Lighting Up Tumors with Receptor-specific Optical Molecular Probes

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Accurate and rapid detection of tumors is of great importance for interrogating the molecular basis of cancer pathogenesis, preventing the onset of complications, and implementing a tailored therapeutic regimen. In this era of molecular medicine, molecular probes that respond to, or target molecular processes are indispensable. Although numerous imaging modalities have been developed for visualizing pathologic conditions, the high sensitivity and relatively innocuous low energy radiation of optical imaging method makes it attractive for molecular imaging. While many human diseases have been studied successfully by using intrinsic optical properties of normal and pathologic tissues, molecular imaging of the expression of aberrant genes, proteins, and other pathophysiologic processes would be enhanced by the use of highly specific exogenous molecular beacons. This review focuses on the development of receptor-specific molecular probes for optical imaging of tumors. Particularly, bioconjugates of probes that absorb and fluoresce in the near infrared wavelengths between 750 and 900 nm will be reviewed.

Key words: Optical imaging, Contrast agents, Molecular probes, Carbocyanine dyes, Cell surface receptors, Somatostatin, Bombesin, Folate, Epidermal growth factor, Neurotensin.

Introduction

Recent advances in fundamental tissue optics, coupled with the rapid development of laser technology, have led to the re-emergence of optical detection methods for medical applications (1, 2). Biomedical optical methods utilize low energy radiation in the visible and NIR wavelengths to detect pathologic conditions and monitor the progression or retrogression of diseases. Planar optical imaging system is a common and simple method to obtain optical images by exciting tissues with a broadband light or lasers and a charge-coupled device (CCD) camera to capture the emitted or transmitted light (Figure 1). With advances in sophisticated image reconstruction algorithms and instrumentation, high-resolution 3D images in real time is becoming a reality (3-10).

Biomedical optical methods provide distinctly new diagnostic capabilities while complementing conventional imaging modalities (11-15). Some advantages of optical methods include the use of non-ionizing radiation, detection of minute amounts of light-absorbing materials in tissue phantoms, capability of continuous data acquisition for real-time monitoring, and the potential availability of low cost, user friendly, and portable imaging systems and endoscopes. Optical

Abbreviations: BBN, Bombesin; CCD, Charge-coupled device camera; EGFr, Epidermal growth factor receptor; GRPr, Gastrin releasing peptide receptor; FR, Folate receptor; ICG, Indocyanine green; NIR, Near infrared; NTR, Neurotensin receptor; STR, Somatostatin receptor.
Molecular Optical Probes and Applications

As progress in molecular medicine continues to reveal specific factors that characterize abnormal tissues, new molecular beacons can be designed to take advantage of these findings for molecular imaging. The use of molecular optical probes can fall into one or more of three categories: (a) detecting tumors at the early stages of formation, (b) differentiating benign from malignant tumors, and (c) staging and monitoring the status of cancer. Interestingly, optical probes can be designed to detect molecular processes in all three applications listed above. Although light scattering agents may play a major role in future, most molecular beacons rely on the absorption and emission properties of the probes to produce contrast between normal and diseased tissue. To be effective, the molecular probes should produce a detectable, quantifiable and discernable signal in response to light absorption or biological activation. In addition, optimizing the in vivo stability, biocompatibility and pharmacokinetics is necessary for their use in living mammals. Presently, fluorescein, indocyanine green (ICG) and fluorescent photosensitizers are widely used as optical molecular antennas for in vivo imaging of tumors, possibly due to their established photophysical properties and safety profile in humans (42-47). Representative classes of common optical probes are summarized below.

Carbocyanine Molecular Probes

Carbocyanine dyes (ICG in particular) are the most widely used NIR optical probes for imaging tumors in small animals and humans (14, 48-52). The basic structural framework of NIR carbocyanine dyes consists of highly conjugated polymethine units flanked by symmetrical or nonsymmetrical aromatic groups (Figure 2).

Homologation of carbocyanine probes results in compounds having a wide range of colors that are useful for multicolor imaging of biological processes. This allows researchers to monitor diseases at different wavelengths without using structurally dissimilar contrast agents that can drastically change their biodistribution profile. Thus, carbocyanine probes are suitable for imaging both superficial and deep tissue diseases. The exceptionally high molar absorptivity of carbocyanine dyes (typically \(>140,000 \text{ M}^{-1} \text{ cm}^{-1}\)) compensate for their low fluorescence quantum yields (typically <20%).

