

Chlorophyll-*a* Analogs for Cancer Imaging and Therapy (Theranostics)

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Abstract The utility of porphyrin-based compounds as photosensitizers in photodynamic therapy (PDT) has been evolving since the mid-1970s. There are a large number of publications including research papers, reviews, and books on basic sciences and clinical applications of PDT. The current research on PDT has been focused on developing agents with enhanced tumor specificity. Efforts are also under way in various laboratories to develop multifunctional agents. In other words, is it possible to use a single agent for cancer imaging and PDT? This chapter briefly describes the recent developments in developing such agents, which could be used for dual-function imaging (e.g., PET/fluorescence, MRI/fluorescence) with an option of PDT. The utility of biocompatible and biodegradable nanoparticles over the synthetic approach for achieving the desired goal is also discussed.

Keywords Image-guided cancer therapy · Photodynamic therapy · Polymer conjugates · Porphyrin derivatives · Theranostics

Contents

1	Introduction	2
1.1	Importance of Imaging	2
1.2	Image-Guided Therapy	5
2	Tetrapyrrole-Based Compounds for Cancer Imaging and Therapy	6
2.1	Fluorescence Image-Guided PDT	9
2.2	MR/Fluorescence Imaging and PDT	13
2.3	PET/Fluorescence Imaging and PDT	14

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3	Multimodality Agents: Advantages of Nanoparticles	18
3.1	Polyacrylamide-Based Nanoparticles	21
4	Conclusion and Future Directions	26
	References	26

Abbreviations

AHM	3-(Acryloyloxy)-2-hydroxypropyl methacrylate
APMA	<i>N</i> -(3-Aminopropyl)methacrylamide
CA	Contrast agent
CE	Contrast enhancement
CLIO	Cross-linked iron oxide
CT	Computed tomography
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
DTPA	Diethylene triamine pentaacetic acid
FDA	Food and drug administration
FDG	Fluorodeoxyglucose
FITC	Fluorescein isothiocyanate
FMT	Fluorescence-mediated tomography
FRET	Fluorescence resonance energy transfer
GQL	Good quality of life
HPPH	3-(1'-hexyloxy)ethyl-3-devinyl pyropheophorbide- α
ICG	Indocyanine green
MB	Methylene blue
MRI	Magnetic resonance imaging
NIR	Near infrared
NP	Nanoparticle
PAA	Polyacrylamide
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PET	Positron emission tomography
PS	Photosensitizer
ROI	Region of interest
SPECT	Single-photon emission computed tomography
US	Ultrasound

1 Introduction

1.1 Importance of Imaging

Heart disease remains the major cause of death among Americans; however, public awareness coupled with improved diet and medicines has led to a drastic reduction in annual number of deaths per 100,000 people [1]. Unfortunately even with

decades of advancements in the development of anticancer compounds, studies still show that age-related death rates for people with cancer have not improved [2]. Next-generation drugs are able to overcome resistances to first-line chemotherapeutics and in many cases improve safety profiles. However, if there is ultimately no improvement in survival rates, then the only effect these new drugs are having on the public is maintaining high health-care costs. Current advancements in medical imaging techniques are aimed at offering the opportunity to make a significant change in cancer treatment, which could produce cancer cures or long-term survival with good quality of life (GQL). While conventional imaging instrumentation is very effective, advancements in machinery and improved understanding of computer models and the physics behind a lot of these technologies have ushered in an era of exponential growth in cancer imaging. It is now the belief of many that while chemotherapeutics hold an undeniable importance in the treatment of cancer, it is through improved imaging systems where the real improvements can be made to lead to the overall reduction in cancer deaths.

Among these conventional imaging modalities, magnetic resonance imaging (MRI) bears the greatest clinical utility due to its versatility [3]. Not only does MRI provide superb spatial resolution and unparalleled soft tissue contrast in living subjects, but it can also detect certain tissue functions and molecular species via the use of specialized MRI techniques, some of which do not rely on the use of target-specific contrast-enhancing agents [4]. Therefore, MRI permits concurrent collection of anatomical and physiological information of the disease state. In addition, MRI has two advantages that make it stand out among other imaging modalities [5]. Unlike positron emission tomography (PET), single-photon emission computed tomography (SPECT), or computed tomography (CT), MRI does not use dangerous ionizing radiation which leads to the second advantage which is that there is no upper limit to the scanning frequency of a patient in a specific time span. However, MRI has very poor sensitivity when compared with other imaging modalities, and this has many manifestations [6]. First, MRI acquisition can take a long time and a large dose of imaging agents to produce adequate signal. As a result, a particular subset of patients is predisposed to potential health complications when using contrast-enhancing agents (insert renal toxicity data). Another result of the poor sensitivity, and therefore high requirement of MRI contrast agents, is that it creates a difficult-to-solve challenge for researchers to design target-specific probes when cellular and molecular targets exist in low concentrations in living subjects [7]. The high amounts of image contrast agents are one of the major reasons why MRI is such an expensive imaging modality. When considering cost, one must also consider not only the high cost of purchasing MRI machines but also the high cost of their maintenance. MRI magnets can only effectively function when they are cooled below their critical temperature. In order to achieve this, liquid helium is used as a coolant; however, with the current global supply of helium shrinking, the cost to operate MRI machines has increased. Overall, MR imaging is extremely potent, although it is not the most sensitive and cost-effective modality. Not only has MRI revolutionized clinical cancer diagnostics, it also adds a powerful tool to

researchers' repertoire in cancer research, enabling them to scrutinize molecular processes in single cells, perfused organs, and intact living animals [8–13].

CT employs tomographic imaging to reconstruct the differential X-ray attenuation by tissues within the body into three-dimensional images reflecting anatomy. It is among the most widely used imaging tools in hospitals [14]. At present, CT is a structural imaging modality, which identifies anatomical patterns, and its use in oncology is that it provides information regarding tumor location, size, and spread based on endogenous contrast. CT contrast agents are predominantly based on iodine-containing molecules, although efforts are under way to develop target-specific gold nanoparticles and enable the application of CT in molecular imaging.

PET and SPECT depend upon the identification or synthesis of target-specific pharmaceuticals, which are tagged with a radionuclide and are used as imaging agents [15]. Through tracing their distribution in the body, PET and SPECT are functional imaging modalities that enable whole-body evaluation of the level of molecular targets/processes within a living subject. Like CT, PET and SPECT employ ionizing radiation, which raise concern about their safety profiles. As a result, there is a limit to how many scans a subject can have in a given time frame.

Ultrasound (US) is another structural imaging modality [16] and utilizes the characteristic properties and behavior of high-frequency sound waves as they travel through biological tissue to reconstruct anatomical images. Although it is primarily used to attain structural information, targeted microbubbles are currently being explored for their feasibility as contrast agents that allow US to image processes such as angiogenesis or inflammation. In comparison to CT, US is more cost-effective, more portable, and much safer. However, US is not suitable for body parts containing bones or air. And although resolution of US can improve significantly with higher frequencies, it comes at the cost of depth of tissue penetration of the sound waves.

In terms of clinical usage, one of the fastest growing imaging modalities is fluorescence optical imaging [17]. Fluorescence optical imaging utilizes the phenomenon of fluorescence and uses a noninvasive propagation of visible or near-infrared (NIR) range light through tissue to develop an image. For decades, fluorescence optical imaging techniques have been used in the academic setting where it has been used to trace movement of cells or organelles, production and reaction rates of proteins, and even recombination events within complex organisms. However, light propagation through biological tissue is limited because light is too easily absorbed, reflected, and/or scattered by biological tissue and their components. This has led to limited use clinically, but advancements in image capturing technology and improvements in image reconstruction algorithms have the potential to change that. Perhaps the biggest advantage of optical imaging is that it avoids the DNA-damaging side effects found with imaging modalities which employ ionizing radiation such as CT or PET which is one reason for the increased desire to transition the optical imaging modality into the clinic. Other advantages of optical imaging include fast acquisition, high sensitivity, and lower cost in comparison to other modalities. However, the resolution of optical imaging is very poor as light has limited depth of penetration.

Fluorescence-mediated tomography (FMT) is a subset of optical imaging that plays a prominent role in the preclinical settings for the investigation of molecular processes in intact animals [18]. A major drawback of older optical reflectance imaging is the lack of quantification in the fluorescent light from the source. Fluorescence-mediated tomography is an alternative modality which allows for quantization of signals. This is accomplished because fluorescence tomography enlists multiple light sources, which are paired with multiple detector pairings to generate numerous experimental data to assist the effort to pinpoint and quantify the fluorescence reporter within the tissue. Another benefit of fluorescence tomographic imaging is the ability to reconstruct three-dimensional images, something that traditional reflectance imaging cannot do. Tomographic images have been overlaid with high-resolution datasets generated from MRI, thereby leading to two very complimentary imaging modalities resulting in superior imaging information. In proof of principle study, mouse brain implanted with human glioma tumor cells was examined using MRI-FMT co-registration and led to an improvement in tumor localization [19, 20].

