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Synthesis of BODIPY-Peptide Conjugates for Fluorescence Labeling of EGFR over-expressing Cells

Ning Zhao¹, Tyrslai M. Williams¹, Zehua Zhou¹, Frank R. Fronczek¹, Martha Sibrian-Vazquez², Seetharama D. Jois³, and M. Graça H. Vicente^{1,*}

¹Louisiana State University, Department of Chemistry, Baton Rouge LA, 70803, USA

²Portland State University, Department of Chemistry, Portland, OR, 97201, USA

³Basic Pharmaceutical Sciences, School of Pharmacy, University of Louisiana at Monroe, Monroe LA, 71201, USA

Abstract

Regioselective functionalization of 2,3,5,6,8-pentachloro-BODIPY **1** produced unsymmetric BODIPY **5**, bearing an isothiocyanate group suitable for conjugation, in only four steps. The X-ray structure of **5** reveals a nearly planar BODIPY core with aryl dihedral angles in the range 47.4° – 62.9°. Conjugation of **5** to two EGFR-targeting pegylated peptides, 3PEG-LARLLT (**6**) and 3PEG-GYHWYGYTPQNVI (**7**), under mild conditions (30 min at room temperature), afforded BODIPY conjugates **8** and **9** in 50–80% isolated yields. These conjugates show red-shifted absorption and emission spectra compared with **5**, in the near-IR region, and were evaluated as potential fluorescence imaging agents for EGFR over-expressing cells. SPR and docking investigations suggested that conjugate **8** bearing the LARLLT sequence binds to EGFR more effectively than **9** bearing the GYHWYGYTPQNVI peptide, in part due to the lower solubility of **9**, and its tendency for aggregation at concentrations above 10 μ M. Studies in human carcinoma HEP2 cells over-expressing EGFR, demonstrated low dark and photo-cytotoxicities for BODIPY **5** and the two peptide conjugates, and remarkably high cellular uptake for both conjugates **8** and **9**, up to 90-fold compared with BODIPY **5** after 1 h. Fluorescence imaging studies in HEP2 cells revealed subcellular localization of the BODIPY-peptide conjugates mainly in the Golgi apparatus and the cell lysosomes. The low cytotoxicity of the new conjugates and their remarkably high uptake into EGFR over-expressing cells renders them promising imaging agents for cancers over-expressing EGFR.

Graphical Abstract

*CORRESPONDING AUTHOR: To whom correspondence should be addressed. Phone: 225-578-7405. Fax: 225-578-3458. vicente@lsu.edu.

Supporting Information Available: ¹H, ¹³C and ¹¹B NMR spectra for all BODIPYs, HPLC traces and conditions, absorption and emission spectra, concentration-dependent dark and phototoxicity graphs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Epidermal growth factor receptors (EGFR) are ligand-stimulated cell surface receptors that play an essential role in the regulation of cellular functions, including in cell proliferation and survival.^{1–4} Over-expression and/or mutation of EGFR can lead to unregulated growth stimulation and tumorigenesis in various tumor types, including lung, breast, brain, prostate, and colon tumors.^{5–8} The frequent activation of EGFR in these cancers makes them highly attractive diagnostic and therapeutic targets. We have been particularly interested in targeting colorectal cancer (CRC), which is the third leading cause of cancer-related deaths in the US, causing approximately 50,000 deaths each year.⁹ Recent studies show that CRC age-specific risk is on the rise, and early screening and diagnosis are essential to address this trend.¹⁰ Current methods for CRC diagnosis, including different types of colonoscopy and radiography, have relatively low sensitivity for detection of small adenomas (< 5 mm) in the early stages of CRC, when there are no outward symptoms in patients.¹¹ Since EGFR is over-expressed on small cancers (< 5 mm) and on the flat, dysplastic, aberrant crypt foci that precede cancer development that are often missed by standard colonoscopy, the molecular targeting of EGFR is a valuable tool in CRC diagnosis and treatment.¹²

In the last two decades, in addition to using the native ligand epidermal growth factor (EGF),¹³ several alternative methodologies have been developed for targeting the extra or the intra-cellular domains of EGFR,² including the monoclonal antibody Cetuximab,^{14–16} single-chain anti-EGFR ScFvEGFR,¹⁷ anti-EGFR affibody,^{18–19} readily available small peptides,^{20–27} and non-peptidic tyrosine kinase inhibitors.²⁸ Among these strategies, peptide ligands with short sequences are particularly attractive for targeting the EGFR protein for a variety of reasons, including their low cost, readily availability, low immunogenicity, relatively fast diffusion rates, ease of modification, as well as their ease of conjugation to various molecules. Two peptides (Figure 1) selected using computer-assisted design or by screening phage display libraries, LARLLT (designated “D4” or EGFR-L1) and YHWYGYTPQNVI (designated “GE11” or EGFR-L2), have demonstrated particularly efficient binding to the extracellular domain of EGFR over-expressed on cancers cells, both *in vitro* (e.g. SKBR-3 and BT-474 cells) and *in vivo* (e.g. mice with A431 tumors).^{20,22–27,29–30} Additionally, computational studies^{20,25–26} have modeled the binding of these two peptides to the extracellular domain at two different binding sites: EGFR-L2

binds to the EGF binding pocket, while EGFR-L1 binds near domain I, away from the EGF binding pocket.

4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene dyes (known as boron dipyrromethenes, or BODIPYs) are versatile compounds with highly tunable structures that allow the modulation of their stability, solubility and spectroscopic properties,^{31–35} making them suitable for various applications, including in fluorescence imaging,^{36–40} photodynamic therapy (PDT),^{41–46} boron neutron capture therapy (BNCT),^{47–48} and in dye-sensitized solar cells (DSSC).^{49–51} Functionalized BODIPYs can be conjugated to peptides or proteins for imaging applications using “click” reactions,^{44,52–54} traditional amide bond formation reactions,^{30,55–58} or solid support methodologies.⁵⁹ However, several challenges have hindered their applications using these conjugation methods. Bio-orthogonal “click” reactions usually provide high yields of the targeted conjugates, however, the introduction of azide or ethynyl groups at the BODIPY or peptide periphery requires several steps, and it is normally expensive and time consuming. On the other hand, conjugation reactions using traditional coupling reagents often require an additional activation step and long reaction times (up to 24 h), giving the targeted conjugates in relatively low yields. In solid-supported synthesis, the resin cleavage step often requires harsh conditions, for example using TFA and/or elevated temperatures, which can remove the BF₂ moiety from the BODIPYs. Perhaps the most attractive alternative strategy consists on the introduction of an isothiocyanato moiety on the BODIPY scaffold as an amino reactive group, for the conjugation of various active molecules to BODIPYs including peptides,^{60–63} proteins,^{54,64–66} and other molecules,^{53,57,67–71} due to the short reaction times and high product yields.^{62,72}

Herein we describe the synthesis of unsymmetric BODIPY **5** bearing a single isothiocyanato group, from pentachloro-BODIPY **1** in only four steps. We show that BODIPY **5** readily reacts with pegylated peptides (3PEG-EGFR-L1 and 3PEG-EGFR-L2) providing EGFR-targeted BODIPY-L1 **8** and BODIPY-L2 **9** in good yields. These BODIPY-peptide conjugates were investigated as EGFR-targeted fluorescence imaging agents. The binding interactions of conjugates **8** and **9** to the EGFR protein were investigated by SPR and modeled using Autodock. The uptake, distribution and cytotoxicity of conjugates **8** and **9** in human carcinoma Hep2 cells over-expressing EGFR were evaluated and compared with those for unconjugated BODIPY **5**.

