OPINION

Immunotoxin therapy of cancer

Ira Pastan, Raffit Hassan, David J. FitzGerald and Robert J. Kreitman

Abstract | Rationally designed anticancer agents that target cell-surface antigens or receptors represent a promising approach for treating cancer patients. However, antibodies that bind these targets are often, by themselves, non-cytotoxic. By attaching potent toxins we can dramatically improve the clinical utility of some anti-tumour antibodies. Here we describe the construction and clinical utility of several recombinant immunotoxins; each of which is composed of antibody Fv fragments fused to powerful bacterial toxins. Results from clinical trials indicate that recombinant immunotoxins and similar agents that are designed to combine antibody selectivity with toxin cell-killing potency will be useful additions to cancer therapy.

The development of monoclonal antibody (MAb) therapy has revolutionized our approach to cancer treatment. Several MAbs are now approved for clinical use and are very effective against some types of cancer. Unfortunately, many cancers are resistant to treatment with MAbs alone, but can be killed when a cytotoxic agent is attached to the antibody. Immunoconjugates of this kind are made by attaching chemotherapy drugs, radioisotopes, enzymes or toxins to the antibody. This Perspective focuses on the use of toxins.

Immunotoxins derive their potency from the toxin and their specificity from the antibody or antibody fragment to which they are attached. We will describe the properties of the commonly used toxins, the principles of immunotoxin design and production, and results from recent clinical trials. Other recent reviews provide additional perpectives on the immunotoxin field[1–5].

Toxins used to make immunotoxins

Clinical trials of recombinant immunotoxins use one of two bacterial toxins. Both *Pseudomonas aeruginosa* exotoxin A (PE, 613 amino acids) and diphtheria toxin (DT, 580 amino acids) are produced as single polypeptide chains, each of which has three functional domains. Domain Ia, which is located at the N terminus, is the cell-binding domain of PE, domain II has translocation activity and domain III, which is located at the C terminus, catalyses the adenosine diphosphate (ADP)-ribosylation and inactivation of elongation factor 2 (EF2), which leads to the inhibition of protein synthesis and cell death (FIG. 1). A minor domain called Ib has no known function and is usually deleted. PE binds the α-2-macroglobulin receptor (also known as LRP1), which is expressed in many cell types, and is then internalized through clathrin-coated pits into the endocytic compartment where several processing steps occur (FIG. 1). Following proteolytic cleavage between amino acids 279 and 280 and reduction of the disulphide bond that connects residues 265 and 287, a 37 kDa fragment (amino acids 280–612) that is derived from the C terminus is transported to the endoplasmic reticulum and is then translocated to the cytosol, where it encounters and inactivates EF2. PE-based immunotoxins are currently produced by removing domain Ia (amino acids 1–253) and a large portion of domain Ib, and replacing domain Ia with a targeting moiety such as the Fv portion of an antibody or a growth factor (FIGS 2, 3). This modified form of PE is known as PE38 to reflect its molecular weight.

For DT, the ADP-ribosylation activity occurs at the N terminus and its cell-binding domain is at the C terminus (amino acids 482–539). The receptor for DT is the heparin-binding epidermal growth factor (EGF)-like precursor[6]. After cell binding, DT undergoes internalization, proteolytic activation and disulphide bond reduction, similar to PE, but translocation to the cytosol occurs directly from the acidic endocytic compartment[7]. Chimeric toxin molecules are made by replacing the cell-binding domain at the C terminus of DT with a growth factor or the Fv fragment of an antibody (FIG. 4).

Early immunotoxin molecules were often constructed using plant toxins. Ricin and similar toxins that inhibit protein synthesis by attacking ribosomal RNA were chemically attached to antibody molecules. For various reasons that are related to inefficient cellular processing and release of the toxin from the Fv fragment, the conversion from chemical conjugation methods to recombinant expression has not favoured plant toxins. In addition, the use of ricin-A-chain immunotoxins has been associated with vascular damage. Recent efforts have focused on the development of genetically altered ricin-A-chains with more favourable properties[8].

The development of immunotoxins

First-generation immunotoxins that were prepared by chemically conjugating whole toxins to antibodies were often not effective in animal models because the toxin also killed normal cells. The removal of the cell-binding domain from the toxin and attachment of this modified toxin to various antibodies produced immunotoxins that were much better tolerated by animals. Several of these second-generation immunotoxins have been evaluated in phase I trials in cancer patients. Some anti-tumour activity has been observed but these agents are costly to produce, chemically heterogeneous and, because of their large size, penetrate slowly into bulky tumours. Third-generation immunotoxins overcome these difficulties.

Using recombinant DNA techniques and the principles of protein engineering, immunotoxins are now designed to contain only the elements required to recognize and kill the tumour cells. This is accomplished by replacing the cell-binding domain of the

