IJPSR (2012), Vol. 3, Issue 10 (Review Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 26 June, 2012; received in revised form 01 August, 2012; accepted 12 September, 2012

ANTIBODY ENGINEERING AND IMMUNOCONJUGATES FOR CANCER THERAPY

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Keywords:
Monoclonal antibodies (MAb's),
Effector functions,
Delivery of cytotoxic agents,
Prodrug therapy

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ABSTRACT

In the past three decades, monoclonal antibodies have become increasingly important class of disease modifying drugs for numerous indications including cancer, inflammatory diseases, and viral infections. Antibody therapy can be used in a variety of ways either alone or combination with cytotoxic compounds. Molecular antibody engineering technologies coupled with advances allow the generation of better antibodies with fully human sequence and optimized function thereby increases the antibody efficacy. Recombinant antibodies and its fragments have been fused to radioisotopes, drugs, toxins, enzymes and antibody-directed pre-targeting followed by prodrug activation (ADEPT) has an alternative strategies for promising therapy of cancer. Various monoclonal antibodies (mabs) have been developed and approved as immunotherapeutic agents. This review briefly describes new development of antibodies and immunoconjugates for broader applications.

INTRODUCTION: Cancer is characterized by uncontrolled growth of cells, ability to spread other parts of body through the blood and lymph system, treatment is a major challenge for modern medicine. However, in most cases current cancer treatment strategies like localized tumors removed by surgery and radiation, chemotherapy were not sufficient to control metastatic cancer.

Therefore effective treatment to be needed for every organ of the body to control the progressive metastatic cancer. Most of the cancer associated antigens are growth factor receptors, which are overexpressed in number of cancers. Targeting of these molecular cell surface markers by antibodies are now important biological agents for the treatment of cancer. Immunotherapy of cancer has been explored over a century but in recent years monoclonal antibodies have become an attractive treatment model for many metastatic cancer patients because of their ability to bind specific antigens, long circulating half-life and less toxic than chemotherapy and radiation. The production of murine monoclonal antibodies by hybridoma technology was first reported by Kohler and Milstein in 1975 with interesting biological properties [1]. In middle of the 1980 murine mabs had entered into clinical studies of human use including Muromonab (Orthoclone OKT3) anti CD3 antibody approved in prevention and treatment of acute rejection of solid organ transplants but not have high success rate for commercialization [2,3].

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Murine monoclonal antibodies are relatively easy to produce but are severely restricted for therapeutic use due to inadequate recruitment of host effector functions, Human Anti Mouse Antibody (HAMA) response in humans, which leads to clearance of the antibody and limitations in its efficacy [4]. Repeated administration of murine antibodies also leads to formation of allergic reactions up to anaphylactic shock. There are currently eight therapeutic mabs already approved by FDA in oncology, around 150 in clinical developments and is one of the most active areas of clinical research. In this review i discuss briefly on different antibody engineering technologies and immunoconjugates for various human diseases.

Antibody Engineering: Antibodies have been engineered by a variety of methods to suit a particular therapeutic use. Molecular engineering has improved the prospects of antibody based therapeutics by different approaches like chimeric, humanized and human antibodies as shown in **Figure 1**, to reduce the immunogenicity. Chimeric mabs consist approximately 65% human and 35% mouse and humanized versions consist of 95% human and 5% mouse protein sequence. Chimeric mabs comprise human heavy and light chain constant regions recombinantly fused with murine variable heavy and light chain domains, like cetuximab targeted extracellular domain of the human EGFR and has been approved for use in patients with colorectal cancer [5].

