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Peptides as targeting elements and tissue penetration devices for nanoparticles

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Abstract

The use of nanoparticles in medicine (nanomedicine) has recently become an intensely studied field. Nanoparticles carrying drugs and imaging agents have already reached the clinic, but they are essentially passive delivery vehicles, not what are referred to as “smart” nanoparticles. An important function to add to make nanoparticles smarter is active homing to the target tissue. It makes nanoparticles accumulate in the target tissue at higher concentrations than would be the case without this feature, increasing therapeutic efficacy and reducing side effects. This review discusses the recent developments in the nanoparticle targeting field with emphasis on peptides that home to vascular “zip codes” in target tissues and provide a tissue- and cell-penetrating function.

Introduction

Nanoparticles (NPs) are thought to have great potential as drug delivery vehicles in medicine. The first NP drugs are already in the clinic. Examples include a NP composed of albumin-paclitaxel complexes (Abraxane) and liposomes loaded with doxorubicin (Doxil), which are both cancer therapeutics. However, these NPs are essentially passive drug delivery vehicles that do not fully exploit the potential of NPs. By virtue of being particles, NPs can accommodate multiple functions, such as being able to zero in to their target in the body. The targeting can be accomplished by coupling onto the NP a homing element, such as an antibody or peptide that specifically binds to the target tissue. The concept of targeted drug delivery is an old one. The idea is appealing because this approach has some of the advantages of topical application of drugs: high local concentration at the site of the disease process and low systemic exposure. Some of the reasons for the modest success of the approach so far include the early focus on targeting the parenchymal (tumor) cells, which has been largely stymied by poor penetration of the probes into extravascular tissue. The realization that the vasculature is more accessible to molecular probes has been a significant advance. Moreover, the recent emergence of NPs as delivery vehicles and the identification of specific targets in the vasculature have rekindled interest in the targeting approach. We call this mode of drug delivery “synaphic” (Gr. syn, together; aphic, affinity) targeting; it is also referred to as ‘active’ or ‘pathotropic’ targeting.

Peptides are particularly well suited for NP targeting because they are small, easy to synthesize and typically non-immunogenic, and because the multivalent presentation of a peptide on a NP provides high avidity for the target. We screen phage libraries in live mice

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to identify peptides that direct phage homing to a specific target, such as a tumor or the vascular bed of a certain tissue. As the phage is a NP, it is essentially confined to the vessels, and the screening primarily targets differentially expressed endothelial cell markers (vascular "zip codes"; [1]). Endothelial marker molecules are readily available for NP binding, and quite frequently the parenchymal cells express the same marker as the endothelium. However, NP access to the extravascular tissue is limited. Recently discovered tumor-penetrating and tissue-penetrating peptides provide a solution to this problem, as they are capable of taking a payload deep into extravascular tissue. Remarkably the NP or drug does not even have to be coupled to the peptide; the peptide activates a bulk transport system that sweeps along any compound that is present in the blood[2],[3]. Treatment studies in mice show improved anti-tumor efficacy and less damage to normal tissues. In this review, I discuss the recent advances in NP delivery focusing on the use of molecular markers in the vasculature as the primary target, NP exit from the blood vessels, and their transport through the target tissue.

Molecular specialization of the vasculature

Peptides detecting vascular zip codes—Studies designed to examine the possibility that tissue-specific metastasis of tumors depended on a specific affinity of circulating tumors cells for the vessels of the preferred host tissue suggested molecular heterogeneity of the endothelium[4]. In the early 1990's, I decided to explore the proposed molecular heterogeneity and it role in metastasis by using *in vivo* phage display. A library of peptides expressed as fusions to a phage surface protein and typically containing about 1 billion different peptides is intravenously injected into live mice under anesthesia, and 5–10 minutes later, the tissue of interest is collected for phage isolation. By repeating the process a number of times, one obtains a pool of phage enriched in phage clones displaying peptides that selectively home to the target tissue. Sequencing the part of the phage genome that encodes the peptide insert in a sample of phage clones from the enriched pool typically reveals repeated peptide sequences; those are the candidate homing peptides. We initially showed that it was possible to identify homing peptides for brain and kidney vessels[5]. Phage screening has since been used to identify homing peptides for many additional tissues, so many that it seems reasonable to conclude that every tissue puts a specific signature on its vasculature (reviewed in ref. [6]). We also used in vivo phage display to identify a breast cancer cell surface protein that recognizes a lung vascular zip code, promoting metastasis to the lungs[7]. These results support the metastasis hypothesis that inspired the initial *in vivo* phage display studies.

Various diseases put disease-specific signatures on the vasculature of the diseased tissue, and *in vivo* phage display has also proven useful in the identification of these disease-specific vascular markers. Cancer, inflammation, atherosclerotic plaques, arthritis, and regenerating tissue are known to induce the expression of new molecular markers in the blood vessels. A major advantage of the *in vivo* phage screening is that it is unbiased in revealing what works *in vivo*. Other unbiased methods, such as antibody-based screens[8], cloning strategies[9], and *in vivo* biotinylation[10] have also been used successfully in analyzing tumor vasculature. A major advantage of phage screening is that recognizes proteins that are expressed at the cell surface in tumors but are entirely intracellular in normal tissues. This expression pattern is quite common in tumors and other activated tissues (see the section on receptors).

