Radiopharmacologist’s and Radiochemist’s View on Targeting the Eph/Ephrin Receptor Tyrosine Kinase System

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ABSTRACT: In the past decade, there have been extensive efforts to open up the Eph/ephrin subfamily of the receptor tyrosine kinase family for diagnostic and therapeutic applications. Besides classical pharmaceutical developments, which focus either on drugs targeting the extracellular ligand binding domains or on the intracellular tyrosine kinase domains of these receptors, there also have been first radiopharmaceutical approaches. Here the focus is on the development of specific and selective probes for molecular imaging, particularly by means of positron emission tomography, and the functional characterization of the Eph/ephrin subfamily in certain target tissues. The aim of this mini-review is to summarize the different approaches toward Eph-targeting radiotracers by using antibodies, peptides, and small molecules and to discuss their radiopharmacological characterization. With regard to the small molecules, further considerations will focus on the design and synthesis of nonradioactive reference compounds and precursors as well as on radiolabeling strategies.

EPH/EPHRIN SUBFAMILY OF RECEPTOR TYROSINE KINASE FAMILY

Eph receptors and their cognate ligands, the ephrins, currently represent the largest subfamily of the receptor tyrosine kinase (RTK) family. The term Eph has been derived from erythropoietin-producing human hepatocellular carcinoma since the first Eph receptor was identified in a screen for oncogenic tyrosine kinases in various carcinoma cell lines. The Eph/ephrin receptor pathway mediates direct cell–cell communication (over short distances) by affecting the cytoskeleton and, thus, cell adhesion and repulsion. This, in turn, plays important roles in many physiological processes, for instance, in development (formation of tissue boundaries, neuronal migration, axon guidance), synaptic plasticity, vasculogenesis, (adult) stem and progenitor cell maintenance, tissue repair and remodelling, and bone homeostasis, to name a few. On the other hand, Eph/ephrin signaling pathways have been characterized as pathophysiologically important in carcinogenesis and cancer progression as well as in neurological and inflammatory disorders.3 Nevertheless, and in contrast to other RTKs that are already theranostically targeted to a greater extent, the development of Eph-/ephrin-directed drugs or tracers is still in its infancy.

Structure, Function, And Signaling Concepts. Currently, 14 Eph receptors and 8 ephrin ligands have been identified in the human genome. Both receptors and ligands were grouped into class A and class B. EphA receptors preferentially bind ephrin-A ligands, whereas EphB receptors primarily bind ephrin-B ligands. However, also promiscuous binding of EphA receptors and ephrin-B ligands and vice versa has been proven, which leads to the possibility of even greater diversity in downstream signaling.2 The extracellular domain of Eph receptors consists of a ligand-binding domain, followed by a cysteine-rich domain and two fibronectin type III domains (Figure 1). Linked by a transmembrane region, the intracellular domain comprises the juxtamembrane region, followed by the tyrosine kinase domain, a sterile α motif (SAM) domain, and a PDZ-binding motif. The ephrin ligands also are membrane-bound proteins. In the case of class A ephrins, embedding in the membrane takes place via a GPI anchor. In the case of B-type ephrins, the extracellular receptor-binding domain is followed by a transmembrane region and an intracellular C-terminal PDZ-binding motif.1 The highly complex signaling of Eph/ephrins has been reviewed considerably elsewhere.4–6 However, it has to be taken into account also for a correct assessment of the potential but also the advantages, disadvantages, and limits of (radio)-pharmacologically addressing this system.

Received: March 9, 2020
Accepted: May 25, 2020
Published: June 17, 2020

http://pubs.acs.org/journal/acsodf

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membrane-bound ligands, the ephrins. As a receptor activation is based on the interaction with their phosphorylation sites.

Figure 1. Summary of the different approaches toward Eph-/ephrin-targeting radiotracers. Structure of Eph receptors (orange) and ephrin ligands class A (red) or class B (yellow). Potential points of therapeutic intervention are shown, and recent radiopharmacological approaches are highlighted by a “radioactive” label. GPI: Glycosylphosphatidylinositol anchor. PDZ: Postsynaptic density 95-disc large-Zona occludentes-1-protein domain. LBD: Ligand-binding domain. Cys: Cysteine-rich domain. FN: Fibronectin type III domain. K: Tyrosine kinase domain. SAM: Sterile α motif. P: Potential tyrosine phosphorylation sites.

Unlike other RTKs that bind secreted diffusible ligands, Eph receptor activation is based on the interaction with their membrane-bound ligands, the ephrins. As a first special feature, this juxtacrine, or contact-dependent, activation of the Eph receptors thus requires cell–cell contact between neighboring cells. A second important feature of Eph/ephrin signaling, that should be kept in mind, is that Eph/ephrin signaling is always context-dependent on the cell type, the specific receptor, and the cellular context, e.g., the tumor microenvironment.

The classical (canonical) signaling pathway is referred to as “forward signaling”; a membrane-bound Eph ritin ligand binds to the Eph receptor, followed by receptor dimerization and transphosphorylation of the Eph receptor tyrosine kinase domain. After further (auto)phosphorylation in the juxtamembrane region, SH2 domain-containing signaling or adapter proteins are recruited to the complex, and signaling in the Eph receptor-expressing cell is initiated. Effectors of forward signaling are typical intracellular signaling proteins like Rho/Rac GTPases, Ras/MAPK, PI3K/Akt/PK3, Src family kinases, and adapter proteins such as Nck and CrkII. The activated receptors can now undergo oligomerization and further on cluster formation, leading to amplification of forward signaling.

Signaling from the Eph-expressing cell into the ephrin-expressing cell, by contrast, is designated as “reverse signaling”. Similar to Eph receptors, B-type ephrins can be phosphorylated intracellularly on multiple tyrosine sites, providing binding sites for SH2 domain-containing proteins, whereby Src family kinases seem to be the most important ones. Thus, in B-type ephrins a direct reverse signaling is possible. A-type ephrins, on the other hand, are embedded in the plasma membrane by a GPI anchor, which makes direct signal transduction impossible. However, after binding of an Eph receptor also A-type ephrins lead to reverse signaling via interaction with other transmembrane proteins.