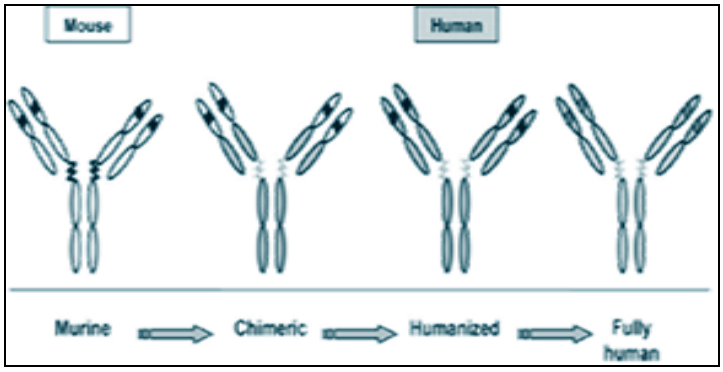

FIG. 1: THE EVOLUTION OF MONOCLONAL ANTIBODY TECHNOLOGIES

Antibodies with human constant region are potential to activate immunological effector functions of the human immune system to create more effective therapeutic agents. Chimeric mabs are superior to murine antibodies but still moderate risk of immunogenicity to patients from murine residual components. These can be further reduced by another approach called "Humanization" of antibodies. Humanized mabs include 6 complementarity determining regions (CDRs) from murine variable domains recombinantly grafted into the human frame works with constant region to create reshaped human antibody is considered as CDR graft. CDRs are likely to interact with antigen by the support of frameworks, determines antigen specificity and grafting has been widely used to retain the original antigen binding property [6, 7, 8].

During the process humanization antibodies may loss antigen binding property and additional engineering steps may be required in human frameworks with key residues from parent antibody to restore binding affinity. Bevacizumab is a humanized monoclonal antibody targeting vascular endothelial growth factor (VEGF) and has been recently approved by the US FDA as a first-line therapy for widespread metastatic colorectal cancer [9]. These developments of mab products would lower the HAMA response in humans. Solution for the immunogenicity problems associated with murine mabs is to be the development of human mabs. Thus antibody based therapeutics has been emerged as important components of therapies for an increasing number of human malignancies and numerous indications as shown in **Table 1**.

Fully human sequence derived antibodies have no murine sequence is produced by two approaches: (a). Antibody Phage display technology and (b). Transgenic mice model. Phase display technology involves the generation of antigen binding fragments of antibodies with huge library of human antibody genes from B cells of immune system. These libraries of antibody genes genetically fused to the phase surface coat protein in Phagemid and subsequent expression on filamentous bacteriophage. Antigen specific phase displayed antibodies are selected from billions of different antibody genes by multiple rounds of affinity selection to enrich for antigen specific phase antibodies and process called panning. Once the antigen specificity is obtained, the genes of antibody variable regions can be isolate and cloned into human IgG to produce fully human monoclonal antibodies [10]. Adalimumab is fully human antibody specific for TNF alpha developed by phage display of human VH and VL sequences and approved for therapeutic use [11, 12].

The latest technology is transgenic mice carrying genetic modifications (XenoMouse), in those, murine immunoglobulin genes were replaced with the functional human immunoglobulin genes, capable of producing human antibodies to the immunization of specific antigen. These genetic modifications carried out in mouse embryonic stem cells by gene targeted deletions.

XenoMouse strains allow the generation of wide diversity of high-affinity human antibodies with desired effector functions and many of which are progressing in clinical development [13, 14, 15]. Panitumumab is the fully Human antibody against EGFR developed by transgenic mice technology and approved by FDA and represents milestone for future antibody developments. The current trend is clearly towards clinical study of humanized and human mabs rather than murine and chimeric antibodies, because more human sequence makes better mabs for effective treatments of cancer [16]. Antibodies are also function as carriers of cytotoxic substances, such as radioisotopes, drugs, and toxins. In non-hodgkin lymphoma immuno-conjugate like anti-CD20 antibody-radioconjugates have superior antitumor activity than their unconjugated antibody counterparts with less hematologic toxicity. In addition, antibody fragments have advantages especially in terms of the rate of solid penetration & require 16 hours to move 1 mm into solid tumor whereas intact Ig G needed 54 hours to reach the same distance [17]. These findings are strong incentives to continue the pursuit of immunoconjugates for cancer therapy [18]. Here we focus on engineered Fc variants with improved effector functions and development of immunoconjugates for cancer therapy.