The idea behind creating dual-function or multifunctional compounds lies in the fact that no imaging technique is perfect and all have some drawbacks. Simply, if one technique has a disadvantage and another modality can compensate for this, then the two modalities could be complementary to each other. In case of MRI, the aforementioned cost, sensitivity, and acquisition times are major hindrances, but it excels in resolution and depth of penetration and provides physiological information. Optical imaging on the other hand uses inexpensive probes and has excellent sensitivity, requires small doses, can be used for image-guided therapy, but yields very poor spatial resolution and has limited clinical usage due to depth of penetration. Therefore, it becomes necessary to develop long-wavelength optical imaging agents and combine its use with other imaging techniques.

1.2 Image-Guided Therapy

Real-time imaging of the entire tumor is the goal of image-guided therapy [21]. Conventionally, imaging is done preoperatively, then surgical resection of the tumor is performed, and possibly postsurgical imaging is performed to ensure that the entire tumor has been removed. However, imaging modalities such as fluorescence optical imaging, due to its very short acquisition time and cancer specific probes, offer the ability to imaging during the surgical resection process, thereby ensuring that the entire tumor is removed. Van Dam et al. [22] illustrated the importance of image-guided therapy in their work with intraoperative fluorescence imaging of ovarian cancer in which they used folate-FITC to determine what areas of the patients' ovaries contain cancer which needed to surgically be removed and which areas were normal tissue. In addition to surgery, other methods for tumor ablation can work well with image-guided therapy. One such method is photodynamic therapy (PDT) which currently serves as either a primary or adjunctive



Fig. 1 General structures of porphyrin-based compounds: porphyrins, chlorins, and bacteriochlorins

treatment for solid cancers of the head and neck, brain, lung, pancreas, intraperitoneal cavity, breast, prostate, or skin [23].

2 Tetrapyrrole-Based Compounds for Cancer Imaging and Therapy

Tetrapyrrole-based compounds (e.g., porphyrins, chlorins, and bacteriochlorins, Fig. 1) have shown great potential in the area of photodynamic therapy (PDT) [24–31], which consists of three components: a photosensitizing drug (photosensitizer), light of specific wavelength, and oxygen. Photosensitizer or the prodrug, injected intravenously (i. v.), intraperitoneally (i. p.), or applied topically, circulates systemically and is retained in tumor preferentially compared to normal tissues. When the accumulation of photosensitizer in tumor is optimal, the tumor is illuminated by monochromatic laser light of a specific wavelength.

The photosensitizer, when irradiated with the light, promotes one of its electrons from a ground triplet state to an excited singlet state. This electron can either relax back to the ground state to emit a photon (fluorescence) or undergo intersystem crossing and convert from the excited singlet ($^1\text{PS}^*$) to the excited triplet state ($^3\text{PS}^*$). The excited triplet state can then transfer energy to the surrounding molecules directly in a type I reaction, producing radicals, or it can transfer energy to molecular oxygen in a type II reaction, producing singlet oxygen ($^1\text{O}_2^*$). Although both reaction types are known to happen, experimental evidence suggests that the type II reaction is dominant during PDT [32] (Fig. 2). Singlet oxygen is cytotoxic, damaging cellular molecules and structures directly and decreasing membrane stability, triggering cell death through either necrosis or apoptosis. Furthermore, it destroys the microvasculature, cutting off the tumor oxygen supply, leading to hypoxia in tumor tissues posttreatment. In addition, PDT has been shown to initiate inflammatory and immune responses attacking tumor cells, which serves as an auxiliary mechanism [33].

The class of photosensitizers known as porphyrins holds the greatest promise in image-guided therapy because they can easily be modified to improve target

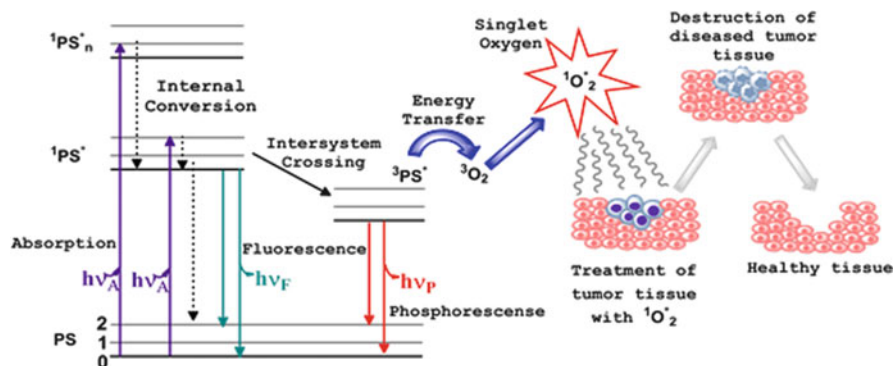


Fig. 2 Ablation of tumor cells after the three essential components (PS, light of the appropriate wavelength, and oxygen) of photodynamic therapy are combined. This usually results in the destruction of diseased tissue without affecting normal tissue. Jablonski diagram for the electronic states of the photosensitizer (PS) is shown on the left [32]

specificity and also their tetrapyrrolic structure offers great stability. Porphyrins and their derivative macrocycles, which contain four pyrrole rings joining one another via methane bridges, are by far the most commonly used photosensitizers in PDT. The most notable porphyrin-based photosensitizer is Photofrin™, the FDA-approved photosensitizer currently in use for cancer treatment. By altering the saturation of the bonds by reduction of one or both of the two peripheral cross-conjugated double bonds in the opposite pyrrolic rings that does not affect the macrocycle's aromaticity, porphyrin derivatives are formed. Reducing one of the double bonds of a pyrrole ring yields chlorins; reducing one double bond on two of the pyrrole rings yields bacteriochlorins. The difference in the extent of conjugation has significant effects on their properties as photosensitizers. First of all, porphyrins, e.g., Photofrin™, exhibit weak absorption maxima around 630 nm, while chlorins and bacteriochlorins have strong absorption maxima around 650 and 710 nm, respectively. Since the longer the wavelength of visible light the deeper into human tissue it can penetrate, chlorins and bacteriochlorins are more effective in treating deep-seated tumors than Photofrin™. Furthermore, previous studies have shown that some of the analogs of chlorins and bacteriochlorins have much reduced skin phototoxicity in comparison to Photofrin [34–36]. Therefore, chlorins and bacteriochlorins are better candidates for photosensitizer development than porphyrins. Among the long wavelength-absorbing porphyrin-based photosensitizers (660–800 nm), developed in our laboratory, HPPH stands out because of its tumor avidity, outstanding PDT efficacy, and much reduced skin phototoxicity than Photofrin (an FDA-approved agent and other porphyrin-based compounds) and is currently in phase I/II human clinical trials [37–46].

In this review, we will outline several imaging modalities that are currently being used clinically as well as other underutilized modalities which offer great promise in the future (summarized in Table 1). In addition to imaging, other promising therapeutic approaches to cancer treatment centered on tetrapyrrolic

Table 1 Advantages and limitations of various imaging modalities (table adapted from [7])

Modality	Temporal resolution	Spatial resolution	Depth of penetration	Sensitivity	Cost	Safety profile
Computed tomography	Minutes	High	Limitless	Low	\$\$	Ionizing radiation
Magnetic resonance imaging	Minutes to hours	High	Limitless	Low	\$\$\$	Non-ionizing radiation
Positron emission tomography	Seconds to minutes	Reasonable	Limitless	High	\$\$\$	Ionizing radiation
Single-photon emission tomography	Minutes	Reasonable	Limitless	High	\$\$	Ionizing radiation
Ultrasound	Seconds to minutes	Reasonable	Poor	High (w/ microbubbles)	\$	Good
Optical fluorescence imaging	Seconds to minutes	Poor	Poor	High	\$	Good
Optical bioluminescence imaging	Seconds to minutes	Poor	Poor	High	\$	Good

systems are also discussed. Tetrapyrroles are a very diverse class of chemical compounds that offer the ability to be easily manipulated and can serve as an effective scaffold to make multifunctional compounds which allow for both therapy and imaging within a single compound.

2.1 Fluorescence Image-Guided PDT

As with other noninvasive techniques, fluorescence imaging has the potential for performing *in vivo* diagnosis *in situ*, with real-time display of the resulting information. Optical tomographic techniques are being devised to visualize the fluorescent probes within tumor volumes. Optical imaging instruments may be simpler and less expensive to operate than those required for other imaging technologies permitting their eventual application by less specialized medical centers. Therapeutically, in applications such as endoscopic examination, fluorescence imaging can allow precise assessment of the localization and a size of the tumor and provide information on its invasiveness. During debulking surgery, where malignant loci can be difficult to identify, the presence of fluorescent signal might assist the surgeon in identifying the diseased site.

