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Review Article

A Review on Therapeutic Proteins: Therapeutic Modalities and Production Challenges

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ABSTRACT

Therapeutic proteins have become one of the most important breakthroughs in modern medicine, providing highly specific and effective treatment for a variety of diseases. They have opened a new era in the treatment of diabetes mellitus, cancer, autoimmune diseases, hematological diseases, infectious diseases, and several genetic deficiencies and defects. The advances made in recombinant DNA technology, protein engineering and biopharmaceutical manufacturing have allowed for the production of safe, effective, quality therapeutic proteins whose properties are enhanced for the benefit of the patient. This review aims to give a detailed coverage of the therapeutic proteins starting with their basic concepts, their classification and the main sources of therapeutic proteins such as the natural proteins, recombinant proteins, and genetically engineered proteins. Different classes of therapeutic proteins, their various clinical applications and representative examples are also included in the article. Specific focus on proteins of therapeutic value like hormones, cytokines, monoclonal antibodies, enzymes, blood coagulation factors, growth factors and fusion proteins in the context of various acute and chronic disorders.

INTRODUCTION

Therapeutic proteins are one of the most significant class of biopharmaceuticals and are being used in modern medicine for treatment of many diseases. Proteins which are engineered in the laboratory for pharmaceutical use are referred as therapeutic proteins. There are many diseases and syndromes in which peptides and proteins have been shown to have a great therapeutic potential. There have been numerous

recombinant proteins approved by the FDA as biotechnology medicines, antibody drug conjugates, vaccines, enzymes, natural/recombinant cytokines, and interferons [1].

Recent developments in biotechnology enable the selection and preparation of new macromolecular compounds (e.g., peptides, proteins, DNA analogues, made by Hybridoma cell technology and Recombinant DNA technology) which can be used as drugs (e.g.,

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hormones, monoclonal antibodies, vaccines and diagnostic tools) for several purposes, including diagnosis, prophylaxis, treatment and cure.

Therapeutic antibodies have been of particular interest as they are highly specific and effective. A number of antibody-based drugs are approved to treat cancers, autoimmune diseases, metabolic diseases and infectious diseases. Monoclonal antibodies (mAbs) are produced through engineered B-lymphocytes by using recombinant DNA technology to recognize and bind target molecules that play a key role in therapeutics and disease diagnosis. The advantage of the monoclonal antibodies of being able to target diseased cells and avoid healthy ones has made them a large part of the therapeutic choice for many chronic and life-threatening diseases. The recently developed bispecific antibodies are also unique, in that they are simultaneously able to bind two different antigens or targets. The dual targeting has been useful in treating complex diseases like acute lymphoblastic leukemia and hemophilia A, and has improved the therapeutic effect.

Another type of therapeutic proteins is enzymes. In many cases of inherited metabolic disorders, the enzymes that are needed for normal cellular function are deficient or totally absent. In these cases, the missing enzyme may be administered to the patient as Enzyme Replacement Therapy (ERT), in which recombinant enzymes, which are enzymes made by the genetic engineering process, are given to the patient. ERT has greatly enhanced the lives and clinical outcomes of people with lysosomal storage disorders, a group of rare genetic disorders where substrates are not fully metabolized in the lysosomes. There are currently a number of enzyme replacement therapies available for the treatment of several lysosomal storage disorders: Gaucher disease, Fabry disease, and Pompe disease. ERT can help to control the symptoms of the disease and slow the pace of disease progression, but cannot cure the disease since the genetic defect is not changed. Patients typically have to take these therapeutic enzymes for years, making long-term treatment difficult and expensive, but it is one of the most successful protein-based therapeutics in clinical practice.

Over the past few decades, therapeutic proteins have emerged as a promising treatment option for various

diseases, particularly cancer, cardiovascular disease, diabetes, and others. The formulation of protein-based therapies is a major area of research, however, a few factors still hinder the large-scale production of these therapeutic products, such as stability, heterogeneity, immunogenicity, high cost of production, etc.

Biologics, specifically recombinant biologics, have been extensively used as 'medicine of choice' in many different disease areas, largely because of their specificity in identifying and modifying disease related pathways [2].

