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NRP1 transport of cancer therapeutics mediated by tumor-penetrating peptides

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Summary

Whereas uptake of low molecular weight agents is generally inhibited in tumors due to high interstitial pressure, tumor uptake of macromolecules is increased due to enhanced permeability and retention (EPR). Small molecule drugs alone or incorporated in nanoparticles (NP) have largely been dependent on such physical tumor uptake (passive) for therapeutic activity. Although passive targeted NP such as Stealth Liposomal Doxorubicin (Doxil ®) are effective with improved safety, drug delivery to tumors is still significantly limited. To improve tumor delivery and efficacy, tumor-penetrating peptides (TPP), which contain sequences that target the tumor and activate the neuropilin-1 receptor (NRP1), have either been co-administered with or conjugated to both small and large therapeutic molecules. In this review, we will discuss TPP-mediated therapeutics which target the NRP1 transport system of tumors.

Keywords

neuropilin-1 receptor; tumor-penetrating peptides; tumor; endocytosis; transport

Introduction

By targeting tumors and their angiogenic vessels, tumor penetrating peptides (TPP) have primarily been isolated by an *in vivo* screening T7 phage display method (1–8)(see review by (9)). In 2002, Laakkonen and colleagues utilizing the T7 phage identified the LyP-1 peptide as a TPP. With this screening approach, the phage displaying LyP-1 accumulated 15- and 60-fold higher in KRIB and MDA-MB-435 tumors, respectively, than a control non-targeted phage (10). Fluorescently labeled LyP-1 peptide or phage particles injected intravenously had widespread intratumoral distribution in different tumor models, with a particular affinity toward tumor lymph vessels. In contrast to control phages, rapid distribution of LyP-1 targeted phage particles within the tumor indicated a transport process other than simple diffusion (1, 10). Subsequently, the CendR sequence (R/KXXR/K) within

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DISCLOSURE

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LyP-1 was identified as a critical component enabling vascular and tumor transport of macromolecules (8, 11). Despite different patterns of localization within tumors than LyP-1, other tumor penetrating peptides such as iRGD share the important property of rapid and widespread tumor distribution (8), and further corroborated the importance of the CendR sequence. With recognition of the essential role of CendR and its activation of the NRP1 pathway, further studies were designed to understand this pathway and develop improved tumor-penetrating peptides. Here, we review TPP-mediated therapy associated with the NRP1 transport system, that is upregulated in angiogenic tumor blood vessels and frequently in tumor cells.

Tumor-Penetrating Peptides

Since LyP-1, there have been two additional tumor penetrating peptides, iRGD and iNGR, which have been identified. Whereas iRGD was also isolated by phage display, iNGR was designed de novo combining an angiogenic targeting NGR peptide with the CendR sequence (1, 4, 7, 8, 11, 12). These TPP had an essential role validating the importance of the CendR sequence in the widespread intratumoral distribution of TPP. Sequences of LyP-1, iRGD, and iNGR are CGNKRTRGC, CRGDKGPDC, RNGRGPDC, respectively, with the CendR motif underlined. With the exception of the truncated linear form of LyP-1 (tLyP-1: CGNKRTR) (12), cyclic forms of the peptides give optimal binding and enhanced tumor specificity. For use as ligands for targeting small and large molecules, TPP generally require three motifs: one for tumor homing, one for enzymatically processing, and one for tumor penetration (Figure 1). Moreover, for tumor homing of nanoparticles, the iRGD, iNGR, and LyP-1 peptides initially bind to $\alpha_v\beta_3/5$ integrins, CD13, and p32 receptors, respectively, on angiogenic vessels of tumor tissues (13, 14). Interestingly, in contrast to the cyclic LyP-1, the truncated linear form does not bind p32, yet it still targets the lymph vessels of tumors (12). In addition to these TPP, there are other peptides with the CendR sequence which appear to have similar tumor penetrating properties (15, 16).

