, Affibodies, Nanobodies, Peptides and RNA interference (RNAi) for EGFR targeting.

2.9 Clinical Status of EGFR Inhibitors

Due to potential use of TKIs and mAbs targeting EGFR in cancer treatment, a number of new molecules targeting EGFR are being developed. Apart from this, shortcomings of the currently available EGFR inhibitors have speeded up the research in this field to put forth new agents that can overcome these problems. Various agents have

entered clinical trials and others are on their way to clinical trials. **Table 2.6** briefs the clinical outcomes of established EGFR inhibitors, either as a single agent therapy or as a combination therapy in various cancer types.

Table 2. 6 Clinical Status Different EGFR inhibitors (18)

Type of Cancer	Phase of the Clinical Study	Drug	Regimen	Results
Metastatic CRC	Phase II	Cetuximab	Oxaliplatin, Leucovorin, and Fluorouracil (FOLFOX-4) with or without Cetuximab	Addition of cetuximab led to increased response rate and decreased rate of disease progression
	Phase II	Cetuximab	Cetuximab alone, Cetuximab + Irinotecan	Clinically significant activity of cetuximab alone and in combination in irinotecan-refractory colorectal cancer
	Phase II	Cetuximab	Capacitabine and Oxaliplatin (XELOX) with or without Cetuximab	Improved outcomes with cetuximab addition
	Phase III	Cetuximab	Cetuximab	Better overall survival and progression-free survival
	Phase III	Cetuximab	Irinotecan alone, Cetuximab and Irinotecan	Similar overall survival with improved

				progression-free survival, response rate and quality of time on addition of cetuximab
	Phase III	Cetuximab	irinotecan, Fluorouracil, and Leucovorin (FOLFIRI) with or without Cetuximab	Cetuximab reduced the risk of progression of disease but benefit was limited to KRAS wild type cancer
	Phase III	Panitumumab	Panitumumab	Improved progression-free survival in chemo-resistant colorectal cancer
HNSCC	Phase II	Cetuximab	Cetuximab, Cetuximab and Cisplatin	Combination was active in refractory SCCHN, Cetuximab associated with skin rashes and occasional serious allergic effect
	Phase II	Cetuximab	Cetuximab and Cisplatin	Combination is active and well tolerated in patients with platinum-refractory or metastatic HCCHN

	Phase II	Cetuximab	Cisplatin + 5-Fluorouracil + Cetuximab	Effective in recurrent and metastatic HCCHN
	Phase II	Cetuximab	Cetuximab + Docetaxel (pretreatment with Cisplatin)	Combination was active in HCCHN No superiority in comparison with single agent therapy Activity was independent of cisplatin sensitivity
	Phase III	Cetuximab	Radiation therapy +Cetuximab	Improved locoregional control and reduces mortality without increasing the common toxic effects associated with radiotherapy
	Phase III	Cetuximab	Cisplatin/Carboplatin+5-Fluorouracil Cisplatin/Carboplatin+5-Fluorouracil +Cetuximab	Improved overall survival in cetuximab group
NSCLC	Phase II	Gefitinib	Gefitinib	Clinical activity and symptomatic relief by gefitinib as second and third line agent

	Phase II	Gefitinib	Gefitinib	Improved disease related symptoms in pretreated patients
	Phase II	Gefitinib	Gefitinib Docetaxel	Non-inferior survival as compared to docetaxel
	Phase III	Gefitinib	Gefitinib+ Paclitaxel+ Carboplatin	Significantly prolonged survival in patients with ≥90 days therapy showing maintenance effect of gefitinib No added benefit to standard therapy in survival, time of progression and response rate Confirmed safety profile
	Phase III	Gefitinib	Gemcitabine Cisplatin Gemcitabine + Gefitinib Gefitinib + Cisplatin	No significantly different improvement as compared to single agent therapy
	Phase III	Gefitinib	Gefitinib	Significant improvement in patients of Asian origin with

				pretreated advanced disease
	Phase III	Gefitinib	Gefitinib	Different survival outcomes in different group of patients Benefit in non-smokers and in Asian patients
Metastatic Breast Cancer	Phase III	Lapatinib	Paclitaxel Lapatinib + Paclitaxel	Combination improved outcomes in HER-2 positive breast cancer No benefit in HER-2 negative and HER-2 untested patients
	Phase III	Lapatinib	Capacitabine Lapatinib + Capacitabine	Combination was superior in HER-2 positive patients with progressed disease pretreated with anthracyclines, taxanes and trastuzumab
	Phase III	Lapatinib	Lapatinib +Letrozole Letrozole	Improved progression-free survival and clinical benefit rates in patients that co-express

				hormone receptors and HER-2
Pancreatic Cancer	Phase I, IB	Erlotinib	Gemcitabine + Erlotinib	Biologically active and well tolerated combination for treatment of advanced pancreatic cancer
	Phase III	Erlotinib	Gemcitabine Gemcitabine +Erlotinib	Improved survival rate and progression-free survival by adding erlotinib to gemcitabine

2.10 Development of Resistance to EGFR Inhibitors

The use of EGFR inhibitors is limited owing to the development of intrinsic or acquired resistance in cancer therapy (93). It was reported that the resistant phenotype unaffected by the treatment with C225 (mAb against EGFR) is due to the intrinsic activity of those pathways (121, 122). Persistent activation of downstream signaling steps such as MAPK and PI3K/AKT could promote cell proliferation, survival, differentiation and motility (123). Moreover, the upregulation of the vascular endothelial growth factor (VEGF) in human cancer cells by EGF and TGF-α causes the increase in angiogenesis and promotes resistance to EGFR inhibition.

2.11 Potential Predictive Markers of Response to EGFR

The extensive growth in EGFR targeted therapies has improved the efficacy of conventional chemotherapy in both preclinical and clinical studies. However, the biological heterogeneity has affected the response to these specific treatments in various malignancies. As a result, patients with different types of advanced cancer retort differently to currently available drugs, generally ranging from 10% to >90%, corresponding to partial and complete stabilization, while many patients do not benefit from anti-EGFR therapy (124). It appears that integrity of the complex EGFR-activated

downstream intracellular signal transduction machinery shapes the response to these drugs; thereby, as illustrated in recent experiments, the cancer cell may evade from growth suppression by using alternative growth mechanisms or by constitutive activation of downstream signaling effectors (17). Therefore, identification of specific markers able to predict the patients' response to anti-EGFR therapy appears necessary for the goal of improving treatment strategies for cancers and reducing treatment costs (125). List of predictive markers of response to EGFR are given in **Table 2.7**.

Table 2. 7 Predictive Markers of Response to EGFR (18)

Malignancy	**Marker**	**Anti-EGFR agent**	**Response type**
Colorectal Cancer, lung cancer	KRAS mutation	Cetuximab, Panitumumab	Presence of mutation led to absence of response
Colorectal Cancer, Metastatic Melanoma	NRAS mutation	Cetuximab	Presence of mutation led to absence of response
Colorectal Cancer	B-raf mutation	Cetuximab, Panitumumab	Presence of mutation led to absence of response
Colorectal Cancer, Metastatic Breast Cancer, head and Neck Cancer	PTEN/PI3K/AKT signaling network	Cetuximab, Trastuzumab, Gefitinib	PIK3CA mutations and PTEN loss in tumours were significantly associated with lack of response
Colorectal Cancer	Activated/ phosphorylated EGFR (pEGFR)	Cetuximab	Higher disease control in patients with high levels of pEGFR
Colorectal Cancer	EGFR amplification	Panitumumab, Cetuximab	No clear association between tumor EGFR expression and

			response to EGFR-targeted therapy
Colorectal Cancer	VEGF	Cetuximab, Gefitinib	Cetuximab induced acquired resistance via the increase in VEGF production
Colorectal Cancer	Loss of p21	Gefitinib	Loss of p21 expression was found in 43% of cases and was associated with a higher response rate.
Colorectal Cancer. Lung cancer	JAK/STAT pathway	Cetuximab, Gefitinib	Slight increase in STAT phosphorylation in samples of responder patients during cetuximab treatment; gefitinib may be most effective in patients with basal Akt activation
Colon cancer, head and neck cancer, NSCLC	Dinucleotide repeats polymorphism	Gefitinib	In CRC, patients with lower number of CA repeats frequently developed skin toxicity.
Colon cancer	Cyclin D1 A870G polymorphism	Cetuximab	Patients with favorable genotypes (CCND1 any G allele) showed long survival
Colon cancer	EGF A61G polymorphisms	Cetuximab	Patients with favorable genotypes (EGF any A allele) showed long survival
Colon cancer	Pharmacokinetic variability	Cetuximab	Observation needs to be confirmed
Colorectal, pancreatic, head and neck tumors	Skin rash	Cetuximab, Erlotinib	Intensity and severity of skin rash and response are related.

