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Review

Current strategies for the development of peptide-based anti-cancer therapeutics¶

CORINA BORGHOUTS, CHRISTIAN KUNZ and BERND GRONER*

Georg-Speyer-Haus, Institute for Biomedical Research, Paul-Ehrlich-Strasse 42-44, D-60596 Frankfurt am Main, Germany

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Abstract: The completion of the human genome sequence and the development of new techniques, which allow the visualisation of comprehensive gene expression patterns, has led to the identification of a large number of gene products differentially expressed in tumours and corresponding normal tissues. The task at hand is the sorting of these genes into correlative and causative ones. Correlative genes are merely changed as a consequence of transformation and have no decisive effects upon transformation. In contrast, causative genes play a direct role in the process of cellular transformation and the maintenance of the transformed state, which can be exploited for therapeutic purposes. Oncogenes and tumour suppressor genes are prime targets for the development of new inhibitors and gene therapeutic strategies. However, many target oncogene products do not exhibit enzymatic activity that can be inhibited by conventional small molecular weight compounds. They exert their functions through regulated protein–protein or protein–DNA interactions and might require other compounds for efficient interference with such functions. Peptides are emerging as a novel class of drugs for cancer therapy, which could fulfil these tasks. Peptide therapy aims at the specific inhibition of inappropriately activated oncogenes. This review will focus on the selection procedures, which can be employed to identify useful peptides for the treatment of cancer. Before peptide-based therapeutics can become useful, it will be necessary to increase their stability by modifications or the use of scaffolds. Additionally, various delivery methods including liposomes and particularly the use of protein transduction domains (PTDs) have to be explored. These strategies will yield highly specific and more effective peptides and improve the potential of peptide-based anti-cancer therapeutics.

Keywords: peptide therapy; aptamers; cancer; intracellular drug targets; protein transduction; liposomes; scaffolds; cellular targeting

INTRODUCTION: ONCOGENES AS THERAPEUTIC TARGETS

Genetic mutations and epigenetic alterations are being considered as the cause of cancer [1–3]. The genetic events associated with tumorigenesis can be associated with the gain and loss of entire chromosomes, specific chromosomal translocations, gene amplifications, deletions or point mutations [4]. Epigenetic events are manifested in alterations in DNA methylation patterns and in secondary modifications of chromatin components [5]. These events can lead to the gain of functions of oncogenes or to the loss of functions of tumour suppressor genes and are responsible for the acquired features of tumour cells. Autonomous cellular proliferation, immortalisation, deficiencies in differentiation, the induction of angiogenesis, the propensity for invasion, the resistance to apoptosis induction and increased genomic instability are common characteristics of tumour cells.

The focus of current cancer research is how these insights can be translated into advances in prevention, diagnosis and treatment. For therapeutic purposes, it is most important to know which of these genetic alterations are rate limiting and possibly reversible. The answer to these questions will determine which genes or gene products will become the most promising drug targets in the future. Weinstein [6] postulated that cancers become 'addicted' to individual signalling components as a function of oncogene activation. This could even include downstream signals, which mediate the effects of oncogenes on the gene expression level [6]. Signalling molecules, which contribute directly to cellular proliferation, dedifferentiation or survival as a function of oncogene activation, could become indispensable. Their inactivation could lead to cell-cycle arrest, induction of differentiation, apoptosis and tumour regression.

Many cancers exhibit features that suggest the existence of such rate-limiting factors. The consequences of oncogene inactivation have been studied in a number of transgenic mouse models, in which the expression of the oncogene is conditional, i.e. it can be exogenously manipulated [7,8]. These experiments have shown that inactivation of various oncogenes in different

* Correspondence to: B. Groner, Georg-Speyer-Haus, Institute for Biomedical Research, Paul-Ehrlich-Strasse 42-44, D-60596 Frankfurt am Main, Germany; e-mail: groner@em.uni-frankfurt.de
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types of tumours indeed lead to tumour regression, confirming that they are persistently required to maintain the transformed phenotype. Examples are oncogenes, which act as transcription factors like MYC [7,9], ligands such as WNT-1 [10], intracellular signalling molecules like RAS [11,12] or receptors, e.g. MET or ErbB2/NEU [10,13–15]. Reversibility has also been shown for tumours with mutations in regulators of apoptosis or in genes modulating DNA repair or genomic stability such as ABL and the SV40-T antigen [16,17]. In these studies, it was possible to reverse the phenotype of haematopoetic tumours, epithelial tumours and sarcomas. Even genomically unstable tumours can undergo regression upon oncogene inactivation [9,18]. These results demonstrate that in a multitude of experimentally induced tumours, oncogenesis is reversible. This principle applies to many tumours, but it does not seem to be generally true and exceptions have to be taken into consideration [19,20].

Oncogene inactivation as a therapeutic strategy is conceptually and practically appealing, as it may induce tumour regression directly through the restoration of proper cell-cycle control or the induction of differentiation. It might also influence the sensitisation toward apoptosis inducers or influence the ability of tumours to sustain angiogenesis and cell–cell or cell–matrix interactions [11,21]. From the therapeutic point of view, this is of paramount interest if it becomes practicable to exploit oncogene products as therapeutic targets [22].

THE USE OF PEPTIDES FOR THE MANIPULATION OF PROTEIN FUNCTIONS

On the basis of the insights into the genetic defects of cancer cells, new technologies are being developed to extend the repertoire of therapeutic approaches for cancer therapy. These include the replacement of defective versions of tumour suppressor genes or the specific inhibition of inappropriately activated oncogenes. The spectrum of genetic tools used to interfere with the function of a given gene product includes antisense and siRNA, intracellular antibodies, dominant-negative proteins and RNA aptamers. Selected inhibitory peptides can extend this spectrum. There are multiple examples in nature, where peptides are used as ligands to influence the function of specifically binding target proteins. Also, cellular regulatory mechanisms are dependent on numerous inhibitory proteins that function through allosterism or inhibition of protein interactions.

