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Monobodies as possible next-generation protein therapeutics – a perspective

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Summary

Over the past two decades, hundreds of new somatic mutations have been identified in tumours, and a few dozen novel cancer therapeutics that selectively target these mutated oncoproteins have entered clinical practice. This development has resulted in clinical breakthroughs for a few tumour types, but more commonly patients' overall survival has not improved because of the development of drug resistance. Furthermore, only a very limited number of oncoproteins, largely protein kinases, are successfully targeted, whereas most non-kinase oncoproteins inside cancer cells remain untargeted. Engineered small protein inhibitors offer great promise in targeting a larger variety of oncoproteins with better efficacy and higher selectivity. In this article, I focus on a promising class of synthetic binding proteins, termed monobodies, that we have shown to inhibit previously untargetable protein-protein interactions in different oncoproteins. I will discuss the great promise alongside the technical challenges inherent in converting monobodies from potent pre-clinical target validation tools to next-generation protein-based therapeutics.

Keywords

targeted therapies; cancer-cell signalling; oncoprotein; protein engineering; cellular delivery

Introduction

Tumorigenesis is a multi-step process that is accompanied by numerous genetic changes in tumour cells, some of which result in the activation of oncogenes and the loss of tumour suppressor genes. These genetic and ensuing epigenetic changes contribute to the acquisition of functional hallmark capabilities in cancer cells, including sustained cell proliferation, resistance to cell death, and replicative immortality [1]. While most cancer chemotherapeutic agents are generally non-specific in their action to impair growth of rapidly dividing cells, including tumour cells, several targeted anti-cancer drugs have been

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developed over the past 15 years and entered clinical practice. Up to now, targeted cancer therapeutics come in two main flavours: therapeutic monoclonal antibodies (mAbs) target the extracellular side of cell surface receptor proteins on tumour cells or on cells in the tumour microenvironment, whereas small-molecule chemical inhibitors of protein kinases and a few other enzymes (e.g., the proteasome, poly ADP ribose polymerase (PARP), histone deacetylases (HDACs)) act inside cancer cells [2, 3]. In total, more than a dozen mAbs and 34 kinase inhibitors have received regulatory approval and inhibit the signalling of key oncoproteins in specific tumour types. Targeted cancer therapeutics are used clinically as single agents, or have been added to conventional chemo- and radiation-therapy regimens. In a few cases, best exemplified by the use of the BCR-ABL tyrosine kinase inhibitor imatinib (Gleevec®) and its successors to treat chronic myeloid leukaemia (CML), a strong increase in overall survival of cancer patients was observed, which has converted CML from a fatal disease to a manageable chronic condition with a life expectancy that is not different from that of the general population [4]. In contrast, for most of the other targeted therapies, the general lessons learned over the past 15 years have unveiled several severe limitations of these drugs.

The limitations of targeted cancer therapies

1. The observed clinical responses with targeted inhibitors are often short-lived due to the rapid development of evasive and adaptive resistance that is caused by multiple molecular mechanisms [5]. Often, no benefit in overall survival is observed. A main reason for this phenomenon is the enormous genetic heterogeneity that has been revealed by cancer genome sequencing studies. On one hand, patients with the same tumour type often display a large genetic heterogeneity resulting in failure of targeted agents in certain patient subsets with a particular genetic make-up. On the other hand, even within one particular tumour in the same patient, several subclones with a very different composition of genetic driver mutations may exist. Therefore, although (single-agent) targeted therapies may be able to eradicate a dominant clone with a particular driver mutation, the “tail” of sometimes many dozen additional subclonal mutations can be selected and result in relapse and disease progression [6].

2. Only a few kinase inhibitors, such as imatinib and the EGFR inhibitor lapatinib, are highly selective, whereas most other approved kinase inhibitors have between 10 and 100 off-targets, which can include both kinases, other enzymes, but also proteins from other families [3, 7]. This lack of selectivity results in dose-limiting adverse events that decrease therapeutic efficacy [8]. Furthermore, adverse events are a main driver of poor medication-regime adherence among patients, which provides fertile ground for development of resistance [9].

