Minimizing side effects, maximizing returns: what makes a smart therapeutic design?

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Symptoms of cancer can be devastating. However, the side effects of cancer treatments aimed at leaving the patient tumour-free, often inflict their own brutal attack on the body. The same treatments that kill rapidly dividing tumour cells will just as easily kill any other rapidly dividing cells in the body, wreaking havoc on the lining of our intestines, our replenishing blood cells and our usually industrious hair follicles. This is an extreme example of a common problem: drugs have frequent, unintended and largely unpleasant side effects as a result of indiscriminate binding to both their desired targets and a multitude of other sites. How can synthetic biologists help?

Every day the average person makes 200 trillion new red blood cells (RBCs) to replenish those lost by wear and tear. If a person has suffered recent blood loss, that number climbs to even greater heights. Unsurprisingly, when these cells dwindle people are left feeling exhausted. Normally, the body regulates our levels of RBCs through a simple mechanism: the kidney, in addition to filtering out waste from the blood, keeps track of flow and when it detects that our oxygen carriers are low it sends out a signal to make more. That signal is a protein called erythropoietin (EPO). Epoetin alfa is a recombinant version of EPO—that is, we synthesize it in a lab by intentionally inserting DNA into cells, instead of extracting it from natural sources.

In many ways, this drug was one of the first big successes of recombinant DNA research. When a patient has low levels of RBCs, a condition called anaemia, injecting recombinant EPO helps. Anaemia is common in menstruating women, those with kidney disease, cancer patients and more. Over the course of a decade, this drug went from clinical approval to doctors worldwide dispensing over 20 million doses per week (Figure 1).

However, EPO does not just signal new red cell production. It might be more accurately described as a traumatic blood loss hormone than a drug to treat anaemia—the distinction being that a person with blood loss needs platelets and clotting factors and possibly new vessel growth in addition to new RBCs. EPO users were found to have significantly higher rates of devastating clot-based side effects including strokes, heart attacks and pulmonary embolisms. Cancer patients were found to have better vascularization in their tumours, making those tumours more robust and easier to spread. Unsurprisingly, EPO use was curtailed. In 2007, the Food and Drug Administration (FDA) issued a black box warning label on EPO products alerting, ‘INCREASED MORTALITY, SERIOUS CARDIOVASCULAR EVENTS, THROMBOEMBOLIC EVENTS, STROKE and INCREASED RISK OF TUMOR PROGRESSION OR RECURRENCE’ (Figure 2). Today, the use of EPO is restricted to those who either cannot make their own EPO or who suffer from incurable, lethal cancer. Despite these restrictions, EPO remains a popular medication with an enormous market.

Current protein therapeutics: their strengths and limitations

Since recombinant DNA technology was developed in the 1970s, the creation and commercialization of protein therapeutics has grown very rapidly. The first protein drug, recombinant human insulin, was approved by the FDA in 1982. These drugs have become a mainstay of modern therapy. As of 2018, seven out of the top 10 money-making drugs are proteins.

Protein therapeutics have several advantages over
most other types of drugs. Protein–protein interactions are potent and highly specific. They can be further modified or engineered to mimic or improve natural protein interaction and fine-tune target specificity. Our knowledge of evolution and structure–function relationships of proteins enables the rational design of protein therapeutics with desired properties. Unlike the majority of small molecule drugs, proteins are naturally found in our body, so it is easier to predict their behaviour, such as how long they will circulate in our body (‘pharmacokinetics’) and if they will trigger any bad immune response or other side effect (‘toxicity’). Compared with small molecules and cell-based therapeutics, the manufacturing process for proteins is simpler, more controllable and more predictable.

One class of protein therapeutics are cytokines and hormones. Both are signalling proteins that can modulate immune responses or regulate cell maturation and growth. Cytokines normally act locally, such as at the site of a wound or in a specific tissue, while hormones act throughout the body. When given as drugs, they can provide a replacement for endogenous proteins that a patient lacks or makes incorrectly due to a genetic disorder. They can also supplement or augment cytokine/hormone activity in a patient who produces an insufficient amount of endogenous proteins or needs more than standard levels. Insulin (Humulin®) and erythropoietin (EPO, Epogen®) are used to treat diabetes and anaemia, respectively, by providing a replacement for or supplementing the endogenous protein. Interleukin-2 (IL-2, Proleukin®), tumour necrosis factor alpha (TNFα, Beromun®) and interferon alpha (IFNα, Intron A®) are examples of recombinant human cytokines that augment normal immune responses to treat cancer.

Another class of protein therapeutics is monoclonal antibodies, which are large proteins produced by white blood cells to neutralize or attack specific targets as part of our adaptive immune system. Borrowing from these natural drugs, monoclonal antibody-based therapeutics have been developed to block undesired native signalling or trigger immune responses in patients (Figure 3). Adalimumab (Humira®), for instance, is an anti-TNFα antibody that mitigates excessive immune responses in rheumatoid arthritis by inhibiting the binding of
this inflammatory signalling molecule to its receptor. Rituximab (Rituxan®) is an antibody to CD20, a cell surface marker on B cells, and mediates killing of B cells to treat B cell lymphomas, autoimmune diseases and rheumatoid arthritis. Because of their large size, monoclonal antibodies have long half-lives in the body (proteins with a molecular weight of less than about 50 kilodaltons (kDa) filter through the kidney and disappear into the urine). Because of their highly specific target affinity, they are optimal for neutralizing undesirable antigens while avoiding most off-target side effects.

