De Novo Design of a Tumor-Penetrating Peptide

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Abstract

Poor penetration of antitumor drugs into the extravascular tumor tissue is often a major factor limiting the efficacy of cancer treatments. Our group has recently described a strategy to enhance tumor penetration of chemotherapeutic drugs through use of iRGD peptide (CRGDK/RGPDC). This peptide comprises two sequence motifs: RGD, which binds to αvβ3/5 integrins on tumor endothelia and tumor cells, and a cryptic CendR motif (R/KXXR/K-OH). Once integrin binding has brought iRGD to the tumor, the peptide is proteolytically cleaved to expose the cryptic CendR motif. The truncated peptide loses affinity for its primary receptor and binds to neuropilin-1, activating a tissue penetration pathway that delivers the peptide along with attached or co-administered payload into the tumor mass. Here, we describe the design of a new tumor-penetrating peptide based on the current knowledge of homing sequences and internalizing receptors. The tumor-homing motif in the new peptide is the NGR sequence, which binds to endothelial CD13. The NGR sequence was placed in the context of a CendR motif (RNGR), and this sequence was embedded in the iRGD framework. The resulting peptide (CRNNGPDC, iNGR) homed to tumor vessels and penetrated into tumor tissue more effectively than the standard NGR peptide. iNGR induced greater tumor penetration of coupled nanoparticles and co-administered compounds than NGR. Doxorubicin given together with iNGR was significantly more efficacious than the drug alone. These results show that a tumor-specific, tissue-penetrating peptide can be constructed from known sequence elements. This principle may be useful in designing tissue-penetrating peptides for other diseases.

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Introduction

The vasculature of each tissue is unique in terms of protein expression and these molecular differences are referred to as ‘vascular zip codes’ (1). The selectively expressed proteins provide targets for specific delivery of diagnostic and therapeutic compounds to the vasculature of desired tissues. Currently, a variety of tumor-targeting peptides are in preclinical and clinical development. However, vascular abnormalities, fibrosis, and contraction of extracellular matrix contribute to an increased interstitial fluid pressure inside the tumor, which impedes drug delivery into the extravascular tumor tissue (2).

Our group has recently reported the identification of the CendR motif (R/KXXR/K) that is capable of increasing the penetration of peptides, chemicals, and synthetic and biologic nanoparticles into tissues through the engagement of neuropilin-1 (NRP-1; ref. 3). Specific penetration into tumors was achieved through the use of an iRGD peptide (CRGDK/RGPDC; ref. 4). iRGD, identified by in vivo phage display for tumor-homing peptides, combines targeting to tumor vessels and tumor parenchyma through an RGD motif with the cell-internalizing and tissue-penetrating properties of a CendR motif RGDK/R in the peptide (4). iRGD mechanism of action involves 3 steps. First, the RGD sequence binds to αvβ3/5 integrins. Then, a proteolytic cleavage by a yet-to-be-identified host protease(s) exposes the CendR motif, which is now able to interact with NRP-1 to trigger the internalization process. This strategy allows the activation of the CendR motif only in a targeted tissue, avoiding NRP-1 activation in normal vasculature. Interestingly, iRGD triggers a specific tumor penetration of not only iRGD-coupled compounds but also drugs co-administered with free iRGD peptide (5). The CendR motif also activates the penetration pathway through binding to NRP-2 (6).

Potentially, the addition of a cryptic CendR motif could increase the penetration of other tumor targeting peptides, providing more tools to overcome the poor delivery of drugs to tumors. We set out to test this hypothesis using the NGR tumor-homing motif. The NGR sequence was identified by in vivo phage display in tumor-bearing mice (7). Initially, it was thought to bind one or more of the integrins selectively expressed in angiogenic vessels (7, 8). This idea was further supported by the discovery that the asparagine in the NGR
motif undergoes a spontaneous deamidation reaction that yields iso-aspartic acid (isoDGR), generating an RGD mimetic (9, 10). However, the unaltered NGR motif also specifically homed to tumor vessels, where it binds to an isomer of aminopeptidase N (CD13; refs. 11, 12). NGR peptides have been used to target a variety of agents into tumors; an NGR conjugate of human TNF-α is in advanced clinical trials for cancer therapy (13–16). Here, we combined the NGR motif with a CendR motif to create a new tumor-homing peptide with tissue-penetrating properties.

