Advanced Drug Delivery Reviews 110–111 (2017) 3–12

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Tumor penetrating peptides for improved drug delivery☆

Erkki Ruoslahti *

Cancer Research Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA
Center for Nanomedicine, Department of Cell, Molecular and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA, USA

ARTICLE INFO

Article history:
Received 11 February 2016
Received in revised form 16 March 2016
Accepted 18 March 2016
Available online 1 April 2016

Keywords:
CendR pathway
Tumor vasculature
Synaphic targeting
Endocytosis
Integrins
Neuropilins
Cell-penetrating peptides
Nanomedicine

ABSTRACT

In vivo screening of phage libraries in tumor-bearing mice has been used to identify peptides that direct phage homing to a tumor. The power of *in vivo* phage screening is illustrated by the recent discovery of peptides with unique tumor-penetrating properties. These peptides activate an endocytic transport pathway related to but distinct from macropinocytosis. They do so through a complex process that involves binding to a primary, tumor-specific receptor, followed by a proteolytic cleavage, and binding to a second receptor. The second receptor, neuropilin-1 (or neuropilin-2) activates the transport pathway. This trans-tissue pathway, dubbed the C-end Rule (CendR) pathway, mediates the extravasation transport through extravascular tumor tissue of payloads ranging from small molecule drugs to nanoparticles. The CendR technology provides a solution to a major problem in tumor therapy, poor penetration of drugs into tumors. Targeted delivery with tumor-penetrating peptides has been shown to specifically increase the accumulation of drugs, antibodies and nanotherapeutics in experimental tumors *in vivo*, and in human tumors *ex vivo*. Remarkably the payload does not have to be coupled to the peptide; the peptide activates a bulk transport system that sweeps along a drug present in the blood. Treatment studies in mice have shown improved anti-tumor efficacy and less damage to normal tissues with drugs ranging from traditional chemotherapeutics to antibodies, and to nanoparticle drugs.

Contents

1. Introduction . . . 3
2. Discovery of tumor-penetrating peptides . . . 4
   2.1. *In vivo* phage display screening for peptides . . . 4
   2.2. Molecular basis of iRGD activity and the CendR motif . . . 4
   2.3. Family of tumor-penetrating CendR peptides . . . 4
3. The CendR pathway . . . 6
   3.1. The cell biology of the CendR pathway . . . 6
   3.2. Cell-penetrating peptides and the CendR pathway . . . 6
4. Tumor-penetrating CendR peptides in drug delivery: general considerations . . . 7
   4.1. Advantages in drug delivery . . . 7
   4.2. Pharmacokinetics considerations . . . 7
   4.3. On-target side effects . . . 8
5. Tumor-penetrating nanoparticles . . . 9
6. Tumor-penetrating peptides in pre-clinical treatment studies . . . 9
7. Conclusions and future prospects . . . 10
Conflict of interest statement . . . 10
Acknowledgement . . . 10
References . . . 10

1. Introduction

Compounds that selectively recognize target molecules in tumors are potentially valuable reagents for targeted delivery of diagnostic and therapeutic agents into tumors (synaphic, active, or ligand-

☆ This review is part of the *Advanced Drug Delivery Reviews* theme issue on "Peptides and Peptide Conjugates in Medicine"

* Sanford Burnham Prebys Medical Discovery Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA.
E-mail address: ruoslahti@sbpdiscovery.org.

http://dx.doi.org/10.1016/j.addr.2016.03.008
0169-409X/

directed) targeting). There are numerous targets in tumors, both in tumor blood vessels, and on tumor cells and stromal cells within tumors. Examples include certain integrins, fibrin deposits, and tumor antigens, such as prostate specific membrane antigen (PSMA) and carcinoembryonic antigen (CEA). The targeting ligand used can be an antibody, a peptide or a natural ligand of a receptor preferentially expressed in tumors (*e.g.* the folate receptor). The rationale of synaphic targeting is that a drug coupled to a targeting ligand will preferentially accumulate in the tumor, resulting in greater activity and fewer side effects elsewhere in the body [1,2,3]. Despite this simple rationale and vast amount of preclinical work, progress in bringing targeted compounds into the clinic for the treatment of solid tumors has been slow. The new tumor-penetrating peptides may overcome some of the limitations of the targeting technology; they deliver drugs deep into tumor tissue and enable enhanced drug delivery even without coupling of the drug to the peptide. This review focuses on the discovery of tumor-penetrating peptides, their mechanism of action, and their use in drug delivery.

2. Discovery of tumor-penetrating peptides

2.1. In vivo phage display screening for peptides

Phage display makes use of libraries of peptides that are expressed at the surface of a phage particle, such that each phage particle expresses one peptide, and the whole library typically contains up to 10^9 different peptide sequences. The phages carrying a peptide with the desired activity are selected from the library based on their ability to bind to the desired target (unfortunately, functional screens are not possible). Sequencing the part of the phage DNA that encodes the peptide then allows identification of the peptides. *In vivo* phage library screening follows the same principles, but the screening is done in live animals, selecting for phages that accumulates at the desired target tissue [4,5]. The *in vivo* screening has a built-in negative screen in that phages that bind indiscriminately will not significantly accumulate at the target tissue because they will also bind somewhere else. This circumstance gives an advantage to those phages that only bind at the target tissue. Because the phages are a nanoparticle (T7 phage, diameter ~ 40 nm; filamentous phage dimensions, 6 nm × 900 nm), they do not readily penetrate beyond the vascular wall, and *in vivo* phage screening mostly probes the vasculature. Indeed, the method has revealed so much molecular heterogeneity in the vasculature of normal and diseased tissues that we have coined the term “vascular zip codes” for it [2].