In spite of being 'natural' molecules, protein drugs can cause serious side effects. For example, since naturally formed cytokines act locally, when they are given as drugs and distribute throughout the body, this is not a ‘natural’ situation. Most cytokines are associated with severe dose-limiting toxicities that come from on-target binding to non-target cell types. For example, IL-2 causes a general upregulation of the immune system and fever, and IFNα causes ‘flu-like symptoms’ that can be difficult to bear when this drug is given for a year as is typical in treatment for hepatitis viruses. As mentioned above, the anaemia-relieving RBC replenishment caused by EPO is paired with clotting and vessel growth—these last two effects are adaptive features in the natural setting of wound repair but are extremely risky in generic anaemia treatment. When EPO is given for years to patients with kidney failure, these effects cause an increase in heart attacks, strokes and deep vein thrombosis. Cancer patients receiving EPO may get relief from their anaemia-induced exhaustion but have increased growth of the blood supply to their tumour. The challenge for synthetic biologists is to manipulate the properties of these drugs through careful, biologically inspired design so that side effects can be minimized.

### Fusion proteins for cell-targeted delivery

Thanks to recombinant DNA technology and protein engineering, researchers have learned to mutate, truncate or attach protein parts to enhance the clinical potential of protein therapeutics. Etanercept (Enbrel®) was the first fusion protein drug to be approved by the FDA, in 1998. It is composed of a fragment of TNF receptor and the dimeric Fc domain of an antibody, putting two receptor domains into a binding configuration so that they can soak up TNF, a profoundly inflammatory cytokine. Etanercept also has a longer plasma half-life compared with the unfused receptor fragment because of its high molecular weight. Whole antibody-cytokine fusion proteins do even more than extend half-life – they are composed of the target (‘antigen’)–binding domain of an antibody, connected via a linker to the active cytokine domain, such that the antibody fragment directs the cytokine signalling specifically to the desired target cell types (Figure 4). In one preclinical study, an antibody-IL12 fusion protein directed to the tumour microenvironment showed potent activity in various mouse cancer models at a dose that was more than 20-fold lower than the unfused cytokine alone. However, the cytokine domain is not prohibited from binding to its receptors on non-target cell types (Figure 4). Clinical studies suggest that side effects are as dose dependent as desired effects, and result from cytokine action on non-target tissues. Attempts to increase half-life, by definition, cause longer exposure times, and improved tumour penetration may require higher doses, both of which mean more opportunity for undesired side-effect signalling.
Protein engineering strategies to remove side effects—’chimeric activators’

A ‘chimeric activator’ is a type of antibody fusion protein that incorporates a mechanism to prevent the activating cytokine/hormone domain from binding to non-target cell receptors. How might this be achieved? There are a lot of ways to convey information and some of them are better than others. Imagine I want to tell you a secret. It would be very silly of me to, say, just shout that secret in a crowded room over and over until you acknowledge me, and there might well be some negative side effects from shouting my secrets to a roomful of strangers. Treating someone with an unaltered cytokine is like taking the random shouting approach, where any cells in the vicinity can pick up the message and take action. A cytokine-antibody is a little bit better, but it is somewhat like a shout where you are sure your friend is nearby. A chimeric activator is intended to act like a whisper to a friend who has been pulled close to exchange information.

This is achieved by weakening the cytokine molecule so that signalling is reduced—to use the analogy above this turns the message from a shout to a whisper. To be scientifically precise, the binding is tuned by mutation to be too weak to activate its receptor in most tissues, and activity is rescued only when the cytokine is highly concentrated at its desired target location. This rescuing effect is produced by an antibody element that binds to a second protein on target cells, causing high drug concentrations right at those target cell surfaces. This is like grabbing your friend and pulling them close—suddenly that quiet whisper is loud enough to hear due to proximity.

When the chimeric activator has bound itself via the antibody element to its target cells, that antibody domain anchors drug to the target cell surface, causing an increased local concentration of the cytokine domain. This anchoring thereby shifts the equilibrium towards the receptor-bound state (Figure 4). This design allows cell targeted delivery, target cell-specific activity and removal of side effects mediated by on-target signalling on non-target cells.

Targeted EPO for the treatment of anaemia

‘Targeted EPO’ is an example of how configuration as a chimeric activator can improve the specificity of an existing protein drug that otherwise leads to various side effects. Recall that EPO stimulates the maturation and proliferation of RBCs in the bone marrow. Recombinant EPO was widely used to treat anaemia until 2007, when it received an FDA black box warning for pro-thrombotic side effects. EPO receptors are found on many tissues in our body, and therefore, these side effects might be avoided...
by targeting EPO action specifically to RBC precursors and away from other cell types. Targeted EPO consists of a mutation-weakened EPO fused via a flexible linker to an antibody domain that binds glycophorin A, which is a surface protein abundant on RBC precursors. In mice, non-targeted forms of EPO stimulate production of both RBCs and also platelets. Targeted EPO stimulates RBC production but not platelet production, demonstrating that it can perform cell-targeted activity while avoiding signalling via non-target cell receptors. This molecule thus may be a safer, less thrombotic drug for anaemia treatment. If further studies bear positive results, this advance would allow doctors to resume providing anaemia relief to the patients who were excluded from treatment due to risks with the first iteration of recombinant EPO.

Future perspectives

The first wave of protein drugs was recombinantly synthesized cytokines, hormones and monoclonal antibodies that were essentially identical to proteins found in the human body. A new generation of protein drugs combines two or more protein parts to improve therapeutic effect, drug half-life, biodistribution and target specificity. Chimeric activators are one example, along with bispecific antibodies (a fusion of two antibody fragments) and the engineered proteins that are part of CAR-T cells [Editor’s note: be sure to check out our February 2019 issue for more on CAR-T cells from Peter Abel]. Increasing knowledge in protein engineering will continue to drive smarter design strategies for safe and effective drugs and is limited only by our understanding of disease mechanisms and the creativity of protein engineers.

Further reading

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