RESULTS AND DISCUSSION

1. Synthesis

We have recently reported the synthesis of versatile polyhalogenated BODIPY platforms, and investigated their reactivity under different reaction conditions.^{73–76} Such BODIPY platforms contain halogen groups with distinct reactivity, allowing for the regioselective functionalization of the BODIPY periphery using various Pd(0)-catalyzed coupling or nucleophilic elimination/addition reactions. For example, the reactivity order of the chloro groups in pentachloro-BODIPY **1** was shown to be: 8-Cl > 3,5-Cl > 2,6-Cl, which allows for the stepwise and regioselective functionalization of the 8-position, followed by the 3 and/or 5 positions, and finally the 2 and/or 6 positions of this BODIPY platform.^{76–77}

The starting material 2,3,5,6,8-pentachloro-BODIPY **1** was prepared by chlorination of 8-chloro-BODIPY using TCCA/ACOH, as previously reported.⁷⁶ The key precursor BODIPY **5** bearing an isothiocyanato group was prepared from BODIPY **1** in four steps, as shown in Scheme 1. A Suzuki-type coupling reaction between **1** and 5 equiv of 4-methoxyphenylboronic acid, in the presence of Pd(PPh₃)₄ and 1M Na₂CO₃(aq), gave 3,8-disubstituted BODIPY **2** as the major product, in 45% isolated yield, after 5 h reflux in toluene. This reaction was carefully monitoring by TLC and mass spectrometry, which showed the initial formation of the 8-substituted BODIPY, prior to that of the targeted product. The 4-nitrophenyl group was then introduced at the 5-position of BODIPY **2** using another Suzuki coupling reaction using 5 equiv of 4-nitrophenylboronic acid and Pd(PCy₃)G2 as the catalyst, to provide BODIPY **3** in 60% yield. The use of Pd(PPh₃)₄ in place of Pd(PCy₃)G2 gave lower yields of the targeted product. This is probably due to the relatively lower reactivity of 4-nitrophenylboronic acid, and the slower oxidative addition step under the catalysis of Pd(PPh₃)₄, as previously observed.⁷⁶ The reduction of the nitro group of BODIPY **3** using Pd(0)/C and hydrazine in ethanol,⁷⁸ provided the amine-functionalized BODIPY **4** in 92% yield. Treatment BODIPY **4** with an excess amount of 1,1'-thiocarbonyldi-2(1*H*)-pyridone (TDP) in CH₂Cl₂ gave the desired isothiocyanato-BODIPY **5** in 88% yield. The structures of all BODIPYs were confirmed by ¹H NMR, ¹³C NMR, ¹¹B NMR and HRMS (ESI-TOF) methods.

A suitable crystal of BODIPY **5** for X-ray analysis was obtained by slow evaporation from chloroform. The result is shown in Figure 2, the first structure in the Cambridge Structural Database of an isothiocyanato-functionalized BODIPY. The 12-atom BODIPY core is reasonably planar, exhibiting a mean deviation of 0.061 Å. The 8-methoxyphenyl substituent is twisted out of the BODIPY plane, and it forms a dihedral angle of 47.4° with the BODIPY core, as is characteristic of 8-aryl substituents on 1,7-unsubstituted BODIPYs. On the other hand, the substituents at the 3 and 5 positions form slightly larger dihedral angles with the BODIPY core, that for the NCS-phenyl being 54.5° and for the OMe-phenyl being 62.9°. The average C-Cl distance is 1.706 Å. As expected the NCS group is essentially linear, with N-C-S angle 174(2)°, but it bonds slightly nonlinearly to the phenyl group, the angle about N being 168(2)°. It also tilts slightly out of the plane of the phenyl group, with the N atom 0.08 Å out of plane, the C atom 0.30 Å and the S atom 0.68 Å.

Two peptide ligands, EGFR-L1 and EGFR-L2, have been shown to specifically target EGFR over-expressing tumor cells both *in vitro* and *in vivo*.^{26,27} We have previously reported the conjugation of EGFR-L1 and EGFR-L2 to a porphyrin⁷⁹ and a phthalocyanine²⁶ macrocycles, using both short (up to 5-atom) and long (up to 20-atom) linkers. PEG linkers, particularly the low molecular weight triethylene glycol, were shown to increase the solubility and cellular uptake of the conjugates, while preserving the fluorescent properties of the dye, due to their relatively low flexibility compared with higher molecular weight PEG groups. Furthermore, PEG linkers have been shown to increase the specificity for affinity-based molecules for surface sites.⁸⁰ In addition, the triethylene glycol linker is expected to decrease the steric effects of the *N*-terminus of the peptide with the BODIPY-NCS **5**, leading to higher yields of the targeted conjugates, and to favor extended conformations of the BODIPY-peptide conjugates that could lead to enhanced EGFR

binding affinity.^{26,81–82} The pegylated peptides tri(ethylene)glycol-LARLLT (PEG3-EGFR-L1, **6**) and tri(ethylene)glycol-GYHWYGYTPQNV (PEG3-EGFR-L2, **7**) were synthesized by solid phase peptide synthesis (SPPS) on a Fmoc-Pal-PEG-PS resin using TBTU, HOBt, and DIEA as the coupling reagents, to yield the parent peptides LARLLT and GYHWYGYTPQNV, respectively, as previously reported.^{11g,35} The resulting peptides were then reacted with Fmoc-NH-PEG3-propionic acid to yield peptides **6** and **7**. In the final step, the peptide was deprotected of the Fmoc group using a 20% piperidine solution, and then cleaved from the resin with a cocktail consisting of TFA (94%), Millipore water (2.5%), liquefied phenol (2.5%) and Tris (1%). Purification by reverse phase HPLC gave the corresponding pegylated peptides in 38–39% yields.

The pegylated peptides **6** and **7** reacted with BODIPY-NCS **5** in anhydrous DMF and in the presence of TEA, at room temperature for 30 min, to provide the corresponding conjugates **8** and **9** in 80 and 50% yields, respectively, as shown in Scheme 2. Longer reaction times (up to 24 hours) led to lower product yields, by favoring side reactions. Under these reaction conditions the BODIPY platform was preserved, as confirmed by ¹¹B-NMR, which showed triplets for the BF₂ groups at $\delta = 0.368$ and 0.374 ppm for **8** and **9**, respectively. The BODIPY-peptide conjugates were further characterized by ¹H NMR, HSQC, and MALDI-TOF.

2. Spectroscopic properties

The spectroscopic properties of BODIPYs **3**, **4**, and **5** in dichloromethane, and of peptide conjugates **8** and **9** in DMSO, including their maximum absorption (λ_{abs}) and emission (λ_{em}) wavelengths, Stokes' shifts, fluorescence quantum yields (Φ_f), and molar extinction coefficients ($\log \epsilon$), were investigated and the results are summarized in Tables 1 and S1. Figures 3 and S1 show the normalized absorption and emission spectra for the BODIPYs and the conjugates. All BODIPYs showed characteristic strong absorption bands ($\log \epsilon = 4.3 - 4.7$) in the range of 613–634 nm attributed to the S₀–S₁ (π – π^*) transitions. The weaker absorption bands centered around 450 nm are attributed to S₀–S_n ($n \geq 2$) transitions. The Stokes shift was smallest for **4** (19 nm) bearing the electron-donating amino group, and in the range of 41–48 nm for all other BODIPYs. As previously reported, the largest molecular orbital coefficients are located at the BODIPY 3 and 5 positions.³⁴ Therefore, the reduction of the nitro group of BODIPY **3** led to 36 and 14 nm red-shifts in the absorption and emission bands of **4**, respectively, due to the electron-donating character of the *para*-aminophenyl group. In addition, this reduction also caused a significant decrease in the BODIPY's fluorescence quantum yield due to reductive photoinduced electron transfer (a-PeT).³³ On the other hand, BODIPY-NCS **5** shows only slightly red-shifted absorption and emission bands (by 3 nm) compared with **3** bearing a *para*-nitrophenyl group (see Figure S1).

