Bioorganic & Medicinal Chemistry Letters 22 (2012) 3632–3638

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A specific molecular beacon probe for the detection of human prostate cancer cells

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ARTICLE INFO

Article history:
Received 2 February 2012
Revised 3 April 2012
Accepted 10 April 2012
Available online 19 April 2012

Keywords:
Molecular beacon
Fluorescent probe
Fluorogenic
Chromogenic
Detection
Prostate cancer
Carboxylesterase activity
Biomarker
Porcine liver esterase

ABSTRACT

The small-molecule, water-soluble molecular beacon probe **1** is hydrolyzed by the lysate and living cells of human prostate cancer cell lines (LNCaP), resulting in strong green fluorescence. In contrast, probe **1** does not undergo significant hydrolysis in either the lysate or living cells of human nontumorigenic prostate cells (RWPE-1). These results, corroborated by UV–Vis spectroscopy and fluorescent microscopy, reveal that probe **1** is a sensitive and specific fluorogenic and chromogenic sensor for the detection of human prostate cancer cells among nontumorigenic prostate cells and that carboxylesterase activity is a specific biomarker for human prostate cancer cells.

Prostate cancer is the most common malignancy in American men and the second leading cause of cancer mortality.[1] There are no symptoms during this cancer's early stage; patients may die due to lack of early detection and treatment.[2] Therefore, there is an urgent need to develop effective chemical methods for the detection of prostate cancer in its early stages, potentially allowing us to control the cancer from spreading and, ultimately, cure the aggressive disease.[2]

At present, a chemical test measuring the level of prostate-specific antigen (PSA) in serum is the most effective method for the early detection of prostate cancer; its positive predictive value is, however, only 35%.[3] Therefore, the majority of patients will receive false-negative results, making them likely to lose the possibility of early detection, prevention, and treatment. Another method for the clinical diagnosis of human prostate cancer is through biopsy, which checks the patient's prostate tissues for cancerous cells.[4] The diagnosis and prognosis of prostate cancer might also be possible through analysis of the carboxylesterase activity in the cells; some reports have described increased carboxylesterase activity in the cells of other cancer types, such as human breast cancer,[5,6] relative to that in normal tissues. We are, however, unaware of any previous reports of increased carboxylesterase activity in human prostate cancer cells for the diagnosis and prognosis of prostate cancer cells.[7]

In a previous study, the carboxylesterase activity in breast cancer cells was determined through fluorescence monitoring, with fluorescein diacetate used as the substrate for the enzyme.[5] This activated aryl acetate is, however, labile (high automatic hydrolysis rate), making it unstable in water or buffer.[8] As a result, the fluorescence background was high and the signal to noise ratio was low.[5] Fluorescein diacetate also possesses limited water solubility.[8] One approach toward overcoming the limitations of that probe would be to develop a specific, water-soluble, ester-based fluorescent probe, such as an alkyl aliphatic acid ester, with a low rate of automatic hydrolysis.[8]

Recently, water-soluble 2-(2′-phosphoryloxyphenyl)-4-(3*H*)-quinazolinone ($Q_{2\text{-}p}$) derivatives have been applied to detect the activity of extra-cellular phosphatases around prostate cancer cells.[9] In addition, a couple of large molecules and fluorescent probes containing pyrenyl oligonucleotides have been employed in the detection of intra-cellular carboxylesterase activity in HeLa cells.[8] Notably, such pyrenyl probes have not been used for the detection of human