The optimal wavelength range for *in vivo* fluorescence excitation and emission is determined by tissue optical properties. Hemoglobin has strong absorption at wavelength less than 600 nm and there can be significant background fluorescence from endogenous biomolecules up to about 680 nm. At longer wavelengths into the NIR, tissue absorption and scattering decrease with wavelength [47, 48]. There is a large increase in light penetration as wavelength increases from 600 to 800 nm. In addition, the difference between the optical imaging fluorophore's absorption and emission bands (i.e., its Stokes shift) should be at least 20 nm, to readily discriminate between the excitation and emission light. Many NIR fluorescent dyes are based on carbocyanine molecules such as indocyanine green (ICG), an FDA-approved agent with a 730 nm excitation and 830 nm emission maxima. A challenge has been to deliver the dyes selectively and in high enough concentration to detect small tumors. Use of ICG alone to image hypervascular or "leaky" angiogenic vessels around tumors has been disappointing, due to the dye's limited intrinsic tumor selectivity. Multiple approaches have been employed to improve optical probe localization, including administering it in a quenched form that is activated within tumors, or coupling the fluorescent agents to antibodies, or small molecules such as receptor ligands. Some of the peptides and folic acid analogs of certain ICG derivatives have shown some tumor specificity. A few years ago, Achilefu's group [49] reported that certain multicarboxylate-containing carbocyanine probes used as optical scaffolds not only serve as fluorescent antennae but also participate in structural assembly of the multivalent molecular construct. However, none of these compounds were designed for both tumor detection and therapy.

In addition to their therapeutic functions, porphyrin-based structures are also viable fluorophore for optical fluorescence imaging [50, 51]. Because of their tumor avidity, porphyrins can differentiate normal tissues and tumors, which enable them to enlist the help of imaging to locate tumor and guide treatment efforts with surgery and PDT. Porphyrins, therefore, fall into the category of theranostic agents, which combine diagnostic and therapeutic functionalities and are capable of simultaneous diagnosis of cancer and treatment. Furthermore, porphyrin structures have several functional groups on their periphery that are accessible for further chemical modifications that introduce additional imaging functionalities. This enables the use of porphyrin-based compounds for multimodal imaging, the synergistic use of multiple imaging modalities to complement detailed gross anatomy with disease-state physiology [52]. However, most of the porphyrin-based compounds (with a few exceptions) are not ideal candidates for optical imaging due to limited Stokes shift(s) between longest wavelength absorption and emission. Finally, most of them have relatively short absorption wavelengths, <800 nm, which are not optimal for tissue penetration.

In our attempts to investigate the utility of tumor-avid porphyrin-based agents as vehicles to deliver non-tumor-specific cyanine dyes (CD), we linked it to a clinically relevant photosensitizer, the hexyl ether derivative of pyropheophorbide-a (HPPH) [53, 54]. The resulting candidate (HPPH-CD) was found to be an efficient tumor-imaging (fluorescence imaging) and PDT agent. Interestingly, compared to HPPH, the HPPH-CD conjugate produced a significantly higher uptake in tumor than skin, with limited skin phototoxicity. However, there was a significant difference between the imaging and therapy dose. The reason being a part of the singlet oxygen (a key cytotoxic agent in PDT) produced by exciting the PS with light (665 nm) was quenched by the cyanine dye moiety. Therefore, several PS-CD conjugates were designed with strategies such as increasing the length of linkers joining the PS and CD moieties and introducing the CD at variable position of the PS [55, 56]. Such strategies showed an indirect correlation between FRET and singlet oxygen producing efficiency. In other words, conjugate with more FRET showed less singlet oxygen production and reduced PDT efficacy. In another approach, increasing the number of HPPH moiety in HPPH-CD conjugate from 1 to 2 (Fig. 3) further enhanced its PDT efficacy with similar tumor-imaging ability. The development of tumor imaging or efficient PDT agent by itself represents an important step, but a dual-function agent provides the potential for tumor detection and targeted PDT (Scheme 1).

Among the purpurinimide-CD conjugates in which the CD containing a carboxylic acid functionality was covalently linked with N-substituted purpurinimides bearing variable number of N-alkyl amines (Fig. 4), the conjugate containing a linker with two carbon chains ($\text{CH}_2\text{-CH}_2$) was most effective and also showed excellent tumor uptake and fluorescence-imaging ability at 24 h postinjection in BALB/c mice bearing Colon26 tumors [57].

To investigate the impact of certain metallated PS-CD conjugates in fluorescence imaging and PDT, we synthesized a series of purpurinimide-CD conjugates in which the CD was introduced at position-20 of the tetrapyrrolic system [58]. In

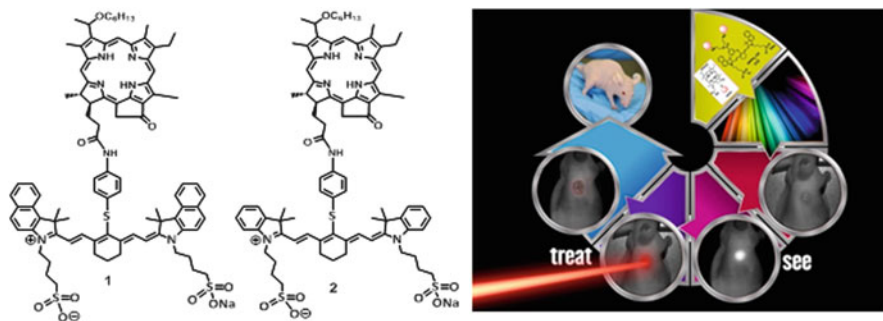
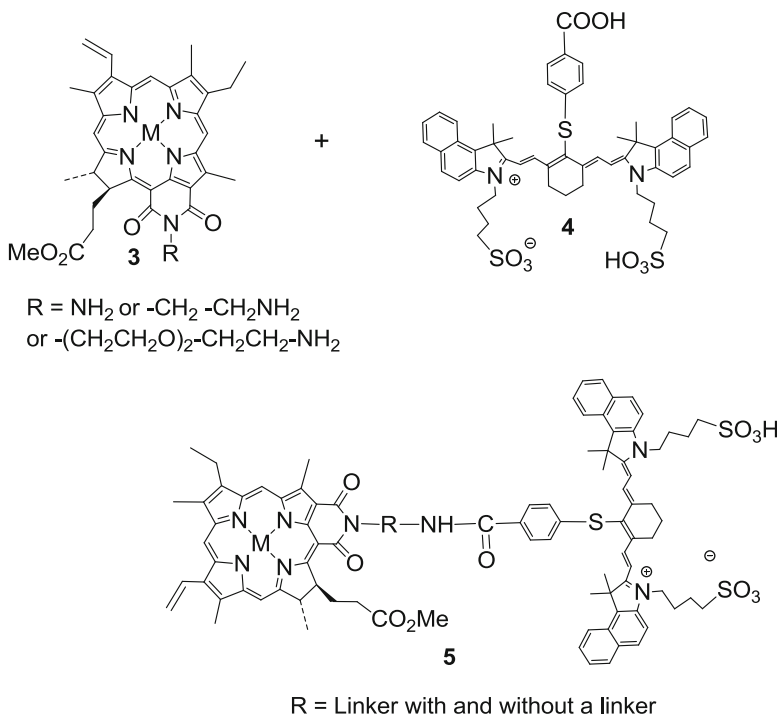


Fig. 3 Structures of dual-function agents in which the HPPH is conjugated to a cyanine dye containing bis-benzoindole and bis-indole moieties with amide linkages



Scheme 1 Purpurinimide-CD conjugates. Among these analogs, the conjugate X (R = CH₂-CH₂) showed improved PDT efficacy than the HPPH-CD conjugate in BALB/c mice bearing Colon26 tumors

brief, the reaction of *meso*-substituted purinimide with *N*-bromosuccinimide regioselectively introduced a bromo-functionality at position-20, which on further reacting with a variety of boronic acids under Suzuki reaction conditions yielded the corresponding *meso*-substituted analogs (Scheme 2).