Materials and Methods

Animal use

All procedures on the animals, including those to ensure minimizing discomfort, have been carried out according to the protocol approved at the Sanford-Burnham Medical Research Institute (La Jolla, CA).

Preparation of compounds

Synthetic peptides (4), peptide-coated nanoworms (NW; ref. 17), and peptide-expressing T7 phage (18) were prepared as described elsewhere. Doxorubicin was purchased from Sigma-Aldrich. Evans Blue was purchased from MP Biomedicals.

Cell lines and tumor models

Human umbilical vein endothelial cells (HUVEC; Lonza) were cultured in complete EGM-2 medium from Lonza. 4T1 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Thermo Scientific) supplemented with 10% FBS and penicillin/streptomycin (Gibco). All tumor cell lines were bought and authenticated by American Type Culture Collection. Orthotopic 4T1 breast tumors were generated by injecting 1010 plaque-forming units (pfu) of phage particles into the tail vein of tumor-bearing mice and allowed to circulate for 40 minutes. The mice were perfused through the heart with PBS containing 1% BSA under deep anesthesia. The tumors and tissues were excised and mechanically homogenized for phage titration or fixed in 4% PFA for immunostaining. In some cases, 50 μg of anti-NRP-1 antibody or control IgG was intravenously injected 15 minutes before the phage injection. For the peptide-homing studies, 100 μL of 1 mmol/L FAM-labeled peptides were intravenously injected into tumor mice and allowed to circulate for 1 hour. In case of the nanoworms, particles at a dose of 5 mg/kg of iron were injected into the tail vein of tumor mice and allowed to circulate for 5 hours. After perfusion of the mice, tissues were collected, macroscopically observed under UV light (Luminatool Bright Light System LT-9900), and processed for immunostaining. Intratumoral accumulation of nanoworms was quantified with ImageJ.

Biodistribution of peptides, phage, and nanoworms

In vivo tumor-homing experiments with peptides, phage, and nanoworms were conducted as described (4, 17). Briefly, 107 pfu of phage particles were injected into the tail vein of tumor-bearing mice and allowed to circulate for 40 minutes. The mice were perfused through the heart with PBS containing 1% BSA under deep anesthesia. The tumors and tissues were excised and mechanically homogenized for phage titration or fixed in 4% PFA for immunostaining. In some cases, 50 μg of anti-NRP-1 antibody or control IgG was intravenously injected 15 minutes before the phage injection. For the peptide-homing studies, 100 μL of 1 mmol/L FAM-labeled peptides were intravenously injected into tumor mice and allowed to circulate for 1 hour. In case of the nanoworms, particles at a dose of 5 mg/kg of iron were injected into the tail vein of tumor mice and allowed to circulate for 5 hours. After perfusion of the mice, tissues were collected, macroscopically observed under UV light (Luminatool Bright Light System LT-9900), and processed for immunostaining. Intratumoral accumulation of nanoworms was quantified with ImageJ.

Immunofluorescence

Immunofluorescence on cells was conducted using 10 μmol/L FAM peptides and following the protocol previously described (19). Immunofluorescence on frozen sections was conducted as described earlier (4) using the following antibodies at 1:200 dilution: rat anti-mouse CD31 Alexa-594 (Invitrogen), rabbit anti-T7 phage (3), and donkey anti-rabbit Alexa 488 (Invitrogen). Images were taken using a Fluoview confocal microscope (Olympus).

In vivo systemic permeability assay

Tumor-bearing mice were injected intravenously with 4 μmol/kg of peptide combined with 1 mg of Evans Blue, 10 mg/kg of free doxorubicin, or 5 mg iron/kg of CGKRK-coated nanoworms (20). After indicated time of circulation, the mice were perfused with the heart with PBS supplemented with 1% BSA and tissues were collected. Evans Blue was extracted by incubating the tissues in 1 mL of 2.2 N-methylformamide overnight at 37°C with mild shaking. After centrifugation, the optical density (OD600) of the supernatant was measured. To assess the extravasation of CGKRK-NWs, tissues were fixed in 4% PFA overnight at 4°C and subjected to immunostaining for CD31-positive blood vessels. For doxorubicin quantification (21), excised tissues were mechanically homogenized in 1 mL of 1% SDS containing 1 μmol/L H2SO4 and frozen overnight in 2 mL of chloroform/isopropanol alcohol (1:1, v/v). The samples were melted, vortexed, and centrifuged at 16,000 × g for 15 minutes, and OD299 of the organic (lower) phase was measured.