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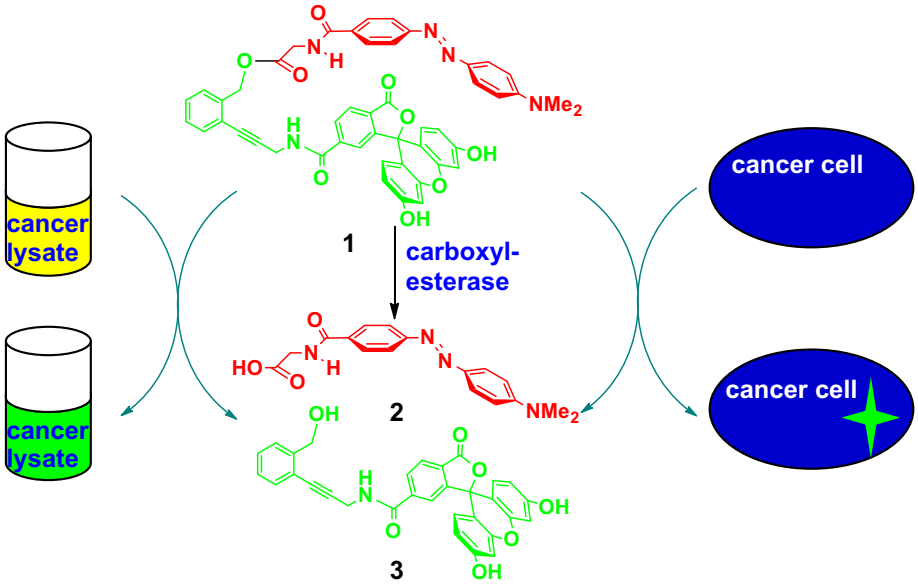
http://dx.doi.org/10.1016/j.bmcl.2012.04.055

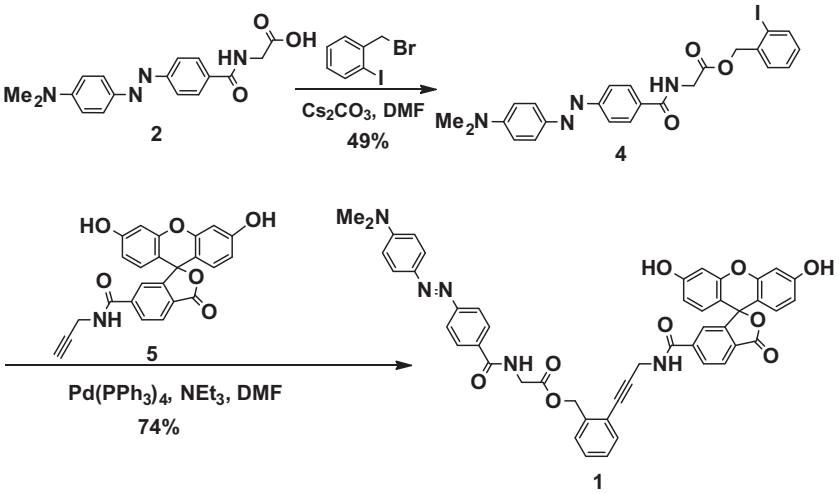


Scheme 1. Schematic representation of the operation of probe **1** for detection of human prostate cancer cells.

prostate cancer cells. Therefore, a small-molecule with better cell permeability and a probe with specific fluorescence appear necessary for monitoring the carboxylesterase activity in human prostate cancer cells.

We have designed the molecular beacon probe **1** as a substrate for monitoring carboxylesterase activity (Scheme 1). This molecule features one aliphatic ester group and two aromatic acid amide groups; only the ester group will be hydrolyzed readily by carboxylesterase.[10] Aryl acid amide groups are typically less-reactive substrates for most carboxylesterases, amidases and proteases.[11,12] In addition, we expected this probe to be resistant to hydrolyzes catalyzed by intra- and extra-cellular phosphatases, nucleases, and phosphodiesterases in prostate cancer cells due to probe **1** is non phosphate salt/ester. Furthermore, probe **1** incorporates a fluorophore—fluorescein, a water-soluble unit[13] at physiological pH (7.4)—and a typical fluorescence quencher (dabcyl group).[14] In addition, the structure features an ortho-substituted phenyl group that acts as a bridge to bring the fluorescein and dabcyl units together. This design makes it possible to ensure effective fluorescence quenching of the fluorescein moiety by the dabcyl group. Taken together, we expected these features to make probe **1** a specific substrate for carboxylesterases. After hydrolysis of **1** by a carboxylesterase through acyl substitution, *N*-dabcylglycine (**2**) will be expelled, causing the fluorescein unit to become fluorescent (i.e., no longer quenched). We synthesized the molecular beacon probe **1** from the dabcyl derivative **2** through Williamson ether synthesis[15] and palladium-catalyzed Sonogashira coupling[16,17] with compound **5** (Schemes 2 and S1–S3). We also prepared compounds **3** and **6** for NMR spectroscopic studies (Fig. 1, Schemes S2 and S4).