As shown in Figure 3, BODIPY-peptide conjugates **8** and **9** have slightly broader absorption and emission bands compared with their precursor BODIPY-NCS **5**, and red-shifted by 11–13 nm, caused by the replacement of the linear isothiocyanato group with a thioamide group. As a consequence, both conjugates **8** and **9** show emissions at 634 nm in the near-infrared region of the optical spectrum, characterized by reduced autofluorescence and absorption by

tissues, and with better penetration through tissues than visible light. However, the fluorescence quantum yield for **8** and **9** decreased by 5 to 10-fold compared with **5**, also due to the introduction of the flexible pegylated peptide chains that lead to increased non-radioactive decay.⁸⁴ Nevertheless, conjugates **8** and **9** were still efficient in cell imaging investigations (vide infra).

3. SPR studies

To evaluate the binding of peptides and BODIPY-peptide conjugates to the EGFR protein, SPR^{85–86} studies were carried out and the results are shown in Figures 4 and S10. Pegylated peptides **6** and **7** exhibited binding to EGFR with fast kinetics of association and dissociation as shown in Figure 4a,b. For peptide **6** at 100 μM the response was around 20 units, whereas for peptide **7** at the same concentration the response was around 150 units, suggesting a significantly higher binding affinity for **7** compared with **6**. In the case of conjugate **8** two modes of binding were observed. At lower concentrations up to 100 μM the response increased by 400 units and saturation was reached. However, further addition of compound **8** resulted in a drastic increase of the SPR signal (nearly 2000 units), as seen in Figure 4c. At higher concentrations, the kinetics of association and dissociation suggest specific binding of **8** to EGFR, as seen by the slow increase in the SPR signal. On the other hand, in the case of conjugate **9**, the SPR signal was saturated at only 10 μM concentration of the compound, and the relative increase in response was less than 10 units (Figure 4d), suggesting weak or non-specific binding of this conjugate. To verify the non-specific binding, BODIPY **5** was also used as an analyte in SPR studies (see Supporting Information, Figure S10). BODIPY **5** also indicated binding similar to conjugate **9**, suggesting that the BODIPY probably binds to EGFR non-specifically, along with peptide **7**. However, peptide **7** exhibited specific binding as shown in Figure 4b. Therefore, the addition of BODIPY to peptide **7** appears to reduce the specificity of binding to EGFR. This could be due to the poor solubility of conjugate **9**, and its tendency for aggregation at concentrations above 10 μM . We have previously observed that zinc(II) phthalocyanine-EGFRL2 conjugates linked via short 5-atom carbon or tri(ethylene)glycol spacers also show reduced EGFR-targeting and poor solubility in aqueous solutions.²⁶

4. HEP2 Cell Studies

Human squamous cell carcinoma HEP2 with EGFR over-expression^{11g,87} is a convenient model cell line for evaluation of the cytotoxicity, cellular uptake, and subcellular distribution of BODIPY **5** and the peptide conjugates **8** and **9**. The Cell Titer Blue assay was used to evaluate the cytotoxicity, both in the dark and after exposure to 1.5 J/cm² light dose, and the results obtained are summarized in Table 2, and shown in Figures S2 and S3. The least toxic BODIPY was unconjugated **5**, with IC₅₀ values, obtained from dose-response curves, above 100 μM . The BODIPY-peptide conjugates were slightly more toxic, particularly **8**, although both conjugates still revealed low cytotoxicities in the dark (IC₅₀ > 98 μM) and upon exposure to low light dose (IC₅₀ > 74 μM). The slightly higher cytotoxicity of conjugate **8** may be due to its positive charge on the peptide sequence, which favors a fast and efficient intracellular uptake, in agreement with previous observations with phthalocyanine-EGFR-L1 conjugates.²⁶ The low dark and photo cytotoxicities observed for the new conjugates are an

important feature for their application as molecular imaging agents for early detection of cancers over-expressing EGFR.

The time-dependent cellular uptake for BODIPYs **5**, **8** and **9** was performed at the concentration of 10 μ M in HEP2 cells, and the results are shown in Figure 5 and Table S2. At concentrations higher than 10 μ M conjugate **9** has tendency for aggregation, as observed in the SPR investigations. Both BODIPY-peptide conjugates **8** and **9** showed remarkably higher cellular uptake compared with unconjugated BODIPY **5**, at all time points investigated, suggesting that these conjugates actively target the EGFR over-expressed in this cell line. Indeed, after 1 h, conjugates **8** and **9** accumulated 90-fold and 60-fold higher, respectively, than unconjugated BODIPY **5**, and after 24 h both conjugates accumulated about 30-fold higher than **5**. These remarkable differences indicate that both peptide sequences, and in particular EGFR-L1, have a strong effect on the cell targeting and uptake ability of the BODIPY conjugates.⁸⁸ In addition, the presence of the triethylene glycol groups in **8** and **9** and their enhanced solubility compared with **5**, favor their cellular uptake. Conjugate **9** bearing a longer peptide sequence with 11 hydrophobic and two polar amino acids (Figure 1) has increased hydrophobicity compared **8** bearing the shorter peptide sequence with 4 hydrophobic, one polar, and one cationic amino acids.

Conjugates **8** and **9** showed different uptake kinetics, with **8** bearing the cationic EGFR-L1 peptide, accumulating at a much faster rate in the first 8 h, after which a plateau was reached. On the other hand, conjugate **9** containing a longer and more hydrophobic EGFR-L2 sequence, accumulated at a slower rate during the first 8 h, but its cellular uptake continued to increase during the time investigated, and up to the 24 h evaluated in this study. This is likely due to the enhanced ability of conjugate **8** bearing the shorter EGFR-L1, to quickly permeate across the negatively charged plasma membrane, as a result of its cationic charge, as well as its lower molecular weight and increased solubility compared with conjugate **9**.

Fluorescence microscopy was performed to evaluate the major and minor sites of localization of BODIPY **5** and conjugates **8** and **9** in HEP2 cells, and the results are shown in Figures 6, 7, and S4, and summarized in Table 2. Despite the low fluorescence quantum yields, a clear fluorescent signal is observed by microscopy, suggesting that these conjugates can be used for fluorescence imaging of EGFR-expressing cells. The observed fluorescence signal was mainly intracellular, indicating that the BODIPY conjugates do not appear to localize on the plasma membrane, probably as a result of their fast internalization. In the co-localization experiments, the organelle-specific ER Tracker Blue/White (ER), BODIPY Ceramide (Golgi), MitoTracker Green (mitochondria), and LysoSensor Green (lysosomes) were used. The BODIPY-peptide conjugates localized mainly in the Golgi apparatus and the cell lysosomes (Figures 6f,j and 7f,j), whereas BODIPY **5** was preferentially found in the ER and the Golgi apparatus (Figure S4d,f). Indeed, BODIPY dyes are often found in ER and Golgi,^{47,89} and conjugates **8** and **9** also localized, to a smaller extent, in the ER (Figures 6d, 7d). Interestingly, conjugates **8** and **9**, in contrast to BODIPY **5**, were also found in the cell lysosomes. Lysosomal localization is often observed in fluorophore-peptide conjugates, probably as a result of an endocytic mechanism of cell internalization for this type of conjugate, as we have previously observed for a phthalocyanine conjugated to EGFR-L1 and

EGFR-L2.^{11g} In addition, conjugate **8** was found to localize to a smaller extent in the mitochondria, and this might explain its observed slightly higher cytotoxicity compared with **5** and **8** (Table 2).