3. Although the overall number of approved targeted cancer drugs may sound impressive, multiple drugs target the same proteins or pathways, as there is a strong tendency among pharmaceutical companies to focus on the same targets/pathways (“me-too drugs”). Currently, five or more drugs are approved year that target BCR-ABL, EGFR or VEGF/VEGFR. A similar development can be

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expected for inhibitors of BRAF/MEK (currently four approved drugs) and PD-1/PD-L1 (currently five approved antibodies) pathways. Overall, not more than 20 targets are covered by the current collection of targeted therapeutics, in contrast to the ~700 “cancer genes” that have been found recurrently mutated in tumours. The untargeted cancer proteome does include tens of readily targetable kinases and surface receptors involved in cancer [10], but for the most part non-kinase cytoplasmic or nuclear oncoproteins, including small GTPases of the Ras superfamily, transcription factors, epigenetic regulators, metabolic enzymes, phosphatases and enzymes of the ubiquitylation machinery.

4. Finally, while mAbs are potent and in general more specific than small-molecule drugs, and can be engineered to target virtually any epitope on proteins, their large size and hydrophilicity preclude their use in targeting intracellular proteins. In addition, mAbs often possess limited tumour tissue penetration.

In summary, broadening the spectrum of targeted oncoproteins is urgently needed to increase efficacy, decrease adverse events, and limit resistance of cancer therapies. This may provide us with a more efficacious armamentarium for a more personalised cancer treatment using targeted therapeutics and/or more effectively combination with chemo- and radiation-therapy approaches. Notwithstanding the great promise of immunotherapy strategies, in particular using immune checkpoint inhibitors, one should not overlook the severe adverse events that are frequently observed, and there are several non-immunogenic oncogenes that cannot be targeted. Furthermore, it is not clear if immune checkpoint inhibitors will work well for other tumour types beyond chronic lymphocytic leukaemia, metastatic melanoma, bladder cancer and squamous non-small-cell lung cancer for which superior clinical efficacy has been demonstrated.

Synthetic binding proteins from non-antibody scaffolds: monobodies

Engineered binding proteins derived from non-antibody scaffolds may be promising candidates to overcome many of the shortcomings of targeted cancer therapy described above, and complement the repertoire of mini-immunoglobulin scaffolds, such as scFvs, Fabs and nanobodies (table 1). Engineered non-antibody scaffolds can be developed readily to bind with higher affinity and higher selectivity than most small chemical inhibitors to virtually any protein target. In addition, their much smaller size promises better tissue penetration and may enable an easier intracellular delivery as compared to mAbs. In general, these synthetic binding proteins are selected by directed evolution techniques from large combinatorial libraries, in which several surface-exposed amino acid residues of a stable molecular scaffold are mutated. Several scaffolds on which high-affinity binders can be engineered, including ankyrin repeats (DARPins), leucine-rich repeats (Repebodies), Protein A (Affibody), SH3 domain (Fynomer) and lipocalins (Anticalins), have been developed and characterised over the past years to bind a variety of target proteins [11–13] (table 1). A class of non-antibody scaffolds that is characterised in great detail is based on the fibronectin type III (FN3) domain. These binders are termed monobodies and are extensively used in my laboratory [14]. Monobodies were invented and pioneered by Dr. Shohei Koide (University of Chicago, now at New York University Langone Medical Center) with whom my

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laboratory is closely collaborating. Monobodies can bind with low nanomolar affinity to their target proteins after phage- and yeast-display selection from large combinatorial libraries. They are only ~10kDa in size i.e., less than a tenth of the size of an IgG antibody, and free of cysteine residues (table 1). The lack of disulphide bridges enables the expression and activity of monobodies in the reducing environment of the cytoplasm and facilitates their recombinant production in bacteria. Monobody development encompasses the following steps: production of the recombinant target protein, phage- and yeast display selection, and monobody clone characterisation. The most recent combinatorial monobody library is the 'loop-and-side' library (fig. 1A) [15]. In this library, 17-24 amino acid positions are diversified and map to the FG- and CD-loops, which are located on opposite ends of the FN3 scaffold, and also involve residues in the βC and βD strands (fig. 1A). This results in a diversity of ~1.5×10^{10} monobody variants. When compared to earlier libraries, the positioning of the diversified positions provides improved shape complementarity with convex target surfaces [15], [16]. For further details on monobody combinatorial library design, selection and the exciting structural details of how monobodies interact with their target proteins, I refer to excellent review articles [14–17].