Currently, proteins and peptides experimentally selected for high-affinity intracellular interactions with pre-determined target structures are emerging as important molecules, which could serve to extend conventional druggability. In a few model systems, peptides have already been used to manipulate crucial regulatory networks in cancer cells [23–29]. They can target specific intracellular proteins required by cancer cells for proliferation and invasion. Additional essential signalling components in cancer cells are being discovered and it is conceivable that individual peptides can be derived to inhibit their function in a targeted fashion. These peptides can be used for mono therapy or in combination with conventional chemotherapeutic agents. Since multiple pathways are dysfunctional in different cancers, and cancer cells accumulate oncogenic mutations as they progress, the greatest and most durable benefit will likely be achieved by combining therapeutic agents, which address different hallmarks of cancer. This concept, also called 'multi-focal signal modulation therapy' (MSMT), is promising, since combinations of signal modulators have already achieved dramatic suppression of tumour growth [30–32].

It might become possible to derive peptides that act as inhibitors of all individual oncogenic signalling aspects. Many crucial problems, of technical and conceptual nature, have to be addressed to reach these goals. Which signalling component is able to mediate a phenotype defined as a hallmark of cancer and can serve as a worthwhile target? How can specific peptide ligands that interfere with the signalling functions of the target proteins be found? How can these peptides be delivered to reach and bind to their intracellular targets?

THE IDENTIFICATION OF INHIBITORY PEPTIDES

Natural Peptides

Inhibiting peptides can be designed from naturally occurring binders. Usually crystallographic data are required for this approach, to study the interaction surface between the target protein and the binding partner. This is exemplified by the design of a peptide binding to TRAF6 (tumour necrosis factor (TNF) receptor associated factor 6), which was derived from the sequence of two natural binders. TRAF6 participates in signal transduction mediated by TNF receptors as well as the IL-1 receptor family and plays a role in immunity and bone homeostasis. This peptide inhibits TRAF6 signalling and osteoclast differentiation and has the potential as a therapeutic modulator for the treatment of osteoporosis or cancer-induced bone lesions.

A second report used structural data to define a peptide, which is able to bind to BCL-6 with similar affinities as the natural binding partners NCoR and SMRT [33]. In other studies, a domain was more arbitrarily chosen, without knowing the exact amino acids responsible for the interaction. In one case the SH2-domain of PLC-γ1 was chosen as a peptide inhibitor to block PLC-γ1 activity, which is an adapter protein involved in growth factor-mediated tumour cell

migration. The peptide was able to reduce migration up to 75% compared to the controls [34]. Similarly, peptides were derived from the Mdm-2 binding domain of p53 [35]. Binding of Mdm-2 to p53 targets it for ubiquitination and degradation. These p53-derived peptides prevent binding to Mdm-2 resulting in a prolonged half-life of the tumour suppressor p53 and a reversion of cell-cycle deregulation that leads to cellular transformation. The accumulation of p53 eventually caused cell death of the cancer cells [36]. Two other studies used the BH4-domain of the anti-apoptotic Bcl-x_L protein, preserving mitochondrial integrity, or a peptide that blocks the interaction of NEMO with IκB-kinase complex, selectively inhibiting the activation of transcription factor NF-κB, which is critical for regulating responses to immune challenges [37,38]. These peptides are interesting for the development of drugs treating diseases involving accelerated apoptotic death and mitochondrial dysfunction or of drugs preventing pro-inflammatory activation of the IκB-kinase complex, respectively.

These findings indicate that peptides with high affinity already exist for various target genes, which can be used for therapeutic approaches without the necessity to screen peptide libraries. The use of small endogenous domains not only provides for a high affinity, but also circumvents problems encountered with larger exogenous proteins, such as lower stability and higher immunogenicity if used *in vivo.*

Peptide Aptamers

If the structure of the target protein or the interface with its natural interaction partners is unknown, inhibitory peptides of specific targets can also be selected from synthetic peptide libraries of random sequence. Such libraries can comprise an enormous number of different individual peptide sequences serving as a source from which individual peptides with specific and desired target binding properties can be selected. Random peptide libraries with inserts of 12–20 amino acids in length have been generated. The high complexity of these libraries, i.e. the large number of different peptide sequences present, provides for a high probability that specific binders to pre-determined targets might be included. These so-called peptide aptamers can serve as ligands for distinct domains within a particular protein, e.g. a domain known to mediate the interaction with a second protein required for its function. Many proteins only assume their function upon homo- or heterodimerisation. The binding of the peptide aptamer might mask the dimerisation domain and thus interfere with the formation of the productive protein dimers. The inhibition of protein functions by peptide aptamers might be relatively subtle. It is possible to affect only a single function in a protein with multiple functional domains, e.g. the DNA-binding domain or a phosphorylation site of the target protein. This might result in the inhibition of only one property of the protein and might leave other ones intact. An advantage of the aptamer selection procedure is that they can be selected without prior knowledge of the structure of the target protein or the requirement for known binding pockets of related ligands. However, it has to be mentioned that the structure might be helpful in finding druggable moieties. Selection of peptide aptamers usually occurs through screening of high complexity libraries either *in vitro* using phage display or *in vivo* using genetic complementation methods based on protein–protein interactions [39]. Various phage display approaches have been described [40,41] and most applications have been employed for the identification of peptides binding to extra-cellular target proteins such as ligands (e.g. IGF-1, EGF, MCP-1, PSA), matrix proteins like metalloproteases or to cell surface molecules including extra-cellular domains of membrane proteins and receptors (e.g. VEGF receptor, CCR5, CXCR4) [42–48].

