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REVIEW ARTICLE

Peptide-based molecular beacons for cancer imaging and therapy

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Abstract Peptide-based molecular beacons are Förster resonance energy transfer-based target-activatable probes. They offer control of fluorescence emission in response to specific cancer targets and thus are useful tools for in vivo cancer imaging. With our increasing knowledge about human genome in health and disease, peptide-based "smart" probes are continually developed for in vivo optical imaging of specific molecular targets, biological pathways and cancer progression and diagnosis. A class of fluorescent photosensitizers further extends the application of peptide beacons to cancer therapeutics. This review highlights the applications of peptide beacons in cancer imaging, the simultaneous treatment and response monitoring and smart therapeutics with a focus on recent improvements in the design of these probes.

Keywords Molecular beacons · Optical imaging · Photodynamic therapy · Activatable probes · Proteases

Introduction

Important developments over the past decade have established molecular imaging techniques as potential front-runners in the early detection and characterization of cancer, cancer biology insight and the evaluation of treatments. Molecular imaging presents more specific parameters with regard to tumor environment such as detection of premalignant molecular abnormalities, growth kinetics, angiogenesis growth factors, tumor cell biomarkers and metastatic markers. Combining molecular imaging with targeted therapies, we may potentially assess therapeutic outcome at a molecular level long before phenotypic changes occur, and gain insight into the pathogenesis of intact cancer microenvironment. Finally, molecular imaging has the potential to provide real time, three-dimensional information non-invasively that can be applied readily for image-guidance purposes.

A popular molecular imaging technique is fluorescence imaging which is proving to be a powerful tool for cancer diagnostics and treatment. Near-infrared wavelengths (650–900 nm) are optimal for fluorescence imaging as it provides deeper tissue penetration due to the low tissue absorption and low autofluorescence resulting in higher signal-to-noise ratios (Weissleder and Ntziachristos 2003; Fragioni 2003). A major appeal of fluorescence imaging is the development of molecular beacons, a Förster resonance energy transfer (FRET)-based target-activatable probes. They offer control of fluorescence emission in response to specific cancer targets and thus are useful tools for in vivo cancer imaging. With our increasing knowledge about human genome in health and disease, peptide-based "smart" probes are continually developed for in vivo optical imaging of specific molecular targets, biological pathways and cancer progression and therapeutics. This review aims to summarize the recent accomplishment, challenges and developments of peptide-based molecular beacons and their role in cancer applications.

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Proteases

Proteases are catalytic enzymes, involved in essential chemical reactions of cellular function. They break down

proteins by hydrolyses of peptide bonds (Turk 2006; Law and Tung 2009). As tumors develop, they must pass through several important stages that require the action of proteases. Thus, many tumors have been shown to have overexpressed levels of proteolytic enzymes. Numerous studies have shown a correlation between cancer and the overexpression of a protease where protease expression is often associated with cancer aggressiveness and/or staging (Turk 2006; Law and Tung 2009). For example, a growing body of evidence shows that matrix metalloproteinases (MMPs) play a major role in cell proliferation, migration, differentiation, angiogenesis, apoptosis and host defense (Cicek and Oursler 2006; Scherer et al. 2008). MMP expression is upregulated in almost all type of human cancers and is often associated with a more aggressive phenotype of tumors with an overall poor prognosis. Thus, MMPs are believed to play key roles in cancer invasion and metastasis as they enable malignant cells to cross the basement membrane and digest extracellular matrix (Scherer et al. 2008; Ii et al. 2006). MMP-7, matrilysin, is distinct due to its epithelial origin, minimal domain organization and is over-expressed by tumor cells themselves (Ii et al. 2006). It has been shown to be overexpressed in invasive cancers of the esophagus, stomach, colon, liver, pancreas, lung, skin, breast, prostate, and head and neck (Ii et al. 2006). Thus, MMP-7 is a potential useful biological marker and therapeutic target for aggressive tumors (Ii et al. 2006). The urokinase plasminogen activator (uPA) is a protease shown to facilitate cancer progression, angiogenesis and metastasis (Law et al. 2004; Hsiao et al. 2006). The high expression level of uPA has been correlated with poor prognosis in breast, gastrointestinal and urogenital cancers (Law et al. 2004; Hsiao et al. 2006). The proteolytic activity of uPA has been reported to play a role in regulating downstream proteases by activating certain MMPs, vascular endothelial growth factor, hepatocyte growth factor and transforming factor-β, all factors involved in tumor development (Law et al. 2004; Hsiao et al. 2006). Thus, similar to MMPs, uPA is a potential target for tumor diagnosis and therapy. Cathepsin expression also plays a role in cancer development and is highly upregulated. They increase neoplastic progression with direct roles in tumor growth, migration, invasion, angiogenesis and metastasis (Mohamed and Sloane 2006). Another protease family of interest is caspases which are expressed during apoptosis, a mode of programmed cell death critical for maintaining tissue homeostasis. Imaging of caspases is attractive as many cancers have deregulated apoptotic mechanisms (Bullok and Piwnica-Worms 2005; Maxwell et al. 2009). Not all relevant biological targets are expressed by the tumor cells themselves. For example, the fibroblast activation protein (FAP) is a cell-surface serine protease that is expressed by cancer-associated fibroblasts in over 90% of human epithelial cancers (breast, ovarian, bladder, colorectal, lung, etc.) (Lo et al. 2009), but its expression is limited in normal fibroblasts and other normal tissues (Lo et al. 2009).

Protease-targeted therapies have been in development for the past half a century with some success (Turk 2006). As we better understand the role of proteases in normal and diseased states, interest in protease-targeted therapies continually grows. There is promise in early detection of disease as a result of the identification of a number of protease substrates and the ability to image the molecular change of protease expression (Turk 2006). By directly imaging the underlying alterations of disease, the efficacy of protease inhibitors and the effect of therapies can be determined shortly after treatment initiation. Cancer diagnosis and progression can be evaluated in vivo with the ability to repeatedly and non-invasively map protease expression in real time (Law and Tung 2009; Scherer et al. 2008). Protease imaging also serves as in vivo screening tools for drug development and aids in the understanding of how protease activities are regulated in an intact environment (Law and Tung 2009; Tung 2004). As proteases recognize and cleave specific amino acid sequences, peptide-based probes are excellent candidates for protease-targeted imaging. In addition, peptide-based probes have a high delivery yield, low toxicity and immunogenicity, rapid clearance, and are easily synthesized and modified (Law and Tung 2009; Tung 2004).

