Production of novel protein therapeutics to improve targeted cancer therapy
Al-Qahtani, Alanod

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Chapter 2:

Strategies for the production of long-acting therapeutics and efficient drug delivery for cancer treatment

Alanod D. AlQahtani\textsuperscript{1, 2}, David O’Connor\textsuperscript{3}, Alexander Domling\textsuperscript{2}, Sayed K. Goda\textsuperscript{4, 4}

\textsuperscript{1}. Anti-doping Lab-Qatar, Research Department, Protein Engineering unit, Doha, Qatar.
\textsuperscript{2}. Drug Design Group, Department of Pharmacy, University of Groningen, Antonius Deusinglaan, AV Groningen, The Netherlands.
\textsuperscript{3}. Xi’an Jiaotong-Liverpool University, Department of Biological Sciences, Science and Education Innovation District, Suzhou 215123, China
\textsuperscript{4}. Cairo University, Faculty of Science, Giza, Egypt.

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## Contents

1. **Abstract** .......................................................................................................................... 23

2. **Keywords** .......................................................................................................................... 23

3. **Abstract Figure** .................................................................................................................. 25

4. **Introduction** ....................................................................................................................... 27

5. **The need for modified therapeutic proteins and why they need to last longer in the body** ........................................................................................................................................ 29

6. **Advantages of modified proteins over unmodified ones** ........................................ 30

7. **Strategies for producing long-acting protein therapeutics** ........................................ 31

8. **Protein PEGylation using polyethylene glycol (PEG)** .................................................. 33

9. **Fusion to Human Serum Albumin** .................................................................................. 38

10. **Diseases that have been treated with PEGylated proteins** ........................................ 40

10.1. **PEGylation to improve drug delivery and targeting of cancer cells** ......................... 42

11. **Diseases which have been treated with proteins linked to HSA** .................................. 44

11.1. **HSA fusion to improve drug delivery and targeting of cancer cells** ......................... 46

12. **Immune responses of patients towards the modified drugs** ........................................ 47

12.1. **Effects of the PEG moiety on protein immunogenicity and stability** ......................... 47

12.2. **Effects of the HSA moiety on protein immunogenicity and stability** ......................... 53

13. **Advantages of PEGylation and HSA fused drug** .......................................................... 55

14. **Nanonization and Drug improvement** ........................................................................... 60

15. **Conclusions** ...................................................................................................................... 61

16. **Acknowledgements** ......................................................................................................... 62

17. **References** ....................................................................................................................... 62
1. Abstract

Protein therapeutics play a significant role in treating many diseases. They, however, suffer from patient’s proteases degradation and antibody neutralization which lead to short plasma half-lives. One of the ways to overcome these pitfalls is the frequent injection of the drug albeit at the cost of patient compliance which affects the quality of life of patients.

There are several techniques available to extend the half-life of therapeutics. Two of the most common protocols are PEGylation and fusion with human serum albumin. These two techniques improve stability, reduce immunogenicity, and increase drug resistance to proteases. These factors lead to the reduction of injection frequency which increases patient compliance and improve quality of life. Both techniques have already been used in many FDA approved drugs.

This review describes many technologies to produce long-acting drugs with the attention of PEGylation and the genetic fusion with human serum albumin. The report also discusses the latest modified therapeutics in the field and their application in cancer therapy. We compare the modification methods and discuss the pitfalls of these modified drugs.

2. Keywords

PEGylations, Human Serum Albumin, Targeted cancer cells, Drug Delivery, HalfLife Extension, Protein Immunogenicity.
Strategies for the production of long-acting therapeutics and efficient drug delivery for cancer treatment.

**Therapeutic Proteins**
- Pitfalls:
  - Short half life
  - Suffer proteases degradation
  - Antibody neutralization
  - Lack Solubility

**Strategies to modify Therapeutic Proteins**
- Engineered Fc
- HESylation
- Fe fusion
- Albumin binding
- Polysialylation
- IgG binding
- Nanoparticles
- O-glycosylation

- **A** Produce Biobetter drug
- **B** To deliver drug to cancer cells

**Addition of a modifier**
- Or with linker

**New modification**
- **A**

- Increase drug bioavailability
- Reduce immunogenicity
- Increase stability
- Extends drug half life
- Minimizes loss of activity

**Therapeutic protein**

**Stability barrier**

**Extracellular space**

**Cleaves at the linker**

**Intracellular space**

**Cancer cell death**

**3. Abstract Figure**
4. Introduction

Proteins therapeutic can be defined as proteins that are either naturally produced in the body or created in the laboratory and introduced into the patient with the aim of improving or curing a pathological condition. They are usually acquired from either microbial cells or by genetically modifying an animal or plant, and their uses range from oncology to inflammation to infectious diseases. Proteins therapeutic also have the advantage that they function naturally as either pharmacokinetic or pharmacodynamic drugs, as they usually serve to replace an absent protein, and the body responds as if the protein is naturally occurring.

Proteins often have multiple highly specific and complex functions that cannot be mimicked by simple chemical compounds. However, in common with small-molecule drugs, there are three major parameters influence their therapeutic efficacy: time (t1/2 or half-life), toxicity and targeted binding.

The body produces many diverse proteins that are used as therapeutics. In the case of diseases caused by the mutation or deletion of a protein-coding gene, the protein therapeutic generally replaces the abnormal or missing protein in question without the need to go through gene therapy. Protein therapeutics have multiple advantages over small-molecule drugs. In particular, the clinical development and approval time of protein therapeutics by national drug approval agencies such as the Food and Drug Administration (FDA) is generally faster than that of small-molecule drugs.