While ICG has been used for numerous contrast agent-mediated optical imaging studies (see below), its disulfonate group does not react with bioactive molecules under mild reaction conditions. Consequently, several reactive derivatives of ICG have been developed (48, 53-57). Some of the newer cyanine probes are also more stable and have higher fluorescence quantum yields than ICG (58, 59). The endemic low yield problems of isolated pure compounds encoun-
tered during scale-up synthesis of NIR carbocyanines appears to be resolved by modifying the conventional synthetic pathways (60-62). For example, a recent report showed that pre-acetylating the reactive intermediate, glutaraldehyde dianil, during the synthesis of NIR carbocyanine dyes, facilitates the preparation of the pure molecular beacons in >70% yield without the need for HPLC purification (60). Using this procedure, the reaction of activated benzoindoles with a series of polymethine dialdehydes gave compounds that absorb and fluoresce from about 550 to 850 nm in >65% yield of the pure dye (Figure 3). The carboxyl group of the new carbocyanine dyes reacts with bioactive ligands to form stable NIR probe labeled bioconjugates.

Because of the need to identify molecular beacons that have similar spectral properties and blood clearance profile as ICG, the chromophore chain (n) and the alkyl group (m) were modified until a carbocyanine dye [bis(propanoic acid) indotricarbocyanine, Figure 3, \( m = 2, n = 3, \text{ cypate} \)] that has similar absorption and fluorescence emission properties as ICG was identified (48). In vivo and ex vivo evaluations of cypate in male Lewis rats showed that cypate also have similar blood clearance profile (Figure 4a, \( \tau = 115 \) seconds), and excretion pathway (Figure 4b, hepatobiliary) as ICG. Although carbocyanine molecules such as ICG are relatively small, their binding to serum albumin and immunoglobulin in blood converts them into large macromolecules that are subsequently retained in the liver (63-66). The use of ICG analogues for the preparation of tumor-spe-
specific NIR molecular probes may facilitate regulatory approval for human use of the new optical contrast agents. A variety of structurally similar analogues of ICG has been prepared and representative examples are shown in Figures 2 and 3.

A practical problem with ICG is that it degrades in aqueous solutions, resulting in a wavelength shift of the main absorbance peak, and a concurrent decrease in its fluorescence intensity (65, 67). Confronted with this problem, Rajagopalan et al. (68) performed a series of studies and found that addition of polyaspartic acid (PAA) to aqueous ICG solution can stabilize the formulation for >24 days. Thus, formulating aqueous ICG in the presence of PAA could enable researchers to perform longitudinal assays and imaging studies with the same batch of aqueous ICG solution. Stabilization and enhancement of ICG fluorescence emission can also be achieved by adsorbing the probe on metal surfaces (51). In view of the advantages of carbocyanine optical probes, this class of compounds will continue to play a major role in contrast-mediated optical imaging of tumors in small animals and humans.

**Xanthene Molecular Probes**

Fluorescein and its derivatives are the most widely used xanthene probes in optical imaging studies (69-74). These probes typically absorb and emit radiation in the visible wavelengths and hence, are useful for imaging superficial or endoscope-accessible deep tissues. A variety of reactive fluorescence intermediates is commercially available (Figure 5) and this class of compounds is particularly attractive for their exceptionally high fluorescence quantum yields (e.g., 0.8 for fluorescein). Additionally, most laboratory instruments that rely on optical methods such as microscopes and fluorescence-assisted cell sorters (FACS) are optimized for use in the visible wavelengths. For this reason, fluorescein derivatives facilitate the use of the same probe to perform high-resolution microscopy with labeled cells or tissues and tumor detection in living organisms without changing the essential features of the molecular beacons.

![Figure 5: Fluorescein and derivatives.](image)

**Photosensitizer Molecular Probes**

Photosensitizers are generally used for photodynamic therapy (PDT) of human diseases (75-80). The chromophore system allows the photosensitizers to absorb light and, through intersystem crossing, generate singlet oxygen radicals that are toxic to surrounding tissue. Some PDT agents also emit detectable fluorescence that is useful for imaging studies (15, 81-84). Such molecules can serve the dual role of imaging and therapeutic agents. Doubts persist, however, about the efficacy of this two-pronged use of PDT agents for tumor detection and treatment because: (i) the molar absorptivity and the fluorescence quantum yields of PDT agents are usually low to be useful for in vivo imaging; (ii) care must be taken to decouple the therapeutic from diagnostic light doses to minimize phototoxicity to normal tissue during fluorescence imaging with PDT agents; and (iii) once PDT light dose is applied, the chromatophore system may no longer be available for fluorescence imaging because of photo-damage. These limitations can be overcome by integrating a diagnostic and a therapeutic chromatophore system in the same molecule to form diapeutic (tandem diagnosis and therapeutic) agents. To be effective, the chromatophore system for imaging should preferably absorb at a longer wavelength than the photosensitizer component to enable selective activation of the diapeutic agent for fluorescence intensity or lifetime imaging of tumors without eliciting PDT effect on normal surrounding tissue (85, 86).