Protease-activatable probes

In the 1940s, Theodor Förster published a series of papers that contributed to the formulation of Förster Resonance Energy Theory (FRET). FRET describes the process where a chromophore (donor) in its excited state non-radiatively transfers its energy to another chromophore (acceptor) in the ground state through long range dipole–dipole interaction (Forster 1946). This results in quenching of the fluorescence of the donor and/or appearance of the characteristic fluorescence of the acceptor. The efficiency of FRET depends on the extent of overlap between the emission spectrum of the donor and absorption spectrum of the acceptor. The yield of transfer decreases with the sixth power of the distance between the chromophores (Carmel et al. 1973). Generally, efficient FRET in known donor–acceptor pairs allow for separation distances ranging up to 50 Å and becomes insignificant at distances greater than 100 Å (Matayoshi et al. 1990).

By combining the mechanisms of protease targeting and FRET-based activation, a probe with an extremely high level of target specificity, peptide-based molecular beacons (MBs), is created. These MBs consist of a donor and an acceptor attached by a protease-specific peptide linker

resulting in an intramolecular energy transfer that effectively quenches the fluorescence of the donor. Enzymatic cleavage of the linker separates the donor and acceptor moieties, leading to a drop in the effect of FRET, resulting in the activation of the donor's fluorescence (Carmel et al. 1973). MBs use FRET to deactivate/quench fluorophores. Therefore, the selection of an efficient donor/acceptor pair is critical for obtaining a large change in fluorescence signal for each activated MB (Carmel et al. 1973; Matayoshi et al. 1990).

Peptide-based MBs for cancer imaging are generating a great amount of promise with the continual discovery of cancer biomarkers and the high expression levels of proteases associated with this disease. MBs are attractive as their activation is confined to tissues overexpressing the protease target, whereas MBs remain inactive in non-expressing tissues. Other than the specificity of MBs, another advantage of protease activation is the high signal amplification as a small amount of enzyme can continually cleave and activate a countless number of MBs. Protease-activated probes creates a reduced background as a result of the quenching of the inactive form. Protease specificity is achieved by virtue of different peptide substrates and the MB template can be applied to various other enzymes (Weissleder and Ntziachristos 2003; Tung 2004). Therefore, MBs are emerging as promising tools in cancer diagnostics, image guidance and therapy and can be classified into three categories: (1) MBs for cancer imaging, (2) MBs for simultaneous treatment and response monitoring and (3) MBs as smart therapeutics.

MBs for cancer imaging

Molecular beacon can be divided into several categories when discussing their role in cancer imaging: (1) classic protease probes, (2) polymer-based peptide beacons and (3) nanoparticle-based peptide beacons (Fig. 1).

Classic protease probes (peptide beacons)

Classic protease probes consisted of two chromophores attached to the same molecule by an enzymatic substrate (Fig. 1a). An intramolecular interaction occurs wherein the excited chromophore (the donor) transfers its excitation energy to another chromophore (the acceptor) resulting in quenching of the fluorescence of the donor, and appearance of the acceptor characteristic fluorescence. Enzymatic cleavage of these compounds results in the separation of the donor and acceptor moieties by diffusion, decreasing the yield of energy transfer, enhancing the fluorescence of the donor (or reduction of the fluorescence of the acceptor). These first classical protease probes were primarily used as

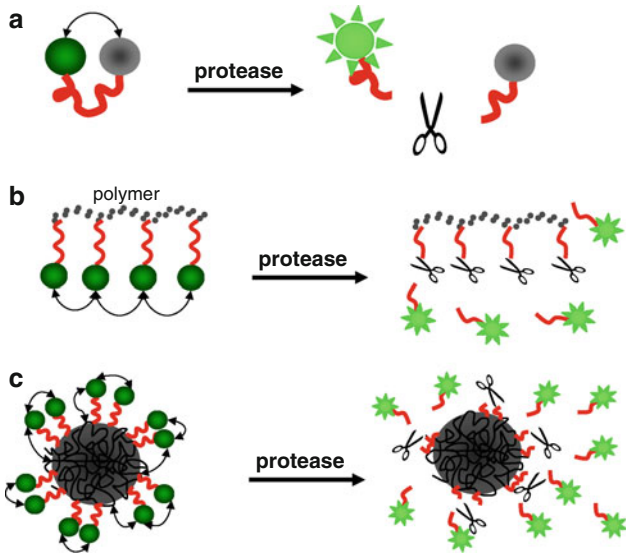

Fig. 1 Schematic diagram of the **a** classic peptide probe, **b** polymer-based peptide probe and **c** nanoparticle-based peptide probe

a kinetic assay studying enzymatic cleavage characteristics (Carmel et al. 1973). Proteolytic activity can be continuously monitored using MBs by the increase in fluorescence intensity over time. The transient enzyme–substrate complex is a characteristic feature of enzyme catalysis, and provides an insight into the definition of enzyme mechanisms; however, these enzyme-catalyzed reactions have extremely rapid reaction rates (Carmel et al. 1973; Lobb and Auld 1979). The advantage of MBs is their ability to visualize this transient enzyme–substrate complex directly providing the capability to determine the number of intermediates, the type of reaction mechanism and the individual rate and binding constants of specific enzymes (Matayoshi et al. 1990; Lobb and Auld 1979).

Not until 1999, were MBs applied in vivo and the true potential of MBs realized (Weissleder et al. 1999). Not only can MBs image specific tumor-associated protease activity in tumors, it can be used to assess protease inhibitor therapy at a molecular level, used as an image-guidance tool distinguishing tumor from healthy tissue and elucidate the functional contribution of specific proteases involved in tumorigenesis, metastatic spread and angiogenesis (Weissleder et al. 1999; Bremer et al. 2001). MB imaging has also been aided by the use of near-infrared (NIR) fluorophores and the concurrent development of in vivo NIR imaging systems (Weissleder and Ntziachristos 2003; Zheng et al. 2004). The advantage of using fluorophores excited in wavelengths in the NIR region (650–900 nm) is their ability to penetrate deeper into tissues. NIR fluorophores are poorly absorbed by biomolecules of tissues, such as oxyhemoglobin, deoxyhemoglobin, lipids and water, and therefore have the ability to penetrate several centimeters (Fragioni 2003; Chance 1998; Ntziachristos et al. 2003).

Furthermore, NIR light reduces tissue autofluorescence which optimizes the signal-to-background ratio (Fragioni 2003; Chance 1998; Ntziachristos et al. 2003). Following the classic design of MBs, a MMP7-targeted MB consisting of a NIR fluorescence emitter (Cy5.5) and a NIR fluorescence absorber (NIRQ820) has the ability to assay enzyme activity both in vitro and in vivo and screen various inhibitors for cancer therapy (Pham et al. 2004). A caspase-targeted MB has the capacity to image apoptosis and provides a non-invasive diagnostic tool to guide therapeutic choices and monitor treatment (Bullok and Piwnica-Worms 2005).