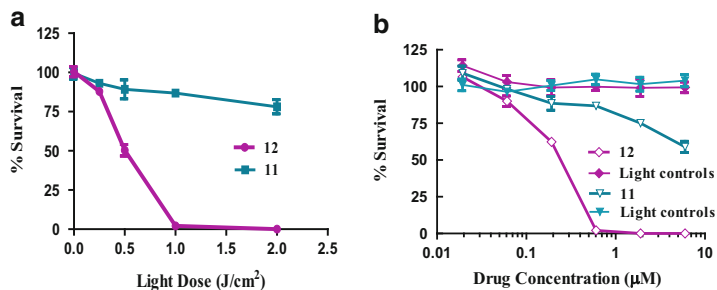
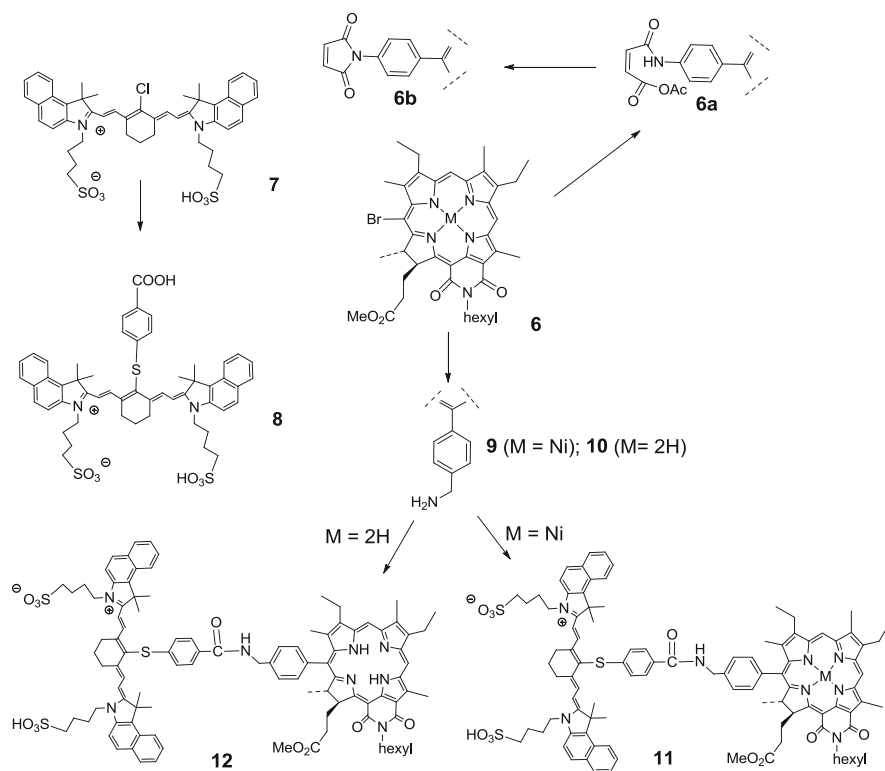


Fig. 4 In vitro photosensitizing efficacy (MTT assay) of Ni(II) purpurinimide-cyanine dye conjugate **11** and the corresponding non-metallated analog **12**; (a) PDT efficacy at a fixed concentration of the photosensitizers, but the cells were exposed to variable light doses, (b) PDT efficacy at a fixed light dose, but at variable concentrations. On exposing the cells with light without incubating with photosensitizers **11** and **12**, no dark toxicity (cell kill) was observed. The cells were incubated for 24 h before the light exposure



Scheme 2 Synthesis of metallated [Ni(II)] and free-base mesopurpurinimide-cyanine dye conjugates

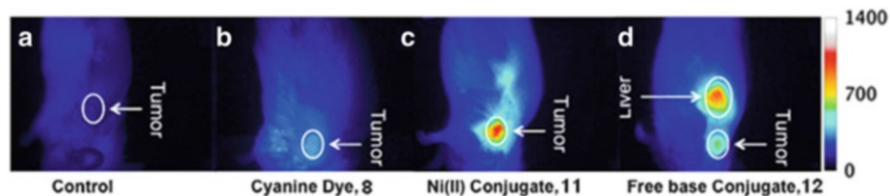


Fig. 5 Whole-body fluorescence imaging of (a) control mouse, (b) cyanine dye **8**, (c) conjugate **11**, and (d) conjugate **12** at a dose of 0.3 $\mu\text{mol/kg}$, 24 h post-intravenous injection

The free base showed significant *in vitro* PDT efficacy, but limited tumor avidity in mice bearing tumors, whereas the corresponding Ni(II) derivative did not produce any PDT-mediated cell kill but showed excellent tumor-imaging ability at a dose of 0.3 $\mu\text{mol/kg}$ at 24, 48, and 72 h postinjection (Figs. 4 and 5). The limited PDT efficacy of Ni(II) analog could be due to its inability to produce singlet oxygen. Based on electrochemical and spectroelectrochemical data in DMSO, the first one-electron oxidation and the first one-electron reduction of both the free base and the corresponding Ni(II) conjugates were centered, while the second one-electron reduction of the two conjugates is assigned to the purpurinimide part of the molecule. Reduction of the CD unit is facile and occurs prior to reduction of the purpurinimide group, suggesting that the CD unit could be a driving force to quench the produced singlet oxygen as an oxidant. An obvious interaction between the CD and the purpurinimide group is observed for the free-base conjugate, as compared to a negligible interaction between two the functional groups in the case of the Ni(II) conjugate. As a result, the larger HOMO–LUMO gap of the free-base conjugate and the corresponding smaller quenching constant is considered to be a reason to avoid singlet oxygen quenching to some degree.

2.2 MR/Fluorescence Imaging and PDT

Currently, the most widespread MRI contrast agents (CAs) are gadolinium (Gd)-based nonspecific agents such as Magnevist[®] (Gd-DTPA) for contrast enhancement (CE). While providing powerful contrast efficacy and excellent safety in patients without severe dysfunction, these CAs lack real specificity for depicting certain tissue, organ, and disease. These characteristics have limited the diagnostic capacity of contrast-enhanced MRI in both clinical and experimental settings.

Therefore, the use of tumor-avid porphyrin-based compounds as vehicles to deliver the Gd(III) ion to tumor has been investigated in various laboratories. Since the ring structure of porphyrins and chlorins are too small to adequately accommodate Gd, Gd-labeled porphyrins and metalloporphyrins were difficult to synthesize and found to be unstable. However, in expanded porphyrin systems, the Gd can be inserted with a high stability of the resulting product, and certain Gd analogs, e.g., Gd(III) Texaphyrin [59], are under clinical trials with promising

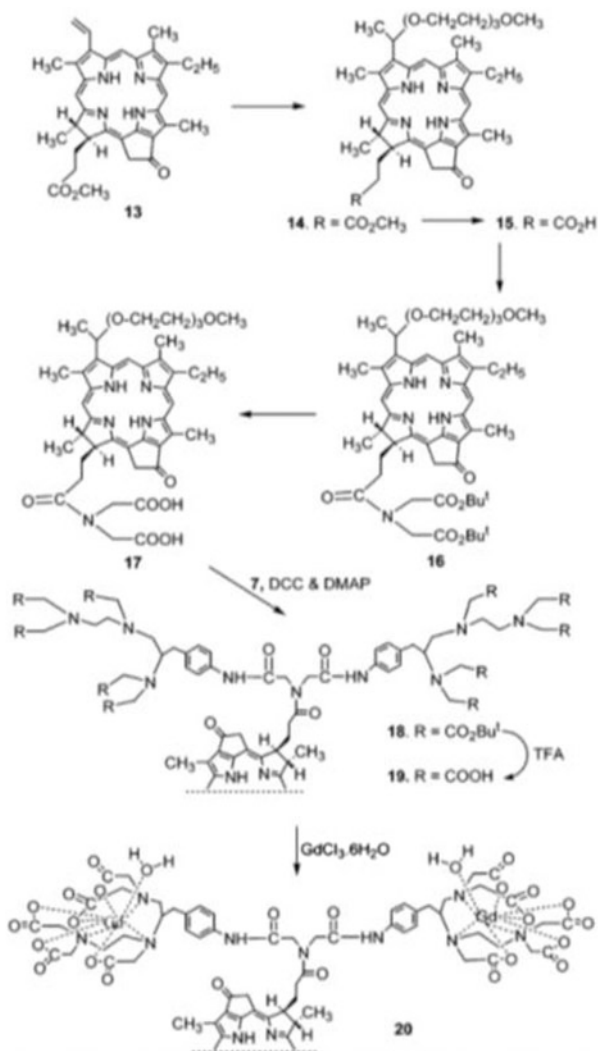
results. The approach which has been quite successful in incorporating Gd to porphyrin-based compounds has been to link the tetrapyrrolic system to diethylene triamine pentaacetic acid (DTPA) or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) in which Gd can easily be chelated. Gadoporphyrin-2 [60], in which the paramagnetic metal was chelated to DTPA side chains, showed a favorable safety profile with high stability. Additionally, it showed remarkable target specificity to necrotic tumor tissue. For the last few years, the Roswell Park group has been developing dual-function imaging agents (MR/fluorescence) with an option of NIR PDT. In their initial study, HPPH was conjugated with variable number of Gd-DTPA [61]. The synthetic methodologies for HPPH-2Gd(III)DTPA and HPPH-3Gd(III)DTPA are illustrated in Schemes 3 and 4, respectively. It was observed that by increasing the number of Gd(III)DTPA, moiety enhances the tumor contrast to some extent (Table 2). Among the conjugates investigated containing 2 to 6 Gd(III)DTPA moieties [62, 63], both 2- and 3-Gd(III)DTPA-HPPH conjugates showed excellent tumor-imaging (MR and fluorescence) and PDT efficacy in tumored mice and rats (Figs. 6, 7, 8, and 9). However, the conjugate with 3-Gd(III)DTPA was easier to formulate in PBS and was selected for a detailed investigation. Interestingly, the conjugate even at 8-fold higher than the imaging dose did not show any normal organ toxicity. Compared to Magnevist (current clinical standard), the MR imaging dose of HPPH-3Gd(III)DTPA was 10-fold lower and provides a unique opportunity to develop a single agent for both cancer imaging and therapy.

