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Near infrared active heptacyanine dyes with unique cancer-imaging and cytotoxic properties

Maged Henary [a,*], Vaishali Pannu [b], Eric A. Owens [a], Ritu Aneja [b,*]

[a] Georgia State University, Department of Chemistry, Petit Science Center, 100 Piedmont Ave., Atlanta, GA 30303, USA
[b] Georgia State University, Department of Biology, Petit Science Center, 100 Piedmont Ave., Atlanta, GA 30303, USA

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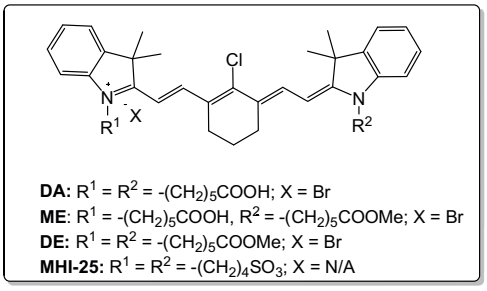
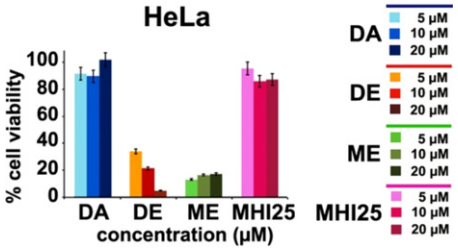
ABSTRACT

Three near-infrared fluorescent heptacarbocyanine dyes have been synthesized using a facile one-pot synthetic approach. The reaction methodology afforded a mixture of three symmetric and unsymmetric heptacyanines containing various *N*-indolenine substituents, a dicarbocyclic acid (DA), a monoester (ME), and a diester (DE). These compounds were isolated, purified, characterized and biologically investigated for tumor cell cytotoxicity and uptake selectivity. Using cell viability and in vitro proliferation assays, we found that the esterified dyes (monoester, ME and diester, DE) were selectively cytotoxic to cancer cells and spared normal fibroblast cells. Additionally, confocal fluorescence imaging confirmed selective uptake of these dyes in cancer cells, thus suggesting tumor cell targeting.





Cyanine dyes display distinguishable characteristics and possess two nitrogen-containing heterocyclic groups connected by an electron deficient conjugated methine chain, as shown by their general structure in Figure 1.

Essentially, cyanine nomenclature directly corresponds to the number of methine groups located in the bridge connecting the two heterocycles. Dyes containing $n = 0$, 1, 2, and 3, are classified as mono-, tri-, penta-, and heptacarbocyanines, respectively.[1] Electron delocalization provides cyanine dyes with their characteristically wide range of absorption spectra in the visible and infrared regions; additionally, they also exhibit narrow absorption bands and high extinction coefficients.[2] These properties combined with low tissue auto-fluorescence, the ability to deeply penetrate tissue, and an imaging window of 650–1000 nm have made cyanine dyes promising for use in cancer-imaging in vivo.[3] Fluorescence imaging

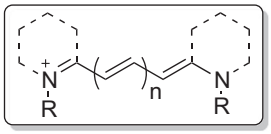


Figure 1. General structure of cyanine dyes.

* Corresponding authors. Tel.: +1 404 413 5566; fax: +1 404 413 5505.
E-mail address: mhenary1@gsu.edu (M. Henary).

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techniques with near-infrared dyes generally display excellent sensitivity and selectivity. An increase in the fluorophores' rigidity upon binding directly induces a significant increase in fluorescence. Commonly, near-infrared emission agents are conjugated with ligands to target specific tumors and to be used in optical imaging applications.