Monobodies are potent and selective inhibitors of key oncoproteins

Over the past few years, we have carefully assessed the use of monobodies as antagonists for oncoprotein signalling. As a benchmark, we have selected Src-homology 2 (SH2) domains, which are a large class of modular protein-protein interaction domains. Its 120 members can be found in 110 human signalling proteins with various functions, including kinases, phosphatases, adaptor and scaffold proteins, as well as cytoskeletal and small GTPase regulators [18–20]. Many SH2-containing proteins are classical oncogenes. The key function of SH2 domains is to recognise tyrosine-phosphorylated peptide sequences through two conserved pockets. One pocket binds the phospho-tyrosine (pY) sidechain, and a second pocket dictates selectivity by recognising the +3 sidechain downstream of the pY residue (fig. 1B) [21]. Binding of SH2 domains to pY ligands is critical for inter- and intra-molecular regulation of key oncogenic enzymes and for productive growth factor-, immune- and cytokine signalling. Targeting of SH2 domains with dominant negative peptides, peptidomimetics and small molecules has proven challenging, mainly because high selectivity has been very difficult to achieve [22, 23]. Over recent years, we have developed monobodies to target inter- and intramolecular protein-protein interactions mediated by the SH2 domain of the BCR-ABL kinase at two different interfaces [24–26], both SH2 domains of the oncogenic SHP2 tyrosine phosphatase [27], and the SH2 domain of all eight members of the Src family of tyrosine kinases [28]. These publications established monobodies as potent and selective antagonists that can inhibit signalling and oncogenicity of these oncoproteins. Binding affinities of <20nM to the target SH2 domains were readily achieved after selection and for several independent clones. Using unbiased affinity purification-mass spectrometry methods, SH2-targeting monobodies showed outstanding specificity in different cancer cell lines, and some were even found to be monospecific, making them superior to almost all small-molecule drugs [27]. Detailed structural information from a dozen co-crystal structures of SH2 domain-monobody complexes showed dominant targeting of the pY binding pocket, but with great structural variations that explained the

outstanding selectivity of SH2-targeting monobodies [28] (fig. 1B–E). Interestingly, for SH2 domains and other targets, a majority of the characterised monobody clones were found to target hotspots of protein-protein interactions [16]. Upon expression of monobodies in cancer cell lines, using plasmid transfection or retro-/lentiviral gene transfer, perturbation of oncogenic signalling, attenuation of oncogenic transformation and induction of apoptosis was observed [25–28]. In addition, impressive results were obtained with monobodies, which act as allosteric inhibitors or specificity modulators of different enzyme classes, antagonists of pY and PDZ ligand interactions, and crystallisation chaperones [29–33].

Conceptually, monobodies are novel precision perturbation tools, which provide complementary and additional information to genetic loss-of-function studies. Genetic knockouts and all RNAi-based approaches ultimately remove the complete protein, which is biologically fundamentally different from inhibition of a particular domain interaction or enzymatic activity of the target. This is possibly best illustrated by the growing number of examples where drugs such as kinases inhibitors show paradoxical and unexpected behaviour in cells, which does not mirror the phenotype obtained in knock-out/knock-down experiments [3, 34]. Even CRISPR/Cas9-mediated introduction of point mutations into the genomic locus of endogenous proteins may alter protein stability and protein-protein interactions beyond the intended perturbation on the targeted domain.

The great promise of monobodies to target oncoprotein signalling

The examples above provide strong arguments that monobodies can be engineered to bind different oncoprotein targets with high affinity and outstanding selectivity in cells, so that they may act as potent antagonists of proteinprotein, protein-ligand or enzyme-substrate interactions to perturb their functions precisely. One can envisage that monobodies will be able to target a larger spectrum of oncoproteins hitherto declared “undruggable”, such as transcription factors, small GTPases, adaptor/scaffold proteins, and others. Still, there are three major roadblocks that will be discussed in detail in the following paragraphs and need to be addressed to stimulate a possible clinical translation, and progress monobodies to next-generation protein-based therapeutics:

1. In order to reach oncoproteins in the cytoplasm or nucleus of cancer cells, methods to enable the efficient delivery of monobody proteins across the plasma membrane need to be developed.
2. Possible unfavourable pharmacokinetic and immunogenic properties of monobodies need to be studied in detail and overcome by modern protein engineering approaches.
3. To minimise the activity of monobodies on healthy cells and tissues, tumour-cell selectivity needs to be engineered.