Shim and coworkers [64] extended this approach and synthesized a series of Gd(III)DTPA-based purpurinimide analogs, in which one or two moieties of purpurin-18-*N*-(2-aminoethyl) were conjugated with DTPA, which on further reaction with gadolinium chloride yielded the desired product (Scheme 5). However, the imaging and PDT efficacy of the Gd-complexes are under investigation.

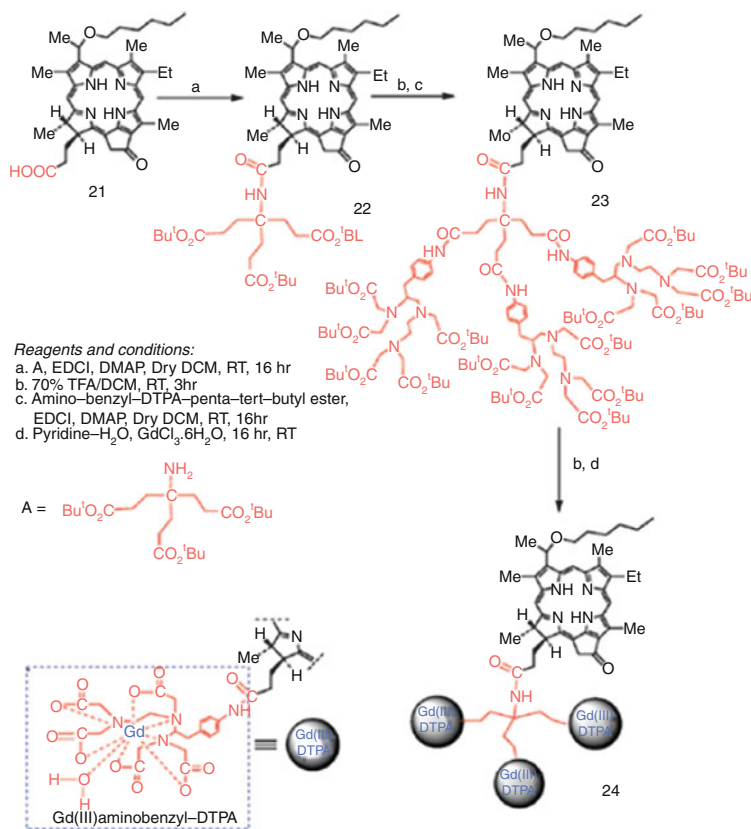
2.3 *PET/Fluorescence Imaging and PDT*

In recent years, multimodality systems for in vivo imaging of small animals have become important tools for modern biomedical research, as they offer advantages of combining complimentary characteristics of different modalities. Representative examples of multimodality system are the combination of MRI and CT (computed tomography), CT and fluorescence, and CT and positron emission tomography (PET). A system with PET or SPECT and fluorescence offers advantages because the co-registration of PET and fluorescence images is quite convenient. In recent years, several agents combining both fluorescence and PET-imaging abilities have been developed in various laboratories and have definite advantages over the use of two independent agents with different pharmacokinetic profiles. However, in developing nuclear imaging agents, the selection of radionuclide plays very important role, and it should coincide with the pharmacokinetic properties of the molecule in which the radionuclide is attached.

Scheme 3 Synthesis of HPPH-2 Gd(III)DTPA conjugates



The strategy of employing tumor-specific molecules as a vehicle to carry radioactive tracers to target tissue turned out to be very successful and has led to the development of many novel tumor-imaging radiopharmaceuticals [65]. In this regard, ¹¹¹In-complexes of certain porphyrins have shown significant potential for SPECT imaging. Roswell Park group was the first to show the utility of tumor-avid chlorophyll-*a*-based NIR photosensitizer for dual imaging (PET/fluorescence) with an option of photodynamic therapy [66–70]. Most of the porphyrin-based compounds show optimal tumor uptake at 24–48 h post-administration. Therefore, the radionuclide initially selected was ¹²⁴I- with a half-life of 4 days, which provides several advantages: (1) the ¹²⁴I-labeled agent can be synthesized in high yield and



Scheme 4 Synthesis of HPPH-3Gd(III)DTPA conjugates

Table 2 Comparative T₁ and T₂ relaxivity of HPPH-Gd (DTPA) complexes

	T ₁ relaxivity	T ₂ relaxivity
HPPH-di-DTPA-Gd(III)	10.7	58.6
HPPH-tri-DTPA-Gd(III)	14.14	85.32

specificity, (2) unlike ¹⁸F-FDG, it can be transported to various distances, (3) it is not necessary to have cyclotron at each center, which may reduce the PET-imaging cost significantly, (4) ¹⁸F-FDG is not recommended for PET imaging of diabetic, pancreatic cancer patients, and (5) because of high glucose metabolism in normal brain, more efficient brain cancer-imaging agents than ¹⁸F-FDG are needed.

For developing improved cancer-imaging agents with an option of PDT, we used two different approaches. In our first approach, chlorophyll-*a* was converted into methyl pyropheophorbide-*a*, which in a sequence of reactions was converted to both radioactive and nonradioactive iodo-analogs (Scheme 6). To investigate the impact of carbohydrates in tumor selectivity, we prepared the corresponding galactose, glucose, lactose (glucose + galactose), and cellobiose (glucose + glucose) analogs of pyropheophorbide-*a*. These compounds were synthesized to investigate

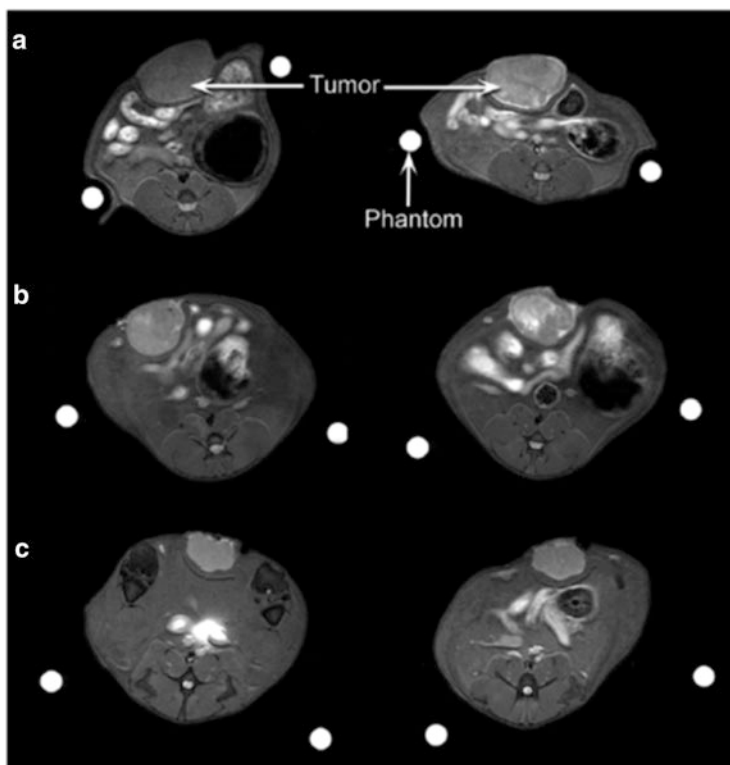


Fig. 6 Visible increase in signal intensity was seen in rat Ward colon tumors (*arrow*) from preinjection (*left*) to 24 h postinjection (*right*) of HPPH-3Gd(III)DTPA at 10 $\mu\text{mol/kg}$ (**a**) and at 5 $\mu\text{mol/kg}$ (**b**). Tumor contrast enhancement of HPPH-3Gd compares favorably to (**c**) Gd-DTPA (current MRI standard)

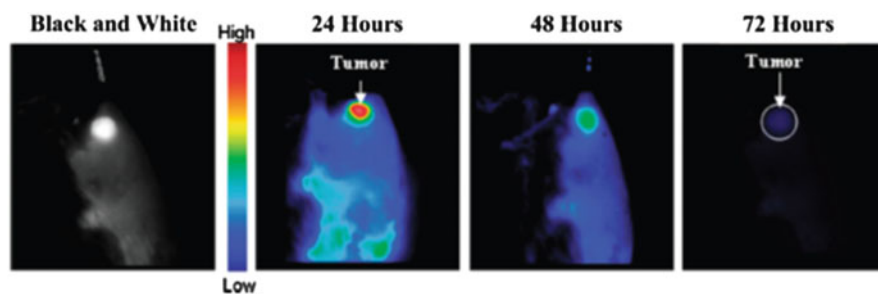


Fig. 7 In vivo fluorescence images of HPPH-3Gd in BALB/c mice at 24, 48, and 72 h with an imaging and therapeutic dose of 10 mmol/kg . Spectral unmixed images are presented with a false color representing fluorescence intensity of the imaging agent. Best tumor images were obtained at 24 h postinjection. A pre-analysis black and white image at 24 h is given for comparison with the false-color images provided

Fig. 8 In vivo biodistribution of ^{14}C -labeled HPPH-3Gd(III) DTPA in Ward colon tumors (3 rats/group). At 24, 48 after injection, 3 rats/time point were sacrificed. Preferable uptake of the conjugate in the tumor was seen at 24 and 48 h after compared to most normal tissues