Optical imaging is becoming increasingly recognizable as an imaging technique that produces high-resolution imaging of fluorophores in cancerous tissue.[4] Employing near-infrared (NIR) dyes as contrast agents in optical imaging is becoming popular.[5] This non-radiative technique[4] is safer than current radiological techniques. In addition, this imaging method is favorable due to its low tissue absorption and minimal auto-fluorescence of NIR light.[6] Conversely, NIR fluorescence may be able to provide a fast, inexpensive screening for breast cancer as well as other cancers.[4–7] Although indocyanine green (ICG)[8] has been clinically approved for testing in fluorescence angiography and ophthalmology and has been shown to be a potent contrast agent for the detection of tumors in animal models and humans, the application of cyanine dyes in cancer diagnosis and detection is yet to be fully explored.[9,10] The long range goals are to synthesize cyanine dyes that will natively target specific tumors without requiring molecular conjugation.[3,11]

Two heptamethine cyanine dyes MHI-148 and MHI-25 (IR-783; Fig. 2) containing a rigid cyclohexenyl ring within the methine bridge were synthesized in our laboratory, and were shown to selectively target cancer cells.[11] We had reported cancer detection properties of these dyes that were found to accumulate specifically and selectively in cancer cells while sparing normal cells.[11]

Recently, our laboratory discovered that MHI-148 is a mixture of three analogous compounds. The three dyes were carefully isolated using flash chromatography and a gradient elution of dichloromethane/methanol. The structure of each dye was identified, and they were characterized as containing various *N*-substituted functionalities including a monoester, a diester, and a diacid. Subsequent testing concluded that the monoester and the diester specifically target and induce cellular death of human cervical (HeLa) and prostate (PC-3) cancer cells. However, our trypan blue exclusion data show that the diacid derivative and MHI-25 were found to have no cytotoxic activity in these cells. In light of these interesting data, further in vitro and preclinical research is warranted to evaluate the antiproliferative and proapoptotic activity of similar dyes with modified esters in order to evaluate their potential usefulness as anti-cancer agents.

In this Letter, we describe a simple methodology for synthesizing symmetrical and unsymmetrical heptamethine cyanine dyes as potential precursors for developing functionalized near-infrared labels for proteins and peptides. We discovered that MHI-148 contains three separate dyes. The dyes **4**, **5**, and **6** were isolated from the previously tested MHI-148 sample.[11] They were identified and characterized as the diester (**4**, DE), monoester (**5**, ME) and diacid (**6**, DA) as depicted in Scheme 1. The cell viability and anti-proliferative activity of these dyes were evaluated and compared with MHI-25 using the trypan blue and MTT assays, respectively (Figs. 3 and 4).[12–15] As shown in Scheme 1, the indolenine **1** was reacted with 6-bromohexanoic acid in boiling acetonitrile for 18 h under a nitrogen atmosphere to afford quaternary salt **2**. Compound **2** was allowed to react with Vilsmeier–Haack reagent[16] in acetic anhydride for 3 h in the presence of sodium acetate. Subsequently, when the reaction mixture was quenched with methanol, it furnished a mixture of three heptamethine cyanine dyes **4**, **5**, and **6** which were isolated by column chromatography.[17] It should be noted that the reaction of salt **2** with Vilsmeier–Haack reagent in boiling ethanol under basic conditions yielded only dye **6**.