The pharmaceutical industry has a growing category of medicines that are based on protein, the fastest-growing type of medicine [3]. More than 170 protein therapeutics are used around the world, and most are recombinants. Recombinant human insulin was the first recombinant therapeutic protein and a major achievement in Biotechnology. Prior to this introduction diabetes was treated with animal-derived insulin, but was known for being limited in availability and immunogenic. The introduction of recombinant human insulin (Humulin) by the U.S. FDA in 1982 ushered in the modern era of protein therapeutics and spurred the creation of a variety of recombinant biopharmaceuticals.

The most obvious difference between a small molecule drug and therapeutic protein is their size. The therapeutic proteins are much larger (approximately 100 to 1000 times), than the drug molecules. Due to their size, complex nature and large molecular size, they cannot be synthesized in laboratories, but can be manufactured by living cells. The major factors that affect the biological production of these proteins in this context are cell characteristics, such as cell line, culture conditions and origin (species) of cells.

As the proteins are more likely to elicit an immune response in individual recipients, thus this is an important safety issue related with the therapeutic applications of these biomolecules. The three dimensional secondary, tertiary and quaternary structure of these therapeutic proteins are occurring and hence the characterization of these molecules can be done only by sophisticated analyses that measure their physico-chemical, biological and immunochemical properties. The drugs which are genetically engineered and



recombinantly modified human proteins are called therapeutic proteins.

Types of therapeutic proteins:

Therapeutic proteins are proteins used in the prevention, treatment or diagnosis of a variety of diseases. Their high specificity, effectiveness and power to target complex mechanisms of disease make them an essential tool in modern pharmaceutical therapy. Therapeutic proteins may be presented in different dosage forms (injection solution, lyophilized/formulated powder, sustained release dosage form, and advanced drug delivery systems) and include a variety of protein classes such as monoclonal antibodies (mAbs), hormones, enzymes, cytokines, growth factors, blood factors, fusion proteins, and recombinant vaccines depending on the clinical need.

• Antibody based drugs:

Conjugates of antigen specific monoclonal antibody and a drug covalently coupled by chemical linkers which are very target specific. Mylotarg is a commercially available antibody drug conjugate which is used as an antitumor agent.

• Anti-coagulants:

Substances which inhibit blood clotting. They are given to patients as a way to lower the risk for having strokes and heart attacks. Antithrombin (recombinant) is a FDA Approved Anti-coagulant.

• Engineered protein scaffolds:

The ability to be used as a framework to generate antibodies with target specificity, with flexible binding sites for target molecules. Ecallantide (Kalbitor®) is a proven plasma kallikrein inhibitor.

• Growth factors:

A family of proteins that have growth promoting effects. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) are commonly used to promote white blood cell recovery in cancer patients, following chemotherapy treatment.

• Interferons:

Antiviral and anti-tumor cytokines pro and anti-inflammatory that can play an active role.

• Thrombolytics:

Enzymes or drugs to treat thrombotic diseases. Thrombolytics are cost effective over traditional methods of clot removal. Streptokinase is one of the earliest thrombolytics found and used for treatment in a patient suffering from pulmonary embolism.

• Fc-Fusion protein:

Synthetic hybrid of the crystallizable portion of a monoclonal antibody (mAb) and a protein being used in therapeutic applications that could provide more target specificity and delivery. Romiplostim is an Fc-Fusion peptide of the type of peptides that are used as enhancers for platelet production.

• Bone morphogenetic proteins:

A group of growth factor proteins recently found to potentially aid in healing a spinal injury or other reconstructive surgery. BMP-2 is an osteo-inductive growth factor that is widely used.

• Enzymes:

Commonly applied in the industrial and pharmaceutical areas, biocatalysts. Recombinant enzymes have several applications, such as therapeutic use in enzyme deficiency disorder, as anti-inflammatory agents, as antibiotics, as anticoagulant agents and as thrombolytic agents. Lysosomal storage disorders are treated with marketed recombinant enzymes, like Myozyme® (α-glucosidase).

• Hormones:

Chemicals produced by cells that form multicellular glands. For patients with a hormonal deficiency disorder like diabetes, recombinant hormones are produced and given to patients.

• Interleukins:

Pro-inflammatory cytokines which can be used for the modulation of inflammatory responses in allergy and infectious diseases. Aldesleukin/Proleukin is a human



recombinant interleukin-2 used for the treatment of cancer patients.