After the tumor-specific components of the TPP such as iRGD binds to a specific receptor on the surface of the tumor endothelium (i.e., $\alpha_v\beta_3/5$ integrins), limited proteolytic degradation of the cyclic peptide exposes the CendR sequence. It is likely that proprotein convertases, such as furin or furin-like enzymes (i.e., PACE4), proteolytically cleave cyclic TPP (17, 18). This is based on several lines of evidence including 1) recognition and cleavage of the CendR sequence by furin; 2) elevation of furin in more aggressive cancers (19); and 3) processing of class III semaphorin by furin with subsequent activation of NRP1. Trypsin-like enzymes (i.e., matriptase), elevated in many cancers (20), may also have a role in processing TPP, peptides, and proteins to activate NRP1 (8, 20). After proteolytic cleavage, TPP with a K/RXXK/R motif containing a C-terminal carboxyl end (11) bind and activate NRP1 (9, 21) on tumor vessels and on tumor cells, leading to marked accumulation of TPP within the tumor. Similarly, conjugates of TPP, that include a therapeutic agent, can bind and activate the pathway of NRP1, thereby enhancing extravasation of the agent into the tumor. Once past the endothelial barrier, the iRGD peptide primarily targets NRP1-expressing tumor cells (22), whereas LyP-1 targets p32 enriched elements of the tumor such as lymph vessels and macrophages, as well as tumor cells (1, 10, 13). The distinct distribution pattern of LyP-1 in the tumor may also be due that the protease-processed

peptide (similar to tLyP-1) binds not only to NRP1 but also to the NRP2 homologue, which has increased expression on the surface of lymph vessels (12).

In addition to TPP, cell penetrating peptides may bind to NRP1 and in selected cases, activate the NRP1-mediated endocytic pathway. Kadonosono et al. found that cell penetrating peptides fused to fluorescent proteins had reduced accumulation in tumors (about 50%) of mice that had received a prior injection of an antibody to NRP1 (23). Interestingly, no increase in vascular permeability was observed with these cell penetrating peptides *in vivo*, suggesting that these peptides were able to bind NRP1 without activating NRP1-transport pathways. Recently, Pang et al. determined that specific cell penetrating peptides and TPP, based on their C-terminal sequences, may share the same macropinocytosis pathway (21). By examining cell penetrating peptides such as TAT with different C-terminal ends (TAT-CO₂H and TAT-NH₂) and a NRP1-dependent peptide (RPARPAR-CO₂H), two distinct endocytic pathways were discovered to be responsible for the uptake of the cell penetrating peptides. One macropinocytosis transport pathway was dependent on NRP1 whereas the classical macropinocytosis pathway was dependent on heparin sulfate (HS). Whereas TAT-NH₂ was taken into the cell solely by the HS pathway, TAT-CO₂H was transported into the cell by both pathways. They found that peptides which had a CendR motif (i.e., TAT-CO₂H) enhanced vascular permeability, yet those lacking the CendR motif did not (TAT-NH₂) (21).

NRP1 in Tumor Pathology

NRP1 is a 120–140 KDa transmembrane pleiotropic receptor with multiple domains that primarily exists as a homodimer. It functions as a co-receptor for a number of extracellular ligands including some isoforms of VEGF, class 3 semaphorins, hepatic growth factor, FGF and TGF- β (see reviews of Graziani and Lacal, (24); Guo and Kooi, (25)) (26–29). Whereas class 3 semaphorins bind to the a1/a2/b1 domains of the NRP1 receptor, growth factors such as VEGF₁₆₅ bind exclusively to its b1/b2 domains. It is also the b1/b2 domain which TPP bind and activate the NRP1 co-receptor. Moreover, growth factor receptors such as VEGFR interact with NRP1 through dimerization in their extracellular domains. Whereas levels of NRP1 are markedly increased in the angiogenic vessels of tumors (30), levels of NRP1 in tumor cells are elevated with more invasive and advanced stages of cancer. Because NRP1 is universally elevated in angiogenic tumors and is associated with invasiveness of the tumor, several therapeutic strategies including siRNA, monoclonal antibodies, and small molecule inhibitors have been used to inhibit the activity of NRP1 in tumors (31–33). For instance, in murine tumor models with low NRP1-expressing tumor cells yet elevated NRP1-expressing angiogenic vessels, monoclonal antibodies, that target NRP1 and VEGF-A, had additive effects in reducing tumor growth and prolonging the survival of tumor-bearing mice (33).

