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Targeted therapy of cancer using diphtheria toxin-derived immunotoxins

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The mortality rate in cancer patients demands novel therapy. One of the novel approaches developed in recent decades includes immunotoxins. Cancer cells frequently have specific growth factor receptors/antigens overexpressed on their surface; this is the principle of selective targeting of immunotoxins. Ligands recognizing these receptors and antigens can be conjugated to modified toxins. Continuous efforts are being made (i) to investigate molecules exclusively expressed on cancer cells, (ii) to improve the specificity and efficacy of these immunotoxins, (iii) to eliminate side effects (iv) to decrease immunogenicity and (v) to improve pharmacokinetics and ensure better drug delivery.

Introduction

A neoplasm, or tumor, is an abnormal mass of closely packed cells characterized by uncoordinated, uncontrolled growth. The cancer cells may, or may not, invade neighboring cells and accordingly they are termed as invasive or noninvasive. In the case of invasive tumors, they may spread to remote tissues, either through the circulatory or lymphatic systems, a process termed as metastasis. Progression depends on molecular and chemical signals received by the 'transformed' cell. Treatment of cancer depends on grade, stage and type of cancer. Tumor grading indicates the level of differentiation, which is represented by the Roman numerals 0–IV, where increased values indicate increasing loss of differentiation. Staging of tumors, on the other hand, is more uniform as it indicates the severity of the disease.

The conventional therapies (chemotherapy, radiation therapy and so on) used today target uncontrolled cell division, which is the most important feature of a transformed cell. The rate of tumor growth depends on factors such as (i) growth fraction – the number of cells in the tumor that are capable of proliferation, (ii) the time required to complete the cell cycle (iii) the rate at which cells within the tumor are shed. Most chemotherapeutic agents target the proliferating pool, so rapidly growing tumors respond very well [1,2]. By the time of diagnosis, however, due to tumor progression, the growth fraction decreases, resulting in a poor response to chemotherapy. Conventional therapies have their own limitations, for example radiation therapy affects not only the tumor, but also the surrounding normal tissue. Most chemotherapeutic agents and radiation cause DNA damage, leading to genomic instability and a susceptibility to mutations that, again, predisposes to cancer [3]. Surgery is applicable only at the initial stages of non-hematological tumors. In many, if not most, cases, however, by the time of diagnosis, the disease can have advanced to metastasis. All the above limitations of conventional therapies show the need for novel therapy, with a wider margin for safety and good selective toxicity to the tumor. New modes of therapy include proton therapy, photodynamic therapy, biologic therapy and so on, most of which are targeted therapies, selectively aimed at cancerous tissue and not at normal tissue, thereby minimizing toxicity and enhancing potency [4]. Immunotoxins, a class of biologic therapeutics, can potentially provide a more effective and specific treatment for cancer than other contemporary methods [5]. Surface-targeted biologic therapies, such as unlabelled MAbs (monoclonal antibodies), kill cells after binding, through apoptosis induction, antibody dependent cytotoxicity and complement dependent cytotoxicity [6]. Immunotoxins are a better option for those with malignant cells resistant to apoptosis and whose immune systems will not perform antibody or complement-dependent cytotoxicity [7]. Radioimmunotherapy is limited by the potency of the radionuclide molecules that can be conjugated to each MAb molecule [8]. There are limitations to

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1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2008.06.017

various surface-targeted strategies, but immunotoxins are distinct from these approaches and target the surface of cancer cells with considerable potency, using protein toxins that kill the cell with a single molecule.

Selective toxicity

Cancer cells usually have specific growth factor receptors or antigens overexpressed on their surface, compared to normal cells [9]. Ligands corresponding to these receptors or antibodies raised against antigens can be conjugated to toxins, these conjugates will selectively bind to these overexpressed molecules and kill the tumor cells. Several groups are working on detecting molecules exclusively expressed on cancer cells, which will definitely be of immense use for selective targeting.

Tumor immunology

The immune system recognizes tumors as foreign through antigens overexpressed on their surfaces. Tumor antigens were initially classified into tumor-specific antigens and tumor-associated antigens, but later it was found that tumor-specific antigens were not actually specific and recent classification has evolved, based on the source of origin of the tumor. Antigens are also used in diagnosis, prognosis and to follow response to therapy. Some of the common tumor antigens include oncofetal antigens, antigens produced by oncogenic viruses, protein products of mutated genes, oncoproteins and so on. The immune system does recognize and fight cancer cells, but the degree of recognition and response seems to be far less than workers had originally expected; it has been argued that the reason for this is that tumor cells are not viewed as particularly foreign to the body. Activation of similar immune responses by the use of adjuvants, like recombinant IL-2 or *ex vivo* stimulated LAK cells, is one practical approach [10]. In a similar process, IL2 ligated to toxin (immunotoxin) can also stimulate immune response. The immune response is of two types, that is non-specific and specific. Anti-tumor activity in the host comes is an example of specific immunity, as it is triggered specifically to the existing tumor; by contrast, the non-specific immune response is general and present throughout life. The lymphatic and immune systems, consisting of lymph, lymphatic vessels, lymphatic tissue, and bone marrow are important for anti-tumor activity; their other functions include transport of nutrients and draining of interstitial fluid. Lymphatic tissue contains a large number of lymphocytes; two important lymphocytes that mediate immune response are B and T cells. They are referred to as immunocompetent because of their ability to fight external invading microbes like bacteria, virus, fungi as well as abnormal tissue. Anti-tumor activity/tumor immune response involves both cell mediated and humoral immunity [11,12].