Tumor treatment studies

Mice bearing orthotopic 4T1 breast tumors at 50 mm3 received intravenous injections of free doxorubicin (3 mg/kg) with PBS, the tumors were lysed in 1 mL of LB/NP-40 for phage titration. In some cases, the tumors were fixed in PBS containing 4% paraformaldehyde (PFA) and processed for immunostaining.

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or PBS, combined with 4 µmol/kg of peptide every other day. Tumor growth and body weight were monitored every other day. The tumor volume was calculated using the following formula: volume (mm$^3$) = ($d^2 	imes D$)/2, where $d$ is the smallest and $D$ is the largest tumor diameters.

**Statistical analysis**

All data were analyzed with one-way ANOVA. Tumor treatment studies were analyzed with 2-way ANOVA for repeated measurements.

**Results**

**Design of iNGR peptide**

We used 3 elements to create the iNGR peptide (CRNGRGPDPC): the NGR motif, a CendR motif (RNGR) overlapping with the NGR motif, and a cleavable consensus (GPD) from the iRGD peptide. The cyclic conformation required for high affinity binding of NGR to CD13 (22) was obtained through the addition of cysteines at the N- and C-terminus of the peptide. We also prepared the truncated version of iNGR that is expected to result from proteolytic activation of INGR (CRNGR), which we refer to as INGRt. The conventional NGR (CNGRC), RGD (CRGDC), iRGD (CRGDKGPDPC), and activated iRGD (CRGDK) peptides were used as controls. The peptides used in this study are summarized in Supplementary Table S1.

**iNGR and NGR bind to the same primary receptor**

HUVECs express on their surface both the NGR receptor CD13 and the CendR receptor NRP-1 (Supplementary Fig. S1). iNGR bound to HUVECs as efficiently as CNGRC, whether tested as FAM-labeled peptide (Fig. 1A) or displayed on phage (Fig. 1B, black columns). As expected, the INGR phage did not bind CD13$^+$ U937 monocytes (Supplementary Fig. S2A–S2C), as previously reported for the CNGRC peptide (11). UV-inactivated phage displaying CNGRC inhibited the HUVEC binding of the iNGR phage in a dose-dependent fashion, whereas inactivated CRGDC did not differ from insertless control phage in this regard (Fig. 1C). CNGRC phage did not inhibit the binding of iRGD phage to HUVECs (Supplementary Fig. S3A). Moreover, only in vitro deamidated CNGRC and iNGR phage showed binding to immobilized αvβ3.
integrin (Supplementary Fig. S3B). These results suggest that CNGRC and iNGR bind to the same primary receptor through the NGR motif and that the conversion of asparagine to aspartate does not take place during phage production, purification, storage, or during the incubations. Therefore, the binding of CNGRC and iNGR to cells is not due to isoDGR interacting with αvβ3/5 integrins.

