Protein-driven nanomedicines in oncotherapy

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Abstract

Proteins are organic macromolecules essential in life but exploited, mainly in recombinant versions, as drugs or vaccine components, among other uses in industry or biomedicine. In oncology, individual proteins or supramolecular complexes have been tailored as small molecular weight drug carriers for passive or active tumor cell-targeted delivery, through the *de novo* design of appropriate drug stabilizing vehicles, or by generating constructs with different extents of mimesis of natural cell-targeted entities, such as viruses. In most of these approaches, a convenient nanoscale size is achieved through the oligomeric organization of the protein component in the drug conjugate. Among the different taken strategies, highly cytotoxic proteins such as microbial or plant toxins have been conveniently engineered to self-assemble as self-delivered virus-like, nanometric structures, chemically homogeneous, that target metastatic cancer stem cells for destruction of metastasis in absence of any partner vehicle.

Keywords

Nanomedine; drug delivery; cancer; recombinant proteins; virus; viral mimetics

Introduction

Proteins are essential macromolecules in life that combine structural and biological functionalities including catalysis, signaling and precise interaction with targets. By recombinant DNA technologies or by chemical synthesis, full-length proteins or short peptides are produced and engineered to mimic natural versions or to generate novel activities. In this context, proteins are used in industrial catalysis, in research and development and as vaccines or drugs for clinical applications [1]. Most drug proteins are substitutes of endogenous enzymes or hormones absent in the patient's body. However, proteins are also explored as partners of conventional small molecular weight drugs and nucleic acids, to confer stability, nanoscale size and cell penetrability or specific targeting, of which conventional medicines are generically devoid. Nanoscale size of the vehicle-drug complex is desirable not only to improve the EPR effect and cell penetrability but also to minimize renal filtration, that shows a cut-off of 6-8 nm [2]. Cell-targeted drug delivery is specifically demanded in the case of cytotoxic agents (mainly used in oncology), that lacking selectivity cause severe side effects [3]. This leads to minimized doses to keep toxicity within acceptable levels, with the consequent reduction of the actual effectiveness. To reach nanoscale and targeting, drug designers exploit natural or bioinspired agents such as natural viruses or virus-like particles, *de novo* designed protein oligomers, engineered polypeptides and short peptides. Under these premises, the most explored protein-based drug delivery approaches in oncology are summarized in the following sections and schematized in Figure 1.

Protein and polyamino acid-based nanoparticles

Albumin-based nanoparticles use Nab coacervation technology to increase solubility, half-life, stability and safety of the payload drug, although without conferring any cell targeting in the delivery process [4]. Nab-paclitaxel (Abraxane®) is an albumin-stabilized nanoparticle of paclitaxel approved by the FDA (2005) and EMA (2013) for advanced breast cancer therapy [4]. Its combination with carboplatin or gemcitabine is used for non-small cell lung (NSCLC) [5] and

pancreatic [6] cancer therapy, respectively. Paclitaxel enters tumor cells and stabilizes microtubules, preventing their depolymerization and yielding antineoplastic activity through inhibition of cell motility, mitosis and replication. Despite the induction of neutropenia, fatigue and neuropathy, Abraxane™ increases tolerance compared to free paclitaxel, allowing its administration at higher dosage with greater efficacy [7]. Other albumin-stabilized nanoparticles are Nab-docetaxel and Nab-rapamycin. Nab-docetaxel incorporates the taxane microtubule inhibitor Docetaxel and is in preclinical testing [8]. Nab-rapamycin binds water-insoluble rapamycin to the albumin nanoparticle. Its antineoplastic activity is mediated by rapamycin-induced immunosuppressant and antiangiogenic effects after tumor and endothelial cell uptake [9]. Nab-Rapamycin is being tested in a phase II clinical trial in advanced malignant perivascular epithelioid cell tumors, and in Phase I/II for non-muscle invasive bladder cancer therapy.