Protein therapeutics are categorized as having either an enzymatic or regulatory activity. They can have specifications based on their pharmacological activity, in which they
replace a protein that is deficient or abnormal. Alternatively, they can augment an existing pathway, provide a novel function or activity; interfere with a molecule or organism; or deliver other compounds (including other proteins), such as a radionuclide, cytotoxic drug, or effector protein.4

The first promoted recombinant therapeutic protein was human insulin (Humulin R) which was first produced in 1982 and has become one of the best-selling biologics worldwide after FDA approval.5 There are now multiple approved protein therapeutics, and many of these proteins have molecular mass below 50 kDa and a short terminal half-life in the range of minutes to hours.6 These limitations have led to the development and implementation of half-life extension approaches to lengthen the time that these recombinant proteins remain in the blood and to improve their pharmacokinetic properties as well.7 To achieve therapeutically effective concentration over a prolonged period of time, the drug is typically applied at a local region or subcutaneously so that it is only slowly absorbed into the bloodstream. Thus, factors such as the clearance rate, volume of circulation and the bioavailability of the therapeutic drug all influence its effective half-life.7

This review discusses some key strategies to extend the half-lives of therapeutic proteins and their applications. In particular, it focuses on two approaches, the attachment of polyethylene glycol moieties to proteins (protein PEGylation) and fusion with human serum albumin, as these are most often used and have proved especially useful.
5. The need for modified therapeutic proteins and why they need to last longer in the body

Chemical and structural changes in therapeutic proteins are possible and are carried out frequently to accomplish pharmacological or clinical benefit. Such modifications are essential as the drug needs to pass through various membrane barriers, e.g. to reach a tumor. Active targeting of a drug is typically achieved by conjugating it to a target entity that improves bioavailability and reduces systemic toxicity.8

Half-life extension technologies are now entering the clinic. Importantly, they are allowing the implementation of new biologic therapies, especially those involving shortacting therapeutic agents that would otherwise require frequent dosing profiles, which is particularly beneficial for the treatment of chronic conditions.

Modified therapeutic proteins can also be applied in a technique called the Antibody Directed Enzyme Prodrug Therapy (ADEPT) for cancer targeted therapy. ADEPT therapies are designed to generate toxic chemotherapeutics at the site of malignancy, potentially improving efficacy and reducing side effects.9, 10 The design of the modified therapeutic proteins aims to produce enzyme variants with good catalytic efficiency, high levels of stability and reduced immunogenicity. Such extra features will often increase the protein’s circulatory half-life, i.e. the time that the protein will circulate in the blood. This lead to the decrease of the number of doses required to be given to the patient, thereby reducing the possibility that the patient will generate antibodies to the modified protein and limiting the time available for the targeted cancer cells to mutate and hence avoid or resist the treatment as in case of glucarpidase. It has been shown that
protein modification using PEGylation or HSA gene fusion of glucarpidase produces forms of the enzyme with a much longer half-life and more resistant to proteases.11

6. Advantages of modified proteins over unmodified ones

In contrast to small-molecule drugs, proteins are readily amenable to site-specific alterations through genetic engineering. In principle, therefore, it is possible to build in features that allow them to remain active for longer in the body and or to improve their tolerance. These features include: resistance to proteolysis; delayed clearance; reduced capacity to cause local irritation; increased half-life; lower toxicity; increased stability and solubility, and decreased immunogenicity.12, 13

Many of protein therapeutic drugs have now been developed and approved. Many exhibit short half-lives in plasma and hence strategies to improve their pharmacokinetic properties, which influence distribution and excretion,13 are becoming increasingly important. Increasing the size and hydrodynamic radius of the protein, or peptide aims to decrease kidney filtration and to increase the net negative charge of the target protein or peptide has a similar effect, as the net charge of the protein contributes to renal filtration. It has been suggested that the proteoglycans of the endothelial cells and the glomerular basement membrane contribute to an anionic barrier, which partially prevents the passage of negatively-charged plasma macromolecules.14

Another approach is to increase the degree to which the therapeutic peptide or protein interacts with serum components, e.g. albumin or immunoglobulins, which tends to increase the half-life of the circulating targeted protein.15, 16 Both serum albumin and
immunoglobulins (particularly IgG1, IgG2 and IgG4) have extraordinarily long half-lives – around 19 days - in humans. Use of neonatal Fc receptor is another approach that can be used to promote interactions with albumin or with the Fc region of IgG in a pH-dependent manner. FcRn binding can protect albumin and IgG from degradation in the lysosomal compartment and redirects them to the plasma membrane. Thus, such binding can extend or modulate the half-life of the protein that is attached to it.

7. Strategies for producing long-acting protein therapeutics

Significant effort has been expended to discover different approaches to extend the half-lives of protein drugs, not least by evading or interfering with their common clearance pathway. Modifications to protein drugs that prolong their half-lives include conjugation or fusion to specific moieties and the discovery of variants of the therapeutic protein drugs.

Figure 1: Different strategies to extend the half-life of therapeutic proteins.
These strategies also include chemical coupling of polymers and carbohydrates, posttranslational modifications such as N-glycosylation, and fusion to recombinant polymer mimetics. On the other hand, changes in structure or sequence of protein molecules (e.g. through glycosylation or PEGylation) may cause changes in the pharmacokinetic properties of these compounds. The size of a therapeutic protein may hinder its passage across a biological membrane. Other factors that affect its half-life include its immunogenicity, the level of the corresponding endogenous protein, the period of drug administration, and the rate and site of drug delivery.

Gene modification can be used to create therapeutic proteins with altered isoelectric points and protein dynamics. Such mutations can also modulate both enzyme selectivity and the intrinsic activity of the enzyme. In one example, both the activity and the specificity of Neprilysin, a protease that degrades amyloid beta and hence might be of use in the treatment of Alzheimer’s disease, were altered through site-specific mutagenesis. The engineered Neprilysin double mutant G399V/G714K showed a ~20-fold increase in activity on amyloid beta 1–40 but a ~3,200-fold reduction in activity on other peptides. Further, this therapeutic drug is therefore, a promising candidate for the in vivo treatment of Alzheimer's disease.

One strategy which is different from the above is to isolate a similar enzyme to the one under study which will not be recognized by the antibody of the original protein. This approach will lead to prolonging an enzyme’s activity. For example, a novel variant of Carboxypeptidase G2 (CPG2), which has been used in drug detoxification and ADEPT is used in targeted therapy for cancer, especially in the ADEPT strategy mentioned above. A leading approach to improving the half-life of a protein therapeutic is to reduce its
renal clearance rate, e.g. by increasing its size above the renal cut-off of 40–50 kDa. Several ways can achieve this, including chemical and post-translational modification as well as by genetic engineering. Table 1 lists different modifications that can create favorable new features in therapeutic proteins. Two of the wildly used approaches to extend the half-life of therapeutics and improve drug delivery, are PEGylation and albumination, this review will focus on the use of the two techniques and discuss their application in cancer therapy. This part will include our recent work on the glucarpidase PEGylation and albumination.