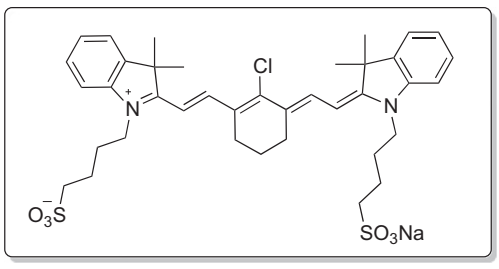
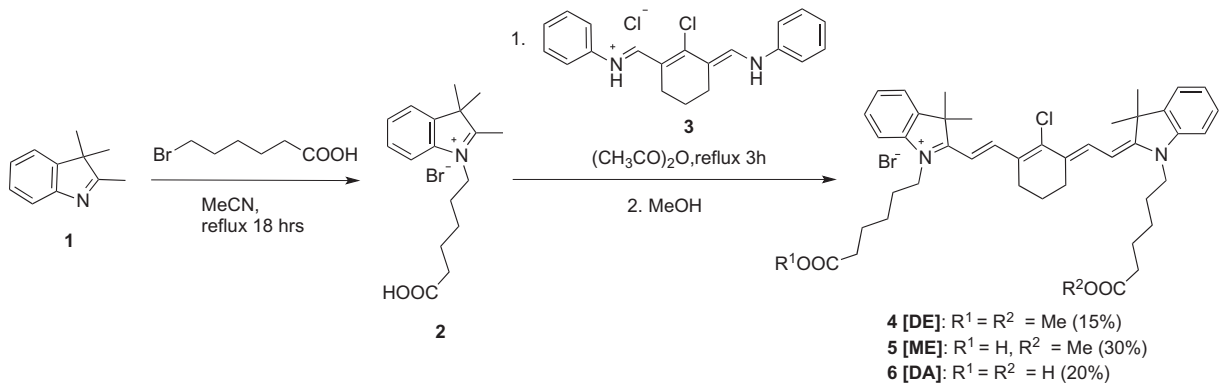


Figure 2. Structure of MHI-25 (IR-783).

We have previously reported the unique tumor imaging and targeting properties of the heptamethine cyanine dyes MHI-25 and MHI-148. It has been shown that these NIR dyes are actively taken up by and accumulate within cancer cells but not normal cells.[11] They were found to be superior for cancer detection compared to other cyanine dyes, such as indocyanine green[7] and noncyanine dyes, such as rhodamine 123. This was particularly significant because imaging with NIR dyes can yield much higher signal/noise ratios with minimal interfering background fluorescence.

Since MHI-148 was found to be a mixture, we optimized the purification process that yielded the diacid (DA), diester (DE), monoester (ME).[17] Although these heptamethine dyes show selective tumor targeting attributes, some of them have been reported to be cytotoxic with specificity to cancer cells without affecting normal cells. Thus, we first asked if these dyes killed cancer cells while sparing normal cells. We employed two representative cancer cell lines viz., HeLa (cervical) and prostate (PC-3).[18] Primary human dermal fibroblast (HDF) cells were used as non-cancerous normal cells. Our trypan blue exclusion data demonstrated that the esterified dyes (both monoester, ME and diester, DE) were cytotoxic to cancer cells but spared normal cells (Fig. 3).[13,14] The DE at concentrations ranging from 5-20 μM killed >90% of Hela and PC-3 cells at 24 h whereas HDF cells were unaffected at the same dose levels. This demonstrated remarkably significant selectivity of DE for cancer cells while normal cells showed 100% cell viability. Along similar lines, ME was cytotoxic to cancer cells, with HeLa cells showing enhanced sensitivity compared to PC-3 at the concentrations studied. ME treatment however compromised cell viability of HDFs. Lower concentrations (5–10 μM) resulted in 10–15% cell death, whereas 20 μM affected viability of ~80% HDF cells, indicating cytotoxicity at this concentration. On the other hand, ME at 5 and 10 μM concentrations displayed significant tumor cell selectivity with only ~20% cell survival in HeLa and PC-3 cells. This was in stark contrast to HDF cells which were ~85% viable at the same concentration. The diacid (DA) and the disulphonate MHI-25, did not exert any cytotoxic effects in cancer or normal cells under the concentration conditions used in our experiments.

We next performed an in vitro cellular proliferation assay to determine the IC_{50} of these dye forms in cancer (HeLa and PC-3) and normal (HDF) cells as shown in Figure 4.[15] Essentially, the IC_{50} value represents the concentration at which 50% of cell proliferation is inhibited. Our MTT data show that the IC_{50} of DE and ME were 0.8 and 1.0 μM, respectively, in HeLa cells (Fig. 4). The IC_{50} values for DE and ME were higher in PC-3 cells (3 and 2 μM, respectively) indicating lower sensitivity of prostate cancer cells compared to cervical cancer cells (Fig. 4). The IC_{50} of DE in normal HDF was 10 μM, indicating a wide 'therapeutic window' (Fig. 4).[15] However, the therapeutic window for ME was much narrower compared to DE. Since DA and MHI-25 did not affect the proliferation of cancer or normal cells, we were unable to deduce the IC_{50} of these two dye forms over a wide range of concentration in the cell lines included in our study (data not shown).