To demonstrate whether the designed and synthesized **1** has a hairpin like structure and whether it is a new molecular beacon probe, and to determine whether fluorescence quenching was possible between the fluorescein and dabcyl units in **1**, we investigated their intramolecular hydrogen bonding and π–π stacking interactions using ^{1}H NMR spectroscopy. We monitored the chemical shifts of the protons of the probe **1**, its synthetic precursor **6**, and its related compound **3** under the same conditions (in acetone-d_6, at a concentration of 38.0 mM, and at a temperature



Scheme 2. Synthesis of **1**.

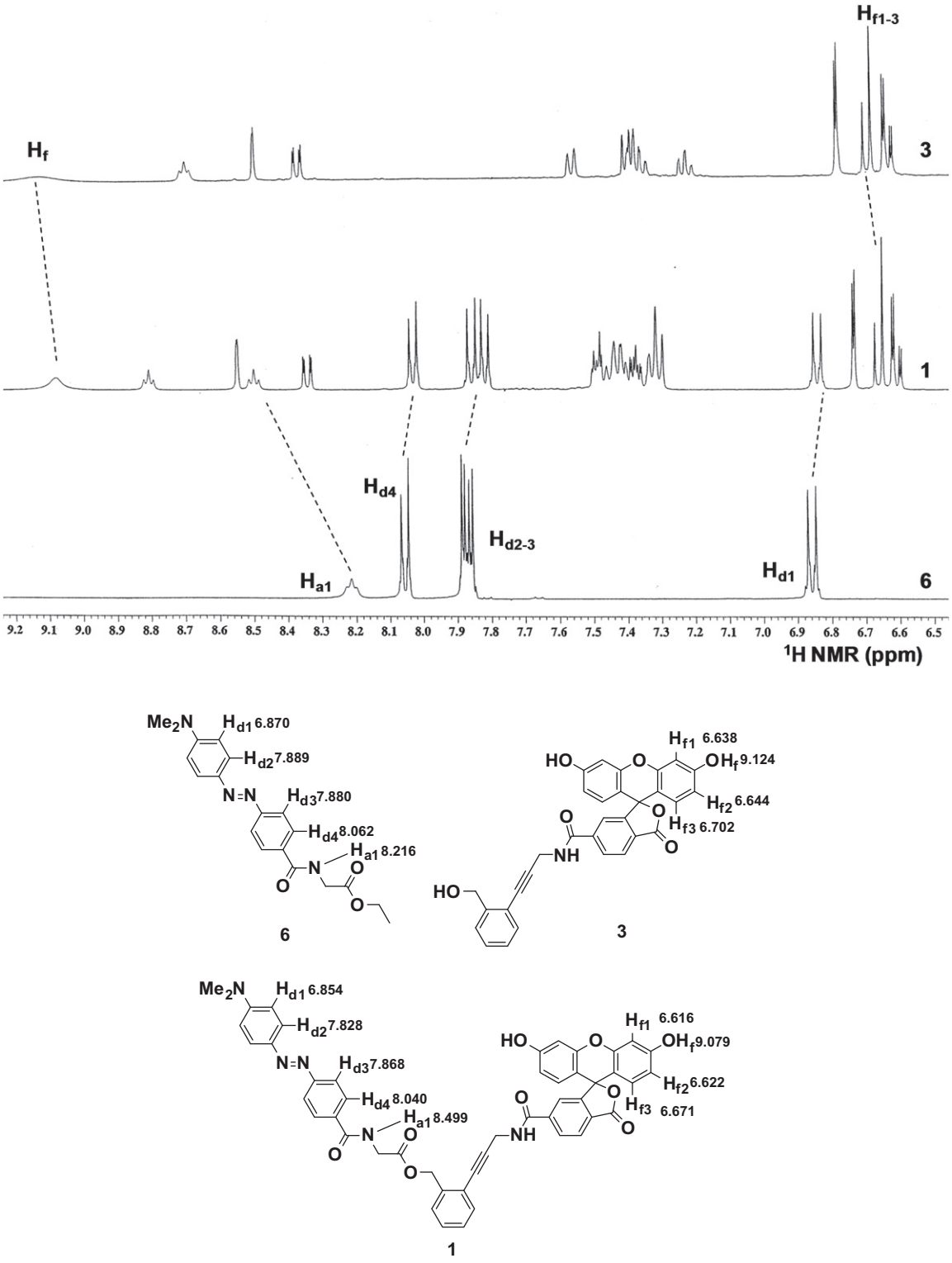


Figure 1. Partial ^{1}H NMR spectra of compounds **1**, **3**, and **6** at 38.0 mM in acetone-d_6, with assignments of the chemical shifts (ppm) of selected protons of OH, NH, and aromatic groups.

of 25 °C (Fig. 1). Relative to its position in the NMR spectrum of **6**, the signal for the amide proton H_{al} of **1** had shifted downfield by 0.283 ppm, suggesting that this hydrogen atom was involved in significant intramolecular hydrogen bonding (Fig. 2A). Furthermore, the signals of the OH proton and aromatic protons $H_{dl\text{-}4}$ and $H_{f1\text{-}3}$ of **1** were