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Review Article

Imaging ligands targeting glypican-3 receptor expression in hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality. Early detection of HCC is important since potentially curative therapies exist in the initial stages of HCC; no curative therapies exist for late-stage HCC. However, the initial detection of HCC remains challenging due to the lack of symptoms during the early stage of the disease. Other methods of screening and detecting HCC, including blood serum tests and conventional imaging methods, remain inadequate due to genetic differences between patients and the high background activity of liver tissues. Thus, there is a need for an accurate imaging agent for the diagnosis, staging, and prognosis of HCC. Glypican-3 (GPC3) is an oncofetal receptor responsible for regulating cell division, growth, and survival. GPC3 is a clinically relevant biomarker for imaging and therapeutics, as its expression is HCC tumor-specific and absent from normal and other pathological liver tissues. The development of novel GPC3-targeting imaging agents has encompassed three classes of biomolecules: peptides, antibodies, and aptamers. These biomolecules serve as constructs for diagnostic imaging (demonstrating potential as positron emission tomography [PET], single-photon emission tomography [SPECT], and optical imaging agents) and HCC treatment delivery. More than 20 unique ligands have been identified in the literature as showing specificity for the GPC3 receptor. Although several ligands are currently under clinical investigation as therapies for HCC, clinical translation of GPC3-targeting ligands as imaging agents is lacking. This review highlights the current landscape of ligands targeting GPC3 and describes their promising possibilities as imaging agents for HCC.

Keywords: Glypican-3, hepatocellular carcinoma, liver, tumor targeting, diagnostics

Introduction

Hepatocellular carcinoma (HCC) is the leading cause of primary liver cancers and the third most frequent cause of cancer-related mortality worldwide [1]. However, early detection of HCC remains difficult due to the lack of specific symptoms during the early stages of the disease and the inadequate sensitivities of the two primary surveillance methods, ultrasonography and alpha-fetoprotein serum levels [2]. Therefore, diagnosis of HCC often requires more advanced imaging techniques. Although effective therapeutics, including surgical resection, exist for early-stage HCC [3], only 40% of this aggressive cancer is diagnosed in earlier stages, resulting in poorer prognoses [4, 5]. Therefore, an early and accurate diagnosis of HCC is essential.

Though serum and histochemical biomarkers have been identified to detect the presence of HCC with varying accuracy [6], more sophisticated methods of tumor imaging *in vivo* remain elusive [7]. Exploiting the high uptake of glucose by tumor cells, positron emission technology/computerized tomography (PET/CT) using the radiotracer ^{18}F-fluorodeoxyglucose (^{18}F-FDG) has proven advantageous in the diagnosis, monitoring, and prognosis of many cancers [8, 9]. However, ^{18}F-FDG PET/CT is limited in the detection of HCC, with a sensitivity of only 40% [10]. ^{18}F-FDG's limited accuracy may be mediated by genetic differences in the expression of β-hydroxy β-methylglutaryl-CoA reductase degradation 1 (HRD1) enhancing the degradation of glucose transporter 1 (GLUT1) and subsequently decreasing uptake of ^{18}F-FDG [11]. Furthermore, the liver is a primary clearance

organ of exogenous molecules, such as radiotracers, resulting in high normal liver uptake and limiting the use of certain imaging agents [12]. Thus, HCC imaging agents must overcome high background liver uptake in visualizing tumors.

Glypican-3 (GPC3) is a surface heparan sulfate proteoglycan expressed in approximately 80% of hepatocellular carcinomas [13] and is associated with poorer prognoses [14]. It is postulated that GPC3 plays a role in the regulation of cell division and growth through the signaling pathways of Wnt, hedgehog, bone morphogenic protein, and fibroblast growth factor [15]. GPC3 is a promising biomarker for HCC due to its histochemical and serologic presence in liver tissue of HCC patients and absence in normal and other pathological liver tissues [16]. Additionally, the GPC3 receptor is expressed during the early stage of hepatocarcinogenesis, potentially allowing for earlier detection of HCC [17].