2.12 Combinatorial Approaches for EGFR Targeting

The rationale behind the combinatorial approach derives from the basic fact that the malignant nature of any tumor is rarely dependent on just one expressed receptor abnormality or signaling pathway. Additionally, signaling network and different regulatory pathways of cell proliferation exhibit a high level of compensatory "cross-talk" among receptors (126). Therefore, understanding the principles of combinatorial approach through interdisciplinary approach can help on how these strategies may be translated to clinical practices. The most preferred approach is to target same receptor using a combination of cytotoxic agents or combination of anti-receptor therapies directed at different members of the ErbB family of receptor. The former can be used for those tumors with a level of dependence on a given receptor. In the case of EGFR, a combination of anti-EGFR mAbs and low-MW EGFR TKIs has been reported to be promising in preclinical activity (127, 128). Furthermore, a combination of MAbs against the same receptor binding to different epitopes of the receptor could be a useful tool to get different mechanism of action. In addition, a variety of tumors have shown concurrent expression of more than one member of the ErbB receptor family (129). The combinations of EGFR and HER2 inhibitors were proposed to be synergistic against EGFR-positive; in this regard trastuzumab in combination with gefitinib or erlotinib was tested in HER2-overexpressing tumors in patients with breast cancer.

2.13 Single chain antibodies (scFvs)

Almost four decades ago the generation of monoclonal antibodies was started from mouse B-cell hybridomas (130) and continuously increasing a role of monoclonal antibodies in research, diagnosis and as therapeutics due to their ability to bind specifically to the antigen and block antigen synthesis or function. The natural immunoglobulin (antibody) molecules are composed of two heavy and two light chains and each chain of immunoglobulin consists either of one variable and one constant region (light chain) or one variable and several constant regions (heavy chain). Antigen binding sites are located in the Fv (variable fragment region of protein sequences) and each Fv fragment comprises of two domains (a heavy chain variable domain (VH) and a light chain variable domain (VL)) that are responsible for specific attachment to an antigen.

Advances in recombinant technologies have facilitated the manipulation, cloning and expression of antibody-encoding genes in various hosts (131) and the recombinant

antibodies can be cloned and expressed as intact antibodies, monovalent antigen binding fragment (Fab), single chain fragment variable (scFv), variable-region fragment (Fv: smallest unit of immunoglobulin molecule with function in antigen-binding activities), (132). Recently, nanobody, a single-domain antibody (sdAb) is also useful in research, therapeutic and diagnostic area. It is an antibody fragment consisting of a single monomeric variable antibody domain only from heavy chain with a molecular weight of only 12–15 kDa, sdAb are much smaller than scFv (~25 kDa, two variable domains, one from a light and one from a heavy chain) and selectively binds to a specific antigen. But rapid clearance from the blood circulation before reaching their target site due to smaller size is the major drawback of nanobodies. All intact antibodies and derivatives of antibodies retain their full binding function to the antigen. Different types of monoclonal antibodies, their fragments with molecular weight shown in **Figure 2.5**.

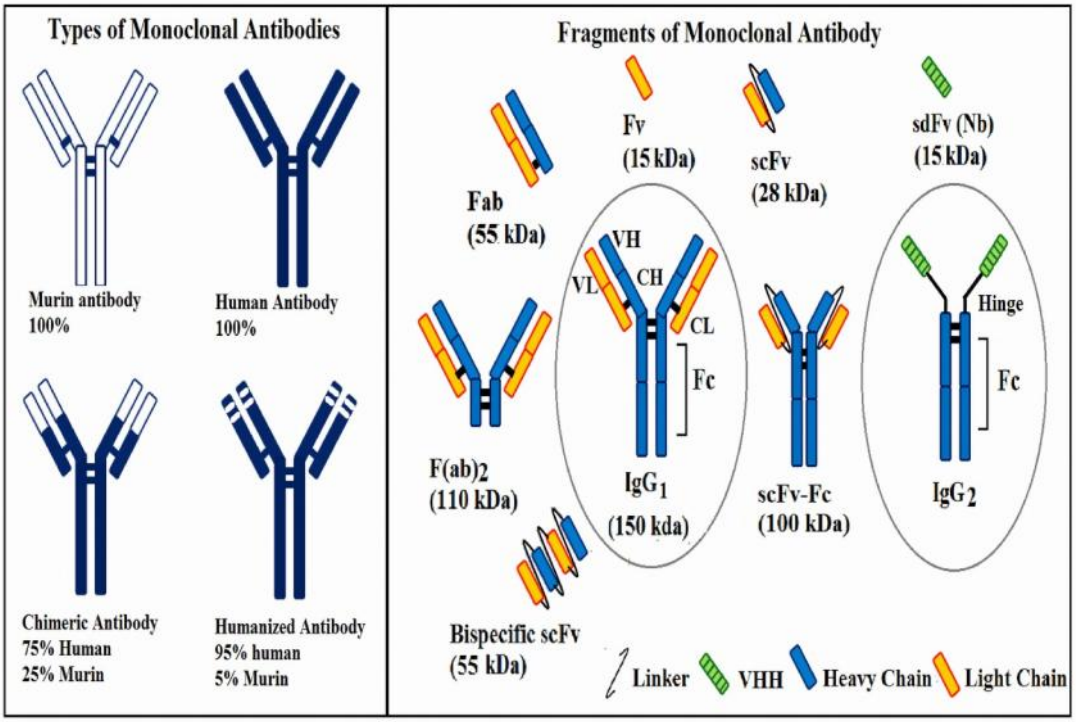


Figure 2. 5 Types of monoclonal antibodies and their fragments.
IgG: immunoglobulin, Fc: constant region, VH: variable heavy chain, VL: variable light chain, CH: constant heavy chain, CL: constant light chain.

ScFv is one of the most popular form of antibodies consists of variable regions of heavy and light chains fused together by a flexible peptide linker (133) and it can be easily expressed in functional form in E. coli. It has been estimated that the peptide linker

must span 35°A between the carboxy terminus of the variable domain and the amino terminus of the other domain without affecting the ability of the domains to fold and form an intact antigen-binding site and the length of the flexible DNA linker used to link both of the V domains is critical in yielding the correct folding of the polypeptide chain (134). In the design of a flexible linker peptide the amino acid composition plays an important role in addition to the length of linker. In order to avoid intercalation of the peptide within or between the variable domains throughout the protein folding the amino acid sequence must be hydrophilic (135). Currently the stretches of Gly and Ser residues meant for flexibility and charged residues of Glu and Lys interspersed to enhance the solubility are the most extensively used designs (136). scFv encoded by a single gene and is expressed as a single-function polypeptide chain that makes scFv very useful. Functional scFv expression in E.coli allows protein engineering to increase affinity and alter the specificity (137).

scFv can be produced intracellularly in eukaryotic cells that make them different from the natural immunoglobulins secreted by plasma cells extracellularly. scFv can be reformed for targeted expression in various intracellular compartments by using intracellular trafficking signals including endoplasmic reticulum (ER) through SEKDEL sequence (138) in the nucleus via simian virus 40 (SV-40) nuclear localization signals (139) or in the cytoplasm (140) where no trafficking signal is needed. Due to molecular engineering scFv molecule is continued to diversity, resulting in paired scFvs that bind to one another through complementary regions to form bivalent molecules (diabodies), complementary scFvs themselves produced as a single chain (tandem scFvs or tascFvs), and bispecific tandem scFvs (bis-scFvs), among others. (141). Better tissues penetration and rapid clearance is the major advantage of scFv fragments over whole immunoglobulins due to their minimized size. scFv also lacks the Fc region, leading to low immunogenicity. In addition, scFvs can be cloned and expressed in bacterial and mammalian cells, making it possible to produce large quantities easily and cost-effectively.