The iNGR CendR motif interacts with NRPs and promotes cell internalization

We constructed phage displaying a truncated version of iNGR in which the CRNGR CendR motif occupies a C-terminal position (iNGRt). This phage bound avidly to HUVECs, likely due to an interaction with NRP-1 (Fig. 1A and B). Indeed, iNGRt phage binding to HUVECs was reduced by preincubation with a UV-inactivated phage expressing a prototypic CendR motif peptide (RPARPAR) or a neutralizing NRP-1 antibody (Fig. 1B), indicating involvement of the CendR/NRP-1 pathway. Preincubation with a phage displaying a peptide with a blocked CendR motif (RPARPARA), and a control antibody, had no effect on iNGRt binding to HUVECs. The binding of intact iNGR was not affected by the UV-inactivated RPAPR phage or NRP-1 antibody showing that NRP-1 is not involved in the initial binding of the peptide. Measuring phage internalization by incubating phage with HUVECs at 37°C, followed by a wash with a low pH buffer to inactivate extracellular phage, showed stronger internalization of iNGR and iNGRt than CNGRC (Fig. 1D). Inhibition experiments showed that the internalization was mediated by the interaction of the CendR motif with NRP-1. These results suggest that HUVECs express a protease capable of activating the cryptic CendR motif embedded in the iNGR peptide. Indeed, mass spectrometry showed that upon incubation of HUVECs with FAM-iNGR, only the cleaved FAM-CRNGR fragment (m/z: 1076.527) but not the full-length peptide (m/z: 1445.065), was present inside the cells (Supplementary Fig. S4). FAM-CNGRC peptide (m/z: 1020.020) did not penetrate into the cells. Direct proof that the CendR motif within the iNGR peptide is capable of binding to NRPs was provided by CRNGR phage binding to immobilized NRP-1 (Fig. 1E). This phage also bound to NRP-2 (Fig. 1F). In agreement with the finding that the motif R/KXXR/K has to be in a C-terminal position to bind to NRPs (the Cend-Rule; ref. 3), only phage expressing the iNGRt, and the analogous iRGDt, bound to the NRPs, whereas the corresponding full-length peptides showed only background binding. Interestingly, iNGRt showed higher affinity for NRP-1 and NRP-2 than iRGDt, suggesting that a C-terminal arginine residue (CRNGR) provides higher affinity than lysine (CRGDK).

iNGR penetrates deeper into tumors than NGR

We have previously shown in ex vivo tumor penetration assays that iRGD uses a CendR-mediated active transport system to cross tumor barriers (5). To investigate the iNGR-mediated penetration pathway, we conducted an ex vivo tumor penetration assay of phage using explants of orthotopic 4T1 murine breast tumors (positive for expression of CD13 and NRP1/2; Fig. 2A). To evaluate the extent of tumor penetration, we titrated the phage recovered from tumors (Fig. 2B) and determined the distribution of phage immunoreactivity (Fig. 2C). The CendR-containing phage (iNGR and iRGD) penetrated into the explants with similar patterns, whereas non-CendR phage (CNGRC and control) remained at the...
outer rim of the explants. These results suggest that iNGR and iRGD share a similar CendR-mediated transport mechanism to penetrate tumor tissue.

**Systemic iNGR selectively accumulates and penetrates into tumors**

Having determined that the CendR motif within the iNGR peptide can be proteolytically activated to trigger interaction with NRPs and penetration into cells and tissues, we assessed the homing of iNGR in vivo. Intravenously administered iNGR phage accumulated within the tumor and penetrated into the tumor stroma more than CNGRC phage (Fig. 3A and B, top). The iNGRt phage also showed high tumor penetration, presumably because of the high expression of NRP-1 on tumor vasculature and tumor cells, but this phage also accumulated in lungs and heart of the tumor mice. iNGR penetration could be blocked by concomitantly administering a neutralizing anti-NRP-1 antibody, but not a control antibody (Fig. 3B, bottom). Vascular targeting of iNGR was not inhibited by the anti-NRP-1 treatment (arrows, Fig. 3B, left bottom), supporting the notion that the CendR activation occurs after iNGR accomplishes NGR-dependent vascular targeting. Intravenously injected FAM-iNGR peptide also accumulated in 4T1 breast tumors (Fig. 3C) and BxPC-3 pancreatic tumors (Supplementary Fig. S5A) more strongly than FAM-CNGRC. FAM-iNGR extravasation within tumor tissue was greater than...
that of FAM-CNGRC (Fig. 3D). FAM-iNGR selectively penetrated into tumors and not into control organs (Fig. 3E). Elongated iron oxide nanoparticles (nanoworms) coated with iNGR also showed higher extravasation than CNGRC-NWs (Fig. 3F and Supplementary Fig. S5B). The nanoworms were less efficient than phage in penetrating the tissue, likely because they are larger in size (nanoworms, 30–70/200 nm; phage, 55 nm).