Vascular specificities are often shared among cancer, atherosclerosis, inflammation and tissue regeneration. The apparent reason is that they all involve angiogenesis, the sprouting of new blood vessels from existing ones[11]. However, that does not mean that the specificity profiles in different diseases are identical. A striking example of the dependence

of vascular marker expression on the nature of the lesion is what happens during tumorigenesis: the vessels of premalignant lesions differ from those of fully malignant lesions (while both differ from normal vessels). *In* vivo phage display in two *de* novo transgenic mouse tumor models yielded peptides that recognized the vessels of premalignant lesions, but no longer bound to the vessels in lesions that had turned into fully malignant tumors in the same tumor model. Other peptides displayed the opposite binding pattern, and a third set recognized the vessels in both pre-malignant and fully malignant lesions[12],[13]. In addition to the interesting biology, being able to specifically target pre-malignant lesions has potentially important diagnostic and therapeutic implications. It may be possible to image very early incipient malignancies and target them for destruction. Also, when a target receptor and targeting probe are chosen for tumor imaging and therapy, it will be important to know whether the system also recognizes pre-malignant conditions. Comparison of tumor vessels and vessels from regenerating liver by gene expression analysis revealed distinct differences[14]. Similarly, *in vivo* phage display with tumors as a target has produced different sets of peptides than when wounds[15] or arthritic joints[16] were targeted. One of the wound-homing peptides, a cyclic peptide with the sequence CARSKNKDC, also recognizes tumor vessels, but is much more potent as a homing peptide for wounds and inflamed tissues[15],[17].

Target molecules (receptors) for homing peptides—A homing peptide identified by phage screening can be used to identify the corresponding receptor. The most commonly used method for receptor identification is affinity chromatography or “pull-down” on the peptide, followed by mass spectrometry analysis of the bound proteins. Some receptors for tissue-specific vascular homing peptides have been identified[18],[19],[20]. More is known about the receptors in tumors. An early phage screening study[21] yielded a tumor-homing peptide with an RGD sequence motif. As RGD is an integrin-binding motif[22], and RGD-binding integrins such as αvβ3 and αvβ5 are specifically expressed in tumor endothelia[23], this result confirmed the validity of *in vivo* screening.

New targets identified with homing peptides in tumor vasculature include a form of aminopeptidase N (CD13), which binds peptides containing the NGR motif[24],[25]. A peptide representing a 31-amino acid fragment of human high mobility group protein 2, termed F3, is an example of a novel tumor-homing peptide identified by *in vivo* phage screening of protein fragments encoded by cDNAs[26]. The receptor for F3 is nucleolin expressed at the cell surface. Nucleolin is ubiquitous as an intracellular protein, but is specifically expressed at the cell surface of endothelial cells and tumor cells *in vivo* ([27], [28]. Nucleolin was the first example of what appears to be a common phenomenon, expression of intracellular proteins at the cell surface of tumor cells and tumor endothelial cells. Phage display is particularly well suited for the discovery of markers, the accessibility of which at the cell surface makes them tumor specific, rather than high overall expression. Other examples of such markers in tumor vasculature include the cytoplasmic proteins annexin1[29],[30] and plectin-1[31], and the mitochondrial protein p32 (also known as gC1q receptor, and hyaluronic acid binding protein). This protein is the receptor for the tumor homing peptide LyP-1, also originally discovered using *in vivo* phage display[32]. LyP-1 targets p32 on the cell surface of lymphatic, myeloid, and cancer cells in tumors, whereas normal tissues only express p32 inside the cells, where it is not available for peptide binding[33].

The fibrin-fibronectin complex in blood clots is a well-recognized target in vascular thrombosis. Less appreciated, but perhaps even more important, is the presence of such complexes in tumors and atherosclerotic plaques. The walls of tumor vessels and the interstitial spaces in tumors contain products of blood clotting, presumably as a result of plasma protein seepage from leaky tumor vessels. Fibrinogen leaked from blood vessels is

converted to a fibrin meshwork by tissue procoagulant proteins such as tissue factor [34],[35],[36]. Other plasma proteins, plasma fibronectin in particular, become covalently linked or otherwise bound to the fibrin meshwork. These fibrin-fibronectin complexes can be accessed with peptides such as the 9-amino acid cyclic peptide CLT-1[36],[37] and the pentapeptide CREKA[38]. Subtle clotting also takes place on the surface of atherosclerotic plaques, and has been made use of in delivering cargo to plaques[39].