Establish efficient intracellular delivery methods

A variety of techniques to deliver macromolecules, such as nucleic acids, peptides, proteins or drugs with insufficient cellular penetration, to the cytoplasm of cells have been proposed over the past two decades. In particular, the delivery of recombinant proteins has remained a

major challenge, but the field has clearly gained momentum in the past few years with several refined techniques indicating successful delivery of recombinant proteins to the cytoplasm of (tumour) cells. An important caveat of many published studies on protein delivery is the lack of quantification of uptake. For therapeutic applications, a concentration of the delivered protein above the K_d to its target needs to be reached. Furthermore, many studies lack a detailed elucidation of the uptake mechanism, and the precise subcellular localisation of the delivered cargo is often not well calculated. It is particularly important to exclude entrapment of the cargo protein in endosomes or other organelles of the secretory pathway. Finally, to validate delivery, many studies solely use either fluorescent proteins (e.g., GFP) or enzymes (e.g., luciferase or Cas9) as cargos, of which very small amounts suffice to produce a signal in the respective read-out assay. Depending on the intended application and cargo, such evidence may not suffice to conclude efficient delivery. As a notable exception, the development of a generic biotin ligase-based assay, in which the cargo protein is fused to an Avi-tag biotinylation sequence and only results in target biotinylation if the cargo is present in the cytoplasm, enables the objective quantification of cytosolic delivery [35]. Three protein delivery strategies that may be suitable for the delivery of monobody proteins will be discussed here (fig. 2). I refer to a number of excellent review articles that discuss protein delivery and its mechanisms in great detail [36–41].

Delivery by cell-penetrating peptides (CPPs), cell-penetrating poly(disulphide)s (CPDs) and supercharged proteins

CPPs are 8–20 amino acids long and, when linked covalently or non-covalently, enable the delivery of proteins to the cytoplasm of the cell by various mechanisms, including endocytosis, micropinocytosis or direct penetration [37] (figs 2 and 3). More than 3000 publications report on diverse CPP sequences to deliver various cargos. CPPs can be derived from natural sequences (such as HIV TAT and antennapaedia CPPs) or artificial model peptide sequences, and are either mainly poly-cationic (rich in arginines or lysines) or amphipathic, or a combination of both. Clinical trials with different CPPs have been performed to facilitate intracellular delivery of drugs, therapeutic peptides and siRNAs, but none of these products has received regulatory approval [37, 42]. Still, there is considerable scepticism in the cancer research field about whether CPPs can be used for the delivery of therapeutically relevant proteins, such as monobodies. A possible superior alternative to CPPs are cell-penetrating poly(disulphide)s (CPDs). CPDs can be regarded as arginine-rich CPPs with a poly(disulphide) instead of a polyamide backbone [43]. CPD uptake is centred around dynamic covalent disulphide exchange chemistry on cell surfaces with thiols. CPDs covalently attach to the membrane during uptake and are released in the cytosol by disulphide exchange with glutathione [43, 44]. CPDs have been shown to mediate non-endosomal uptake of organic dye molecules, peptides and quantum dots, but have not been tested for protein delivery [43, 45, 46]. Lastly, naturally supercharged proteins with a high net positive or negative charge were shown to be able to enter mammalian cells (figs 2 and 3) [47]. Likewise, supercharged proteins can also be engineered on ‘normal’ proteins to enable cytoplasmic delivery, as elegantly demonstrated for different supercharged GFP variants [48].

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Delivery using bacterial toxin subunits

A second possible approach hijacks a natural mechanism for protein uptake. Many bacteria have sophisticated multi-subunit machineries to deliver specific toxins to their host cells. Bacterial toxins have evolved to target specific cells and can enter the cell efficiently by endocytosis followed by endosomal escape. Different toxins have been employed for intracellular protein delivery. These include Exotoxin A (ETA) of *P. aeruginosa* [35] and Anthrax Toxin Protective Antigen [49], as well as a combination of the receptor binding domain of *E. coli* Shiga-like toxin and the translocation domain of Exotoxin A (ETA) of *P. aeruginosa* [50]. The binding/translocation domains of theses toxins were fused to different cargo proteins to replace the enzymatically active domains of the toxins that are responsible for cytotoxicity (fig. 3). With these systems, delivery of DARPins, repebodies and monobodies into model cell lines was achieved. Still, it can be expected that these recombinant toxin fusions might be strongly immunogenic, which may limit their use *in vivo*.