GPC3 is a novel target for the development of high-affinity probes. Molecular labeling of such probes, for instance with Cu-64 or Lu-177 [18, 19], may allow integration of diagnostic imaging and targeted therapeutics, a term coined "theranostics". Thus, a probe targeting GPC3 may enable the theranostic management of HCC patients. This review intends to summarize the current landscape of research into imaging ligands targeting the expression of GPC3 for diagnosing, monitoring, and predicting prognoses of HCC, specifically those of peptide, antibody, and aptamer constructs.

Ligands targeting GPC3 receptor

Biomolecules

Ligands targeting GPC3 have utilized three basic structures - protein, antibody, and aptamer - each with its own unique set of advantages and disadvantages as imaging agents. Protein ligands have seen extensive use as an imaging modality due to their small size, high affinity, *in vivo* stability, ease of synthesis and modification, and low immunogenicity. However, due to their small size, receptor binding affinity and pharmacokinetics are heavily dependent on the type of imaging label and are potentially subject to enzymatic degradation [20]. Antibody constructs show excellent antigen specificity and sensitivity and can be labeled for diagnostic and/or therapeutic purposes. Several anti-GPC3 antibodies are currently being assessed in clinical trials for their therapeutic potential in HCC, as they have previously been shown to inhibit HCC growth [21]. However, unlike proteins, antibodies are large molecules that suffer from long clearance times and half-lives, resulting in greater radiation exposure to the patient and potentially limiting their use as imaging agents [22]. Aptamers are short oligonucleotides between 15 and 40 bases and research on their use to target GPC3 is limited. However, aptamers have demonstrated potential as an imaging agent due to their stability, high affinity, and low immunogenicity [23]. As such, aptamer-based ligands are a versatile tool designed to tackle the inherent disadvantages of peptide and antibody-based ligands for targeting the GPC3 receptor in HCC [24, 25].

Imaging

Non-invasive imaging modalities serve a crucial role in cancer, relaying valuable information regarding tumor size, location, and regional physiologic and chemical processes. PET is a powerful imaging tool that uses positron-emitting radiopharmaceuticals and can be coupled with CT or magnetic resonance imaging (MRI) to detect and localize abnormal tissue pathologies [26]. SPECT is a nuclear imaging modality that is more accessible than PET and uses gamma-emitting radioisotopes [27]. Together, PET and SPECT have a complementary role in oncology, allowing the identification of tumors expressing biomarkers, including specific receptors, *in vivo* [28]. Optical imaging uses bioluminescent or fluorescent probes to reveal cellular and molecular functions in cancer. Optical imaging plays a significant role in the development of preclinical drugs and tracers due to its low-cost and noninvasiveness of fluorescent probes to determine *in vitro* and *in vivo* performance. Although limited in its ability to be used at a tissue depth beyond several centimeters, optical imaging may still have clinically translatable importance in surgical resection and tissue staining [29]. Several ligands have been reported in the literature showing affinity for GPC3 and some are currently under investigation as HCC therapy in humans [30]. Ligands with potential for diagnostics or theranostics will be mentioned.