Due to the capacity of both Eph receptors and ephrins to act simultaneously as receptors and ligands, further signaling modes, designated as “bidirectional signaling”, “parallel signaling”, and “antiparallel signaling”, occur. In addition, noncatalytic functions are mediated by Eph receptors. These include regulatory effects mediated by Eph receptors with only scaffolding function for downstream signal molecules, e.g., for kinases of the Src family, by Eph receptor clusters involving other membrane-bound RTKs or proteases, and by shortened isoforms of Eph receptors without functional intracellular kinase domains or true pseudokinases, such as EphA10 and EphB6.

Furthermore, proteases like a disintegrin and metalloproteases (ADAMs), matrix-metalloproteases (MMPs), and γ-secretase play an important role for Eph/ephrin signaling by the formation of proteolytic products of Eph receptors and ephrins. Lastly, Eph/ephrin signaling can be regulated (and terminated) by endocytosis. Of note, both mechanisms, shedding of Eph/ephrins, leading to high levels in serum of patients, and, on the other hand, endocytosis, leading to the reduction of accessible extracellular targets, can interfere with theranostic targeting. In addition to (i) protease activity and (ii) Eph/ephrin endocytosis, as mentioned above, also (iii) conformation of the tyrosine kinase domain (active vs inactive), (iv) coclustering of Eph/ephrin complexes, as well as (v) effective elimination processes out of intact cells might influence the accessibility of the Eph/ephrin system for targeted functional imaging. However, at this point it must be stated that we are still far from a clear understanding of the precise mechanisms and possibilities of their modulation, in particular, in the context of various pathologies.

Role in Cancer. It is difficult to assign the Eph/ephrin system general functions in cancer. Both overexpression and downregulation of Eph receptors were detected in tumor tissue and found to be associated with both scenarios, tumor promotion and tumor suppression. A detailed description of Eph/ephrin expression in cancer was reviewed in detail elsewhere. Eph/ephrin signaling is always context-dependent and strongly associated with a complex signaling network, which connects numerous pathways. The expression level of Eph/ephrins on cancer cells and the concomitants of different Eph receptors have different consequences. Furthermore, one Eph receptor can mediate tumor-promoting effects with one ephrin ligand and opposite tumor-suppressing effects with another ephrin ligand. Eph/ephrin signaling in cancer functions via direct effects, e.g., on cell proliferation, cell survival, cellular motility, and invasiveness. On the other hand, Eph receptors and ephrins are also expressed in the tumor microenvironment, e.g., on vascular or stromal cells, leading to indirect tumor-promoting or -suppressing effects, such as support of tumor angiogenesis, promotion of epithelial–mesenchymal transition (EMT), tumor stem cell propagation and maintenance, and stimulation of the immune system. In light of their outstanding role in tumor progression and therapy response, most efforts in developing diagnostic or therapeutic agents have been done toward EphA2, EphB2, and EphB4.

Very briefly, EphA2 shows predominantly tumor-promoting effects. EphB2 has tumor-promoting as well as tumor-suppressing effects, depending on cell type and cellular context. In the case of EphB4, the forward signaling induced by ephrin-
**Table 1. Antibodies Binding Selectively to One Eph Receptor**

| target | mAb | tracer | imaging modality | chelators per mAb | antigen | radiolabeling for imaging |
|--------|-----|--------|-----------------|-----------------|--------|---------------------------|
| EphA2  | IC1 | ^6^Cu-DOTA-IC1 | PET | ~19 | | Cai 2007 |
| EphA2  | 4B3 | ^6^Cu-NOTA-4B3 | PET | 0.5 | | Puttick 2015 |
| EphA2  | DS-8895a | ^11^In-CHX-A^*^-DTPA-DS-8895a; ^12^I-DS-8895a | SPECT | | | Burvenich 2016 |
| EphA2  | DS-8895a | ^9^Zr-Ds-Bz-NCs-DS-8895a | PET | | | Burvenich 2016 |
| EphA3  | 3A4 | ^11^In-CHX-A^*^-DTPA-3A4; ^12^I-3A4 | SPECT | ephrin-binding domain | | Vearing 2005 |
| EphB4  | hAB47 | ^6^Cu-DOTA-Lys-hAB47 | PET | ~3.6 | fibronectin type III domain 2 | Liu 2013 |
| EphB4  | hAB131 | ^6^Cu-DOTA-Lys-hAB131 | PET | ~1.9 | fibronectin type III domain 1 | Li 2013 |
| EphB4  | hAB131 | hAB131-F(ab′)^3^-Cy5.5 | NIRF | | fibronectin type III domain 1 | Li 2013 |
| EphB4  | hAB131 | hAB131-Fab-Cy5.5 | NIRF | | fibronectin type III domain 1 | Li 2013 |

**Table 2. Peptides Binding Selectively to One Eph Receptor**

| target | peptide | IC_{50} peptide | IC_{50} cells | developed by | radiolabeling for imaging |
|--------|---------|----------------|--------------|-------------|---------------------------|
| EphA2  | YSAYPDNVYMM | 1 μM (agonist) | | Koolpe 2002 (Pasquale group) | |
| EphA2  | SWLAVPGAVSYR | 1 μM (agonist) | | Koolpe 2002 (Pasquale group) | Preote 2014 (Pietzsch group) |
| EphA4  | KYPYWPVPLSLL | 1 μM (antagonist) | 15 μM | Muri 2003 (Pasquale group) | |
| EphA4  | APyC4YRGSWJS | 1 μM (antagonist) | | Muri 2003 (Pasquale group) | |
| EphA4  | VTMEAINLAPFG | 1 μM (agonist) | | Muri 2003 (Pasquale group) | |
| EphB1  | EWLSPLAPLSR | ~10 μM (antagonist) | >200 μM | Koolpe 2005 (Pasquale group) | |
| EphB2  | SNMWQFPRLPQHI | ~15 μM (antagonist) | 100 μM | Koolpe 2005 (Pasquale group) | Kuchar 2012 (Pietzsch group) |
| EphB4  | DHNNHLYNWPRL | ~200 μM (antagonist) | | Koolpe 2005 (Pasquale group) | |
| EphB4  | TNYLSPNGPIPA | ~150 μM (antagonist) | >350 μM | Koolpe 2005 (Pasquale group) | |
| EphB4  | TNYLSPNGPIARA(W) | ~15 nM (antagonist) | | Koolpe 2005 (Pasquale group) | Li 2013 |

*The eponymous amino acid motif within the entire peptide sequence is delineated in bold.