TABLE 1: FOOD AND DRUG ADMINISTRATION (FDA) APPROVED MONOCLONAL ANTIBODY DRUGS

Generic Name (Trade Name)	Mab type	Isotype	Antigen	Indication	Approval year
Murine mabs					
Muromonab (Orthoclone-OKT3)	Murine	m Ig G2a	CD3	Transplant rejection	1986
Irbitumomab (Zevalin)	Murine	m Ig G1 (Antibody conjugate)	CD 20	Non-Hodgkin lymphoma	2002
Tositumomab (Bexxar)	Murine	m Ig G2a (Antibody conjugate)	CD 20	Non-Hodgkin lymphoma	2003
Chimeric mabs					
Abciximab (Reopro)	Chimeric	h Ig G1(Fab)	Gp IIb/IIIa	Cardiovascular Disease	1994
Rituximab (Mabthera, Rituxane)	Chimeric	h Ig G1	CD-20	Non-Hodgkin lymphoma	1997
Infliximab (Remicade)	Chimeric	h Ig G1	TNFα	Inflammatory diseases	1998
Basiliximab (Simulect)	Chimeric	h Ig G1	CD25	Transplant rejection	1998
Cetuximab (Erbitux)	Chimeric	h Ig G1	EGFR-1	Colorectal cancer	2004
Brentuximab (Adcertis)	Chimeric	h Ig G1 (Antibody conjugate)	CD 30	Hodgkin lymphoma	2011
Humanized mabs					
Dacilizumab (Zenapax)	Humanized	h Ig G1	CD 25	Transplant rejection	1997
Trastuzumab (Herceptin)	Humanized	h Ig G1	HER2	Brest cancer	1998
Palivizumab (Synagis)	Humanized	h Ig G1	RSV F	Prevention of RSV	1998
Gemtuzumab (Mylotarg)	Humanized	h Ig G4 (Antibody conjugate)	CD 33	Acute myeloid leukemia	2000
Alemtuzumab (Campath)	Humanized	h Ig G1	CD 52	Chronic lymphocytic leukemia	2001
Efalizumab (Raptiva)	Humanized	h Ig G1	CD 11a	Psoriasis	2003
Omalizumab (Xolair)	Humanized	h Ig G1	Ig E	Asthma	2003
Bevacizumab (Avastin)	Humanized	h Ig G1	VEGF	Colorectal cancer	2004
Natalizumab (Tysabri)	Humanized	h Ig G4	Integrin-α4	Inflammatory diseases	2006
Ranibizumab (Lucentis)	Humanized	h Ig G1(Fab)	VEGF	Macular degeneration	2006
Eculizumab (Soliris)	Humanized	h Ig G2/4	CSP-C5	Paroxysmal noctumal hemoglobinuria	2007
Certolizumab (Cimzia)	Humanized	Fab(Pegylated)	TNFα	Crohns disease	2008

Tocilizumab (Actemra)	Humanized	h Ig G1	IL-6R	Rheumatoid arthritis	2002
Human mabs					
Adalimumab (Humira)	Human	h Ig G1	TNF α	Auto-immune disorders	2002
Panitumumab (Vectibix)	Human	h Ig G2	EGFR	Colorectal cancer	2006
Canakinumab (Ilaris)	Human	h Ig G1	IL-1β	Cryopyrin associated periodic syndrome	2009
Ofatumumab (Arzera)	Human	h Ig G1	CD-20	Chronic lymphocytic leukemia	2009
Golimumab (Simponi)	Human	h Ig G1	TNFα	RA, Psoriatic arthritis	2009
Denosumab (Prolia)	Human	h Ig G2	RANK-L	Bone loss	2010
Belimumab (Benlysta)	Human	h Ig G1	BLyS	Systemic Lupus erythematous	2011
Ipilimumab (Yervoy)	Human	h Ig G1	CTLA-4	Metastatic melanoma	2011

Mechanism of Mab Action: Monoclonal antibodies have significant potential as therapeutic agents because they can be used as naked molecules or combination with cytotoxic compounds. Antibodies are glycoproteins with dual functionality by having two antigen binding fragments $(Fab)_2$ encoded by complementary determining regions (CDR) and Fc region is responsible for binding to ligands or complements through which activates clearance mechanisms. Most of the therapeutic antibodies are human Ig G1 and Ig G3 isotype, which can potentially induce strong ADCC and CDC when compared with other isotypes of human antibodies [19].

The mechanisms of antibodies are referred to as antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), which involves the recognition of the antibody that bound to antigen on target cell to effector cells or complement proteins leads to cell destruction or it can initiate signalling mechanisms in the target cell and induces the cells to undergo apoptosis [20].

Antibodies can also leads to inactivation of cellular pathways by binding to cancer cell surface receptors such as EGFR-1and EGFR-2 resulting in decreased cell growth. Effector-cell-mediated mechanisms significantly contribute to antibody efficacy *in vivo*. Activation of effector mechanisms is dependent on the amino acid sequence present in the CH2 domain of the constant region and glycosylation of the antibody. Glycosylation can significantly affect the in vivo safety, stability and efficacy profile of therapeutic mabs [21].

Several approaches are currently persued to improve the interaction between Fc receptor-expressing effector cells and tumor target antigens.