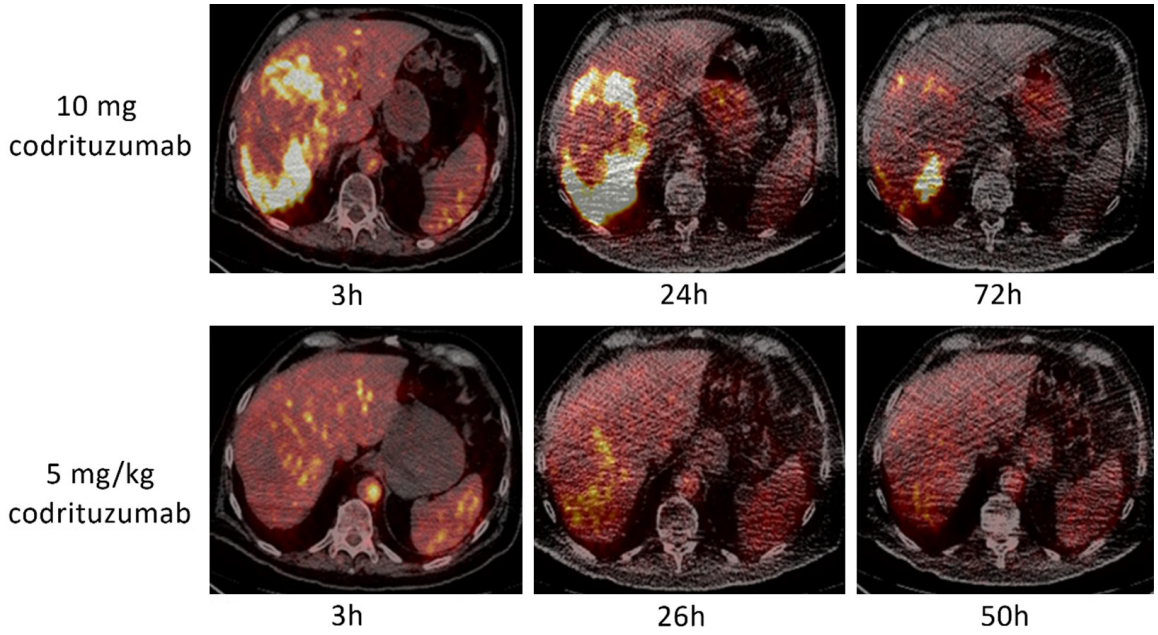
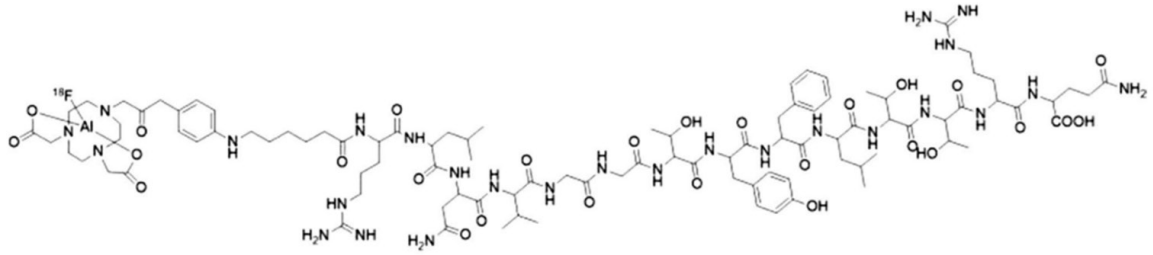


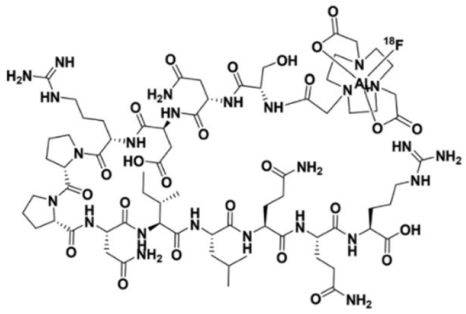
Figure 1. Representative imaging data for I-124 codrituzumab-PET/CT in a patient with HCC (adapted from the literature [32]).