B2 predominantly leads to an inhibition of tumor cell proliferation, whereas in the absence of the ligand EphB4 shows tumor-promoting properties, which also indicates a strong influence of the cellular context. As in other B-type Eph/ephrins, reverse signaling from the EphB4-expressing cell to the ephrin-B2-expressing cell also plays an important role, e.g., by acting in a tumor-suppressive manner in some cancers, and is indispensable for regulation of tumor angiogenesis.1,3,6

**Role in Nonmalignant Disorders.** First clinical observational studies also deal with the role of Eph/ephrin systems as biomarkers, e.g., in cardiomyopathy, chronic transplant glomerulopathy, and the craniofrontonasal syndrome, in these cases mainly concerning ephrin-B1. Based on a large number of preclinical observations, initial clinical evidence has been presented to show that Eph receptors and ephrins are indeed involved in the etiology, manifestation, and progression of nonmalignant diseases. In addition to the above-mentioned, this includes neurologic disorders like traumatic brain injury and spinal cord injury, neurodegenerative diseases such as Alzheimer’s disease, and motor neuron diseases but also inflammatory disorders from arthritis to various pathological vascular changes. Involvement of Eph receptors and ephrin ligands in these disorders is comprehensively reviewed elsewhere.7 At the moment, it is not foreseeable which thanerotic approaches are promising and will be clinically applied. A fundamental challenge here is to clearly differentiate the pathologically relevant changes from the physiologically significant effects and to develop strategies to address only the former while sparing normal tissue. In principle, this also applies to tumor therapy, but here the molecular differences between the tumor and normal tissue with regard to target density and activity are often significantly greater.

**Eph/ephrin system as a therapeutic target in cancer**

The central role of Eph receptors and ephrins in many pathological conditions has triggered the development of therapeutics for a broad range of applications, ranging from anticancer therapeutics to modulators of synaptic plasticity, as well as for the control of regenerative and pathological neoangiogenesis, bone homeostasis and remodeling, immune modulation, glucose homeostasis, and stem cell biology.7 In general, treatment strategies for tumor diseases differ significantly from those for other diseases. In the former case, the aim is, among other things, to directly cause tumor cell death, to inhibit tumor-associated processes such as angiogenesis, or to support other forms of therapy, e.g. by chemo- or radiosensitization (e.g., sEphB4-HSA; NCT04091867). The ultimate goal is a complete destruction of the tumor or associated tissue. The therapy of neurological or inflammatory diseases, in contrast, serves to regenerate and reconstitute the tissue or to minimize collateral damage. A nuclear medical diagnostic approach with radiotracers has to be justified in terms of radiation exposure but is, in principle, applicable to all these conditions provided that knowledge of the functional expression of a target molecule is relevant for the treatment of a patient.

Therapeutic agents targeting the Eph/ephrin system, as schematically illustrated by Figure 1, have been comprehensively summarized by Boyd and colleagues in 2014 and comprise ephrin-mimetic peptides, peptide vaccines, interfering RNAs (e.g., EphA2-targeting DOPC-encapsulated siRNA; NCT01591356), recombinant fusion proteins (e.g., sEphB4-HSA; NCT01642342, NCT04033432), monoclonal antibodies (mAb; e.g., anti-EphA2; NCT02004717), and small-molecule tyrosine kinase inhibitors (TKI; e.g., sitravatinib;
With regard to the last, it has to keep in mind that when using TKIs in these studies mostly those with lower selectivity are used, whose main targets are then a few other RTKs but which also address Eph receptors. Exemplarily, JI-101 targets EphB4 besides the growth factor receptors VEGFR2 and PDGFRβ. For therapeutics, such multikinase inhibitor approaches may be useful, but in most cases they are rather unsuitable for tracer applications.

Currently developed therapeutics for patients with various advanced or recurrent solid tumors and certain hematologic neoplasms are in Phase I (dose escalation and safety) at best, but most are still in the recruitment phase. Moreover, it is noticeable that the number of newly initiated clinical studies, compared to other forms of therapy such as CAR-T cell approaches, has not increased above average during the last six years.

### EPH/EPHRIN SYSTEM AS A DIAGNOSTIC TARGET IN CANCER

In addition to their role as potential therapeutic targets, Eph receptors and ephrins are also candidate prognostic markers and, therefore, in focus for the development of molecular imaging agents. In general, an intended radiopharmacological approach toward the Eph/ephrin system has two main objectives. On one hand, suitable radiolabeled compounds allow the (radio)pharmacological characterization of lead structures and certain derivatives with respect to their distribution, specificity and selective accumulation in the target tissue, stability, metabolism, and elimination in disease models by in vivo imaging. In addition to pharmacokinetics, quantitative information on pharmacodynamics can also be obtained, depending on the model and the study design. Moreover, it is noticeable that the number of newly initiated clinical studies, compared to other forms of therapy such as CAR-T cell approaches, has not increased above average during the last six years.

The successful development of chemical leads may allow for the development of theranostic approaches by using matched pair radionuclides, for instance, copper-64 (diagnostic β+ emission for PET imaging) and copper-67 (therapeutic β− emission). If successful in animal experimental settings, these...
approaches can be easily translated to the clinic and contribute to the clarification of the individual molecular signature of tumors and metastases in the respective patient before, during, and after a targeted therapy. Then, therapeutic decisions can be derived from this information, and insights into the response or failure of the therapy can be gained. This is particularly
hypoxic metabolism (\([18F]\)FMISO), or increased proliferation (\([18F]\)FLT). Furthermore, hydroxy groups can be functionalized with chelator units and labeling windows are required for the accumulation in target tissue. For this purpose, functionalization with chelator units and labeling of these molecules are promising candidates as chemical leads to develop radionuclide-based compounds for noninvasive tumor imaging.