8. Protein PEGylation using polyethylene glycol (PEG)

Polyethylene glycol (PEG) is a neutral polyether polymer. Because it is water soluble, nonionic and biocompatible, it is widely employed in the field of polymer-based drug delivery. PEG moieties are made from multiple units of ethylene oxide that create long chains of amphiphilic inert molecules. In 1990 the FDA approved the first PEGylated product, and ever since it has been extensively used in post-production modification methodology to improve the physicochemical properties, and hence the biomedical efficacy, of therapeutic proteins. PEGylated pharmaceuticals have proven their applicability and safety over many years. Thus, PEGylation plays an essential role in prolonging the residence time in the circulation of the relatively small therapeutic drugs such as peptides, proteins, nanobodies and scaffolds, which is achieved by increasing their molecular size to above that needed for half-life extension. As indicated above, a key advantage of using PEGylated proteins is that patients require fewer doses to maintain the necessary therapeutic levels in the circulation.
More recently, releasable PEG moieties have been developed that can be removed from a therapeutic protein under controlled conditions. This strategy allows administration of the protein in a pro-drug format prior to reconstitution of the native protein under appropriate conditions. A wide range of biologically important molecules have been conjugated to PEG to take advantage of its advantages (Table 1). Moreover, site-specific PEGylation offers opportunities to create novel proteins and peptides of medicinal interest.

It is essential to add a functional group to the PEG at one or both termini which will enable its conjugation to a protein. By choosing the functional group judiciously, it is possible to attach PEG moieties to specific amino acid side chains or to the N-terminus of a protein (Figure 2).

Table 1: Strategies to modify the half-lives of therapeutic products

| Strategy       | Target                  | Examples                                      | Effect                                               | Treatment                           |
|----------------|-------------------------|-----------------------------------------------|------------------------------------------------------|-------------------------------------|
| PEGylation     | Small molecule          | Metal Nanoparticle Surfaces                   | Reduction in Nonspecific Cell Uptake                | brain glioma cancer cells          |
|                |                         |                                               |                                                      |                                     |
| Affinity ligands | protein A              |                                               | Improved selectivity in affinity chromatography     | Staphylococcus aureus Disease      |
| Peptide        | glycosaminoglycan (GAG)-binding enhanced transduction (GET) | Improved safety profile and efficient gene transfer of a reporter luciferase plasmid, enhanced gene expression, and enhanced transfection efficiency | Lung gene therapy                   |
| Protein | Avoidance of the immune system and increased the half-life. | Targeted Cancer Treatment\textsuperscript{11} |
|---------|-----------------------------------------------------------|-----------------------------------------------|
| Carboxypeptidase G2 (glucarpidase) | interferon β-1a | Increased half-life and hence decreased frequency of administration. | multiple sclerosis \textsuperscript{28} |
| Saccharides | radix ophiopogonis polysaccharide (ROP) | Suppression of elimination from plasma | myocardial ischemia\textsuperscript{29} |
| Oligonucleotides | interleukin-17A (IL-17A) | Better stability in blood circulation. | systemic inflammatory disease\textsuperscript{30} |
| Lipids | Synthetic highdensity lipoprotein nanoparticles (sHDLs) | Increased half-life | Targeted Cancer Treatment\textsuperscript{31} |
| Liposomes and PARTICLES | DC-Chol/DOPE cationic liposomes | Enhanced silencing of the target gene at tumor sites and substantial suppression of tumor growth. | ovarian cancer therapy\textsuperscript{32} |
| Polysialylation | protein | Erythropoietin | Significantly prolonged circulating half-life, improved stability against proteases and thermal stress, reduced clearance, and enhanced in vivo efficacy | anemia\textsuperscript{33} |
| Fc fusion | Protein | Thymosin alpha 1 (Tα1) | Increased half-life and stronger activity. | Melanoma and Breast Cancer\textsuperscript{34} |
| | recombinant human growth hormone (rhGH) | Increased half-life, and less dosage | human growth hormone |
|                     |                  |                                                                 |                                                                 |
|---------------------|------------------|------------------------------------------------------------------|------------------------------------------------------------------|
| **Engineered Fc**   | protein           | proprotein convertase subtilisin kexin type 9 (PCSK9)            | Increased serum half-life and enhanced efficacy in vivo, enabling less frequent or lower dosing into the blood. |
|                     |                   |                                                                  | lower plasma LDL levels                                           |
| **IgG binding**     | protein           | interferon-α                                                      | Increased serum half-life with significantly                      |
|                     |                   |                                                                  | viral infections                                                 |
|                     |                   |                                                                  | improved bioavailability                                         |
| **Albumin fusion**  | protein           | factor VIII and factor IX                                         | Increased half-life replacement therapy in hemophilia A and B     |
| **Albumin binding** | protein           | Triclocarban (TCC)                                               | Transport, and distribution                                       |
|                     | Peptide           | [177Lu]LuDOTATATE peptide receptor                               | Enhanced residence time in blood.                                |
|                     | saccharides       | Ganoderma lucidum polysaccharides (GLP)                          | Increased tumor uptake, and a higher kidney uptake.              |
|                     |                   |                                                                  | Toxix effect of humans                                            |
|                     | saccharides       |                                                                  | Targets Tumers                                                   |
| **Nanoparticles**   | protein           | recombinant tissue plasminogen activator (rtPA)                  | Increased thermal stability                                       |
|                     | Small molecule drugs | paclitaxel (PTX)                 | targeted therapy and increased half-life                           |
|                     |                   |                                                                  | osteosarcoma targeted therapy                                    |

| **Figure 2:** | Modification of the protein. A) Using PEG derivatives carrying appropriate functional groups, it is possible to target specific sites / amino acid residues within a protein. Alternatively, the PEG moiety can be attached via an enzyme-mediated |
reaction.\textsuperscript{48}  

B) The three main modifications to the serum albumin protein used to improve drug delivery (modified as mentioned elsewhere).\textsuperscript{49}

PEGylation of proteins can be performed by chemically reacting a specific chemical functionalities within a protein (e.g. the side chains of lysine, histidine, arginine, cysteine, aspartic acid, glutamic acid, threonine, tyrosine, and serine as well as the N-terminal amino and the C-terminal carboxylic acid groups) with a suitable PEGylation reagent.\textsuperscript{16} As the degree of modification increases, the likelihood of antigenicity generally decreases whereas the circulatory half-life of the therapeutic protein is extended. Due to reactions with different nucleophilic groups on the protein, even mono-PEGylation leads to positional isomers that can differ significantly in their biological and biomedical properties mainly in body residence time and immunogenicity. However, it should be noted that conjugation might sometimes lead to the formation of new epitopes as a consequence of, e.g., partial protein denaturation after conjugation or the use of an inappropriate spacer between protein and PEG chain.\textsuperscript{45}
PEG derivatives are often attached to the amino groups of lysine and the N-terminus of polypeptide molecules. PEG derivatives are suitable for amine modifications includes N-hydroxysuccinimidyl-activated esters, which produce an amide linkage between PEGepoxide, and PEG-aldehyde, PEG-tresylate and PEG-carbonyl imidazole, which will provide a urethane linkage. The activated PEG compound will react with one or all exposed free amino groups contained within the protein groups, with regards to steric hindrance. By regulating the concentration of the reagents whether through the protein, or reaction conditions, in reference to the standard methods of amine condensation, one can control the degree of PEGylation of the free amino groups exposed on the folded protein.