In tumors and their vessels, the most studied interaction of the NRP1 co-receptor is with VEGFR1/2 and VEGF₁₆₅, resulting in promotion of tumor angiogenesis, cell invasion, and metastases. The complex of VEGF₁₆₅-VEGFR2-NRP1 also has a role in the development of lymph vessels in tumors (34–36), although the interaction of VEGFC/VEGFD-VEGFR3-NRP2 has a much more essential role in their development and proliferation. In addition to the VEGF-mediated pathway, other growth factors (i.e., FGF, HGF, TGF- β) and receptors

may interact with NRP1 on tumor and stroma cells (i.e., immune and endothelial cells, fibroblasts) to promote tumor aggressiveness (26–29). Independent of growth factors and their cognate receptors, TPP through interaction with NRP1 reduced tumor cell migration and metastases (discussed in next section) (37).

Tumor-penetrating Peptides Decrease Metastasis

Since TPP activate NRP1-mediated transport including markedly enhancing vascular permeability of the tumor, concerns have been raised about whether TPP might increase metastases. However, these potential fears about TPP increasing metastases from primary tumors have recently been allayed by the finding that the peptides reduced metastases (37). In a murine tumor model that demonstrated spontaneous metastases, both iRGD and iNGR–treated mice had a marked reduction of metastases compared to controls. Also, the reduction was shown in both prostate and pancreatic murine tumor models. In contrast, the size of the primary prostate and pancreatic tumors was not significantly affected by every other day treatment with iRGD. The decrease in the number of metastases did not seem to be mediated by either the RGD or NGR component of the tumor penetrating peptide but rather by the CendR motif. As a result, peptides without the CendR motif such as cRGD or cNGR had little effect on the number or extent of metastases. Moreover, blockade of NRP1 with an antibody reduced the anti-metastatic activity of the infiltrating peptides. Upon treatment with iRGD, cell surface protrusions and migration of tumor cells were markedly reduced in contrast to cells treated with peptides that lack the CendR motif (cRGD). The investigators further determined that NRP1 regulates $\beta 1$ integrin expression by the tumor cells, which may have a significant role in the reduction of metastases (37).

In a subsequent study, Hamilton and colleagues compared the efficacy of the iRGD peptide with iRGD-conjugated nanoworms in preventing brain metastases (38). Administration of iRGD-nanoworm conjugates, six days after inoculating breast cancer cells, reduced significantly the number of brain metastases, whereas administration of iRGD had no statistical effect. Consequently, the nanoworm provided a template to accentuate the anti-metastatic effects of the iRGD peptide. Moreover, the iRGD nanoworms were markedly more effective at reducing the number of brain metastases compared to cyclic RGD (cRGD) peptide targeted-nanoworms (38). Similar to its importance in reducing metastasis with iRGD in the above mentioned study (37), the CendR motif has a significant role in the reduction of metastases with iRGD targeted nanoworms.

It is also likely that the LyP-1 peptide reduces tumor metastases. LyP-1, which has cytotoxic effects and induces apoptosis in tumor cells, significantly reduced the size of primary tumor xenografts when administered intravenously. More germane to metastasis, LyP-1 markedly reduced the number of lymph vessels in the primary tumors, a common route for the spread of tumor cells (10).

NRP1-mediated Transport of Molecules through Vessels and Tumors

Since NRP1 is universally upregulated on the surface of tumor angiogenic vessels, TPP binding and activation of NRP1 provides an explanation for their transport through the

endothelium with subsequent widespread distribution in tumors. The blood vessel endothelium is a major barrier for efficient delivery and transfection of tumor cells within tumor tissues, and NRP1-mediated transport of CendR molecules through the endothelium is at least necessary for widespread distribution in the tumor. It has been postulated that endothelial NRP1-mediated transport enables nutrients to be taken up in calorie-restricted tissues such as tumors (39). Independent of influences by the tumor, Teesalu and colleagues determined that the CendR peptide, RPAPAR, injected intradermally, induced vascular permeability as evidenced by accumulation of Evans blue at the site of the peptide injection (11). To determine the role of this peptide and NRP1, pre-injecting with NRP1 antibody intravenously blocked the vessel permeability induced by RPAPAR. Similar results of increased vascular permeability were observed with class 3 semaphorin which also contains a CendR motif, providing additional evidence that vessel permeability is mediated by NRP1.