Delivery using nanocarriers

Lastly, several nanocarriers systems have also been tested for protein delivery [40] (fig. 2). While liposomal carriers in particular have been extensively used for more than two decades for the delivery of nucleic acids such as plasmid DNA and siRNAs to cultured cells and whole animals, their potential for protein delivery has only been investigated more recently. In particular, fusogenic liposomes, which are easy to produce, versatile and can be easily engineered were shown to enable delivery of proteins with a variety of sizes and properties [51] (fig. 3). Alternatively, exosomes, polymers and different nanoparticles, such as gold nanoparticle-DNA aptamer composites, mesoporous silica particles and carbon nanotubes are among a wide variety of materials that have been successfully tested for the delivery of biological macromolecules [36, 40, 52, 53].

A possible strength of these three approaches is that they can be combined modularly (fig. 2). The addition of a CPP or CPD to either cargo-bacterial toxin fusions or to cargo-nanocarrier complexes may result in synergistic cellular uptake. Likewise, encapsulation with nanocarriers can enhance CPP-mediated uptake by increasing interactions with target cells. Lastly, the continuing refinement of potent viral delivery strategies using adeno-, AAV-, vaccinia- and lentiviral vectors may also be suitable to deliver monobodies and to target tumour cells *in vivo*.

Overcoming immunogenicity and pharmacokinetics issues

An innovative strategy to limit immunogenicity of engineered proteins that could be applied in monobodies is the development of mirror-image proteins that are entirely composed of D-amino acids. Mirror-image D-proteins have been shown to be nonimmunogenic, metabolically stable and to have a longer half-life in circulation *in vivo* compared to their L-protein counterparts [54]. A major reason for these properties is that peptide bonds between D-amino acids are not substrates of proteases. In order to generate a therapeutic D-protein such as a D-monobody binding to an L-target protein, the following strategy must be used: the target protein is first produced in D-configuration by total chemical synthesis based on

native chemical ligation of peptide segments covering the entire protein, refolded, and subjected to standard selection with a combinatorial (L-) monobody library. The mirror image of the retrieved (L-) monobody binding with high affinity to the D-target proteins will then be synthesised with D-amino acids and re-folded. The resulting D-monobody will consequently bind to the initial L-target protein. Such a strategy was used to produce a high-affinity D-binding protein to VEGF [55]. Advances in native chemical ligation of peptides now allow the production of proteins of up to 120 amino acids by ligating 2-4 peptide segments.

A second obstacle is the small size of monobodies, as it can be expected that they will be cleared quickly *in vivo* as is commonly observed for peptides and other small therapeutic proteins. Different ways to increase plasma half-life are now well established, and include PEGylation or conjugation to an albumin-binding peptide [56, 57].

Increasing tumour cell selectivity and penetration

The systemic administration of cancer therapeutics inevitably results in toxicity to non-tumour tissue that decreases the therapeutic window and may limit treatment efficacy. For antibody-based therapies, several approaches to increase cell tropism have been developed, such as the use of bi-specific antibodies that contain a targeting arm that binds to a cell surface marker on the target cell, aside from its effector arm. Alternatively, tumour-penetrating peptides are successfully used for tumour targeting and to increase tissue penetration. One of the best studied examples is the RGD motif or its cyclic derivative Cilengitide that binds with high specificity to the αVβ3 and αVβ5 integrins upregulated on many tumours, and which are critical for tumour angiogenesis [58]. To increase tumour-cell selectivity and penetration, a nanoparticle-packed monobody formulation can for example be decorated with a tumour-homing peptide or an antibody/antibody fragment that binds to certain tumour-cell selective antigens, such as Ep-CAM, HER2, CD20, PSMA and others.

Outlook

Overall, once the roadblocks discussed above have been addressed, monobodies might be a valuable addition to the armamentarium of targeted cancer drugs given their unique properties and superior selectivity. Thereby, monobodies could be combined with other targeted cancer therapeutics, including conventional small-molecule drugs and therapeutic antibodies, as well as chemo-, radiation-, and immunotherapy approaches. In particular, the ability of monobodies to potently perturb intracellular protein-protein interactions that are difficult to target with small-molecule drugs is a key asset that may enable the development of efficient precision therapeutics for several hitherto untargetable oncoproteins.