**Fluorescent and Bioluminescent Protein Molecular Reporters**

The fluorescent and bioluminescent reporters are gene products from cells that are transduced with the corresponding reporter genes. They play important roles in elucidating the molecular basis of pathogenesis. Unlike optical probes prepared by chemical synthesis and administered into organisms, cells encoding photoproteins are particularly useful for longitudinal studies without a significant loss of detectable signal in small animals and cell cultures (87-90). This advantage arises from replication of the photoprotein cDNA with each cell division. Two major photoproteins that represent this class of optical probes are the green fluorescent proteins (GFP) (23, 26, 89) and the bioluminescent luciferases (21, 87, 91, 92).

GFP contains 238 amino acids and utilizes oxygen to rearrange its primary structure to generate the chromatophore unit (89, 93). To improve the spectral properties of GFP, a variety of genetically engineered mutants have been developed (89). For example, GFP mutants that absorb at longer wavelengths have been reported and shown to improve fluorescence detection in tissue (89, 94-98). Tissue autofluorescence, which can confound GFP spectral signature, photobleaching and cell damage can be minimized by the use of two-photon excitation techniques (99, 100). Exposure of organisms or cells possessing GFP to the appropriate wavelength yields fluorescence emission that serves as an antenna for studying biological processes. GFP and its mutants can furnish useful information on protein-protein interactions, cancer growth and metastasis, and the efficacy of new anticancer drugs in live animals and in cell cultures.
Particularly, GFP fluorescence can be used to localize tumor cells in intact animals by optical tomography and morphological changes from the same tumor tissue can be analyzed \textit{ex vivo} by high-resolution confocal microscopy. Once stably transfected cells are grafted into the host animal, excitation light is used to activate the photoprotein. However, because of the rapid attenuation of light in the visible wavelengths, biomedical applications involving these fluorescent proteins will be most useful for imaging superficial tissues with good resolution. Additionally, a recent report cautions the use of GFP in cancer studies because the organization of its chromophore system rely on the presence of molecular oxygen that may be lacking in certain tumors (93).

In contrast to fluorescent proteins, bioluminescent reporters emit radiation when they interact with their substrate (22). Luciferases of different origins (bacteria, firefly, click beetle, and Renilla) are the most widely used bioluminescent reporters for biomedical applications. Typically, the process of light emission requires the consumption of ATP, oxygen, and an enzyme substrate, D-luciferin. This approach is highly sensitive because external excitation light is not required for activation, among other reasons. A comparative study between the sensitivity of GFP and luciferase-expressing tumor cells shows that the latter can be detected within one day postinjection of the cells relative to seven days with GFP-expressing cells (101). This finding has been corroborated by a recent study, which demonstrated that the bright fluorescence signal from fluorescent reporters in the visible wavelengths does not translate into high sensitivity compared with bioluminescent proteins because of the “noise” from background autofluorescence (102). Using subcutaneously implanted dual-labeled fluorescent and bioluminescent reporter cells in mice, the investigators showed that the bioluminescence detection was achieved with about 500 cell population compared with 50,000 cells needed for the fluorescence detection. This exceptionally high sensitivity is a major reason for the wide use of bioluminescence techniques in molecular imaging. The high specificity of bioluminescence imaging arises from selective interaction of the enzyme-substrate complex that must occur before light generation. With current interest in developing strategies for gene therapy, reporter proteins may be useful for monitoring the efficacy of this new treatment paradigm (24, 103). A major challenge is to develop stable human-based reporter genes for clinical application.

\textbf{Nanomaterials, Metal Chelates, and Microbubbles}

Nanoparticles and quantum dots are small particulates that can circulate in blood vessels and accumulate in target tissue by specific or nonspecific mechanisms. Their spectral properties can be optimized for a specific application by varying the particle size, which typically affects their spectral properties. These materials are the ultimate optical probes for tumor imaging because of the potential to obtain products that combine strong light absorption, emission, and scattering contrast effects. Additionally, the surface of these materials can be modified, thereby allowing the amplification of target delivery system through synergistic action of multiple biomolecules per particle (104). Because the nanoparticles are more photostable than organic dyes, they can be used to monitor biological processes longitudinally (104). The development and applications of these probes have been extensively reviewed in the literature (32-34, 36, 105).

A recent study by Geddes \textit{et al.} (106) showed that conjugation or binding of carbocyanine dyes to metal surfaces such as silver colloids is a reliable approach to alter the fluorescence intensity and lifetimes of the dye. The remarkable increase in fluorescence intensity and photostability, and a significant decrease in the fluorescence lifetime of the ICG provide a mechanism to interrogate molecular processes with the same probe. The ability of some nanoparticles such as magnetic iron oxide (used for MRI) to quench the fluorescence of fluorochromes have been used to perform highly specific functional optical-MRI multimodal imaging (107).