Scheme 1. Synthetic route for the one pot preparation of symmetrical and unsymmetrical heptacyanine dyes **4**–**6**.[17]

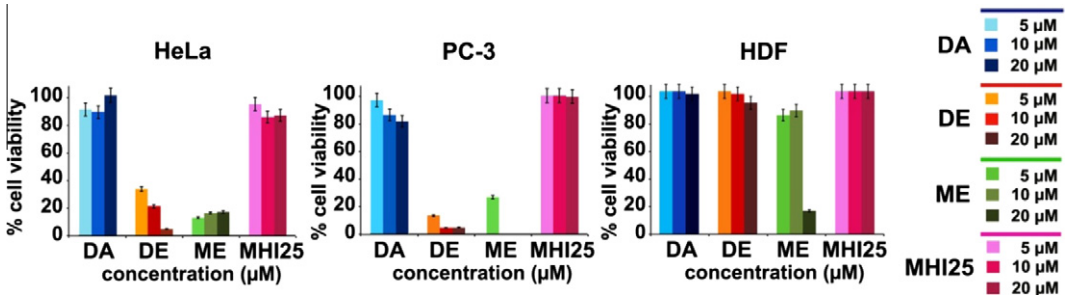


Figure 3. HeLa, PC-3 and HDF cells were treated with three different dose-levels (5, 10 and 20 μM) each of DA, DE, ME and MHI-25 for 24 h. The percentage of dead cells upon various treatments was measured using trypan blue staining. The corresponding bar-graphs show percentage cell viability determined by cell counting upon each treatment. The values and error bars shown in all the graphs represent average and standard deviations, respectively, of three independent experiments (p <0.05). Note: Nearly 100% of cell death was observed with 10 and 20 μM ME in PC-3 cells. Thus, the bars are not visible for percent cell viability at indicated concentrations of ME.[13,14]