Conjugation to polymeric nanoparticles is another strategy to increase solubility while reaching a nanometric size, minimizing renal clearance and expanding the drug half-life in blood. The polymer Paclitaxel-polyglutamate links the drug to this biodegradable, water-soluble polymer that allows the delivery of higher doses than paclitaxel alone [10]. Phase I/II clinical trials are ongoing for the treatment of NSCLC, glioblastoma, and ovarian and head and neck cancers. Pegaspargase (Oncaspar®) conjugates polyethylene glycol to the enzyme L-asparaginase to limit clearance by the mononuclear phagocytic system. L-Asparagine is critical for protein synthesis in leukemic cells, unlike normal cells [11]. Asparaginase hydrolyzes asparagine, thereby depleting cells of asparagine and blocking protein synthesis to induce tumor cell death. Oncospar® was approved by the FDA (2006) and EMA (2016) as a component of a multi-agent chemotherapeutic regimen for the treatment of patients with acute lymphoblastic leukemia.

Virus-based strategies

Nanoscale cancer drugs include genetically modified viruses, whose activity is based on three different therapeutic approaches, namely gene therapy, vaccination and oncolysis. Viral gene therapy utilizes replication incompetent viruses to transfer genetic material to cancer cells, either to restore tumor suppressor gene expression, to reduce the expression of activated oncogenes or to express suicide genes. Current strategies have been demonstrated to be safe in early clinical trials but mostly show low therapeutic effect due to due to the small number of cancer cells that are infected and to immune system blockade [12]. Only Gendicine® (rAd-p53) was approved in 2003 in China for systemic cancer (head and neck) therapy. Its combined administration with chemo and radiotherapy yields higher response rates than standard treatments [13].

Viral-based cancer vaccines are immunotherapeutics that use non-replicating viruses encoding tumor antigens, co-stimulatory molecules or cytokines [14]. Ongoing Phase III clinical trials are evaluating adenovirus Instiladrin® (rAD-IFN/Syn3) and vaccinia virus PROSTVAC®, for the treatment of bladder [15] or prostate [16] cancer, respectively. The clinical progress of virus-based vaccines has been hampered, however, by tumor immune evasion and heterogeneous immune responses among patients [17].

Oncolytic viruses (OVs) appear to solve virus-based gene therapy and cancer vaccines limitations since they are competent to replicate in tumor cells, but inactive in normal cells. Subsequently, they trigger cancer cell lysis and progeny virus spread that infects nearby cells. OVs show also an immunostimulating effect against cancer cells, by recruiting antigen-presenting cells or T cells to the tumor, reducing microenvironment-mediated immunosuppression and enhancing tumor

antigen presentation in cancer cells [18]. The efficacy and reduced toxicity of specific OVs in preclinical studies, prompted their testing in clinical trials.

In 2015, the FDA approved the first OV, a modified HSV named Imlygic® (T-VEC), for the treatment of melanoma patients [19]. Ongoing Phase III clinical trials are evaluating vaccinia virus Pexa-Vec® for liver cancer therapy, adenovirus CG0070 for bladder cancer, and reovirus Reolysin® for HNC and retrovirus Toca 511® for glioblastoma (www.clinicaltrials.gov). Additional approaches using different virus types are showing promising results in early clinical phases (www.clinicaltrials.gov; [20]. Despite their potential advantages, the therapeutic efficacy of OVs as single agents is hampered by their reduced systemic delivery, and limited spread or maintenance in the tumor microenvironment [18, 21]. Multimodality approaches combining OVs or virus-based vaccines with chemotherapy, radiotherapy or immune checkpoint inhibitors hold the greatest promise for clinical success.