2.14 Generation of scFvs

scFv is a noncovalent heterodimer and for the generation of recombinant scFv fragments the VH and VL genes of mAbs can be used that are obtained by isolation of mRNA from hybridoma (142) spleen cells from immunized mice (143), B lymphocytes from human (144) and bone marrow. mRNA was first isolated from hybridoma, reverse

transcribed into DNA and then the antibody genes are amplified by PCR. For the recognition of any type of antibody gene requires oligonucleotide primers and this process crated the opportunities for generating large libraries containing a diverse range of antibodies with VH and VL genes (145). Generation of scFv by different technologies graphically represented in **Figure 2.6**.

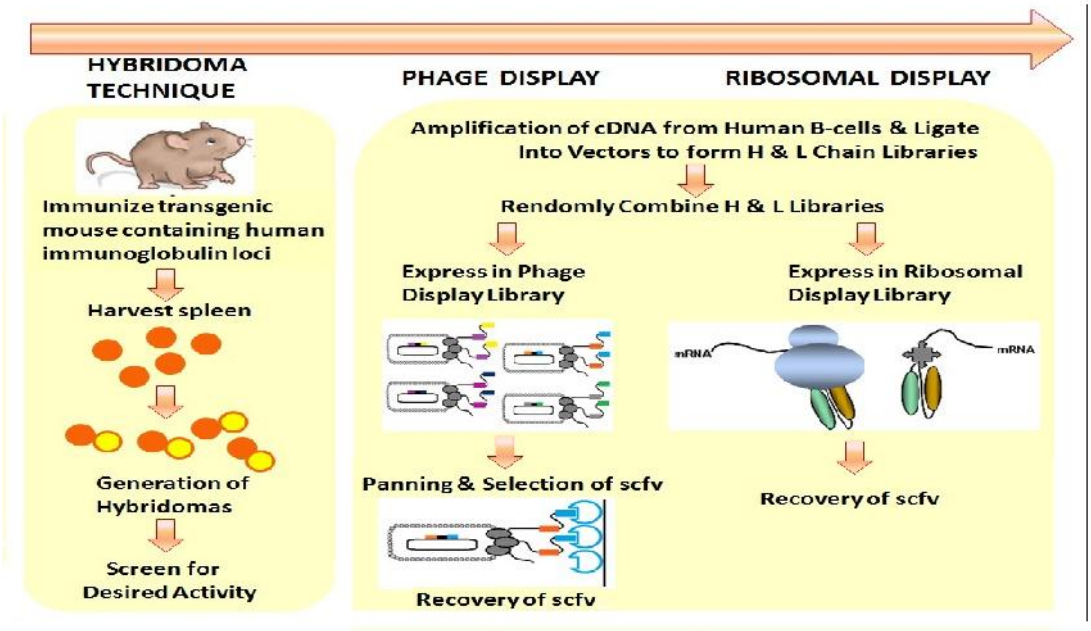


Figure 2. 6 Generation and selection of scFv by hybridoma, phage display and ribosomal display technologies.

Prospect for in vitro isolation of scFv from large libraries of V-genes by skirting the traditional hybridoma technology was opened with the construction of a phage carrying recombinant antibody genes and phage display (displaying antibody variable fragments on its surface) (146), together with the new technique of affinity selection of phage-displayed antibodies on tubes coated with antigen (147). Different types of phase libraries with their feature, advantages and drawbacks are given in **Table 2.8.**

Table 2. 8 Different type of phase libraries

Types of libraries		
A. Phage Display Libraries		
Immune	**Naïve**	**Synthetic**
• Constructed from V-segments of B-	• Constructed from B cells non-immunized	• Created from non-immune sources and are

cells IgG genes obtained from different kinds of immunized animals such as mice (143) , camels, sheep (148), and humans (149). • Biased towards the antigen. • Creates a large panel of high affinity antibodies. • **Drawback:** Need to construct a new library for each antigen.	donors obtained from a pool of IgM mRNA V-genes (150). • Not biased towards any antigen • Used for isolation of antibodies against different kinds of antigens. • Especially useful for the production of antibody fragments against non-immunogenic or toxic antigens. • **Drawback:** Not easily generated by hybridoma technology.	also widely used and yield high affinity mAbs. • Prepared artificially by combining germ line gene sequences with randomized complementary determining regions (CDR) responsible for antigen binding (151). • **Drawback:** Synthetic antibody libraries construction mainly focused on randomizing CDR3 regions mainly responsible for antigen binding that is the major challenge.
B. Ribosomal Display Libraries		
• *In vitro* scFv isolation is possible directly without using phages and bacteria by this method. • scFv DNA library is transcribed and translated *in vitro.* • mRNA–ribosome–scFv protein linked complex is created and used for selection on immobilized antigen. • The mRNA belonging to specifically bound scFv is eluted, reverse transcribed and the regenerated DNA pool enriched with binders is used further for selection (152). • **Drawback:** Method has the potential to further simplify and shorten scFv selection but is relatively new and thus not yet much in use.		

The order of the domains can be either VL-linker-VH (153) or VH-linker-VL and both orientations have been applied in construction of scFv (154). Several scFv (single-chain fragment variable) have been generated against carbohydrate (155), hapten (156),

protein (154), receptor (142), tumor antigen (157), and viruses (158) and all these scFv having application many fields such as therapeutics, diagnostic and imaging.

2.15 Expression of scFv

Most popularly the scFv fragments are expressed in bacteria *E.coli* (132), but Fab, Fv and scFv (recombinant antibody fragments) can also be expressed in mammalian cells (159) yeast (160), insect (161), and plant (162). Ability to fold and secrete the scFv proteins are depends on the expression system. It can be expressed as correctly folded and directly active proteins or as aggregates requiring in vitro refolding to make them active. Flow chart for the expression of scFv in bacterial and mammalian cells shown in **Figure 2.7.**

Each host having different advantages and disadvantages in production of active scFv fragments that are considered during design of vectors and expression system used with the different hosts (131). Bacterial expression system is most commonly used for the production of scFv fragments compared to other expression strategies. Because bacteria lack a system of post-translational modifications hence scFv can be functionally produced in bacteria as scFv does not have to be glycosylated unlike natural immuno-globulins. Oxidizing environment of the secretory compartment is mainly responsible for correct folding of scFv antibodies contain two disulfide bonds while insoluble inclusion bodies are formed when scFv expression in reducing environment of bacterial cytoplasm. Denaturing agent urea used to create proper disulfide bonds and restore binding activity by solubilizing and refolding of insoluble inclusion bodies (163). Cloning of the bacterial leader sequence (pelB, ompA) in frame to the N-terminus of scFv (164) is useful method to avoid the refolding process. It generates the properly folded scFv fragments by target expression of scFv in the periplasmic space (compartment between the inner and outer membranes of bacteria). Synthesis of both chains in an equal amount in the Fab fragment produced in *E. coli* due to expression of a discistronic operon unit in both of the genes (L and Fd) are controlled by the same promoter.