Cytokines are a group of immunomodulatory agents, which include interleukins, lymphokines, interferon, monokines, chemokines and colony stimulating factors. They are proteins, or glycoproteins, acting at very low concentrations and they exhibit pleiotropy, synergism, antagonism and redundancy. They are classified, based on their origin and function, into two types. The first type modulates cell-mediated immune response and are derived from type I helper T cells; they include IL2, IFγ, IL12 and TNFβ. The second type mediate humoral immune response and are derived from type II helper T cells and include IL4, IL5, IL6, IL10 and IL13. Cytokines display a dual role with regard to cancer, a few inhibiting tumor progression and others assisting tumor progression, The two most important cytokines with anti-tumor activity approved by FDA for cancer treatment are IL2 and type I IFN. IL2 promotes natural killer cells, T cells and acts as a mitogen, interferes with blood flow to tumor, hence it is best-suited as an adjuvant [13]. Low dose recombinant IL2 is proved to activate antitumor immune response in advanced malignancies [14–16]. IL2 exhibits its activities by binding to its various receptors. IL2 receptor has three subunits α, β and γ, of which the γ subunit is most common in the interleukin family. The high affinity receptor has all α, β and γ subunits, whereas the low affinity receptor has only the α subunit and the intermediate affinity receptor has β and γ subunits. The α subunit is important to cement the complex, the β and γ subunits are important for internalization and signal transduction [17–19]. The high affinity receptor is transiently expressed on T and B cells but not on normal tissue, thereby facilitating selective targeting. IL2 receptor-targeted therapies were used in treatment of neoplasia, autoimmune diseases and transplantation [20]. Interferon is a substance produced in response to viral infection but it is not a virus-specific protein. Apart from its antiviral properties it also has anti-proliferate, immunomodulatory and anti-angiogenic properties; interferon leads to activation of B and T cells and increases antigen receptor expression. It can be used as an adjuvant in cancer therapy [21]. Type I IFN, that is IFN α and IFN β were approved by FDA for cancer therapy, whereas IFN γ is not approved [22].

Immunotoxins

Immunotoxins are proteins that contain a toxin conjugated to an antibody or growth factor by genetic fusion or by chemical ligation; the antibody or growth factor provides the ability to target specifically the conjugated toxic payload to the cancer cells. Designing effective immunotoxins is based on utilizing the cellular entry mechanism inherent in the parent toxin to cross the plasma membrane barrier to the cytosol, where it exerts its effect. Protein toxins that are used in cancer treatment along with targeting molecules are plant toxins (such as ricin, abrin, saporin, gelonin), bacterial toxins (such as diphtheria toxin, pseudomonas exotoxin) [23,24], fungal toxins (such as restrictocin) [25] and animal toxins (e.g. hemolytic toxin from sea anemone) [26]. The immune system components that have been used as targeting molecules include cytokines, monoclonal antibodies, immunoglobulins and growth factors.

The immune response against cancer is poor and inadequate because of the lack of specific antigens. Moreover, tumor cells develop mechanisms to escape immune response by antigen masking, decreased expression of MHC (major histocompatibility complex), decreased production of co-stimulators and so on. Moreover, the immune system acts to eliminate highly immunogenic cells, that is, cells expressing more antigen, leaving behind the pool of cells that are relatively less immunogenic. Inducing or activating the immune system to fight against cancer is one practical approach being considered in recent years. Some of the approaches that have been tried include:

- Administration of cytokines like IL-2 (proleukin) [27], IFN, TNF [13].

- LAK (lymphokine-activated killer cells), lymphocytes are isolated from patient's blood, activated in the laboratory by IL-2 and re-infused back [28–30].
- TIL (tumor-infiltrating lymphocytes), which are similar to LAK [27–33].
- Monoclonal antibodies; these are raised against tumor specific antigens and are used either alone, or in a conjugated form, the host humoral immune response reduces the half life of MAbs raised in mouse, therefore humanized antibodies are the product of choice [31,32]. Antibodies mediate their action either by binding to the respective antigen against which it was raised or by blocking sites to which ligands bind, thereby inhibiting growth and proliferation signals.
- Fusion proteins/immunotoxins; these are chimeric protein molecules constructed by fusing a protein toxin to a cell-selective ligand. Such chimeric molecules exhibit selective targeting of tumor cells. The mechanism of action of fusion proteins depends on the toxin employed, most of which are protein synthesis inhibitors, for example Ontak [34,35].