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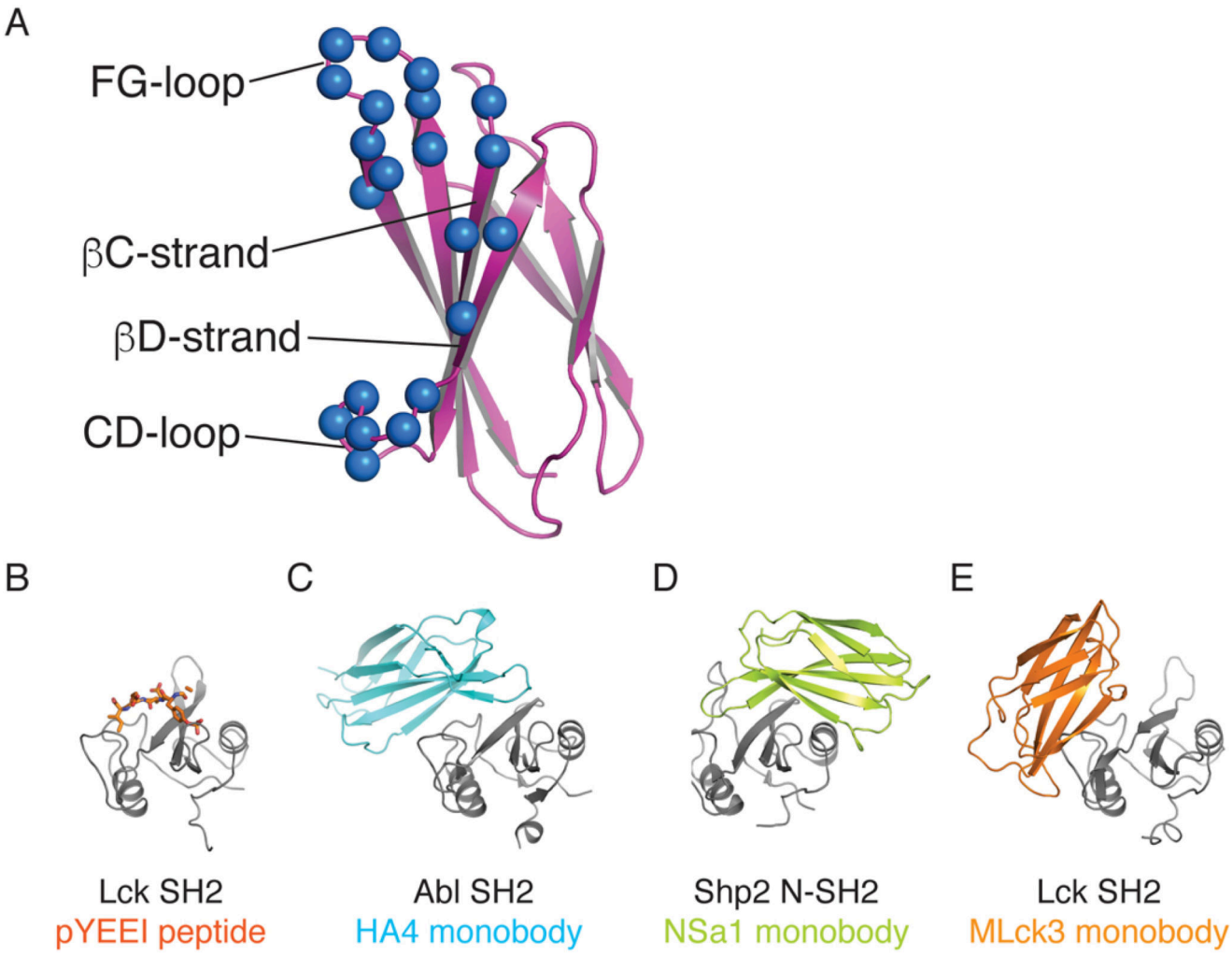

Figure 1.

(A) The structure of the FN3 scaffold of a monobody is shown in magenta cartons. The location of the diversified residues in the side-and-loop combinatorial library is shown as blue spheres. (B-E) Co-crystal structures of monobodies targeting three different SH2 domains (panels C, D and E), as well as the pYEEI peptide Lck complex structure (panel B) showing the canonical interaction of an SH2 domain with a phosphotyrosine (pY) peptide, are shown. The SH2 domains are depicted in grey whereas the monobodies and the pYEEI peptide are shown in different colours. The following PDB entries were used to draw this figure: 1LKK (pYEEI peptide-Lck SH2), 3K2M (HA4-Abl SH2), 4JE4 (NSa1-Shp2 N-SH2) and 5MTM (MLck3-Lck SH2).