Polymer-based peptide beacons

Modifications of the classic peptide-based probes are not surprising since small peptides have unfavorable pharmacokinetics posing a concern for in vivo applications. Poor accumulation in target tissues results due to the short plasma half-life and limited tissue penetration of small peptides (Funovics et al. 2003). Therefore, several strategies were developed to address these concerns including the conjugation of a long circulating polymer that would enhance high tumoral delivery (Fig. 1b) (Weissleder et al. 1999; Funovics et al. 2003; Krinick et al. 1994). Polymer conjugation is a popular approach for cancer delivery because polymers with high molecular weights tend to accumulate at tumor sites (Funovics et al. 2003). This is a result of the enhanced permeability retention effect or so-called passive targeting. The first reported polymer backbone for MB modification is a methacrylamide polymer backbone (Krinick et al. 1994). Cathepsin B-targeted peptide sequences were conjugated to the polymer and fluorophores were conjugated to the peptide linkers. Silencing of fluorescence was achieved by self-quenching of the fluorophores (Krinick et al. 1994). Currently there are two other polymer backbones used in polymer-based peptide beacons; one being a poly-L-lysine backbone (Weissleder et al. 1999; Campo et al. 2007; Gabriel et al. 2007), the other being a dendrimeric polyamido amino scaffold (McIntyre et al. 2004). The poly-L-lysine backbone increases the circulation time of the probe and provides a multitude of unmodified lysines serving as sites for enzyme cleavage with Lys-Lys specificity. Thus, multiple fluorophores can be conjugated to the polymer backbone, increasing the probe's payload (Weissleder et al. 1999; Campo et al. 2007; Gabriel et al. 2007). However, these polylysine MBs are limited to enzymes that cleave at lysine residues such as cathepsins.

To increase the specificity of activation, protease-specific short peptide linkers were attached to the polymer backbone. Short peptides were used to target enzymes with trypsin-like activity on the poly-L-lysine backbone where cleavage of the peptide linker resulted in a 34-fold increase in fluorescence (Campo et al. 2007). Narrowing the specificity to a single target, an uPA peptide sequence was conjugated to the copolymer of poly-L-lysine and methoxypoly(ethyleneglycol) resulting in a 6-fold fluorescent increase after uPA activation (Law et al. 2004). The uPA-specific polymer-based MB was specifically activated in vivo by uPA expressing tumors (Law et al. 2004). Concurrently, a MMP7-specific peptide sequence was conjugated to a dendrimeric polyamido amino scaffold and a 17-fold fluorescent increase occurred after cleavage of the peptide linker (McIntyre et al. 2004). This MMP7 polymer-based MB possessed the capacity to detect and image MMP7 activity in vivo (McIntyre et al. 2004). Although a high payload of fluorophores for a single probe can be achieved with these polymer-based MBs, this high payload decreases the water solubility of the probes. Furthermore, although a high payload of fluorophores results in more efficient quenching, enzymatic digestion is decreased because the cleavage sites required for enzyme recognition are extensively modified (Gabriel et al. 2007). On the other hand, low fluorophore-loading leads to less efficient quenching, resulting in relatively poor signal/noise ratio after enzymatic cleavage (Pham et al. 2004). Therefore, a balance between effective quenching and efficient activation is required for polymer-based MBs (Gabriel et al. 2007).

Nanoparticle-based peptide beacons

Another strategy to improve the delivery of peptide beacons to target tissues is nanoparticle-based MBs (Fig. 1c). Through the enhanced permeability retention effect, nanoparticles ranging in size from 20 to 150 nm preferentially accumulate in tumor tissues (Law and Tung 2009). Examples of nanoparticle-based MBs include an uPA-activated nanofiber MB whereby an uPA-specific peptide linker was inserted into the core of the nanofiber. Only in the presence of uPA was a 4-fold increase in fluorescence intensity observed (Law et al. 2007). A MMP-activated nanoparticle MB consisting of a fluorophore and a quencher tethered to a tumor-homing polymeric nanoparticle effectively detected and imaged MMP activity in vivo (Lee et al. 2009). A strong correlation between the expression of MMPs and the fluorescent intensity was observed in ex vivo studies (Lee et al. 2009). Other than delivery enhancement, the incorporation of nanoparticles presents three distinct advantages. First, if conjugates are composed of superparamagnetic iron oxide particles, multimodality imaging is enabled as both can act as magnetic resonance and optical imaging contrast agents. Protease-activatable nanoparticles are attractive as they have the capacity to report spatial and anatomical information through their magnetic properties, while still providing optical information with regard to their environment

(Josephson et al. 2002; Bremer 2008; Kircher et al. 2003). Secondly, different fluorophores conjugated to tunable peptide spacers attached to the nanoparticle allow for simultaneous multichannel assessment of several characteristics (Lee et al. 2009; Josephson et al. 2002; Bremer 2008; Kircher et al. 2003). Finally, maximum quenching efficiency can be achieved by incorporating a number of quenchers and/or multiple types of quenchers (Lee et al. 2009; Josephson et al. 2002; Bremer 2008; Kircher et al. 2003).

Protease-activated quantum dot probes have also been developed. Using the energy transfer that occurs between a quantum dot (donor) and an acceptor such as a gold nanoparticle (Chang et al. 2005) or quenchers (Medintz et al. 2006) or fluorescent proteins (Suzuki et al. 2008), a quantum dot probe is optically suppressed in its native state and becomes highly luminescent after proteolysis of the peptide linker (Chang et al. 2005). The utilization of quantum dots overcomes many of the shortcomings of organic fluorophores. Quantum dots have strong luminescence, good photostability against photobleaching and physical environments (such as pH and temperature) and optical tunability (Chang et al. 2005; Medintz et al. 2006; Suzuki et al. 2008). The tunability of quantum dots is especially attractive as the quantum dots photoemission can be size-tuned to optimally overlap spectrally with a particular acceptor (Medintz et al. 2006). The size of quantum dots is also an advantage as multiple acceptors can be attached to a single quantum dot as it acts as a central FRET donor (Medintz et al. 2006). Thus, quantum dots are ideal for optical imaging and hold great potential in both in vitro and in vivo biological labeling. A comprehensive discussion regarding protease-activatable optical imaging in cancer is discussed elsewhere (Mahmood and Weissleder 2003).