Classification of Therapeutic Proteins

The therapeutic proteins can be grouped according to their function, to their modes of use (clinical) and to their pharmacological properties. Therapeutic proteins can be categorized into four groups [1-4].

Group-I: Therapeutic proteins with enzyme/regulatory activity. This is further subdivided in 3 subgroups: Ia, Ib and Ic.

- **Ia:** includes the strategy of replacing the deficient enzyme or protein that cause a particular disease or disorder, such as human growth hormones and insulin etc.
- **Ib:** The therapeutic proteins of Ib subcategory involves in enhancing the magnitude or modulating the expression timing of a particular normal protein. RBC production is stimulated in the bone marrow, for instance, by recombinant erythropoietin produced by kidney, especially in patients with chemotherapy induced anaemia [5]. Recombinant

follicle stimulating hormone that leads to maturation of follicles [6].

- **Ic:** The therapeutic proteins of this category have the approach to add or modify the function or activity of the naturally occurring proteins. For instance, the leftover DNA in neutrophils is removed by recombinant human deoxyribonuclease I (DNase I). Enzymes from bacteria and yeast, called collagenase, are used for chronic dermal ulcers and severe burns [7]. Botulinum toxin is used for dystonia and cosmetic uses [8].

Table 1:

Therapeutic Protein	Category	Role / Function	Clinical and therapeutic Role
Insulin	Ia	Replacement	Diabetes mellitus [9]
Growth Hormones	Ia	Replacement	Growth failure, turner syndrome [10]
Factor VIII	Ia	Replacement	Hemophilia A [11]
β - Glucocerebrosidase	Ia	Replacement	Cystic fibrosis, Pancreatic insufficiency [12]
Erythropoietin	Ib	Augmenting of existing protein	Anemia due to renal failure or chemotherapy [5]
Human chorionic gonadotropin (HCG)	Ib	Augmenting	Assisted IVF [6]
Botulinum toxin type	Ic	Augmenting	Dystonia [8]
Human deoxyribonuclease I	Ic	Augmenting	Cystic fibrosis, respiratory diseases [13]
Alteplase	Ic	Augmenting	Myocardial Infarction [14, 15]

Group II: Therapeutic Proteins with Special Targeting Activity

The therapeutic proteins of this group are divided into 2 subcategories:

- **IIa** – Proteins which interfere with other molecules in the body.
- **IIb** – Molecules that are delivered at a specific site in the body.

They are both performed with the help of monoclonal antibodies (mAbs). Monospecific antibodies are produced by rDNA technology with only one type of B cells.

Mabs are particularly useful for elucidating their function in a variety of diseases such as autoimmune diseases, multiple sclerosis, rheumatoid arthritis or lupus erythematosus, due to their high affinity and selectivity to their molecular antigenic targets.



Therapeutic Protein	Category	Role / Function	Clinical and therapeutic Role
Cetuximab-Monoclonal antibody	IIa	activate a signalling pathway	colorectal, neck and head cancer [16]
Ibritumomab tiuxetan (recombinant murine IgG1 kappa monoclonal antibody)	IIa	deliver proteins specifically and on-target	non-Hodgkin's lymphoma and persistent cutaneous T-Cell lymphoma [17]
Hepatitis B immune globulin	IIa	Mab binds with surface components of Hepatitis B Virus, deliver drugs to the target cells	Prevention through Hepatitis [18]
Raxibacumab	IIa	Mab that targets the protective antigen of Anthrax toxin to prevent intracellular entry of the anthrax lethal factor and edema factor	Prophylaxis and treatment of anthrax [19]
Brentuxmab	IIb	Chimeric Mab	Hodkin's Lymphoma and systemic anaplastic large cell lymphoma [20]
Trastuzumab	IIb	binding to HER2 proteins on the surface of cancer cells	Treatment of HER2-positive breast cancer and HER2-positive stomach (gastric) cancer. [21]

Group III: Therapeutic Protein Vaccine

The therapeutic proteins of this group have 3 approaches:

- **IIIa** – Protection against deleterious agents
- **IIIb** – Treatment of autoimmune diseases