Another option is to use the thiol groups of cysteine residues, which can be modified by use of PEG-maleimide and vinyl sulfone. However, changes in PEGylation interactions or reaction conditions can result in changes in the functional properties of the therapeutic proteins. 50-52

A study was conducted to optimize site-specific PEGylation of Exendin-4 (Ex4-Cys), an analogue of glucagon-like peptide-1 (GLP-1) with anti-diabetic properties, using a highmolecular-weight trimeric PEG. PEGylation of the C-terminus (C40-tPEG-Ex4-Cys) was carried out using Ex4-Cys and activated trimeric PEG. The resulting C40-tPEG50K-Ex4Cys derivative had a better t1/2 in circulating blood (7.53-fold increase) and its AUC_{inf} (a measure of total exposure to the drug) relative to Ex4-Cys was increased over 45-fold. Further, its hypoglycemic duration, a measure of its pharmacologic activity, was increased 8-fold relative to that of native Ex4-Cys, with a dose of 25 nM/kg.52
9. Fusion to Human Serum Albumin

Human serum albumin (HSA) is one of the best-characterized proteins in the pharmaceutical field. It is responsible for transporting endogenous and exogenous compounds and has a long average half-life (around 19 days). In part, this is due to its size – it is around 66 kDa, which is almost at the boundary of the kidney’s filtration capacity – and also the fact that it is the most abundant protein in plasma. It tends to accumulate around tumors and inflamed tissues sites, and this feature opens the potential of fusing albumin to a target protein to aid targeting to the therapeutic site of interest. It is widely used as an excipient, especially for biotechnology products. Recombinant versions of the protein are available, which alleviate any potential concerns about the transmission of infectious agents associated with the human plasma-derived protein.

Many researchers have developed methods to improve novel albumin-based drug carriers and these can generally be categorized into three main categories: (1) low-molecularweight proteins fused with albumin; (2) polymerization; (3) surface modification (Figure 2). I has recently emerged as an adaptable carrier for drug delivery to transport therapeutic peptides and proteins against diabetes, cancer, and infectious diseases.

Therapeutic compounds have been pharmaceutically enhanced by multiple techniques using albumin to improve their distribution, bioavailability and the half-life. For example, non-covalent interactions allow the binding of the albumin to a broad range of endogenous and exogenous ligands. Albumin dimerization in particular has significant potential and advantages for clinical applications, as both a plasma expander and as a
drug carrier. Such dimers are present at elevated levels in the circulating blood of patients with chronic renal disease and also result from oxidative damage in the blood. Many molecules of therapeutic interest bind to endogenous albumin in the blood through its fatty acid binding sites, thereby prolonging their half-life and bioavailability. For example, the human insulin analogue, Detemir (marketed by Novo Nordisk as Levemir), is longacting due to the myristic acid moiety bound to the Lys residue at position B29 of insulin. The attached fatty acid facilitates binding to albumin thereby prolonging the circulatory half-life of this insulin derivative in blood.

Covalent binding of a drug to albumin can be achieved either through direct chemical conjugation or via the use of a small molecule to link the two components. Alternatively, it can be achieved through gene fusion to create a chimeric protein that is expressed in a suitable host, resulting in the production of a single polypeptide. The gene fusion approach has been used to attach albumin to the N- and or C-termini of several proteins of therapeutic interest, to extend their half-life. Examples of therapeutic proteins that have been attached to HSA include interferons, growth factors, hormones, cytokines, coagulation factors, and antibody fragments.

Various domains of the HSA molecule have also been used to make bioconjugates with increased stability, better targeting properties, and/or extended half-lives in blood. For example, domain I of HSA has been used in the preparation of antibody conjugates. This was achieved through the use of a cyclohexene sulfonamide compound that siteselectively labels Lys64 in this HSA domain. Similarly, the half-life the of granulocyte colony stimulating factor (G-CSF) was prolonged by genetic fusion to domain III of I to its N-terminus.
10. Diseases that have been treated with PEGylated proteins

Several PEGylated molecules have been approved for clinical use. For example, PEGylated interferon for such infections, PEG-interferon alfa-2b, was approved by the FDA in August 2001. Table 2 lists some PEGylated products that have received FDA approval.8

Table 2: FDA approved PEGylated and albuminated protein therapeutics 8, 53, 54

| Trade name | Conjugate            | FDA Approval date | FDA approved date/clinical trial status and use                                      |
|------------|----------------------|------------------|--------------------------------------------------------------------------------------|
| Sylatron™  | PEG-interferon α-2b  | March 9, 2011    | Approved as adjuvant therapy for resected stage III melanoma                          |
| Pegasys®   | PEG-interferon α-2a  | October 2002     | Phase I for melanoma and phase II as for chronic myelogenous leukemia                 |
| Neulasta®  | PEG-filgrastim        | January 31, 2002  | Used to treat neutropenia during chemotherapy                                         |
| Oncaspar® | PEG-asparaginase      | July 24, 2006     | February 1994, acute lymphoblastic leukemia, and on July 24, 2006 the first-line treatment for acute lymphoblastic leukemia |
10.1. PEGylation to improve drug delivery and targeting of cancer cells