To identify peptide aptamers targeting intracellular oncoproteins, different *in vivo* approaches (e.g. yeast-two-hybrid systems or mammalian screens) have been described and summarised [49–53]. Various peptides have been isolated using these methods, which inhibit cancer-relevant intracellular proteins involved in cell-cycle progression (e.g. CDK2, E2F1), cell signalling (STAT3, EGFR, RAS), cell dynamics (TRIO, MMP-9), or viral oncoproteins (HPV16-E6, -E7, HBV core protein) [23,27,39,42,54–58]. The efficacies of various peptide aptamers have been evaluated *in vitro* and have consistently shown excellent anti-tumour effects. Apart from being used directly as therapeutic agents, interfering peptides can also be used as lead structures to guide the synthesis of chemical compounds with similar functional capabilities, e.g. peptidomimetics [59,60].

INCREASING THE STABILITY OF THERAPEUTIC PEPTIDES

Primary and Secondary Modifications

The use of peptides as drugs is limited by a number of biological properties, which have to be altered and manipulated in order to enhance efficacy. These include their metabolic instability, the inability to cross cell membranes and their potential immunogenicity. In the past years, considerable progress has been made to convert peptides into therapeutically useful molecules. This includes the increase in stability by chemical modifications. Peptides assembled partly or totally from D-amino acids are more stable and less susceptible to proteolytic degradation than peptides made from natural L-amino acids. D-peptides are obtained by synthesising D-amino acids in the reverse order from

those in the parent peptide and replacing L-amino acids. They usually closely resemble the structure of the L-variants or even show increased affinity [61–63]. Short peptides (<40 mer) can be chemically synthesised on a small scale. Chemical modifications of the peptides can be also incorporated to increase the binding efficacy. This has been achieved with non-naturally occurring or phosphorylated amino acids or by protection of the *N*- and *C*-terminus. This improved the systemic and intracellular stability of the recombinant peptides. However, synthesis of longer and highly structured peptides is laborious and expensive. *E. coli*, *S. cerevisiae* and insect systems are now available, which allow peptides and proteins to be obtained in a recombinant setting. With such systems it is even possible to incorporate non-natural amino acids into proteins or to modify coded ones by post-translational modifications [64,65]. The recombinant systems allow cost-effective production of milligrams of protein. Production can be easily scaled-up to obtain hundreds of grams of peptide per batch cycle.

As mentioned above, if peptides are not recombinantly expressed, but chemically synthesised, non-natural amino acids (e.g. α-amino-isobutyric acid, 1-amino-cyclopropanecarboxylic acid) or chemically modified amino acids (phosphomethyl-phenyl-alanine, 6-chloro-tryptophan) can be directly incorporated. Cyclisation might also be used to stabilise or to increase binding affinity. However, after every modification peptides need to be tested for their binding affinity, as not every change leads to an increase in binding affinity. This was nicely shown for the peptide interfering with binding of Mdm-2 to p53. As a starting point, a peptide was derived from p53; this sequence was optimised by phage display to obtain a 29-fold more potent binding affinity [67]. Subsequent truncations and the substitution with non-natural amino acids did not further improve IC_{50} values but kept them in the μM range [68]. The resulting peptide served as a template for the introduction of non-natural amino acids. They can help organise the structural conformation in solution (entropy effect) before binding, and can alter chemical characteristics that directly affect binding (enthalpy effect). To further pre-organise the peptide in solution, their cyclisation further improved their potency. Finally, by substitutions with two modified amino acids forming a salt bridge with Mdm-2 and occupying a small hydrophobic cavity the affinity was increased more than 400-fold [68,69]. Nevertheless, the design of inhibitors of protein–protein interactions, as discussed here, is still a challenge in medicinal chemistry.

Another way of increasing intracellular stability was shown by Roisin *et al.* (2004). This group associated a small peptide to a stabilising protein SUMO-1 (small ubiquitin-related modifier). The SUMO-1 protein is small, ubiquitously expressed, abundant and of human origin. In this way, application of small amounts of peptides fused to SUMO-1 did not interfere with the endogenous cellular function of this protein. Several other, maybe more effective, proteins can be used as a stabilizing domain, but SUMO-1 was the first reported, and worked well lacking cytotoxic effects [66].

Use of Scaffolds

A peptide molecule exists in either a compact folded state or a variable and open unfolded state. One way of shifting the equilibrium toward the folded state is by inserting peptides with both ends in a platform or a so-called scaffold protein or by attaching them on one side to a support protein (like SUMO-1). This not only increases stability but also constrains the conformation, improving binding affinities by decreasing their flexibility in solution (entropy) before binding [70]. For most screening methods mentioned above, peptides are usually presented in a constrained setting. Critical factors in the development of effective scaffolds that can be used in cancer therapy include absence of regions prone to aggregation or susceptible to proteolysis. The scaffold should also offer low immunogenicity, high affinity and specificity, solubility and stability to the peptide. Meanwhile multiple scaffolds have been described, each displaying their own advantages and applicabilities. However, most scaffolds meet only a limited number of the described criteria (see Table 1).