5. Modeling and Docking

To visualize the binding interaction of conjugates **8** and **9** with EGFR, three-dimensional structures of the compounds were generated using InsightII molecular modeling software (Figure 8) and the conjugates were docked to the EGFR structure using Autodock software.⁹⁰ The peptide LARLLT in conjugate **8** is known to bind to EGFR near domain I, as indicated in previous studies.^{25–26} The lowest energy docked structure of compound **8** is shown in Figure 7A with docking energy of -4.19 kcal/mol. The docked structure formed three hydrogen bonding interactions with the EGFR protein, namely the peptide NH of L5 formed an hydrogen bond with the carbonyl group of Ser222 from EGFR, the NH of L1 formed an hydrogen bond with the carbonyl group of Gln59, and one oxygen atom of the PEG linker formed an hydrogen bond interaction with Asn119 of EGFR. The BODIPY core of conjugate **8** formed hydrophobic interactions with the following residues on EGFR: Leu186, Pro171, Lys185 side chain, Ile189 and 190. The BODIPY group was extended away from the peptide binding pocket, likely forming non-specific hydrophobic interactions with the EGFR surface.

Figure 7B shows the binding of conjugate **9** to EGFR near the EGF binding pocket.²⁰ Compound **9** binds to the lower side of the EGF binding pocket with a docking energy of -6.07 kcal/mol, forming three hydrogen bonds. The G6 and T8 side chain of the peptide formed hydrogen bond interactions with the side chain of Arg310 on the EGFR. The peptide residue Y2 also formed an hydrogen bond interaction with Val312 of EGFR. The PEG chain was folded in a loop structure and it was not involved in any hydrogen bond interactions with the receptor. The BODIPY core of conjugate **9** is located on a hydrophobic pocket near Phe335 and His334 of EGFR. The alpha 4-methoxyphenyl group on the BODIPY is enfolded in the hydrophobic pocket of EGFR, stabilizing the structure of conjugate **9** in the EGF binding pocket. The BODIPY core extends out of the EGF deep binding pocket, while the peptide chain remains inside the EGF binding site.

SPR and docking studies suggest that conjugate **8** bearing the EGFR-L1 sequence binds specifically to EGFR, at a site away from EGF binding pocket. On the other hand, conjugate **9** binds near the EGF binding pocket, with the BODIPY core buried in a hydrophobic pocket and extended out of the pocket. These results are in agreement with previous studies²⁰ suggesting that the EGFR-L2 peptide binds near the EGF binding pocket, partially overlapping with the EGF binding pocket. Our docking studies demonstrate that conjugate **9** binds to the lower side of EGFR, in the EGF binding pocket. Furthermore, SPR studies indicated that BODIPY **5** binds to EGFR non-specifically (Figure S10) and the binding of conjugate **9** seems to be non-specific at concentrations greater than $10\text{ }\mu\text{M}$ (Figure 4d). This may be due to the lower solubility and tendency for aggregation of **9** at concentrations above $10\text{ }\mu\text{M}$, and to the binding of the BODIPY core to the hydrophobic pocket near the EGF binding pocket. Furthermore, modeling studies suggest that in the case of conjugate **8**, the PEG linker and BODIPY core do not mask the amino acid functional groups in the peptide,

whereas in conjugate **9** the PEG linker folds back onto the peptide surface, masking some or part of the amino acid side chains in the EGFR-L2 peptide (Figure 7). Therefore, conjugate **8** appears to be superior for the specific targeting and binding to EGFR, which is also in agreement with the SPR and cellular studies.

CONCLUSIONS

We report an expedite synthesis of BODIPY **5**, bearing an isothiocyanate group, in four steps from 2,3,5,6,8-pentachloro-BODIPY **1**, via two regioselective Suzuki cross-coupling reactions, followed by reduction with Pd(0)/hydrazine in ethanol, and conversion to the isothiocyanate functionality using 1,1'-thiocarbonyldi-2(1*H*)-pyridone in CH₂Cl₂. The structures of all BODIPYs were confirmed by NMR, HRMS, and in the case of **5**, by X-ray crystallography. The reaction of the isothiocyanate-BODIPY **5** with two pegylated EGFR-targeting peptides, 3PEG-LARLLT and 3PEG-GYHWYGYTPQNVI, proceed smoothly at room temperature for 30 min, providing the corresponding BODIPY-peptide conjugates **8** and **9** in 50 – 80% isolated yields. The structures of **8** and **9** were investigated by NMR, HSQC, MALDI-TOF and molecular dynamics. BODIPY conjugates **8** and **9** showed absorption and emissions in the red and near-infrared regions of the spectrum, at ca. 588 and 634 nm respectively, and fluorescence quantum yields in the range $\Phi_f = 0.01$ – 0.033 in DMSO. SPR investigations indicated that while conjugate **8** binds specifically to EGFR at concentrations up to 250 μ M, conjugate **9** showed non-specific binding to EGFR at concentrations above 10 μ M due to its tendency for aggregation. The binding modes of BODIPY-peptide conjugates were investigated using Autodock. These studies suggest that **9** binds to the lower side on the EGF binding pocket group, while **8** binds near domain I of EGFR, away from the EGF binding pocket. The BODIPY moiety in these conjugates is involved in non-specific hydrophobic interactions with the EGFR surface. Studies in human HEp2 cells over-expressing EGFR revealed low cytotoxicity for the BODIPY conjugates, and remarkable EGFR-targeting ability and cellular uptake, up to 90-fold that of unconjugated BODIPY **5**. The Golgi apparatus and cell lysosomes were the main sites of intracellular localization for the conjugates, whereas the unconjugated BODIPY localizes mainly in the ER and the Golgi apparatus. Our studies show that these BODIPY-peptide conjugates, in particular **8**, effectively target and bind to EGFR, and despite their relatively low fluorescence quantum yields, can be used for fluorescence imaging of cells over-expressing EGFR. Further investigations of EGFR-targeted molecular imaging agents with red-shifted emissions for increased penetration through tissues and reduced interference from auto-fluorescence, are currently being investigated in our laboratories.