**Targeting with Antibodies.** One of the first attempts for Eph-selective radionuclide-based *in vivo* imaging has been performed in 2007 by Cai and colleagues. For this purpose, the humanized monoclonal antibody (mAb) 1C1, which recognizes bothmurine and human EphA2, was conjugated with the DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) chelator and radiolabeled with copper-64 (\(^{64}\)Cu-DOTA-1C1; Table 1). With this labeled mAb in hand, the authors were able to noninvasively visualize and quantify the EphA2 expression in eight tumor models (murine CT-26; rat C6; human HT-29, A375, PC-3, DU145, SKOV3, and U87MG) by PET imaging. This may allow for a more efficient treatment monitoring and dose optimization of EphA2-targeted therapies since treatment-induced changes in the EphA2 expression level may occur much earlier than any anatomical changes can be detected by conventional medicinal imaging modalities. In general, noninvasive detection of molecular markers of diseases can enable earlier diagnosis, earlier treatment, and better prognosis.

Despite its promising potential for molecular imaging, a phase I clinical trial (NCT00796055) investigating the safety of an 1C1 drug conjugate with the microtubule inhibitor auristatin (1C1-maleimidocaproyl-auristatin phenylalanine, mcMMAF) had to be terminated because of adverse side effects (drug-related bleeding and coagulation events).

Especially for glioblastoma, an improved diagnosis by means of molecular imaging is urgently needed. For this purpose, the mAb 4B3, specific to only human EphA2, was conjugated with ...
the NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) chelator and radiolabeled with copper-64 (\(^{64}\text{Cu-NOTA-4B3}\); Table 1). To constitute more pathophysiologically relevant conditions, \(^{64}\text{Cu-NOTA-4B3}\) was tested in three intracranial glioblastoma models (human U87MG, RN1, and WK1), where the radiotracer had to penetrate the blood–brain barrier. PET images obtained with \(^{64}\text{Cu-NOTA-4B3}\) were superior regarding the tumor-to-brain contrast compared to the current clinical standards \([18\text{F}]\text{FDOPA PET}\) and gadolinium contrast-enhanced MRI. In addition, imaging results obtained with antibody-based tracers such as \(^{64}\text{Cu-NOTA-4B3}\) could be significantly improved by intravenous administration of a blocking dose of nonspecific IgG, especially when using mouse models like NOD/SCID, which do not naturally express IgG. Nonspecific IgG binds to Fc receptors and, thereby, reduces the number of receptors available for (unspecific) tracer binding but leaves the targeted receptor free.

Recently, Burvenich and colleagues explored the radiolabeling of another EphA2-specific mAb DS-8895a with iodine-125 using the Iodogen method, with indium-111 using CHX-A″DTPA (N-(2-amino-3-(4-isothiocyanatophenyl)-propyl)-trans-cyclohexane-1,2-diamine-pentaacetic acid) and with zirconium-89 using Df-Bz-NCS (p-isothiocyanatobenzyl-desferrioxamine) as chelators, suitable for SPECT (iodine-125 and indium-111) or PET (zirconium-89) imaging (Table 1). Neither chelation nor radiolabeling altered the structural integrity or immunoreactivity of the conjugates, resulting in high uptake of \(^{111}\text{In-CHX-A″-DTPA-DS-8895a}\) and \(^{89}\text{Zr-Df-Bz-}

Despite their subnanomolar affinities, both iodine-125- and indium-111-labeled ephrin-A5 Fc’s were not suitable for EphA3-specific visualization of tumor xenografts (human HEK293-EphA3, SK-MEL28, and LK63). This can be explained by rapid blood clearance and by the fact that ephrin-A5, despite its preferential interaction with EphA3, indeed binds most EphA receptors as well as EphB2. 11 In-ChX-A’-DTPA-3A4, by contrast, showed a significant uptake and retention (up to 10 days) in EphA3-positive tumors. 12-13 3A4, on the other hand, was also not suitable for EphA3-specific tumor imaging since internalization of mAb 3A4 led again to deiodination of the radioconjugate and release of iodine-125, whereas the radioconjugate with indium-111 was retained within the cells much longer. 11 For KB004, the humanized version of 3A4, a Phase I safety and bioimaging trial is currently ongoing in patients with recurrent glioblastoma (NCT03374943) combining an initial PET imaging sequence using 89Zr-KB004 followed by the therapeutic application of KB004. Initially, promising clinical responses in the absence of any dose-limiting toxicities or drug-related side effects had been reported. 2

In 2013, Liu and colleagues used the two humanized mAbs hAb47 and hAb131, developed by them three years before, which specifically bind to EphB4 with high affinity (Kd ~ 1 nM), for radiolabeling with the PET nuclide copper-64. 12 Whereas hAb47 binds to both human and murine EphB4, hAb131 is only affine to human EphB4. Within this study, hAb47 was conjugated to the DOTA chelator with three different methods using (i) the amino groups on the lysine side chain (64Cu-DOTA-Lys-hAb47; Table 1), (ii) the sulfhydryl group on cysteine after partial reduction of disulfide bonds (64Cu-DOTA-Cys-hAb47), or (iii) the aldehyde groups after oxidation of oligosaccharide (64Cu-DOTA-Sug-hAb47). 12 Although all three conjugation methods provided radioconjugates with high radiochemical yield (RCY) and mostly retained antibody recognition potential as well as antigenic characterization, both iodine-125- and indium-111-labeled ephrin-A5 Fc’s were not suitable for EphA3-specific visualization of tumor xenografts (human HT-29 and MDA-MB-231). In direct comparison, 64Cu-DOTA-Lys-hAb131 enabled a clear visualization of EphB4-positive tumor xenografts (human HT-29 and MDA-MB-231). In direct comparison, 64Cu-DOTA-Lys-hAb131 was superior to 64Cu-DOTA-Lys-hAb47, most likely due to steric reasons, since 64Cu-DOTA-hAb131 binds to the upper fibronectin type III domain 1 and, thereby, might have lower steric hindrance than 64Cu-DOTA-hAb47, which binds to the lower fibronectin type III domain 2).