One of the most popular scaffolds for peptide library display is the bacterial thioredoxin A protein (Trx). This protein is characterised by a stable structure and can be easily purified in large quantities from *E. coli* extracts. Efficient purification is an important parameter for determination of aptamer structure as well as for protein transduction experiments (see following text). It might become the basis for cost-effective production of potential therapeutics. Numerous peptide aptamers integrated into this scaffold have been identified, which bind to various target proteins [23,27,56,71]. It was even possible to show that the binding domain of a natural interaction partner can be displayed in a proper interacting conformation by the Trx scaffold [93]. Other proteins that are suitable for the presentation of peptides such as GFP, a catalytically inactive derivative of *Staphylococcus* nuclease, and the cellular transcription factor SP1 have been described [93–95].

Randomised sequences have also been introduced into surface loops of highly constrained small proteins such as the *Streptomyces tendea* α-amylase inhibitor tendamistat [96], human pancreatic secretory trypsin inhibitor [97] and other members of the protease inhibitor family like EETI-II [87]. These scaffolds are characterised by several intra-molecular disulfide bonds resulting in a very compact and rigid structure. Random peptide libraries can be inserted into a loop

Table 1 Characteristics of selected scaffolds used for presentation of peptide aptamer libraries

Scaffold	Screening system	Characteristics	References
Trx	Yeast-two-hybrid Phage display Bacterial display	• Highly constrained conformation. Peptides are inserted into the active site of the thioredoxin protein. • Immunogenic • Size: 108 amino acids • Easy to produce/isolate from bacteria, but too large for synthesis.	[56] [67] [71] [72] [73]
CCSL	Phage display	• Anti-parallel coiled-coil stem loop structure depending on intra-molecular interactions between positively and negatively charged amino acids. Formation of loop structure partly depends on the peptide insert. • Non-immunogenic • Size: 56 amino acids, but variable for other coil scaffolds. • Production by synthesis or bacterial expression.	[74] [75] [76] [77]
Dimerisation domain EFLIVKS	Intracellular expression systems	• Peptide library inserted between two dimerising EFLIVKS repeats forming β-sheets. Whether monomeric dimers are formed largely depends on the peptide sequence. For intracellular expression in target cells or for synthesis. Proteolysis insensitive and contains no disulfide bonds. • Non-immunogenic • Small size: 14 amino acids • Production: synthesis	[78] [79]
GFP	Mammalian screen Yeast-two-hybrid	• Peptide library inserted into a solvent-exposed loop. Used for monitoring intracellular expression levels of peptides in yeast or mammalian cells. Cells expressing soluble and protease resistant peptides can be sorted before library construction and screening. • Immunogenic • Size: 239 amino acids • Production: bacteria	[51] [80] [81]
Nanobodies	Immunisation and subsequent phage display or directly by phage display	• Single-domain heavy-chain *Camelidae* antibodies with a highly constrained conformation and a stable structure. Evolutionary design for random sequence display. Also human V_H domains can be 'camelised'. • Low immunogenicity due to 80% homology to human IgM, • Size: *ca* 130 amino acids • Production: Easy to produce/isolate from *E.coli*	[82] [83] [84] [85] [86]
EETI-II	*E. coli* surface display	• *Ecballium elaterium* trypsin inhibitor II. Compact structure (cysteine knot) with three disulfide bonds. Peptides are displayed in the *N*-terminal inhibitory loop. Easily refolds correctly. • Immunogenic • Size: 28 amino acids • Production: Easy to produce/isolate from *E. coli* or to synthesise	[87] [88] [89]
No scaffold	any	• Unconstrained conformation might reduce binding affinity and increase protease sensitivity. Easy to synthesise and to modify, which increases protease resistance. • Not immunogenic (small) • Size: not applicable • Production: Easy to synthesise	[56] [89] [90] [91] [92]

structure of these proteins. This makes them less sensitive to proteases and the highly constrained conformation increases binding efficacy to the target. Libraries inserted into these scaffold resemble cyclic peptide libraries obtained by introducing cysteine residues flanking the variable peptide sequence [98,99].

Antibodies can be considered as scaffolds displaying paired variable domains able to bind specifically to an antigen. They have been selected by nature to provide optimal scaffolds for peptide presentation. However, attempts to generate single-domain minibodies (minimised antibodies e.g. Fab, scFv) were hampered by poor solubility, low expression yields, aggregation and reduced affinities. By coincidence, it was discovered that the humoral immune response of *Camelidae* is based largely on heavy-chain antibodies in which the light-chains are totally absent [100]. These antibodies display only one variable domain (VHH) for antigen interaction. Owing to their small size of 15 kDa, we refer to these entities as nanobodies. Synthetic libraries of camel VHH scaffolds with randomised CDR3 loops could constitute a valid alternative to immune libraries to retrieve single-domain antigen binders [82,101]. The recombinant VHH nanobodies that are selected from such libraries are well expressed in bacteria, highly soluble and very robust [83]. Furthermore, because of proline and glycine residues the variable loop is able to form protrusions that can fit into catalytic clefts of enzymes [83,102]. The six hypervariable regions in conventional antibodies create a larger (flat) interaction surface and usually do not inhibit enzyme activity [103]. Other advantages are their high binding affinities (in nM range) and their low complexity facilitating the design of peptide mimetics.

The structure of these nanobodies resembles that of the human family 3 variable heavy-chain domains (VH3), which might reduce their immunogenicity. The core of the antibody is formed by anti-parallel β-sheets that are stabilised by an intra-chain disulfide-bond. However, this disulfide-bond seems to be protected from the solvent by a tryptophan that covers it with its aromatic side-chain [84]. From these data, it is clear that nanobodies could become useful for the recognition of extra-cellular as well as for intracellular targets [85]. Synthetic libraries of camelised human V_H scaffolds with a randomised CDR3 could constitute a valid alternative to retrieve non-immunogenic single-domain antigen binders [86].