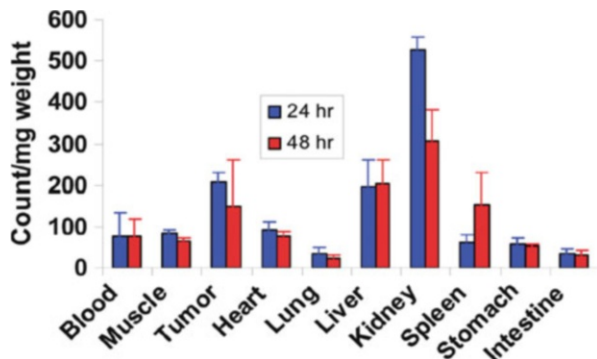
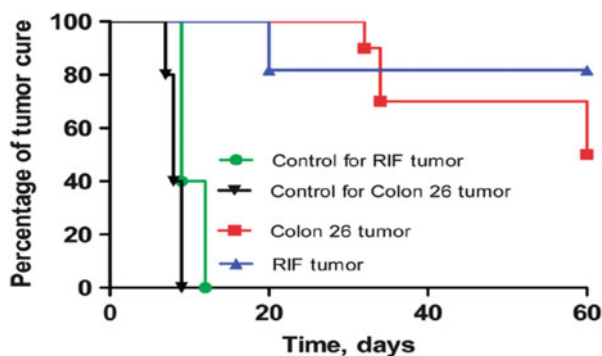


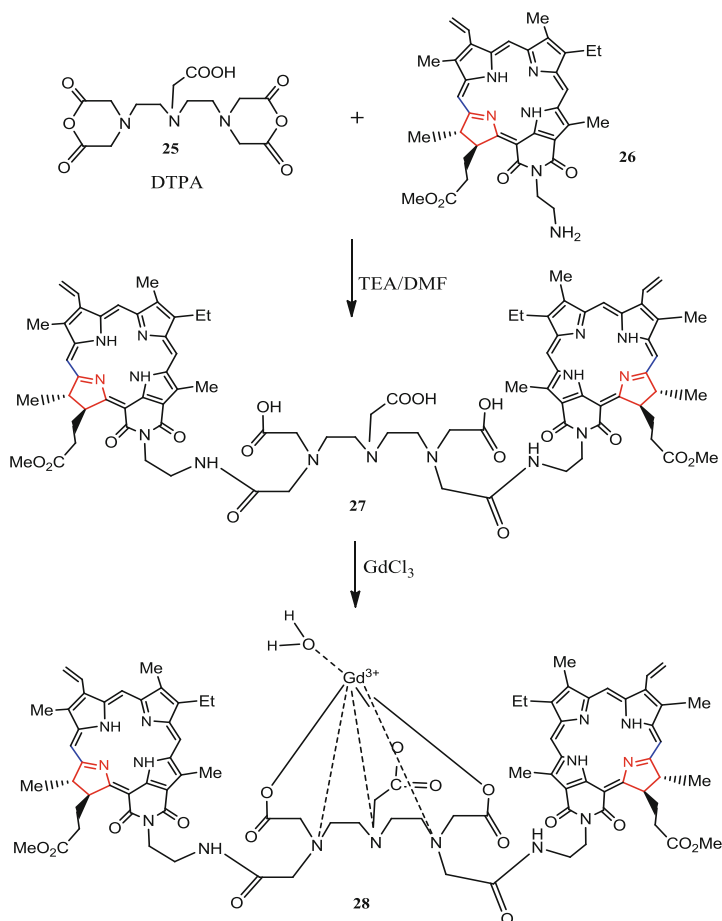
Fig. 9 In vivo PDT efficacy of HPPH-3Gd(III) DTPA in (a) C3H mice bearing RIF tumors and (b) BALB/c mice bearing Colon26 tumors at an imaging dose (10 mmol/kg). Mice were irradiated with a laser light (665 nm, $70 \text{ J}/\text{cm}^2$, $70 \text{ mW}/\text{cm}^2$) and the tumor size was measured daily



their target specificity with similar lipophilicity. For example, glucose and galactose analogs showed similar overall lipophilicity, but a significant difference in PDT efficacy and tumor specificity. The galactose and glucose conjugates were also evaluated for in vivo imaging and PDT. Among the analogs tested for PET imaging, the noncarbohydrate and the galactose analogs showed higher tumor imaging. However, the galactose analog retained in tumor for a longer time, but it also showed a significantly high uptake in liver. On the basis of detailed in vivo studies for cancer imaging (PET/fluorescence and PET) and photodynamic therapy, the noncarbohydrate photosensitizer in combination of ^{124}I -radioactive and nonradioactive (^{127}I -) analogs proved to be an excellent candidate for cancer diagnosis (Fig. 10) and fluorescence image-guided therapy. Efforts are under way to advance this product to phase I human clinical trials.

3 Multimodality Agents: Advantages of Nanoparticles

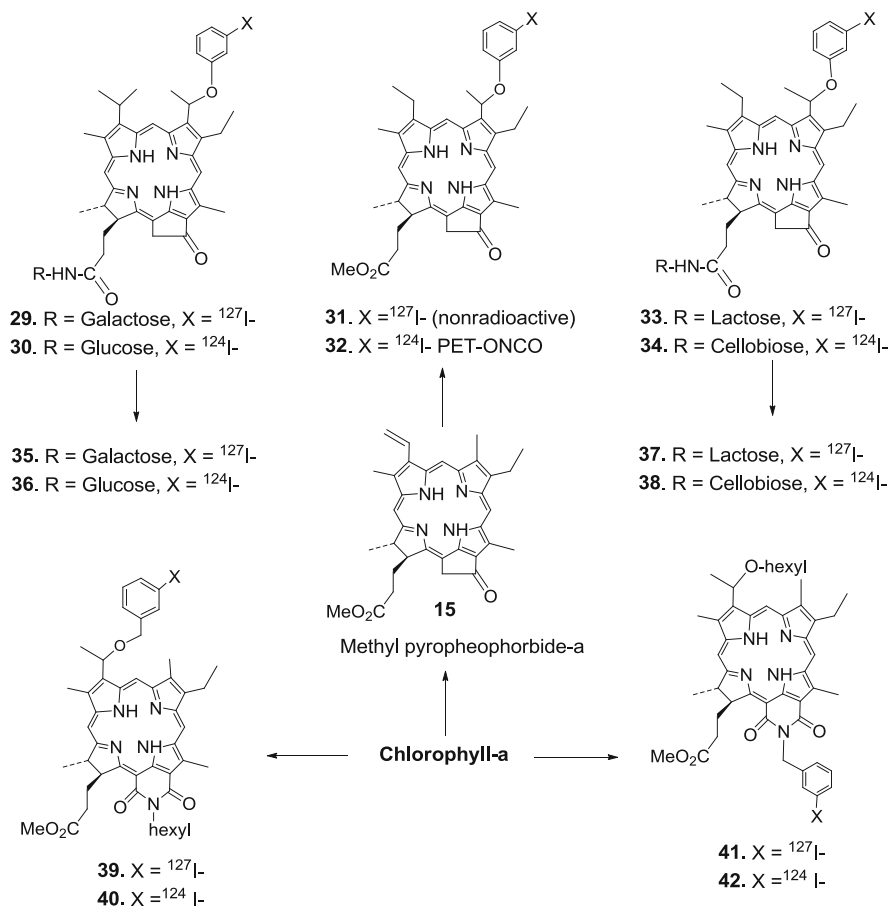
Although nanoplatforms and nanovectors (i.e., a nanoplatform that delivers a therapeutic or imaging agent) for biomedical applications are still evolving, they show enormous promise for cancer diagnosis and therapy.



Scheme 5 Synthesis of purpurinimide-N-Gd(III)DTPA conjugate imide methyl ester

Therapeutic examples include NP containing PDT agents, boron containing dendrimers for neutron capture, and NP-directed thermal therapy. Diagnostically NP formulations have been used for imaging and therapy (Theranostics). In nanotechnology approach various disciplines, e. g., chemistry, physics and biology play important roles [71] (Fig. 11).

This particular article is focused on polyacrylamide-based NPs and can be utilized as carriers of photosensitizers (PS) for photodynamic therapy (PDT) by means of encapsulation of PSs into the NPs, post-loading of PSs, or via covalent conjugation of PSs to the functional groups at the surface of the NPs. Most PSs are hydrophobic and aggregate easily under physiological conditions; therefore, under the suboptimal preparation that most acceptable pharmaceutical formulations offer, the PDT efficacy of the PS can be hindered. On the other hand, some NPs have been



Scheme 6 Synthesis of iodinated agents for PET imaging with an option of cancer therapy by PDT

shown to increase the hydrophilic nature of compounds when they are coupled to them in any of the aforementioned loading methods. Therefore, the use of NPs can circumvent the issue of PS hydrophobicity. Additionally, NPs have been shown to accumulate within tumors and tumor vasculature via the enhanced permeability retention (EPR) effect [72]. Furthermore, increases in tumor-specific localization have been shown by the addition of tumor-targeting ligands (i.e., peptides, monoclonal antibodies) which can be covalently attached to surface functional groups of the NPs. For these reasons, NPs present the benefit of improving the therapeutic effectiveness of PDT PSs.