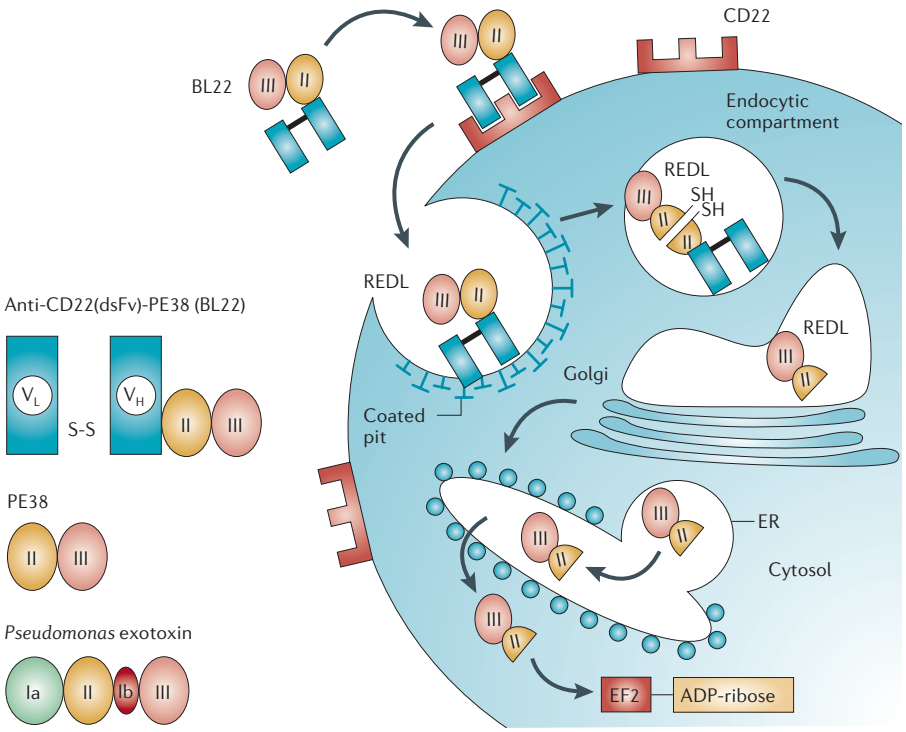

Figure 1 | **BL22.** BL22 is composed of the variable region of the light (VL) and heavy (VH) chains of an anti-CD22 antibody that is joined by a disulphide bond and connected to PE38 by a peptide bond. PE38 is a truncated portion of *Pseudomonas aeruginosa* exotoxin A (PE) that lacks domain Ia. BL22 binds to surface CD22 and then enters the cell through clathrin-coated pits. In the endocytic compartment PE38 is cleaved into two fragments. The C-terminal fragment, which is composed of domain III and a portion of domain II, is transported to the endoplasmic reticulum (ER), presumably through the REDL sequence (REDL functions as a KDEL ER-targeting motif sequence for ER retrieval). From the ER the toxin translocates to the cytosol and adenosine diphosphate (ADP)-ribosylates elongation factor 2 (EF2). This modification causes the inhibition of protein synthesis and leads to cell death.

toxin with the Fv portion of an antibody and retaining the translocation and cell-killing domains (FIG. 2). These recombinant chimeric proteins are produced in *Escherichia coli*. Large amounts of protein can be made economically from this source. The heavy and light chain portions of the antibodies that are used to make these immunotoxins are either linked by a peptide linker (scFv) or the Fv is stabilized by a disulphide bond (dsFv).

Another way to target toxins to cells is to replace the cell-binding domain with a growth factor or cytokine. Examples include interleukin 2 (IL2), transforming growth-factor α (TGFα), granulocyte-macrophage colony stimulating factor (GMCSF) and IL13.

The attraction of using an antibody–toxin or a cytokine–toxin fusion protein to kill cancer cells is the high potency of the toxin. One disadvantage is that toxins are foreign proteins, and patients with solid tumours and normal immune systems develop neutralizing antibodies, which prevents retreatment. Fortunately, neutralizing antibody formation is infrequent in patients with lymphomas and leukaemias, because the diseases are frequently immunosuppressive and because the chemotherapy these patients receive also damages the immune system.

Toxicity associated with immunotoxins can be either nonspecific or targeted. An example of nonspecific toxicity is vascular leak syndrome (VLS), in which there is fluid leakage from capillaries, a fall in serum albumin, fluid retention, oedema and weight gain. This toxicity, owing to endothelial cell damage caused by the high concentration of immunotoxins, can usually be managed by adequate hydration, although severe vascular collapse has been observed at high doses with ricin-based immunotoxins.

The second type of toxicity is caused by targeting the toxin to normal tissues that contain the same target antigen as the cancer cell. This is not a problem if immunotoxins are targeted to antigens on B- or T-cell malignancies, because normal B and T cells can be regenerated from antigen-negative stem cells. But it is a serious problem if solid tumours are targeted, as the antigen can be present on vital organs such as the kidneys, the liver or nerve cells, and the immunotoxin will kill normal cells in these tissues[9–11]. It is essential that the target antigen be restricted in its expression to non-essential normal cells.

Clinical trials of immuntoxins

This section summarizes recent and ongoing clinical trials of various immunotoxins in both haematological malignancies and solid tumours (TABLE 1). Because different immunotoxins can target the same antigen on cancer cells, we have summarized these clinical trials on the basis of the antigens that are being targeted.

Immunotoxins targeting the IL2 receptor. Murphy and colleagues have used recombinant DNA techniques to target DT to cells that express the high affinity IL2 receptor (IL2R) by replacing the cell-binding domain of DT with IL2 (FIG. 4). DAB389IL2 (denileukin diftitox or Ontak) is approved for the treatment of persistent or recurrent cutaneous T-cell lymphoma (CTCL). Denileukin diftitox produced response rates of 30%, which includes 10% complete remissions (CRs)[12–14]. Most patients had objective improvement in skin lesions. Common toxicities included fatigue, VLS with hypotension and low albumin, transaminase elevations and rashes. VLS can be controlled with steroid prophylaxis[13,15,16]. The retinoid bexarotene increases IL2 expression in CTCL, and with denileukin diftitox produced 3 CRs and 3 partial responses (PRs) in 12 patients with CTCL[17]. Denileukin diftitox produced response rates of 33% and 25% in chronic lymphocytic leukaemia (CLL)[18] and non-Hodgkin lymphoma (NHL)[19]. In addition to its use in the treatment of cancers that express IL2R, denileukin diftitox is being studied to eliminate regulatory T cells that express CD25 to increase the anti-tumour activity of cancer vaccines[20].

Clinical trials of anti-CD25 immunotoxins. LMB-2, which targets the α subunit of IL2R, contains the Fv of the anti-CD25 MAb anti-tac[21,22] fused to the N terminus of PE38 (FIGS 2,3). LMB-2 was given to patients with chemotherapy-resistant leukaemia, lymphoma and Hodgkin disease (HD) in a phase I trial. In this trial there were 7 PRs and 1 CR in the 20 patients who received a total dose greater than 60 μg per kg per cycle. The response rate in hairy-cell leukaemia (HCL) was 100%, with 1 CR and 3 PRs[23], and patients with CLL, adult T-cell leukaemia (ATL), CTCL and HD had PRs[24]. Dose-limiting toxicity

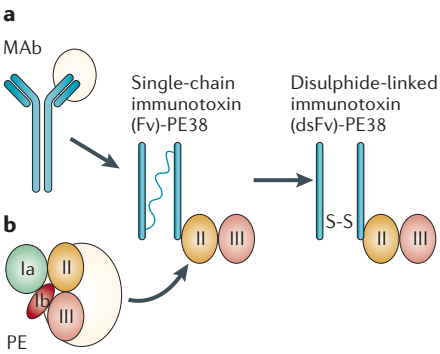

Figure 2 | **Strategy used to make single-chain or disulphide-linked immunotoxins. a** | DNA sequences that encode the heavy and light chains of an antibody are cloned and assembled into a single Fv in which a peptide linker or a disulphide bond connects the two chains of the Fv. **b** | Domain Ia and almost all of domain Ib of *Pseudomonas aeruginosa* exotoxin A (PE) is removed and the resultant 38 kDa fragment is connected to the Fv by a peptide linker.

occurred at 63 μg per kg, included reversible cardiomyopathy or transaminase elevations, and was associated with a fever that was consistent with cytokine release[25,26]. Antibodies that neutralized LMB-2 and prevented retreatment developed in 6 of 35 patients, although none of the 12 CLL patients made neutralizing antibodies. Phase II trials are currently underway in patients with CLL or CTCL. Patients with HCL are now being treated with the anti-CD22 recombinant immunotoxin BL22 (see below).