Improving the effector function of mabs by engineering: Early studies in the field of antibody engineering focused on evaluating panels of antibodies with same variable domains and different constant domains of Ig G isotypes. Currently such studies are towards Fc engineering and glyco-modifications for potential therapeutic mabs.[22] Tumor cell killing by effector mechanism is triggered by mostly human Ig G1 through the interaction between the Fc region of an antibody bound to a tumor cell, and the Fcγ receptors Fcγ RIII (CD16), Fcγ RII (CD32) and Fcγ RI (CD64) on immune effector cells such as natural killer cells, neutrophils and macrophages or complement component C1q [23].

These interactions are enhanced by altering amino acids in the Fc domain by advanced Fc engineering or by modifying Fc linked glycosylation, which enhances the efficacy of effector functions. The clinical importance of the interaction between Fc, FcγR and complement for antitumor activity was shown for Herceptin and Rituxan antibodies [24]. Amino acid residues necessary for Clq and FcγR binding of human IgG1 are located in the constant region of the CH2 domain [25]. Amino acid residues comprising in human Ig G includes Leu234–Ser239, Asp265–Glu269, Asn297–Thr299, and Ala327–Ile332 are involved in binding to Fcγ receptors [26, 27]. Antitumor activity of Herceptin was attenuated by a mutation (D265A) that impairs binding to FcγRIII and FcRγII.

Substitution of the Leu 235 to Glu, Leu 235 to Ala and Gly 237 to Ala in chimeric human IgG1 anti-HLA-DR antibody abolished the FcγRI binding by several fold [28]. These data suggested that IgGs interact with FcγR in slightly different manner. The studies indicate the potential for increase the antitumor activity of an antibody by manipulating the Fc region to increase. It also increases its affinity for the activation of receptors.

Glycosylation of IgG molecules at Asn297 (using the kabat EU index numbering system) on each heavy chain helps to maintain the tertiary structure of their CH2 domains, and is necessary for effector functions. Antibodies with oligosaccharides bearing a bisecting N-acetyl glucosamine (GlcNAc) lacking core fucose is more efficient at recruiting ADCC [19]. The company Glycart generated CHO cell line transfected with the GNTIII enzyme and produced an anti- neuroblastoma antibody product bearing bisecting GlcNAc residues that exhibited a 15- to 20- fold improvement in ADCC [28].

CDC is mediated through series of complements triggered by the binding of C1q to the Fc region of antibody which bound to antigen on cancer cell. This classical pathway is initiated by activation of C1q (close proximity to cell) which is bound to antibody results in the deposition of C3b, C5b, C6, C7, C8 subsequently leads to polymerization of C9 on cancer cell, which allows the formation of cytolytic membrane attack complex (MAC). Once MAC forms on cell membrane induces the target cell lysis through influx of ions and water by generation of membrane penetrating pores.[29] Several studies indicate that few residues around L235, D265 & P331 in human Ig G constant region are important for CDC activity [30].

Antibodies with improved ability to support CDC have been created in Rituximab by mapping binding sites for the complement component C1q on Fc domain for enhanced C1q binding. One example is site-directed mutations of anti CD-20 Rituximab with Alanine at specific positions of D270A, P329A, and P331A were assessed for CDC activity but mutants showed abolition of C1q binding and CDC activity [31]. Glycosylation is one of the most common post-translational protein modifications and oligosaccharides are attached via an N-glycosidic bond to asparagine residue on each heavy chain, represent average of 2 to 3% of the total antibody mass. Glycane on heavy chain consists of a biantennary core sugar structure consisting of *N*-acetyl glucosamine, mannose and variable additions of terminal and branching sugar residues such as *N*-acetyl glucosamine, fucose, sialic acid, and galactose. Particular glycoforms are plays important role in cytotoxic effector functions of mabs and glyco patterns differ according to expression systems [32, 33].

The positive correlation of CDC activity with glycosylation has been found in the anti-CD20 antibody Rituximab, where C1q binding to the Fc region increases with the percentage of galactosylation, and similar effects have been found in other CDC-dependent therapeutic antibodies [31, 34]. These kind of approaches for enhancing CDC in rituximab may be applicable to wide range of monoclonal antibodies and several glycoengineered antibodies are currently investigated in clinical trials.

Antibody conjugates: Chemotherapy selectively kills more rapidly proliferating cancer cells but has less effect in patients whose malignant cells are resistant to drugs. Due to the complex nature of cancer, combination of mAbs agents with other target therapeutics will be the best approach in clinic. Antibodies well suited as targeting agents to deliver cytotoxic compounds to cancer cells.[35] Most of the targeted cancer associated antigens are growth factor receptors, which are overexpressed in number of cancers. Activation of these receptors induces tumor cell growth and insensitivity to chemotherapeutic agents [36].