Based on studies thus far, it is highly likely that tumor cells which express NRP1 are important for efficient distribution of TPP and associated molecules. At least four lines of evidence support that NRP1-expressing tumor cells are essential for widespread intratumoral transport. First, with NRP1 positive tumor cells, fluorescently labeled silver nanoparticles showed widespread distribution in cells grown as 3D spheroids compared to monolayers (39). Second, by restricting nutrients to the tumor and thereby up-regulating NRP1 on the surface of the tumor spheroids and tumor tissue slices (39), transfer of silver particles was markedly enhanced between cells. Consistent with the *in vitro* experiment, an inhibitor of the GLUT1 glucose transporter, WZB117, administered intratumorally enhanced tumor accumulation of a fluorescently labeled TPP targeting NRP1. Third, the rapid and widespread distribution of the nanoparticle in the tumor matrix with no increased accumulation near its blood vessels suggests that the mechanism may involve more than tumor endothelial cells (11). Fourth, dextran (10 KDa) accumulation in a low NRP1-expressing tumor (SUIT-2) was similar with and without co-administration of iRGD (22). In contrast, iRGD markedly increased dextran accumulation and penetration in pancreatic tumors that expressed high levels of NRP1. Nonetheless, more studies would be of value comparing accumulation of iRGD-mediated agents in tumors expressing varying levels of NRP1.

Mechanism of NRP1-mediated transport

Although it appears that the uptake of TPP in tumor tissues is by a macropinocytosis-like mechanism, the energy-dependent and rapid trans-tissue penetration of these molecules is not fully understood (7, 39). The macropinocytosis-like pathway activated by NRP1 appears to be separate from other well-defined endocytotic pathways. Blockade of NRP1 with antibodies reduces endocytosis of CendR-containing macromolecules known to activate NRP1 in tumor and endothelial cells. Upon binding of CendR peptides, including conjugates with therapeutic cargo, to NRP1, the NRP1/TPP-cargo is taken up by endocytosis into cells. Through a genome-wide RNAi strategy, the interaction between the GIPC1/synectin and the cytosolic domain of NRP1 was determined to be necessary for endocytosis of the TPP-cargo (39). If the fate of NRP1, once endocytosed is similar to after binding with pleiotropin, then NRP1 is at least partially recycled to the cell surface (29). Beyond NRP1-mediated endocytosis, the intracellular pathway of TPP or the TPP-cargo is largely unknown.

Two mechanisms have been offered to explain enhanced vascular permeability by tumor-penetrating peptides. While NRP1 transport of macromolecules may be the result of an increase paracellular route (i.e., vessel leakiness) in tumors, there is evidence to support that transport is at least in part by the transcellular route (i.e., transcytosis) (39, 40) (Figure 2). As discussed above, more effective transfer of particles in spheroids vs monolayers suggests transcytosis with possible intercellular transfer of particles is a plausible mechanism. Further support for transcytosis with intercellular or exosome transport of TPP-silver particles comes from studying the transfer of these particles between cells in the presence of an etching buffer. Despite the continuous presence of an etching solution, which removed any extracellular silver particles, the transfer of TPP silver particles between cells in a 3-D spheroid model was not affected, suggesting intercellular transfer of the particles. Although transcytosis could explain the transport processes of TPP and associated macromolecules through the endothelium and tumor tissue, attempts to identify particles in exocytosis vesicles *in vivo* have not yet been successful (9). Interestingly, transcytosis has been implicated in the interaction between NRP1 and VEGF-VEGFR2 based on the formation of an extensive vesicular-vacuolar network in endothelial cells (41). Unlike the uptake of TPP and NRP1 which has a distinct endocytosis pathway, however, the uptake of VEGF-VEGFR2 and NRP1 is by clathrin-mediated endocytosis (41).