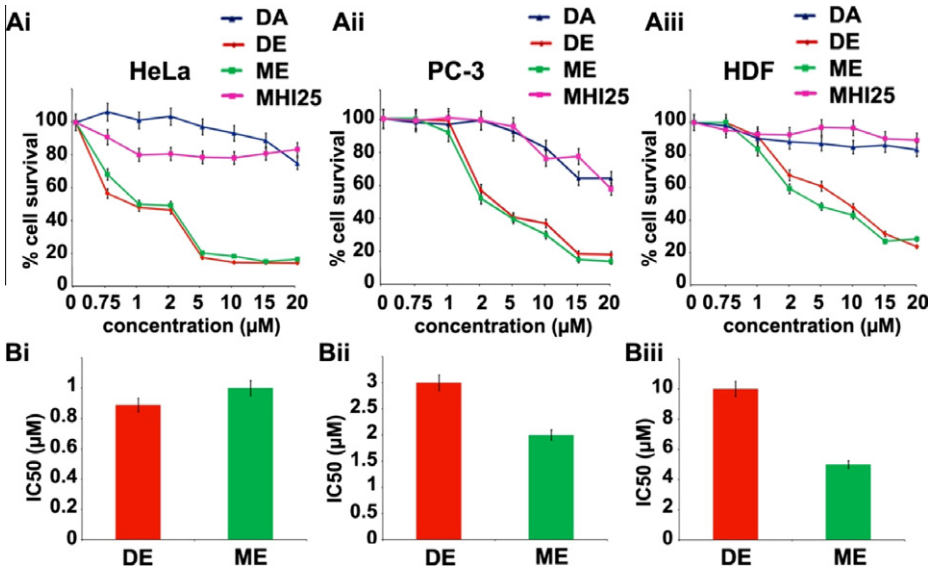


Figure 4. HeLa, PC-3 and HDF cells were treated for 48 h with increasing concentrations (0, 0.75, 1, 2, 5, 10, 15, 20 μM) of DA, DE, ME, MHI25 and cell proliferation was measured using MTT assay. A (i, ii, iii) Line-graph depicting percentage cell survival versus concentration representing the sensitivity profile of corresponding cell lines to each dye. B (i, ii, iii) Bar-graphical representation of IC_{50} values of DE and ME in the corresponding cell lines. The values and error bars shown in all the graphs represent average and standard deviations, respectively, of three independent experiments (p <0.05).[15]

The inability of DA and MHI-25 to inhibit cell proliferation could be attributed to their limited cellular uptake, thus rendering them ineffective. It is also likely that they are taken up by cancer cells but are not cytotoxic. To confirm the intracellular localization of these dyes, we performed fluorescence confocal imaging in PC-3 and HDF cells upon 8 h of dye exposure (Fig. 5).[19,20] Both the esterified dyes (DE and ME) showed considerable cytoplasmic localization in PC-3 cells; however, neither DE nor ME showed accumulation in HDF cells. PC-3 cells demonstrated enhanced uptake of ME as compared to DE, perhaps explaining its lower IC_{50}. DA and MHI-25 however, exhibited negligible intracellular localization in both PC-3 and HDF cells. It is reported that esters are

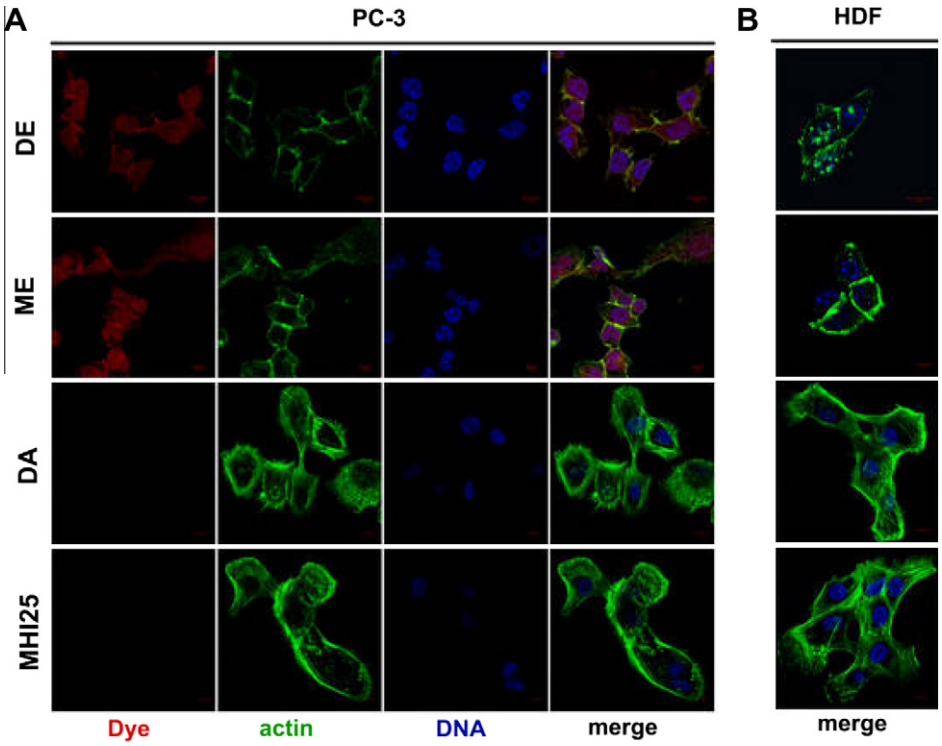


Figure 5. (A) Confocal micrographs showing PC-3 cells treated with DE, ME, DA and MHI-25 (20 μM) for 8 h and stained for actin (green) and DNA with DAPI (blue). Cellular localization of the corresponding dyes is visible in red. (B) Merged micrographs showing HDF cells treated with the corresponding dyes (20 μM) for 8 h, stained with actin (green) and DNA with DAPI (blue). Any intracellular dye localization should appear in red. Scale bar = 10 μm.[19,20]