In general, antibodies are considered as potentially the most specific probes for imaging because they offer an unmatched ability to bind selectively to any conceivable target. Moreover, they have the advantage of a reproducible, large-scale production, which enables researchers to effectively target an enormous number of receptors in a highly specific way. Of course, aspects such as immunogenicity, plasma residence time, undesirable kidney accumulation, etc. must be taken into account. In this regard, due to their high molecular weight, intact antibodies like hAb131 (150 kDa) are characterized by a long plasma residence time in vivo, which leads to unfavorable imaging kinetics and high background at early time points. Antibody fragments, by contrast, exhibit smaller molecular weight, faster clearance rate, and better tumor penetration capability and, thereby, might be more suitable for tumor imaging in vivo. In line with this, hAb131-F(ab’)-2-CysS-S (110 kDa; Table 1) was suitable for a rapid (>4 h p.i.) EphB4-specific tumor (human HT-29) targeting by near-infrared fluorescence.
(NIRF) imaging with good contrast. However, the antibody fragment hAb131-F(ab’)-Cy5.5 did not achieve the excellent tumor uptake found for the full-length mAb hAb131-Cy5.5, albeit at very late time points (>48 h p.i.). The smallest antibody fragment hAb131-Fab-Cy5.5 (50 kDa), which lacks the Fc domain, was not appropriate for EphB4-targeted *in vivo* imaging since tumor uptake was low and mostly attributed to nonspecific targeting.13

**Targeting with Peptides.** Radiolabeled peptides have drawn much attention for diagnostic imaging and targeted radiotherapy, especially in oncology, because of their excellent permeability, high binding affinity, and ease to be synthesized and radiolabeled. In the last years, several 12-amino acid-containing peptides have been discovered by phage display screens bearing a certain similarity to the ephrins’ 15-amino-acid long G–H loop, the sequence which mediates the high-affinity interaction with their cognate Eph receptors. These peptides target the ephrin binding pocket of the Eph receptors and, thereby, antagonize ephrin binding to the Eph receptors. Therefore, these peptides are supposed to be useful for therapeutic applications as selective inhibitors preventing ephrin-induced Eph downstream signaling responsible for different pathological activities of Eph receptors and, moreover, may be suitable as targeting probes for molecular imaging. An overview of these peptides is given in *Table 2*, and detailed information is well summarized by Noberini et al.14

Out of these peptides, only SWL, SNEW, and TNYL-RAW have been used in the past for first radiolabeling and *in vivo* imaging experiments. Obviously, excellent inhibitory activity of most compounds, as shown exemplarily for peptides (*Table 2*) but also for small molecules, is significantly diminished when analyzed in cell-based assays in comparison to protein-based assays. The latter could, among other things, provide indications of a reduced stability of the peptides, which have to be taken into account also for molecular imaging.

Since direct labeling of peptides with fluorine-18 is not amenable, radiolabeling usually requires the synthesis of *F*-containing building blocks, which are then connected to (i) a free primary amine function at the N-terminus and/or a lysine side chain or (ii) a free thiol group of cysteine. For this purpose, different *F*-containing building blocks have been developed in the past like N-succinimidyl 4-[*F*]fluorobenzoate ([*F*]SFB), containing an activated ester for labeling of primary amines, N-(6-(4-[*F*]fluorobenzylidene)aminoxyethyl)-maleimide ([*F*]FBAM) for thiol labeling, or 1-(3-azidopropyl)-4-(3-[*F*]fluoropropyl)piperazine ([*F*]AFP) and 1-(but-3-ynyl)-4-(3-[*F*]fluoropropyl)piperazine ([*F*]BFP) for radiolabeling via click chemistry.

The EphB2-specific SNEW peptide has been radiolabeled by a novel solid phase approach using the *F*-containing building blocks [*F*]SFB and [*F*]FBAM and the bioorthogonal building blocks [*F*]AFP and [*F*]BFP (*Scheme 1A*).15,18,22 Since the key amino acid sequence SNEW, located at the N-terminus of the peptide, is critical to retain the high affinity interaction with EphB2, it is mandatory to selectively radiolabel the peptide’s C-terminus. For radiolabeling with [*F*]SFB, the parent SNEW peptide sequence was synthesized on the solid phase and C-terminally extended by a lysine residue containing an Alloc-protected side chain to allow selective deprotection of this primary amine function.17 In comparison to the standard [*F*]SFB radiolabeling approach in aqueous solution, the solid phase approach enabled a more reliable and efficient site-selective radiolabeling but with a rather low RCY of 5%.17 When using the completely deprotected SNEW peptide for the radiolabeling with [*F*]SFB, a C-terminally labeled product occurred additionally. To overcome this, l-cysteine was connected at the C-terminus instead of l-lysine to enable regioselective radiofluorination using [*F*]FBAM.15 Unfortunately, the [*F*]FBAM-labeled SNEW peptide was instable in rat plasma *in vivo* probably due to reversibility of the Michael addition.18 Therefore, the SNEW peptide sequence was again varied at the C-terminus by the introduction of non-natural (amo) acids containing either an alkylene or an azide residue (4-azido-l-proline, azido-l-norleucine, S-azidovaleric acid, ε-(pent-4-ynamido)-l-lysine). This allows for the efficient, bioorthogonal, copper(I)-mediated azide–alkyne (Huisgen [3 + 2]) cycloaddition (CuAAC) with the novel building blocks [*F*]AFP and [*F*]BFP, respectively, under mild aqueous conditions.16,22 Additionally, both building blocks take advantage of using spiro azetidinium salt precursors (*Scheme 1B*), which are easily prepared from the open-chain compound, having the ammonium residue as an excellent leaving moiety and two sites at the four-membered ring for the nucleophilic attack of [*F*]fluoride under milder reaction conditions compared to convenient nucleophilic fluorinations. Further, precursors and building blocks allow for a fast purification using SPE and provide high RCYs and molar activities in a short reaction time.22 However, radiolabeling trials with [*F*]BFP and the azide-functionalized SNEW peptide were not successful due to the Glaser coupling as a side reaction.18 Thus, switching the functionalities of the peptide precursor and the radiolabeling building block to alkylene and azide, respectively, was mandatory and provided the [*F*]AFP-SNEW peptide in an acceptable RCY and molar activity. Biological evaluation demonstrated an adequate stability of the [*F*]AFP-SNEW peptide in rat plasma *in vivo*. However, PET imaging revealed a very short blood residence time, accompanied by metabolism and rapid renal elimination of the [*F*]AFP-SNEW peptide in rats, making this *F*-tracer not suitable for EphB2 receptor imaging *in vivo*.18