Targeting of these molecular cell surface markers by immunoconjugates is the evaluation of novel therapeutic approach in which antibodies are linked to the cytotoxic compounds such as radioisotopes, drugs, catalytic toxins, cytokines and enzymes [37]. These kind of approaches lead to dual role of antibodies as Killer of cancer cells by effector functions and delivering vehicle for cytotoxic agents. The process of antibody conjugation through the chemical cross linking reagents, dipeptides or directly to the antibody or recombinant methods, strategies may vary with drug, linker and antibody used. Conjugation technologies are critical in generating effective antibody conjugates, can have major impact on activity, antigen binding

property, aggregations in circulation, bio distribution and pharmacokinetics [38]. Target antigen and antibody is likely to be crucial to the success of immuno-conjugate strategies, ideally the target antigen should be universally found on tumor cells of a given type, but absent or present at much lower levels on normal cells [39].

Immunoconjugate targeted cancer associated antigen would also be internalise once bound by antibody, these internalization of immunoconjugate –antigen complex leads to intracellular cleavage and release of cytotoxic drug. Application of proteomic tools and sequencing of human genome for discovery of molecular markers provides valuable approach to identify new targets for cancer therapy.

Antibody conjugates and site-selective prodrug activation is to reduce toxic effects on bystander tissue, is the antibody-directed enzyme prodrug therapy in which an antibody-bound enzyme is targeted to tumor cells, which permits selective activation of a nontoxic prodrug to a cytotoxic agent at the tumor site has resulted promising results in clinical trials and achieved regulatory approval for several drugs now on the market [40]. Significant advancements have been made in the antibody conjugation technology but there are major issues with conjugation technology like impact on antibody affinity, internalization rate, intracellular compartmentalization, and pharmacokinetics.

Radioimmunoconjugates: The addition of a radioisotope to a therapeutic monoclonal antibody can improve its activity through targeted radiation. Antibodies with radionuclides enables them to kill cancer cells, this is because of α, β and γ radiation emitted by commonly used radionuclides as shown in **Table 2** (131 Iodine, 90 Yttrium, 186 Rhenium and 188 Rhenium) are cytotoxic to many cell diameters [41]. Radioimmunotherapy (RIT) has the advantages like radiolabeled antibody does not need to be internalized or radionuclide does not need to be dissociated from antibody as it required for drug conjugation and shows combined effect of radiation induced cell death, immune mediated cellular toxicity [42].

Designing of optimal radioimmunoconjugates must be considered following components like

a) Selection of the antibody and target antigen.

b) Choice of the radionuclide with nature of radiation and

c) Delivery system of the radionuclide to cancer and cell clearance behaviour of the Isotope-complex [43, 44].

d) Half-life of the radionuclide should be long enough to allow the radiolabeled monoclonal antibody to reach the tumor and short enough to limit radiation exposure to normal tissues.

e) Radiolabeled monoclonal antibodies should be specific to the target and easy to be eliminated from the excretory organs [42].

Selection of the radionuclide is important aspect in designing safe and effective therapy for cancers and β emitters have been most widely used in radioimmunotherapy. Radioimmunotherapy (RIT) is one of the therapeutic strategy that uses an antibody recognizes a tumor-associated antigen carries a cytotoxic radionuclide to target and destroy cancer cells. CD20 is the major target used in therapy of B-cell leukemia and lymphomas has an expression limited to B-cells [45, 46].

TABLE 2: RADIONUCLIDES USED IN RADIOIMMUNOTHERAPY

Isotope	Radiation	Half-life(Hrs.)
Copper-67	β	62
Iodine -131	β or γ	193
Rhenium-186	β	91
Rhenium-188	β or γ	17
Lutetium-177	β	160
Yttrium-90	β	64
Actinium-225	α	240
Astatine-211	α	7
Bismuth-213	α	46 min

Irbitumomab tituxetan ($Yttrium^{90}$) and tositumomab ($Iodine^{131}$) both are the radioimmunoconjugate antibodies approved by the FDA for use in patients with recurrent low-grade or transformed low-grade lymphoma [23, 47]. These two radioimmunoconjugates were mouse monoclonal antibodies and humanized forms of these mabs would bind and target not only the CD20 positive target cells but also cells containing Ig G receptors, could do more harm to non-target cell types than targeted cancer cells. The development of

human anti mouse antibody (HAMA) response was low and reported as 8% for I-131 tositumomab and 1% for Y-90 irbitumomab. Both of these radioconjugates have significant antitumor effect by emitting β radiation, β particle have longer path length than diameter of the cancer cells.