• **IIIc** – Cancer treatments

Adaptive immunity is the approach which is utilized to generate protective response against foreign particles like recombinant hepatitis B surface antigen (HBsAg) protein vaccine and Human papilloma virus (HPV) vaccine, which protect from cervical cancer and genital warts. (17)

Therapeutic Protein	Category	Role / Function	Clinical and therapeutic Role
HBsAg	IIIa	Non-infectious protein on surface of Hepatitis B Virus	Hepatitis B Vaccination [22]
HPV Vaccine	IIIa	Major capsid protein	Prevention of HPV infection [23]
Anti-Rh IgG	IIIb	Antibodies that neutralize Rh antigen	Routine antepartum and postpartum prevention of Rh(D) immunization in Rh(D) negative women [24].
Sipuleucel-T	IIIc	Activated Antigen Presenting Cells	In the treatment of Metastatic hormone refractory prostate cancer [25]

Group IV: Therapeutic Protein Diagnostics

Many therapeutic proteins are used only for diagnostic purposes, *in vitro* and *in vivo*. One of the prime examples of this is purified protein derivatives (PPD) test used to detect *Mycobacterium tuberculosis* infection.

TSH is used to diagnose residual thyroid cancer.

Various proteins are used as imaging agents to detect multiple infections and pathological conditions.



Therapeutic Protein	Category	Role / Function	Clinical and therapeutic Role
Recombinant purified protein Derivative (DPPD)	Group IV	Non-infected Protein from Mycobacterium tuberculosis	Diagnosis of Tuberculosis exposure [26, 27]
Glucagon	Group IV	Pancreatic hormone that increases blood glucose level	treating acute hypoglycemia and as a diagnostic aid during gastrointestinal imaging [28]
Secretin	Group IV	Synthetic human peptide	Diagnosis of pancreatic exocrine dysfunction or gastrinoma [29]
Apcitide	Group IV	Imaging agent, synthetic RGD-mimicking polypeptide, which binds to glycoprotein IIb/IIIa receptors on activated platelets.	detection of acute Deep Vein Thrombosis and Pulmonary Embolism [30]

Sources of therapeutic proteins

Depending on their ability to produce the desired chemical, prokaryotic and eukaryotic microorganisms have been utilized as hosts for producing therapeutic proteins. Due to differences in the nature of cells, smaller proteins are normally expressed in prokaryotes, while larger proteins are normally expressed in eukaryotes. The expression of therapeutic proteins is cost-effective, easiest and fastest in prokaryotic systems like *E. coli*. Proteins that need glycosylation are better when expressed in insect systems, mammalian cells and fungi [31], however yeasts [32] could be used to produce higher amount of native glycosylated proteins (~50 kDa) at low cost. Mammalian cell lines are the most frequently-used cell lines for the construction of recombinant mammalian proteins. Animals can also be genetically modified for the production of therapeutic proteins, either in their blood, urine or milk [33].

(i) Bacteria

Some 30% of all recombinant protein production in the last 30 years has been done using the genetic material of *Escherichia coli*, which has been the host of the first recombinant DNA procedures by infecting bacteriophages [34]. However, knowing its transcriptional and translational machinery and the development of new genetic tools make *E. coli* a good host for the large-scale production of proteins, particularly non-glycosylated proteins [35,36]. Simple and inexpensive media components can be used to enhance the productivity of proteins employing promoters of *Escherichia coli*, such as lac, tac and trc [37].

Enzymes and therapeutic proteins can be expressed using either homologous or heterologous expression systems with the *Bacillus* species especially gram-positive bacilli. Recombinant proteins that are expressed in *Bacillus* are likely to be expressed in their native and biologically active form. These bacteria grow and metabolise quickly and do not produce endotoxins and are efficient hosts for the production of therapeutic peptides. Furthermore, it is possible to use proteins secreted directly into the culture medium, thus avoiding the process of disrupting the cells and making it easier to purify in the downstream process. Vaccine development has involved expression of the proteins and virus-like particles of viruses particularly in *E. coli* [38]

Bacterial cells have two main advantages over other cells; however, they are easily cultured in the lab or in industry, and are ideal for large-scale production [39]. However, due to the limitations in bacterial expression systems, lack of post translational modifications and glycosylation, eukaryotic expression systems including yeast cells, fungal cells and algal cells were developed to produce therapeutic proteins [40]. As cells are used as factories to produce recombinant proteins and the potential of these proteins explored, they:

In particular, *Escherichia coli* has been the host for the expression of viral proteins and virus-like particles for vaccine development.