PET ligands

In the past decade, many ligands have been preclinically assessed for their use in PET imaging of GPC3 in *in vitro* and *in vivo* studies. The only clinically researched PET ligand targeting GPC3 is iodine-124 (I-124) labeled codrituzumab (also known as GC33, K_D=0.673 nM). This 2009 phase Ib study (NCT00976170) assessed the humanized antiGPC3 antibody, codrituzumab, in combination with sorafenib in 41 HCC patients to determine efficacy. 14 patients underwent subsequent I-124 codrituzumab imaging to explore biodistribution and pharmacokinetics, 13 of which showed positive scan findings of an HCC tumor [31]. Low tumor accumulation in one patient with negative HCC detection may have been due to antigen heterogeneity and low GPC3 expression [32]. **Figure 1** shows a patient's representative I-124 codrituzumab PET/CT cross-section. Sham et al. demonstrated the targeting capability of antibody fragments using ^{89}Zr-αGPC3-F(ab')2 (K_D=0.03 nM) to improve upon their previous monoclonal antibody, ^{89}Zr-αGPC3 (K_D=0.03 nM), which suffered from suboptimal imaging pharmacokinetics, poor tumor penetration, and increased immunogenicity [33]. ^{89}Zr-αGPC3-F(ab')2 demonstrated faster blood clearance and allowed earlier detection of tumors but suffered from lower absolute tumor uptake than ^{89}Zr-αGPC3 [34]. An et al. introduced the GPC3-specific single domain antibody (sdAb), G2 (K_D=1.297 nM), labeled with Ga-68 and F-18, which clearly diagnosed HCC tumors. Further modification with the albumin-binding domain significantly improved imaging pharmacokinetics [35]. There are no published chemical structures for antibody PET ligands targeting GPC3. The first protein-based PET ligand targeting GPC3 reported in the literature is ^{18}F-AlF-NOTA-MP-6-Aoc-L5 (K_D=101 nM) by Wang et al. using the novel 12-mer peptide ligand L5 (sequence: RLNVGGYYFLTTRQ, K_D=44.7 nM), which was first identified by Lee et al. in 2011. Although the probe demonstrated selectivity for GPC3 expressing tumor cells and clear visualization *in vivo*, the authors concluded that ^{18}F-AlF-NOTA-MP-6-Aoc-L5 requires further chemical modification to achieve higher tumor-to-liver ratios [36, 37]. Furthermore, Berman et al. showed that *in vitro* performance of L5 and another promising peptide, TJ12P1 (sequence: DHLASLWWGTEL, K_D=280.4 ± 33.51 nM) [38], demonstrated a lack of selectivity or potency, failing to bind to GPC3 at concentrations in the range of their published K_D potentially due to their relative hydrophobicity. Therefore, the authors concluded that TJ12P1 and L5 should no longer be developed in their current forms



^{18}F-AIF-NOTA-MP-6-Aoc-L5; K_D= 101 nM

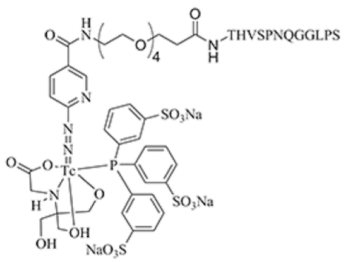


^{18}F-AIF-NOTA-TJ12P2; K_D= 158 ± 26.25 nM

Figure 2. PET imaging ligands targeting GPC3.

Table 1. PET ligands reported in the literature targeting GPC3

Ligand	Biomolecule	K_D (nM)	Reference
^{89}Zr-DFO-1G12	Antibody	0.41 ± 0.05	[12]
^{90}Y-DOTA-αGPC3	Antibody	0.03	[50]
^{89}Zr-DFO-αGPC3	Antibody	0.03	
^{89}Zr-ERY974	Antibody	1.5	[51]
^{89}Zr-Df-H3K3	Antibody	3.89 ± 0.23	[52]
GP2076 (sequence: RLNVGGTYFLTTRQ)	Protein	101	[53]
GP2633 (sequence: GGGRDNRLNVGGTYFLTTRQ)	Protein	63.3	
^{68}Ga-DOTA-F3 (sequence: *not reported*)	Protein	*Not reported*	[54]



^{99m}Tc-(tricine)-(TPPTS)HyNIC-PEG4-GBP; K_D= *similar to GBP (735.2 ± 53.6 nM)*

Figure 3. SPECT imaging ligand targeting GPC3.

as molecular imaging agents [39]. Optimizing the methods used to identify TJ12P1, TJ12P2 (sequence: SNDRPPNILQKR, K_D=158 ± 26.25 nM) was isolated and subsequently labeled with F-18 to produce ^{18}F-AIF-NOTA-TJ12P2. Unlike TJ12P1, TJ12P2 showed improved affinity and decreased normal liver uptake and is a promising candidate for translation as an HCC imaging agent [40]. The chemical structure of ^{18}F-AIF-NOTA-MP-6-Aoc-L5 and ^{18}F-AIF-NOTA-TJ12P2 are depicted in **Figure 2**. Other GPC3-targeting PET ligands published in the literature are summarized in **Table 1**.