The anti-CD25 MAb RFT5 and the anti-CD30 MAb Ki-4, which is attached to a deglycosylated ricin-A-chain known as dgA, have both been tested in a phase I and II setting in patients with HD and significant clinical activity has been observed[27–31].

Targeting CD22 for treatment of HCL and other haematological cancers. To target CD22, which is expressed in B-cell leukaemias and lymphomas, the variable domains of a MAb to CD22 (RFB4) were cloned and used to make a disulphide-linked recombinant immunotoxin RFB4(dsFv)–PE38 or BL22 (FIG. 1). BL22 was found to be cytotoxic to cell lines that express CD22 and causes the complete regression of CD22-positive tumours in mice[32,33]. In a phase I trial, BL22 was given to 46 patients with various B-cell malignancies: HCL, CLL and some B-cell lymphomas. All patients were drug resistant before they entered the trial, and patients with HCL had received 1–6 separate courses of cladribine and other standard therapies. A total of 265 cycles of BL22 were given, which totalled up to 33 cycles per patient. In HCL[34], 61% CRs and 19% PRs were achieved in 31 patients[35]. All 25 responders benefited clinically after one cycle of treatment. Neutralizing antibodies developed in 11 out of 46 patients, all with HCL, and only in 1 patient after 1 cycle. Mild VLS and transaminase elevations were not dose limiting. Four HCL patients (12%) in the phase I trial developed a reversible haemolytic uremic syndrome during the second or third cycle, which was successfully treated with plasmapheresis. All of these patients responded well to BL22. The maximum tolerated dose (MTD) was established as 40 μg per kg intravenously every 2 days × 3 for cycle 1, with no serious toxicity observed in any of the treated patients. The median duration for CR was 36 months, and 8 remained in CR at 45 months. A higher percentage of patients (73% of 30 patients) achieved haematological remission, which lasted a median of 37 months. BL22 is the first agent that has been reported to induce a high rate of CRs in HCL patients that have resistance to purine analogues. Moreover, the phase I response rates (61% CR, 81% overall response) that were reported in the 31 patients with HCL that were tested were higher than those of any other targeted toxin, including the approved agent denileukin diftitox for CTCL (11% CR, 32% overall response in 106 phase I and II patients). Activity from BL22 was also observed in CLL, which included a durable haematological response that was maintained for nearly 5 years in one patient receiving 33 cycles of BL22. BL22 is currently undergoing phase II testing in HCL and phase I testing in both CLL and in paediatric acute lymphoblastic leukaemia (ALL).

Immunotoxins targeting the GMCSF receptor. DT–GMCSF (DTGM) is a recombinant toxin that targets the GMCSF receptor (GMCSFR) that is present on acute myeloid leukaemia (AML) cells[36–38]. DTGM induced 1 CR and 2 PRs in 31 chemo-resistant patients with AML[39]. Unfortunately, the further development of this agent has been complicated by liver toxicity, which has been attributed to targeting GMCSFR expressed on Küpffer cells with subsequent damage to hepatocytes[26,39,40]. An alternate approach to treat patients with AML is to target the IL3R using the recombinant toxin DT388–IL3 because this chimeric protein does not target macrophages or Küpffer cells[41,42].

Immunotoxins targeting the Lewis Y antigen for treatment of epithelial cancers. The Lewis Y antigen has been found to be highly expressed in many epithelial tumours[43]. The first clinical activity of PE-based immunotoxins was observed in a phase I trial in which MAb B3 was chemically attached to PE38 (FIG. 2). This immunotoxin, which is named LMB-1, produced a PR in a patient with colon cancer and a CR in a patient with breast cancer[44]. Dose-limiting toxicity was ascribed to endothelial damage because small amounts of the Lewis Y antigen are present on endothelial cells[45].

Several recombinant immunotoxins that target the Lewis Y antigen have also been tested in the clinic. B3(Fv)–PE38 (LMB-7) was produced by fusing the Fv of the MAb B3 to the N terminus of PE38. In a phase I clinical trial of LMB-7 two significant toxicities were observed. The first toxicity was a targeted toxicity: severe gastritis caused by the killing of normal cells that expressed Lewis Y antigen in the stomach. This toxicity was prevented by blocking acid secretion with the proton pump inhibitor Omeprazole accompanied by antacids. At higher dose levels renal toxicity developed and was dose limiting. This toxicity was probably because of small amounts of Lewis Y present on some tubular cells in the kidney[43]. B3(dsFv)–PE38 or LMB-9, which is a stable disulphide version of LMB-7, was also tested in a phase I trial. In this trial, which used Omeprazole to prevent stomach toxicity, renal toxicity was also dose limiting (I.P., R.H, D.J.F and R.J.K,

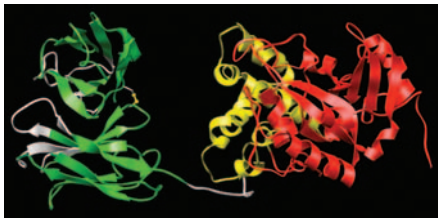

Figure 3 | **Model of a recombinant immunotoxin.** A model of a recombinant immunotoxin in which the heavy and light chains (green) are connected by a disulphide bond (yellow). The complementary determing regions (CDRs) are shown in grey. The Fv is connected by a peptide linker (grey) to the 38 kDa fragment of *Pseudomonas aeruginosa* exotoxin A (PE) as PE38. The translocation domain is shown in yellow and the adenosine diphosphate (ADP)-ribosylation domain in red. Model prepared by J. Vincent, Laboratory of Molecular Biology, US National Cancer Institute.