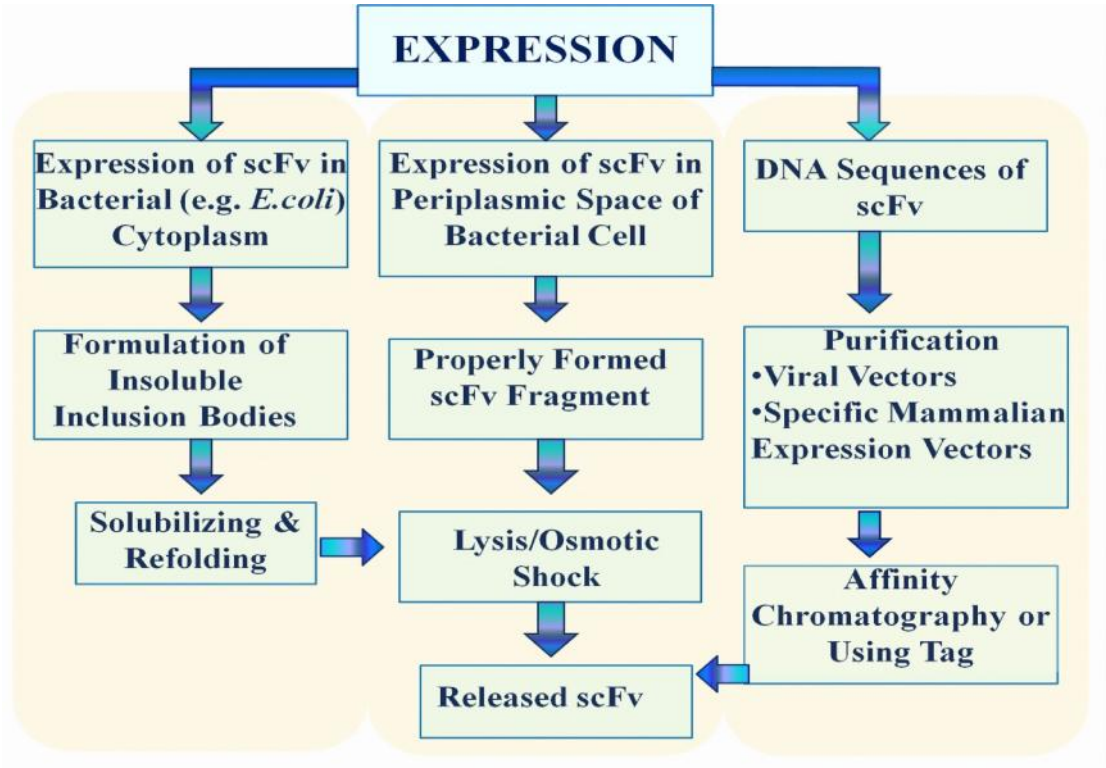


Figure 2. 7 Expression of scFv in bacterial and mammalian cells.

Cells lysis or osmotic shock is used to isolate the correctly folded antibodies released in periplasmic space. Jurado et al reported that *E. coli* strains carrying mutations allowing the oxidation and folding of functional scFv antibodies in the cytoplasm (165). Natural environment for the expression of soluble scFv fragments in eukaryotic cells that have a post-translational modification system. Insect and plant system also have the potential to generate scFv fragments for therapeutic purposes whereas the yeast expression system combines the advantages of a microorganism and a eukaryotic cell. System used for expression of scFv for therapeutic application must be free from potentially dangerous oncogenic and viral agents and the mammalian expression systems having this drawback (166) but the mammalian cells possess a complex post-translational modification system plus a secretory path-way, including chaperones makes natural environment for expression and folding of scFv. Antibody can be directed to different compartments of the cell such as the nucleus, endoplasmic reticulum, cytoplasm or it can be secreted after attaching the proper localization signal in frame to the scFv DNA sequence (167). Viral vectors or specific mammalian expression vectors used to transfer the scFv DNA sequence to mammalian cell. Various components of vectors ensure the proper transcription, translation (168) of transfected DNA sequences, and selection of

antibody expressing cell. Expression in mammalian cells is transient or stable. The transient expression commonly performed in COS cells; it takes around 1 to 4 days and is mainly used to test the ability of expression construct to produce protein. While the stable expression performed in stably expressing cell line and it takes more time compared to transient expression (weeks) and it requires the integration of antibody gene into the chromosomal DNA of the transfected cell. General steps involved in production and purification of scFvs shown in **Figure 2.8.**

2.16 Purification of scFvs

There are various methods currently available for purification for scFv antibody fragments but most widely purification is carried out by immobilized metal affinity chromatography (IMAC) which employs chelating metal ions as ligand. Generally N- or C-terminal end of recombinant scFv tagged with an artificial oligohistidine that having high binding affinity for transition metals such as Ni^{2+}, which are chelated by the resin of the columns. During passage through the column the oligohistidine-tagged scFv are bound to the resin, while impurities are eluted and latter purified scFv is removed from the resin by using chelation competitors EDTA or by lowering the pH. Different techniques used for purification of scFvs are given below.

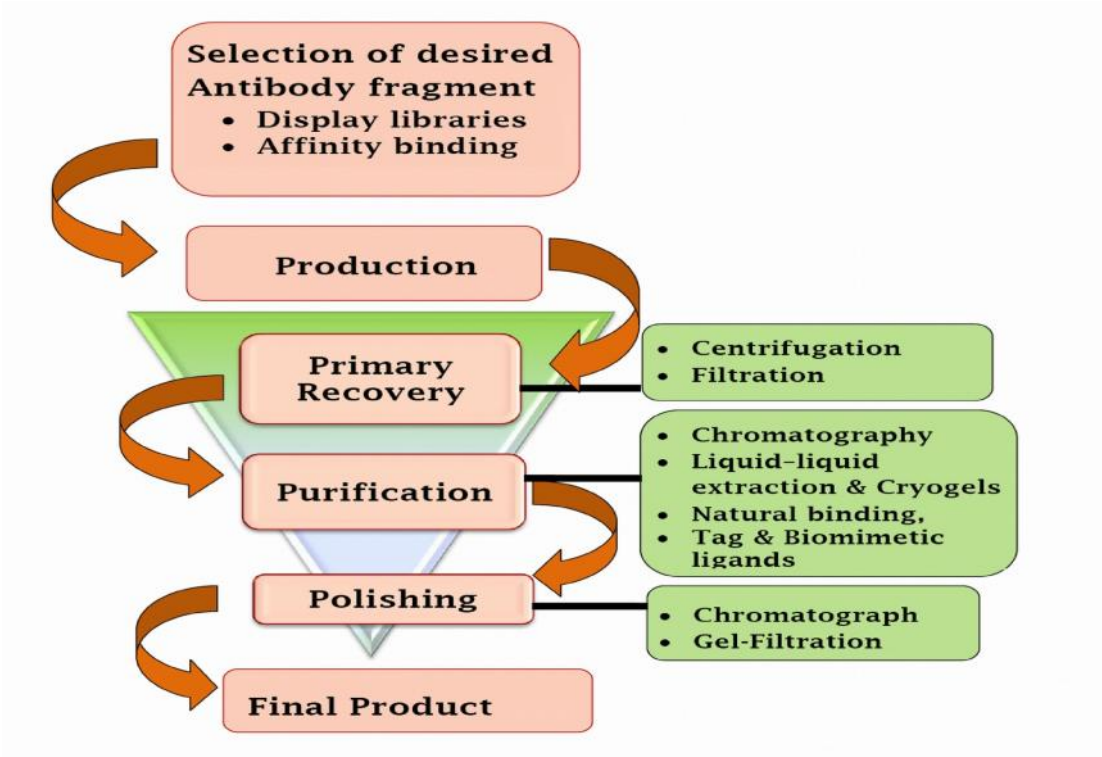


Figure 2. 8 General steps involved in production and purification of scFv.

2.16.1 Affinity chromatography

In affinity chromatography, purification is based on the principle of specific interaction between the antibody molecule and a complementary ligand (169). This method reduces the non-specific interactions, increases operational yield and provides high purity products because it eliminates the undesirable contaminants, even from diluted extracts. Different types of ligands used in affinity chromatography are given below.

2.16.1.1 Tag ligands

A short polypeptide sequences or whole proteins co-expressed as fusion partners with the target proteins having ability to bind with high affinity to both natural and synthetic protein stretch (170). Binding takes place around neutral pH and elution can be carried out by either reducing pH or using competitors (e.g. imidazole, EDTA) (171). Applicability under denaturing conditions is a distinctive advantage of IMAC over other affinity techniques and also useful for recombinant proteins over-expressed in the form of inclusion bodies (172). Ligand stability, low cost, high protein loading, mild elution conditions and easy regeneration (172) are other advantages of this technique. Whereas lower separation performances, extensive work to process, controlled oxidative reduction conditions inside the column, metal-induced cleavage that may damage the protein backbone, toxicity of metal ions leaching from the solid support and additional steps required for removing tags are the drawbacks of this technique.

2.16.1.2 Natural binding ligands

Peptostreptococcal protein L (PpL) having high affinity for the Fab portion of antibodies. Probe reagent to detect antigen–scFv complexes in immunoassays since it interacts with light chains practically without affect the antigen binding property of scFv (173) is advantage of this technique. Whereas not all mammalian immunoglobulins present a high PpL affinity is the drawback of this technique (174).