Homing peptides in the clinic—Remarkable success in delivering the cytokine tumor necrosis factor α (TNFα) into tumors has been reported with RGD and NGR peptides; the targeted cytokine was effective in doses as much as 1,000-fold lower than the usual dose, mitigating side effects of this highly toxic cytokine[40]. A TNFα-NGR fusion protein is currently in phase 3 clinical trials[41]. The reasons for this success are likely to be twofold: TNFα is a trimer and the NGR peptide is attached to each subunit, enhancing binding through an avidity effect. In addition, the chimeric protein has two receptors potentially acting in concert the receptors for TNFα receptor and the NGR peptide. The same RGD and NGR peptides have also been used to deliver tissue factor in human patients to induce blood clotting specifically in tumor blood vessels, with resulting occlusion of the vessels and tumor infarct[42].

Tissue-penetrating peptides—We have recently described a tissue-penetrating transport pathway that can be activated with peptides and can be made disease-specific. The key element in the tissue-penetration is the sequence motif R/KXXR/K, which binds to neuropilin-1 (NRP-1), a co-receptor for VEGF. We have named R/KXXR/K the C-end Rule (CendR) motif, because the second arginine or lysine residue has to be C-terminal for the motif to bind to NRP-1[43]. When the CendR peptide binds to NRP-1, a cell internalization and trans-tissue transport pathway is activated. This pathway can transport payloads ranging from small molecules to NPs. Most importantly, it can be activated in a tissue-specific manner by making the CendR motif cryptic and delivering it to the target tissue. The tissue specificity (in this case tumor-specificity) was accomplished with the prototypic peptide of this class, iRGD[2],[3]. The sequence of iRGD is CRGDKGPD (the lysine residue can also be an arginine, and the aspartic acid a glutamic acid) and it is cyclized through a disulfide bond between the cysteine residues. This peptide homes to tumors because it contains the integrin-binding RGD motif. What makes it special, however, is the basic residue after the RGD, which creates an internal, cryptic CendR motif (RGDR or RGDK). The multiple steps that make a cryptic CendR peptide a tissue-specific activator of the NRP-1-dependent pathway are as follows[2]: (1) a peptide containing an internal R/KXR/K motif binds to a vascular receptor specific for a target tissue (RGD binding to αvβ3 and αvβ5 in the case of iRGD); (2) as a result of this initial binding at the target, the peptide is proteolytically processed to convert the internal R/KXR/K motif into a C-terminal one; (3) the C-terminal R/KXXR/K motif binds to NRP-1, inducing the transport pathway out of the blood vessels and through the extracellular tissue (Fig. 1). The peptide can carry a payload that is either covalently coupled to the peptide, or the payload can simply be administered together with the peptide because the endocytic bulk transport pathway triggered through neuropilin-1 sweeps along bystander molecules[3].

The cell surface binding mediated by the integrin binding is needed for the proteolytic step to occur in cell culture[2], explaining why the CendR motif of iRGD is only activated in tumors *in vivo*. The protease that activates iRGD and other cryptic CendR peptides has not been identified. In fact, more than one protease capable of cleaving after a basic residue may be involved, as these peptides can be activated *in vitro* by trypsin[43]. However, furins are likely candidates because the CendR motif conforms to their consensus substrate. Interestingly, furin expression has been shown to be elevated in tumors and has been used

for tumor targeting[44]. NRP-1 is often also highly expressed in tumors[45]. Thus, high furin and NRP-1 may add to the tumor specificity created by the RGD-integrin interaction.

A number of homing peptides described earlier may be tumor-penetrating peptides similar to iRGD. That this is the case with LyP-1 (CGNKRTRGC; [32]) has been shown [46]. Surprisingly, the truncated version of LyP-1 with an active CendR motif exhibited a degree of tumor selectivity[46]. Although RGD peptides with a basic residue following the RGD motif bind poorly to integrins[2], a peptide resembling the CendR fragment of iRGD (RGDK) has been reported to selectively home to tumors [47]. It may be that a combination of over-expression of neuroplin-1, which is common in tumors, with even a weak binding to a tumor-specific component can render a peptide partially selective for tumor homing. Two other tumor-homing peptides, F3 (KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK; Porkka et al., 2002) and CRGRRST [13] also contain potential CendR sequences (underlined). We used quantum dots coated with the LyP-1 and F3 peptides in our early NP studies to show the feasibility of *in vivo* targeting[48].

Many of the well-known cell-penetrating peptides (CPPs), such as the Tat peptide, contain CendR motifs. However, the CPPs are not cell-type specific[49], they enter into all types of cells. Likely reasons for the lack of cell type-specificity include the following: First, The CendR motif of a CCP may be irrelevant, because the CPP uses a ubiquitous pathway different from the CendR pathway. Second, The CendR motif may be active (C-terminal) in the CPP causing internalization to all cells expressing NRP-1 (or NRP-2). Third, a CPP may bind to a ubiquitous primary receptor causing activation of its cryptic CendR motif at the surface of all cells. Tumor-homing variations of CPPs have been engineered. In one peptide, the cell-binding activity of the positively charged Tat peptide is neutralized by a tethered negatively charged amino acids, until a tumor protease cleaves the tether, reversing the blockade[50]. Others have combined a CPP with a tumor-homing peptide, and somewhat surprisingly, the homing peptide activity overrides the universal internalizing properties if the CPP and the chimeric peptides become selective for tumors[51],[52].