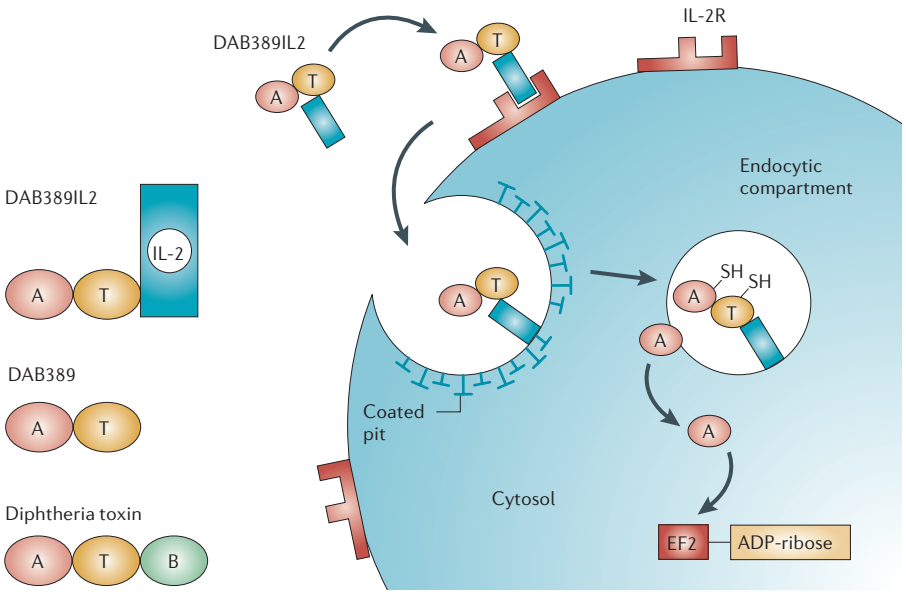

Figure 4 | **DAB389IL2.** DAB389IL2 (denileukin diftitox) is composed of the enzyme active and translocating domains of diphtheria toxin (DT) fused to human interleukin 2 (IL2). To make the construct, the binding domain of DT is deleted and replaced with IL2. Denileukin diftitox binds with cell-surface IL2R and then enters cells through clathrin-coated pits and into endocytic vessels. The acidic pH in the endocytic compartment causes a conformational change that enables the translocation of the A chain of DT to the cytosol. Similar to PE38, DT modifies elongation factor 2 (EF2) by adenosine diphosphate (ADP)-ribosylation, which leads to inhibition of protein synthesis and cell death.

unpublished observations). No significant anti-tumour activity was observed with either LMB-7 or LMB-9. A molecule similar to LMB-7, but made with a different anti-Lewis Y antibody, termed BR96sFv–PE40 (SGN-10), was tested in 46 patients with Lewis Y-expressing metastatic carcinomas[46]. Omeprazole was not used with this trial, and the dose-limiting toxicity was gastrointestinal (nausea, vomiting and diarrhoea). Clinical activity with immunotoxins that target Lewis Y has been limited, which is in part due to the low affinity of the immunotoxin for this carbohydrate antigen, and the presence of this antigen on cells of the kidney.

Immunotoxins targeting mesotheliomas, ovarian and pancreatic cancers. Mesothelin is a differentiation antigen that is expressed on the cell membrane of normal mesothelial cells, and is highly expressed on several types of cancer, such as mesothelioma, ovarian and pancreatic cancer[47–49]. An immunotoxin (SS1P) with a high affinity for mesothelin has been produced by a combination of antibody phage display and Fv mutagenesis[50,51]. Because this immunotoxin produced CR of mesothelin-bearing tumours in mice and was well tolerated by monkeys whose cells bind SS1P, two phase I clinical trials were initiated at the US National Cancer Institute. In one trial, which has just been completed, SS1P was given by continuous infusion over 10 days[52]. Continuous infusion was tested as an approach to increase tumour uptake. In the other trial, SS1P was given by 30 minute infusion every 2 days for 3–6 doses[53]. The significant dose-limiting toxicity in both trials was pleuritis ascribed to the targeted killing of normal mesothelial cells in the pleura. VLS characterized by weight gain and a fall in serum albumin also occurred, but was not dose limiting[53]. Minor but significant anti-tumour responses were observed after either method of administration, both by computerized tomography and positron emission tomography. The formation of antibodies that neutralized SS1P occurred in over 70% of patients and prevented further treatment cycles. Nevertheless, there was sufficient anti-tumour activity after only one cycle of SS1P to justify a phase II trial. In the phase II trial that is currently being planned, patients with mesothelioma will receive a combination of SS1P and gemcitabine, because this combination is very effective in an animal model (I.P., R.H, D.J.F and R.J.K, unpublished data).

Immunotoxins targeting IL4, IL13 and epidermal growth factor receptors. The term 'immunotoxin' has been extended to include proteins where cytokines are fused to toxins. The efficacy of denileukin diftitox in some leukaemias and lymphomas has already been described. Truncated PE has been fused to several cytokines and growth factors. These include TGFα–PE38 (which targets cells that express epidermal growth factor receptors (EGFR)), IL4(38–37)–PE38KDEL and IL13–PE38QQR. These variants of PE38 share the same cytotoxic activity with the exception of PE38KDEL, for which increased cytotoxic activity has often been noted. The receptors for these cytokines are present on many normal cells so that systemic therapy results in unacceptable toxic side effects. For example, in a phase I trial of IL4(38–37)–PE38KDEL in patients with advanced solid tumours that expressed IL4R, the dose-limiting toxicity was liver damage and no objective responses were observed[54]. These agents are better suited for local therapy, and 3 of these agents that target the EGF, IL4 and IL13 receptors have been evaluated for the therapy of glioblastoma. During treatment, the proteins are slowly infused into or next to the brain tumour by continuous infusion over many hours. This technique of enhanced-convection delivery was initially developed for the administration of a conjugate of transferrin and DT into brain tumours[55]. Phase I and II trials of IL4(38–37)–PE38KDEL showed a few CRs and PRs, but the associated toxicity was unacceptable and development was abandoned[56,57]. By contrast, both TGFα–PE38 and IL13–PE38QQR are much better tolerated and have shown CRs in some patients during phase I and phase II trials[58–61]. Their development is being actively pursued.

Improving affinity and activity

The importance of affinity in immunotoxin activity was comprehensively shown in a study in which many different antibodies to CD30 were produced and used to make immunotoxins[62]. A statistically significant correlation of affinity with cytotoxic activity was observed.