The number of therapeutics involving drug delivery has increased markedly, especially for cancer treatment (Table 3). While most of the PEGylated products to date are nonprotein-based, the use of peptide- and protein-based PEGylated products is now being investigated. In principle, PEGylation of proteins, due to its enhanced permeability and retention (EPR) effect, is an excellent way to achieve a longer circulation time and for drug delivery to a tumor site.
For example, a succinimide-activated PEG derivative has been used to PEGylate the ε-amino groups of lysine residues of xanthine oxidase, which mediates anticancer activity because of its ability to generate cytotoxic reactive oxygen species. In animal studies, this derivative exhibited 2.8-fold higher tumor accumulation at solid tumors when compared to the native enzyme in a 24 hr injection period.65

Bispecific antibodies have been studied as a method in cancer immunotherapy, and the use of PEGylation is an effective method to improve their antitumor efficiency. Sitespecific PEGylation has been used to modify a bispecific single-domain antibody-linked Fab (S-Fab), which was designed to link an anti-carcinoembryonic antigen (anti-CEA) nanobody with an anti-CD3 Fab. The resulting construct, polyethylene glycol-S-Fab (PEGylated S-Fab), had slightly decreased tumor cell cytotoxicity in vitro when compared to the free S-Fab, but an increased half-life (t1/2) - 12-fold – resulting in effective inhibition of tumor growth in vivo.68

PEGylation can be combined with other strategies to improve drug delivery. For example, it has been used in conjunction with niosomes, i.e. non-ionic surfactant-based vesicles that can carry various drugs within them, to improve cell targeting. Niosomes are first rendered magnetic with Fe₃O₄@SiO₂ nanoparticles prior to modifying their surface by PEGylation. In this case, the role of PEGylation increases the bioavailability of niosomes, and magnetization makes them capable of targeting specific tissues. In one application, carboplatin, an antitumor drug, was loaded into PEGylated magnetic niosomes, leading to an increased drug release rate (Figure 3). Moreover, using an external magnetic field significantly increases their toxicity towards cancerous cells.69
In addition to the use of drug encapsulation using a vesicular carrier, drugs can be delivered to a tumor site by attaching them to a drug delivery module via acid-cleavable linkers, which can be hydrolyzed in the acidic environment of the tumor. Alternatively, some other type of specialized linkage can be used that permits the drug to be released in situ within the tumor microenvironment. Thus, both pro-drug and active targeting strategies can be used. To minimize the loss of activity reversible PEGylation has been developed and a large number of cleavable linkages, mediated in vivo by specific enzymes, hydrolytic cleavage or reduction, have been identified.

The use of pH sensitive cleavable PEG has proved to be an effective approach in which cleavage of a PEG-lipid moiety is triggered in the vicinity of the tumor. In order to achieve a tumor-specific cleavable PEG system, the enzymes specifically expressed in the tumor have also been exploited for cleavage, e.g. matrix metalloproteinases (MMP). Another comparable example in facilitating drug delivery to tumor cells is the peptide-loaded pHsensitive PEGylation to liposomes (PEG-PpHL) which are characterized and delivered.
to cis-platinum resistant ovarian cancer C13 cells. The carrier entraps the drug and exhibits a pH-dependent release in the tumor site. Moreover, the PEGylated PpHL behaved differently against macrophage cells due to its ability to protect liposomes from the cells of the reticuloendothelial system.\textsuperscript{72}

\section*{11. Diseases which have been treated with proteins linked to HAS}

A number of therapeutic products conjugated to HSA have now been approved for clinical use (Table 2). For example, fatty acid derivatives of human insulin bound to HSA have applications in the treatment of diabetes while paclitaxel-HSA nanoparticles have been used to treat various cancers such as metastatic breast cancer and advanced pancreatic cancer. It has even proved possible to use HSA to deliver a bioactive gas, nitric oxide (NO), to treat ischemic/reperfusion injury, cancer, and bacterial infections. While endogenous S-nitrosated HSA occurs naturally in blood plasma and serves as a NO donor, analogues have been developed in which the HSA molecule has many conjugated SNO groups (polySNO-HSA). Interestingly, while SNO-HSA inhibits apoptosis, poly-SNO-HSA possesses very strong pro-apoptotic effects against tumor cells.\textsuperscript{73}

Albiglutide, a glucagon-like peptide-1 agonist (GLP-1 agonist) for the treatment of type 2 diabetes (marketed as Tanzeum and as Eperzan and the US and Europe, respectively), was one of the first HSA-peptide or protein fusion product to be approved for clinical use. Whereas the half-life of pharmacologically active native GLP-1 is 1–2 min, albiglutide's half-life is 4–7 days, which allows it to be administered weekly rather than more frequently.\textsuperscript{74} IL-2, which is used in passive cancer immunotherapy, is another successful example. One of the IL-2 limitations is its low serum half-life, which necessitates high
doses that have severe side-effects. To overcome these issues, Adabi et al. therefore fused IL-2 to an albumin-binding domain from streptococcal protein G. The resulting fusion protein, ABD-rIL-2, binds to serum albumin, and had a three-fold increase in its terminal half-life in serum relative to recombinant IL-2, when tested in BALB/c mice.75

TV-1106 is a recombinant form of human growth hormone (rhGH) that has been genetically fused to recombinant HSA. Again, this fusion resulted in a long plasma half-life for rhGH in the systemic circulation. In the case of GH deficiency, TV-1106 has been developed for the treatment of this disease. This modified drug provides sustained exposure which will lead to the reduction of the frequency of injection and therefore will improve patient’s compliance and quality of life. A phase 1 clinical trial demonstrated that the TV-1106 is well tolerated, has a prolonged plasma half-life, and is hormonally active in GH-deficient adult patients. The side effects of GH therapy were reported to be rare and it was shown to have a favorable overall safety profile.76

11.1 HSA fusion to improve drug delivery and targeting of cancer cells

Fusion of therapeutic proteins to HSA has proved to be a viable and effective way to increase the solubility and/or delivery of molecules for cancer therapy.77 The physicochemical properties of HSA, which facilitate coupling to drugs, and its preferential uptake in tumor tissue make it an almost ideal carrier for drug delivery.78

In pancreatic cancer chemotherapy the gemcitabine (GEM) nanocarriers have received extensive attention in recent years. Linking HSA to GEM/IR780 resulted in a complex
that had elevated levels in blood and a long-term circulation in tumor tissues when compared to the free IR780. 79