We have shown, particularly for the prototype cryptic CendR peptide iRGD, that the delivery of therapeutic and diagnostic agents specifically into tumors can be greatly enhanced with these peptides[2],[3],[53],[46]. This difference is particularly striking when the vascular homing peptide also binds to the cells in tumor parenchyma, as this helps retain the peptide and drive its further spreading within the extravascular tumor tissue. For example, the receptors for iRGD, integrins and NRP-1, are expressed in tumor vessels, as well as on the various other cell types present in the tumor mass, including usually the tumor cells[2]. The studies described above focused on tumor-homing CendR peptides. However, homing peptides for other diseases and tissues exist[54], and the neuropilin CendR receptors are ubiquitously expressed, albeit that the expression tends to be higher in tumors[45],[46]. Thus, there seems to be no reason why disease- and tissue-specific CendR peptides could not be obtained for purposes other than cancer targeting. For example, in addition to tumors, LyP-1 specifically homes to atherosclerotic plaques and penetrates into them, but does not recognize normal vessels[55].

A major advantage of the CendR system is that by allowing effective extravasation and tissue penetration, a CendR peptide makes more of the target tissue (such as a tumor) available for a therapeutic agent than would be the case with targeting elements that lack the CendR properties. The ability of CendR peptides to promote tissue entry and accumulation of compounds that are not conjugated with the peptide (by-stander effect) provides additional unique advantages: First, it is not necessary to create a new chemical entity to target a drug by CendR peptide co-administration, as is the case when a drug is coupled to a targeting element. This greatly simplifies the path to clinical application. Second, the

amount of any given receptor in a target tissue is likely to be quite low, and this is a major limitation of synaphic targeting of drug conjugates[56]. For example, if one assumes that a gram of tumor tissue contains 10^9 cells, and they express an average of 100,000 receptors per cell, there would be roughly 170 pmoles of the receptor in that tumor mass. Only a fraction of the total receptor is likely to be available for the binding of a targeting probe. For many drugs, the required tissue concentration is much higher than a few picomoles per gram of tissue, which means that only a limited amount of a covalently coupled conjugate can be specifically targeted. The by-stander effect does not have this limitation, as the targeted receptor is only used to activate the CendR transport pathway.

The CendR pathway has only been partially characterized so far. It is an endocytic pathway, and marker studies suggest that the CendR endosomes are not related to any of the well-known endosomal vesicles[43]. The fact that this pathway, once activated, sweeps along by-stander molecules and even Nps, suggests a relationship to pinocytosis. Although this has not been directly observed, the CendR vesicles may also be exocytic because it would be difficult to explain the tissue penetration if the payload would only enter cells and not come out of them. The pathway is an active transport pathway; it requires energy and is much faster than what could be explained on the basis of diffusion[3].

NP targeting

Homing peptide-targeted NPs—NPs can incorporate unique functions that cannot be engineered into simple drugs. One such function is selective homing to a target. Coating the surface of NPs with a targeting element, such as a homing peptide, confers the NPs specific affinity to the intended target tissue. NPs with a surface studded with binding elements have been dubbed nanoburrs[57], a term that coveys the idea of binding, but does not quite cover the specificity of the binding.

Vascular targeting is particularly advantageous with NPs, which have limited ability to exit the vasculature. There is a vast literature on passive NP targeting to tumors through the so-called enhanced permeability and retention (EPR) effect, but EPR is not very effective, and its size-dependency, slow time frame, and variability from tumor to tumor limit its usefulness ([58],[59]. High interstitial pressure and fibrosis constitute additional barriers to the access of NPs to the interior of tumors[60],[61],[62]. Furthermore, EPR is unlikely to operate in non-tumor vascular beds. For these reasons, it is important to design systems that initially target the vasculature, rather than the parenchymal cells.

The luminal side of vessels is fully accessible to NPs circulating in the blood and the vessels can serve as a gateway to the interior of the targeted tissue. NPs are an ideal payload for homing peptides because the presentation of multiple copies of the peptide on the NP surface makes possible multivalent binding. The high avidity resulting from the multivalency compensates the generally moderate affinity of peptides. In addition, elongated shape, such as that of iron oxide nanoworms can enhance homing peptide-mediated binding of NPs to the surface of cells, presumably because more interactions between the two surfaces are possible than when the NP is spherical[63]. Since we use phage display to find homing peptides, and the phage is a NP and the display is multivalent[6], the peptides identified in this manner are already selected for NP homing.

The “dark” side of NP multivalency is that all surface features in them are presented in a multivalent fashion, providing potential recognition signals for the reticuloendothelial system (RES). RES, which is also known as mononuclear phagocyte system (MPS), eliminates foreign materials, such as NPs from the circulation by capturing them into the liver and spleen. Minimizing NP uptake by RES is critical to ensure effective drug delivery. Various ‘stealth’ coatings, and modifying the shape and size of the particles can mitigate this

problem (e.g. ref. [64]), but only delay the inevitable; NPs eventually end up in the RES. However, any delay in the RES uptake of NPs is important because it gives them more time to reach the intended target. The RES remains a major problem in nanomedicine (reviewed in ref. [65]),[56], and better solutions for this problem are likely to come from more complete understanding of the RES phagocytosis process at the molecular level.