MBs for simultaneous treatment and response monitoring

A recent development upon MBs is their use as multifunctional probes. For example, the MB concept has been extended for therapeutic purposes by exploiting the intrinsic fluorescence capabilities of photosensitizers (PS) (Stefflova et al. 2006a, b, 2007). Photodynamic therapy (PDT) is a minimally invasive treatment that destroys cells when light activates a PS in the presence of oxygen due to the production of singlet oxygen ($^1O_2$) (Wilson and Patterson 2008). Since most porphyrin-like PS can emit both fluorescence and produce $^1O_2$ when activated by light and the concentrations required for killing and imaging cells are in the same range, NIR fluorescent imaging and PDT can be integrated in a single molecule. Poyrphyrins are also known for their affinity for tumors and their ability to modulate the pharmacokinetics of fluorophores (Chen et al. 2005), small peptides (Zheng et al. 2007) and other biomolecules (Chen et al. 2008; Zheng et al. 2009). Thus, by substituting the fluorophore for a porphyrin-like PS, classic peptide-based MBs can be converted as dual NIR imaging and PDT agents (Stefflova et al. 2007) with improved biodistribution and pharmacokinetics. The therapeutic function of PS enhances the capabilities of MBs in which they can be used for simultaneous treatment and response monitoring.

A PDT agent with a built-in apoptosis sensor (PDT-BIAS) was designed to detect PDT-induced apoptosis in real time (Stefflova et al. 2006a). It possesses a caspase 3 peptide linker holding a PS and a quencher in close proximity that enables FRET and silences the fluorescence (Stefflova et al. 2006a). Once light activates the PDT-BIAS that has entered a cell, the PS produces $^1O_2$ that begins damaging the cell. If the apoptotic cascade is initiated, caspase 3 is processed to its active form indicating the initiation of irreversible apoptotic death. Caspase 3 will then activate PDT-BIAS specifically cleaving the peptide linker, separating the quencher from the PS and restoring its fluorescence (Stefflova et al. 2006a). Thus, PDT-BIAS can act as an early self-evaluation of its PDT therapeutic outcome in vivo in apoptotic cells using real time NIR fluorescence imaging. Apoptotic death is validated by an 8-fold increase in fluorescence after cleavage of the caspase 3 peptide linker (Stefflova et al. 2006a). In vitro results indicated that intact PDT-BIAS can enter cells (confocal microscopy), initiate PDT cell destruction (MTT assay), be specifically cleaved by caspase 3 and detect apoptosis (confocal microscopy overlay with an Apoptag TUNEL assay) (Fig. 2) (Stefflova et al. 2006a). Distinguishing between apoptosis and necrosis is advantageous in the evaluation of drugs and the optimization of PDT treatment protocols.

Although PDT-BIAS is a promising therapeutic and response monitoring tool, it lacks cancer-targeting capabilities. The folate receptor is a common cancer biomarker shown to be overexpressed in a multitude of cancers including breast, ovarian, kidney, lung and colorectal (Kelemen 2006; Salazar and Ratnam 2007; Sega and Low 2008). A targeted PDT agent with a built-in apoptosis sensor (TaBIAS) was designed to address the cancer targeting challenges of PDT-BIAS (Stefflova et al. 2006b). A tumor-homing folate molecule was incorporated into the original built-in apoptosis PDT agent to target the folate receptor. In vitro and in vivo results indicated that TaBIAS preferentially accumulated in cancer cells and xenografts overexpressing the folate receptor (Stefflova et al. 2006b). Monitoring the fluorescence intensity after PDT treatment in folate receptor positive versus folate receptor negative cells and xenografts, significant fluorescence was only detectable in folate receptor positive cells and xenografts. Furthermore, little fluorescence was observed in both folate receptor positive/negative cells with heat-induced necrosis

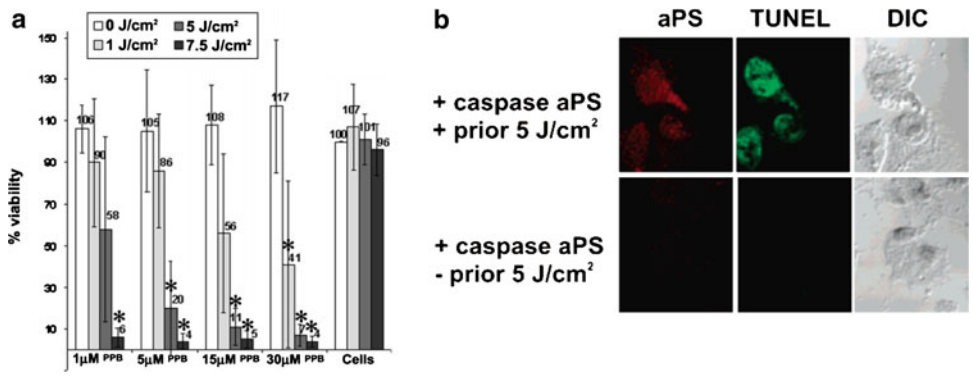

Fig. 2 **a** PDT efficacy of PDT-BIAS determined by a cell viability (MTT) assay. Viability of HepG2 cells after treatment with PDT-BIAS at four different concentrations (1, 5, 15 and 30 μM) and with three different PDT light doses (1, 5 and 7.5 J/cm^2) compared with cells alone that serve as the 100% viable reference. **b** Apoptosis inducing and caspase detecting capability of PDT-BIAS. PDT-BIAS incubated with HepG2 cells and treated with 5 J/cm^2. HepG2 were then stained with Apoptag after light treatment and both Fluorescein and Pyro fluorescence coexist. This confirms that it is the apoptosis causing the PDT-BIAS cleavage (modified with permission from Ref. Stefflova et al. 2006)