Tumor blood vessels are morphologically and molecularly quite different from normal blood vessels [1], and lymphatic vessels in tumors differ from normal lymphatic vessels [6,7]. *In vivo* phage screening has uncovered many of these differences, and this method has also produced the first tumor-penetrating peptides, which are the topic of this review.

Using an *in vivo* screening procedure designed to probe tumor lymphatic vessels, we identified a peptide that specifically accumulated in tumor lymphatics and not in normal lymphatics [6]. We now know that this peptide, LyP-1, primarily accumulates in a myeloid cell/macrophage in tumors, when intravenously injected into tumor-bearing mice. Some of these cells incorporate into tumor lymphatics, causing LyP-1 accumulation in the endothelium of these vessels [8]. Endothelial cells of tumor blood vessels and tumor cells also bind LyP-1, but much less of the peptide accumulates in these cells than in tumor macrophages. The macrophages are particularly abundant in hypoxic areas of tumors, which are low on blood vessels but contain abundant, albeit dysfunctional lymphatic vasculature [9]. Remarkably, the phage carrying the LyP-1 peptide reaches these areas within minutes of systemic injection. The ability of this peptide to reach poorly vascularized parts of tumors remained a mystery for several years, until we discovered another peptide with similar tumor-penetrating properties, and set out to uncover the underlying mechanism.

The new peptide, iRGD, was identified in a screen for peptides that home to tumor metastases [10]. It is a 9-amino acid cyclic peptide (sequence: CRGDKGPDC). iRGD has the integrin-binding RGD motif, but it was immediately obvious to us that this peptide was different from standard RGD peptides; the iRGD phage and the free iRGD peptide spread much more extensively into extravascular tumor tissue than other RGD peptides, which tend to accumulate only around tumor vessels.

2.2. Molecular basis of iRGD activity and the CendR motif

The iRGD peptide homes to tumors and accumulates in them through a 3-step process (Fig. 1): First, the integrin-binding RGD sequence motif binds to αvβ3 and αvβ5 integrins, which are specifically expressed in tumor endothelial cells. Other cells in tumors also express these integrins, which is likely to be important for the spreading of the peptide within tumor tissue, but the vascular endothelium is the gateway to the tumor for the peptide. Second, a protease cleavage event activates the CendR motif (R/KXXR/K). This protease(s) has not been identified, but is likely a furin or furin-like enzyme because the CendR motif is a preferred recognition motif for these proteases. In principle, any protease that cuts after a basic residue can activate iRGD. We have used trypsin and urokinase *in vitro* for this purpose [11]. The protease cleavage requires the integrin binding; a peptide that has the CendR motif but does not bind to integrins (CRG**E**KGPDC) is not activated. The requirement for integrin binding limits the activation of iRGD to tumors. Third, the CendR motif binds to neuropilin- 1 (NRP-1) or neuropilin-2 (NRP-2), and the interaction activates an endocytotic/exocytotic transport pathway named the CendR pathway [10,11]. This pathway is responsible for the enhanced transport of drugs into tumors triggered by iRGD.

2.3. Family of tumor-penetrating CendR peptides

Examination of the amino acid sequence of LyP-1 (CGNKRTRGC) shows that it also contains a CendR motif, and this peptide also uses the CendR pathway [12]. The primary receptor for LyP-1 is a mitochondrial protein p32/gC1qR/HABP, which acts as a chaperone in the mitochondrial oxidative phosphorylation pathway [8,13,14]. For reasons that are not understood, p32 is expressed at the cell surface in highly activated cells, such as tumor endothelial cells, tumor macrophages, and tumor cells, whereas it remains intracellular in normal cells [8]. A truncated form of LyP-1 (CGNKRTR; tLyP-1) is also a tumor-specific CendR peptide, even though it has an active CendR motif [12]. Although RGD peptides with a basic residue following the RGD motif bind poorly to integrins [15], a peptide resembling the CendR fragment of iRGD (RGDK) has been reported to selectively home to tumors [16]. Generally, such peptides, (*e.g.* RPARPAR) home to all tissues, the lungs in particular, because NRP-1 is expressed in all vessels, not just tumor vessels [11]. It may be that a combination of over-expression of neuroplin-1, which is common in tumors [12,17] with even weak binding to a tumor-specific component, can render a peptide partially selective for tumor homing. tLyP-1 may be a useful peptide with characteristics complementary to those of iRGD.

Having determined the salient properties of the tumor-penetrating CendR peptides, we designed such a peptide *de novo;* we converted a cyclic peptide with an NGR tumor-homing motif into a cryptic CendR peptide by adding and arginine residue to create the CendR motif RNGR [18]. These examples above show that tumor-penetrating CendR peptides can bind to different primary receptors and then converge to the CendR pathway through NRP-1 binding. Two other tumor-homing peptides from our phage library screens, F3 (KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK; [19] and CRGRRST [20] also contain potential CendR sequences (underlined). Whether these peptides actually act as CendR peptides has not been determined, but at least F3 shows internalization into cells [19] and has been used to target an oligonucleotide therapeutic into tumors [21].