Amplified tumor homing of NPs—While both drugs and NPs can be targeted, NP targeting can be enhanced by engineered more complex, cooperative targeting functions into them. We have described NPs that self-amplify their own homing to tumors. Superparamagnetic iron oxide NPs (SPIO) are coated with a pentapeptide (sequence: CREKA) that binds to fibrin-fibronectin complexes deposited in tumor blood vessels (and tumor stroma) as a result of subtle clotting[38]. These deposits are not present in normal vessels, making the homing tumor specific. The initial accumulation of the CREKA-SPIO in tumor vessels causes additional clotting in these vessels, which creates new binding sites for additional NPs, which causes more clotting, and so on. This self-amplifying homing system occludes about 20% of tumor vessels, and the enhancement factor from the amplification is about 6 fold[38]. The amplified NP homing greatly enhanced tumor imaging, but the level of vessel occlusion was not sufficient for significant inhibition of tumor growth. Recent improvements to the system have made it possible to occlude 60–70 % of tumor vessels, resulting in tumor necrosis and growth inhibition[66]. A clotting activator (tissue factor) that is targeted to tumors with a homing peptide has been introduced into clinical trials[42]. The CREKA system has the advantage that the NPs could be loaded with a drug to further enhance its anti-tumor potency.

Integrating multiple functions into a single NP can reduce the efficacy of the individual functions. For example, we have found that a two-fold reduction of homing peptide density on an NP can drastically reduce the efficacy of tumor homing[66]. Dividing the functions between two NPs that then cooperate in their functions can circumvent these problems. In one study, we coated two different tumor homing peptides with partially non-overlapping selectivity for tumor vessels onto separate SPIO NPs. Surprisingly, when the two NPs were injected together into the circulation of tumor mice, they completely colocalized producing a wider distribution of each NPs than was obtained with either one alone. This phenomenon, which presumably depends on an attractive force between the NPs, allowed us to greatly increase the number of tumor vessels occluded by the CREKA-SPIO[66]. The clotting promoting activity of CREKA-SPIO in tumors requires three elements: (1) The NP has to be slightly thrombogenic, as is the case with SPIO. Micelles coated with CREKA home to tumors, but do not cause additional clotting. (2) The CREKA peptide is necessary; SPIO coated with another tumor-homing peptide also home to tumors, but have no clotting activity in tumor vessels. (3) The pro-coagulant tumor environment is needed. CREKA-SPIO NPs accumulate in the liver RES, but no occlusion of liver vessels has been observed. CREKA-SPIO also bind to the endothelium over atherosclerotic plaques, where subtle clotting produces fibrin-fibronectin complexes, but cause no additional clotting[39],[66]. Thus, this approach to occlude tumor vessel occlusion seems to be safe.

In another strategy, photothermal heating mediated by tumor-targeted gold nanorods increased the expression of the receptor for a homing peptide coated onto a second, drug-carrying NP[67],[68]. The clotting cascade also lends itself to cooperating NP designs[69]. A combination therapy with NPs that carry different drugs would be another possible application of cooperating NPs. Designing NPs that aggregate under the influence of a feature of the target tissue, such as the expression of an enzyme, is another way of constructing an amplified targeting system, as aggregated NPs are not likely to wash out from a target tissue. Interestingly, one such system utilizes furin cleavage as the triggering

mechanism for NP aggregation[70],[71], which may benefit both from the aggregation and a CendR effect.

In some cases, it will be possible to replace nanosystems that use multiple NPs with multi-compartment NPs, which enable co-presentation of dissimilar properties on the same particle. For example, the requirement of high homing peptide concentration on a NP for optimal homing could be satisfied by coating a compartment of a NP with the peptide, leaving the rest of the surface available for other function[72].

NP biocompatibility—*In vivo* use of NPs for medical purposes places strict requirements on biocompatibility and lack of toxicity. Iron oxide NPs are clinically approved for imaging applications and for the treatment of anemia caused by iron deficiency. Organic NPs (Abraxane and Doxil) are being used in cancer therapy. However, many of the inorganic NPs now studied in animals, such as various types of quantum dots and carbon nanotubes are likely to face high regulatory hurdles. Porous silicon NPs exhibit a favorable toxicity profile as they degrade *in vivo* producing silicic acid, which is a physiological compound eliminated through the urine[73]. The non-toxicity, high capacity for cargo, and quantum dot-like optical properties make porous silicon NPs a promising material for the design of next generation NPs.

Tissue-penetrating NPs—As discussed above, tissue-penetrating peptides can induce exit from the blood vessels in the target tissue and transport through that tissue, particularly when the receptor for the peptide is shared between the vascular and parenchymal cells. The tumor-homing iRGD peptide is an example of a probe that fulfills these criteria. The results with NP targeting have been particularly striking because NPs are much larger than peptides or proteins, and that hinders their diffusion out of the blood vessels.