Fusion with HSA can also be used to target and inhibit essential intracellular pathways. It has been recently reported that a fusion protein consisting of HSA linked to p53-reactivating peptide (p53i) interferes with at least four intracellular targets, making it a viable therapeutic protein for the treatment of a variety of cancers. It retains the ability to bind to MDM2 and MDMX, resulting in p53 transcription-dependent apoptosis, and additionally, is able to bind and neutralize anti-apoptotic Bcl-2 family proteins, Bcl-xL and Mcl-1.77

HSA has evolved to bind many natural ligands and this propensity can be exploited to allow it to carry anti-cancer agents. For example, it readily binds the chemotherapeutic entity CuII nalidixic acid–DACH. It should be noted, however, that binding in this case results in significant shape-changes in HSA as evidenced by UV–vis, fluorescence, CD, FTIR spectroscopy. It remains to be seen if such changes result in the production of new epitopes in HSA. If they do then such conjugates may provoke adverse immune reactions following repeated administration. 80

12. Immune responses of patients towards the modified drugs

12.1 Effects of the PEG moiety on protein immunogenicity and stability

Conjugation of PEG to a protein inevitably results in a new macromolecule with significantly changed physicochemical characteristics. These changes are typically
reflected by reduced immunogenicity. The PEG ‘tail’ is quite flexible and can shield a protein from recognition by the immune system. Additionally, it can reduce the chance of reticuloendothelial clearance, sterically hinder binding to cellular receptors, and reduce the degradation by proteolytic enzymes. These properties collectively lead to decreased renal, enzymatic, and cellular clearance, resulting in prolonged circulation half-lives in the bloodstream. In the case of antibodies, although the PEG moiety is chemically linked to a position as far as possible from the antigen binding site, it is still possible that the flexible polymer sterically blocks the binding interface via interactions that change the plasticity or surface charge distribution of the molecule. Similar principles apply once a PEGylated antibody molecule binds to its antigen on a surface - the polymer tail acts intermolecularly to hinder binding of antibodies to adjacent antigen molecules.

It was demonstrated that very high doses of PEG-protein conjugates might induce renal tubular vacuolization. However, this phenomenon is not associated with functional abnormalities and disappears after the treatment has been completed. Therefore, PEGprotein conjugates are regarded as immunologically safe and non-toxic.

In one study the effect of PEGylation on the antibodies was monitored via competitive ELISA. In this method, the modified or unmodified antibodies were mixed with HRP-attached polyclonal antibodies, specific to human serum IgG (hsIgG) or to IgY, and incubated in ELISA plate coated with the unmodified hsIgG or IgY. The concentration of the free unmodified antibodies competing with the modified antibody lowered the resulting OD to a value of 0.4, which is considered to reflect 100% detection by secondary antibody. The percentage of the reduction of detection by secondary antibody was calculated using the ratio of modified/unmodified free antibody concentration needed to
lower the OD value to 0.4. Conjugation with PEG5 and PEG20 reduced the detection of hsIgG to 12.5% and 3.1% of unmodified hsIgG, respectively, and that of IgY to 2% and 1.6%, of unmodified IgY respectively. This implies that in vitro the PEG molecules mask some of the exposed epitopes on the hsIgG and IgY, and possibly sterically prevent detection and binding of the antibodies to the relevant epitope on the PEGylated protein. However, this is not the case in vivo. Unexpectedly, PEGylation of IgY was found to elevate the immune response via both administration routes (i.v. and i.m.) investigated. PEGylation of hsIgG with PEG5 did not reduce the primary or secondary immune response following administration in BALB/c mice. Interestingly, PEGylation with PEG20 significantly increased the antibody titer when administrated i.m., and significantly decreased it when administrated i.v. The immune response to hsIgG carrying PEG5 was also tested in C57BL/6 mice. In this strain, in contrast to BALB/c, PEG induced an elevation in antibody response.

There are various factors that can influence the properties of PEG such as the number of PEG chains attached to the polypeptide, the structure of PEG chains attached to the polypeptide, the location of the PEG sites on the polypeptide and the chemistry used to attach the PEG to the polypeptide.

PEGylation is considered as one of the best approaches for passive targeting of anticancer therapeutics, based upon the concentration gradient between the intracellular and extracellular space that is created due to the high concentration of the drug in the tumor area.

The clearance of the PEG chain depends on its seizes. The molecule less than 400 Da would be degraded by alcohol dehydrogenases and lead to the formation of toxic
metabolites. The elimination mechanism of longer PEG chains, depends on their molecular mass. PEG below 20 kDa, are eliminated by renal filtration. The PEGylated proteins conjugated with PEG molecule larger than 20kDa are cleared by different pathways such as liver uptake and degradation of the protein part by proteases. It is also the same mechanism for clearing of large protein molecules with molecular masses above 70 kDa.84

Generally the elimination half-life and the absorption of the PEG-protein is directly proportional with the PEG chain. It was shown that branched PEGs have longer elimination half-life than linear PEGs of the same molecular weight.45 However, the stabilizing effect of PEGs on proteins is a delicate balance between the two opposing effects: stabilizing effect due to steric exclusion and destabilizing effect due to hydrophobic interaction.87

It has been established that despite all advantages of the protein PEGylation as drug life extender the patient immune system produces antibodies against the PEG moiety (antiPEG Abs), including both pre-existing and treatment-induced Abs.88 This unfortunately has been correlated with loss of therapeutic efficacy and an increase in adverse effects in several clinical reports examining different PEGylated therapeutics.88

The reason(s) for the presence of pre-existing antibodies specific for PEG in individuals who have never received any formal treatment with PEGylated therapeutics remains largely unknown. However, as a ‘generally regarded as safe’ (GRAS) product, PEG is widely used in cosmetics, processed foods, pharmaceuticals, agriculture, and industrial manufacturing. Because PEG is found in so many domestic and hygiene products, it is reasonable to assume that repeated exposure to PEG could lead to the development of
anti-PEG Abs. However, the constant exposure does not clearly clarify the real mechanism underlying anti-PEG immunity. Due to the abundant presence of PEG, it is very likely to be present at or introduced to the inflammation site. The occurrence of PEG in close vicinity to highly active immune cells may be enough to elicit the stimulation of anti-PEG Abs. Successive exposure to PEG-containing products could prompt a robust memory immune response to the polymer.89

The presence of both pre-existing and induced anti-PEG Abs is a significant challenge to the clinical efficacy of PEGylated therapeutics.90 There is now a large body of evidence indicating that potent and specific antibody reactions against the PEG polymer, which reduce the circulatory half-life circulation and lower the therapeutic efficacy of the PEGylated drugs in both animal models and humans. Addressing this challenge should be a priority in future studies in this area 89