EXPERIMENTAL SECTION

1. Synthesis

Commercially available chemicals were purchased from VWR or Sigma-Aldrich and used without further purification. Thin layer chromatography (TLC) was performed on the precoated silica gel plates (0.2 mm, 254 indicator, polyester backed, 60Å, Sorbent Technologies) to monitor the reactions. Preparative TLC plates (60G, VWR) and silica gel (60Å, 230–400 mesh, Sorbent Technologies) were used for liquid chromatography and

column chromatography. ^1H NMR and ^{13}C NMR spectra were collected on a Bruker AV-400 liquid, or AV-500 spectrometer at 300K. The chemical shifts (δ) are provided in parts per million (ppm) in CDCl_3 (7.27 ppm for ^1H NMR and 77.0 ppm for ^{13}C NMR) and DMSO (2.54 ppm for ^1H NMR). The coupling constants (J) are reported in Hz. High-resolution mass spectra (HRMS) and MALDI (TOF) spectra were obtained on a 6210 ESI-TOF Mass Spectrometer (Agilent Technologies) under negative mode and Applied Biosystems QSTAR at the LSU Mass Spectrometry Facility. Normal-phase HPLC was performed on a Dionex system (organics system) including a P680 pump with a UVD 340 detector and a fraction collector III. This system is connected to a Dynamax axial compression column in the packing of irregular silica gel (Rainin 60 Å). Reversed-phase HPLC analysis was performed with a Waters 2485 Quaternary Gradient Module, Waters Sample Injector, and 2489 UV/Visible detector which are controlled by Waters Empower 2 software. Separations were completed on an X-Bridge BEH300 Prep C18 (5 μm , 10 $\times$ 250 mm) with an X-Bridge BEH300 Prep Guard cartridge 300 Å (5 μm , 10 $\times$ 10 mm) at a 4 mL/min flow rate with UV detection for peptides at 220 nm, and for BODIPY conjugates at 580 nm. Fractions of HPLC purity (> 95%) with the anticipated mass were combined and lyophilized.

2,3,5,6,8-Pentachloro-BODIPY **1** was prepared by using our published procedure.⁷⁶

BODIPY 2—Into a 25 mL round-bottomed flask were added BODIPY **1** (36.4 mg, 0.1 mmol) and 3 mol % of $\text{Pd}(\text{PPh}_3)_4$. The flask was evacuated and refilled with nitrogen three times. Toluene (4 mL) and 2M Na_2CO_3 (aq) (0.5 mL) were added into mixture. (4-Methoxyphenyl)boronic acid (38 mg, 0.25 mmol) was then added portion-wise. The final mixture was stirred at 100 °C under nitrogen for 5 h. The reaction was stopped when the targeted di-coupled product was the major product according to TLC, and the mixture was poured into saturated NaHCO_3 (aq) (15 mL) and extracted with CH_2Cl_2 (10 mL $\times$ 3). The organic layers were combined, washed with brine (30 mL) and water (30 mL), and passed through anhydrous Na_2SO_4 . The organic solvents were removed under reduced pressure and the resulting residue was subjected to column chromatography or prep TLC using CH_2Cl_2 /hexanes (1:2) for elution, yielding the desired product **2** (22.8 mg, 45%). mp 210–212 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.74–7.76 (d, J = 8.7 Hz, 2H), 7.49–7.51 (d, J = 8.6 Hz, 2H), 7.04–7.09 (m, 4H), 6.97 (s, 1H), 6.81 (s, 1H), 3.93 (s, 3H), 3.90 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 162.3, 161.4, 156.5, 143.5, 139.2, 133.4, 132.3, 132.00(t), 130.9, 129.7, 126.3, 125.1, 123.8, 121.2, 120.1, 114.4, 113.7, 55.6, 55.3; ^{11}B NMR (128 MHz, CDCl_3) δ 0.270 (t, J = 28.8 Hz); HRMS (ESI-TOF) m/z calcd for $\text{C}_{23}\text{H}_{16}\text{BCl}_3\text{F}_2\text{N}_2\text{O}_2$ $[\text{M}]^-$ 505.0375; found 505.0392.

BODIPY 3—Into a 25 mL round-bottomed flask were added BODIPY **2** (20.3 mg, 0.04 mmol) and 3 mol % of $\text{Pd}(\text{PCy}_3)_2\text{G}_2$. The flask was evacuated and refilled with nitrogen three times. Toluene (4 mL) and 2M Na_2CO_3 (aq) (0.5 mL) were added into mixture. (4-Nitrophenyl)boronic acid (38 mg, 0.25 mmol) was then added portion-wise. The final mixture was stirred at 100 °C under nitrogen for 3 h. The reaction was stopped when the starting material disappeared according to TLC, and the mixture was poured into saturated NaHCO_3 (aq) (15 mL) and extracted with CH_2Cl_2 (10 mL $\times$ 3). The organic layers were

combined, washed with brine (30 mL) and water (30 mL), and passed through anhydrous Na₂SO₄. The organic solvents were removed under reduced pressure and the resulting residue was subjected to column chromatography or prep TLC using CH₂Cl₂/hexanes (1:1) for elution, yielding the desired product **3** (14.3 mg, 60%). mp 246–248 °C; ¹H NMR (500 MHz, CDCl₃) δ 8.27–8.29 (d, *J* = 8.4 Hz, 2H), 7.84–7.86 (d, *J* = 8.6 Hz, 2H), 7.66–7.67 (d, *J* = 8.7 Hz, 2H), 7.56–5.58 (d, *J* = 8.4 Hz, 2H), 7.10–7.12 (d, *J* = 8.4 Hz, 2H), 7.04 (s, 1H), 6.97–6.99 (d, *J* = 8.5 Hz, 1H), 6.91 (s, 1H), 3.95 (s, 1H), 3.85 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 162.3, 161.3, 157.0, 148.6, 148.1, 144.5, 136.2, 133.6, 132.9, 132.4, 131.9, 131.5, 130.0, 127.0, 125.6, 124.4, 123.1, 121.2, 120.9, 114.4, 113.6, 55.6, 55.3; ¹¹B NMR (128 MHz, CDCl₃) δ 0.507 (t, *J* = 30.4 Hz); HRMS (ESI-TOF) *m/z* calcd for C₂₉H₂₀BCl₂F₂N₃O₄ [M][−] 592.0934; found 505.0909.

BODIPY 4—BODIPY **3** (11.9 mg, 0.02 mmol) was dissolved in EtOH/THF (3 mL/0.5 mL). N₂ was purged into the mixture for 10 min. 10% Pd/C (26.6 mg, 1.25 eq) and hydrazine monohydrate (9.7 μL) were added. The mixture was stirred and refluxed under N₂ atmosphere for 2 h. The reaction was stopped when the starting material disappeared according to TLC. A celite cake was used to remove the catalyst, eluting with ethyl acetate. The residue was subjected to prep TLC using ethyl acetate/hexanes 1:1 for elution to provide the desired product **4** (10.9 mg, 92%). mp 227–229 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.64–7.67 (d, *J* = 8.7 Hz, 2H), 7.52–7.67 (dd, *J* = 10.7, 8.6 Hz, 4H), 7.07–7.09 (d, *J* = 8.7 Hz, 2H), 6.95–6.97 (d, *J* = 8.8 Hz, 2H), 6.91 (s, 1H), 6.87 (s, 1H), 6.69–6.70 (d, *J* = 8.6 Hz, 2H), 3.94 (s, 3H), 3.85 (s, 3H); ¹³C NMR (126 MHz, CDCl₃) δ 161.8, 160.7, 155.2, 153.1, 148.2, 142.6, 133.0, 132.6, 132.2, 131.9, 128.4, 127.3, 126.1, 122.9, 122.0, 119.1, 114.2, 114.1, 113.5, 55.6, 55.2; ¹¹B NMR (128 MHz, CDCl₃) δ 0.63 (t, *J* = 30.4 Hz); HRMS (ESI-TOF) *m/z* calcd for C₂₉H₂₂BCl₂F₂N₃O₂ [M][−] 562.1192; found 562.1175.