Antibody-Drug Conjugates

To gain selectivity for tumor cells, Antibody Drug Conjugates (ADCs) are designed to specifically deliver highly cytotoxic drugs. ADCs consist of a monoclonal antibody covalently linked to an antitumor agent. Their effectiveness depends mostly on the differential expression of the target antigen in tumor cells compared to normal cells, the stability of the linker and the payload drug selection [22]. Most ADCs in clinical development use derivatives of potent antimitotic microtubule-disrupting agents (e.g., auristatins or maytansinoids) or highly cytotoxic DNA damaging agents (e.g., calicheamicins or duocarmycins). Despite the fact that more than 80 ADCs have entered clinical trials, over the last 15 years, only four reached the market.

Gemtuzumab ozogamicin (Mylotarg®) was in 2000 the first FDA-approved ADC. This is a calicheamicin-antibody conjugate that targets the myeloid antigen CD33. It was used as single agent to treat old acute myeloid leukemia patients in first relapse but was withdrawn from US and Europe in 2010 due to toxicity concerns. However, in 2017, the FDA reapproved it with a lower recommended dosage [23]. Brentuximab vedotin (Adcetris) was approved in 2011 for the treatment of Hodgkin's lymphoma and systemic anaplastic large cell lymphoma, which is a conjugate of monomethyl auristatin E and an anti-CD30 antibody [24].

The first and only ADC approved for solid neoplasias is Ado-Trastuzumab Emtansine (Kadcyla), an anti-HER2 antibody conjugated to the maytansinoid DM1 through a non-cleavable linker, for the treatment of HER2-positive metastatic breast cancer [25]. Recently, Inotuzumab ozogamicin (Besponsa®), a calicheamicin targeted to CD22 B-cell antigen, has been approved for the treatment of adults with relapsed or refractory B-cell precursor acute lymphoblastic leukemia [26]. Although several ADCs have demonstrated effectiveness in clinical trials, resistance to these drugs usually happens as with other antitumor agents. The mechanisms of acquired resistance are diverse and include changes in the expression levels of the antigen recognized by the mAb, alteration in the ADC internalization and trafficking pathways, impaired lysosomal function and drug elimination by efflux pumps [27]. Consequently, different strategies are also under evaluation to overcome resistance to ADCs, such as the development of new mAb formats (e.g. bispecific or biparatopic) that target HER2 and/or prolactin [28, 29]. Another promising approach is the combination of ADCs with immunotherapies, such as PD-L1 and PD-1 inhibitors, expected to show synergistic antitumor activity [30].

Virus-like particles

To combine the advantages of viral drug carriers and ADCs, namely protein cages and cell targeting, virus like particles (VLPs), mostly explored as vaccine components, have been adapted as drug delivery systems [31]. Since they are produced by recombinant DNA technologies, selectivity can be incorporated by inserting a targeting protein domain into an exposed surface loop of the viral capsid protein [32, 33]. Cargo drug molecules can be encapsulated within VLPs through different approaches, including external display by direct genetic fusion or bioconjugation, or internal drug encapsulation by direct genetic fusion, internal bioconjugation, nucleic acid driven non-covalent direct encapsulation, electrostatic or protein domain driven interactions or physical entrapment during in-vitro re-assembling [34].

Different molecules have been encapsulated within VLPs for targeted delivery in cancer. In this sense, MS2 phage coat protein VLPs displaing either TAT or transferin were used to deliver microRNA or siRNA respectively to human carcinoma cells [35, 36]. Also, ricin toxin (A-chain) has been encapsulated in targeted MS2 phage VLPs and delivered to human hepatocelular carcinoma cells inducing potent caspase 3 apoptosis activation [37]. Phage derived VLPs (Qβ, P22 and MS2 among others) have been also adapted to deliver active enzymes. For example, cytochrome P450 was successfully encapsulated in P22 VLPs and selectively delivered to human cervix and breast carcinoma cells via folate receptor for pro-drug activation in target cells [38]. JC polyomavirus VLPs were explored to deliver a suicide gene to human lung adenocarcinoma [39]. Johnson grass chlorotic stripe mosaic VLPs produced in tobacco cells have been recently loaded with antitumor doxorubicin [40], and targeted Rous sarcoma VLPs produced in Silkworm specifically delivered encapsulated doxorubicin in colon carcinoma cell lines [41]. In a more innovative approach, Rous sarcoma VLPs were double functionalized with a targeting and therapeutic moiety to specifically deliver Interleukin-2 (IL-2) to colon carcinoma cell lines and induce macrophage attraction [42].