PEGloticase, a PEGylated form of recombinant porcine uricase, is approved for the treatment of refractory gout. In a phase one study, 13 of 30 patients (43%) produced antibodies against PEGloticase that were specific to the PEG component rather than the uricase moiety. Such antibodies caused rapid elimination of the PEGloticase from plasma, which in turn resulted in a loss of efficacy and a doubled risk of infusion reactions. The anti-PEG antibodies appeared after the first dose, but 5 of the 10 responders had preexisting antibodies, even though they had not previously been exposed to PEGloticase. In phase 3 trials, high levels of antibodies to PEGloticase was the main reason for the loss of efficacy. 91

As indicated above, the enzyme-linked immunosorbent assays (ELISA) technique is an efficient method to analyze anti-PEG antibody responses. Direct and competitive ELISAs
can be used in combination to determine the PEG-specificity of Ab responses induced after treatment with a PEGylated protein (PEG-Pr), as well as pre-existing anti-PEG Abs. Both anti-PEG IgM and IgG can bind to polymers composed of repeated subunits.89

PEG-modified recombinant mammalian urate oxidase (PEG-uricase), a treatment for patients with chronic gout, was investigated for the presence of anti-PEG antibodies. In 5 of 13 patients, low-titer IgM and IgG antibodies against PEG-uricase were detected. This correlated with the plasma uricase activity being not detectable beyond ten days after injection. As in the other study 106, the elicited antibodies were directed against the PEG moiety rather than the uricase protein. Conversely, the relatively low titer antibodies did not inhibit uricase catalytic activity. Instead, the uricase activity was decreased due to rapid clearance of the circulating uricase. This is due to the binding of the antibody against the unabsorbed PEG-uricase at the injection site after dosing. (Figure 4) 92
Due to the immunogenicity problem towards the PEG it is essential to consider effective therapeutic options for patients exhibiting anti-PEG antibodies. One possibility is the infusion of a compound that can block or suppress anti-PEG before the administration of a PEG-conjugated drug. In many clinical cases, where anti-PEG antibody response is induced, the clearance rate of the PEG-conjugated drug should be carefully monitored.
and doses adjusted to compensate. Alternatively, it may be possible to replace the PEGconjugate with a non-PEGylated version of the therapeutic agent.93

12.2 Effects of the HSA moiety on protein immunogenicity and stability

HSA is the only therapeutic protein that is stable as a liquid at room temperature. This stability is primarily due to the presence of 17 disulfide linkages present in the molecule. The stability of albumin makes its storage and handling easier than typical proteins, thus making itself well suited as an excipient. The high stability of the protein also allows it to be heated at a temperature of 60°C for 10 hours, without significant denaturation, which facilitates virus inactivation during manufacturing. HSA is used as a stabilizer for proteins due to its amphiphilic properties, which makes it appropriate as an additive to prevent adsorption of the active protein, via the competitive adsorption mechanisms. HSA may also stabilize the native conformation of the active molecule, thereby helping it to maintain its bioactivity throughout the product shelf life.94

While HSA is largely non-immunogenic, the same cannot be said of proteins that bind to HSA. For example, the albumin binding domain of streptococcal protein G is of concern due to its bacterial origin. Accordingly, it has been engineered to reduce its immunogenicity by removing the T-cell epitopes. Based on the existing literature and use of in silico programs for predicting T-cell epitopes, several derivatives have been produced and one (ABD094) is currently being clinically evaluated.95 Besides, its long serum half-life, HSA has been found to accumulate in many tumors as a result of their enhanced vascular permeability and the increased retention of albumin in tumor interstitium.96, 97 These findings have been validated by radiolabeled or
dyeconjugated albumins, which have been shown to have high uptake into tumors.\textsuperscript{98} Based on this property, HSA is considered to be a suitable system for drug delivery to tumor tissue.\textsuperscript{99, 100} By implication, it is assumed that fusion proteins bearing albumin binding domains will also accumulate inside tumors following their association with HSA. In the case of constructs such as ABD-rIL-2, this induces the recruitment of cytotoxic T cells to tumor sites.\textsuperscript{75}

Studies indicate that the position of the fusion has an essential influence on the subsequent activity of the therapeutic protein. Human brain natriuretic peptide (BNP), which is used in the treatment of acute decompensated congestive heart failure, illustrates this point. Four fusion proteins, BNP-HSA, (BNP)\textsubscript{2}-HSA, (BNP)\textsubscript{4}-HSA, and HSA-(BNP)\textsubscript{2}, were constructed, with different numbers of BNP molecules and fusion orientations. Fusion with HSA successfully prolonged the bioactivity of BNP, stimulating intracellular cGMP expression over 24 h and activating human natriuretic peptide receptor A (NPRA). The HSA-(BNP)\textsubscript{2}, with two BNP molecules fused in tandem at the C-terminus of HSA, had the highest and most prolonged BNP bioactivity in activating human NPR-A.\textsuperscript{101} In contrast, the other three fusion proteins only slightly increased the activity of NPR-A. Currently, there is no way of predicting which fusion structure will be most effective – it is necessary to use trial and error to test different constructs and determine which is best.

Similarly, although serum albumin has a half-life in humans of about 19 days, the halflives of therapeutic proteins fused with HSA is generally much lower. For example, the
fusion protein proalbiglutide, a drug marketed for diabetes 2, only has a circulatory half-life of about 5 days. Other proteins tested such as CTP, ELP, or XTEN, their fusion proteins have not shown any better half-life than 2.5, 4–5, and 4–5 days, respectively.74

13. Advantages of PEGylation and HSA fused drug

Rival strategies often have complementary advantages and disadvantages; PEGylation and linkage to HSA are no exception.