One of the main attractive features of radio-immunotherapy is the crossfire effect which allows the ability to damage neighbouring cells from the site of antibody binding cell in tumors. There have been numerous studies of radioimmunotherapy with α particles. Alpha emitters travel only few diameters of cells with their short path length and deposit high linear energy transfer (LET) with in the target leads to highly selective killing of tumor cells [48, 49].

Antibodies conjugated with radionuclides such as 212 Bismuth, 213 Bismuth and 211 Astatine emits α-particles that are highly cytotoxic to many cell types but difficult to identify α-emitters that have physical half-lives suitable for radioimmunotherapy. Radio-immunotherapy with α emitting radionuclides is particularly interesting approach for cancer treatment, but difficult to use in clinically due to short half-life and difficult for homogenous production with proper yield. Different chemical pathways have been developed for conjugation of radionuclides to the monoclonal antibodies. Yttrium nuclide linked through chelating agent MX-DTPA (tiuxetan) to the lysine and arginine amino acids in the Fc portion of the Irbitumomab and Iodine nuclide directly conjugated to tyrosine amino acids via covalent bonds to tositumomab [44 ,50].

In most cases, antibody radiolabeling is accomplished either by direct iodination of tyrosines or indirect conjugation with more stable metal chelators such as DTPA (diethylenetriaminepentaacetic acid) and its derivatives or DOTA (1, 4, 7, 10-tetraazacyclo-dodecane-1, 4, 7, 10-tetraacetic acid) to the antibody molecule. These bifunctional chelating agents contain reactive group for conjugation of antibodies and strong metal chelating group for radiolabeling.

Radioimmunoconjugate will bind to target antigen on cancer cell, the chelators function is to chelate the radionuclide from antibody where it can function in a therapeutic manner to destroy it [51]. However there are limitations in development of radio immunoconjugates like mabs do not deliver sufficient radiation doses to the solid tumor site, complicated chemistry in conjugation and potential toxic effects on normal tissues [52]. The use of antibodies with radionuclides, drugs, toxins and improved conjugation technologies will expand as the next generation of mab-based products for cancer therapy.

Antibody Drug Conjugates: Unconjugated antibodies alone can be effective, but more often, antibody conjugate with cytotoxic compounds enhances the efficacy of the standard treatment. Antibody drug conjugates are proteins that contain drug along with an antibody, bind specifically to antigen and to be internalized by the target cell, involves release of drug from the antibody and leads to target cell lysis [53].

Antibody conjugates can be divided into two types, chemical conjugates and recombinant methods. Development of antibody drug conjugate involves selection of antibody, stability of the linker, and the cytotoxic drug potency. Currently maytansinoids, auristatins, anthracyclins, duocarmycins are being conjugating with antibodies as highly potent cytotoxic drugs with diverse mechanism of action and several antibody conjugates are in various stages of clinical development [54].

Several structural aspects of the antibody permit conjugation of small molecule drugs by partial reduction of interchain disulphide bonds or formation of free thiol groups without changing the antigen binding property and structural characteristics of the antibody. Choosing the right crosslinking reagent is essential for proper drug delivery into target tumor cells for selective killing.

Most of the antibody conjugates constructed through the reaction of drug, crosslinking reagent with solvent accessible reactive amino acids such as lysine side chain amines and cysteine sulfhydryl groups by reducing interchain disulfide bonds in antibody. Both of these conjugation technics may yield heterogeneous products with distinct *in vivo* pharmacokinetics, efficacy, safety profile and consistent batch-to-batch production would be difficult to control [55]. Most of the antibody drug conjugates evaluated by hydrazone or disulfide linkage and drug is released from antibody by

reduction or acid hydrolysis within lysosomes after internalization.

Conjugation technologies by hydrazone or disulfide have short stability half-life than antibody in circulation, leads to toxicity for normal cells by released drug and masking of tumor antigen with mab devoid of drug [56]. Gemtuzumab ozogamicin (Mylotarg) is a conjugate of a humanized anti-CD33 Ig G4 linked to cytotoxic antitumor antibiotic, Nacetyl-γ-calicheamicin dimethyl hydrazine, (isolated from a bacterium, *Micromonospora echinospora* sp. *Calichensis*) by acid-hydrolysable linker acetyl phenoxy butanoic acid and has been approved for treatment of CD33-positive acute myeloid leukaemia(AML) [57].