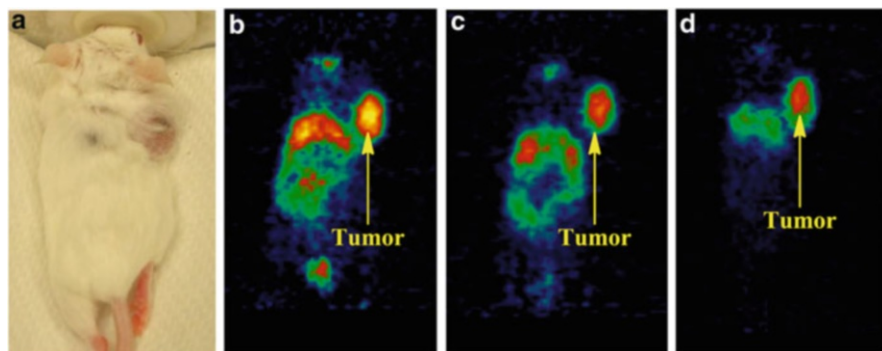
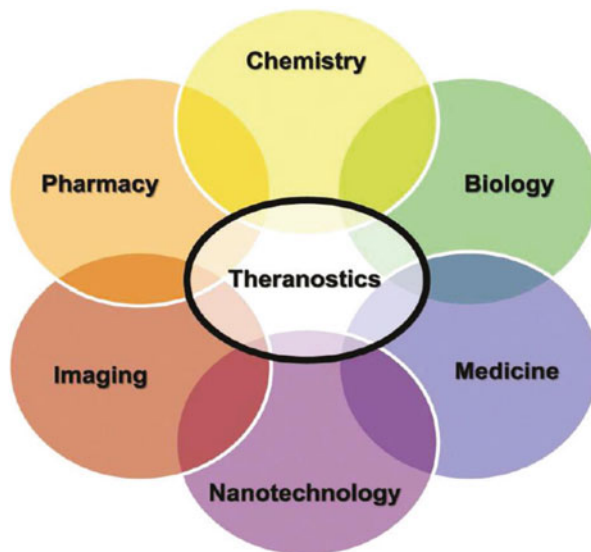


Fig. 10 Whole-body PET images of a BALB/c mouse bearing Colon26 tumor with ^{124}I -compound taken at 24 (B), 48 (C), and 72 h (D) postinjection. The maximum uptake of the PS was observed at 24 h postinjection. However, the best contrast was obtained at 72 h after injecting the ^{124}I -agent

Fig. 11 Various fields encompassing development of “theranostics” for cancer



3.1 Polyacrylamide-Based Nanoparticles

Initially, Kopelman et al. [73] developed and characterized PAA NPs encapsulated with methylene blue (MB). MB is a promising PS with high quantum yield of $^1\text{O}_2$ generation, long excitation wavelength, and low toxicity. However, the clinical use of MB was hampered primarily because it is enzymatically reduced to by-products with negligible PDT efficacy post-administration. To realize MB’s potential, PAA NPs seemed to be an ideal carrier because they can spare MB from such reduction and conserve its PDT effectiveness. Included in Kopelman’s preliminary study

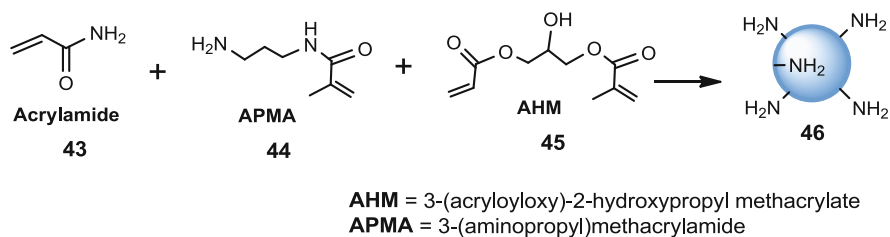


Fig. 12 A general method for the synthesis of amino-functionalized PAA NPs

were MB-loaded nanoparticles composed of PAA, sol-gel, and ORMOSIL. Upon comparison, it was observed that of the possible NP vehicles, PAA NPs showed the most efficient delivery of $^1\text{O}_2$, but its loading efficiency of MB was the lowest. In the follow-up study, Kopelman et al. confirmed that the MB-encapsulated PAA nanoparticle allowed minimal enzymatic reduction of MB and it demonstrated good PDT efficacy in vitro [74]. To address the issue of loading efficiency, Kopelman covalently conjugated MB with the precursor of PAA nanoparticle, *N*-(3-Aminopropyl)methacrylamide (APMA), and designed two nanoplatforms in a more recent study [75]. In addition, these PAA nanoparticles were PEGylated to afford longer blood circulation and were conjugated to targeting F3-Cys peptides. The nanoplatform thus resulted showed much improved targeting efficiency as well as PDT efficacy, an indicator of better loading efficiency, in vitro.

A general synthetic approach for amino-functionalized PAA NPs, its structure, SEM images, and a systemic presentation of target-specific multimodality platform is shown in Figs. 12, 13, and 14.

The most common designs of tumor-specific nanosized imaging agents were cross-linked iron oxide (CLIO) nanoparticles, in which iron oxide core is coated with the cross-linked dextran, and the incorporation of iron oxide into polymer matrices such as PAA. Although either of these designs can be further functionalized with targeting moieties, to specifically enhance the T_2 contrast in MRI, the ease to add surface functional groups for further modification made the latter design slightly advantageous. Moffat et al. first developed an iron-oxide-encapsulated PAA particles functionalized with PEG and reported significant enhancement of R_2 and R_2^* relaxivity in the tumor region [76]. In comparison to iron oxide coated by dextran, these PAA nanoparticles contain more crystals of iron oxide per particle. They were also able to better shield the iron oxide from interacting with tumor vascular component to avoid interruption to their imaging functionalities.

In addition to the applications of PAA in designing improved PDT and imaging agents, the dual operation of diagnostic imaging is also possible, using PAA as the platform to carry both the PS and imaging agents (e.g., MRI/fluorescence or PET/fluorescence). In a preliminary report, Reddy et al. described such an attempt in designing a PAA nanoparticle that carries (1) Photofrin (a PS for PDT) (which exhibited suboptimal selectivity in terms of tumor-to-normal tissue ratios), (2) iron

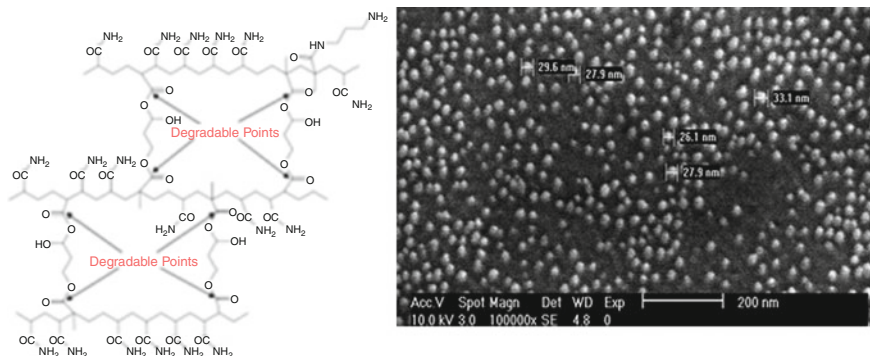


Fig. 13 Structure of amino-functionalized PAA NPs and its scanning electron microscopy (SEM) image (size: 28–30 nm in diameter)

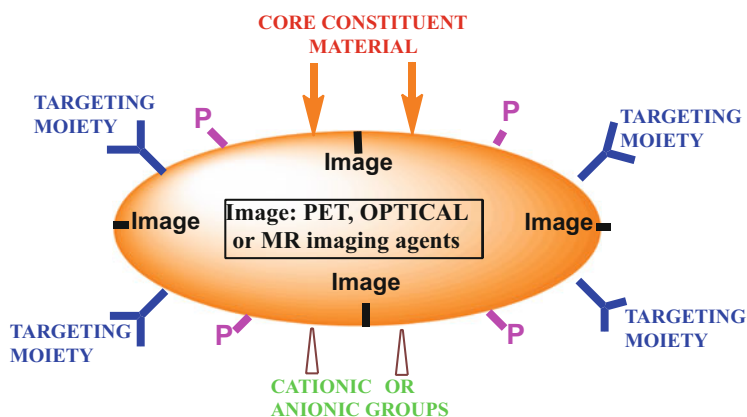


Fig. 14 A simple representation of multifunctional, targeted PAA nanoparticle for cancer imaging and photodynamic therapy (PDT). P = Photosensitizer

oxide (MRI), (3) F3-peptide (which specifically binds to nucleolin, a receptor highly expressed on many cancers), and (4) PEG (for improved lifetime during circulation) [77]. In vitro analysis of singlet oxygen production, targeting efficiency of nucleolin cell surface receptor, and conferral of phototoxicity revealed that the developed nanoparticles were indeed bound, internalized, transported, and concentrated within the tumor cell nuclei which lead to loss of cell viability upon photoactivation. In vivo studies using 9 L glioma rat model showed outstanding R_2 relaxivity in tumor, and using the developed nanoparticle as PS, there was a significantly increased survival time compared to the use of nontargeted Photofrin nanoparticle or Photofrin alone.