(ii) Yeast system

Yeasts are attractive hosts for the production of heterologous proteins. The eukaryotic subcellular organisation of their cells allows them to perform numerous



post-translational folding, processing and modification events that are necessary to produce "authentic" and bioactive mammalian proteins, which are not possible in prokaryotic systems. They also possess the other properties of the unicellular microorganism such as quick growth and genetic manipulation. Yeast have the same desirable properties as the unicellular microorganism with regard to their rapid growth and ease of genetic manipulation. Most studies of yeast expression have utilised the well characterised baker's yeast *Saccharomyces cerevisiae*. Due to the advancement of DNA transformation technologies, however, an increasing number of non-*Saccharomyces* yeasts are also now available as hosts for the production of recombinant polypeptides. They consist of *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, *Schizosaccharomyces pombe*, *Schwanniomyces occidentalis* and *Yarrowia lipolytica* [41].

Therapeutic proteins such as urate oxidase, granulocyte-macrophage colony-stimulating factor (GM-CSF), insulin, glucagon, platelet-derived growth factor, hepatitis B surface antigen, and hirudin have all been produced by *S. cerevisiae* [42].

Non-conventional yeast, such as *Pichia pastoris*, and traditional baker's yeast *Saccharomyces cerevisiae* are the most commonly used expression host systems [43] but also the non-conventional yeast *Hansenula polymorpha* and *Yarrowia lipolytica* are used.

P. pastoris is genetically modified and is used to produce antimicrobial peptides (AMP) [44]. A non-conventional yeast, *H. polymorpha* has been used for commercial production of the recombinant hepatitis B vaccine [45].

(iii) Fungi

The ability to efficiently secrete proteins is hampered by certain limitations of yeast as they tend to hyperglycosylate secreted proteins, which affects their half-life in vivo and efficacy. Also, the expression levels and plasmid stability were found to be low, limiting their use, for example- in *S. cerevisiae*.

High yields of post-translationally modified therapeutic proteins [46] can be produced by filamentous fungi, like *Aspergillus niger*. The *Trichoderma reesei* is one

of the most important hosts of enzymes that yield good amounts of enzymes and can be produced efficiently.

(iv) Microalgal source

Microalgae offer a number of intrinsic advantages such as rapid growth, low and cost-effective photobioreactor input requirements (light source, water source, carbon source, supplementary nutrients, and photoautotrophic nature). The time it takes to transform and produce the recombinant therapeutic proteins in microalgae is comparatively short [47].

Three transformable sites in microalgae (chloroplast, nucleus, mitochondria) and of these sites, nucleus and chloroplast sites are now attracting the attention of the scientific community for recombinant proteins production [48]. The total soluble protein (TSP) production yield of the model microalga via its chloroplast, *Chlamydomonas reinhardtii* is even higher than in plants, with > 5% of TSP [49, 50].

Production of Therapeutic Proteins has some challenges:

The production of such complex molecules, however, is beset with numerous challenges that need to be surmounted to be manufactured safely, efficiently and cost effectively.

The oral delivery of peptide and protein drugs has been difficult for the pharmaceutical scientists because of their several unfavourable physicochemical properties, including high molecular weight, enzymatic sensitivity, short plasma half-life, ion permeability, immunogenicity, tendency to aggregation, adsorption and denaturation [51].

(i) Biological life and Stability.

The short half-life was the main issue with the therapeutic proteins, leading to their degradation. These therapeutic protein molecules are also quickly cleared by the kidneys and are clearly enzymatically active in the blood as a whole, so that their lifetime in the blood is short.

To compensate for this, more may be required to reach the desired concentration in the blood serum for the desired duration. So, a few technologies have been proposed and tested for the extension of half-life of



therapeutic proteins such as amino acid manipulation, bio-conjugation/

They include genetic fusion, post-translational modification through attaching a peptide to natural/synthetic polymers (PEGylation, polysialylation, HESylation) and carrier-mediated delivery [52].