In 2014, the EphA2-specific SWL peptide has been modified with an alkylene function for subsequent radiolabeling with the bioorthogonal radiolabeling building block [*F*]AFP (*Table 2, *Scheme 2A*).15 The [*F*]AFP-SWL peptide was obtained with a RCY of 11 ± 2% and in a high radiochemical purity (RCP) of >98% but was not stable in rat plasma *in vitro* and in a BALB/c mouse *in vivo*. A very rapid blood clearance of the radiotracer and a high and continuous accumulation in the kidneys, followed by elimination into the bladder, were revealed by PET imaging.15 In the same year, Liu and colleagues used the SWL peptide for conjugation with the linker hydrazinonicotinic acid (HYNIC), followed by radiolabeling with the SPECT nuclide technetium-99m ([*99mTc*]-HYNIC-SWL; *Table 2, Scheme 2B*).16 The radiolabeled peptide bound with high affinity to EphA2-positive A549 cells *in vitro* (KD ~ 2.6 nM) and enabled EphA2-specific SPECT imaging of tumor xenografts (human A549) *in vivo* as early as 30 min p.i.16 Comparable to [*F*]AFP-SWL, [*99mTc*]-HYNIC-SWL was characterized by rapid blood clearance (30 min p.i., ~1.45% ID/g; 4 h p.i., ~0.44% ID/g) and, as typical for short peptides, by renal excretion.16 The EphB4-specific peptide TNYL-RAW (*Table 2*) was most comprehensively used for imaging purposes and was first time conjugated with either FITC for fluorescence imaging or the DOTA chelator for subsequent radiolabeling with the PET radionuclide copper-64 (*Scheme 3A*).19 Ephrin-B2 binding to EphB4 was inhibited by TNYL-RAW with a low IC50 value of 15 nM. This is, thereby, comparable to dimeric Fc-fused ephrin-B2
(IC₅₀ ~ 9 nM). Fortunately, Kᵦ values of TNYL-RAW (~3 nM) and ⁶⁴Cu-DOTA-TNYL-RAW (~2 nM) were comparable and lay in the low nanomolar range. Moreover, TNYL-RAW has a slow dissociation rate (~1.3 x 10⁻³ s⁻¹), which is a better indicator for the prospects of an in vivo imaging agent than the binding affinity, at least for reversible inhibitors.¹⁹ In this regard, a slow dissociation from the target or, at best, an irreversible binding to the target enhances target-to-background ratio and extends the time window for functional in vivo imaging. The proof-of-principle for the suitability of TNYL-RAW as a targeting unit for molecular imaging and, in light of this, also for drug delivery applications was shown using EphB4-positive cells (human PC-3M and mouse CT26) in comparison to EphB4-negative cells (human A549). As expected, both FITC- and ⁶⁴Cu-labeled TNYL-RAW peptides bound to EphB4-positive cells with a higher extent compared to EphB4-negative cells, and moreover, cellular binding was efficiently blocked by an excess of unlabeled TNYL-RAW.¹⁹ Additionally, a significantly higher tumor uptake in EphB4-positive tumor xenografts (human PC-3M) in comparison to EphB4-negative tumors (human A549) was observed, which could also be significantly blocked by the unlabeled TNYL-RAW. PET experiments revealed a promising tumor-to-muscle ratio of ~10 but also a high tracer retention in the liver, kidneys, and spleen. Moreover, the ⁶⁴Cu-labeled TNYL-RAW peptide started to be metabolized in vitro after 2 h in mouse serum and revealed a short blood residence time in vivo, two facts which certainly diminish the accessibility of the radiotracer for EphB4-dependent tumor targeting in vivo. Therefore, the group of Li aimed at the optimization of the peptide pharmacokinetics by conjugating the TNYL-RAW sequence to long-circulating, polyethylene glycol-coated core-cross-linked polymeric micelles (CCPM; diameter, 23 nm) dually labeled with the fluorophore Cy7 and the SPECT isotope indium-111 with a longer half-life compared to copper-⁶⁴.²⁰ Systemic clearance of the ¹¹¹In-labeled TNYL-RAW-CCPM (Scheme 3B) was significantly slower compared to the free circulating TNYL-RAW peptide. Moreover, the EphB4 targeting should be improved in comparison to ⁶⁴Cu-labeled TNYL-RAW since each CCPM nanoparticle contains approximately 60 TNYL-RAW molecules on its surface. On the other hand, the widely distributed nature of the TNYL-RAW peptide was significantly impaired after micelle formulation, but at least in part, it was also attributed to the observed higher concentration of ¹¹¹In-TNYL-RAW-CCPM in the blood.²⁰ As expected, EphB4-positive cells and tumors (human PC-3M) could clearly be discriminated from EphB4-negative cells and tumors (human A549). The significant blocking effect of the unlabeled TNYL-RAW suggests an EphB4-dependent binding of TNYL-RAW-CCPM to the surface of EphB4-positive cancer cells both in vitro and in vivo. Tumor uptake value as well as tumor-to-blood ratio confirmed that prolonging the blood residence time of TNYL-RAW improves the signal level in the tumor and, thereby, makes TNYL-RAW-CCPM superior for noninvasive molecular imaging of cancer.²⁰ By the combination of highly sensitive and quantitative PET or SPECT imaging together with fluorescence imaging, which by contrast allows for target detection even on a cellular level due to its outstanding high spatial resolution, multimodal imaging approaches allow for extraction of as much diagnostic information as possible from one examination, which might be advantageous especially in cancer diagnostics.²⁰ Further improvement for imaging purposes was achieved using TNYL-RAWK, a derivative of TNYL-RAW (Scheme 3C).²¹ TNYL-RAWK was not only characterized by an improved Kᵦ value of ~0.1 nM, but moreover, after labeling with both ⁶⁴Cu-DOTA and the NIRF dye Cy5.5, it allows for dual-modality imaging (PET/optical imaging). Thereby, a specific detection of EphB4 expression could be achieved in orthotopic glioblastoma tumor xenograft models (human U251 and U87), not only in the inoculated cancer cells but also in the EphB4-expressing tumor vasculature.²¹ Especially in patients with glioblastoma, a combination of nuclear imaging (PET or SPECT) together with intraoperative NIRF imaging allows for a safer resection of tumors and, thereby, led to an improved progression-free survival.²¹ Despite the promising imaging results observed with TNYL-RAW(K), peptides might also have drawbacks with regard to biomedical imaging purposes such as (i) receptor endocytosis as a result of Eph activation/phosphorylation, resulting in a reduced target availability and (ii) instability toward proteases.