located upfield—by 0.040, 0.016, 0.061, 0.012, 0.022, 0.022, 0.022, and 0.031 ppm, respectively—relative to the corresponding protons of **6** and **3**, indicating their π–π stacking interactions occurred between the fluorescein and dabcyl groups.[18,19]

To investigate the intramolecular interactions suggested by the NMR spectra further, we performed a structural calculation for **1** at

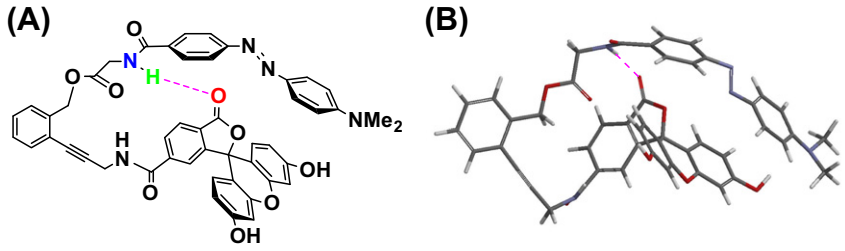


Figure 2. (A) Chemical structure of the intramolecular hydrogen bond N^G-H···O^F (G, glycine; F, 6-carboxamide fluorescein). (B) Calculated structure of probe **1**. Dotted line: N^G-H···O^F hydrogen bond.

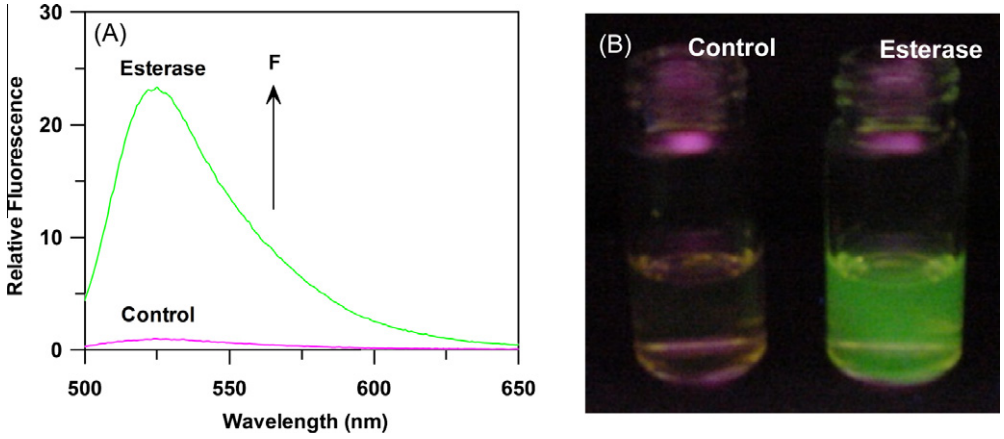


Figure 3. (A) Fluorescence spectra of probe **1** (10.0 μM) after 5 h at room temperature in 0.10 M tris–maleate buffer at pH 8 in the presence (green) and absence (purple) of porcine liver carboxylesterase (PLC, 1.10 μM). (B) Green fluorescence (right) generated from the probe **1** upon hydrolysis with PLC. In the control reaction (left), no or little green fluorescence was observed. The picture was taken under irradiation with UV light (350 nm). All the fluorescence measurements were recorded on a fluorometer by an excitation at 490 nm.