Generations of immunotoxins

Immunotoxins can be classified into generations, based on their date of discovery, construction method employed and potency.

First-generation immunotoxins

These are relatively primitive molecules, made up of the entire toxin moiety with mutations to render the receptor-binding domain non-functional and are fused to the ligand by chemical means using cross-linking agents to introduce disulfide bonds, or establishing an amide bond between two proteins. The ligand employed is the whole antibody or monoclonal antibody. The drawbacks are: a poor tumor uptake; extended half-life and difficulties in production due to their large size.

Second-generation immunotoxins

These are modified versions of first generation immunotoxins, which have been constructed to circumvent some of the drawbacks of the first generation molecules. Here, the receptor-binding domain has been completely deleted and is fused to ligand by genetic engineering. The ligand employed is a fragment of recombinant antibody, or cDNA encoding growth factors or cytokines corresponding to molecules overexpressed on the tumor cell surface [36,37].

The most recent immunotoxins are the bispecific immunotoxins; they can probably be classified as third generation immunotoxins. Bispecific immunotoxins are designed to recognize two separate determinants on tumor surface. As a result of their dual specificity they exhibited greater activity and reduced toxicity [38,39].

Toxins

The mechanism of cytotoxicity of immunotoxins depends on the toxin employed: toxins used in the construction of fusion toxins are generally protein synthesis inhibitors and share common features like structure, mode of action and so on. Here we elaborate upon diphtheria toxin. Native diphtheria toxin binds to heparin

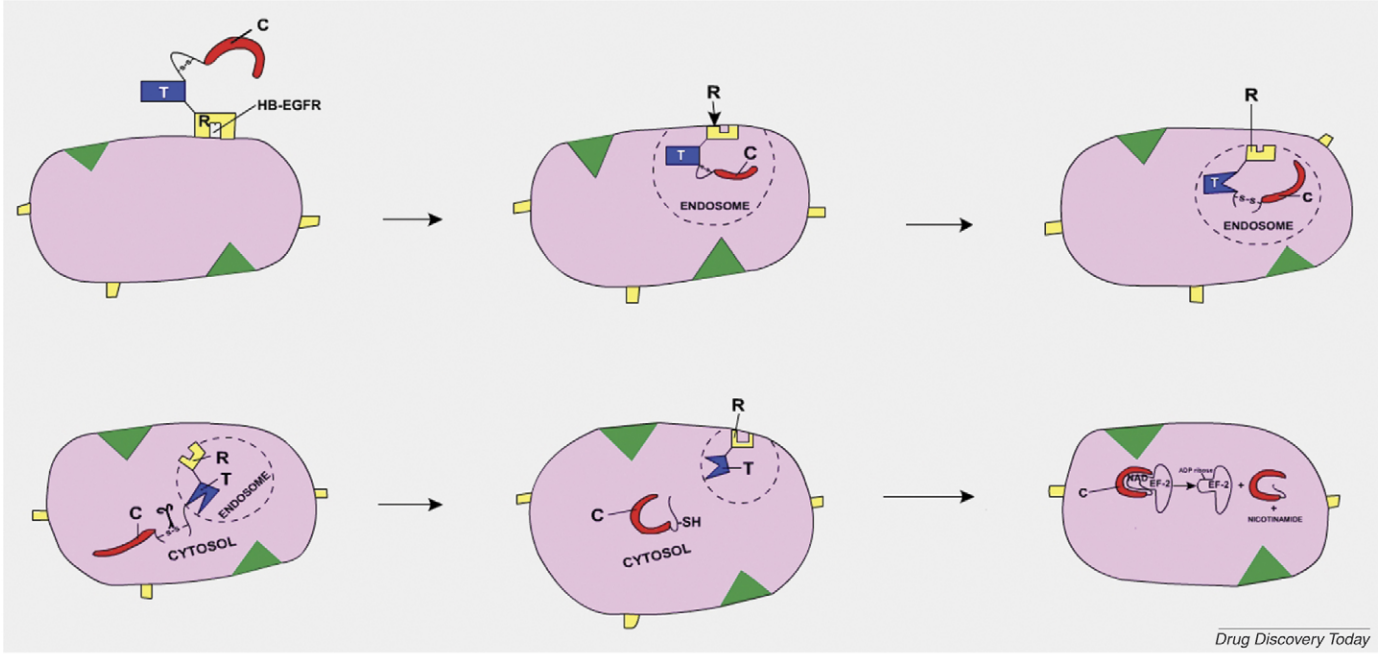

FIGURE 1

Schematic representation of mechanism of action of diphtheria toxin in its native state. C: catalytic domain, T: translocation domain, R: receptor binding domain, HB-EGFR: heparin binding epidermal growth factor receptor. The R domain of diphtheria toxin binds to HB-EGFR on the cell surface and undergoes receptor-mediated endocytosis. In the endosome at acidic pH furin nicks the loop connecting the T domain and C domain and induces conformational change in T domain to facilitate membrane insertion and translocation of C domain into cytosol. In the cytosol the disulfide bond linking the C and T domains is reduced resulting in release of free C domain. The C domain inactivates EF2 by transfer of ADPR moiety from NAD thus halting protein synthesis.