more readily transported across cellular membranes.[21] This might explain the enhanced cellular uptake of the esterified dyes (ME and DE) compared to the diacid and MHI-25. It is also likely that the partition coefficient (log*P*) values of the mono- and diesterified dyes is optimal to facilitate cellular uptake. Our data thus underscore the usefulness of DE and ME as potential tumor-specific imaging as well as tumor targeting dye candidates. Although the accumulation properties of MHI-25 have previously reported[11] in different cancer cell lines including prostate (LNCaP, C4-2, C4-2B, ARCaPE, ARCaPM, and PC-3), lung (H358), breast (MCF-7), cervical (HeLa), leukemia (K562), renal (SN12C, ACHN), bladder (T24), and pancreatic (MIA PaCa-2), our data show minimal cellular uptake of MHI-25 in HeLa and PC-3 cells under our experimental conditions.

In conclusion, a unique synthesis of asymmetric carbocyanine dyes has been presented that is important for the preparation of NIR dyes containing monofunctional groups for biomolecule conjugation. Furthermore, this one-pot synthesis appears feasible with a variety of carboxylic acid alkylated salts. Interestingly, the synthesized mono- and diester dyes were selectivity taken up and accumulated in tumor cells but not normal cells, providing the advantage of tumor-specific targeting that does not require receptor conjugation of the imaging dyes.

Supplementary data

Supplementary data associated with this Letter can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.070.

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13. *Cell viability by trypan blue-exclusion assay*: The loss of membrane integrity in dead and dying cells allows preferential uptake of labels like trypan blue. We used trypan blue assay to determine cell viability upon treatment with dyes.[14] At the end of incubation times with the dyes, PC-3, HeLa or HDF cells were pelleted and washed with PBS. Well-suspended cells were mixed with equal volume of 0.4% trypan blue in 1× PBS, pH 7.4, followed by incubation at room temperature for 5 min. Cells were examined under the microscope and blue-stained cells were considered non-viable.
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15. *Cytotoxicity assay*: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to evaluate the proliferative capacity of cells as described previously.[12] Essentially, MTT is a colorimetric assay, which utilizes the colorless tetrazolium dye and converts it into a colored formazan salt, which can be quantified by measuring absorbance at 570 nm. Briefly, a 96-well format was used to seed 100 mL medium containing cells at a density of 5×10^3 cells per well. After incubation with dyes, cells were treated with gradient concentration of the test compounds, which were dissolved in DMSO. The final concentration of DMSO in the culture medium was maintained at 0.1%. After 48 h of incubation with dye, the spent medium was removed and the wells were washed twice with PBS. Hundred milliliter of fresh medium and 10 mL of MTT (5 mg/mL in PBS) were added to the wells and cells were incubated at 37 °C in the dark for 4 h. The formazan product was dissolved by adding 100 mL of 100% DMSO after removing the medium from each well. The absorbance was measured at 570 nm using a Spectra Max Plus multi-well plate

reader (Molecular Devices, USA). We first performed MTT with a broader concentration range (0.1–100 μM with a 10-fold increment) to determine the IC_{50} of the dyes. Since the IC_{50} range was between 1 and 10 μM for DE and ME, respectively, in PC-3 and below 1 μM in HeLa, we decided to take a narrower range to precisely determine the IC_{50} values. The IC_{50} table for HeLa, PC-3 and HDF is shown in Table 1 in Supplementary data.