Metal chelates are another class of molecular probes that are useful for optical imaging (108-112). These compounds have very largest Stokes shift (>250 nm) that minimizes interference from excitation light and long fluorescence lifetimes that are distinguishable from endogenous absorbers in tissue. Further, different metals possessing paramagnetic or radioative properties can be used to produce multimodal imaging agents (108). Although some of the chelates have high fluorescence quantum yields (0.6), their molar extinction coefficients are low. This feature calls for the use of high concentration of the probe to obtain sufficient signal for \textit{in vivo} imaging of whole animals or human tissue. Ideally, probes for molecular imaging should be capable of generating detectable and discernable signals at low doses (e.g., nanomole/kg body weight) because of the low levels of molecular targets in tumors. Nevertheless, these beacons could be useful for endoscope-assisted detection and monitoring of diseases (110).

Because ultrasound imaging operates by a similar mechanism as the high-resolution optical coherence tomography (OCT), microbubbles that are typically used for ultrasound imaging can serve as contrast agents for OCT (113). Formulation of microbubbles with fluorescent probes will provide a strong contrast for both fluorescence tomography and ultrasonography. Similarly, the scattering properties of encapsulating gold microspheres have been used for OCT applications (114). Overall, the translation of these emerging optical probes from small animal feasibility studies to human use will depend on progress towards optimizing the \textit{in vivo} pharmacokinetics. For quantum dots, human use will require additional assurance that a leak-proof shielding of its toxic constituents has been developed.
Delivery Mechanisms

The utility and effectiveness of contrast effectors in probing molecular processes and cellular functions depend on the efficiency of their delivery to target tissues. Delivery of optical molecular probes to tumors is currently mediated by nonspecific mechanism (2, 14, 39, 48, 115, 116), nonspecific followed by a secondary activation mechanism (117-120), and specific mechanism.

Nonspecific Delivery Mechanism

Molecular probes that operate by this mechanism rely on the differential accumulation of the probe in pathologic tissue based on impaired tumor capillary permeability, increased interstitial fluid, or other structural aberrations. The conditions for nonspecific retention of contrast agents in tumors are usually manifest at advanced stages of tumor develop-
ment. ICG is the major nonspecific optical contrast agent used for a variety of animal and human imaging studies (2, 14, 48, 115, 121, 122). Particularly, neovascularization of tumors can be used to differentiate benign from malignant tumors after bolus injection of ICG. For example, Gu et al. (123) showed that ICG can be used as a blood pool agent by imaging tumor vasculature in 9L tumor xenograft in rodents with a 3D optical imaging system. Additionally, Reynolds et al. (116) demonstrated selective uptake of ICG in spontaneous canine mammary tumors and lymph nodes. As with many nonspecific contrast agents, retention of ICG in tumors can be sporadic. For example, comparison of the retention of ICG in rats bearing different tumor xenografts (DSL 6/A, Dunning R3327H, and CA20948) shows that at 30 minutes postinjection, ICG was retained in certain tumors (e.g., Dunning R3327), and barely visible in others (DSL6/A and CA20948; Figure 6) (48). The retention of ICG in Dunning R3327H is mediated by nonspecific mechanism.

A new analogue that overcomes the rapid hepatobiliary clearance of ICG from blood has been developed (124). This probe was obtained by coupling two glucamine derivatives to the aromatic groups of indotricarbocyanine disulfonate. The slow clearance of the probe from blood provides ample time for it to accumulate in tumors, thereby enhancing the tumor uptake as a function of time. Generally, the low specificity and sensitivity of the nonspecific optical contrast agents such as ICG limit their use as blood-pool agents for optical imaging of tumors.

Non-specific Delivery Coupled with a Secondary Activation Mechanism

Molecular beacons that use this mechanism combine non-specific delivery with a “pro-drug” approach, where the fluorescence emission is pre-quenched (stealth molecular probes) until the probe encounters a target physiological event that results in the emission of detectable fluorescence. A variety of physiological processes can be targeted by this mechanism, including enzyme activity, hypoxic microenvironment, signal transduction pathways, and metabolic processes. The use of stealth probes to image the activity of proteases in vivo is typical of this approach and offers enormous benefit for the early detection and monitoring the status of cancer by visualizing aberrant gene expression (118, 120). Upon activation by enzyme-catalyzed hydrolysis in tumors, the fluorescence intensity increases several folds relative to surrounding normal tissue, which improves detection sensitivity. The fluorescence intensity is then correlated with the expression level of the diagnostic or prognostic enzymes. The transport and activation mechanisms and the clinical potential of these important protease-sensitive stealth molecular probes approach have been extensively reviewed in several publications (29, 30, 125, 126).