Analyses of cells from patients revealed that patients with HCL have 5,000–100,000 CD22 sites per cell and are sensitive to BL22 (inhibitory concentration 50% (IC_{50}) <10 ng ml^{-1}). By contrast, cells from CLL patients have only 300–3,000 sites per cell and are much less sensitive to BL22 (IC_{50} usually >100 ng ml^{-1}). To make a more active immunotoxin we used a combination of antibody phage display and mutagenesis of complementary determining regions (CDRs) to increase the

Table 1 | **Recently completed and ongoing immunotoxin clinical trials**

Antigen targeted	Disease	Immunotoxin and characteristics	Clinical benefit/responses	Side-effects	Clinical status	Refs
IL2 (denileukin diftitox); IL2 fused to DT	CTCL, CLL and NHL	DAB389IL2	10% CR and 20% PR in patients with CTCL; 33% PR in patients with CLL; and 7% CR, 18% PR in patients with NHL (phase II study)	VLS and allergic reactions	DAB389IL2 approved by FDA for CTCL treatment	16–19
CD25 (subunit of IL2)	HCL, CLL, ATL, CTCL and NHL	Anti-tac(scFv)–PE38 (LMB-2); Fv portion of anti-CD25 antibody fused to PE	20 (out of 35) patients received a dose >60 μg per kg per cycle, resulting in 7 PRs and 1 CR (phase I study)	Fever and transaminase elevations	Phase II ongoing for HCL, CLL and CTCL at the NCI	23,24
CD25	HD	RFT5–dgA; anti-CD25 antibody linked to deglyco-sylated ricin-A-chain	2 PRs in 27 patients (phase I study)	VLS, fatigue and myalgia		27,29, 31,68
CD22	HCL, CLL and NHL	RFB4(dsFv)–PE38 (BL22); disulphide linked Fv portion of anti-CD22 antibody fused to PE	19 CRs and 6 PRs in 31 treated patients with HCL (phase I study)	HUS (reversible)	Phase II studies for HCL and CLL open at NCI	34,35
GMCSFR	AML	DT388–GMCSF; GMCSF fused to DT	1 CR and 2 PRs in 31 treated patients (phase I study)	Liver toxicity		39
Lewis Y	Adenocarcinomas	LMB-1; MAb B3 chemically attached to truncated PE	1 CR, 1 PR and 3 minor responses in 35 treated patients (phase I study)	VLS secondary to endothelial damage		44
Lewis Y	Adenocarcinomas	B3(Fv)–PE38 (LMB-7); single chain Fv of MAb B3 fused to PE	Not yet reported	Gastritis and renal toxicity		*
Lewis Y	Adenocarcinomas	B3(dsFv)–PE38 (LMB-9); disulphide stabilized Fv of MAb B3 fused to PE	Not yet reported	Renal toxicity		*
Lewis Y	Adenocarcinomas	BR96(sFv)–PE40 (SGN-10); BR96 scFv fused to PE40	Minor response in 1 out of 42 patients (phase I study)	GI (nausea, vomiting and diarrhoea)		46,69, 70
Mesothelin	Mesotheliomas, ovarain cancer and pancreatic cancer	SS1(dsFv)–PE38 (SS1P); anti-mesothelin Fv fused to PE38	Anti-tumour responses were seen in some patients using a continous infusion (completed) and bolus schedule (ongoing) (phase I studies)	Pleuritis	Planned NCI phase II studies in combination with chemo-therapy	52,53
IL4	Solid tumours that express IL4R and glioblastomas	IL4(38-37)–PE38KDEL (NBI-3001)	Systemic administration — no response noted in 12 evaluable patients (phase I study); unacceptable level of toxicity in local therapy for glioblastoma (phase I and II study)	Liver toxicity		54,56, 57
IL13	Glioblastomas	IL13–PE38QQR	Local tumour perfusion		Phase III	61,71
IL13	Renal cell	IL13–PE38QQR			Abandoned	
EGFR	Glioblastomas	TP-38; TGFα fused to PE38	Local tumour perfusion		Phase II	58,60

* I.P., R.H, D.J.F and R.J.K, unpublished data. AML, acute myeloid leukaemia; ATL, adult T-cell leukaemia; CLL, cytotoxic lymphocytic leukaemia; CR, complete remission; CTCL, cutaneous T-cell lymphoma; DT, diphtheria toxin; EGFR, epidermal growth factor receptor; FDA, US Food and Drug Administration; GMCSF, granulocyte macrophage-colony stimulating factor; HCL, hairy-cell leukaemia; HD, Hodgkin disease; HUS, haemolytic uremic syndrome; IL2, interleukin 2; IL4R, IL4 receptor; MAb, monoclonal antibody; NCI, National Cancer Institute; NHL, non-Hodgkin lymphoma; PE, *Pseudomonas aeruginosa* exotoxin; PR, partial response; TGFα, transforming growth factor-α; VLS, vascular leak syndrome.

affinity of the Fv. The replacement of amino acids SSY by THW in the CDR3 of the heavy chain of the Fv of BL22 produced a mutant immunotoxin with a 10-fold increase in affinity for CD22 and a 10–100-fold increase in potency against malignant CLL cells from patients[63]. This new immunotoxin, HA22, is currently being produced for phase I clinical testing in CLL, NHL, HCL and ALL.

Vitetta and colleagues have used mutagenesis to improve the properties of ricin-based immunotoxins by mutating the toxin so that it has diminished toxicity to endothelial cells[8,64,65].

Decreasing immunotoxin immunogenicity

Clinical responses to immunotoxins have mainly been observed in haematological malignancies and not solid tumours. One reason for this is that cancer cells located in the blood, bone marrow and spleen are readily accessible to the immunotoxin, whereas

cancer cells in solid tumours are not. A second reason is that the immune system in haematological malignancies is impaired and damaged by previous chemotherapy so that anti-immunotoxin antibodies are not readily produced and therefore more than one cycle of treatment can be given. Two unsuccessful clinical trials have been conducted to try and prevent the formation of neutralizing antibodies in patients with solid tumours: one with rituximab that eliminates B cells in the blood and the other with high-dose steroids (I.P., R.H, D.J.F and R.J.K, unpublished data). Another approach under investigation to reduce immunogenicity is to chemically modify the immunotoxin with high molecular weight polyethylene glycol[66]; this approach has been successful in reducing or eliminating the immunogenicity of adenosine deaminase and asparaginase[67].