An even smaller scaffold to constrain the conformation of peptides is the dimerisation domain EFLIVKS. This domain was originally isolated from the neuropeptide head activator protein, involved in head-specific growth of the fresh-water polyp *Hydra attenuta*. Peptides of identical sequence have also been isolated from tissues of higher-organism, e.g. the human hypothalamus. It was reported that this domain dimerises with high affinity ($K_D = 1$ nM) via anti-parallel β-sheet formation. Two dimerisation domains can be attached to both ends of a peptide. The dimerisation and formation of a loop structure largely depends on the insert sequence [78]. However, recent studies have shown that β-sheet formation is rather weak [79]. This restrains applicability but could be useful to generate heterogeneous synthetic peptide libraries with both folded and non-folded members. Furthermore, this scaffold cannot be recommended for recombinant expression as the peptides might easily form aggregates.

The use of scaffolds for the stabilisation and the increase of affinity of peptides has been shown in several studies. The number of scaffolds that can be used for this purpose is still growing and it is very likely that scaffolds which meet most demands mentioned above will be developed in the near future [104].

INTRACELLULAR DELIVERY OF PEPTIDES

Most peptides that are being considered for therapeutic purposes up to now exert their action on the cell surface. Peptides bind to cell surface proteins and act by inducing or inhibiting one or multiple signal transduction pathways. We would like to extend the use of peptides and deploy them intracellularly as modulators of cytoplasmic or nuclear signalling components. For this purpose, peptides need to be internalised in order to exert their action by binding to an intracellular protein involved in oncogenesis.

Various methods have been developed for the intracellular delivery of peptides and proteins. For example, short peptide sequences (so-called protein transduction domains (PTDs)) have been fused to the peptides or proteins of interest. This enables the endosomal uptake of the fusion protein. There are vesicle-like compounds (liposomes) or polymeric colloidal particles (nano-particles), which have been used to incorporate peptides, proteins, DNA and water-insoluble drugs. Liposomes or nanoparticles are able to fuse with or penetrate through the cell membrane, releasing their cargo intracellularly. In the sections 'The use of protein transduction domains (PTDs)' and 'Delivery of peptides by incorporation into liposomes', we present a summary of applications in which PTDs or liposomes have been employed.

The Use of Protein Transduction Domains (PTDs)

Currently, the efficient delivery of therapeutic compounds, peptidyl mimetics and proteins into cells *in vivo* can be achieved only when the molecules are small, typically less than 600 Da. Delivery of bioactive peptides across the blood-brain barrier is generally restricted to even smaller peptides of six amino acids or less [105]. To overcome this problem, the fusion of therapeutic

peptides with PTDs is being exploited. Specific peptide sequences present in a small number of proteins mediate rapid translocation into the interior of cells and even in the nucleus. These domains act autonomously and their ability can be conferred to fused, heterologous proteins. Three PTDs have been used extensively for this purpose. The Antennapedia peptide derived from a family of *Drosophila* homeoproteins [106], the structural polypeptide VP22 forming the major component of the herpes simplex virus (HSV-1) [107] and the TAT peptide derived from the HIV-TAT protein, which is a regulator of transcription of latent HIV, are essential for HIV replication [108]. Various other PTD sequences have been described, but because of its short length (10 amino acids) and its good efficacy in crossing cell membranes of numerous different cell types, the PTD of TAT appears to be most popular. Fusion of this domain to peptides of interest has shown efficient and rapid uptake into all kinds of different target cells, and even *in vivo* data have been obtained [25,109–112].

Uptake mechanism of PTD-fusion peptides. The first step in protein transduction is induced by electrostatic interactions between positively charged amino acids in the PTD and negatively charged glucosaminoglycans in the cell membrane [113]. The subsequent mechanism of internalisation has been a topic of discussion for some years. Initially, it was observed that the uptake is insensitive to low temperature and to inhibitors of endocytosis. This was interpreted as a direct translocation mechanism of the cationic peptides through the plasma membrane [106,114,115]. More recent data suggest that the fixation of the cells might have been responsible for the uptake of PTD-fusion peptides and caused erroneous interpretations. Fluorescence microscopy on live, unfixed cells suggested that peptides are taken up by the endosomes [116]. Now most studies confirm that peptides are internalised by an endocytosis-dependent process called lipid-raft-dependent macro-pinocytosis, which is independent of receptors or transporters [117–119]. This is a special form of endocytosis characterised by detergent-resistant membrane fractions rich in sphingolipids and cholesterol. After uptake, peptides end up in the cytoplasm (Figure 1). One experiment showed that partly denatured peptides can be detected inside cells within 15 min after transduction, whereas biological activity of the peptide was measured after a period of 2 h. This indicates, that after their uptake, the transduced peptides undergo refolding in cytoplasm and become active [111].

How peptides eventually escape from the endosome and enter the cytoplasm is still unresolved. An endosomal escape mechanism must play a role that prevents the inclusion of peptides in the lysosome and their degradation. Two observations confirm this notion. First, the use of lysosomal inhibitors did not increase the amount of functional peptide in the cytoplasm, indicating that PTD fusions circumvent lysosomal degradation [120]. Second, the addition of substances such as chloroquine, which is an ion-transporting ATPase inhibitor, prevents the acidification of endosomes. This causes swelling and disruption of the vesicles and enhances the realease of peptides into the cytoplasm [121]. Unfortunately, chloroquine is toxic at its effective dose and cannot be used in a therapeutic context as a transduction enhancer. Several viruses that have evolved endosomal escape mechanisms are known. These sequences also act autonomously and their action can be conferred to linked proteins. The addition of peptide sequences derived from such viruses, e.g. the amino terminal domain of the influenza virus hemagglutinin subunit HA-2, was shown to increase the release of PTD-fusion peptides in the cytoplasm [118,122]. At pH 5.0, the carboxy-terminal side of this V-shaped peptide undergoes a conformational change, resulting in helix formation that allows deeper insertion of the peptide into the lipid bilayer [123]. This insertion into the endosomal membrane is thought to cause destabilisation and the release of peptides into the cytoplasm.