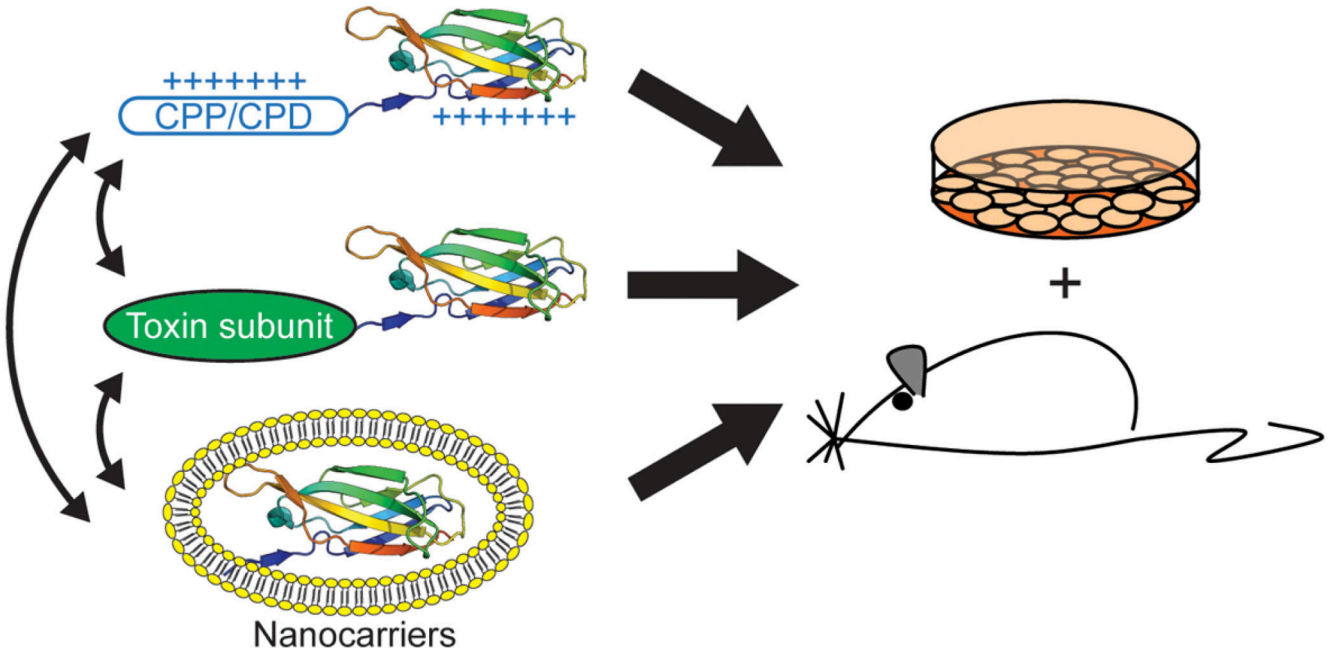

Figure 2.
Overview of the three main intracellular delivery strategies for monobodies that are discussed in this review. A cartoon structural representation of the monobody is shown in rainbow coloursCPP: Cell-penetrating peptide, CPD: cell-penetrating poly(disulphide).