(ii) Heterogeneity of proteins

The therapeutic proteins may be heterogeneous and another challenge in the production of the therapeutic proteins is their heterogeneity. Biologically produced therapeutic proteins differ from small molecule products in that they do not undergo variations in their post-translational modification, folding and aggregation.

The heterogeneity could affect the protein stability, pharmacokinetics and immunogenicity, leading to variations in efficacy and safety [53, 54]. To tackle this, several methods have been developed such as the use of advanced cell lines, optimized culture conditions and novel purification techniques.

(iii) Higher production Cost

One of the major difficulties in the production of therapeutic proteins is the cost of production, this is because they cannot be easily accessed by patients. The complexity of the manufacturing process, regulation, and intellectual property [55] are some of the factors that influence the cost. Several approaches have been investigated to lower production costs, such as process intensification, cell line engineering, and optimization of the process with the help of advanced analytics and automation.

(iv) Host expression system

There exist some challenges with the scalability and manufacturing capacity for production of therapeutic proteins also [56]. Many of the biologics are made with mammalian cells which require large scale cell culture facilities and specialised equipment, making it difficult to produce sufficient quantities of proteins to keep up with the increasing demand. To address this challenge, other expression systems such as microbial cells and plant cells were investigated [57].

(v) Patient Safety and associated Immunogenicity

Safety and clinical efficacy, which depends on several factors such as disease state, target biology, potency, safety margin, dosing and selection of patient population, are the most important feature of these therapeutic proteins.

One of the key challenges for protein therapeutics is their immunogenicity. A modification in the protein's structure, which might happen as a result of post-translational changes such as product's administration, storage, or manufacturing process, sets off the immune response [58]. While a vaccine induced immune response is desired clinical response while immune response due to therapeutic proteins is undesirable.

(vi) Targetability

Another important consideration in the development of protein-based medicines is targetability. The ability to target drugs to specific tissues (e.g., cancer cells) or organs (e.g., brain) is highly desirable both from the standpoint of lowering therapeutic dose as well as reducing side effects. For instance, antibody-drug conjugates can be used to guide the drug to cells which have a specific receptor as the antigen. Many protein-based structures tend to bind in liver, kidney and spleen [59]. Targeting moieties are being incorporated into strategies that promote delivery to sites outside of these organs is an active research area [60]. The majority of current protein-based pharmaceuticals have extracellular targets. Most proteins are not permeable to the cell-membrane due to their relatively large size as well as the diverse surface charges. Intercellular delivery is very desirable; if the ability to get hold of unwanted intracellular processes can be used to create very effective medicines. Cell-penetrating peptides [61], supercharging [62] and dense grafting of DNA [63] are emerging chemical strategies that have been promising in this area.

Chemical changes in protein structure can provide several beneficial properties but can also be detrimental to the activity or potency of the drugs [64].

Future Perspectives

The basic principles of therapeutic proteins, their classification, sources and general clinical applications



have been summarized here, along with a few examples. It also underscores the impressive strides made in the design and manufacture of protein-based therapeutics, including enhanced efficacy, specificity, pharmacokinetics and safety accounts. However, there are several issues to consider: immunogenicity, protein instability, production complexities, delivery issues, and the price of biologic medicines. Many of these limitations are likely to be overcome with further research on advanced protein engineering, new drug delivery methods, biosimilars, AI-driven protein design, and personalized medicine.

CONCLUSION

Therapeutic proteins have revolutionized the field of disease treatment by offering a highly selective and effective therapy option for a wide variety of life-threatening and chronic diseases. The development of biotechnology, recombinant DNA technology, and protein engineering has greatly increased the range of proteins that are available and used in the clinic, and these proteins are essential to current medical practice. The success of recombinant proteins, monoclonal antibodies, enzymes, cytokines, hormones, growth factors and fusion proteins has helped to significantly enhance clinical benefit in patients for a variety of therapeutic areas, including oncology, endocrinology, immunology, haematology, infectious disease and rare genetic disorders. To summarise, therapeutic proteins are one of the fastest-growing, and most innovative, classes of pharmaceuticals. With continued advancements in technology and growing therapeutic potential, their clinical impact and availability are likely to continue to improve, leading to better patient care and the emergence of new generation precision medicines.

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