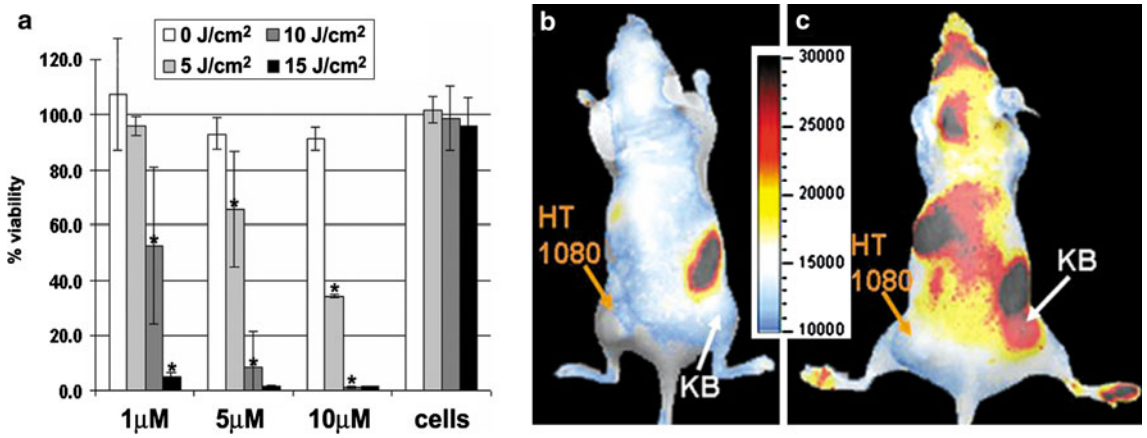

Fig. 3 **a** PDT efficacy of TaBIAS determined by a cell viability (MTT) assay. Viability of KB (folate receptor positive) cells after treatment with TaBIAS at three different concentrations (1, 5 and 10 μM) and with three different PDT light doses (5, 10 and 15 J/cm^2) compared with cells alone that serve as 100% viable reference. TaBIAS has minimal dark toxicity and good PDT efficacy. In vivo induction and detection of apoptosis in a mouse bearing KB (folate receptor positive) and HT 1080 (folate receptor negative) tumors after PDT (90 J/cm^2) using an 80 nmol intravenous injection of TaBIAS (**b**, **c**). Xenogen images of a mouse bearing an HT 1080 tumor on the left side and a KB tumor on the right side **b** before injection of TaBIAS and **c** after PDT (23 h after PDT and 41 h after intravenous injection of TaBIAS) (modified with permission from Ref. Stefflova et al. 2006)

(Fig. 3) (Stefflova et al. 2006b). TaBIAS can effectively trigger and monitor apoptosis with useful applications in the evaluation of the properties and efficacy of other PDT agents and apoptosis-inducing drugs specifically targeting cancer cells.

MBs as smart therapeutics

Peptide beacons can be further extended to create smart therapeutic MBs by exploiting the multifunctional properties of PS; fluorescence as an image-guidance and diagnostic tool and its PDT capabilities for therapeutics. The cytotoxic molecule of PDT is $^1O_2$, which is generated by the light-activated PS, causes cell damage. However, $^1O_2$ action is non-specific and localized due to its short lifetime (Wilson and Patterson 2008; Moan 1990; Dougherty et al. 1998). Therefore, the site of PS accumulation is the site of $^1O_2$ production which, in turn, is the site of cell destruction. By controlling the delivery of light and PS to target tissue, some degree of PDT selectivity is achieved. A thorough discussion regarding PDT can be found elsewhere (Wilson and Patterson 2008; Dougherty et al. 1998). Current PSs are limited by their non-specific accumulation in normal tissues often leading to normal tissue damage. The optimal treatment schedule for patients is also difficult in determining as PS accumulation at the target site is unknown

(Wilson and Patterson 2008). Recently, PDT MBs (PMBs) that mimic the concept of classic imaging MBs have been developed to control the ability of a PS to generate $^1O_2$ only in target cancer cells. PMBs consist of an enzyme-specific peptide linker with a PS and quencher conjugated at its opposite ends. The PS's photoreactivity and fluorescence are inactivated until the peptide is cleaved allowing the PS and quencher to dissociate from one another. Once activated, PMBs restore its photo-reactivity and fluorescence production providing real time image guidance (Lo et al. 2009; Chen et al. 2004, 2005, 2007). Thus, this fluorescence provides a seamless "see and treat" approach in PDT. The protease-specific linker regulates the activation of the PS where only after PMB activation and specific irradiation will $^1O_2$ be generated.

The generation and deactivation of $^1O_2$ by conventional PS has been the focus of most current research. $^1O_2$ quenching can be achieved several ways: (1) direct quenching of $^1O_2$, (2) $^1O_2$ scavenging, (3) quenching by ground-state complexing formation, (4) deactivation of PS excited singlet state and (5) deactivation of PS excited triplet state (Lee et al. 2009; Chen et al. 2004, 2007; Lovell et al. 2009). Although PMBs offer another level of control of fluorescence emission and selective $^1O_2$ generation of PS, this control is highly dependent on correct quencher selection. The benefits of PMBs are nullified if $^1O_2$ quenching is not sufficient in the inactive form, resulting in killing of cells lacking the target. In MBs, fluorescence is effectively quenched through both FRET and the formation of ground-state complexes. Sustained and direct contact between a fluorophore and quencher are required for ground-state complex formation which is a difficult mechanism to design in MBs. However, FRET may be predicted based upon the spectral overlap of fluorophores and quenchers within specific distance ranges (Lovell et al. 2009). The principles of FRET can also be applied to quenching the $^1O_2$ production of PS. Different quenchers with varying absorption spectra were covalently conjugated to a PS. When a short linker was used, the PS fluorescence and $^1O_2$ generation were quenched regardless of the amount of spectral overlap. Extending the linker with a polyproline peptide, only the quenchers with absorption spectra that effectively overlapped with the fluorescence spectra of the PS could sustain effective $^1O_2$ quenching (Fig. 4) (Lovell et al. 2009). Although not directly related, as differences in the extent of fluorescence and $^1O_2$ quenching are observed, measurement of the fluorescence of PMBs is a good indicator for $^1O_2$ production status (Lovell et al. 2009). The fluorescence response and $^1O_2$ response were found to be similar with quenchers of varying efficiency degrees (Fig. 4). However, this relationship between spectral overlap and quenching is only applicable up to 90% fluorescence and $^1O_2$ quenching

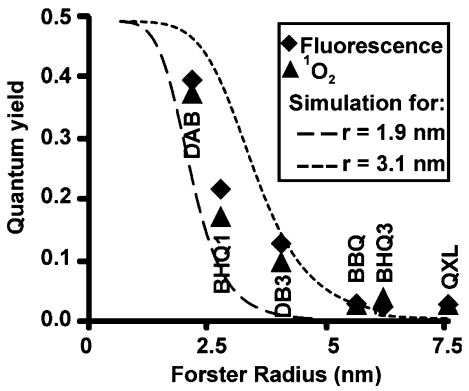

Fig. 4 Fluorescence and singlet oxygen yields as a function of Forster radius. Disperse Blue 3 (DB3) and Black Hole Quencher 1 (BHQ1) effectively quenched the PS when in direct contact, but extending the peptide linker caused a decrease in the quenching efficiency. Blackberry Quencher (BBQ), Black Hole Quencher 3 (BHQ3) and QXL-680 (QXL) maintained greater than 90% quenching of the PS due to their large Forster radii (modified with permission from Ref. Chen et al. 2004)

(Lovell et al. 2009). Not only is effective quenching imperative in the inactive state, but also effective unquenching is equally desired in the activated state. Therefore, quenchers and PSs that have a large predicted Förster radius should be avoided. If the activated beacon is not separated by a distance greater than the quencher or PS's Förster radius, quenching of the PS could occur even in the activated state. Consequently, this leads to a reduction of the activated signal (Lovell et al. 2009). PMB design is a careful process but if ideal PSs and quencher pairs are selected, it is possible to successfully control the fluorescence production and $^1O_2$ generation of PSs.