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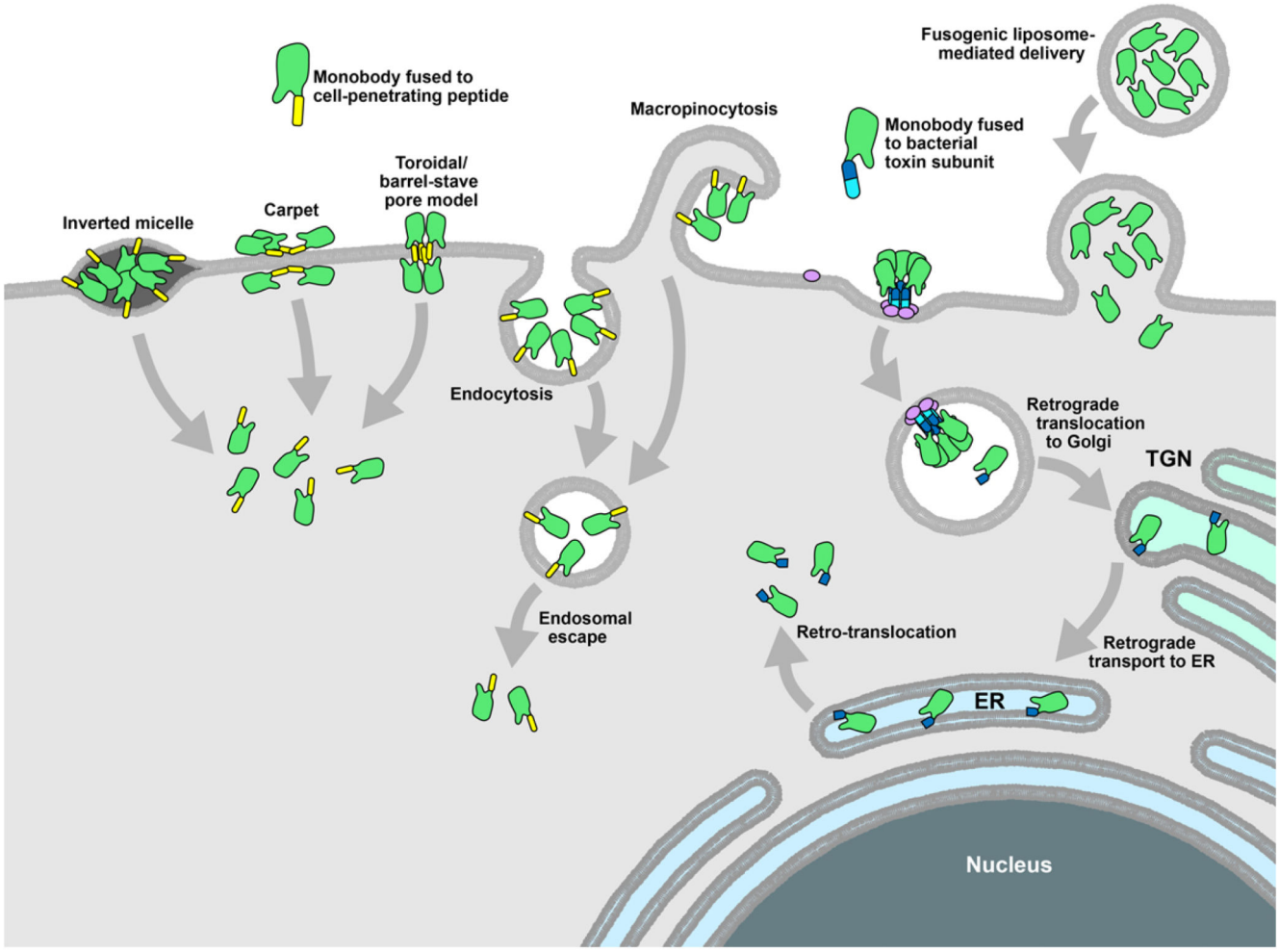

Figure 3.
Overview of uptake routes and mechanisms for possible different approaches for monobody cellular delivery.

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Table 1

Commonly used non- and mini-immunoglobulin scaffolds and their properties.

Scaffold name	Scaffold structure	Size (kDa)	Disulphide bonds	Selection techniques	Recombinant expression system	Expression yields
Non-immunoglobulin scaffolds						
DARPin	Ankyrin repeat	~18	No	Ribosome display	*E. coli* (cytoplasm)	++++
Repebody	Leucine-rich repeat	~28	Yes	Phage display	*E. coli* (cytoplasm)	++++
Affibody	Protein A	~6.5	No	Phage display	*E. coli* (cytoplasm)	++++
Anticalin	Lipocalin	~20	Yes	Phage display	*E. coli* (periplasm)	++
Fynomers	SH3	~7	No	Phage display	*E. coli* (cytoplasm)	++++
Monobody	FN3	~10	No	Phage and yeast display	*E. coli* (cytoplasm)	++++
Mini-immunoglobulin scaffolds						
scFv	Mouse/human Ig	~25	Yes	Phage display	*E. coli* (periplasm), mammalian cells	++
Fab	Mouse/human Ig	~50	Yes	Phage display	*E. coli* (periplasm), mammalian cells	+
Nanobody	VHH (camelid Ig)	~15	Yes	Phage display	*E. coli* (periplasm), mammalian cells	++