Coating of Abraxane with the iRGD or LyP-1 peptide resulted in enhanced accumulation and several-fold higher activity than seen with the original drug[53],[2]. It was also shown that the iRGD effect on Abraxane, and on doxorubicin liposomes, could be achieved by simple co-administration of the peptide with the NPs, without having to couple the two together[3].

Recently, we have used iRGD in the co-administration mode to construct a nanoplatform for the delivery of a drug into glioblastomas[74]. The system consists of elongated iron oxide NPs (nanoworms; NWs; [63], which are coated with a bifunctional peptide through a PEG linker. One branch of the peptide, CGKRK is a tumor-specific vascular homing element[13], and the other branch is $_{D}$[KLAKLAK]$_2$, a membrane perturbing pro-apoptotic D-amino acid peptide, which serves as a drug. We have previously shown that the $_{D}$[KLAKLAK]$_2$ peptide can be targeted to tumors and other disease sites by directly conjugating it to a homing peptide[75],[76]. The conjugates were effective, but also highly toxic. Unexpectedly, we found that the NP-bound $_{D}$[KLAKLAK]$_2$ was hundreds of times more potent in killing cells in culture than the soluble form. Standley et al., (2010)[77] have also reported high efficacy of multivalent $_{D}$[KLAKLAK]$_2$. The greatly increased specific activity of the NP-bound pro-apoptotic peptide was important in that it made it possible to reduce the dose of the peptide. The lower dose in turn brought the dose needed into the range that would not be expected to overwhelm the CGKRK receptors. The resulting improvement in targeting reduced the side effects to a moderate increase in enzyme markers of liver damage, which was reversible upon termination of the treatment. Another significant feature of the CGKRK-$_{D}$[KLAKLAK]$_2$ system that the homing peptide directs the pro-apoptotic $_{D}$[KLAKLAK]$_2$ peptide to the subcellular organelle this pro-apoptotic peptide acts on, the mitochondria. CGKRK binds to mitochondria, and it apparently also has cell

penetrating properties, because the NWs were seen in association with the mitochondria of the target cells[74].

The CGKRK-$_D$[KLAKLAK]$_2$ nanosystem eradicated the tumors in one glioblastoma model and significantly extended the life span of the mice in another, more aggressive model [74]; Fig. 2). When the nanosystem was combined with iRGD injections, there was a further, highly significant extension of survival of the tumor mice with the aggressive tumors. CGKRK is an internalizing peptide, but it lacks tissue-penetrating properties. Accordingly, the NWs, when injected alone, stayed in the blood vessels. However, when co-injected with iRGD they extravasated (Fig. 3). Thus, the likely reason for the improvement of the therapy results is that by using iRGD, we had made the tumor cells a secondary target in addition to the vasculature. Importantly, these results also suggest that iRGD can help a therapeutic agent penetrate the blood-brain barrier in glioblastomas, albeit that the barrier may be somewhat compromised in the tumors. A somewhat analogous nanosystem consisting of the membrane-disrupting toxin melittin targeted to tumors with perfluorocarbon NPs coated with an RGD-mimic compound has been described[78]. Interestingly, melittin contains a cryptic CendR motif. It would be interesting to know whether activation of the CendR system contributed to the anti-tumor activity of these NPs.

Subcellular targeting of NPs—The CGKRK-$_D$[KLAKLAK]$_2$ described above is an example of combined targeting that encompasses systemic delivery of a NP payload to a target tissue (tumor vessels), internalization into cells, and further delivery to a specific subcellular organelle. The entire process was accomplished with one peptide[74]. Similar targeting has been accomplished in cell cultures by coating NPs with cell-penetrating peptides fused with organelle localization signals for the nucleus (e.g. KKKRK[79]) and mitochondria[80]. An extremely important question concerns the delivery of NP payloads into the cytoplasm. Therapies that rely on nucleic acids, such as siRNA, have tremendous potential in making previously “undruggable” molecules accessible as treatment targets. Unfortunately, the application of this technology has been hampered by the unsolved problem of delivering the compounds to the target. The main problem is the instability of these compounds *in vivo*. A likely solution will come from nanotechnology. NPs can protect the compounds, carry them to the target cells and deliver the intact compound into the cytoplasm. An encouraging advance has been the recent report of successful delivery of siRNA to the tumor of a human patient[81].

Beyond tumor penetration: Tissue penetration by peptides has also been observed in tissues other than tumors. LyP-1, which has been shown to be a tumor-penetrating peptide that depends on the CendR mechanism[46], also homes to atherosclerotic plaques, penetrating into the plaque interior[55]. Iron oxide NPs and protein cage NPs have been show to enter plaques when coated with LyP-1[55, 82]. Moreover, peptides in a panel of heart-homing peptides, which were also found to penetrate into extravascular heart tissue, contain CendR sequences[20], and likely also use the CendR pathway. Finally, it is striking that a number of peptides reported to cross the blood-brain barrier also contain cryptic CendR sequences[83],[84],[85]. Taken together with the results on glioblastoma discussed above this suggests that CendR sequences activated at the brain endothelium may be able to cross the blood-brain barrier and take a payload, even NPs, with them. Table 1 lists the many types of NPs and tumor-penetrating peptides that have been used to deliver compounds to various targets.