Targeting with Small Molecular Weight (Tyrosine-Kinase) Inhibitors. Identification of small molecules capable of disrupting protein–protein interfaces for therapeutic attempts is a challenging endeavor due to the often large size of protein-interacting surfaces.¹⁴ However, for the development of radiotracers for imaging purposes such interfaces indeed provides access. To bind to their target, small molecules typically require the presence of suitable cavities such as (i) the extracellular ephrin-binding pocket or (ii) the intracellular ATP-binding pocket of Eph receptors.¹⁴ Since such cavities are not evident in both ephrins and all other domains of Eph receptors, like the extracellular receptor–receptor interfaces, the transmembrane domain, or the cytoplasmic regions, they are less suitable for targeting with small molecules.²⁴ Nevertheless, for targeting of the extracellular ephrin-binding pocket, a few compounds such as salicylates or lithocholic acid have been developed.²⁵ However, their binding potential is found only in the micromolar range and without receptor selectivity. Therefore, these compounds are not suitable as leads for the development of radiotracers.

The second set of small molecular weight compounds, known as tyrosine–kinase inhibitors (TKI), binds to the intracellular ATP-binding pocket within the kinase domain of Eph receptors, some of them with excellent affinity in the nanomolar range. Such compounds are the ideal basis for the development of carbon-¹¹- or fluorine-¹⁸-containing radiotracers. Nevertheless, high conservation of the ATP-binding pocket represents the main challenge resulting in a usually low selectivity of such compounds. For therapeutic purposes, a high selectivity may not always be desirable, and inhibitors that target multiple kinases involved in tumor angiogenesis or cancer progression, for instance, may have an increased effectiveness and a better ability to circumvent a resistance mechanism.²⁶ For molecular imaging, by contrast, it should be well-considered if, e.g., multi-Eph targeting is desired, and this, of course, largely depends on the kind of tumors to be investigated and their molecular fingerprint, especially with regard to up- or downregulation of the individual Eph receptors. An overview of inhibitors used for radiotracer development is pointed out in Table 3.

Further considerations will focus on the design and synthesis of nonradioactive reference compounds and precursors as well as on strategies of radiolabeling. In general, an isotopic exchange, e.g., with carbon-¹¹, is favored for radiolabeling since carbon belongs to the skeleton atoms of organic compounds. Mostly, carbon-¹¹ is introduced as a C1-building block like [¹¹C]methyl iodide at the end of the synthesis.
procedure because of its rather short half-life of 20 min. Thus, strategies to change methyl groups are the focus. Fluorine is often not part of the molecule structure. Thus, an isosteric labeling changing H, OH, or CH₃ groups is state of the art. For this purpose, a starting material, also referred to as a precursor, with a good leaving group is mandatory, as fluorine-18 is mostly introduced in the form of [^{18}F]fluoride by nucleophilic substitution reactions. Due to its medium half-life of 109.5 min, the introduction of fluorine-18 should be the last step of the synthesis as well. When fluorine is inserted into a molecule, its physicochemical and biochemical as well as its pharmacological properties like hydrogen bonds or other interactions are changed. To overcome this obstacle, docking studies are accomplished beforehand to allow the design of ^{18}F-labeled radiotracers with still high affinity comparable to the lead compound without fluorine.

In 2012, our group started to develop a ^{18}F-radiotracer based on the benzodioxolopyrimidine lead compound 1 published by Bardelle et al. in 2010 with an IC₅₀ of 90 nM for EphB4 (compound 1 of the citation). Based on the previous publication, it seemed to be possible to change the methyl group of the sulfonfyl moiety in compound 1 to a fluoropropyl group without changing the affinity to the EphB4 receptor dramatically. To synthesize the nonradioactive reference compound 8 and the precursor 7, the lead structure was divided. First, the pyrimidyl part 4 was prepared and, afterward, the respective sulfonfyl part 3 containing the fluorine. Compound 2 was synthesized for the introduction of the leaving group as pointed out in Scheme 4. Radiolabeling of precursor 7 was accomplished under standard labeling conditions, and the desired ^{18}F-tracer [^{18}F]8 was obtained with a RCY of 16% and a RCP of >95%. The radio-pharmacological characterization of [^{18}F]8 showed a cellular uptake of up to 40% ID/mg of protein over a period of 120 min at 37 °C in A375 melanoma cells with both a low basal EphB4 expression (human wild-type and mock-transfected A375) and a significantly enhanced EphB4 expression (human EphB4-transfected A375). Furthermore, small animal PET studies showed no substantial accumulation of [^{18}F]8 in EphB4-positive tumor xenografts (human A375-EphB4). Radioactivity concentration found in the tumors was low and comparable to radioactivity concentration in the blood. Both dynamic PET studies in vivo and autoradiography ex vivo (120 min p.i.) revealed that the main fluorine-18 activity was quickly accumulated in the liver and almost completely eliminated via the hepatobiliary route into the intestine.

To progress the search for an Eph-specific radiotracer, the indazolopyrimidine derivative 9, characterized by a higher affinity to EphB4 (Kᵟ ~ 1.3 nM, compound 20 of the citation) was chosen as the lead compound. To find the best position for radiolabeling with fluorine-18, docking studies were accomplished beforehand, pointing out either the change of the methylene group to a fluoropropylamine group or the change of one of the morpholine residues to a 3-fluoropropylpiperazine group. As a main result, the secondary amine was mandatory to remain free. This anilino NH function forms hydrogen acceptor and donor bonds to the hinge region at Met696 in the ATP-binding pocket and, thus, is necessary for a high affinity binding.