In conclusion, these studies show that it is possible to introduce functional peptides into cells. Although not all aspects of protein transduction have been elucidated, this process can be exploited for therapeutic purposes. This is also summarised below, where PTDs have been used for *in vitro* as well as for *in vivo* applications.

The use of PTD fusions in vitro. Various studies have shown, that upon transduction from the extra-cellular environment, peptides or proteins are functionally active in the cytoplasm and even in the nucleus. Polo *et al.* (2004) interfered with the action of the BCL-6 oncogene product. This transcription factor is frequently deregulated in B-cell lymphomas. Transduction of a peptide binding to the BTB domain of BCL6 prevented the recruitment of co-repressors to this factor, normally preventing the expression of target genes. In BCL-6 positive lymphoma cells, blockage by TAT-fusion peptides reactivated target genes of BCL6, causing apoptosis and cell-cycle arrest [33]. In another example, two peptides that were derived from a basic-aromatic and an acidic leucine-rich domain of PURα were used. PURα is a DNA-binding protein modulating transcription and replication of cellular and viral DNA. It forms complexes with hyper-phosphorylated pRb and associates with E2F-1, suggesting a role in cell-cycle progression and proliferation. Results from colony formation assays using glioblastoma cells treated with the TAT-PURα-derived fusion peptides showed a reduction in proliferation of about 45%. These TAT-fusion peptides were able to enter the nucleus and could be detected for up to 24 h after treatment [124].

We have used the epidermal growth factor receptor (EGFR) as a target for peptide aptamers. This receptor is over-expressed in various human tumours [125,126].