PEGylation creates relatively simple changes in a protein’s structure. However, such changes can have significant effects on functions such as signaling, targeting, catalysis, and catabolism, circulation time in the blood, the degree of immunogenicity/antigenicity, body-residence time and stability. In part these effects are due to the fact that PEG shields the protein surface from degradative agents and from the immune system. Additionally, the increased hydrodynamic radius that results from PEGylation usually decreases the efficiency of kidney clearance of the protein in question.16

The widespread acceptance of PEG conjugates can be attributed to the exceptionally favorable combination of physicochemical and biological properties of the polymer. This includes its solubility in aqueous and most organic solvents, biocompatibility, lack of toxicity and (usually) low immunogenicity.67 The favorable properties of PEG also result in peptide and protein conjugates that are soluble and active in organic solvents and that have reduced levels of absorption to surfaces. This last property is particularly useful in the case of PEGylated liposomes and niosomes, greatly increasing their utility for drug delivery.47
The FDA approval of several PEGylated therapeutic proteins highlights their advantages. Some of the most important advantages are their prolonged body-residence time, which allows a drug to be administered less frequently, which arises from their increased resistance to degradative agents such as proteases or nucleases, and decreases in immunogenicity. Given these advantages, it is perhaps unsurprisingly that PEGylation has allowed the creation of blockbuster products such as Pegasys (peginterferon alfa-2a) and PEG-Intron (PEG-Intron (Peginterferon alfa-2b).16

The unusually long circulation time of HSA (19 days) has similarly encouraged researchers to use it to prolong the serum half-lives of other proteins either through genetic fusion or by chemical conjugation.102. It has been known for some time that HSA’s longevity in serum is due in large part to its electrostatic repulsion in kidneys and to FcRn mediated recycling in the endothelium.14-16, 103 However, it was initially unclear if fusion with HSA would increase the longevity of other proteins attached to it or if this would simply result in a decrease in HSA longevity. Fortunately, subsequent investigations proved these concerns to be largely unfounded.

In contrast to PEGylated proteins which tend to have reduced absorption in the body relative to their native counterparts, proteins conjugated to albumin tend to accumulate in certain locales in vivo. This means that albumin-based drug carrier systems have particular applications in the field of chemotherapy as they can improve the passive tumor targeting properties of anti-cancer drugs. Proliferating tumor cells utilize albumin and other plasma proteins for their nutrition and take up albumin by fluid phase endocytosis at a greater rate than normal tissues. After lysosomal digestion, the derived amino acids serve as a source for nitrogen and energy in the tumor cells. These favorable properties make albumin an attractive choice as a drug carrier where the conjugates enjoy the same favorable tumor targeting properties as albumin itself, e.g. high tumor uptake rates, low liver uptake rates and a very long biologic half-life.
Both approaches have the ability to conjugate to proteins without comprising the critical property of the target protein. In mice, the serum half-life using the HSA and the PEG was typically around 9- and 7-fold greater, respectively, than that of the sfGFP-WT. Although the binding affinity of HSA to a mouse is much greater than that of a human, it is still much greater than that of a PEG-conjugated protein in human. A disadvantage in both techniques is that the handling and chemical modification of HSA during modification can lead to slight denaturation which may generate a significant immune response.21

The hydrophobic moieties present on the polymers can bind to proteins through hydrophobic interactions (e.g., PEG with aromatic groups). Additionally, these polymers can also destabilize the native protein conformation by stabilizing unfolded protein conformations. Protein excipients, for example human serum albumin (HSA), stabilize biopharmaceuticals by competitively adsorbing to surfaces and interfaces and preventing interface induced aggregation of the drug product.55

The production of long-acting protein therapeutics using techniques such as PEGylation, and others to overcome the patient’s immunogenicity has been established and covered extensively in the literature. We successfully produced two forms of long-acting glucarpidase using PEGylation and HSA fusion with glucarpidase. The two forms produced are more resistant to proteases than the free enzyme (Figure 5). They also less immunogenic than the free glucarpidase (Figure 6).11
**Figure 5:** Stability of different CPG2 Forms after incubation in normal human serum. The samples were collected on 0, 10 and 15 days, which are 1, 2, and 3 respectively. Lane M, SeeBlue Plus2 Prestained ladder (198-10 kDa); a: serum only as control; b: XenCPG2; c: PEG-Xen-CPG2; and d: HSA-Xen-CPG2. The samples were analyzed by western blotting using the anti Xen-CPG2 antibody.

**Figure 6:** Immunogenicity testing using healthy Human PBMC proliferation assay. The positive control, Lipopolysaccharide (LPS) showed a significant increase when compared with all control groups with the vehicle only. The negative control, PEG Xen-CPG2 treated cells in all groups showed no significant increase in proliferation. The cells treated with HSA Xen-CPG2 gave a similar result except in two donors. P < 0.05 was considered statistically significant, ** P < 0.01, *** P < 0.0001.
In our recent work, we established a different and new strategy to overcome the immunogenicity problem. We showed that isolation of a novel form of the protein used with different epitops could also minimize the patient immune response. We showed that the antibody of one form of the enzyme does not neutralize the other form (Figure 7). We therefore proposed that the two forms of the proteins or the enzymes could be used consecutively instead of using one form of the enzyme.

Figure 7: Dot blot using anti his tag and anti Xen CPG2 antibodies. 1-3 concentrations 0.05, 0.1, and 0.2 mg/mL of pure Xen CPG2 and Ps CPG2

14. Nanonization and Drug development

We discussed different strategies to overcome the immunogenicity of drugs and many problems related to drug development and their clinical applications.

Another major obstacle to drug development is poor water solubility of many therapeutics. This poor water solubility affects the drug bioavailability which in turn reduce its efficiency. This presents another major pitfall to their clinical applications.

Many conventional formulations to improve solubility may lead to poor pharmacokinetics and low or loss of bioavailability. Drug PEGylation, in addition to many advantages, may increase solubility.
Nanonization techniques, however, offers an efficient way to overcome problems related to drug development.

Different methods of drug nanonization have been established.\textsuperscript{104-108} The use of nanonization to improved drug development and drug delivery and to overcome the solubility problem have been successful in several drugs.\textsuperscript{105, 109-117}

In summary, the use of techniques such as drug PEGylation and fusion with human serum albumin followed by drug nanonization could solve many problems related to drug development such as immunogenicity, proteases degradation and drug bioavailability.

15. Conclusions

Modification of therapeutic molecules by chemical conjugation with Human serum albumin (HSA), polyethylene glycol (PEG) or other known molecules has been established to enhance the drug pharmaceutical properties. This has been shown in the successful approval of more than 12 modified drugs by the FDA. Despite the fact that the added moiety improves the pharmacologic and pharmaceutical properties of the drug, most of the adverse effects of the modified drugs are due to the active part of the medicine and not to the added moiety. The use of external moieties, such as PEG and HSA, as delivery of the drug to the cancer cell is an added advantage of the process and will pave the way for more research in this area.
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