Although transcytosis appears to have a role in NRP1-mediated transport, paracellular trans-tissue transfer as a possibility has not been eliminated. The recent finding that iRGD downregulates β 1 integrin on the surface of the tumor cells (37) suggests that β 1 interaction with the extracellular matrix *in vivo* may be similarly affected. It will be of interest to determine if the effects of iRGD on β 1 expression on tumor cells also occurs with endothelial cells. Moreover, interaction of an analogue of semaphorin with NRP1 has been reported to loosen cellular junctions (42). Neither mechanism of paracellular or transcellular transfer of particles induced by activation of NRP1 are mutually exclusive. Clearly, much remains to be elucidated as to the pathways activated by NRP1 to transport CendR peptides and their cargos so that more effective therapies can be developed.

Enhancement of Chemotherapy

Several groups have demonstrated that tumor delivery and antitumor efficacy of chemotherapy is enhanced by targeting NRP1 with TPP (4, 7, 43–46) (Table 1). A significant problem with the accumulation and distribution of chemotherapy within tumors is increased institutional tumor pressure. Although enhanced permeability and retention (EPR) may enhance accumulation of nanoparticles greater than 10 nm, the distribution of nanoparticles throughout the tumor is still limited. Transport pathways such as NRP1, which are less dependent on diffusion of particles, may circumvent problems of distribution and augment delivery of both low and high molecular weight molecules to tumors. When iRGD was co-administered, several antitumor agents, including doxorubicin (Dox), Dox-liposomes, Abraxane, and trastuzumab, exhibited significantly greater antitumor efficacy in murine xenograft models of human breast (BT474 cells) and prostate (22Rv1) cancers (7). Concomitant with increased efficacy of these chemotherapies with iRGD was their greater accumulation within the tumor. For example, iRGD augmented accumulation of Abraxane and trastuzumab in tumors by 12 and 14-fold, respectively. Although most iRGD therapies

inhibited the growth of tumors in murine models, only iRGD-mediated trastuzumab therapy caused regression of tumor xenografts (BT474). Moreover, iRGD co-administered with Abraxane gave similar tumor inhibition as iRGD-conjugated to Abraxane (7). Compared to cRGD-mediated conjugates, iRGD mediated polymer-doxorubicin conjugates (or their co-administration) showed widespread accumulation of polymer-doxorubicin in an orthotopic C6 glioma model and resulted in significant increases in mouse survival (44). Specifically, co-administration of iRGD and its polymer-doxorubicin conjugate to mice bearing orthotopic C6 gliomas had a median survival of 61 days compared to polymer-doxorubicin-treated mice that had median survival of 24 days. The finding that co-administration of TPP with antitumor agents augments chemotherapy may facilitate evaluation of targeted therapies and may reduce potential problems with synthesis and long-term stability of TPP-conjugated antitumor agents.

In addition to iRGD, other TPP co-administered with chemotherapy have shown efficacy. For instance, Alberci et al. showed that co-administering iNGR and doxorubicin reduced tumor growth significantly more than doxorubicin alone (4). Moreover, Karmali et al. showed, after multiple intravenous injections, an approximate 40% further reduction in the size of MDA-MB-435 derived tumors occurred with the LyP-1-Abraxane conjugate compared to the non-targeted Abraxane (47).

NRP1-Mediated Delivery of Nucleic Acids to the Tumor

Despite several studies showing TPP improve antitumor efficacy of small molecule drugs or NPs incorporating such drugs (4, 12), few studies have been reported using TPP with siRNA nanoparticles (48). Ren and colleagues demonstrated that LyP-1 siRNA NPs accumulated 3-fold more in tumors compared to untargeted NPs (48). Moreover, location of the targeted NP within the tumor is extravascular in contrast to the intravascular location of the untargeted NP. With a LyP-1 containing NP carrying an siIDF (inhibitor of DNA binding 4), ovarian cancer xenografts implanted subcutaneously were reduced by 80 to 90% compared to tumors treated with the siGFP control group. In a second murine model which had intra-abdominal dissemination of the ovarian malignancy, 80% of the siIDF NP treated mice survived more than 80 days whereas 100% of the saline-treated mice died before day 60.