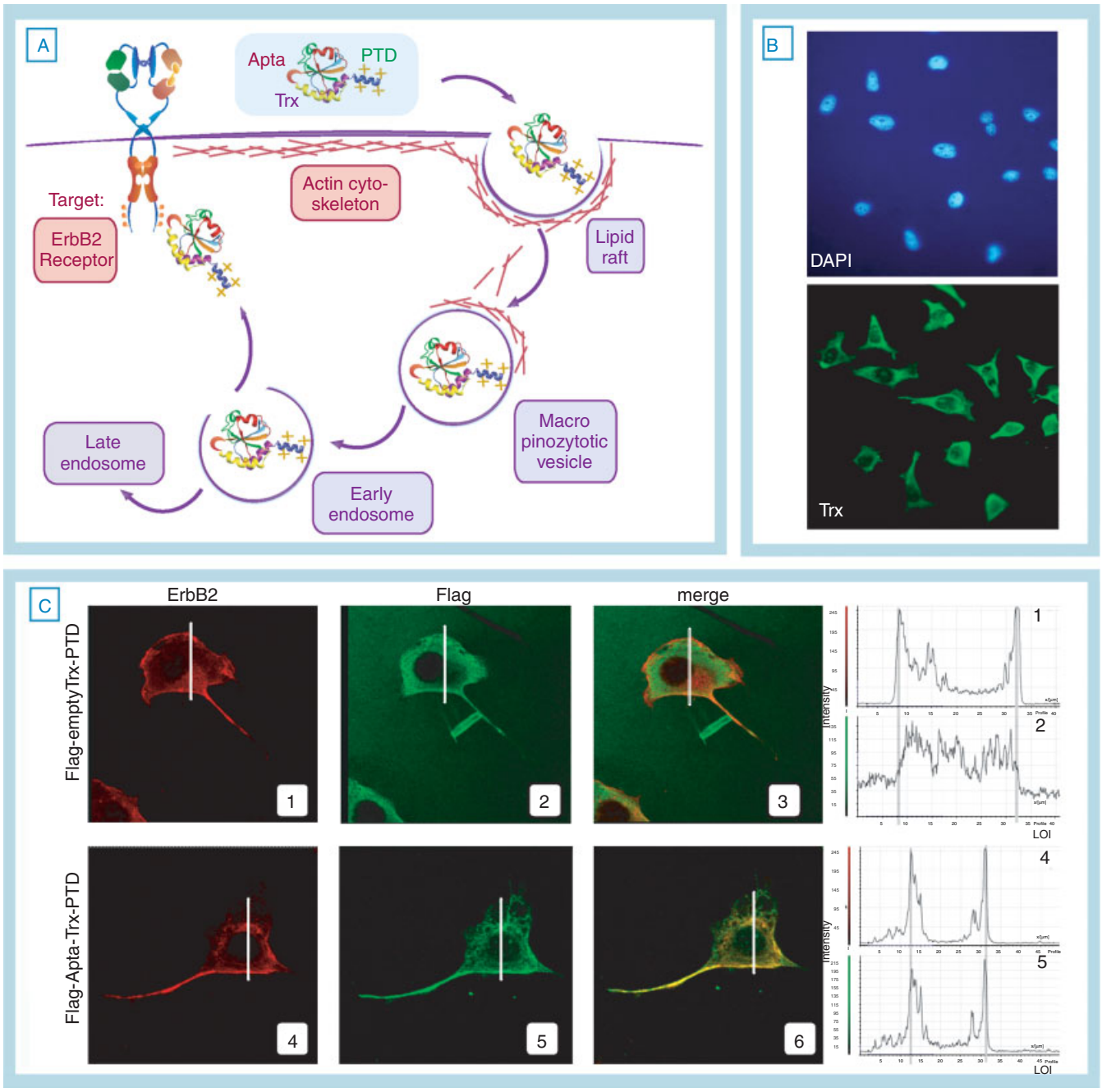

Figure 1 Intracellular delivery of peptides using protein transduction domains. (A) Protein transduction mechanism: A protein transduction vehicle (PTV) consists of a specific peptide aptamer (Apta), a scaffold protein, e.g. Thioredoxin (Trx) and a positively charged protein transduction domain (PTD). The PTV binds to the cell membrane via electrostatic interactions and is taken up by the cell via lipid raft-dependent macro-pinocytosis. Release from the endosome is mediated by an unknown mechanism than enables PTVs to bind to their intracellular targets as shown here for the ErbB2 receptor. (B) Fluorescence microscopy studies with Trx-PTD (green) after transduction (4 h). Protein transduction is a highly efficient process leading to 100% transduced cells as confirmed with DAPI staining of nuclei (blue). (C) Target-aptamer interaction after protein transduction. Cells were transduced with either an aptamer in a thioredoxin scaffold or an empty thioredoxin scaffold protein as a negative control. Before immunofluorescent staining, an acid wash was performed to remove proteins bound to the cell surface. Confocal microscopy analysis was used to visualise the intracellular localisation of the transduced proteins. The control PTV is taken up by the cells (2), but does not interact with the ErbB2 receptor (3). The aptamer-PTV (5) enters the cell and co-localizes with the ErbB2 receptor (6). The diagrams on the right show the intensity of the fluorescence at a line of interest (LOI = white line) drawn in the corresponding pictures on the left. These graphs underline the co-localization of the aptamer and the ErbB2 receptor. This can be explained by an efficient interaction of the aptamer with the target protein.

It activates signalling pathways involved in proliferation, angiogenesis and survival. Over-expression is associated with poor response to treatment and rapid disease progression. We also used the STAT3 signalling molecule as a target. This signal transducer and activator of transcription transfers signals from cell surface receptors to the nucleus, where it participates in gene transcription. STAT3 has also been found to be deregulated in its activity in various primary tumours and tumour cell lines [127]. Purified and refolded PTD-aptamer fusion proteins were added to the medium of various cancer cell lines. Transduction of the recombinant peptides and subsequent immunofluorescence experiments revealed co-localisation of the EGFR and the aptamer at the plasma membrane (see also Figure 1c for similar experiments with aptamers targeting the ErbB2 receptor). We found that application of PTD-aptamers significantly inhibit proliferation *in vitro*. The aptamer binding the DNA-binding domain of STAT3 even caused the induction of apoptosis in STAT3-dependent cells. Peptides inhibiting STAT3 or EGFR pathways have shown that transducible peptide aptamers are able to enter cells, inhibit cancer-relevant signalling pathways and cause cell-cycle arrest and the induction of apoptosis [23,27].

In each of these cases, TAT appears to transport sufficient amounts of the blocking peptides into cells to achieve significant inhibition of relevant protein–protein or protein–DNA interactions, usually with K_D values in the range of 0.5 to 100 μM.

The use of PTD-fusions in vivo. An *in vivo* study using PTD-fusion peptides was first reported with β-galactosidase as a cargo protein. Intra-peritoneal injection of this large protein (120 kDa) resulted in the delivery of the fusion peptide to all tissues including the brain as shown by staining numerous tissue sections with X-Gal [111]. Soon afterwards, various other studies confirming this observation followed [25,38,98,109,112]. For example, in the majority of patients with renal cell carcinomas (RCC), the von Hippel-Lindau (VHL) tumour suppressor gene is mutated. The β-domain is able to inhibit insulin-like growth factor I receptor-mediated signalling essential for tumour growth and invasion of RCCs. Daily intraperitoneal injections of a β-domain-TAT fusion caused partial regression of renal tumours that were implanted in the dorsal flanks of nude mice [128]. In a second report, attempts were made to stabilise the TAT-fusion peptide only in tumour cells. For this the ODD domain of HIF-1α, which usually controls degradation of HIF-1α under normal O_2 conditions, was used. ODD stabilises HIF-1α under hypoxic conditions as found in solid tumours. In a similar way, fusion of the ODD domain to a transducible TAT-caspase-3 peptide resulted in a preferential stabilisation of the peptide in tumour cells. The attached caspase-3 activity caused a significant reduction in tumour size [129].

In a recent study, a transducible peptide was used to restore tumour suppressor activity. A D-isomer of a peptide composed of the *C*-terminal of p53 was used, previously shown to activate specific DNA binding by p53 by an unknown mechanism. This peptide activates wild-type p53 only in cancer cells and thus induces apoptosis. Intra-peritoneal administration of this peptide in mice bearing subcutaneous tumours resulted in a distribution of the peptide throughout the tumour. Tumours exposed to peptides were significantly retarded in their growth and reached a volume of less than 50% when compared to control mice [130]. A reduction in tumour volume after injecting tumours with, e.g. pro-apoptotic PTD-fusion peptides was also previously observed [37,128,129].