Co-specific delivery of diverse cargos has also appeared as very promising strategy against cancer. For instance, potent synergic effects were achieved over human hepatocellular carcinoma by the co-administration of doxorubicin conjugated and small anionic nucleotide conjugated adenovirus dodecahedron VLPs [43]. Targeted delivery of a cocktail of small molecular weight drugs by MS2 phage VLPs also selectively killed human hepatocellular carcinoma cells in a very efficient way [37]. Finally, theranostic approaches have been also explored, where naturalized hepatitis B core protein VLPs have been adapted for targeted delivery of indocyanine green molecule into human glioblastoma cells for imaging and photothermal therapy [44].

Even though the use of VLPs as protein nanocontainers for targeted drug delivery is widely explored, their intrinsic immunogenecity has often limited their use in nanomedicine [45]. In this context, many efforts have been made to reduce VLP immunogenicity for drug delivery approaches. As an example, an *E. coli* based cell-free protein synthesis platform produced modified hepatitis B core protein VLPs with no immunogenicity in mice [46].

Peptides and proteins

In a simpler approach, peptides and proteins as drug targeting moieties show advantages over antibodies or VLPs, since they have high tumor penetrability linked to their smaller size, and get to a lesser extent trapped by the reticuloendothelial system (RES). Peptides are less

immunogenic than full-length proteins or viral capsids but more prone to be degraded. This issue can be overcome by designing peptides with D-isoform amino acids, cyclation, the use of the retro-inverso version or by different modifications on their chemistry. Over the last decade, the application of combinatorial phage display, one-bead one-compound libraries and molecular modeling have rendered new tumor homing peptides (THPs). Those collected until 2012 are available at TumorHoPe database (http://crdd.osdd.net/raghava/tumorhope/). Furthermore, the term aptide has been coined recently to refer to artificial aptamer-like peptide ligands that can mimic the DNA recognition site of basic leucine zipper (bZIP) proteins [47]. Moreover, the *in silico* design of tumor homing peptides through a computational approach is now feasible [48].

From the extensive peptide catalogue obtained, a bunch of proteins and peptides have been assessed in preclinical studies, as nicely summarized [49]. A number of peptides have been incorporated into cargo-bearing therapeutic nanoparticles of varying types, to target not only tumor cells through specific receptors overexpressed on the cell surface, but also components of the tumor microenvironment like blood vessels (for instance RGD, iRGD and ATN-161 that bind integrins, Qa-based peptide analog of sLex and Esbp that target E- and P- selectin, NGR towards CD13, F3 to nucleolin, CREKA to fibrin-fibronectin complexes, F56 to VEGFR-1, A7R to NRP-1 receptor, SP5-52 with unknown receptor, etc) [50] and lymphatic vessels (Lyp-1 and Syp-1 directed to p32 protein). This has been very well documented [51], and some examples will discussed in other reviews within this special section. Apart from THPs, a few natural proteins intrinsically target nanomedicines to tumoral tissues. Transferrin binds and internalizes through the transferrin receptor overexpressed in different tumors, wheat germ agglutinin (WGA) binds sialic acid and other carbohydrates, the synthetic ankyrin repeat proteins bind the epithelial cell adhesion molecule (EpCAM) and chlorotoxin recognizes $\alpha_v\beta_3$, annexin-A2 and MMP2 [52, 53].