Similar to siRNA, few studies about TPP plasmid-based NP have been reported. Different synthetic approaches have been explored for addition of iRGD and other peptides to pegylated cationic lipids (49). At least for synthesis, the cyclic azide-alkyne cycloaddition was found to be particularly useful for coupling disulfide-bridge peptides such as iRGD to a PEGylated-lipid. However, once synthesized and in aqueous solution, the disulfide iRGD modified liposomes may be still susceptible to reduction, with subsequent oxidation of adjacent sulfide groups. Although both linear and cyclic targeting peptides increase cell associated fluorescence, a significant portion of RGD particles (RGD or iRGD) were attached to the cell surface, indicated by their fluorescence quenched by Trypan blue. In contrast, RPARPAR particles, with only the CendR motif, were not quenched, suggesting rapid internalization of these particles. Notably, intracellular fluorescence varied significantly between the different unlabeled and TPP-labeled particles, perhaps due to their different pathways of endocytic uptake.

For antitumor studies, Shen and colleagues demonstrated that iRGD NPs carrying both paclitaxel and a plasmid-based shRNA targeting survivin reduced the size of tumors (50). The size of the tumors treated with the iRGD tagged NPs remained unchanged during the course of therapy (~280 mm³). iRGD-NPs inhibited tumors compared to untargeted NP or saline by about 70% and 90%, respectively.

Recently, histidine-lysine (HK) plasmid polyplexes injected intravenously were shown to have high specificity toward tumors. Although linear HK peptides are 20-mers and are not TPP, these two classes of peptides likely share transport systems that are mediated by NRP1. By injecting a NRP1 antibody prior to administering HK polyplexes (with luciferase-expressing plasmid), tumor transport of HK polyplexes was shown to be mediated by NRP1 with the marked reduction of luciferase expression (96%) in the tumor (15, 16). Although the linear HK (H2K) has multiple –KHHK-repeats, its C-terminal end has a –KHK-sequence with an amide cap, which consequently would prevent H2K from binding to NRP1. As a result, H2K peptide must be enzymatically processed to expose –KHHK-OH to activate the NRP1 transport mechanism in the tumor endothelium and matrix. Similar to protease(s) which may process iRGD (8, 18), furin-like and trypsin-like enzymes may also process H2K peptides, which would cleave these peptides on the C-terminal side of lysine. In contrast to H2K, the branched peptide H2K4b, which also has a -KHHK-motif, does not transport plasmids effectively to tumors. In addition to differences in biodistribution, reduced enzymatic processing of H2K4b, which has a high affinity for DNA, may explain the inability of branched peptides to be effective carriers *in vivo* (15, 16). Moreover, since H2K polyplexes achieve tumor specificity without targeting ligands, multiple factors, including increased NRP1-expression and/or EPR, may be necessary for the specificity of polyplexes.

Conclusion and Future Directions

NRP1 targeted therapy using tumor penetrating peptides is a promising approach that allows greater penetration and accumulation of the drug within the tumor. Despite the promise of offering a significant advance in cancer therapeutics, no clinical trials have been reported utilizing TPP. At least part of the reason for the lack of trials may be that the field is relatively young. Moreover, until recently, one concern about TPP has been that the increased vascular permeability induced by them at the tumor site may enable not only greater drug penetration but also enable metastatic spread. However, two recent reports showed TPP not only did not increase but rather reduced metastases (37, 38). We will discuss some additional areas of interest, a few of which may require further study.

1. Although co-administration may facilitate development of a delivery strategy and prove useful for pre-clinical and clinical trials, one potential problem is that the bio-distribution of TPP is completely different than the nanoparticle. Thus, untoward side effects caused by the TPP such as vascular permeability in non-target organs could limit the TPP-mediated strategy, particularly with the co-administration approach (44). Although, as expected, intradermal injections of linear peptides with an exposed CendR motif did result in enhanced vascular permeability at the injection site of the intravascularly delivered marker, there was no evidence that such permeability in normal tissues occurs with systemic

delivery of TPP-targeted therapy (11). For instance, when iRGD or iNGR were injected intravenously with Evans Blue dye into tumor-bearing mice, an increase in dye occurred only in the tumor and not in normal tissues (spleen, kidney, lung, heart, and liver) (4). In addition to the dye, a marked increase in the uptake of small or large molecules mediated by TPP was only observed in tumors with no difference in accumulation of these molecules in normal tissues between TPP and control PBS co-administered therapies. Moreover, biodistribution of NP in the tumor or in normal tissues is similar whether TPP is directly conjugated or co-administered with the NP (44). Thus, there is no evidence to support that TPP-mediated vascular permeability in non-targeted tissues is a significant problem, but this should be monitored during clinical testing until ruled out.