Delivery of Peptides by Incorporation into Liposomes

Non-specific protein delivery agents are commercially available (e.g. Clariot – by ActiveMotiv or BioTrek – by Stratagene) forming non-covalent complexes with the purified peptide of interest and protecting it from degradation during non-endocytic uptake. These systems were shown to work for most cells as well as *in vivo*. A multitude of alternative nanoscale delivery systems such as encapsulation of proteins into liposomes or other nanoparticles (including solid nanoparticles, polymeric nanoparticles and polymeric self-assemblies) have been developed. In particular, the use of long-circulating liposomes for passive or physiologic targeting of drugs to tumours has been widely studied. Liposomes have the ability to incorporate hydrophilic compounds into their aqueous core and hydrophobic agents into their lipid bilayer. Liposomal delivery of chemotherapeutic agents reduces systemic toxicity by lowering free drug concentrations in the plasma. They are usually cleared by the phagocytic cells, which constitute the reticulo-endothelial system. The composition of liposomes can be adjusted to improve their pharmacokinetics or drug-carrying properties. For example, the incorporation of polyethylene glycol (PEG)-derivatised lipids reduces phagocytic clearance increasing their circulation half-life. Such pegylated or STEALTH liposomes circulate for days as stable constructs and slowly accumulate in neo-angiogenic vessels of tumours. This is mainly due to increased permeability of tumour endothelium and reduced lymphatic drainage. This way it is possible to enhance anti-tumour activity and prolong drug exposure [131]. The use of STEALTH liposomes loaded with doxorubicin has been under intensive investigation for the treatment of breast cancer and other solid tumours [132–134] and has now been approved for clinical use. Owing to the beneficial change in doxorubicin biodistribution, reducing drug levels in the heart, cardiomyopathy caused by doxorubicin toxicity was greatly reduced (reviewed in [135,136]).

Use of Cellular Targeting

The German Nobel prize winner Paul Ehrlich, whom we are particularly fond of as the founder of our institute, described drugs that can selectively kill pathogenic cells without damaging healthy cells as 'magic bullets'. The use of peptides, which are able to target specific cells, tissues or organs, could be an attractive approach to enhance therapeutic effectiveness.

One appealing strategy is the development of antibody-directed liposomes (immunoliposomes) that bind receptors on target tumour cells over-expressing such receptors. Target cell internalisation of these immunoliposomes should allow intracellular drug delivery. A recent study reported the design of immunoliposomes directed against gliofibrillary acidic protein (GFAP). *In vivo* studies showed that these liposomes specifically target rat brain astrocytes and might be valuable for the delivery of drugs to glial brain tumours [137].

Not only antibodies but also peptide ligands can be coupled to liposomes. For example, transferrin-coupled liposomes were developed to target human T-leukemia cells and to promote efficient intracellular delivery of therapeutics in these cells. In this case, the liposomes are internalised by a transferring receptor-dependent endocytosis pathway *in vitro.* This was more effective than non-targeted liposomes [138]. An *in vivo* application with growth factor antagonist targeted STEALTH liposomes showed a 40-fold increased binding affinity for tumour xenografts when compared to non-targeted liposomes. Uptake was thought to be mediated by a receptor-mediated process involving clathrin-coated pits [139].

Another possibility is the incorporation of derivatised folic acid (vitamin B) into the liposomal bilayer. The folate receptor (FR) is over-expressed in many human tumours, whereas its distribution is highly restricted among normal tissues [140,141]. These observations suggest that FRs can be used as a tumour-specific cell surface marker for targeted delivery of cancer therapeutics. Numerous *in vitro* studies have confirmed this idea. FR-targeted liposomes have also been loaded with doxorubicin and showed a 45-fold higher uptake than non-targeted liposomes [142].

Apart from peptide ligands binding to tumour-specific receptors, antibodies or antibody fragments, and growth factors have been conjugated to liposomes to achieve selective targeting of tumour cells [139,143–145]. The modular organisation of targeted-liposome technology enables combinatorial approaches in which a repertoire of targeting entities can be used in conjunction with a series of liposomal drugs to yield a new generation of molecularly targeted agents. Specific targeting can enhance the therapeutic effect of the drugs through their accumulation at the diseased site. Further improvements include the formulation of liposomes designed to resist reticulo-endothelial clearance or, when designed for target cell internalisation, to provide intracellular drug release.

CONCLUDING REMARKS

Therapeutic peptides and proteins, which are currently being investigated, are mainly acting on extra-cellular targets. A great necessity exists to develop new strategies to interfere with intracellular targets. So far, interfering with such targets has been mainly restricted to the inhibition of enzymatic protein functions. However, the development of methods to target intracellular proteins, which do not display enzymatic properties and are components of signalling pathways essential for tumorigenesis, is a challenging task for translational scientists. The blockage of various protein interaction domains is a promising possibility to increase the number of druggable targets. However, before proteins and peptides can be used for intracellular targets, many obstacles still have to be overcome.

The most straightforward approach is to copy nature and use natural binding partners of target proteins and optimise these for clinical purpose. As not every target has its own inhibitor, advanced technology is needed to find new ones. The screening methods available are versatile and have to be adjusted for the target protein of choice. It is also important to consider delivery options of such therapeutics. Linear peptides can be synthesised in high quantities at relatively low costs, but the pharmacokinetical data are often not encouraging. The use of scaffolds can increase the efficacy, but cannot easily be added to a selected linear peptide. The use of endogenous scaffolds with rigid structures enhances refolding properties after purification and could increase plasma half-life of the peptide and reduce immunogenicity. The screening has to be performed with a peptide library inserted into a scaffold. Depending on the size of the scaffold, synthesis of the therapeutic peptide might not be feasible anymore. Recombinant expression methods and subsequent scale-up have to be taken into consideration for production of the therapeutic proteins.

The discovery of PTDs has been extremely valuable for the development of peptide therapeutics for intracellular targets. A final step is now the specific targeting of cancer cells, and current reports using targeting liposomes indicate that there are indeed interesting possibilities to reach this goal. The processing of data obtained from such studies may lead to the development of a new generation of protein drugs, with minimised side effects and increased pharmacokinetic properties.

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