Following the classic strategy of MBs, FAP-targeted PMB (FAP PMB) consists of a fluorophore (pyropheophorbide-α) and a Black Hole Quencher 3 (BHQ3) linked by a FAP-specific peptide sequence (Lo et al. 2009). Complete cleavage of the FAP PMB by FAP results in a 200-fold fluorescence increase and complete fluorescence restoration. The specific activation of the FAP PMB was evaluated by HPLC after the incubation with a number of different but closely related proteases including dipeptidyl peptidase IV and matrix metalloproteinases. Only FAP was able to cleave the FAP PMB demonstrating its activation specificity (Lo et al. 2009). In vitro and in vivo studies further confirmed the specific activation of FAP PMB. FAP PMB is specifically activated in FAP-transfected cancer cells demonstrated by flow cytometry and confocal microscopy studies, whereby it remains silent in FAP-negative cells. Furthermore, only in FAP-positive xenografts was specific activation of FAP PMB observed (Fig. 5) (Lo et al. 2009). The FAP PMB is a promising diagnostic tool for FAP-expressing epithelial cancers and

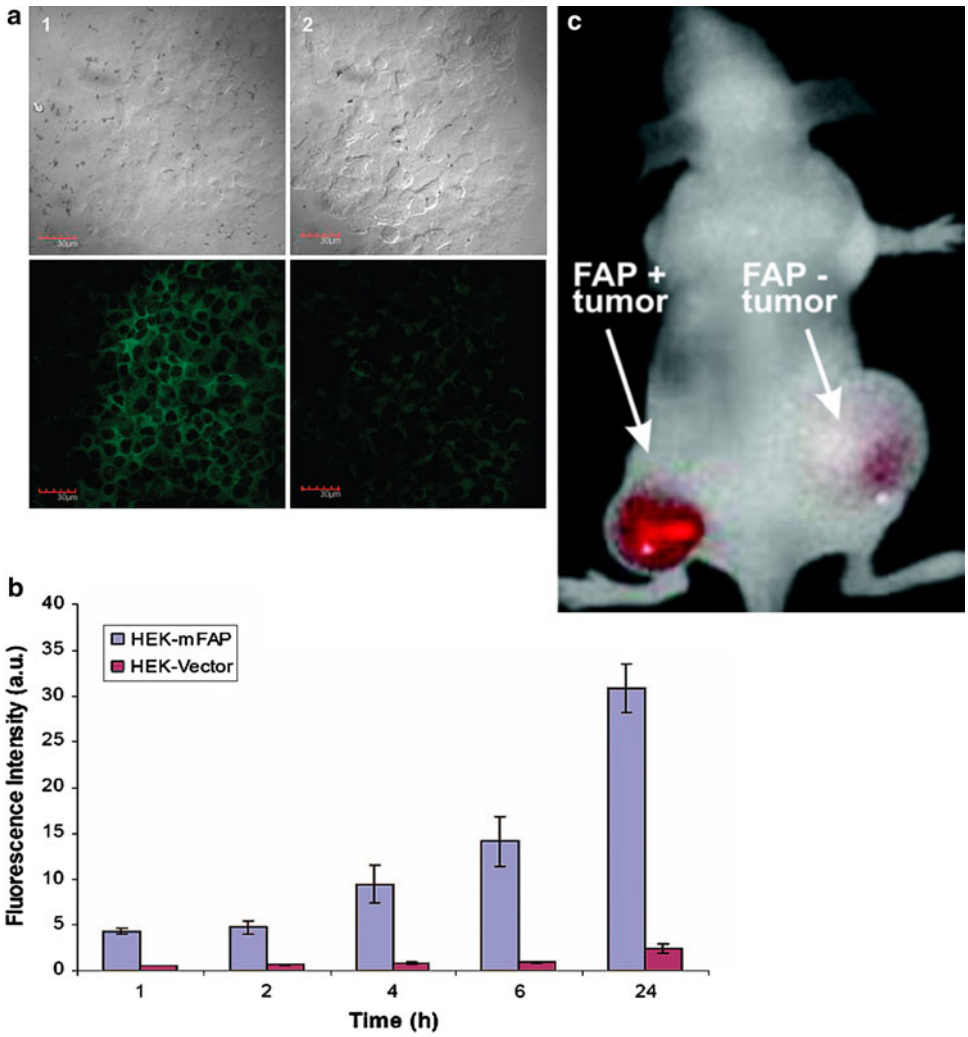

Fig. 5 Validation of FAP-specific activation of FAP PPB in vitro and in vivo. **a** Confocal microscopic images of 25 μM of FAP PMB activation in (1) HEK-mFAP cells (FAP positive) and (2) HEK-vector cells (FAP negative). *Top row* is DIC image, *bottom row* is corresponding fluorescence image. **b** Comparison of intracellular fluorescence intensity of FAP PMB in HEK-mFAP and HEK-vector cells monitored by flow cytometry. **c** In vivo images of a nude mouse bearing FAP expressing and non-FAP expressing xenografts. FAP PMB activation imaged 24 h after an intratumor injection of 25 nmol of FAP PMB (modified with permission from Ref. Lo et al. 2009)

holds promise as a fluorescence-guided surgery in aiding and allowing for complete resection whereby target tissue "lights" up. Furthermore, the PDT capability of FAP PMB can be used as an adjuvant clean up tool to further extend tumor destruction.