The preparation of all ^{11}C- and ^{18}F-tracers as well as the respective precursors and reference compounds afforded the application of a protection group strategy to avoid unwanted alkylations at the nitrogen atoms of the indazolyl part. Out of four different protection groups, the EOE group was chosen and introduced to yield compounds 10 and 11 as first structure parts. A reaction of 10 with 3,5-di(morpholino)aniline 12 as the second part of the structure led to the ^{11}C-precursor 15 (Scheme 5A). Indazolyl compound 11 was further reacted with the modified aniline 13 to obtain the fluorinated reference compound 17 after cleavage of the EOE group. The respective spiro precursor 16 for ^{18}F-labeling was prepared by reacting 11 with 14, followed by an introduction of the mesyl leaving group and final cyclization by heating (Scheme 5A).

Both radiolabeling procedures to [^{11}C]9 and [^{18}F]17 are pointed out in Scheme 5B. The ^{11}C-radiolabeling was accomplished with the commonly used C1-building block [^{11}C]methyl iodide and 15 in DMF with NaOH as base. Afterward, HCl was added to the mixture for cleaving the EOE group. The final ^{11}C-tracer [^{11}C]9 was obtained in 30–35% RCY based on [^{11}C]methyl iodide. For radiolabeling with fluorine-18, precursor 16 containing a fluoramide group was again chosen to exploit the aforementioned advantages of these compounds. The radiotracer [^{18}F]17 was prepared by the reaction of precursor 16 with [^{18}F]F⁻ under standard ^{18}F-labeling conditions, followed by cleavage of the EOE group with HCl. After purification using semipreparative HPLC, ^{18}F-tracer [^{18}F]17 was obtained in 34% RCY and >97% RCP.

A third ^{18}F-radiolabeling approach using xanthine derivatives was published in 2020. Lafleur et al. published the lead compound 18 (compound 66 of the citation, IC₅₀ = 2.3 (EphA2), 1.6 (EphB4)) in 2009. Again, the question of the best labeling position to introduce fluorine-18 was solved by performing docking studies with the EphA3 receptor, representing the similar ATP-binding pocket as EphB4, using crystal structure data and possible fluorine-containing molecules. Based on this result, the two fluorine-containing reference compounds 21a,b were synthesized from 19a,b using Deoxofluor. With this procedure, the only aliphatic OH group of 19a,b is displaced by fluoride under mild conditions (Scheme 6). Furthermore, both precursors 20a,b were obtained by reacting 19a,b with p-TsCl, leading to a 2-fold tosylation of the aliphatic and the aromatic OH group in 19a,b. The aliphatic tosyl group in 19a,b allows for a selective nucleophilic displacement with [^{18}F]fluoride in the following radiolabeling procedure. Radiolabeling of [^{18}F]21a was accomplished in a one-step procedure under microwave conditions using [^{18}F]F⁻ (5–10% RCY). Under these conditions, the aromatic tosyl group is also removed. Normal heating delivered only the tosylated tracer at the aromatic moiety. In contrast, radiotracer [^{18}F]21b was obtained in a two-step procedure using standard labeling conditions, followed by basic cleavage of the tosyl group with NaOH (3–5% RCY). First radiobiological evaluations were done with these tracers in vitro and in vivo showing no radiodefluorination over 60 min in rat blood plasma. However, a complete metabolism into a more hydrophilic species was observed at 60 min p.i., when urine samples of rats were analyzed. Cell uptake studies using A375 cells overexpressing EphB4 revealed a lower uptake of [^{18}F]2 compared to [^{18}F]3, resulting in less and more than 50% ID/mg, respectively.

**CONCLUSION**

This work briefly summarized the different radiopharmaceutical approaches to Eph-/ephrin-targeted functional imaging. These range from antibody-based radioligands to peptides and small molecules. It is not yet possible to estimate which of these approaches has the greatest potential to find their way into the...
clinic. First clinical studies have been initiated, but many of them have not yet progressed beyond the recruitment phase or have been discontinued. Further efforts in the development of radiopharmaceuticals are needed as well as investigations that will lead to a deeper understanding of the importance of the Eph/ephrin system, especially for the progression and treatment response in various cancer entities. Characterization of the functional expression of selected representatives of the Eph/ephrin subfamily by molecular imaging then allows for the corresponding diagnostic conclusions and the derivation of new, also radionuclide-based, therapeutic approaches.

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**ACKNOWLEDGMENTS**

We apologize to those researchers whose works have not been mentioned due to restrictions of length and number of references. We are especially grateful to our former colleagues Bettina Reissenweber, PhD, and Marc Pretze, PhD, as well as Kristin Ebert, Elisa Kinski, Katharina Müller, Paul Vogel, and Jens Wiemer who all received their doctorate or master in the field of Eph/ephrin research at the Technische Universität Dresden, Faculty of Chemistry and Food Chemistry, Germany. The authors are also grateful to Prof. emeritus Jörg Steinbach for his dedicated work and many stimulating and fruitful discussions. The authors thank the staff of the cyclotron and GMP radiopharmaceuticals production units for providing [18F] fluoride/[^18F]fluorine. The expert technical assistance of Julia Aldinger, Mareike Barth, Katrin Baumgart, Kay Fischer, Helge Gläsler, Regina Herrlich, Catharina Knöfel, Uta Lenkeit, Waldemar Herzog, Sebastian Meister, Aline Morgenegg, Peggy Nehring, Andrea Suhr, and Johanna Wodtko is greatly acknowledged. The authors thank the Helmholtz Association for funding a part of this work through the Helmholtz Cross-Programme Initiative “Technology and Medicine – Adaptive Systems”. C.N. and J.P are also thankful to the Deutsche Forschungsgemeinschaft (DFG) for supporting this work within the Collaborative Research Center Transregio 67 “Functional Biomaterials for Controlling Healing Processes in Bone und Skin – From Material Science to Clinical Application” (CRC/TRR 67/3).

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