SPECT ligands

To date, only one SPECT ligand targeting the GPC3 receptor has been published. Using the GBP protein (sequence: THVSPNQGGLPS) isolated by Qin et al. [41], Xu et al. synthesized the SPECT radiotracer ^{99m}Tc-HPG (sequence: ^{99m}Tc-(tricine)-TPPTS)HyNIC-PEG4-GBP, **Figure 3**) and

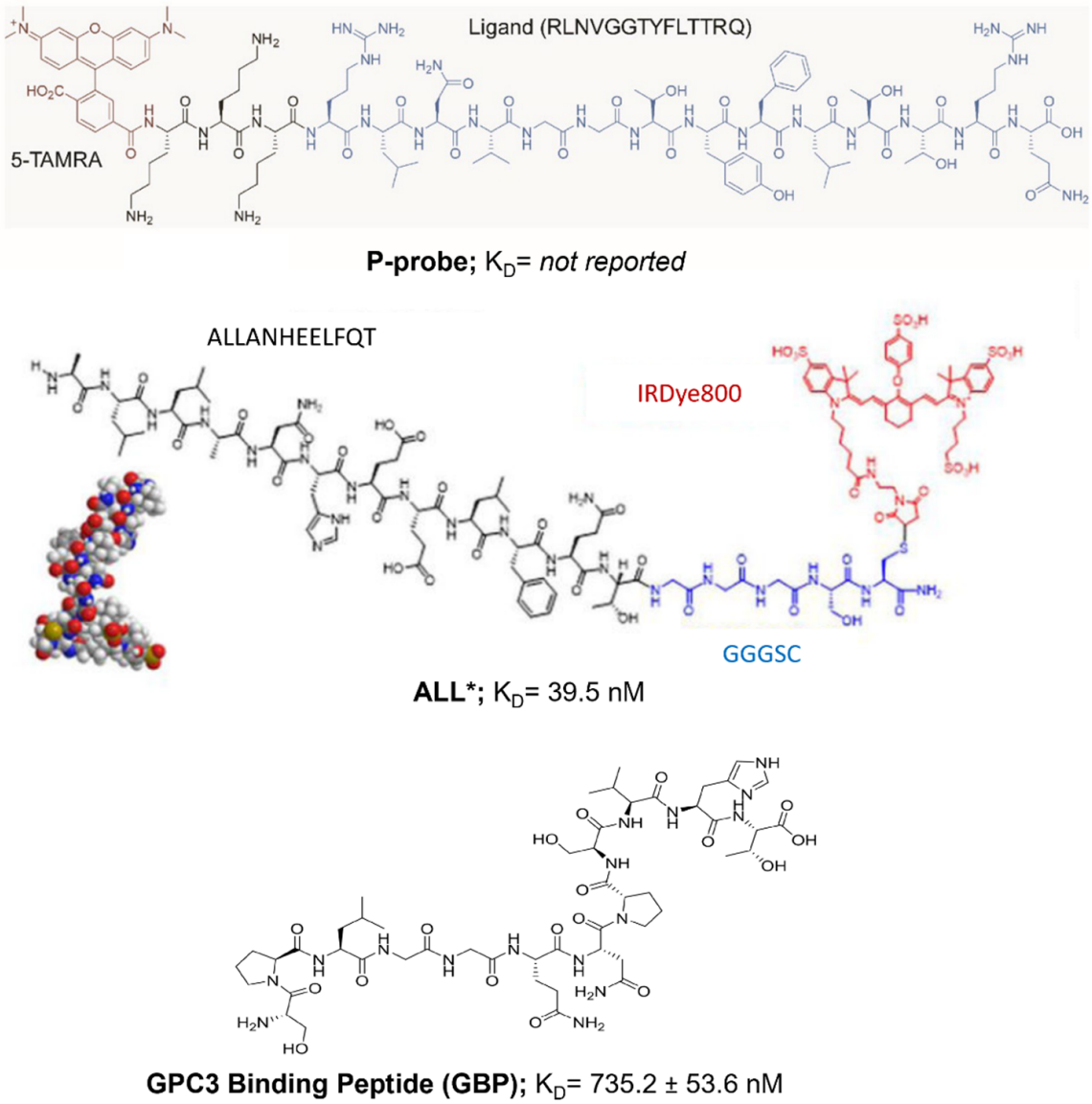


Figure 4. Optical imaging ligands targeting GPC3.