A MMP7-targeted PMB (MMP7 PMB) also demonstrated specific activation and selective PDT efficiency. It consists of a MMP7-specific peptide linker, pyropheophorbide-α (PS) and BHQ3 so that the PS's photoactivity and fluorescence are inactive until MMP7 cleaves the peptide linker unleashing the PS's action (Zheng et al. 2007). Incubation with MMP7 resulted in a 17 fluorescence fold increase and an 18-fold increase in $^1O_2$ production. Only MMP7-positive cells effectively cleaved the MMP7 PMB activating the fluorescence production. MMP7 PMB efficiently killed cells that expressed MMP7 in a light dose and PMB dose-dependent manner. Cells that did not express MMP7 remained viable regardless of the light dose or PMB concentration. When MMP7-positive cells were incubated with a control PMB (non-MMP7 specific), cells still remained viable regardless of light or control PMB dose. These results were mirrored in vivo using a MMP7-positive xenograft where activated MMP7 PMB accumulation at the tumor site was fluorescently imaged and the tumor completely regressed after PDT treatment without any signs of regrowth (Fig. 6) (Zheng et al. 2007). The potential of MMP7 PMB lies in the beacons' MMP7-specific killing capacity on a molecular level while protecting non-target cells from PDT damage.

New directions

As medicine is moving towards personalized treatment, MBs can play a pivotal role in its inception into the clinic. Although new biomarkers targeting cancer are emerging every day; ensuring that the activation of MBs is specific becomes incredibly significant. A challenge still lies in ensuring that peptide substrates are specifically cleaved by only the target protease. These peptide linkers must be able

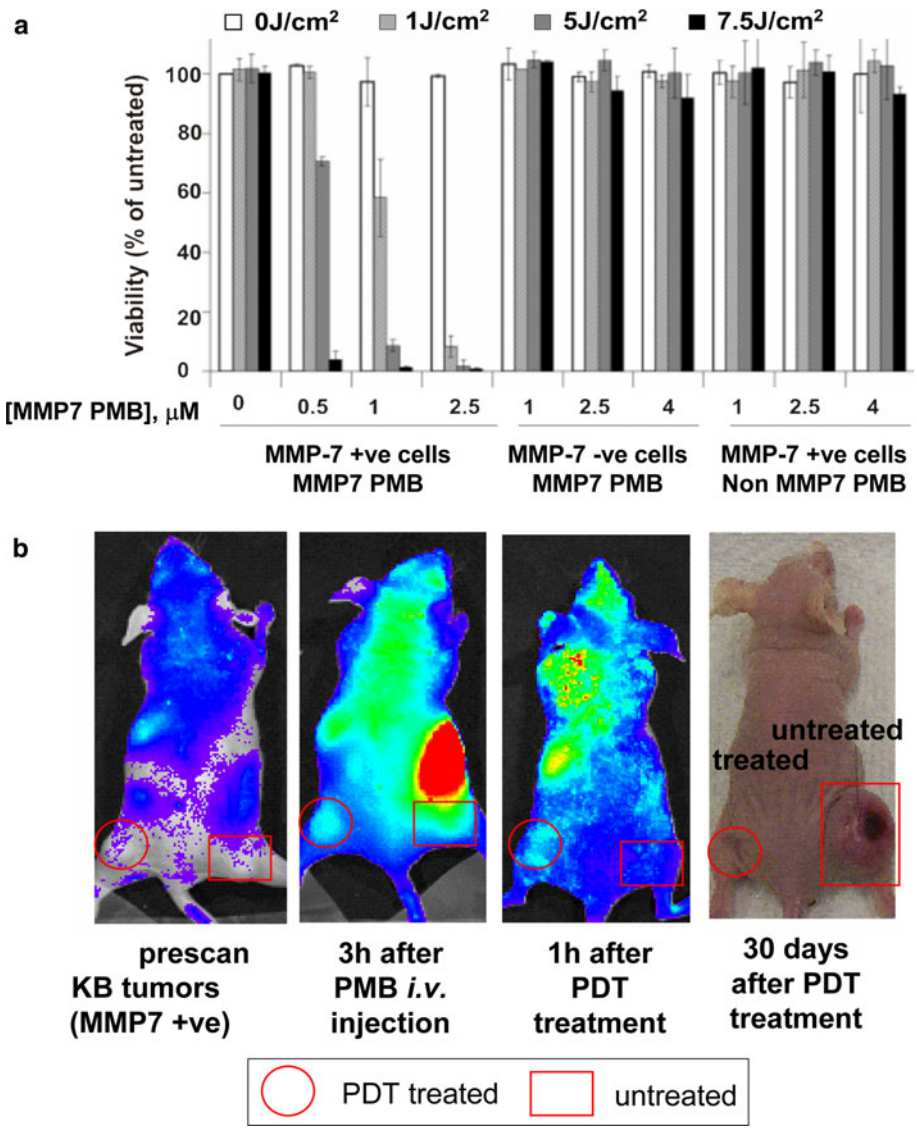

Fig. 6 **a** Viability of cells treated with different concentrations of MMP7 PMB and with different light doses. PDT-induced toxicity is only seen in cells expressing MMP7. **b** In vivo images of nude mice bearing MMP7-positive xenograft tumors before, 3 h after MMP7 PMB injection, 1 h after PDT treatment and 30 days after PDT treatment. Tumor regression is seen in the treated tumor (modified with permission from Ref. Zheng et al. 2007)

to withstand non-target cleavage by other enzymes. Difficulties may arise if the MB is taken up into lysosomes or endosomes where a variety of strong enzymes reside. Furthermore, proteases are divided into classes and families, usually based upon their proteolytic characteristics and function; therefore, a family of proteases (such as MMPs) may recognize similar cleavage sites or have overlapping cleavage sites, again, decreasing the specificity of MBs. It should also be noted that intracellular protease targeting still remains a challenge. This is partly due to the difficulty of delivering an intact MB into target cells as often the acceptor/quencher in MBs do not easily cross the cellular membrane. Furthermore, when comparing the fluorescence fold increase after cleavage of the peptide linkers in FAP PMB (200-fold) (Lo et al. 2009), the MMP7 PMB (17-fold) (Zheng et al. 2007) and PDT-BIAS (eightfold) (Stefflova et al. 2006a), the FAP PMB has a much higher quenching efficiency than both MMP7 PMB and PDT-BIAS. The difference in fluorescence release is a result of the differences in the peptide linker's secondary structure and chemical characteristics which ultimately affects the quenching efficiency. Thus, the total dependence of the fluorescence and PDT quenching upon the random folding of the peptide linker before protease cleavage limits the sequences to ones with natural conformations that bring the quencher and PS in close proximity in order for effective silencing to occur. In addition, the non-specific and passive nature of the PS's delivery to target cells after protease cleavage is suboptimal. PMBs are activated extracellularly, so that the activated probes may diffuse to non-target cells/tissues before target cell uptake occurs. This contributes to the background signal and reduced contrast. To address these concerns, activatable cell-penetrating peptides were developed that addressed the passive delivery of PMBs (Jiang et al. 2004). Activatable cell-penetrating peptides achieve high delivery selectivity due to the electrostatic