To investigate the tumor avidity of PAA-based NPs, the amino-functionalized PAA NPs (nonfluorescent) were conjugated with a CD containing a carboxylic acid ($-\text{COOH}$) group by following the standard peptide chemistry (Patel and Pandey,

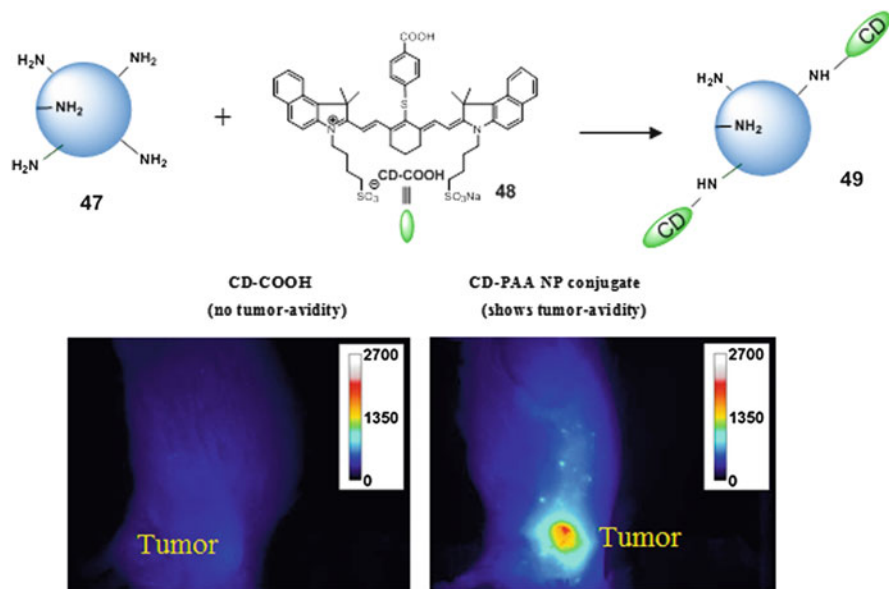


Fig. 15 Comparative tumor uptake and fluorescence-imaging potential of a cyanine dye (CD-COOH) and the corresponding CD-PAA NP conjugate. The cyanine dye was conjugated at the periphery of the nanoparticles (NPs) (BALB/c mice bearing Colon26 tumors at 24 h postinjection, dose of the CD: 0.3 $\mu\text{mol/kg}$)

unpublished results). The free and post-loaded CD-COOH was removed by spin-filter (100 KD) with a continuous wash with ethanol. The release of the CD-COOH was confirmed by measuring the absorption spectra of the filtrates (ethanol wash). The concentration of the CD in nanoparticles was calculated by subtracting the amount recovered in ethanol washes from the total amount used for conjugating to NPs.

The tumor avidity of the free CD-COOH and the corresponding CD-PAANPs conjugate was compared in BALB/c mice bearing Colon26 tumors at similar parameters (dose: 0.3 $\mu\text{mol/kg}$). The mice were imaged at variable time points using the IVIS spectrum (PerkinElmer) by exciting the dye at its longest wavelength absorption. The results summarized in Fig. 15 indicate that the cyanine dye (CD-COOH) alone had limited tumor avidity (1–72 h, only 24 h data is shown), whereas the corresponding NPs showed significant tumor-imaging potential.

More recently, Wang et al. and Gupta et al. [78, 79] conducted a series of studies designing a multifunctional PAA NP which incorporated 3-(1'-hexyloxy)ethyl-3-devinyl pyropheophorbide- α (HPPH), a PS with far greater tumor-targeting efficiency, excellent PDT efficacy, and a longer excitation wavelength; additionally, an NIR CD fluorescence imaging agent was also incorporated (Fig. 16). In the first study, Wang et al. demonstrated that among the NPs synthesized using covalent conjugation, encapsulation, and post-loading (loading of agents into the porous nanoparticle post its synthesis) approach. The post-loaded PAA NP

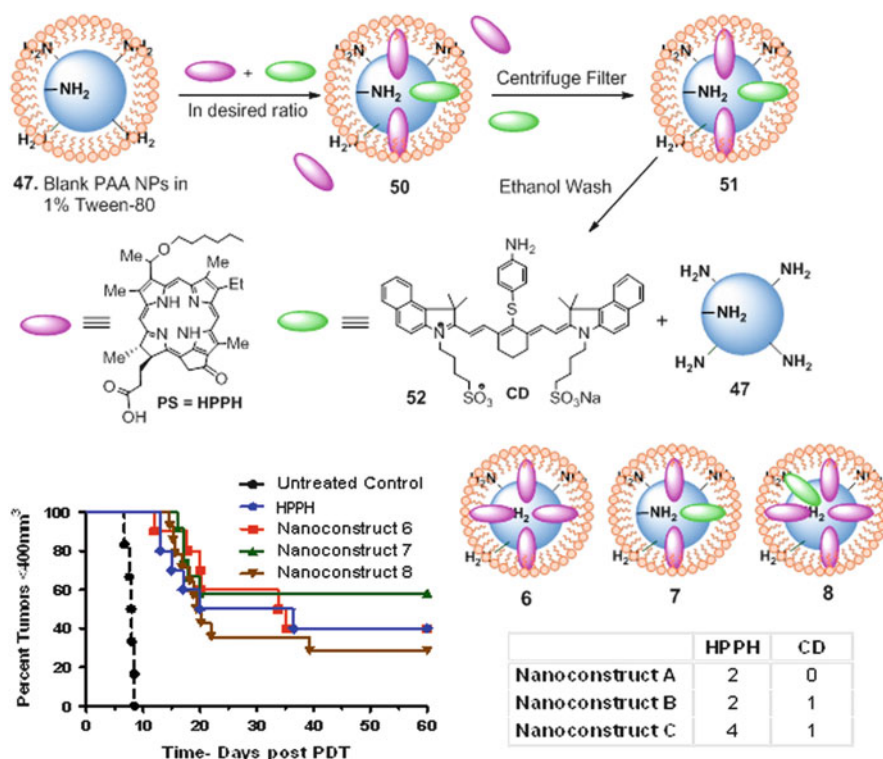


Fig. 16 Post-loading of HPPH and CD-COOH in PAA NPs at variable ratios and their comparative in vivo PDT efficacy in BALB/c mice (10 mice/group). The imaging and therapy dose: $0.47 \mu\text{mol/kg}$. For PDT, the tumors were irradiated with light (665 nm , 135 J/cm^2 , 75 mW/cm^2) at 24 h postinjection

approach best conserved the PDT efficacy of HPPH [80]. In the follow-up studies, different HPPH to CD ratios were examined to minimize the undesirable quenching of HPPH electronic excitation energy due to FRET (fluorescence resonance energy transfer). The finalized nanoparticle incorporated HPPH and CD with an amine functional group in 2:1 ratio and demonstrated excellent tumor-imaging (NIR fluorescence) and PDT efficacy in vivo [79]. A direct correlation between FRET and PDT efficacy was observed. The nanoconstruct that showed higher FRET produced lower long-term tumor cure.

Incorporation of different CD derivatives (either with an amine or a chlorine functional group) was attempted for further optimization. Furthermore, F3 peptide and PEG were covalently conjugated to the surface of the PAA nanoparticle [80].

4 Conclusion and Future Directions

Porphyrins including reduced porphyrins (chlorins and bacteriochlorins) and phthalocyanines are a class of chemically and biologically important compounds that have found a significant application in cancer imaging and therapy. The tumor avidity can be improved by structure-activity relationship studies, and such molecules can also be used for delivering the desired imaging agents (MRI, PET, and fluorescence) to tumors. The imaging and therapeutic agents at a desired ratio in post-loaded or conjugated or combined conjugated and post-loaded forms to biocompatible nanoparticles could generate effective multifunctional nanoplatfroms for both cancer imaging and therapy. The new PDT agents certainly eliminate the problems associated with prolong skin phototoxicity.

Future design strategies for new multimodality agents and nanoplatfroms for the imaging and treatment of cancer should be directed towards tumor specificity/selectivity. Use of PDT in combination with other cancer treatment modalities (e.g., surgery, chemotherapy, virotherapy, photothermal) will certainly help to treat primary and metastatic tumors. Efforts should also focus on developing porphyrin-based agents, which could help to detect the tumors at initial stages, i.e., before metastasis.

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