Besides the outstanding effort to confer active targeting to drugs, there are only a few examples of nanomedicines using proteins/peptides to target tumor tissue in clinical trials. For instance, liposomal/polymeric nanoparticles bound to transferrin (Tf) selectively deliver oxaliplatin or siRNA in cancer cells because of their high Tf receptor overexpression [51]. Apart from that, it has to be considered that tumor homing capabilities through a specific receptor do not necessarily include cell penetration, an essential feature when dealing with intracellular antitumoral effects. In this context it has been observed that THPs with a R/KXXK/R consensus sequence (termed C-end rule, CendR) also show intracellular penetration, fact that boost the creation of iRGD, iNGR, tLyp-1 and other THPs containing CendR sequences [54]. In this context, the concept of tumor homing cell penetrating peptides (THCPPs) with both tumor homing and cell internalization abilities, has been already coined [55].

Viral mimetics

Combining the functionality of short peptides, the versatility in the genetic engineering of full-length proteins and the nanoscale oligomeric organization of viral capsids would be highly desirable when approaching the design of new and more effective antitumoral drugs or drug vehicles. In this context, *de novo* designed oligomeric protein constructs, measuring 12-40 nm and that mimic features of viral capsids without the involvement of infectious material, have been recently generated by using a simple protein engineering platform [56, 57]. These constructs, avoiding potential viral-associated immune responses, can be empowered by genetic fusion with peptidic ligands of relevant tumoral markers such CD44 or CXCR4 [58-60]. Under an ADC-like concept, the multivalent presentation of these ligands dramatically favors

cell penetration and enhances effectiveness of any linked drug [61]. A CXCR4-targeted oligomeric vehicle, conjugated with a polymeric form of 5-fluorouracyl, has been recently shown as extremely potent against metastatic colorectal cancer in mouse models of the human disease [62]. Taking the same principle, several microbial and plant toxins, among those explored as cytotoxic agents in oncology [63], have been engineered to self-assemble as CXCR4-targeted oligomers, that upon systemic injection, result in self-targeted self-delivered drugs. No external nanoscale vehicle is there required, following the emerging concept of vehicle-free nanoscale drugs [2]. Nanostructured diphtheria toxin and ricin derivatives have been proven to be highly potent in colorectal cancer and acute myeloid leukemia *in vivo* models, respectively [64, 65], while the incorporation of human pro-apoptotic factors [66], instead of foreign toxins, would minimize the risk of undesired immune responses to this new category of nanostructured protein-based antitumoral drugs.

Conclusions

Protein-based technologies for nanoscale drug design offer functional and structural versatility that allows high selectivity in the delivery process through specific interaction with cell surface markers. The combination of functional peptides with cage-like supramolecular assemblies allow the generation of smart vehicles and selective drug carriers in form of viral mimetics, but beyond the most conventional use of viruses or VLPs.

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Figure 1. Main protein-based approaches to nanoscale drug delivery in cancer. **Oncolytic viruses** (with edited genomes) and **viral gene therapy vectors** (carrying a therapeutic transgene) exploit natural viral properties as cell-targeted nanoparticles for intracellular delivery of nucleic acids, that either kill the infected cell or restore a cell function. Cell targeting can be additionally modulated by protein engineering. **VLPs** use structural properties of natural viruses for the controlled in vitro packaging of antitumoral drugs (red symbols). Again, targeting can be tailored by protein engineering. Drug **coacervates** are simple non-covalent protein-drug complexes, untargeted, in which proteins mainly have stabilizing roles, apart from increasing the size in which the drug is presented. **ADCs** are simple drug nanoconjugates targeted to a specific cell receptor. Cell targeting can be also achieved with non-antibody proteins, in the form of either **nanocojugates** or as peptide-functionalized cages or **nanovehicles**, including liposomes and polymeric particles. Self-assembling engineered proteins form **virus-inspired nanoparticles** that can be conjugated with conventional drugs or contain therapeutic protein domains, usually with cytotoxic proteins (**self-structured protein drugs**). This last case is the only so far explored example of chemically homogenous nanomedicines in cancer therapies.