binding epidermal growth factor receptor on cell membrane from which it is transported into the endoplasmic reticulum by receptor-mediated endocytosis as a vesicle. A soluble cytoplasmic protein known as dynamin helps in pinching off the vesicle from the membrane. This vesicle is coated with a cage of protein called clathrin towards the cytosolic surface and this coat is discarded before the vesicle fuses to its target. Vesicular ATPases acidify the endosomes leading to a decrease in pH to about 6.0. At this acidic pH, the T domain undergoes a conformational change, resulting in partial unfolding and exposure of hydrophobic sites mimicking transmembrane proteins [40,41]. This helps in membrane insertion and the formation of channel large enough for the C domain to be translocated in an unfolded state and is released into the cytoplasm. The catalytic domain is translocated into the cytosol and receptor is recycled to plasma membrane by exocytosis from early endosome and remnants are degraded. In case of receptor down regulation, receptor is also degraded. In the cytoplasm the disulphide bond is reduced by thioredoxin reductase and Hsp 90 refolds the C domain. The catalytic domain acts by transferring ADPR (adenosine di phosphoate ribosyl) moiety from NAD (nicotinamine dinucleotide) to the post-transcriptionally modified histidine residue at 715 called diphthamide (2-[3-carboxyamido-3-(trimethylammonio) propyl]) in EF2, thus elongation factor is inactivated [42,43]. Inactivating EF-2 by diphtheria toxin results in inhibition of protein synthesis and cell death [44]. This reaction is irreversible and involves an inversion in configuration of NAD from the β to α anomer. This is a sequential ordered mechanism where catalytic domain first binds to NAD and then transfers ADP ribosyl moiety to EF2. EF2 of bacteria, that is EF-G is not susceptible whereas EF-2 of eukaryotes, yeast and archae are susceptible. Mutants that cannot produce diphthamide will be resistant to the toxin.