Binding of Mylotarg with the CD33 antigen results in the formation of a complex that is internalized and the calicheamicin derivative is released inside the lysosomes of the myeloid cells. The released calicheamicin derivative binds to DNA in the minor groove resulting in DNA double strand breaks and cell death by apoptosis [58]. Gemtuzumab conjugate approved for AML in 2000 & withdrawn in 2010 because a post-approval clinical trial raised new questions about the drug's safety and effectiveness. Doxorubicin also one of the most commonly used conjugate in antibody-based applications. Doxorubicin is an anthracycline produces its cytotoxic effects by alkylation of double-stranded DNA resulting in inter strand crosslinking, triggering apoptosis [20].

Optimal number of drugs per antibody is two, four or more through site specific conjugation is more important for efficient antibody function. Increasing the drug/antibody ratio can lead to reduction in antigen binding & aggregate formation, lower in this ratio can be more potent. Brentuximab vedotin is one of the most active antibody drug conjugate consists of monomethyl auristatin E (MMAE) attached with valine citruline dipeptide linker to cysteine residue of hinge region in anti-CD30 antibody. This conjugation strategy is distinguished from earlier methodologies due to significant stability of the linker, selectively cleaved by lysosomal enzymes and highly potent drug component [59].

Antibody drug conjugation through disrupted interchain disulfide bonds may potentially affects stability, in vivo distribution and effector functions. New approaches like design of thiomabs with site specific engineering of cysteine residues in a antibodies for conjugation of drugs can allow development of antibody conjugates without disruption of interchain disulfide bonds [48]. Another example is trastuzumab is very active agent in HER2 expressing breast cancer but majority of the patients who initially respond to trastuzumab develops resistance within one year of treatment. Trastuzumab–DM1 conjugate is designed to combine the biological activity of trastuzumab with targeted intracellular delivery of highly potent antimicrotubule agent maytansine derivative (DM1). DM1 is conjugated to trastuzumab by using SMCC (*N*-Succinimidyl- 4 -(maleimidomethyl) cyclohexane carboxylate) linker is first linked to heavy chain lysine residue of the antibody.

Thiol group in DM1 is then reacted with malemide group of the linker to form more stable nonreducible thioether bond. T-DM1 is the first immuno-conjugate with a noncleavable thioether linker in clinical trials and release active cytotoxic agent lysine-MCC DM1 following internalization into target cells by lysosomes, causes cell death [60, 61]. Antibody drug conjugation with noncleavable linkers may have significant advantage of less toxicity to antigen independent cells than cleavable linkers due to decreased release of free drug into circulation and are currently being testing in clinics.

Conjugates developed by chemical linkage have more complex heterogeneous products and heterogeneity is challenging for process control [62]. Recently reported site specific engineering of cysteine residues in antibody called thiomab technology platform produces more homogeneous preparations of antibody drug conjugates with minimal disruption to the antibody structure and antigen binding site [54]. In addition, development of immunotoxins which contain a toxin with either mutated or deleted cell binding domain that prevents it from binding to normal cells can also improve the treatment of cancer [63].

Example is truncated form of Pseudomonas endotoxin protein conjugate genetically linked to a CD25 or CD22 specific antibody have induced remissions in patients with hairy cell leukemia [43]. Most immunotoxins comprise either a plant toxin, such as ricin A chain or a

bacterial toxin like *Pseudomonas* exotoxin and modified diphtheria toxin conjugated or genetically fused to an antibody or antibody fragments that bind to cancer cells. However, these toxins are foreign proteins and leads to formation of neutralizing antibodies are a concern for repeated use and conjugation procedure was difficult and often of low yield, and the junction between the toxin and ligand could be at many different sites (for example, at any lysine residue). The concept of enhancing the antitumor activity of antibody conjugates in cancer patients has been challenge and it is hoped that future therapies may consists of antibodies with conjugates.

Antibody-Directed Enzyme Prodrug Therapy (ADEPT): Antibody-directed enzyme prodrug therapy (ADEPT) is a therapeutic strategy which aims to improve the selectivity of anticancer drugs. ADEPT is a two-step antibody targeting system in which the antibody-enzyme conjugate component is first targeted and allowed to localize a tumor followed by clearance of the residual circulating antibody. The cytotoxic agent (prodrug) is then administered, diffuses widely but in the ideal case is activated solely at the tumor following contact with the localized conjugated protein [64].