the RM1 level. Figure 2B reveals the presence of an N^G-H···O^F intramolecular hydrogen bond having a hydrogen bond distance of 2.159 Å and angle of 166.4°. These results are consistent with significant hydrogen bonding occurring within the molecule.[18,19] The intramolecular hydrogen bond of probe **1** would promote the formation of a hairpin-like structure, bringing the xanthenyl group of the fluorescein moiety toward the dabcyl group to ensure effective π–π stacking interactions (Fig. 2B).

To test whether probe **1** would be an effective sensor for carboxylesterase activity, we applied it as a substrate for hydrolysis catalyzed by porcine liver carboxylesterase (PLC) at room temperature in 0.10 M tris–maleate buffer at pH 8.0. For the control experiment, we repeated this reaction, but with the absence of PLC. After 5 h, we measured the fluorescence intensity at 525 nm for each sample upon excitation at 490 nm. The spectra revealed a 23-fold increase in fluorescence for the sample in the presence of the esterase, indicating that **1** was sensitive probe for carboxylesterase activity (Fig. 3). The hydrolysis produced compounds **2** and **3**, as detected by mass spectrometry, indicating that the reaction was the hydrolysis of the ester group in probe **1** (Figs. S1 and S2, Scheme 1).

Next, we used probe **1** to detect carboxylesterase activity in the total cellular lysate of human prostate cancer cells under the same conditions as those described above for porcine liver carboxylesterase. For comparison, we also determined the carboxylesterase activity of the total cellular lysate of human nontumorigenic prostate cells and performed a control experiment without any lysates. After 26 h, the fluorescence intensity at 525 nm of the sample from the reaction catalyzed by the lysate of human prostate cancer cells was 3.8-fold greater than that catalyzed by the lysate of nontumorigenic prostate cells (Fig. 4A and B). After subtraction of the fluorescence intensity from the control, the fluorescence intensity of the sample from the reaction catalyzed by the lysate of human prostate cancer cells was 30.8-fold greater than that catalyzed by the lysate of human nontumorigenic prostate cells (Fig. 4C), indicating that probe **1** is a selective and specific sensor for the detection of carboxylesterase activity in the lysate of human prostate cancer cells and that carboxylesterase activity is a reliable biomarker for human prostate cancer. Interestingly, the carboxylesterase activity of the lysate from human prostate cancer cells lasted for a long time (up to 10 days at room temperature), suggesting that **1** can detect the carboxylesterase activity of samples after long periods of storage (Fig 4D), making it a potentially more useful probe.

We also used UV spectroscopy to monitor the hydrolysis of probe **1** in the presence of the lysates of human prostate cancer and nontumorigenic prostate cells (Fig. 5). In the presence of the lysates of human prostate cancer and nontumorigenic prostate cells and in their absence, the wavelengths of maximum absorbance for the solutions were 494, 497, and 497 nm, respectively, indicating that the hydrolysis catalyzed by the cancer cell lysate resulted in a blue shift of the UV absorbance by 3 nm (Fig. 5A). The UV absorbance for a mixture of **2** and **3** also appeared at 494 nm (Fig. 5B), the same wavelength as that for the hydrolysis sample catalyzed by the lysate of human prostate cancer cells, suggesting that the hydrolysis of probe **1** by the lysate of the cancer cells led to the production of compounds **2** and **3** (Scheme 1). In addition, the relative intensities at 494 nm in the spectra recorded in the presence of the lysates of human prostate cancer and nontumorigenic prostate cells and in their absence were 1.29, 1.10, and 1.00, respectively. The increase of absorbance of the sample catalyzed by the lysate of human prostate cancer cells was 29%, which is 2.9-fold of the increase of absorbance (10%) of the sample