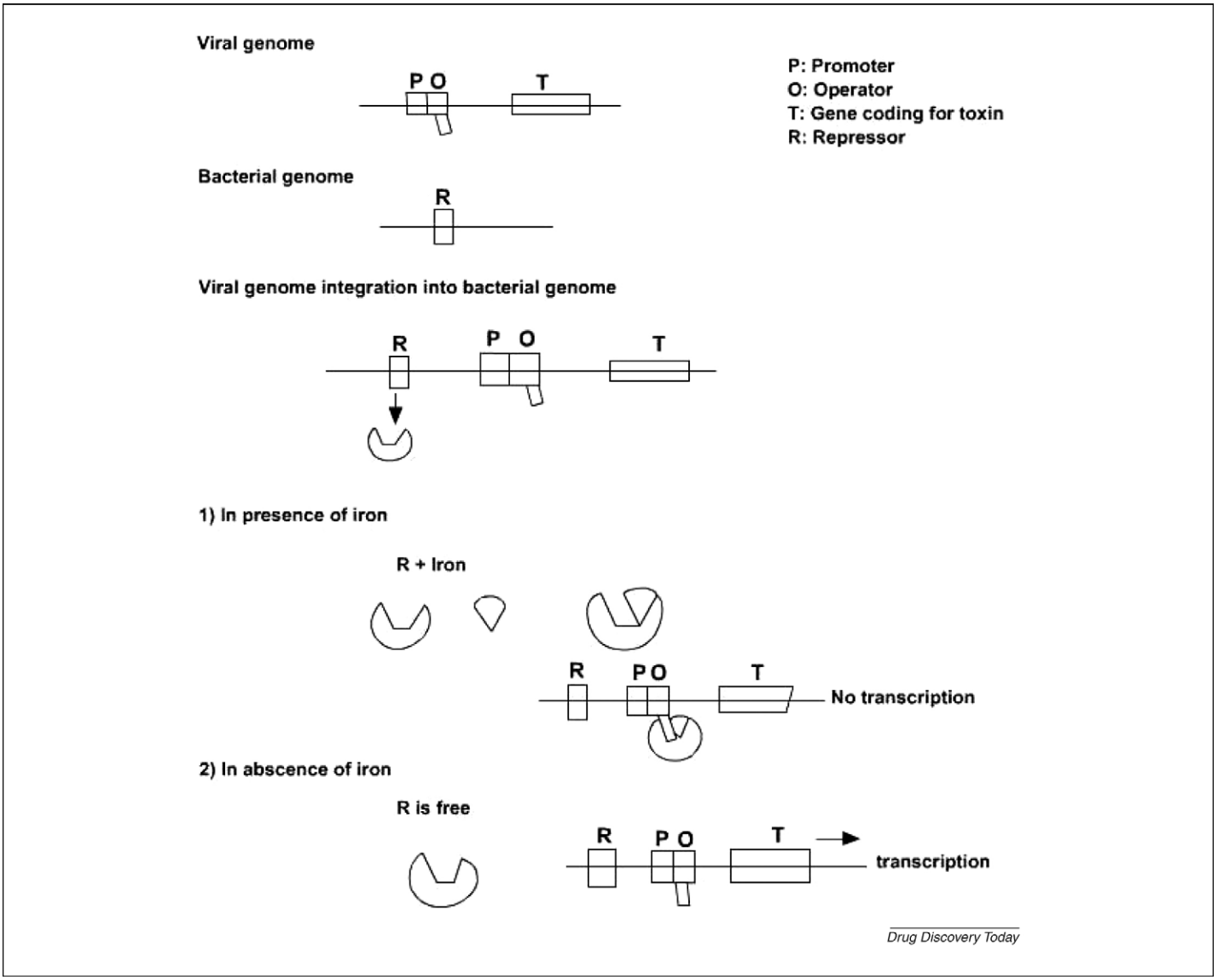

FIGURE 2

Schematic representation of gene regulation of diphtheria toxin in presence and absence of iron. The phage contains the gene for toxin, its promoter and operator sequences whereas bacteria contain sequence coding for repressor. Regulation of this gene depends on iron concentration. In the presence of iron, repressor binds to iron and the complex binds to the operator thereby preventing transcription of the gene. In case of low concentration of iron, repressor is free and cannot bind to operator so transcription of gene proceeds.

The mechanism of action of native diphtheria toxin has been illustrated in Figure 1. The entire process starting from internalization to inhibition of protein synthesis takes about 6 h.

Corynebacterium diphtheriae, a non-motile, non-capsulated, Gram +ve bacillus, can produce 5000 molecules of a potent exotoxin per hour after infection [45]. A single molecule of this diphtheria toxin is lethal to the host cell. The gene for diphtheria toxin is carried by λ phage beside the promoter and operator sequences, but the bacterium carries the repressor sequence, thus regulating gene expression. Regulation of the gene is dependent on iron concentration, as the repressor binds to iron; this complex binds to the operator, preventing transcription of the gene. At low concentrations of iron, the repressor is free and cannot bind to the operator, so transcription of gene proceeds [46,47] Figure 2.

Monomeric diphtheria toxin is compact, globular and toxic, whereas the dimer is non-toxic. Diphtheria toxin, a Y shaped molecule is a monomer of 535 residues in two fragments, A and B. A 14-residue Arg-rich loop connects the two fragments and the structure of this loop differs significantly between monomer and dimer. Fragment A, the amino terminal end, has a catalytic domain (C-domain) consisting of residues 1–193 which for ms 8 β strands and 7 α helices and brings about cytotoxicity by inhibiting protein synthesis. Fragment B, the carboxy terminal end, has two domains, a transmembrane domain (T domain) and a receptor-binding domain (R domain). T-domain (residues 205–379), has nine α helices and helps in translocating the C-domain from endosome to cytosol. The first three helices of the T-domain, constituting the amphipathic region, help in toxin stabilization on the cell surface. Helices 5, 6, 8 and 9 of this domain are non-polar and protonation of anionic residues in these helices results in loss of charge and allows penetration of the C-domain into the membrane [48]. The R-domain, consisting of residues 386–535, forms 10 β strands and helps in the binding of toxin to the heparin-binding epidermal growth factor receptor (HBEGFR) on the cell surface [49]. The diphtheria toxin has four cysteine residues, which make two internal disulphide bonds, linking C186–C201 and C461–C471.

Figure 3 shows the crystal structure of diphtheria toxin [50].

Construction of fusion toxins

Diphtheria toxin conjugate synthesis requires three basic steps

(i) *Inactivating or removing the R domain*: In the case of diphtheria conjugates, molecules with the entire R domain removed (DT386) are reported to have higher activity than conjugates with partially removed R domain (DT486), due to higher binding affinity and the tendency to remain in a monomeric form. Alternatively, conjugates can be synthesized by point mutations in the binding domain, mutating the residues in the binding loop between 510 and 530 [51,52] or a critical S525F [53] or C471Y [54] or L390F. These mutations eliminate the binding function of the toxin to its native receptor (HBEGFR) on normal cells in a fool proof manner [55].