Site-specific Delivery Mechanism

As progress in molecular biology continues to reveal specific factors that characterize abnormal tissues, newly designed optical probes can incorporate the essential features of the molecular basis of tumor pathogenesis to induce specific response or uptake in tumors. This approach takes advantage of the presence or up-regulation of specific factors in pathologic tissues to deliver the contrast agent to a desired target. Some factors that can be targeted for specific delivery by this approach include overexpressed receptors on tumor cell membranes, adhesion factors during angiogenesis, aberrant RNAs, tissue-specific markers such as mammoglobin for breast cancer, and calcium deposits in calcified solid tumors. Several carriers, including macromolecules, saccharides, cells, peptides, antibodies and other proteins, can mediate delivery of optical molecular probes to tumors. Published data have demonstrated the feasibility of using antibody or protein bioconjugates of fluorescein and cyanine dyes to target cell surface receptors that are up-regulated in tumor cells (54, 127-137). However, the efficient targeting of tumor cell membrane receptors with antibodies and large biomolecules or polymers is hampered by low diffusion rate into tumors, rapid uptake by the liver, and the potential to elicit adverse immunogenic reactions (128). Figure 7 illustrates the general approach conventionally applied in designing target-specific optical probes.

Although recent studies on monoclonal antibody fragments may overcome some of these problems (138-143), small bioactive molecular carriers have been successfully used to image tumors by nuclear methods because they are not antigenic and they can rapidly diffuse to tumors than larger biomolecules. Additionally, small molecules are easier to prepare and are amenable to combinatorial synthesis needed in some cases to identify ligands with improved tumor selectivity, pharmacokinetics, and in vivo stability. This approach could also be integrated into stealth molecular probe designs by replacing currently used polymeric carriers with receptor-specific delivery agents. For this reason, the remaining part of this review will highlight some studies performed with fluorescent probe-labeled small bioactive molecules. As state in the Scope of Review section above, studies with somatostatin-avid probes will be discussed in more details because the radiopharmaceutical analogue is currently used in humans. Most of the method strategies and findings with these probes are also applicable to other molecular beacons operating by receptor-mediated mechanism.

Elucidating and Optimizing the Low Energy Conformations of Bioactive Peptides

An important consideration in the preparation of fluorescent probes for optical imaging is that the peptide or small or-
ic molecule carrier may lose their bioactive conformations after conjugation with the optical probe. Fortunately, advances in molecular modeling and NMR structure analyses can be used to predict the impact of adding fluorescent probes to small bioactive molecules before synthesis. Understanding the bioactive conformations of the dye-peptide conjugates would also facilitate the preparation of novel tumor-specific optical molecular probes with improved binding affinity and pharmacokinetics. In a recent study, the proton chemical shifts of octreotate-cypate conjugate were assigned by analysis of TOCSY and NOESY spectra (60). The NMR data of the dye-labeled octreotate suggest that the threonine amino acids at positions 6 and 8 (T6, T8) have a unique \(\beta\)-CH and \(\gamma\)-CH\(_3\) resonance at 3.8-4.2 ppm and at 1.0-1.2 ppm, respectively. By integrating the NMR data into molecular modeling programs, the study demonstrated that the ensemble of low-energy structures deduced by computational modeling fully satisfies the NMR restraints. Two conformers out of a cluster containing 24 structures were shown to possess the most number of individual inter-proton distances satisfying the NMR data (Figure 8). Both possess a \(\beta\)-reversal centered on the D-Trp(4)-Lys(5) fragment that might characterize the bioactive conformation of octreotide and octreotate (60).

This conformational study indicates that some side chains of the peptide that are not involved in somatostatin receptor binding could be modified to increase or reduce the extent of charge density and lipophilicity, among other factors, at well-defined positions distal to the binding site of the ligand. Thus, it is possible to evaluate possible conformational changes in the peptide structure after labeling it with relatively large optical probes. These exciting molecular modeling and NMR data show that structure-activity relationship (SAR) studies can be used to understand and optimize the binding of small molecular beacons to their target receptors.

**Somatostatin Peptide Analogues**

Somatostatin (ST) is a polypeptide with 14 or 28 amino acids units (ST-14 or ST-28) that bind to ST receptor (STR). It regulates the release and activity of digestive enzymes and other hormones such as growth hormones and insulin (144, 145). ST also slows the growth of tumors by interfering with epidermal growth factor and growth hormone release. Five sub-types (STR1, STR2, STR3, STR4, and STR5) of human ST receptors are well-characterized and a putative sixth subtype was recently proposed (144). The expression of STR is high in a variety of tumors (146). However, the use of native somatostatin in therapeutic and diagnostic applications is limited by its biological half-life (2-3 minutes). For this reason, truncated (smaller) ST peptide analogues have been prepared and shown to obviate these problems (147).