For the clinical success of this strategy, the enzyme should be either of non-human origin like bacterial β-lactamase and carboxypeptidase G2 or human protein that is absent or expressed only at low concentrations in normal tissues such as carboxypeptidase A and β-glucuronidase. The prodrug should be good substrate for the enzyme but not to be activated by endogenous enzyme in non-tumor tissues and should not leak into the systamatic circulation [65]. Some of the enzymes and prodrugs used for ADEPT has been given in below **Table 3**. Interval between antibody conjugate and prodrug administration should be optimised so that the conjugate is binding to target cells rather than in blood to avoid systemic toxicity. An example of anti-carcinoembryonic antigen (CEA) single-chain Fv fused to the amino terminus of the enzyme carboxypeptidase G2 (CPG2) has been constructed as a recombinant fusion protein called MFE-23 to achieve ADEPT in CEA-producing tumors [66, 67]. The bacterial enzyme CPG2 converts a prodrug, 4-[*N*,*Nbis*(2-iodoethyl) amino] phenoxycarbonyl L-glutamic acid (ZD2767P), into an active bifunctional alkylating drug (ZD2767) and can act by alkylating the DNA or by formation of cross bridges in the DNA [68, 69].

Another example is Recombinant fusion protein A33scFv::CDy, is phage display-generated anti-gpA33 single chain fragment A33scFv with cytosine deaminase from yeast (CDy), which converts 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) by deamination. 5-fluorouracil (5-FU) is a pyrimidine analogue which inhibits the DNA synthesis and leads to cell cycle arrest and induces apoptosis in target cell [70]. The cytotoxic effect of the drug is ideally confined to the tumor target, so reducing toxicity compared with systematic administration of cytotoxic chemotherapy. Targeting of prodrugs to tumors is particularly attractive in that it has the potential to greatly reduce the systemic toxicity of conventional radio immunotherapy and cytotoxic chemotherapy respectively [71, 72].

ADEPT has proved highly effective in several different tumor xenograft studies, but it is exceedingly difficult to translate into clinical practice because the obstacle has been the immunogenicity of both the enzymes used for prodrug activation and the targeting mab which are both derived from non-human sources [73, 74]. Human enzymes in conjunction with humanized or human mab should greatly reduce this immunogenicity problem. The potential advantage of ADEPT is amplification of cytotoxic activity by conjugated enzyme. Such thoughts inspired the development of enhancing the antitumor activity of antibodies in cancer patients remains an on-going challenge and these problems may be overcome in the near future.

TABLE 3: ENZYMES AND PRODRUG COMBINATIONS USED IN ADEPT STRATEGIES.

Enzyme	Prodrug	Activity
Carboxypeptidase G2	Nitrogen mustards	Cleavage between glutamyl moiety and aromatic nucleus.
Cytocine deaminase	5-fluorouracil	Deamination of cytocine to uracil.
Carboxypeptidase A	Methotrexates	Cleavage of α-glutamyl peptides.
β-Lactamase	Doxorubicin, Paclitaxel, Mitomycin.	Cleavage of lactam ring, elimination of substituents attached to 3' of cephalosporin derivatives.
Alkaline phosphatase	Etoposide, Doxorubicin, Phenol mustard.	Hydrolysis of phosphate groups.

CONCLUSION: Therapeutic monoclonal antibodies (mabs) represent one of the fastest growing areas of the pharmaceutical industry. Variety of strategies has been developed to decrease the toxicity and improve the efficacy of antibody based therapeutics in oncology like generation of chimeric, humanized and fully human antibodies and was consequently approved by FDA. In addition, antibody engineering by amino acid substitutions to increase the efficacy of effector functions and conjugation of cytotoxic compounds like radionuclides, drugs and toxins is expanding as the next generation of mab based therapeutics for cancer. It is hoped that future therapies may consists of a combination of various antibodies targeting different antigens on the cancer cells.

ACKNOWLEDGEMENTS: The author would like to thank Kavitha Iyer Rodrigues and Dr. Sohang Chatterjee for valuable suggestions and helped in all areas of work.

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How to cite this article:
Choudary KB: Antibody Engineering and Immunoconjugates for Cancer Therapy. *Int J Pharm Sci Res*. 3(10); 3618-3629.