BODIPY 5—Into a 25 mL round-bottomed flask were added BODIPY **4** (10.2 mg, 0.018 mmol), 1,1'-thiocarbonyldi-2(1*H*)-pyridone (20.9 mg, 0.09 mmol), and dichloromethane (2 mL). The mixture was stirred at room temperature for 2 h. The reaction was stopped when the starting material was completely consumed, according to TLC. The organic solvent was removed, and the residue was subjected to prep TLC using ethyl acetate/hexanes 1:2 for elution to provide the desired product **5** (9.6 mg, 88%). mp 247–249 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.64–7.69 (m, 4H), 7.54–7.56 (d, *J* = 8.7 Hz, 2H), 7.27–7.29 (s, 2H), 7.09–7.11 (d, *J* = 8.7 Hz, 1H), 6.96–6.98 (m, 3H), 6.90 (s, 1H), 3.95 (s, 1H), 3.86 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 162.2, 161.1, 155.8, 151.0, 144.1, 136.7, 133.3, 132.8, 132.4, 132.3, 131.9, 131.7, 129.2, 128.6, 127.4, 125.8, 125.3, 123.6, 121.7, 121.3, 114.3, 113.6, 55.6, 55.2; ¹¹B NMR (128 MHz, CDCl₃) δ 0.518 (t, *J* = 30.3 Hz); HRMS (ESI-TOF) *m/z* calcd for C₃₀H₂₀BCl₂F₂N₃O₂S [M][−] 604.0756; found 604.0734.

Peptide Synthesis—The amide-terminated peptide sequences LARLLT and GYHWYGYTPQNVI were prepared on a 0.2 mmol scale using standard Fmoc strategy, and reacted with Fmoc-NH-PEG3-propionic acid to create the pegylated sequences: PEG3-LARLLT (**6**) and PEG3-GYHWYGYTPQNVI (**7**). A cleavage cocktail of TFA (94%), Millipore water (2.5%), liquefied phenol (2.5%) and Tris (1%) was added to the resin for 3 – 5 h to remove peptides from resin beads. The peptides were precipitated using cold (−80 °C)

anhydrous ethyl ether (3×45 mL) and centrifuged. The supernatant was decanted and the residue washed with cold ethyl ether. The solid was dissolved in a mixture of Millipore water (A) and acetonitrile (B), freeze-dried and lyophilized. The solvent system for purification of the peptides consisted of Millipore water and HPLC grade acetonitrile with 0.1% TFA.

Peptide 6 (3PEG-LARLLT)—This peptide was obtained as a white solid (67.53 mg, 38%). HPLC (90% A for 5 min, 30% A to 10% A over 13 min, 10% A to 90% A over 2 min at a flow rate of 4 mL/min) and $t_R = 13.95$ min. ^1H NMR (500 MHz, DMSO) δ 8.13 (d, $J = 7.4$ Hz, 2H), 8.00 (dd, $J = 19.6, 7.7$ Hz, 2H), 7.93 (d, $J = 8.1$ Hz, 1H), 7.78 (s, 2H), 7.57 (t, $J = 5.4$ Hz, 1H), 7.38 (d, $J = 8.5$ Hz, 1H), 7.05 (d, $J = 20.9$ Hz, 3H), 4.28 (m, 5H), 4.05 (m, 3H), 3.57 (m, 16H), 3.10 (m, 2H), 2.98 (dd, $J = 10.5, 5.2$ Hz, 2H), 1.64 (m, 4H), 1.49 (m, 10H), 1.20 (d, $J = 7.0$ Hz, 4H), 1.00 (d, $J = 6.2$ Hz, 3H), 0.87 (m, 18H). MS (MALDI-TOF): m/z calcd for $\text{C}_{40}\text{H}_{78}\text{N}_{11}\text{O}_{11}$ $[\text{M}]^+$ 888.588; found 888.542.

Peptide 7 (3PEG-GYHWYGYTPQNV)—This peptide was obtained as a white solid (108 mg, 39%). HPLC (90% A for 5 min, 30% A to 10% A over 13 minutes, 10% A to 90% A over 2 min at a flow rate of 4 mL/min) and $t_R = 12.00$ min. ^1H NMR (500 MHz, DMSO) δ 13.96 (s, 1H), 10.72 (s, 1H), 9.12 (s, 3H), 8.89 (s, 1H), 8.07 (m, 6H), 7.68 (m, 4H), 7.40 (s, 1H), 7.30 (d, $J = 7.9$ Hz, 1H), 7.20 (m, 3H), 7.01 (m, 9H), 6.75 (s, 1H), 6.61 (m, 5H), 4.73 (m, 8H), 4.42 (m, 5H), 4.16 (m, 3H), 4.08 (m, 2H), 3.91 (m, 3H), 3.70 (m, 4H), 3.58 (m, 16H), 2.91 (m, 5H), 2.65 (m, 3H), 2.37 (m, 2H), 2.06 (m, 7H), 1.90 (d, $J = 15.3$ Hz, 3H), 1.74 (m, 2H), 1.43 (m, 2H), 1.25 (s, 3H), 1.10 (m, 4H), 0.84 (m, 10H). MS (MALDI-TOF): m/z calcd for $\text{C}_{86}\text{H}_{119}\text{N}_{20}\text{O}_{23}$ $[\text{M}+\text{H}]^+$ 1799.876; found m/z 1799.972.

General procedure for peptide conjugation—Into a 4 mL vial were added the pegylated peptides (8.79 mg for **6** and 17.82 mg for **7**, 9.9 μmol) and triethylamine (10.3 μL , 74 μmol). The mixture was stirred at room temperature for 5 min. BODIPY **5** (3.00 mg, 4.95 μmol) was added into the vial, and the final mixture was stirred at room temperature for 30 min. TLC was used to monitor the reaction. H_2O (0.5 mL) was added to quench the reaction, and the solvent was removed using a lyophilizer. The residue was then subjected to HPLC ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$ as the eluents for conjugate **8**; $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{TFA}$ (0.1%) as the eluents for conjugate **9**) to provide the desired BODIPY-peptide conjugates **8** and **9**.

Conjugate 8 (BODIPY-3PEG-LARLLT-NH₂)—Yield: 5.91 mg, 80%. HPLC (50% A for 5 min, 10% A to 0% A over 13 minutes, 0% A to 50% A over 2 min at a flow rate of 4 mL/min) and $t_R = 11.00$ min. ^1H NMR (500 MHz, DMSO) δ 9.87 (s, 2H), 8.16 (s, 2H), 8.03 (s, 4H), 7.91 (d, $J = 8.1$ Hz, 1H), 7.72 (d, $J = 8.7$ Hz, 2H), 7.68 (d, $J = 8.6$ Hz, 2H), 7.51 (dd, $J = 14.9, 8.7$ Hz, 4H), 7.40 (d, $J = 7.8$ Hz, 2H), 7.19 (dd, $J = 23.7, 9.7$ Hz, 5H), 7.05 (m, 4H), 6.48 (s, 1H), 4.27 (m, 5H), 4.05 (m, 2H), 3.92 (s, 3H), 3.82 (s, 3H), 3.67 (s, 2H), 3.52 (m, 14H), 3.09 (d, $J = 6.0$ Hz, 2H), 1.58 (m, 4H), 1.48 (m, 8H), 1.21 (m, 7H), 1.00 (d, $J = 6.3$ Hz, 3H), 0.87 (m, 18H); ^{11}B NMR (128 MHz, DMSO) δ 0.368 (t, $J = 30.2$ Hz); MS (MALDI-TOF): m/z calcd for $\text{C}_{70}\text{H}_{98}\text{BCl}_2\text{F}_2\text{N}_{14}\text{O}_{11}\text{S}$ $[\text{M}]^+$ 1493.659; found 1493.726.