(ii) *Identifying ligands corresponding to overexpressed molecules on cancer cell surface*: Receptors for cytokines, MAbs, proteoglycans, transferrin, GMCSF, endothelial growth factor, MSH, TRH, substance P and placental lactogen receptor are overexpressed in tumor cells [24]. Ligands corresponding to these molecules may be conjugated to toxins. Cytokines are widely employed as ligands due to their advantage of having additional cytotoxic properties however their clinical application is limited by toxicities. IL-2 has demonstrated activity against melanoma, lymphoma [34,56,57] and leukemia [35,58] IL-4 shows minimal cytotoxic activity, since it only inhibits proliferating cells. IL-6 has some anti tumor activity but is also a growth factor for myeloma cells. Thus, cytokines are useful both in hematological malignancies and immunogenic tumors. The most commonly used cytokines are IL-2, IL-3, IL-4, IL-12, and GMCSF.

(iii) *Strategy for linking ligand to modified toxin*: This is the most critical step in the construction of fusion toxins, as linking should not alter (a) the ligand's binding affinity to receptor, (b) the endocytosis of toxin, (c) the translocation of the B fragment, (d) the ADP ribosylation of elongation factor two. The ligand is located at the carboxy terminal end of the diphtheria toxin to allow the ADP ribosylation domain to translocate without ligand.

Chemical conjugation is achieved by: (a) forming an amide linkage between the carboxyl group of modified diphtheria toxin and amino groups of cytokines; (b) by cross linking using bifunctional reagents like SDPD, MDS and so on, the cross linkers add disulfide moieties to the two proteins to be fused, so that a disulfide bond can be introduced, however, disulphide conjugation has shown little success [59] (c) the use of acid-cleavable cross-linking agents which mimics intact peptide cleavage and liberation of free toxin into cytosol [60] and (d) through non-reducible thioester bonds which are useful if the ligand is attached to the part of the toxin that does not translocate to the cytosol (Figure 4)

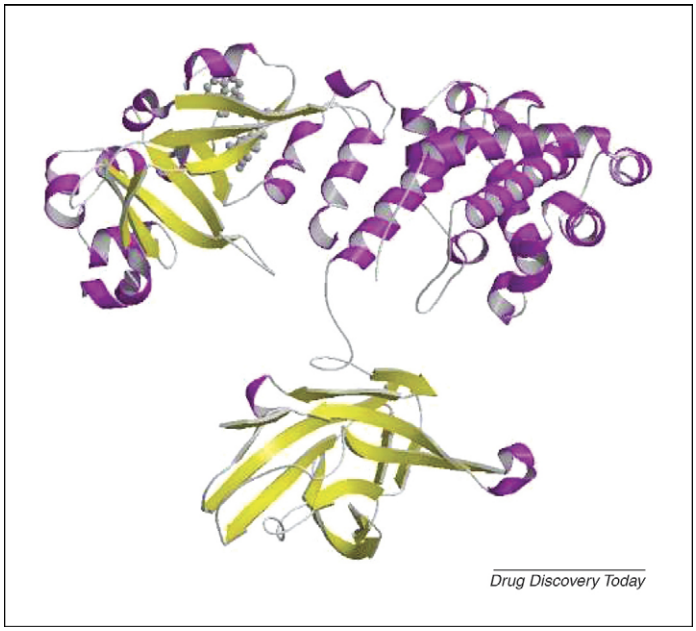

FIGURE 3

The crystal structure of diphtheria toxin in ribbon representation. (PDB ID: 1DDT) The amino terminus has a catalytic domain (C-domain) consisting of 8 β strands and 7 α helices. The carboxy terminal end has two domains, a transmembrane domain (T-domain) consisting of nine α helices and a receptor binding domain (R domain) consisting of 10 β strands. It has four cysteine residues, which form two internal disulphide bonds linking C186–C201 and C461–C471.