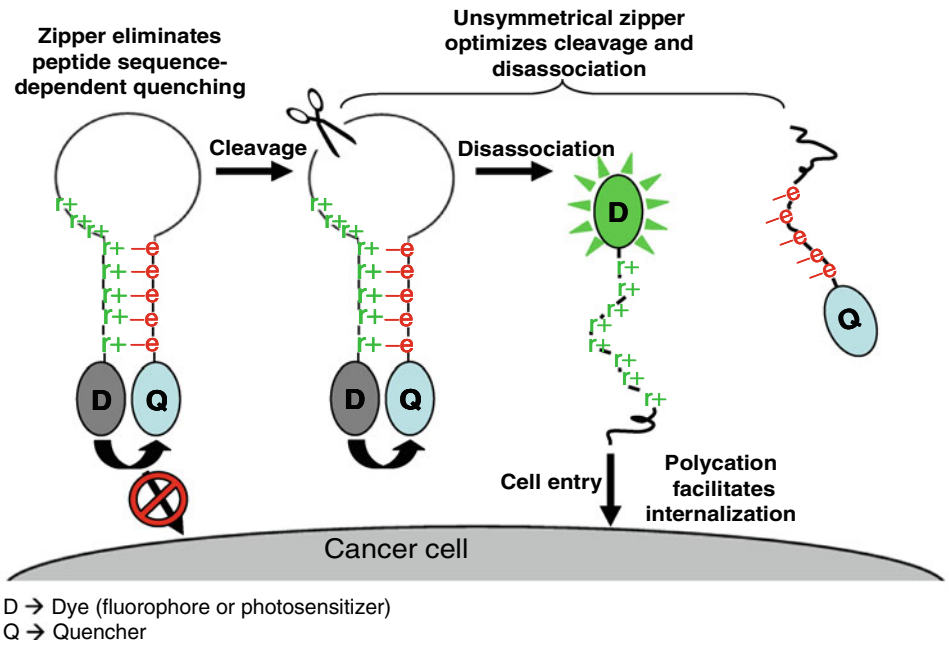

Fig. 7 A dye (fluorophore or photosensitizer) is held in close proximity due to the electrostatic attraction of the zipper which consists of a polycation and polyanion arms. This results in silenced dye activity regardless of the peptide linker sequence. Upon specific cleavage of the peptide linker by the target protease, the dye and quencher dissociate, activating the dye and unleashing the polycation which increases cellular uptake (reproduced with permission from Ref. Chen et al. 2009)

formation of a polycation/polyanion 'zipper' mechanism in which the peptide linker is selectively cleaved by a target protease unleashing the delivery function of the cell-penetrating peptide. This 'zipper' mechanism was incorporated into PMBs and zip PMBs (ZMBs) were developed.

ZMBs consist of four functional groups: a target protease cleavable peptide linker, a polycation and polyanion attached at each end of the peptide linker forming a 'zipper' structure through electrostatic attraction, a PS (pyropheophorbide-α) and BHQ3 (Fig. 7) (Chen et al. 2009). ZMB is functionally reminiscent of PMBs, a target protease specifically cleaves the peptide linker; the PS and quencher dissociate, resulting in PS photoactivity and fluorescence restoration. Cleavage of the peptide linker also unleashes the polycation resulting in increased cellular uptake (Chen et al. 2009). The zipper provides several advantages: (1) an increase in the quenching efficiency occurs due to the electrostatic attraction between the polycation/polyanion that holds the PS and quencher in direct contact, (2) an improvement in the cleavage rate occurs as a result of the zipper causing a "hairpin" conformation of the peptide linker, (3) the function of the cell-penetrating peptide is blocked and the uptake of the entire ZMB into cells is inhibited due to the polyanion arm, (4) cellular uptake of the PS is enhanced after cleavage of the peptide linker due to the polycationic arm and (5) quenching is no longer dependent upon the natural folding of the peptide linker as the zipper is solely responsible for the inactive state of ZMB (Chen et al. 2009). The longer the polycation/polyanion arms, the stronger the electrostatic attraction of the zipper, which results in greater quenching efficiency. However, if the zipper is too stable, after cleavage of the peptide linker, the unleashing of the polycation is on a much slower time scale resulting in decreased specificity (Chen et al. 2009). This is a consequence of the two-step activation observed in ZMBs. The first step is the cleavage of the protease peptide linker; the second step is the dissociation of the zipper's polycation/polyanion arm. The key point of ZMB design is to balance maximal quenching efficiency and optimal two-step activation (protease cleavage with zipper dissociation). An asymmetrical zipper arm ZMB composed of eight consecutive arginines and five consecutive glutamates has been synthesized to achieve the high quenching efficiency and ideal activation rate with enhanced PS internalization (Chen et al. 2009). Importantly, the ZMB concept creates a generalizable approach to improve the functionality of a wide range of diagnostic and/or therapeutic probes through a simple switching of substrate sequences. The increased selectivity, fluorescent production and targeted uptake of a ZMB could lead to more selective, efficient and effective tumor destruction while protecting non-target cells.

Conclusion

Peptide-based MBs show great promise in cancer diagnosis based on the overexpression of proteases and have developed quickly over the past few years. Although MBs are still faced with several obstacles such as selective delivery, specific activation and detection sensitivity, current and future modifications upon the classic protease probe design holds great potential in overcoming these challenges. For example, nanoparticle encapsulation of MBs can ensure high payload

delivery of these probes to the target site and potentially deliver them intracellularly. Incorporating radiolabeled moieties into MBs would aid in the quantification of MBs' biodistribution and pharmacokinetics in vivo (Pandey et al. 2005; Pandey et al. 2009a, b). With the incorporation of iron oxide, gold nanoparticles and radiolabeled PS into the MB design, we are no longer confined to optical imaging techniques in which penetration concerns arise. Activatable NIR contrast agents in conjunction with PET, SPECT, MRI and CT are conceivable, further improving the sensitivity and specificity of early cancer detection.

PMB concept has extended MBs from diagnostics to therapeutics. With the intrinsic fluorescence of PSs, a seamless "see and treat" approach is achieved. Incorporating the zipper mechanism not only increases the cellular uptake of activated probes but also eliminates the peptide sequence dependence of MBs, further refining and generalizing the PMB design. Thus, with the continual discovery of cancer biomarkers, the possibility of MBs is limitless. However, not only is perfecting the design of MBs important, but also the validation of these activatable probes in vitro and in vivo in order for their translation into the clinic. The goal of personalized medicine based upon a patient's cancer signature will be partially realized through MBs as it is possible to create MBs targeting a vast array of cancer biomarkers.

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