iNGR triggers tumor-specific penetration of co-administered compounds

The engagement of NRP-1 increases vascular permeability (23), and iRGD triggers this phenomenon specifically in tumors (5). We found that iNGR significantly increased extravasation and accumulation of the albumin-binding dye Evans blue in 4T1 tumors, but not in nontumor tissues. CNGRC or vehicle alone had no effect on the biodistribution of the dye (Fig. 4A and B and Supplementary Fig. S6). iNGR facilitated tumor-specific accumulation of Evans blue in CT26 colon and LLC lung tumor models as well (Supplementary Fig. S7). We also co-administered iNGR with nanoworms coated with a tumor-homing peptide, CGKRK (20), which brings the nanoworms to tumor vessels but does not trigger extravasation. iNGR allowed the NWs to extravasate into the tumor parenchyma (Fig. 4C).

iNGR enhanced anticancer drug efficacy

Having found that iNGR co-administration increased the local accumulation of doxorubicin within tumors, we investigated the effect of iNGR on the activity of doxorubicin. We treated orthotopic 4T1 breast tumor mice with a combination of doxorubicin (3 mg/kg) and 4 μmol/kg of iNGR, a control peptide, or PBS every other day. As shown in Fig. 5A, iNGR, but not CNGRC, enhanced the antitumor effect of doxorubicin. iNGR alone had no effect on tumor growth. Loss of body weight as an indicator of doxorubicin toxicity was not affected by the peptide co-administration (Fig. 5B). These results show the potential of iNGR as an adjuvant to increase the efficacy of co-administered anticancer drugs.

Discussion

We report here the design of a new tumor-penetrating peptide, iNGR. The peptide was constructed by combining the
The resulting isoDGR peptides, like RGD peptides, bind to deamidation of the asparagine residue into isoaspartic acid. NGR peptides are known to spontaneously undergo slow deamidation by CNGRC, indicating that iNGR binds to the same receptor. The binding of iNGR peptide and phage to cultured cells. It is also unlikely that isoDGR formation affects the in vivo tumor targeting of INGR because the deamidation process takes several hours (24), whereas the half-life of intravenously injected peptides of the size of iNGR is only minutes (25). Thus, iNGR and iRGD bind to different primary receptors on cells.

Figure 5. INGR enhances efficacy of anticancer drugs without affecting side effects. A, mice bearing orthotopic 4T1 tumors were treated every other day with PBS or 3 mg/kg of doxorubicin (DOX) combined with 4 μmol/kg of CNGRC or INGR peptide. Tumor growth was assessed every other day. B, body weight changes of the tumor mice from the treatment studies (A). Percentage of body weight shift is shown. *, P < 0.05; 2-way ANOVA; ***, P < 0.001; 2-way ANOVA. Error bars, SE.

tumor-targeting motif NGR and tissue-penetrating CendR motif into a 9-amino acid cyclic peptide. The INGR peptide, homed to tumor vessels, exited the vessels, and penetrated into the tumor mass. It was able to take both coupled and co-administered payloads with it. When the co-administered payload was a drug (doxorubicin), the efficacy of the drug increased. These results show that it is possible to use the existing knowledge to construct a new tumor-specific, tissue-penetrating peptide.

The mechanisms underlying INGR activity are similar to those described for iRGD (4, 5). The receptor for the tumor-targeting motif NGR is a variant form of aminopeptidase N (12). The binding of iNGR to cultured cells was specifically inhibited by CNGRC, indicating that iNGR binds to the same receptor. NGR peptides are known to spontaneously undergo slow deamidation of the asparagine residue into isoaspartic acid. The resulting isoDGR peptides, like RGD peptides, bind to αvβ3 integrins. Our results exclude integrin involvement in the binding of INGR peptide and phage to cultured cells. It is also unlikely that isoDGR formation affects the in vivo tumor targeting of INGR because the deamidation process takes several hours (24), whereas the half-life of intravenously injected peptides of the size of iNGR is only minutes (25). Thus, INGR and iRGD bind to different primary receptors on cells.