Summary

The finding that immunotoxins that target CD22 and CD25 cause reproducible tumour regressions and even complete remissions in many phase I patients with haematological malignancies establishes that these agents can have a useful role in cancer treatment. These results need to be extended to other tumours and particularly solid tumours. One promising target is the mesothelin protein that is expressed on ovarian cancers and on mesotheliomas. Another barrier that needs to be overcome is the poor uptake of therapeutic proteins by solid tumours. This might be achieved by combining immunotoxin therapy with cytotoxic agents that damage blood vessels and diminish the high interstitial pressure within tumours that slows the entry of therapeutic proteins. Finally, the toxin portion of the immunotoxin needs to be made less immunogenic so that neutralizing antibodies do not develop and more treatment cycles can be given. Useful approaches could include identifying and removing T- or B-cell epitopes, modifying the protein with high molecular weight polyethylene glycol or treating patients with immunosuppressive agents.

Ira Pastan, Raffit Hassan, David J. FitzGerald and Robert J. Kreitman are at Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institues of Health, 37 Convent Drive, Bethesda, MD 20892-4264, USA.
Correspondence to I.P.
e-mail: pastani@mail.nih.gov

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1. FitzGerald, D. J., Kreitman, R., Wilson, W., Squires, D. & Pastan, I. Recombinant immunotoxins for treating cancer. *Int. J. Med. Microbiol.* **293**, 577–582 (2004).
2. Frankel, A. E., Neville, D. M., Bugge, T. A., Kreitman, R. J. & Leppla, S. H. Immunotoxin therapy of hematologic malignancies. *Semin. Oncol.* **30**, 545–557 (2003).
3. Foss, F. M., Saleh, M. N., Krueger, J. G., Nichols, J. C. & Murphy, J. R. in *Clinical Applications of Immunotoxins* (ed. Frankel, A. E.) 63–81 (Springer, Berlin, Germany, 1998).
4. Uckun, F. M. & Reaman, G. H. Immunotoxins for treatment of leukemia and lymphoma. *Leuk. Lymphoma* **18**, 195–201 (1995).
5. Engert, A., Sausville, E. A. & Vitetta, E. in *Clinical Applications of Immunotoxins* (ed. Frankel, A. E.) 13–33 (Springer, Berlin, Germany, 1998).
6. Naglich, J. G., Metherall, J. E., Russell, D. W. & Eidels, L. Expression cloning of a diptheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* **69**, 1051–1061 (1992).
7. Zhao, G. & London, E. Behavior of diphtheria toxin T domain containing substitutions that block normal membrane insertion at Pro345 and Leu307: control of deep membrane insertion and coupling between deep insertion of hydrophobic subdomains. *Biochemistry* **44**, 4488–4498 (2005).
8. Smallshaw, J. E. *et al*. Genetic engineering of an immunotoxin to eliminate pulmonary vascular leak in mice. *Nature Biotechnol*. **21**, 387–391 (2003).
9. Gould, B. J. *et al*. Phase I study of an anti-breast cancer immunotoxin by continuous infusion: Report of a targeted toxic effect not predicted by animal studies. *J. Natl Cancer Inst.* **81**, 775–781 (1989).
10. Pai, L. H. *et al*. Clinical evaluation of intraperitoneal *Pseudomonas* exotoxin immunoconjugate OVB3-PE in patients with ovarian cancer. *J. Clin. Oncol.* **9**, 2095–2103 (1991).
11. Pai-Scherf, L. H. *et al*. Hepatotoxicity in cancer patients receiving erb-38, a recombinant immunotoxin that targets the erbB2 receptor. *Clin. Cancer Res.* **5**, 2311–2315 (1999).
12. Taniguchi, T. & Minami, Y. The IL2/IL-2 receptor system: a current overview. *Cell* **73**, 5–8 (1993).
13. Olsen, E. *et al*. Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. *J. Clin. Oncol.* **19**, 376–388 (2001).
14. Duvic, M., Kuzel, T. M., Olsen, E. & Martin, A. G. Quality-of-life improvements in cutaneous T-cell lymphoma patients treated with denileukin diftitox (ontak). *Clin. Lymphoma* **2**, 222–228 (2002).
15. Railan, D., Fivenson, D. P. & Wittenberg, G. Capillary leak syndrome in a patient treated with interleukin 2 fusion toxin for cutaneous T-cell lymphoma. *J. Am. Acad. Dermatol.* **43**, 323–324 (2000).
16. Foss, F. M. *et al*. Biological correlates of acute hypersensitivity events with DAB(389)IL-2 (denileukin diftitox, ONTAK) in cutaneous T-cell lymphoma: decreased frequency and severity with steroid premedication. *Clin. Lymphoma* **1**, 298–302 (2001).
17. Foss, F., Demierre, M. F. & DiVenuti, G. A phase-1 trial of bexarotene and denileukin diftitox in patients with relapsed or refractory cutaneous T-cell lymphoma. *Blood* **106**, 454–457 (2005).
18. Frankel, A. E. *et al*. A phase II study of DT fusion protein denileukin diftitox in patients with fludarabine-refractory chronic lymphocytic leukemia. *Clin. Cancer Res.* **9**, 3555–3561 (2003).
19. Dang, N. H. *et al*. Phase II study of denileukin diftitox for relapsed/refractory B-cell non-Hodgkin's lymphoma. *J. Clin. Oncol.* **22**, 4095–4102 (2004).
20. Dannull, J. *et al*. Enhancement of vccine-mediated antitumor immunity in cancer patients: depletion of regulatory T cells. *J. Clin. Invest*. **115**, 3623–3633 (2005).
21. Uchiyama, T. A., Broder, S. & Waldmann, T. A. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. *J. Immunol.* **126**, 1393–1397 (1981).
22. Chaudhary, V. K. *et al*. A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature* **339**, 394–397 (1989).
23. Kreitman, R. J. *et al*. Responses in refractory hairy cell leukemia to a recombinant immunotoxin. *Blood* **94**, 3340–3348 (1999).
24. Kreitman, R. J. *et al*. Phase I trial of recombinant immunotoxin anti-tac(Fv)–PE38 (LMB-2) in patients with hematologic malignancies. *J. Clin. Oncol.* **18**, 1614–1636 (2000).
25. Onda, M., Kreitman, R. J., Vasmatzis, G., Lee, B. & Pastan, I. Reduction of the nonspecific toxicity of anti-Tac(Fv)-PE38 by mutations in the framework regions of the Fv which lower the isoelectric point. *J. Immunol.* **163**, 6072–6077 (1999).
26. Onda, M. *et al*. Inhibition of TNFα produced by Kupffer cells protects against the non-specific liver toxicity of immunotoxin anti-Tac(Fv)-PE38, LMB-2. *J. Immunol.* **165**, 7150–7156 (2000).
27. Schnell, R. *et al*. Treatment of refractory Hodgkin's lymphoma patients with an anti-CD25 ricin A-chain immunotoxin. *Leukemia* **14**, 129–135 (2000).
28. Schnell, R. *et al*. Clinical trials with an anti-CD25 ricin A-chain experimental and immunotoxin (RFT5–SMPT-dgA) in Hodgkin's lymphoma. *Leuk. Lymphoma* **30**, 525–537 (1998).
29. Engert, A. *et al*. A phase-I study of an anti-CD25 ricin A-chain immunotoxin (RFT5–SMPT-dgA) in patients with refractory Hodgkin's lymphoma. *Blood* **89**, 403–410 (1997).
30. Schnell, R. *et al*. A Phase I study with an anti-CD30 ricin A-chain immunotoxin (Ki-4. dgA) in patients with refractory CD30+ Hodgkin's and non-Hodgkin's lymphoma. *Clin. Cancer Res.* **8**, 1779–1786. (2002).
31. Borchmann, P., Schnell, R. & Engert, A. Immunotherapy of Hodgkin's lymphoma. *Eur. J. Haematol.* **75**, 159–165 (2005).
32. Mansfield, E., Amlot, P., Pastan, I. & FitzGerald, D. J. Recombinant RFB4 immunotoxins exhibit potent cytotoxic activity for CD22-bearing cells and tumors. *Blood* **90**, 2020–2026 (1997).
33. Kreitman, R. J., Wang, Q. C., FitzGerald, D. J. P. & Pastan, I. Complete regression of human B-cell lymphoma xenografts in mice treated with recombinant anti-CD22 immunotoxin RFB4(dsFv)-PE38 at doses tolerated by cynomolgus monkeys. *Int. J. Cancer* **81**, 148–155 (1999).
34. Kreitman, R. J. *et al*. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. *N. Engl. J. Med.* **345**, 241–247 (2001).
35. Kreitman, R. J. *et al*. Phase I trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with B-cell malignancies. *J. Clin. Oncol.* **23**, 6719–6729 (2005).
36. Hall, P. D., Willingham, M. C., Kreitman, R. J. & Frankel, A. E. DT388-GM-CSF, a novel fusion toxin consisting of a truncated diphtheria toxin fused to human granulocyte-macrophage colony stimulating factor, prolongs survival in a SCID model of acute myelogenous leukemia. *Leukemia* **13**, 629–633 (1999).
37. Hogge, D. E. *et al*. Malignant progenitors from patients with acute myelogenous leukemia are sensitive to a diphtheria toxin-granulocyte-macrophage colony-stimulating factor fusion protein. *Blood* **92**, 589–595 (1998).
38. Frankel, A. E. *et al*. Diphtheria toxin fused to granulocyte-macrophage colony-stimulating factor is toxic to blasts from patients with juvenile myelomonocytic leukemia and chronic myelomonocytic leukemia but is nontoxic to normal myeloid progenitors. *Blood* **91**, 4279–4286 (1998).
39. Frankel, A. E., Powell, B. L., Hall, P. D., Case, L. D. & Kreitman, R. J. Phase I trial of a novel diphtheria toxin/granulocyte macrophage colony-stimulating factor fusion protein (DT388GMCSF) for refractory or relapsed acute myeloid leukemia. *Clin. Cancer Res.* **8**, 1004–1013 (2002).
40. Wescott, M. M. *et al*. Diphtheria toxin-murine granulocyte-macrophage colony-stimulating factor-induced hepatotoxicity is mediated by Kupffer cells. *Mol. Cancer Ther.* **3**, 1681–1689 (2004).
41. Cohen, K. A. *et al*. Safety evaluation of DT388IL3, a diphtheria toxin/interleukin 3 fusion protein, in the cynomolgus monkey. *Cancer Immunol. Immunother.* **54**, 799–806 (2005).
42. Urieto, J. O. *et al*. Expression and purification of the recombinant diphtheria fusion toxin DT388IL3 for phase I clinical trials. *Protein Expr. Purif.* **33**, 123–133 (2004).
43. Pastan, I. *et al*. Characterization of monoclonal antibodies B1 and B3 that react with mucinous adenocarcinomas. *Cancer Res.* **51**, 3781–3787 (1991).
44. Pai, L. H., Wittes, R., Setser, A., Willingham, M. C. & Pastan, I. Treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to *Pseudomonas* exotoxin. *Nature Med.* **2**, 350–353 (1996).
45. Kuan, C., Pai, L. H. & Pastan, I. Immunotoxins containing *Pseudomonas* exotoxin targeting Le^y damage human endothelial cells in an antibody-specific mode: relevance to vascular leak syndrome. *Clin. Cancer Res.* **1**, 1589–1594 (1995).