2. Despite detailed pharmacokinetic studies with TPP-mediated transport of therapeutic agents, additional experiments may provide more insight into improving therapeutic efficacy. We could not find tumor targeting studies that compared pegylated versus non-pegylated NP that were mediated with TPP. To the best of our knowledge, all studies thus far have utilized TPP-mediated NPs which have been pegylated. Nevertheless, these pegylated NPs have shown minimal lung uptake, marked tumor uptake and accumulation, and favorable pharmacokinetics, suggesting that pegylation of TPP-targeted NP may be beneficial. By prolonging blood circulation (plasma half-life) of the NP and reducing non-specific binding, pegylation may allow accumulation of ligand-decorated nanoparticle in tumors above the EPR effect, and in the case of TPP enable widespread intratumoral distribution.
3. To investigate antitumor efficacy in mice, co-administration of TPP and NP has usually been administered every three to four days. Because the maximum effect of TPP is 30 min with an effect lasting approximately 1 hour, repetitive injection of TPP may allow greater accumulation of the NP in the tumor, particularly if the NP has a long half-life in the blood stream. If such an understanding is achieved, it might provide for development of innovative clinical strategies tailored for different cancer indications. Extensive studies with specific preparations will be needed to understand the controlling parameters and provide a foundation for clinical studies.
4. The ratio of TPP to therapeutic agent may need to be optimized for each preparation and each indication. Since calculations have suggested that the number of NRP1 receptors may be limited in both tumor vessels and tumor cells, TPP-conjugates may deliver less drug to the tumor under conditions in which the receptor is saturated compared to the co-administration approach (17). Co-administration, by untethering TPP from the therapeutic drug, may enable TPP to activate the NRP1 pathway and to increase the amount of drug to be delivered to the tumor. The difference in antitumor efficacy is likely to be most marked when the ratio of the TPP-drug conjugate is close to 1:1 (i.e., iRGD: doxorubicin) compared to co-administration of TPP and drug in which ratios could favor delivery of greater amounts of the drug.

There are other considerations as well when the NRP1 is saturated and activated, particularly if a significant route of particle transfer is paracellular and not transcellular with intercellular transfer. For example, once NRP1 is saturated with the TPP-conjugate (or TPP), excess TPP-conjugates may enter and penetrate into tumor tissues through NRP1 activated pathways (i.e. via the paracellular route), which may not be significantly limited by saturation of NRP1 on angiogenic vessels. Moreover, once the TPP-conjugate has extravasated into the tumor matrix, saturation of NRP1 receptors by the conjugate may limit entry into the tumor cell. Under these conditions, however, it is also possible that excess NPs may enter by a less preferred endocytic pathway. Although recent studies with TPP-labeled silver particles or proteins showed that these enter only by the NRP1-mediated endocytosis (39), polyplexes, which are dependent on NRP1 for widespread distribution in the tumor, enter cells (at least *in vitro*) through numerous endocytic pathways (16). How much NRP1 receptor mediated therapy is limited by saturation of receptors on tumor cells may be dependent on multiple factors including the specific TPP-drug (or NP) conjugate, the pegylation pattern, and the level of non-NRP1 mediated endocytosis in the cell.

5. Regardless of the size of the therapeutic agent, TPP-mediated therapy enhanced uptake into the tumor. For instance, Sugahara et al. determined that levels of a doxorubicin conjugate or an NP such as Abraxane accumulated in tumors significantly more with TPP-targeting (7). Still, EPR and/or tumor interstitial pressure may have a role in tumor targeting and biodistribution of molecules mediated by TPP targeting of NRP1 (16, 44). For instance, in addition to targeting NRP1, EPR may provide a necessary component for the increased accumulation of polyplexes that did not contain a targeting ligand (15, 16). Further study of these physical factors will be of interest to determine if they have any effect on the distribution of TPP-mediated cargo within the tumor.