Conclusion and future prospects

Poor tissue penetration of NPs limits the application of NPs to the treatment of disease. The capacity of the receptors at the target tissue poses a further limitation to the number of NPs

that can be specifically targeted to a target tissue. The tumor-penetrating peptides we have recently described can solve these problems. These peptides activate a bulk tissue-specific transport pathway in that once activated is not limited by the availability of specific receptors for the targeted NPs. The specificity of the peptide determines the tissue the pathway is activated in, resulting in target specific delivery of compounds that are co-administered with the peptide. A current challenge that is likely to only be resolved by nanomedical approaches is the delivery of nucleic acid-based therapeutics. Substantial progress has already been made in this area, but efficacious subcellular delivery still remains to be resolved. Finally, a major future advance in nanomedicine would be engineering NPs in which binding to a target would elicit an activity, such as release of a drug.

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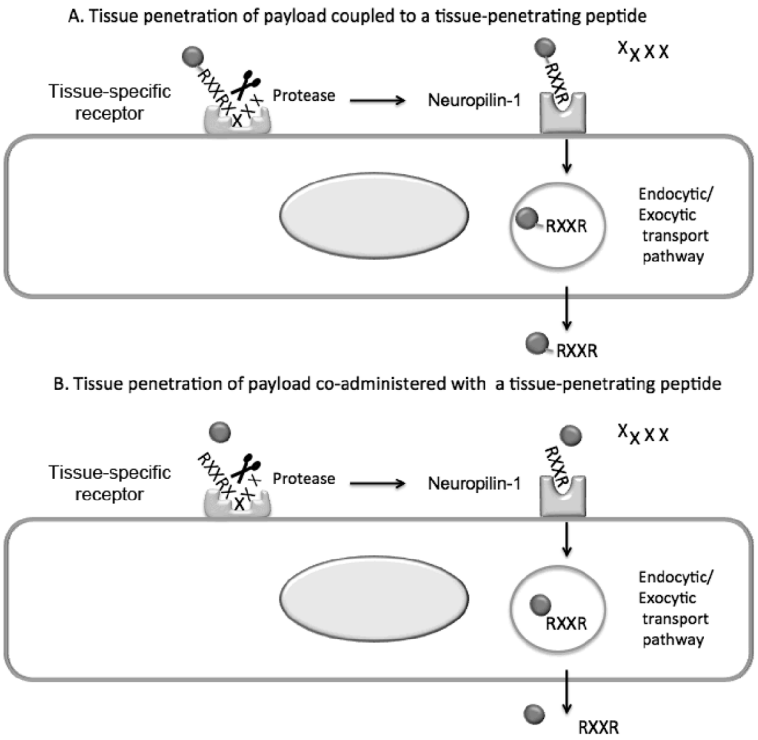

Fig. 1. Schematic representation of the trans-tissue transport pathway induced by tissue-penetrating peptides of coupled and co-administrated payloads[43],[2],[3]

Note that the exosome aspect of the pathway shown in the figure is an inference from the properties of the pathway and has not been directly observed. See the text for detail.

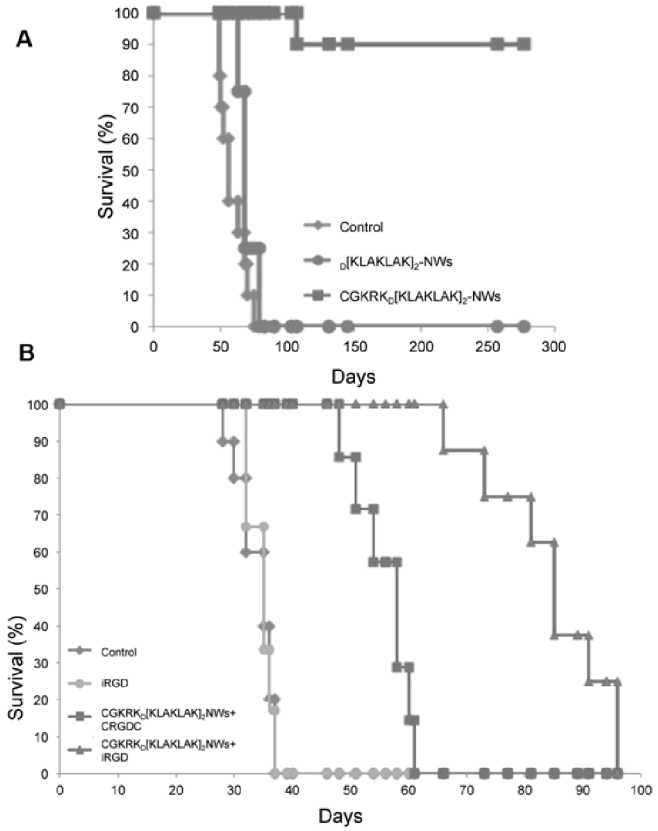

Fig. 2. Glioblastoma treatment with CGKRK$_D$[KLAKLAK]$_2$-nanoworms (NWs) in mice