2.16.1.3 Biomimetic ligands

Synthetic molecules that have emerged from novel technologies includes combinatorial techniques, sophisticated molecular modeling approaches, designing and screening programs (170, 175). These molecules represent low cost and robust alternatives to the use of conventional biological affinity ligands. Triazine based ligand are the most efficient for scFv purification. Capability of binding both fragments and full

sized immunoglobulins of different classes and from sources (176). This property resulted in the isolation of immunoglobulins from crude samples, under non-optimized conditions, thus achieving a high degree of purity (up to 95%) (177). Capability of binding to a wide range of human light chains (176) is the advantage of this technique. Whereas little information about their mechanism of interaction with antibodies, which is necessary to optimize the binding process is the drawback of this technique.

2.16.2 Non affinity chromatography

Ion exchange, size exclusion, and hydrophobic interaction can also be used to purify antibody fragments. Higher percentage of recovery is the major advantage of non affinity-based chromatography.

2.16.2.1 Ion exchange

Researchers (178) successfully purified PEGylated scFv by combining ion exchange and hydrophobic interaction chromatographies, reaching final purities of more than 90% and recoveries higher than 50%. Researchers separated scFv-albumin fusion and obtained better recovery when purified with ion exchange chromatography (IEX) instead of the single scFv protein (179).

2.16.2.2 Hydrophobic interaction chromatography

Removing effectively DNA and host protein contaminants, thus achieving scFv purity near 100% is the advantage of this technique (178). Whereas high salt concentrations, required to elute antibodies and its low yield recovery make its use restricted are the drawbacks of this technique (180).

2.16.2.3 Size exclusion chromatography

It is a widely used technique for scFv purification. Complementary methodology to remove impurities and inactive antibody fragments such as aggregates and degradation products are the advantage of this technique (181). Whereas low selectivity in capturing recombinant antibodies is the drawbacks of this technique.

2.16.3 Non chromatographic methodologies

Precipitation and liquid-liquid extraction methods are important methods due to their simplicity and low cost.

2.16.3.1 Precipitation

Concentrating the sample to a solid, thus offering the maximum degree of volume reduction and benefiting the subsequent downstream purification (180). Conversion of soluble proteins to an insoluble state, which subsequently separated. Changing pH, metal ions, nonionic polymers, organic solvents, specific ligands and polyelectrolytes used to reduce the solubility of proteins are the advantages of this technique (182).

2.16.3.2 Liquid–liquid extraction

Powerful unit operation for the downstream processing of biomolecules. Aqueous two phase systems provide a suitable environment to maintain biological activity and protein solubility due to its high biocompatibility, high water content and low interfacial tension, minimizing product degradation (183, 184) are the advantages of this technique. Whereas complex interactions of the multiple components involved as well as in scaling up. Several systems have been explored to purify immunoglobulins from plants (185) and mammalian cells with purity of 70–95% and recoveries greater than 90% are the drawbacks of this technique (186, 187).

2.17 Applications of ScFv antibodies

2.17.1 Diagnostic

Recombinant scFv antibodies are a better alternative to conventional immunodiagnostic agents (188). Various assay formats can be used to discover the functionality of scFv as immunological agents (189). scFvs can bind to various antigens and they can be used in ELISA. The binding of scFv to antigen is distinguished through a short peptide fused to the C- or N-terminus of scFv which serve as a target for a secondary antibody. E-tag or c-myc is most popular tags. Moreover, scFv can be easily inactivated due to improper folding when coated on micro-titer plate. scFv is kept bonded to the coat protein of a filamentous phage to avoid this problem. Phage may mimic the missing constant domains and may improve the folding (190). Fusion of scFv to other proteins is the second approach to keep scFv more stable and functional (191, 192).

Avidity and stability of the original monovalent scFv would be enhanced by scFv fusion to other proteins (193). Moreover, direct detection with a substrate can be possible by AP fusion without the practice of expensive antibody–enzyme conjugates. Various viruses have been also detected by using recombinant antibody fragments. Sensitivity of fusion protein in detection was found comparable and often higher than polyclonal

antibodies. Potyviruses (194), potato leafroll virus and others are reliably detected by using scFv. HIV p24 protein and Visna virus p25 protein can be detected by scFv (195). It can also be used for differentiation between virulent and avirulent forms of Newcastle disease virus (196). Antigen can be detected and recognized in ELISA or immunoblot detection by phages displaying antibody fragments (197). However, magnification of the detection can be done by fusing a colour generating enzyme to the protein(198). scFv fusion proteins in both detection and capture of plant pathogen by using sandwich-type ELISA (enzyme-linked immunosorbent assay) were also developed (192).

New horizons for exploiting scFvs have then opened up with the emerging concept of fluobodies. Green fluorescence protein (GFP) fused scFv expressed in E. coli was used for direct labeling in flow cytometry and immunofluorescence experiments (199). Fluobodies do not fade after illumination when compared to fluorochrome fluorescein isothiocyanate (FITC) conjugated antibodies. scFv antibodies can also fuse with proteins fluorescing at different wavelengths for simultaneous multi-colored staining against different antigens (200). scFvs can be expressed in bacterial systems that allow their in vitro manipulation to introduce linker groups for coupling to nano particulate system (13). Simultaneous detection measurements revealed that specific antigen binding in this system was proportional to the concentration of antigen. Shen *et al*. (14, 15) utilized an additional cysteine or histidines within the linker peptide of the scFv for antibody immobilization on a gold piezoimmunosensor surface. Highly sensitive detection assays can be developed by using these immunosensors. Recombinant antibody developed by immortalizing the antibody genes from a hybridoma clone is useful for immunodetection of superantigen staphylococcal enterotoxin B. [Construction of a Single-Chain Variable-Fragment Antibody against the Superantigen Staphylococcal Enterotoxin B].

2.17.2 Imaging

Exaggerating field of recombinant antibodies has brought new breakthrough for imaging. Fluorescence imaging is emerging as a sensitive and economic tool to assess in vivo biomarker expression (201). It can be used in preclinical studies for the screening of agents which are to be further evaluated by clinical trials. Clinical application of fluorescence imaging is limited to superficial tissues. Moreover, fluorescent probes could be used during endoscopy and in approved surgical protocols. Numerous optical imaging probes based on target specific antibodies and their fragments have been analyzed for

their targeting stuffs (202-204). There should be balance between the tumor accumulation and system clearance of the probes used for imaging to provide high contrast images for a short period of time after administration. Ligand binding activity of scFv antibodies and other protein fused to O 6-alkyl-DNA alkyltransferase (SNAP-tag) was not affected due to their modification with fluorescent dyes or covalently coupled nano-particles (205). In this study, capability of scFvSNAP fusion protein directed against EGFR was analyzed by in vivo optical imaging [Rapid optical imaging of EGF receptor expression with a single-chain antibody SNAP -tag fusion protein].

Radioisotope labeled antibodies can be used in the detection of tumors in vivo. High tumor uptake and short survival in blood circulation are the prime requirements for the candidate which are to be used in cancer imaging. It subsequently results in low exposure of the healthy tissue (206). Technetium-99m labeled scFv is the first breakthrough for utilization of antibody fragments in cancer imaging (207). Half-life of technetium (6 h), make it more promising candidate for labeling of scFv fragments. Faster blood circulation and enhanced penetration into tumor are two major advantages of small radiolabeled scFv over an intact antibody as a tumor imaging agent. Several preclinical studies showed higher functional affinity of these molecules (208). Higher uptake of Radiolabelled scFv specifically bound to tenascin-c, was took place in infarcted myocardium when compared to the non-infarcted one. Moreover, very low amount of radioactivity was remained at 6 h after 111In-scFv injection (209). Quantum dots conjugated scFv targeted against GRP78 was delivered in a xenograft mouse model (210). Easy visualization of in vivo target can be possible due to quantum dot conjugated scFv. In one more study, anti-tumor scFvs which bind to oncomarkers, were conjugated to quantum dots to empower the visualization of cancer cells (211). Magnetic resonance imaging (MRI) can also be possible with the use of scFv (212). scFv conjugated supramagnetic iron oxide nanoparticles (SPIONs) can be used to improve the sensitivity of MRI. scFvs against carcinoembryonic antigen was attached with SPIONs to improve specificity of targeting cancer cells in MRI.