It has been speculated that the NRP1 pathway serves as bulk nutrient transport system to nutritionally deficient tissues (39). Similarly, tumors have usurped the NRP1 transport system to obtain sufficient nutrients (9). In addition to tumors, viruses containing cryptic CendR motifs, such as hemorrhagic fever virus, may have appropriated the NRP1 pathway to augment their dissemination in tissues (51). It is thus ironic, yet perhaps logical, that the NRP1-mediated transport system utilized by a wide variety of disease processes may enable major therapeutic advances in cancer and other human diseases.

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Figure 1.

A prototypical transporting-penetrating peptide, iRGD, require three components to transport cargo into the tumor matrix. After iRGD binds to $\alpha_v\beta_3/5$ integrins on the angiogenic vessels of tumors, proteolytic cleavage of iRGD (dash line) exposing the KDGR-CendR motif is the second essential component. With reduction of the cystine linkage (solid line), the third component is the binding of the CendR motif to the neuropilin-1 receptor (NRP-1), resulting in the endocytosis of the cargo.

Figure 2.

Transport mechanisms of TPP-cargo into the tumor matrix. After iRGD binds to integrins on tumor endothelial cells (left panel), iRGD is cleaved exposing the KDGR CendR motif (middle panel), activating the NRP1 pathway. Following endocytosis, transport of iRGD-cargo across the endothelial barrier may occur by one or two mechanisms (right panel). First, the endocytosed TPP-cargo may be transported transcellular to the tumor side of the endothelium. This may include intercellular transfer of molecules from endothelial to tumor cells. Second, entry of the TPP-cargo into the tumor may be through a paracellular route by the loosening of cellular junctions. The mediators of the activated NRP1 pathways and the location of these mediators (cell surface, endosomes) that enhance a possible paracellular are unknown. E, endothelial cell, T-tumor cell, RBC-red blood cell, ?-unknown mediators which increase paracellular entry.

Table 1

Cancer therapies enhanced by TPP

TPP	Therapy	Administration of TPP	Tumor	Other Comments	References
iRGD	Doxorubicin Dox-Liposomes Abraxane Trastuzumab	Co-administered	Breast (BT474)Prostate (22Rv1)	Enhanced Antitumor Efficacy with iRGD	Sugahara et al, 2014 (7)
iRGD	Abraxane	Co-administered vs. Conjugate	Breast (BT474)Prostate (22Rv1)	Similar accumulation and antitumor efficacy with the 2 methods of administration	Sugahara et al, 2014 (7)
iRGD	Doxorubicin attached to a Dendrimer	Co-administered vs. Conjugate	Rat Glioma (C6)	Marked antitumor efficacy and mouse survival in orthotopic model with both strategies	Wang et al, 2014 (44)
iNGR	Doxorubicin	Co-administered	Mouse breast cancer (4T1)	Enhanced antitumor efficacy compared to doxorubicin alone	Alberici et al, 2013 (4)
LyP-1	Abraxane	Conjugate	Melanoma (MDA-MB-435)	Increased antitumor efficacy compared to Abraxane aloneTargets tumor lymph vessels	Karmali et al, 2009 (47)
LyP-1	NP carriers of an siRNA targeting iIDF4	Conjugate	Ovarian cancer (OVCAR-4, OVCAR-8)	Enhanced antitumor efficacy of TPP-conjugate in both subcutaneous and orthotopic model	Ren et al, 2012 (48)
iRGD	NP carriers of Paclitaxel and shRNA targeting survivin	Conjugate	Lung cancer (A549)	Enhanced antitumor efficacy of TPP-conjugate	Shen et al, 2014 (50)

Abbreviations: Dox, doxorubicin; iRGD, internalizing RGD; NP, nanoparticle; siRNA, short interfering RNA; iIDF, inhibitor of DNA binding 4