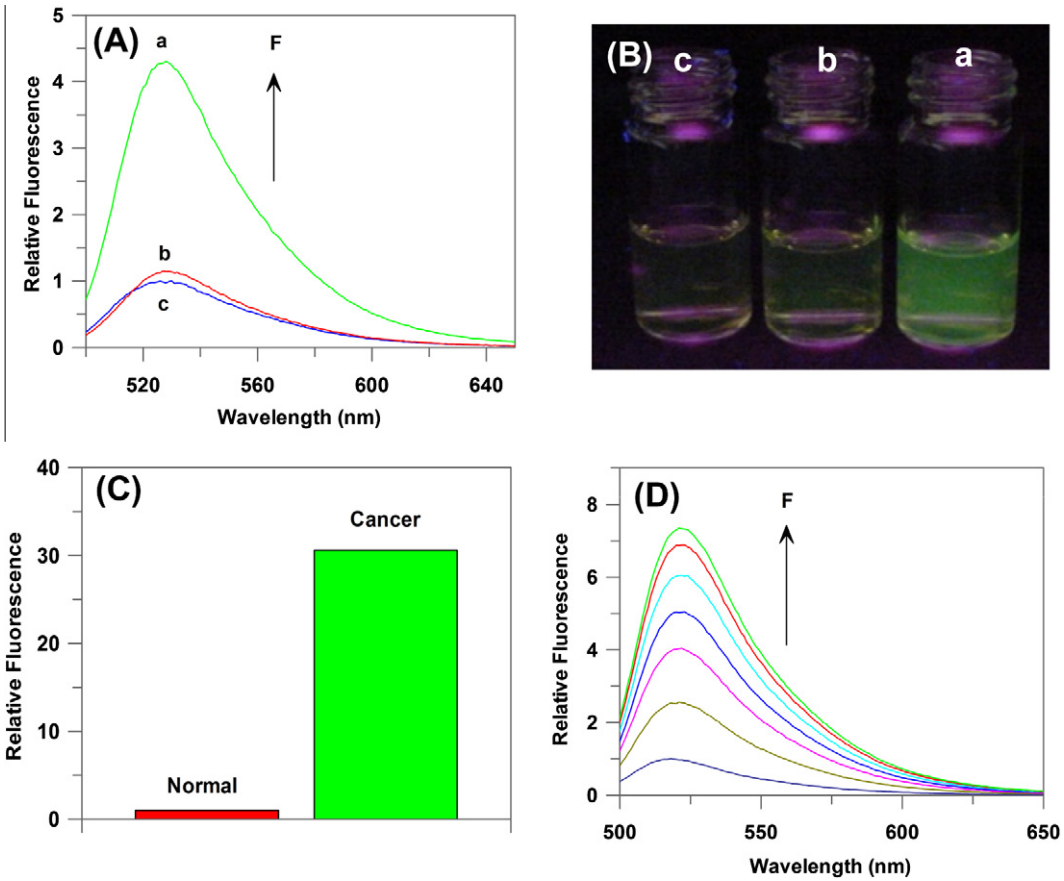


Figure 4. (A) Relative fluorescence intensities upon hydrolysis of probe **1** (10.0 μM) for 26 h at room temperature in 0.10 M tris–maleate buffer at pH 8.0 in the presence of the total cellular lysates (0.60 μg/μL) of (a) prostate cancer cells (LNCaP) and (b) nontumorigenic prostate cells (RWPE-1) and (c) in the absence of cell lysates. (B) (a) Green fluorescence generated from probe **1** upon hydrolysis with the lysate of prostate cancer cells for 26 h. (b) Weak fluorescence observed upon hydrolysis with the lysate of nontumorigenic prostate cells (0.60 μg/μL). (c) In the control reaction, no or little green fluorescence was observed. The picture was taken under irradiation with UV light (350 nm). (C) Relative increase in fluorescence intensity at 525 nm upon hydrolysis of probe **1** catalyzed by the lysates of nontumorigenic prostate and prostate cancer cells for 26 h. (D) Incremental increase in fluorescence at 525 nm upon hydrolysis of probe **1** (10.0 μM) for 0 (indigo), 1 (dark yellow), 2 (purple), 4 (blue), 6 (turquoise), 8 (red), and 10 (green) days, catalyzed by the lysate of human prostate cancer cells (0.60 μg/μL) at room temperature in 0.10 M tris–maleate buffer at pH 8.0. All the fluorescence measurements were recorded on a fluorometer by an excitation at 490 nm.