2.17.3 Proteomics

Recombinant antibodies can also play an important role in the proteomics. Tremendous data concerning nucleotide sequences has been generated due to sequencing of human and other genomes. Posttranslational modification or alternative splicing of mRNA can produce several protein variant from single gene. There is a need to explore

the protein composition of cell in a certain state as proteins are important mediators of cell functions. Protein array method should be used in analysis of protein composition of the cell. By using large number of antibodies, high throughput method can analyze thousands of proteins at the same time. Phage display antibody libraries can easily produce recombinant antibodies in scFv or Fab format against any protein. scFv fragment were employed in the screening of an array of 27648 human fetal brain proteins (213). Proteome can also be analyzed by using antibody arrays. In which, antibodies are arrayed and then proteins from different cell samples (e.g., healthy and disease cells) are applied. scFv antibody libraries were used in the generation of an antibody array (214). Basic and applied research would get breakthrough by using the information obtained from proteome studies, in which antibody fragments will be important object enabling tremendous growth in knowledge of proteomics.

2.17.4 Therapeutics

ScFv fragments exhibit good tissue penetration, but their tumor tissue accumulation is limited due to its rapid clearance from blood (215). However, blood clearance can be minimized due increment in the molecular size and hydrodynamic radius of scFv by their conjugation with polyethylene glycol (216, 217). Improved affinity and reduced renal clearance can also be obtained by dimerization of scFv by inducing C-terminal cysteines in order to form scFv multimers (215). Moreover, increase in size of scFv reduces the blood clearance but, it may also compromise tissue penetration. Blood clearance and tissue penetration of scFv should be balanced for effective tumor therapy. In this regard, diabodies outperform monomeric scFvs with a better tumor blood ratio (215). Thus, better in vivo tumor targeting can be achieved by recombinant antibodies of 60–100 kDa. A long-lasting and high level expression of the antibody was sustained and resulted in anti-tumor activity against CCR4+ bearing tumor cells (218). Recombinant immunotoxins (RIT) have also been recently developed by using scFvs to carry cytotoxic drugs for killing cancer cells (183, 219). Cell binding domain of Pseudomonas exotoxin A is replaced by scFv to binds with an antigen on cancer cells. Clinical trials are being conducted for different types of cancer malignancies by using three different RITs, either alone or in combination with chemotherapy (220).

Chimeric have assessed the effect of IGF-I and IGF-II on IGF 1R signaling through inhibition of their binding to IGF 1R by using scFv. The anti-IGF1R scFv-Fc developed from a mouse monoclonal antibody, 1H7, which blocks binding of IGF-I and

IGF-II to IGF1R [A Chimeric Humanized Single-Chain Antibody against the Type I Insulin-like Growth Factor (IGF) Receptor Renders Breast Cancer Cells Refractory to the Mitogenic Effects of IGF-I].Cystein modified scFv fragments were used for dual targeting (221). Bispecific immunoliposomes were developed by using two recombinant scFv directed against EGFR and CEA. Various cancerous cells over expressed both the antigens (222). Immunoliposomes for dual-targeting were developed by post-insertion method in which antibody fragments were coupled to micellar maleimide-PEG-DSPE lipids. They have showed that dual targeted liposomes are capable of targeting tumour cells overexpressing both or only one antigen. (223). GBM tumor sphere cells targeted by human scFvs were reported by Zhu et al. Efficient internalization of these antibodies by GBM tumor cells were observed (224). Homogenous distribution of radiolabeled scFv with penetratin as well as TAT was observed in excised tumors as compared to control treatment. Live animal imaging enhanced tumor localization with penetratin without any increment in normal tissue uptake. Administration of scFv with penetratin leads to significant improvement in tumor retention of scFv and hence proved the potential of this combination in mAb-based radiopharmaceuticals (225). Colcher et al. developed monovalent and divalent form of MAb CC49 scFv which was reactive with TAG-72 antigen. The divalent CC49 constructs have shown higher tumor uptake as compared to the monomeric scFv form of CC49 when determined in athymic mice bearing xenograft of adenocarcinoma cell line (226). An anti-vascular endothelial growth factor (VEGF) scFv was able to reduce the tumor growth in mice by approximately 50%. Substantial tumor inhibition was the result of systemic administration of recombinant adenovirus encoding scFv V65 (227).

Specific delivery of exogenous DNA into ErbB2(+) cells by using fusion proteins encompassed of the ML39 scFv and a condensed form of protamine leads to 8 to 10 fold up-regulation of the luciferase in ErbB2(+) cells as compared to ErbB2(-) cells. Moreover, vector developed by using DNA, ScFv, protamine, and lipids even more efficiently delivered the reporter gene into ErbB2 (+) cells.F5-scFv targeted doxorubicin liposomes had significantly reduced the tumor size in xenografted mice as compared to non-targeted doxorubicin liposomes. This strategy can be useful in generation of immune-therapeutics for other types of malignancies (228).

Intratracheal administration of scFvs using adeno-associated virus (AAV) vector is main mode in Alzheimer's disease therapy. scFvs with high immunoreactivity against the Aβ were delivered in the brains of transgenic mouse models of AD (229, 230). AAV

can mediate the long term overexpression of scFvs and antibodies were observed in the brain even after a year without causing neurotoxicity (229). Less invasive scFv delivery routes are required for human therapies, and that's why other route of administration has been explored. Reduced amyloid plaques and improvement of cognitive impairment in Alzheimer disease mouse model was observed after intramuscularly-administration of scFvs against Aβ (217). Reduction of Aβ accumulation was observed after nose to brain delivery of scFvs directed against the C-terminus of Aβ. Preclinical and clinical status of various scFv antibodies given in **Table 2.9.**Various applications of scFvs shown in **Figure 2.9.**

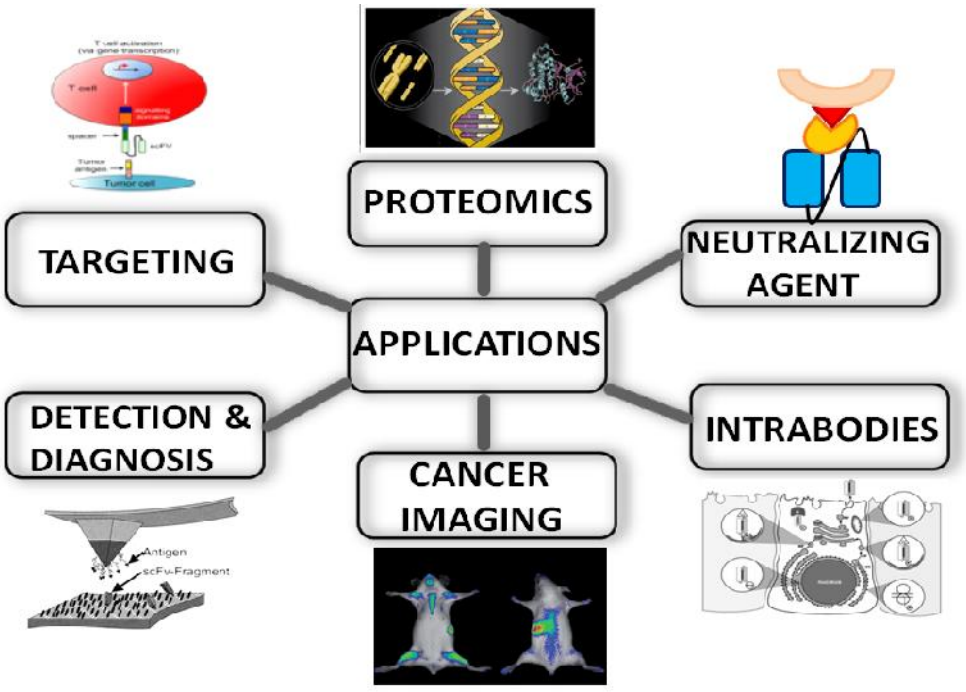


Figure 2. 9 Applications of scFv in different areas as diagnostic, research and therapeutic tool.