Conjugate 9 (BODIPY-3PEG-GYHWYGYTPQNVI-NH₂)—Yield: 5.95 mg, 50%.

HPLC (50% A for 5 min, 10% A to 0% A over 13 minutes, 0% A to 50% A over 2 min at a flow rate of 4 mL/min) and $t_R = 11.99$ min. ¹H NMR (500 MHz, DMSO) δ 13.99 (s, 1H), 10.74 (s, 1H), 9.89 (s, 2H), 9.15 (s, 3H), 8.90 (s, 1H), 8.09 (m, 12H), 7.70 (dd, $J = 26.4, 8.5$ Hz, 5H), 7.51 (dd, $J = 14.2, 8.7$ Hz, 4H), 7.43 (s, 1H), 7.22 (m, 6H), 7.03 (m, 8H), 6.79 (s, 1H), 6.62 (d, $J = 4.2$ Hz, 5H), 6.54 (s, 3H), 4.58 (dd, $J = 12.0, 5.6$ Hz, 4H), 4.38 (t, $J = 7.0$ Hz, 5H), 4.16 (m, 2H), 4.08 (t, $J = 7.8$ Hz, 2H), 3.92 (s, 4H), 3.81 (s, 3H), 3.59 (m, 16H), 3.13 (m, 3H), 2.90 (s, 4H), 2.67 (m, 3H), 2.37 (m, 2H), 1.99 (m, 11H), 1.45 (m, 3H), 1.16 (m, 14H), 0.83 (m, 9H); ¹¹B NMR (128 MHz, DMSO) δ 0.374 (t, $J = 30.2$ Hz); MS (MALDI-TOF): m/z . calcd for C₁₁₆H₁₃₉BCl₂F₂N₂₃O₂₅S⁺ [M+H]⁺ 2405.950; found 2406.018.

2. Spectroscopic Studies

A Varian Cary spectrophotometer and a Perkin Elmer LS55 spectrophotometer were used to measure the UV-Visible and emission spectra at room temperature. Quartz cuvettes (1 cm path length) were used for both measurements. The plots of integrated absorbance vs the corresponding solution concentrations were used to determine the extinction coefficients (ϵ) at the maximum absorption. Different dilute BODIPY solutions with absorbance values between 0.03–0.1 at $\lambda = 550$ nm or 580 nm were chosen and used for the determination of the relative fluorescence quantum yields. Crystal violet perchlorate (0.50 in ethanol) and methylene blue (0.03 in methanol) were used as the external standards.⁸³ Relative fluorescence quantum yields (Φ_f) were calculated using the following equation:⁹¹

$$\Phi_x = \Phi_{\text{std}} \times (\text{Grad}_x / \text{Grad}_{\text{std}}) \times (n_x^2 / n_{\text{std}}^2)$$

Where Φ refers to the fluorescence quantum yields; n represents the refractive indexes of the solvents used for the measurement; Grad refers to the gradient of integrated fluorescence intensity vs the corresponding absorbance; subscript x and std represent the tested samples and the external standards, respectively.

3. Crystallography

The crystal structure of BODIPY **5** was determined from an extremely thin plate using data collected at low temperature with MoK α radiation on a Bruker Kappa Apex-II diffractometer equipped with a Triumph curved monochromator. Refinement was by SHELX2014. Hydrogen atoms were placed in idealized positions and treated as riding. The sample crystal was a twin by two-fold rotation about the reciprocal 0 0 1 direction, and the two twin components were present in nearly equal populations. *Crystal data*:

C₃₀H₂₀BCl₂F₂N₃O₂S, MW = 606.26, triclinic, space group P-1, $a = 8.1751(14)$ Å, $b = 12.972(2)$ Å, $c = 13.740(3)$ Å, $\alpha = 102.792(13)^\circ$, $\beta = 104.575(12)^\circ$, $\gamma = 92.638(11)^\circ$, $V = 1367.2(4)$ Å³, $Z = 2$, $d = 1.473$ g cm⁻³, $T = 90$ K. The total number of reflections measured to $\theta_{\text{max}} = 23.4^\circ$ was 11,182, yielding 3879 independent data, of which only 1931 were observed with $F^2 > 2\sigma(F^2)$, $R_{\text{int}} = 0.130$, $R = 0.141$, $wR(F^2) = 0.389$ for all data and 373 refined parameters. ISOR restraints were necessary to prevent non-positive-definite ellipsoids for some C atoms. The total of 228 restraints was used. CCDC 1523511.

4. Surface Plasmon Resonance

SPR was performed using Biacore X100 from GE Health Sciences. Pure recombinant protein EGFR was obtained from Leinco Technologies (St. Louis, MO). HBS-EP+ buffer and 100 mM glycine at pH 4, 4.5, 5, 5.5 were purchased from GE Health Science. The extracellular domain of EGFR protein was immobilized on a CM5 sensor chip (GE Healthcare Biosciences) at a rate of 10 $\mu\text{L}/\text{min}$ using a traditional amine coupling procedure. The carboxyl groups on the CM5 chip were activated using a solution containing 0.2 M N-ethyl N-(dimethylaminopropyl) carbodiimide (EDC) and 0.05 M N-hydroxysuccinimide (NHS) (35 μL solution, with a flow rate of 5 $\mu\text{L}/\text{min}$). The running buffer for peptides was HBS-EP+ buffer diluted 10 times, containing 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20 at pH 7.5. Peptides dissolved in buffer were used as the analytes. The running buffer for the BODIPY conjugates was HBS-EP+ buffer diluted 10 times, also containing 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20 at pH 7.5, but brought to 4% DMSO concentration. BODIPY **5** and conjugates **8** and **9** were dissolved in 4% DMSO buffer solutions and used as the analytes. SPR sensorgrams were obtained and the association and dissociation rates were obtained from 0 to 250 μM concentrations.

5. Cell Studies

The human HEp2 cell line was purchased from ATCC, and maintained in a DMEM/AMEM (50:50) mixture supplemented with FBS (10%) and Penicillin Streptomycin (1%). All other commercially available reagents used in the cell studies were purchased from Life Technologies.

5.1 Dark Cytotoxicity—Stock solutions (32 mM) of BODIPYs **5**, **8**, and **9** were prepared using 100% DMSO as the solvent. Working solutions with concentrations of 0, 6.25, 12.5, 25, 50, and 100 μM were prepared from the stock solutions. The HEp2 cells were exposed to the different working solutions of BODIPYs **5**, **8** and **9** up to 100 μM and incubated overnight (5% CO_2 , 37 $^\circ\text{C}$). The loading medium was removed, and the cells were washed with PBS solution. The medium containing 20% CellTiter Blue (Promega) was added and the cells incubated for another 4 h. BMG FLUOstar Optima microplate reader was used to measure the dark cytotoxicity by reading the medium fluorescence at 570/615 nm. The fluorescence intensity of the untreated cells was normalized to 100%.

5.2 Phototoxicity—The HEp2 cells were exposed to the different working solutions of BODIPYs **5**, **8** and **9** up to 100 μM and incubated overnight (5% CO_2 , 37 $^\circ\text{C}$). The loading medium solution was removed, and the cells were washed with PBS solution. The fresh medium was added and the cells were then exposed to the light for 20 min. The light source has equipped with a halogen lamp (600 W), a water filter (transmits radiation 250–950 nm) and a beam turning mirror (200 nm to 30 μm spectral range, Newport). The total light dose was approximately 1.5 J/cm^2 . After exposing to light, the cells were then incubated for another 24 h, followed by the measurement of cell viability as described above.