OctreoScan\(^\text{®}\), which is widely used for imaging neuroendocrine tumors in humans, is an \(^{111}\text{In-DTPA}\) conjugate of octreotide (148-150). After conjugation of the radioactive metal chelate to the amino-terminal amino acid (Phe) of octreotide, the peptide retained its STR binding affinity. Recently, an analogue of octreotide with improved pharmacokinetics and much higher STR binding affinity, octreotate, was developed (151). Octreotate has a nine fold higher STR binding affinity than octreotide and does not require cumbersome chemical transformation of the carboxy-terminal threonine to the alcohol analogue, threoninol, as found in octreotide (151). Human studies have shown that radiolabeled octreotide is useful for imaging and treating tumors (151). Based on these observations, the NIR fluorescently labeled conjugates of octreotate have been prepared by solid phase synthesis (48, 74, 152-154). The presence of two carboxyl groups on the ICG analogue, cypate, makes it possible to incorporate one or two bioactive molecules to obtain homogenous or heterogeneous receptor-specific molecular probes (Figure 9).

In a small animal model, a nominal 50 mW collimated solid state laser source and a CCD camera were used to evaluate specific retention of the STR-avid fluorescent probes in male Lewis rats bearing subcutaneously implanted STR-positive CA20948 pancreatic tumor xenograft (Figure 10). Biodistribution of the dyes and receptor-specific optical molecular probe in normal rats were performed by injecting a solution of the compound via the rat’s lateral tail vein. The results show remarkable retention of the STR-avid probe in CA20948 tumors (48, 154).

STR-avid NIR probes could be used in translational research, especially for endoscope-accessible GEP tumors and breast cancers that express exceptionally high levels of STR (144). Particularly, cypate-octreotate optical probes could receive early regulatory approval for use in humans because the analogous peptide (octreotide) and dye (ICG) are currently approved for use in humans by the US FDA.

**Bombesin Receptor-avid Optical Molecular Probes**

Bombesin (BN) is a tetradecapeptide that binds with high affinity to gastrin-releasing peptide receptor (GRPr). This cell surface receptor protein is up-regulated in several tumor cells, including small cell lung, ovarian, pancreatic, colorectal, and prostate cancers (155-166). BN stimulates tumor growth by trans-activating and up-regulating epidermal growth factors and hence, GRPr antagonists are useful for treating BN-positive tumors. Because the native ligands are not stable in biological media for prolonged periods and are rapidly excreted in the liver, efforts to prepare stable compounds have yielded small peptide and non-peptide analogues (158, 159, 161, 167-170). These studies demonstrate that truncated BN peptide analogues retain high GRPr-bind-
ing affinity, even after conjugation with radioisotopes or radiometal chelators. As with other G-protein coupled receptors, internalization of the receptor-ligand complex, followed by the recycling of the receptor back to the cell membrane, can enhance the efficiency of delivering probes to target cells. Initial clinical studies suggest that radiolabeled BN can be used to image prostate and breast cancers in humans (171).

Predicated on these observations, Bugaj et al. (154) conjugated a NIR probe to a truncated BN peptide analogue and showed that the peptide’s receptor binding affinity was retained. In vivo biodistribution studies with a GRPr-positive tumor, AR42J, showed that the probe was selectively retained in the target tumor relative to normal tissues (Figure 11). Interestingly, other studies showed that practically all the invasive prostate carcinomas examined by autoradiography have elevated GRPr expression relative to normal tissues (169, 172). Since human prostate is amenable to endoscope-assisted optical imaging, BN-specific NIR molecular probes may be useful for detecting and staging prostate cancer. However, visualizing the GRPr-positive tumor in rats was difficult until 24 hours postinjection of the contrast agent (154). Additionally, nonspecific uptake in the kidneys and liver were also high. Further work is needed to optimize this class of compounds for in vivo optical imaging.

Neurotensin Receptor

Neurotensin (NT) is a tridecapeptide that functions as a neuromodulator in the central nervous system and has a range of physiological functions in the periphery (173). NT activity is mediated by interacting with specific G-protein coupled receptors (NTR) to trigger the activation of a cascade of events (174). The observation that NTR is expressed at high levels in ductal adenocarcinoma suggests the potential utility of NT as a vehicle to deliver diagnostic and cytotoxic drugs to pancreatic tumors. Recent studies show that >75% of all ductal pancreatic adenocarcinomas from patients were NTR-positive (175, 176). A logical strategy for targeting pancreatic cancer is to label native NT with a signal-generating moiety. Unfortunately, native NT [NT(1-13)] is unstable in vivo, with a short half-life of 1.5 min in human plasma (177). Published reports showed that a truncated NT [NT(8-13)] has a similar NTR binding affinity and a longer half-life (10 min) in plasma than native NT but the peptide degrades rapidly before detectable quantities could selectively accumulate in NTR-positive tumors in vivo (177, 178). This metabolic instability is attributed to the rapid cleavage of the scissile Arg8-Arg9 and Tyr11-Ile12 amide bonds of NT by proteolytic enzymes (179, 180).