Table 2. 9 Preclinical and clinical status of various scFv antibodies (124, 231)

Indication	Specification	Stage
Melanoma	scFv targeting p96 antigen	Preclinical
Breast cancer	scFv for Her2	Preclinical
Ovarian and breast cancer	scFv (dibody) for Her2/Neu	Preclinical

Antiangiogenesis	Dibody for EDB Domain of fibronectin targeting	Preclinical
Colorectal cancer	CEA targeting	Preclinical
B-cell tomors	Bispecific scFv for CD19 & CD3	Phase I
Advanced Unresectable Melanoma	IMCgp100, a monoclonal T cell receptor anti-CD3 scFv fusion protein	Phase 0
Refractory acute lymphoblastic leukemia	anti-CD19 scFv TCR:41BB	Phase II
Advanced Solid Tumours	L19-IL2 constituted of a single chain Fragment variable (scFv) format directed against the ED-B domain of fibronectin	Phase I Phase II
Unspecified Adult Solid Tumor	MFE23 scFv-expressing autologous anti-CEA MFEz T lymphocytes	Phase I
Multiple Myeloma Acute Myeloid Leukaemia	Anti-LeY- scFv-CD28-ζ vector	Phase 1
Malignant Melanoma	IMCgp100	Phase 1
Malignant Melanoma	Bispecific scFv rM28 and autologous PBMCs	Phase 1 Phase 2
Malignant Pleural Mesothelioma: T cells transfected with anti-mesothelin mRNA expressing a scFv linked to the intracellular CD 3 zeta	T cells transfected with anti-mesothelin mRNA expressing a scFv linked to the intracellular CD 3 zeta T cell receptor domain and	Phase 1

T cell receptor domain and the 4-1BB costimulatory domain	the 4-1BB costimulatory domain	
Advanced solid malignomas	scFv(FRP5)-ETA specific for the ErbB2/HER2 receptor	Phase I

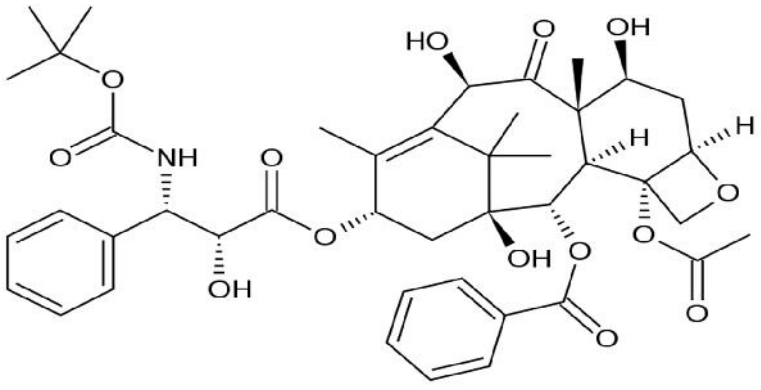
2.18. Drug Profile

2.18.1 Docetaxel

Name: Docetaxel

Description: Docetaxel is a clinically well-established anti-mitotic chemotherapy medication used mainly for the treatment of breast, ovarian and non-small cell lung cancer. Docetaxel binds to microtubules reversibly with high affinity and has a maximum stoichiometry of 1 mole docetaxel per mole tubulin in microtubules. Docetaxel (Taxotere) is an analogue of paclitaxel (Taxol®), obtained by semi synthesis from 10-deacetylbaccatin III, extracted from the needles of the European Yew Tree Taxus baccata. Like paclitaxel, docetaxel exerts its cytotoxic properties by inhibiting microtubule depolymerization and promoting tubulin assembly. Docetaxel has shown excellent anti-tumor activity, in both *in vitro* and *in vivo* models, and has generally been found to be more active than paclitaxel. Docetaxel was first administered to cancer patients in 1990 and clinical phase II studies started in 1992.

Chemical name: (2R, 3S)-N-carboxy-3-phenylisoserine, N-tert-butyl ester, 13-ester with 5(beta)-20-epoxy-1, 2(alpha), 4,7(beta),10(beta),13(alpha)-hexahydroxytax-11-en-9-one 4-acetate 2-benzoate, trihydrate.



Marketed Preparations: Taxotere, Sanofi Aventis, USA.

Physicochemical properties

Docetaxel

Empirical formula: C43H53NO14

Molecular weight: 807.9

Appearance and color: A white to almost white powder

Docetaxel trihydrate

Empirical formula: C43H59NO17

Appearance and color: A white to almost white powder

Molecular weight: 861.9

Melting point: 168.5° (Liao, Ho et al. 2008)

Solubility: Soluble in ethanol, methanol, chloroform, insoluble in water.

Ultraviolet Spectrum in Aqueous acid (ethanol): 230, 275, 283nm

Physicochemical stability

In acidic media or in the presence of electrophilic agents, opening and/or rearrangement the D ring, as well as in the B ring is observed, depending on the conditions employed. In basic media, cleavage of the ester groups at positions 2, 4 and/or 13 is observed. One of the principal paths of degradation observed, be it in alkaline, neutral or strongly acidic media is the epimerization of the hydroxyl group at position 7 which results in the formation of 7-epi-docetaxel by way of a retro aldol reaction. The degradation of docetaxel can result in products which have reduced activity or are completely inactive. They also demonstrate pharmacological and toxicological profiles completely different from the active principle. The importance of these complex transformations has grave consequences when considering the fact that the pharmaceutical formulations are destined for use in human subjects.

Clinical pharmacology

Docetaxel acts by disrupting the microtubular network that is essential for mitotic and interphase cellular functions. It promotes the assembly of tubulin into stable microtubules and inhibits their disassembly, causing inhibition of cell division and eventual cell death. Both docetaxel and paclitaxel bind to the same microtubule site, although the affinity of docetaxel is 1.9-fold higher. Cross-resistance between docetaxel and paclitaxel does not occur consistently. Docetaxel is a radiation-sensitizing agent. It is cell cycle phase-specific (G2/M phase).

Pharmacokinetics

Protein binding: > 95%.

Disposition in the body: Docetaxel is rapidly distributed throughout the body into body tissue and is extensively metabolised by the hepatic cytochromes of the CYP3A group. Excretion is mainly in faeces (75%) as one major and three minor inactive metabolites and a very low amount of the unchanged drug.

Half-life: Half-lives for α, β and γ phases are 4 min, 36 min, and 11.1 hr, respectively.

Volume of distribution: 95 to 150 L/m^2 (from various studies), also reported as 113 L.

Clearance: 17 to 22 L/h/m^2.

Distribution in blood: Little interaction with red blood cells.

Therapeutic concentration: Four patients with solid tumors, both male and female, were administered with an intravenous dose of 100 mg/m^2 docetaxel over 1 to 2 h. A peak plasma concentration of 2.41 mg/L was reached by the end of infusion (Drug-Profile-Clarks 2006). In another study, 7 patients administered with a 100 mg/m2 dose reached peak plasma concentrations of 3.67 mg/L.

Toxicology

Most important dose dependent acute toxicities involved with docetaxel are myelosuppression, peripheral neurotoxicity, moderate immune suppression, febrile neutropenia, hypersensitivity reactions, fluid retention, nausea, diarrhea, mouth sores and alopecia.

Indications and usage

- ✓ *Breast Cancer:* Taxotere (docetaxel) for Injection Concentrate is indicated for the treatment of patients with locally advanced or metastatic breast cancer after failure of prior chemotherapy.
- ✓ *Non-Small Cell Lung Cancer:* Taxotere (docetaxel) for Injection Concentrate is indicated for the treatment of patients with locally advanced or metastatic non-small cell lung cancer after failure of prior platinum-based chemotherapy.

Dosage and administration

Doses between 55 and 100 mg/m^2 body surface are administered for 1 h every 3 weeks; the greater dose is the usual dose. Lower doses are given if adverse reactions are observed during treatment. Patients with hepatic impairment: 75 mg/m^2.

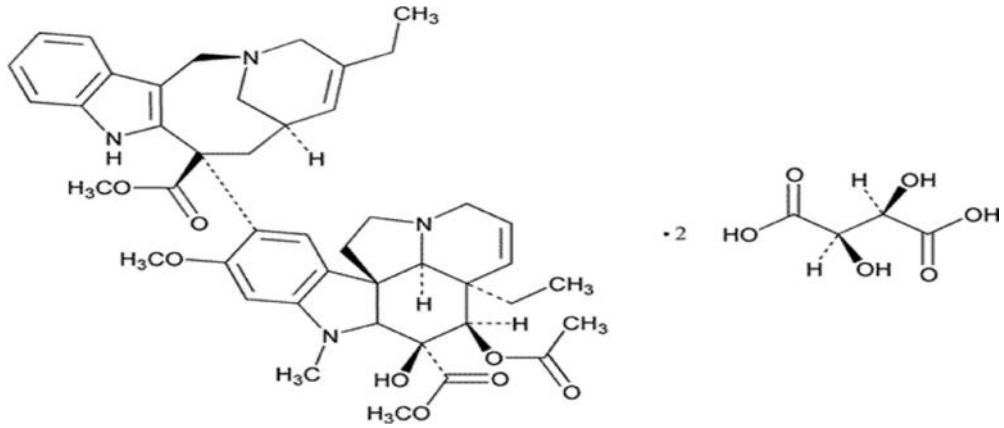
2.18.2 Vinorelbine Tartrate

Name: Vinorelbine Tartrate

Description: Vinorelbine tartrate is a white to off white amorphous powder which is very hygroscopic. Vinorelbine tartrate is freely soluble in water and partially soluble in Methanol. It is insoluble in aprotic solvents.