A. Mice bearing lentiviral (H-RasV12-shp53) induced brain tumors[86],[87] in the right hippocampus were intravenously injected with iron oxide NWs coated with peptides (5 mg of iron per kg). The particles were administered every other day for 18 days, starting 3 weeks post-viral injection. Survival curves (n=8–10 mice per group) shows rapid demise of the mice in the control groups and long-term survival of the group treated with CGKRK$_D$[KLAKLAK]$_2$ NWs. B. Mice bearing orthotopic 005 tumors implanted 10 days earlier (n=8–10 mice per group) received every other day for 3 weeks intravenous injections of either CGKRK $_D$[KLAKLAK]$_2$-NWs (5 mg of iron/kg), or CGKRK $_D$[KLAKLAK]$_2$-NWs (5 mg/kg) mixed with 4 mmol/kg of iRGD or PBS. CGKRK $_D$[KLAKLAK]$_2$-NWs have a strong anti-tumor effect, which is further enhanced when the NWs are co-injected with iRGD. In contrast to the CGKRK$_D$[KLAKLAK]$_2$ nanosystem, a number of other treatments, such as various anti-angiogenic agents have shown no activity in these models[87] (modified from ref. [74]).

* Note: figure 2 above first appeared in the *Proceedings of the National Academy of Sciences*, in an article by Agemy et al 108(42) P. 17450–5, 2011, PNAS October 18, 2011. PMCID: PMC3198371. Copyright PNAS. Permission for use has been granted for this *Advanced Materials* article.

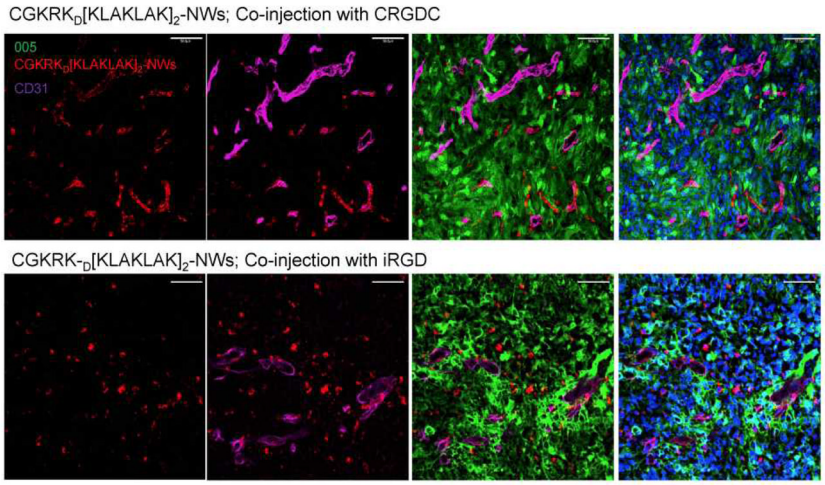

Fig. 3. Enhanced penetration of CGKRK $_D$[KLAKLAK]$_2$-NWs co-injected with iRGD into extravascular glioblastoma tissue

Mice bearing orthotopic 005 glioblastomas[87] were intravenously injected with CGKRK $_D$[KLAKLAK]$_2$-NWs (5 mg of iron/kg) in combination with 4 mmol/kg of either non-labeled CRGDC (upper row) or iRGD (lower row) peptide. The tumors and tissues were collected 5–6 hours later, and analyzed by confocal microscopy. CGKRK $_D$[KLAKLAK]$_2$-NWs (red) are found outside the blood vessels (magenta), associated with tumor cells (green) when co-injected with iRGD, but remained associated with blood vessels when co-injected with the conventional RGD peptide, CRGDC, which lacks tumor-penetrating properties. Nuclei were stained with DAPI (blue). Scale bars, 50 mm. Modified from ref. [74].

* Note: figure 3 above first appeared in the *Proceedings of the National Academy of Sciences*, in an article by Agemy et al 108(42) P. 17450–5, 2011, PNAS October 18, 2011. PMCID: PMC3198371. Copyright PNAS. Permission for use has been granted for this *Advanced Materials* article.

Table 1

Nanoparticle delivery with tumor-penetrating peptides

Nanoparticle	Target	Peptide	Reference
Quantum dots	Tumors, lungs	LyP-1, F3*	[48]
Micelles	Tumors	LyP-1	[53]
Iron oxide NPs	Tumors, atherosclerotic plaques	iRGD, LyP-1, F3	[2], [2, 3] [55] [88]
Protein cage NPs	Atherosclerotic plaques	LyP-1	[82]
Albumin-paclitaxel NPs (Abraxane)	Tumors	iRGD	[2], [3]
Doxorubicin liposomes	Tumors	iRGD, LyP-1	[73] [3]
Bismuth sulfide NPs	Tumors	LyP-1	[89]
Hydrogel NPs	Tumors	F3	[90]

* F3 contains a CendR motif and internalizes into cells, but the involvement of the CendR pathway in F3 activities has not been formally proven.