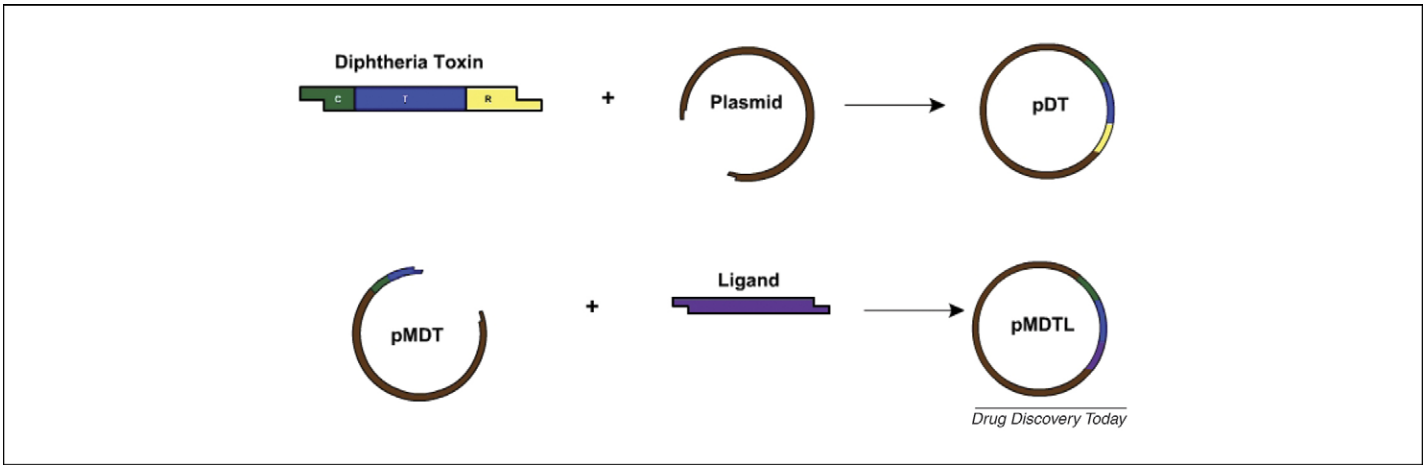

FIGURE 4

The new generation immunotoxins are constructed by recombinant DNA technology. Construction involves two basic steps. (1) The DNA coding region of diphtheria toxin is cloned into plasmid in correct translational reading frame. (2) The receptor-binding domain of diphtheria toxin is replaced by DNA coding for ligand in same reading frame to obtain fusion toxin containing fragment of toxin and ligand as a single protein. C: catalytic domain, T: translocation domain, R: receptor binding domain, pDT: plasmid carrying diphtheria toxin, pMDT: plasmid carrying modified diphtheria toxin with R domain deleted, pMDTL: plasmid carrying modified diphtheria toxin and ligand.

The drawbacks of chemical conjugation include: harsh conditions; batch-to-batch variation and unwanted by-products. Due to these limitations of chemical methods, focus has shifted to recombinant immunotoxin development, where the hybrid gene is produced by gene splicing, overlap extension and ligation into plasmids. After successful cloning, the plasmid is transformed into *Escherichia coli* from where the protein can be harvested and purified. The strategy of linking ligand to toxin genetically has not only overcome the drawbacks of chemical conjugation, but also offers added advantages, like high precision and increased stability. Introducing a linker between toxin and ligand increases the overall efficacy of the compound by facilitating better receptor-ligand interaction [61,62]. This result again varies from one immunotoxin to other depending on the length, composition and location of adapter, fusion point, toxin employed, ligand used and so on [63]. The half-life of a small size molecule can be extended by ligating them to ligands with a longer serum half-life. Immunotoxins are designed, based on selective targeting of molecules expressed on tumor cell surface, which causes minimal, or no, side effects. This strategy exhibits better safety to surrounding normal tissue. The side effects can be minimized if molecules present exclusively on tumor cell surface are further investigated. This experimental approach could lead to better design of immunotoxins that can be used in the future treatment of cancer with better efficacy and safety.

Advantages of fusion toxins

Most conventional therapies currently in use, such as radiation therapy and surgery are applicable to solid tumors, whereas immunotherapy is applicable to solid tumors, hematological malignancies and metastases. Immunotherapy is not known to cause damage to DNA, whereas chemotherapeutic agents and radiation are mutagens and, hence, carcinogens. Combination therapy of immunotoxins with chemotherapy or radiation therapy demonstrated synergy. In the case of chemotherapy and immunotoxin combination therapy, synergy is due to dual targeting of the cell, that is chemotherapy targets cell division and immunotoxins target overexpressed molecules on the cell surface. Similarly, in radiation and immunotoxin combination therapy, the synergy is due to radiation effecting signal transduction pathways, resulting in changes in receptor expression levels on the tumor cell surface, thus making it more vulnerable to immunotoxins [64–66].

Fusion toxins were primarily designed to target molecules overexpressed on the tumor cell surface. Based upon the particular molecules overexpressed on various tumor cell surfaces and the ligands used to target such molecules, these fusion toxins are used in different types of cancer. Response to these fusion toxins vary from patient to patient and with type of disease, for example, CTCL patients respond best to DT-IL2 conjugates. Fusion toxins are also used in the treatment of psoriasis, rheumatoid arthritis, diabetes mellitus amongst other conditions. In the pathogenesis of psoriasis, activated T cells express sufficient numbers of IL2 receptors to allow targeting with fusion toxins [67]. DAB389IL2 showed promising results in rheumatoid arthritis and insulin- dependent diabetes mellitus [68,69]. These chimeric molecules also exhibited activity against the autoimmune disease, pemphigus vulgaris. Immunotoxins have also been employed in allogenic transplantation to target donor T cells that mediate immune response (graft versus host disease) [31]. Low dose interleukin-2 is used in cancer therapy, AIDS therapy, treatment of lepromatous leprosy and in bone marrow transplantation [10]. IL2 receptor targeted therapies are used in treatment of neoplasia, autoimmune diseases and transplantation [20].