5.3. Time-Dependent Cellular Uptake—The HEp2 cells were exposed to 10 μM concentration solutions of BODIPYs **5**, **8** and **9** for different periods of time (0, 1, 2, 4, 8, and 24 h). The loading medium solution was removed, and the cells were washed with PBS

solution. The cells were then solubilized by adding 0.25% Triton X-100 in PBS solution. BODIPY solutions with concentrations of 10, 5, 2.5, 1.25, 0.625, and 0.3125 μM were prepared by diluting 400 μM of each compound with 0.25% Triton X-100 (Sigma-Aldrich) in PBS and used to obtain the standard curves. The concentration of the compounds was determined using a BMG FLUOstar Optima microplate reader at 570/720 nm.

5.4. Microscopy—The human HEP2 cells were incubated in a 35 mm tissue culture dish (CELLTREAT) and allowed to grow overnight. The cells were exposed to each compound at a concentration of 10 μM and incubated for 6 h (5% CO_2 , 37 $^\circ\text{C}$). The following organelle trackers (Invitrogen) were added to the cells: ER Tracker Blue/White (100 nM), BODIPY FL C5 Ceramide (50 nM), MitoTracker Green (250 nM), LysoSensor Green (50 nM). The cells were incubated with each compound and trackers for half an hour, and washed with PBS solution three times before imaging. A Leica DM6B upright microscope equipped with a water immersion objective, and DAPI, GFP, and Texas Red filter cubes (Chroma Technologies) were used to acquire the images.

6. Modeling and docking

The 3D structures of conjugates **8** and **9** were built using InsightII (BIOVIA San Diego, CA) molecular modeling software. The BODIPY structure was obtained from the coordinates of the crystal structure of **5** described in this report. Peptides LARLLT and GYHWYGYTPQNVI were linked to BODIPY **5** via the triethylene glycol linker. The 3D structures generated were energy minimized using 100 steps of the steepest descent method, and then the conjugate gradient method in vacuum. The folded structures of the compounds were used for the docking studies.

For the EGFR receptor 3D structure, the pdb file 1nql⁹² that represents EGFR in a closed conformation was downloaded from the protein data bank, and water and other molecules were removed from the structure file. Conjugates **8** and **9** contain a boron atom in the BODIPY core and forcefield parameters were not defined in the Autodock default parameters file. Parameters for boron can be obtained from the literature,⁹³ however, for docking calculations the boron atom was replaced with a carbon atom, as boron atom is not calibrated in docking studies using Autodock. For conjugate **8** a grid was created around amino acids Glu71, Asn134, and Gly177 on the EGFR, and for conjugate **9** a grid was created near the EGF binding site, as reported in previous studies.^{20,25–26,93} Docking was performed using Autodock 4.⁹⁰ The lowest energy docked structure was represented using PyMol software (Schrodinger LLC, Portland, OR).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BODIPY	boron dipyrromethene
EGFR	epidermal growth factor receptors
CAD	computer-assisted design
CRC	colorectal cancer
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DSSC	dye sensitized solar cells
EDTA	ethylenediaminetetraacetic acid
EDC	N-ethyl N-(dimethylaminopropyl)
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FBS	fetal bovine serum
FDA	food and drug administration
HBSS	Hank's balanced salt solution
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid
HPLC	high performance liquid chromatography
MO	molecular orbital
NHS	N-hydroxysuccinimide
PBS	phosphate buffered saline
PBS	phosphate buffered saline
PDT	photodynamic therapy
SPR	surface plasmon resonance
TCCA	trichloroisocyanuric acid
TDP	thiocarbonyldi-2(1 <i>H</i>)-pyridone
TEA	trimethylamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
Tris	triisopropylsilane

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Figure 2.
X-ray structure of BODIPY 5.

Figure 3. Normalized UV-Vis and fluorescence emission spectra of BODIPYs **5** (yellow), BODIPY-peptide conjugate **8** (blue), and BODIPY-peptide conjugate **9** (red) in DMSO at room temperature.

Figure 4.

SPR sensograms for peptides **6** (a) and **7** (b), and conjugates **8** (c) and **9** (d) binding to EGFR, at concentrations up to 250 μM (burgundy); 200 μM (lime), 100 μM (yellow), 50 μM (orange), 25 μM (green), 10 μM (royal blue), 1 μM (navy blue), 0.5 μM (purple), and bank (mint).

Figure 5. Time-dependent cellular uptake of BODIPYs **5** (blue), **8** (green) and **9** (purple) at 10 μ M in human HEP2 cells.

Figure 6. Subcellular fluorescence of conjugate **8** in HEp2 cells at 10 μ M for 6 h. (a) Phase contrast; (b) overlay of **8** fluorescence and phase contrast; (c) ER tracker Blue/White fluorescence; (d) overlay of **8** fluorescence and ER Tracker; (e) BODIPY Ceramide; (f) overlay of **8** fluorescence and BODIPY ceramide; (g) MitoTracker Green fluorescence; (h) overlay of **8** fluorescence and MitoTracker Green; (i) LysoSensor Green fluorescence; (j) overlay of **8** fluorescence and LysoSensor Green. Scale bar: 10 μ m.

Figure 7. Subcellular fluorescence of conjugate **9** in HEp2 cells at 10 μ M for 6 h. (a) Phase contrast; (b) overlay of **9** fluorescence and phase contrast; (c) ER tracker Blue/White fluorescence; (d) overlay of **9** fluorescence and ER Tracker; (e) BODIPY Ceramide; (f) overlay of **9** fluorescence and BODIPY ceramide; (g) MitoTracker Green fluorescence; (h) overlay of **9** fluorescence and MitoTracker Green; (i) LysoSensor Green fluorescence; (j) overlay of **9** fluorescence and LysoSensor Green. Scale bar: 10 μ m.

Figure 8. Proposed model of interaction of EGFR with A) conjugate **8** and B) conjugate **9**. The BODIPY moiety is highlighted. The peptide amino acids are represented by a single letter code, and the protein amino acids are represented with a three letter code. The EGFR protein is shown in a surface representation.

Scheme 1.
Synthesis of isothiocyanato-functionalized **5**.

Scheme 2.

Synthesis of peptide conjugates BODIPY-L1 (8) and BODIPY-L2 (9).

Table 1

Spectroscopic properties of BODIPYs in DMSO at room temperature

BODIPY	λ_{abs}	λ_{em}	Stokes' shift	Φ_f/a	$\log \epsilon$
5	575 nm	623 nm	48 nm	0.16	4.5
8	588 nm	634 nm	46 nm	0.033	4.3
9	586 nm	634 nm	48 nm	0.014	4.5

$[\alpha]_{\text{Cresyl violet (0.5 in ethanol)}}^{83}$ was used as the standard for compounds **5**, **8**, and **9**.

Table 2

Cytotoxicity for BODIPYs **5**, **8** and **9** using the Cell Titer Blue assay in human HEP2 cells and their major/minor subcellular localization sites

BODIPY	Dark toxicity (IC ₅₀ , μ M)	Phototoxicity (IC ₅₀ , μ M)	major sites of subcellular localization	minor sites of subcellular localization
5	>200	> 100	ER, Golgi	mitochondria, lysosomes
8	98	74	Golgi, lysosomes	ER, mitochondria
9	180	> 100	Golgi, Lysosomes	ER