To circumvent this problem, numerous stabilization strategies have been reported, including the synthesis of non-peptide (181, 182), cyclic peptide (183), and pseudo-NT peptide (177, 180, 184, 185) analogues. A more recent study incorporated metabolically stable amino acid mimics into the structural framework of NT (186). This approach yielded many serum-stable NT peptide analogues with high NTR binding affinity. A representative structure of one of the stable peptide analogues is shown in Figure 12.

Figure 12: Structures of DTPA conjugate of a metabolically stable neurotensin peptide analogue.

Because of the exceptional metabolic stability and high NTR binding affinity of the above compound, its biodistribution was evaluated in NTR-positive HT29 tumor-bearing SCID mice by radiolabeling it with 111InCl3 (186). Apart from the kidneys, which are the primary route of excretion, the radiolabeled peptide was selectively taken up by the NTR-positive tumor tissue. While its accumulation in many organs was negligible at 1 h postinjection (Figure 13), relatively high concentrations of the injected dose were retained in the tumor (2.83 %ID/g) and the kidneys (6.14 %ID/g). To demonstrate that the observed uptake in tumor tissue was receptor-mediated, blocking studies were performed (low specific activity experiment; LSA). The results show that a ten-fold excess of the unlabeled NT peptide analogue inhibited retention of the radiolabeled probe in HT29 tumor but not the kidneys at 4 h postinjection. Some of the compounds were also labeled with fluorescein isothiocyanate (FITC) to

Figure 13: Biodistribution of [In-111]-NT peptide analogue (17) in HT29 tumor bearing SCID mice at 3 hours post injection, and graphed as % injected dose per gram tissue; LSA, low specific activity (0.5 Ci/mmol) radiolabeled NT-peptide used to demonstrate NT receptor specific uptake.
generate the optical probes. Recently a NIR NT peptide conjugate was prepared and preliminary in vivo studies show that it is selectively retained in HT29 NTR-positive tumors (Achilefu S. et al., unpublished work).

A combination of the ease of synthesis, exceptional stability in biological media and high NTR binding affinity provides a unique opportunity to use these molecules as pancreatic cancer-specific delivery vehicles for diagnostic or cytotoxic drugs.

**Folate Receptor**

Proliferating cells such as those of tumors require folic acid for DNA synthesis. Internalization of this vitamin (folate) in cells is mediated by low affinity protein transporter or folate receptor (FR) endocytosis. Folate conjugates are exclusively internalized in cells by binding with high affinity to FR expressed in the plasma membrane and form vesicles that are released into the cytosol (187). FR has been shown to be up-regulated in cell surfaces, especially those of highly proliferating and metastatic cancer cells (187-189). For example, >90% of ovarian cancers have elevated levels of FR (189). Although a small molecule, folate is readily recognized by its receptor, even after conjugation with large macromolecules (190). For this reason, folate conjugates have been used successfully for the delivery of a variety of therapeutic and imaging agents (189-192). An example is the use of radiolabeled $^{111}$In-DTPA-folate conjugate to image malignant ovarian cancer in humans (188). Recently, some investigators have developed FR-avid optical molecular probes and demonstrated enhanced uptake of the probe in FR-positive tumors (193-195). Unlike the receptor-specific molecular constructs described above, folate is not a peptide, and hence, it is not subject to rapid degradation by peptidases. Because folate is an essential vitamin that is present in large amounts in human nutrients, fasting may be needed to minimize the unfavorable competition of the probe with endogenous folate.

**Epidermal Growth Factor**

Although epidermal growth factor receptor (EGFr) is expressed in many tissues, it is upregulated in tumors. The native ligand (EGF) is a polypeptide consisting of 53 amino acid sequence that is capable of stimulating cell proliferation (196). The dye-labeled analogues of EGF has been used to image tumors in rodents (197) or evaluate the biological properties of EGFr (198). By using a continuous wave laser source and an intensified CCD camera, Ke et al. (197) showed that a carbocyanine labeled epidermal growth factor (EGF) specifically localized in a human mammary tumor xenograft in rodents. Control experiments were performed with EGFr positive and negative tumor models. Successful blocking of the uptake of EGFr positive tumors with anti-EGFr monoclonal antibody C225 or unlabeled EGF showed that the tumor uptake was EGFr-specific. Other investigators have developed anti-EGF-antibodies for evaluating tumors (132, 199). For example, Sokolov et al. (199) conjugated anti-EGFr antibody with fluorescent gold nanoparticles for imaging epithelial cancers by endoscope-assisted optical method. Topical application of the agent on epithelial tissues and subsequent in vivo imaging with a high-resolution microscope can furnish valuable diagnostic or prognostic information in real-time. These studies demonstrate the potential of using NIR contrast-mediated optical imaging to monitor and image the expression of EGFr in tumors. Development and labeling truncated peptide analogues of EGF can improve the rapidity of tumor uptake and the blood clearance profile of the dye-peptide conjugates.