46. Posey, J. A. *et al*. A phase I trial of the single-chain immunotoxin SGN-10 (BR96 sFv-PE40) in patients with advanced solid tumors. *Clin. Cancer Res.* **8**, 3092–3099 (2002).
47. Chang, K. & Pastan, I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc. Natl Acad. Sci. USA* **93**, 136–140 (1996).
48. Argani, P. *et al*. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin. Cancer Res.* **7**, 3862–3868 (2001).
49. Hassan, R., Bera, T. & Pastan, I. Mesothelin: a new target for immunotherapy. *Clin. Cancer Res.* **10**, 3937–3942 (2004).
50. Chowdhury, P. S., Viner, J. L., Beers, R. & Pastan, I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. *Proc. Natl Acad. Sci. USA* **95**, 669–674 (1998).
51. Chowdhury, P. S. & Pastan I. Improving antibody affinity by mimicking somatic hypermutations *in vitro*. *Nature Biotech*. **17**, 568–572 (1999).
52. Kreitman, R. *et al*. SS1(dsFv)PE38 anti-mesothelin immunotoxin in advanced malignancies: Phase I study of continous infusion. *Proc. Am. Soc. Clin. Oncol*. **21**, 22b (2002).
53. Hassan, R., Bullock, S., Kindler, H. & Pastan, I. Updated results of the phase I study of SS1(dsFv)PE38 for targeted therapy of mesothelin expressing cancers. *Eur. J. Cancer* **2** (Suppl.), 280a (2004).
54. Garland, L. *et al*. Phase I trial of intravenous IL-4 Pseudomonas exotoxin protein (NBI-3001) in patients with advanced solid tumors that express the IL-4 receptor. *J. Immunother.* **28**, 376–381 (2005).
55. Laske, D. W., Youle, R. J. & Oldfield, E. H. Tumor regression with regional distribution of the targeted toxin TF-CRM107 in patients with malignant brain tumors. *Nature Med.* **3**, 1362–1368 (1997).
56. Weber, F. W. *et al*. in *Local Therapies for Glioma: Present Status and Future Developments* (eds Westphal, M., Tonn, J. C. & Ram, Z.) 93–103 (Springer, Vienna, Austria, 2003).
57. Rand, R. W. *et al*. Intratumoral administration of a recombinant circularly permuted interleukin-4-*Pseudomonas* exotoxin in patients with high grade glioma. *Clin. Cancer Res.* **6**, 2157–2165 (2000).
58. Sampson, J. H. *et al*. Progress report of a Phase I study of the intracerebral microinfusion of a recombinant chimeric protein composed of transforming growth factor (TGF)-α and a mutated form of the *Pseudomonas* exotoxin termed PE-38 (TP-38) for the treatment of malignant brain tumors. *J. Neurooncol.* **65**, 27–35 (2003).
59. Boskovitz, A. *et al*. Monoclonal antibodies for brain tumour treatment. *Expert Opin. Biol. Ther.* **4**, 1453–1471 (2004).
60. Sampson, J. H. *et al*. Sustained radiographic and clinical response in patient with bifrontal recurrent glioblastoma multiforme with intracerebral infusion of the recombinant targeted toxin TP-38: case study. *J. Neurooncol.* **7**, 90–96 (2005).
61. Parney, I. F. *et al*. Neuroradiographic changes following convection-enhanced delivery of the recombinant cytotoxin interleukin 13-PE38QQR for recurrent malignant glioma. *J. Neurosurg.* **102**, 267–275 (2005).
62. Nagata, S. *et al*. Novel anti-CD30 recombinant immunotoxins containing disulphide-stabilized Fv fragments. *Clin. Cancer Res*. **8**, 2345–2355 (2002)
63. Salvatore, G., Beers, R., Margulies, I., Kreitman, R. J. & Pastan, I. Improved cytotoxic activity towards cell lines and fresh leukemia cells of a mutant anti-CD22 immunotoxin obtained by antibody phage display. *Clin. Cancer Res.* **8**, 995–1002 (2002).
64. Baluna, R., Rizo, J., Gordon, B. E., Ghetie, V. & Vitetta, E. S. Evidence for a structural motif in toxins and interleukin-2 that may be responsible for binding to endothelial cells and initiating vascular leak syndrome. *Proc. Natl Acad. Sci. USA* **96**, 3957–3962 (1999).
65. Baluna, R., Coleman, E., Jones, C., Ghetie, V. & Vitetta, E. S. The effect of a monoclonal antibody coupled to ricin A chain-derived peptides on endothelial cells *in vitro*: insights into toxin-mediated vascular damage. *Exp. Cell Res.* **258**, 417–424 (2000).
66. Tsutsimi, Y. *et al*. Site-specific chemical modification of recombinant immunotoxin anti-Tacc(Fv)-PE38 (LMB-2) improves antitumor activity and reduces animal toxicity and immunogenicity. *Proc. Natl Acad. Sci. USA* **97**, 8548–8553 (2000).
67. Molineux, G. Pegylation: engineering improved pharmaceuticals for enhanced therapy. *Cancer Treat. Rev.* **28**, S13–S16 (2002).
68. Castelletti, D. *et al*. A dominant linear B-cell epitope of ricin A-chain is the target of a neutralizing antibody response in Hodgkin's lymphoma patients treated with an anti-CD25 immunotoxin. *Clin. Exp. Immunol.* **136**, 365–372 (2004).
69. Haggerty, H. G. *et al*. BR96 sFv-PE40 immunotoxin: nonclinical safety assessment. *Toxicol. Pathol.* **27**, 87–94 (1999).
70. Damle, B., Tay, L., Comereski, C., Warner, W. & Kaul, S. Influence of immunogenicity on the pharmacokinetics of BMS-191352, a *Pseudomonas* exotoxin immunoconjugate, in rats and dogs. *J. Pharm. Pharmacol.* **52**, 671–678 (2000).
71. Kunwar, S. in *Local Therapies for Glioma: Present Status and Future Developments* (eds Westphal, M., Tonn, J. C. & Ram, Z.) 105–111 (Springer, Vienna, Austria, 2003).