Upon engagement of the iRGD peptide at the plasma membrane of target cells, a proteolytic cleavage by a yet-to-be-identified enzyme(s) exposes the CendR sequence, which subsequently binds to NRP-1 (4). Our evidence indicates that the same mechanism operates with INGR. First, phage displaying the predicted CendR product of iNGR, CRNGRC (iNGRt) bound to NRP-1 and NRP-2, and did so with a higher affinity than CRGDK fragment of iRGD. The reason for the difference may be that a peptide with a C-terminal arginine binds more efficiently to NRPs than a peptide with a lysine C-terminus (26). Comparison of the tumor-homing efficacy of iRGD with an arginine or a lysine (CRGDK/CRGD/CRGD/CRGD) showed that the lysine-containing form was more effective in vivo (4). It may be that other effects of the lysine residue, such as stronger integrin binding or higher susceptibility to protease cleavage, overcome the effect of lower affinity for NRPs. Second, iNGR, both as a synthetic peptide and on phage was taken up by cells in an NRP-dependent manner. Third, we isolated the iNGRt CendR fragment from inside cells treated with the intact iNGR peptide, as has been previously done with iRGD (4). Fourth, the co-injection of iNGR phage with neutralizing anti-NRP-1 antibody resulted in a reduced extravasation of iNGR. These results show that iNGRt, the active form of iNGR, is generated through proteolysis and that the tumor-penetrating properties of iNGR are based on its ability to activate the CendR pathway.

The activation of iNGR into iNGRt appears to take place only in tumors because INGR only accumulated in tumors. In contrast, the truncated iNGRt form, while showing preferential homing to tumors, also accumulated in the lungs and heart. This homing pattern reflects the expression of NRP-1, which is universal in the blood vessels but particularly high in tumor vessels (27). The reason for the selective activation of the cryptic CendR motif in tumors is likely to be that binding to the primary receptor is needed for the activating proteolytic cleavage. Previous work from our laboratory has shown that an iRGD variant that does not bind to integrins, but contains a CendR motif, does not penetrate into cultured cells, whereas iRGD does (4). The nature of the primary receptor does not seem to matter, as long as the receptor is tumor specific. iRGD and iNGR bind to different primary receptors, but both become activated in cell cultures and in tumors. Moreover, we have recently shown that a previously identified tumor-homing peptide, CGNKRTGRC (LyP-1; ref. 28) also penetrates into tumors through the CendR/NRP mechanism (6). The primary receptor for this peptide is p32/gC1qR/HABP1, a mitochondrial protein expressed at the cell surface in tumors (29). Thus, our results show that at least 3 different primary receptors can initiate the sequence of events that leads to the NRP-dependent activation of the CendR pathway in tumors. Importantly, the
difference in the primary receptors allows us to differentially target tumors or tumor areas based on receptor expression patterns, providing multiple options to enhance tumor therapy with tumor-specific CendR peptides.

The experiments with phage, fluorophore-labeled peptide, and nanoparticles showed the ability of iNGR to take coupled payloads into the extravascular tumor tissue. Our results further show that such enhanced delivery and tumor penetration also applies to compounds co-administered with iNGR. Importantly, we showed this for doxorubicin, the antitumor activity of which was increased by injecting the drug together with iNGR.

The co-administration strategy has significant advantages. First, because chemical coupling is not needed, new chemical entities are not created, providing a faster route for clinical development. Second, unlike targeting of compounds chemically coupled to a homing element, the co-administration process is not strictly dependent on the number of available receptors, which seriously limits the amount of a drug that can be delivered to a target (30).

Taken together, our results show that iNGR possesses the same targeting ability as CNGRC, supplemented with cell-internalizing and tumor-penetrating properties. This transformation suggests an important principle: a targeting peptide can be ad hoc improved by the addition of a CendR motif, which endows the peptide with tissue-penetrating properties and allows enhanced delivery of co-administered compounds into a target tissue. Rational optimization of targeting peptides in this manner may also have valuable applications in other diseases.

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Disclosure of Potential Conflicts of Interest

L. Alberici, C. Bordignon, C. Traversari, and G.-P. Rizzardi are employees of MolMed SpA. K.N. Sugahara, V.R. Kotamraju, T. Teesalu, and E. Ruoslahti are shareholders of CendR Therapeutics Inc, which has rights to some of the technology described in the article. No potential conflicts of interest were disclosed by the other authors.

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