**Others**

A variety of other transporter- or receptor-specific carbocyanine molecular probes have been developed for contrast-mediated optical imaging of tumors, including those that target vasoactive intestinal peptide (VIP) receptor subtype 1 (200), hydroxyapatite (201, 202) and glucose transporters (203).

**VIP Receptor:** The effects of structural modification on VIP agonist or antagonist activity are known and can be used to advantage in designing tandem therapeutic and diagnostic drug pairs. Recently, Bhargava et al. (200) used the “spot synthesis” approach on a cellulose support to prepare and screen a limited library of dye-labeled VIP analogues for optical imaging. The study showed that the decrease in VIP receptor binding affinity of indocarbocyanine-VIP conjugates can be overcome by incorporating positively charged amino acids at distal positions to the peptides’ pharmacophore. As progress continues in the clinical evaluation of the radiolabeled VIP analogues, further development of the equivalent NIR molecular probes would facilitate the imaging of VIP receptor-positive tumors by optical methods.

**Hydroxyapatite:** Microcalcification in breast cancers is an important diagnostic parameter in mammograms. A particular form of microcalcification is the occurrence of calcium phosphate deposits (hydroxyapatite) in malignant breast cancer. Zaheer et al. (201) recently developed a NIR optical probe for targeting hydroxyapatite. This was accomplished by conjugating a NIR carbocyanine fluorescent probe (IRDye 78) with a known hydroxyapatite targeting agent (bisphosphonate pamidronate). This study demonstrated that the molecular construct was capable of detecting osteoblastic activity in rodents. To evaluate the application of this probe for imaging breast cancer, Lenkinski et al. (202) developed animal models of breast cancer that forms stable microcalcification. The result demonstrates the feasibility of imaging breast cancer with the NIR probe.
Glucose Transporters: The glycolytic pathway for energy production by cells requires delivery of glucose to the mitochondria, where it is phosphorylated by hexokinase (204). Glucose transporters (GLUTs) facilitate the internalization of the carbohydrate into cells. Because of their high energy needs, proliferating cells overexpress GLUTs to enhance this equilibrium-dependent transport relative to normal cells. Consequently, $^{18}$F-radiolabeled 2-fluoro deoxyglucose (2-FDG) glucose is used to detect cancers in humans (205). An intricate interplay between mitochondrial hexokinase and GLUTs determines the outcome of 2-FDG images. Interestingly, a fluorescent probe-labeled glucosamine derivative, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) was reported as a viable non-radioactive method to monitor glucose transport and uptake in living cells (206). Consequently, Chen et al. (203) and Ye et al. (207) conjugated glucosamine to a NIR carbocyanine dye. The conjugate was injected in athymic mice bearing human hepatoblastoma tumor xenograft and imaged by frequency domain optical method. In contrast to control ICG, the imaging study showed that the glucosamine conjugate was retained in this proliferating tumor. A similar study with a photodynamic therapy agent, pyropheophorbide 2-deoxyglucosamide, also demonstrated specific uptake of the conjugate in tumors (84). These findings may result in the development of NIR surrogate probes for radiolabeled 2-FDG.

Summary

As molecular imaging continues to evolve into a central science in molecular medicine, development of exogenous molecular antennas that respond to pathophysiological processes at the cellular and molecular levels are highly needed. Of particular interest is the re-emergence of optical methods for imaging tumors. This imaging modality is attractive because of its high sensitivity and the use of non-ionizing radiation. While numerous endogenous biomolecules can provide optical contrast for functional imaging, exogenous molecular beacons are well-suited for molecular imaging because their spectral properties can be designed to respond to specific molecular events. Additionally, the same molecular beacon can be used to detect and monitor diseases from cellular level to bedside because of the availability of laboratory and clinical instruments that are amenable to optical data acquisition and analysis. However, exogenous molecular beacons will have to overcome regulatory hurdles prior to use in humans. These hurdles are not insurmountable because ICG and fluorescein are already approved for use in humans by the US FDA. The prevalence of receptor-mediated radiopharmaceuticals in clinical intervention by nuclear methods also provides precedence for using receptor-specific molecular beacons for tumor imaging in humans. Researchers are continuing to make progress towards translating tumor-specific molecular beacons from bench top to bedside by developing biocompatible probes, advanced image reconstruction algorithms, and high-end optical tomography systems. In the meantime, exogenous molecular probes have found immediate use and are providing valuable insight into molecular processes in genomics, proteomics, drug action, high throughput screening, and gene therapy in cells and small animals.

Acknowledgement

Part of the studies described here was funded in part by research grants from the NSF (BES 0119489), Siteman Cancer Center Research Development Award and the US Army breast cancer research award (DAMD17-02-1-0613).

References

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Date Received: February 17, 2004