Conjugated hybrid toxins employ more than one toxin. They act on different substrates by different mechanisms and, hence, are more potent and exhibit an additive effect. Administration (i.v.) of Diphtheria toxin A chain induces temporary renal damage, mimicking sub-acute glomerulonephritis [70]. Some of the modifications made in recent decade to native fusion toxins to increase efficacy are listed below:

(a) Mutating critical residues in the ligand to enhance binding affinity, efficacy of immunotoxins and hence cytotoxicity, for

example IL3 (K116W) [71] (b) Receptor upregulation enhances the efficacy of these compounds, since efficacy is directly proportional to ligand receptor density. Some of the compounds used in receptor upregulation include Bexarotene, Arginine butyrate, Phorbol myristate acetate; Phytohemagglutinin amongst others [72–74] (c) Vitamin A and its analogues play a role in differentiation and proliferation. Retinoids bind to either RAR (retinoic acid receptor) or RXR (retinoid X receptors) and have an important immunomodulatory role on T and B cells by increasing the expression of IL2 receptors on cell surface, especially α and β subunits. It also plays a role in enhancing apoptosis, thus, exposure to retinoids increases susceptibility to IL2 linked fusion toxins [38,75–77].

Toxicities and methods employed to overcome

Receptors for ligands are not specific to tumor cells, but are also present transiently on normal cells, so this mode of therapy is not devoid of side effects. Hepatocyte injury results from non-specific uptake of immunotoxins by hepatic cells or by macrophages that release cytokines, and as a consequence cause direct liver cell damage especially at high doses although this can be overcome by site-directed mutagenesis. They also result in cancer, cachexia, wasting and shock. Non-specific uptake by macrophages results in the release of cytokines that mediate further reactions, leading to VLS (vascular leak syndrome), while uptake by endothelial cells results in the release of nitric oxide that mediates oxidative damage leading to VLS, characterized by hypotension, hypoalbuminemia, increased vascular permeability, mild uremia and pulmonary edema. VLS can be minimized by (a) designing compounds with short plasma half lives and small size (b) suppression by anti inflammatory agents (c) using compounds which prevent binding of toxin to endothelial surface (d) site directed mutagenesis. Elevations in hepatic transaminases, serum lactate dehydrogenases, anti DT antibodies and anti cytokine antibodies are observed, however, there is contradicting evidence as to high antibody titers alter efficacy of immunotoxins or not [57,78]. Immunotoxins also shows some dose related side effects like nausea, vomiting, diarrhoea, inflammatory response, and so on which could be treated by use of prophylactic chemotherapeutics. The covalently linked growth factors raise a humoral immune response, this reduces the half-life of targeted toxin and thereby decreases efficacy. This condition can be handled by (a) pretreatment with immunosuppressive agents [79] (b) altering toxin structure by treating with chemicals like polyethylene glycol which enhances stability, potency, half-life and reduced immune response [80] (c) immunodominant epitope modification (d) site specific mutagenesis of toxin (e) reducing immunotoxins size, improves uptake and pharmacokinetics [81,82]. Other mild constitutional side effects that can be symptomatically treated are fever, chill, headache and flu like syndrome. IL-2 increases lymphoid infiltration effecting function of vital organs, increases vascular permeability, leading to fluid retention and edema. IL2 toxicity can be overcome by using BAY 50-4798, an IL2 agonist that binds selectively to the high affinity receptor, where two amino acids have been modified, resulting in a molecule with similar efficacy but decreased toxicity [83]. Mutating critical amino acids that are highly conserved and those directly involved in interaction with the receptor can alter binding affinity and efficacy, but these mutations should not alter the structure or function of the protein. Other mutations in IL2, which decreased toxicity, are R38G, R38W, R38D and R38Y [84].

Conclusion

Immunotoxins have shown promise both *in vivo* and *in vitro,* but in practice, to date only experimentally and clinically useful *in vitro*. Up to now only one agent, which contains human IL-2 and truncated diphtheria toxin, has been approved for use in cutaneous T cell lymphoma. Furthermore, the half-lives of immunotoxins may be too limited for diffusion to occur into solid tumor masses. The nature of the antigen and disease targeted remain the major determinants of immunotoxins efficacy and resistance. Research in the fields of cancer biology can uncover uniquely expressed antigens/receptors on tumor cell surface, similar discovery of more potent less immunogenic smaller size toxins can bring exciting success in future development of immunotoxins. Moreover protein engineering and mutation studies can be employed wisely to overcome the toxicities of immunotoxins.

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