Chemical Name: Methyl(3aR,4R,5S,5aR,10bR,13aR)-4-(acetyloxy)-3a-ethyl-9 [(6R, 8S)-4- ethyl-8-(methoxycarbonyl)-1,3,6,7,8,9-hexahydro-2,6-methano-2H azacyclo decino[4,3-b] indol-8-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a octa hydro-1H indolizino [8,1-cd]carbazole-5-carboxylate dihydrogen bis [(2R,3R)-2,3-dihydroxy butanedioate].

Structure:



Physicochemical properties

Molecular Weight: 1079.11

Molecular Formula: $C_{53}H_{66}N_4O_{20}$

Mechanism of action

The antitumor activity of vinorelbine is thought to be due primarily to inhibition of mitosis at metaphase through its interaction with tubulin. Vinorelbine binds to the microtubular proteins of the mitotic spindle, leading to crystallization of the microtubule and mitotic arrest or cell death. Like other vinca alkaloids, vinorelbine may also interfere with: 1) amino acid, cyclic AMP, and glutathione metabolism, 2) calmodulin-dependent Ca2+-transport ATPase activity, 3) cellular respiration, and 4) nucleic acid and lipid biosynthesis.

Pharmacodynamics

The vinca alkaloids are considered to be cell cycle phase-specific. Vinorelbine is a vinca alkaloid antineoplastic agent used as a treatment for various cancers including

breast cancer, Hodgkin's disease, Kaposi's sarcoma, and testicular cancer. The vinca alkaloids are structurally similar compounds comprised of 2 multiringed units, vindoline and catharanthine. The vinca alkaloids have become clinically useful since the discovery of their anti-tumour properties in 1959. Initially, extracts of the periwinkle plant (Catharanthus roseus) were investigated because of putative hypoglycemic properties, but were noted to cause marrow suppression in rats and anti-leukemic effects in vitro. Vinorelbine has some immunosuppressant effect.

Pharmacokinetics

After intravenous bolus injection or infusion in patients, the plasma concentration of vinorelbine is characterized by a three exponential elimination curve. The terminal elimination phase reflects a long half-life greater than 40 hours. Linear pharmacokinetics has been shown for intravenously administered vinorelbine up to a dose of 45 mg/m². Vinorelbine is primarily metabolised by CYP3A4 of cytochrome P450. All metabolites have been identified and none are active with the exception of 4-O-deacetylvinorelbine, which is the principal metabolite in the blood. Renal elimination is low (<20% of the dose). Small concentrations of deacetyl vinorelbine have been recovered in humans, but vinorelbine is principally detected as the unchanged compound in urine. Elimination of the active substance is mainly via the bile duct and consists of the metabolites and mainly of unchanged vinorelbine.

Volume of distribution: 25.4 to 40.1 L/kg

Protein binding: ~27%

Route of elimination: Vinorelbine undergoes substantial hepatic elimination in humans, with large amounts recovered in feces after intravenous administration to humans.

Half-life: 27.7-43.6 hours

Clearance: 0.97 – 1.26 L/hr/kg

LD_{50}:

- Oral (Rats: 26-34 mg/kg in, Mouse: 77-89 mg/kg)
- Intravenous (Rat: 11-12 mg/kg Mouse: 32-42 mg/kg)

2.19 Excipient Profile

2.19.1 Human Serum Albumin (HSA)

Empirical Formula and Molecular Weight: Human serum albumin has a molecular weight of about 66 500 and is a single polypeptide chain consisting of 585 amino acids. Characteristic features are a single tryptophan residue, a relatively low content of methionine (6 residues), and a large number of cysteine (17) and of charged amino acid residues of aspartic acid (36), glutamic acid (61), lysine (59), and arginine (23).

Structure

Primary Structure: Human albumin is a single polypeptide chain of 585 amino acids and contains seven disulfide bridges.

Secondary Structure: Human albumin is known to have a secondary structure that is about 55% α -helix. The remaining 45% is believed to be divided among turns, disordered, and β structures. Albumin is the only major plasma protein that does not contain carbohydrate constituents. Assays of crystalline albumin show less than one sugar residue per molecule.

Functional Category: Stabilizing agent; therapeutic agent.

Applications:

Albumin is primarily used as an excipient in parenteral pharmaceutical formulations, where it is used as a stabilizing agent for formulations containing proteins and enzymes. Albumin has also been used to prepare microspheres and microcapsules for experimental drug-delivery systems. As a stabilizing agent, albumin has been employed in protein formulations at concentrations as low as 0.003%, although concentrations of 1–5% are commonly used. Albumin has also been used as a cosolvent for parenteral drugs, as a cryoprotectant during lyophilization, and to prevent adsorption of other proteins to surfaces. Therapeutically, albumin solutions have been used parenterally for plasma volume replacement and to treat severe acute albumin loss.

Description:

The United State Pharmacopeia (USP 28) describes albumin human as a sterile non-pyrogenic preparation of serum albumin obtained from healthy human donors. It is available as a solution containing 4, 5, 20, or 25 g of serum albumin in 100 mL of solution, with not less than 96% of the total protein content as albumin. The solution contains no added antimicrobial preservative but may contain sodium acetyltryptophanate with or without sodium caprylate as a stabilizing agent.

The European Pharmacopeia (PhEur 2005) similarly describes albumin solution as an aqueous solution of protein obtained from human plasma. It is available as a concentrated solution containing 150–250 g/L of total protein or as an isotonic solution containing 35–50 g/L of total protein. Not less than 95% of the total protein content is albumin. A suitable stabilizer against the effects of heat, such as sodium caprylate (sodium octanoate) or N- acetyltryptophan or a combination of these two at a suitable concentration, may be added, but no antimicrobial preservative is added. Aqueous albumin solutions are slightly viscous and range in color from almost colorless to amber depending upon the protein concentration. In the solid state, albumin appears as brownish amorphous lumps, scales, or powder.

Typical Properties

Acidity/alkalinity: pH = 6.7–7.3 for a 1% w/v solution, in 0.9% w/v sodium chloride solution, at 20°C.

Osmolarity: A 4–5% w/v aqueous solution is iso-osmotic with serum.

Solubility: Freely soluble in dilute salt solutions and water. Aqueous solutions containing 40% w/v albumin can be readily prepared at pH 7.4. The high net charge of the peptide contributes to its solubility in aqueous media. The seven disulfide bridges contribute to its chemical and spatial conformation. At physiological pH, albumin has a net electrostatic charge of about. Aqueous albumin solutions are slightly viscous and range in color from almost colorless to amber depending on the protein concentration.

Stability and Storage Conditions

Albumin is a protein and is therefore susceptible to chemical degradation and denaturation by exposure to extremes of pH, high salt concentrations, heat, enzymes, organic solvents, and other chemical agents. Albumin solutions should be protected from light and stored at a temperature of 2–25°C or as indicated on the label.

Safety

Albumin occurs naturally in the body, comprising about 60% of all the plasma proteins. As an excipient, albumin is used primarily in parenteral formulations and is generally regarded as an essentially nontoxic and nonirritant material. Adverse reactions to albumin infusion rarely occur but include nausea, vomiting, increased salivation, chills, and febrile reactions. Urticaria and skin rash have been reported. Allergic reactions, including anaphylactic shock, can occur. Albumin infusions are contraindicated in patients with severe anemia or cardiac failure. Albumin solutions with

aluminum content of less than 200 µg/L should be used in dialysis patients and premature infants.

LD_{50} (monkey, IV) : >12.5 g/kg

LD_{50} (rat, IV) : >12.5 g/kg

Comments

A 100 mL aqueous solution of albumin containing 25 g of serum albumin is osmotically equivalent to 500 mL of normal human plasma.

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