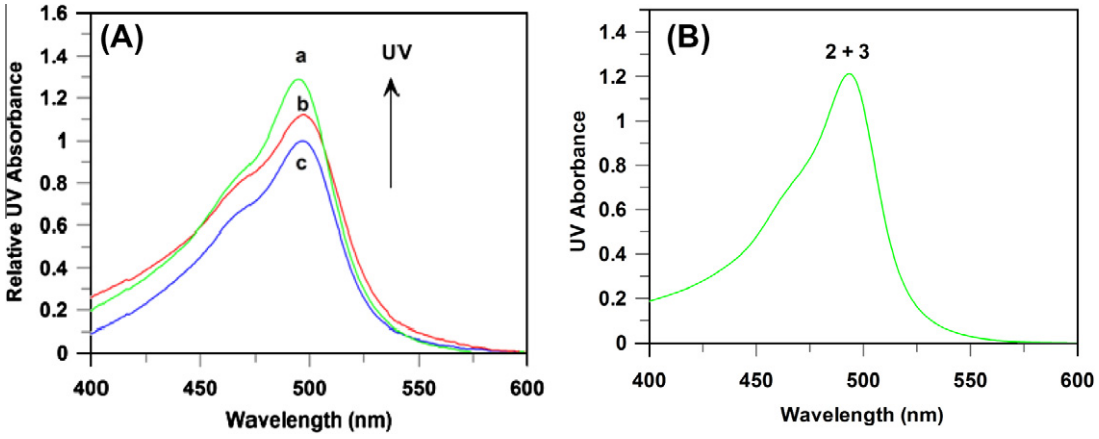


Figure 5. (A) Relative UV absorbance intensities upon hydrolysis of probe **1** (10 μM) for 43 h at room temperature in 0.10 M tris–maleate buffer at pH 8.0 in the presence of the lysates of (a) human prostate cancer cells and (b) nontumorigenic prostate cells and (c) in the absence of any lysates. (B) UV spectrum of a mixture of **2** (10 μM) and **3** (10 μM) in the same buffer.

catalyzed by the lysate of nontumorigenic prostate cells, suggesting that increased UV absorbance occurred significantly upon the cleavage of probe **1** by the carboxylesterase in the lysate of human prostate cancer cells (Fig. 5A). These results are consistent with significant hydrolysis of probe **1** occurring in the presence of the lysate of human prostate cancer cells; the hydrolysis was relatively insignificant when catalyzed by the lysate of human nontumorigenic prostate cells. Thus, the carboxylesterase activity is higher

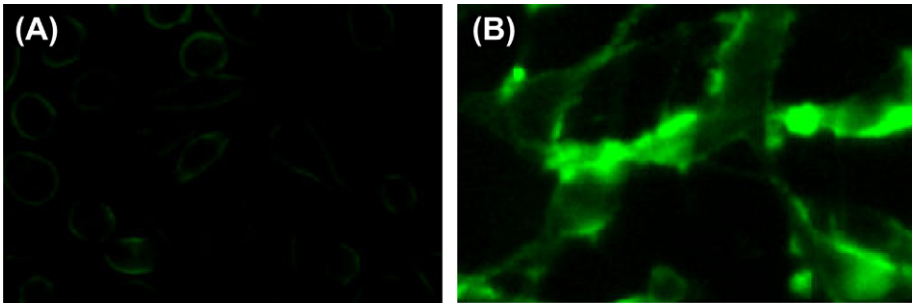


Figure 6. Microscopy images of live human prostate (A) nontumorigenic prostate cells (RWPE-1) and (B) prostate cancer cells (LNCaP) after treatment with probe **1** (10.0 μM) for 30 min in PBS buffer. The fluorescence image was obtained by excitation with 490 nm and detection from 500–560 nm with 200× magnification.

in the lysate of human prostate cancer cells than it is in that of nontumorigenic prostate cells. This experiment confirms that carboxylesterase activity is a biomarker for human prostate cancer cells and that **1** is an effective probe for the detection of the cancer cells.