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Competing interests statement
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OPINION

The US Food and Drug Administration perspective on cancer biomarker development

Steven Gutman and Larry G. Kessler

Abstract | Despite the intense interest in biomarker development for cancer management, few biomarker assays for diagnostic uses have been submitted to the US Food and Drug Administration (FDA). What challenges must researchers overcome to bring cancer-detection technologies to the market and, therefore, into clinical use?

Recent discoveries related to the human genome have brought high expectations for medical advances[1]. Genomic analysis tools are particularly useful in the discovery of biomarkers, which have the potential to revolutionize the diagnosis and treatment of disease — particularly of cancer. The field of biomarkers is beginning to encompass an increasing array of cutting-edge techniques, including tests for genetic alterations, gene-expression assays, proteomic profiles and antibody immunoassays.

In this article we use the term 'biomarker' in a very general sense to describe any measurable diagnostic indicator that is used to assess the risk or presence of disease. This discussion does not address the use of biomarkers as part of general drug discovery and development. Although the focus of discussion is on cancer biomarkers, much of this discussion also applies more generally to biomarkers that are used for other disease processes. The US Food and Drug Administration (FDA) considers the term 'diagnostic' to encompass a broad range of uses, which include determining the future risk of disease, screening for disease and confirming the presence of suspected disease. The term 'diagnostic' also includes uses such as determining the prognosis or staging of disease, monitoring and/or optimizing treatment outcomes by enabling health-care providers to choose, avoid or dose specific treatments with more precision (BOX 1). At the FDA, imaging techniques (such as fluorodeoxyglucose (FDG)-positron emission tomography (PET)) are often also thought of as potential biomarkers, but in this article we will focus on *in vitro* diagnostic procedures for measuring biomarkers in a collected sample.

Although the sequencing of the human genome is likely to have a profound influence on public health, there have not yet been a large number of practical advances based on this information. Several recent