investigated *in vitro* characteristics, obtained SPECT/CT images, and assessed biodistribution in GPC3-positive tumor models. This study showed that the modified SPECT structure of GBP retained the affinity and ability to target GPC3. Furthermore, the probe showed higher uptake in GPC3-positive cells than in GPC3-negative cells. The high tumor-to-background uptake allowed definitive detection of lesions in their HCC tumor transplantation model. Using the same synthesis strategies due to similarities in chemical properties, the authors mention that HPG can utilize a Re-188 label to provide radiation therapy in conjunction with the diagnostic capabilities of ^{99m}Tc-HPG [42].

Optical imaging ligands

Optical imaging is a powerful modality for the molecular imaging of disease and therapy. Fluorescent labeled imaging agents play an important role in the development of imaging tracers and have an opportunity for clinical use. Optical imaging agents can be further developed into PET and SPECT radiotracers. Modifying the L5 peptide construct, Han et al. synthesized the peptide probe (P-probe) and subsequently generated the supramolecular 2D imaging probe (2D probe) that sensitively and selectively imaged GPC3 overexpression *in vitro*. 2D probe (K_D= *not reported)* shows prom-

Table 2. Optical imaging ligands reported in the literature targeting GPC3

Ligand	Biomolecule	K_D (nM)	Reference
GPC-ICG	Antibody	Not reported	[55]
TJ12P1 (sequence: DHLASLWWGTEL)	Protein	390 ± 27.47	[38]
L5 (sequence: RLNVGGYYFLTTRQ)	Protein	44.7	[36, 37]
L5-2 (sequence: YFLTTRQ)	Protein	*Not reported*	
TJ12P2 (sequence: SNDRPPNILQKR)	Protein	158.2 ± 26.25	[40]
MPA-IPA (sequence: DYEMHLWWGTEL)	Protein	225.1	[56]