To determine the effectiveness of probe **1** for the detection of live human prostate cancer cells, we performed experiments using LNCaP cancer cells and RWPE-1 nontumorigenic prostate cells with fluorescent microscopy (Fig. 6). No green fluorescence appeared within the human prostate nontumorigenic prostate cells (Fig. 6A), but green fluorescence appeared within human prostate cancer cells (Fig. 6B), suggesting that **1** is a sensitive probe for the detection of human prostate cancer cells. Furthermore, the green fluorescence appears clear and bright in the image 6B, indicating that **1** is an effective probe for highlighting human prostate cancer cells. The treatment time was only 30 min, consistent with the high reactivity of probe **1** during analysis. Furthermore, the green fluorescence was present within the human prostate cancer cells, suggesting that probe **1** is cell-permeable to human prostate cancer cells. Therefore, probe **1** is specific for the detection of carboxylesterase activity within human prostate cancer cells.

Prostate cancer is one of the leading causes of death in American men; early detection of this deadly disease is necessary for effective treatment. We suggest that unusual carboxylesterase activity in human prostate cancer cells should be listed as another important biomarker, in addition to PSA, for this particular disease. The synthesis of probe **1** is crucial for the measurement of the increase in carboxylesterase activity in human prostate cancer cells. This probe aids the identification of a human prostate cancer biomarker. It is also likely that probe **1** would find applications in clinical trials for the identification of human prostate cancer cells, because human prostate cancer is not diagnosed by a single test, but rather through many tests (including testing for PSA and biopsy). During biopsies, the probe should be useful for testing the carboxylesterase activity, thereby judging the progression and severity of the human prostate cancer. Probe **1** might also be useful for human prostate cancer prognosis, monitoring either the deterioration of the cancer or the patient's recovery. In addition, it is possible that probe **1** might also be used for the detection of other human cancers, including breast cancer. Therefore, further evaluation and application of probe **1** has the potential to improve human health.

This Letter presents the first small-molecule molecular beacon that is useful as a probe for detecting human cancer cells. To reduce the fluorescence background during analysis, we suspect that the quenching dabcyl unit could be substituted by a BHQ-1 unit, which has its maximum UV absorbance near 521 nm and quenches most of the fluorescence of fluorescein at 525 nm; this new quencher might also provide superior quenching efficiency.[20–23] Furthermore, the hydrolysis of the molecular beacon probe **1** results in impressive green fluorescence, a desirable property for biological studies of human prostate cancer cells in vitro and in vivo.[24] Due to complexities associated with the metastasis of cancer and the possibility that blood might also be involved, other fluorescence wavelengths might also be useful.[25] For this purpose, we propose that tetramethylrhodamine (TMR)[26–28] and naphthofluorescein[29,30] units be used instead of the fluorescein unit in probe **1**; these two fluorophores provide bright yellow and deep-red fluorescence, respectively. Indeed, the synthesis of probe **1** is the first step in the development of a series of sensors for carboxylesterase activity in human prostate cancer and other cancers.

In summary, we have synthesized and evaluated a sensitive and specific probe for the detection of human prostate cancer cells among nontumorigenic prostate cells through the analysis of carboxylesterase activity in cell lysates and living cells. We have also determined that carboxylesterase activity is a specific biomarker for human prostate cancer cells. The probe **1** is the first small-molecule, water-soluble molecular beacon for the detection of human prostate cancer cells.

Acknowledgments

This research is supported by the Office of Research and Sponsored Programs (RD09010, RD 0E82016 and RS0026 for Y.L.J. and RD583 for W.L.S.) and SFRA Grants for V.P. and Y.L.J. from the Honors College at East Tennessee State University. This work was also supported in part by NIH grant NIDA020120 to D.Y. We acknowledge funds from the Dishner Chair of Excellence Endowment Funds (KK).

Supplementary data

Supplementary data (^{1}H and ^{13}C NMR spectra of new compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.04.055.

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