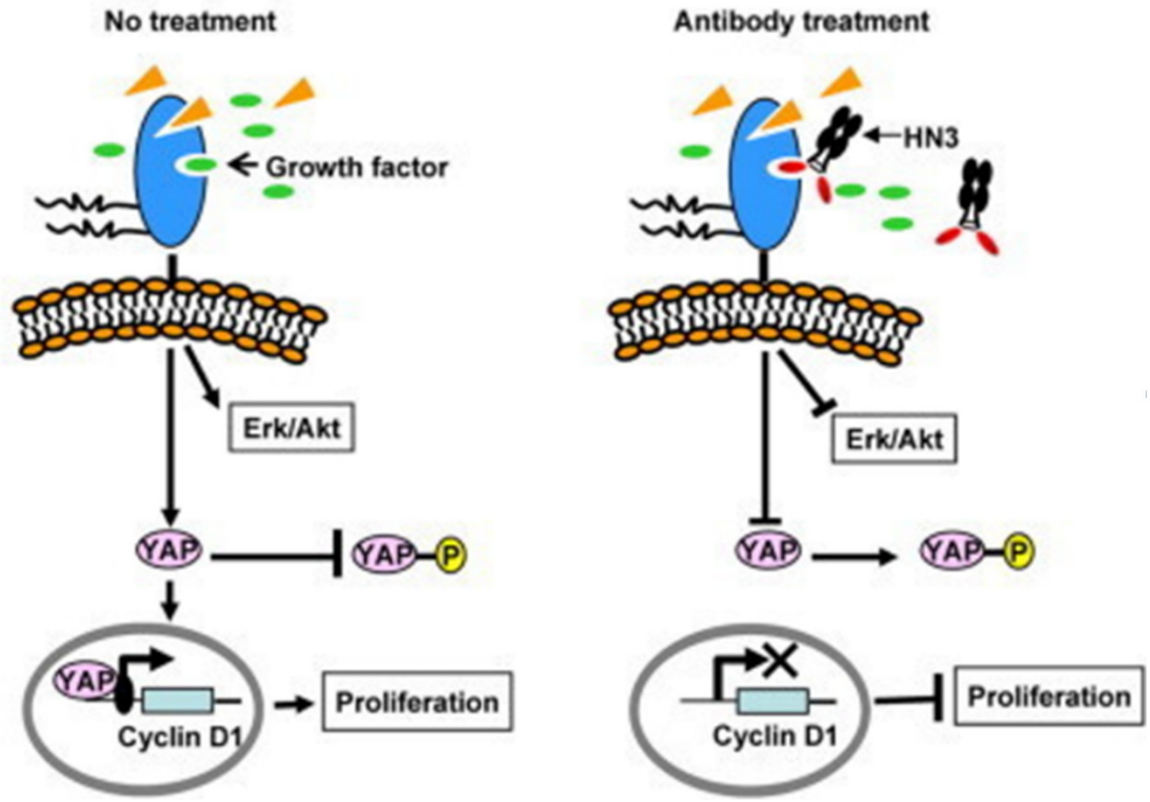


Figure 5. Schematic of the mechanism of action for HN3 (adapted from the literature [48]).

ise as a tumor-section staining method as well as a fluorescence imaging-guided surgery probe [43]. In 2021, Feng et al. synthesized the near-infrared peptide (sequence: ALLANHEELFQT, K_D=39.5 nM) using IRDye800 as a fluorescence label. This peptide showed high affinity, optimal kinetics, and demonstrated visualization of implanted HCC tumors [44]. The GPC3 binding peptide (GBP) was identified by Wang et al. (sequence: THVSPNQGGLPS, K_D=735.2 ± 53.6 nM) and labeled with the near-infrared dye Cy5.5 showing a high accumulation in HCC tumors and not in normal liver tissues *in vivo* suggesting the potential for translation to radiolabeling and clinical studies [41]. Using the fluorescent labels 6-FAM and AF750, the aptamer probe AP613-1 (sequence: 5'-TAACGCTGACCTTAGCTGCATGGCTTTACATGTTCCA-3', K_D=59.85 ± 15.39 nM) was labeled by Zhao et al. and demonstrated high affinity to GPC3 in *in vitro* and *in vivo* studies [45]. Subsequent efforts by Zhao et al. developed the AP613-1 aptamer into an MRI probe using ultrasmall superparamagnetic iron oxide nanoparticles showing high specificity *in vivo* [46]. **Figure 4** depicts the chemical structure of protein optical imaging probes; no chemical structures for aptamer optical imaging ligands have been published. Additional optical imaging probes are summarized in **Table 2**.

Unlabeled and therapeutic ligands

Several therapeutic ligands showing affinity to the GPC3 receptor have been reported but have seen no further investigation as imaging agents. YP7 (K_D=0.3 nM), a mouse anti-GPC3 antibody, was identified by Phung et al. and showed significant inhibition of HCC growth in xenograft tumor mice through antibody-dependent cellular cytotoxicity [47]. Feng et al. isolated the heavy-chain variable domain antibody, HN3 (K_D=0.6 nM), which also significantly inhibited HCC xenograft tumor growth in nude mice through inhibition of YAP signaling and HCC cell proliferation as depicted in **Figure 5** [48]. HS20 (K_D=0.6 nM), a humanized monoclonal antibody, inhibited HCC tumor growth by blocking GPC3 interaction with Wnt/β-catenin signaling [49]. Although holding potential for *in vivo* tumor imaging, YP7, HN3, and HS20 have not been further investigated as imaging agents.

Concluding remark

In summary, we reviewed ligands targeting the GPC3 receptor overexpressed in HCC. The GPC3 receptor is a promising target for detecting and potentially treating HCC. The last decade has seen numerous preclinical studies investigating the ability of ligands to target GPC3. These ligands may serve a future role as diagnostic and therapeutic agents. The clinical value of GPC3 imaging in HCC requires further investigation. Currently, no GPC3-targeting agents for the main purpose of imaging HCC have been translated into human clinical trials. The progression and treatment of HCC may alter GPC3 expression, further necessitating the exploration of novel targets for HCC imaging and therapeutics.

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Disclosure of conflict of interest

None.

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