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17. *Synthetic methods and spectroscopic characterization*: All ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a BRUKER AVANCE-400 NMR spectrometer at ambient temperature with $CDCl_3$ and DMSO-d_6 as solvent and tetramethylsilane (TMS) as an internal standard. High resolution electrospray ionization (ESI) mass spectra were obtained on a Waters Micro-mass Q-TOF micro analyzer in the Department of Chemistry, Instrumentation Facility at Georgia State University. Melting points (open pyrex capillary) were measured on a Thomas Hoover apparatus and are uncorrected. Starting reagents 2,3,3-trimethyl indolenine and 6-bromohexanoic acid were purchased from Sigma Aldrich or Alfa Aesar and were used as received. All of the solvents were at least reagent grade and were used without further purification. *1-(5-Carboxypentyl)-2,3,3-trimethyl-3H indolenium bromide,* ***2***. 2,3,3-Trimethyl-3*H* indolenine **1** (2.0 g, 12.6 mmol) and 6-bromohexanoic acid (7.35 g, 25.1 mmol) were mixed in acetonitrile (15 mL) and heated to reflux for 18 h under nitrogen atmosphere. The mixture was allowed to cool to room temperature. The reaction was concentrated under vacuum to yield an oily residue, which was dissolved in dichloromethane (5 mL). Ether was added dropwise to the dichloromethane solution to give a precipitate, which was filtered and washed with ether to furnish **2** as a pale pink powder; yield 4.4 g (67%); mp 127–129 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.43 (m, 2H), 1.54 (s, 6H), 1.56 (m, 2H), 1.85 (m, 2H), 2.23 (t, J = 7.6 Hz, 2H), 2.86 (s, 3H), 4.46 (t, J = 7.6 Hz, 2H), 7.62 (m, 2H), 7.85 (m, 1H), 7.99 (m, 1H), 11.98 (br s, 1H). *Heptamethine cyanine dyes* **4**, ***5***, *and* ***6***. A solution of salt **2** (2.40 g, 6.77 mmol), Vilsmeier–Haack reagent **3** (1.10 g, 3.39 mmol) and sodium acetate (0.833 g, 10.2 mmol) in acetic anhydride (15 ml) was heated at 90 °C for 3 h under nitrogen atmosphere. The mixture was cooled to room temperature and quenched with methanol (5 ml). After 10 min, the solution began boiling and turned black. The solvent was concentrated under vacuum and the crude solid was dissolved in dichloromethane (20 ml) and filtered to remove sodium acetate. The mixture of dyes was separated by column chromatography on silica gel eluting with methanol-dichloro-methane gradient from 1:30, 1:20, to 1:10. The fractions of each dye were collected together and concentrated under vacuum to give heptamethine cyanine dye diester **4**, heptamethine cyanine dye monoester **5**, and heptamethine cyanine dye diacid **6**. *Chloro-3-[2-[1,3-dihydro-3,3-dimethyl-1-(methylhexanoate)-2H-indol-2-ylidene]-ethylidene]-1-cyclo-1-yl]-ethenyl]-3,3-dimethyl-1-(methylhexano-ate)-3H-indolum bromide (****4****).* Greenish-gold solid, yield 15%, 400 mg, mp 161–163 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 1.54 (m, 4H), 1.73 (s, 14H), 1.86 (m, 4H), 2.00 (m, 4H), 2.36 (t, J_{HH} = 7.6 Hz, 4H), 2.75 (t, J_{HH} = 7.6 Hz, 4H), 3.65 (s, 6H), 4.25 (t, J_{HH} = 7.6 Hz, 4H), 6.26 (d, J_{HH} = 14.0 Hz, 2H), 7.18 (d, J_{HH} = 7.6 Hz, 2H), 7.27 (m, 2H), 7.40 (m, 4H), 8.34 (d, J_{HH} = 14.0 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 173.8, 172.2, 150.2, 144.2, 142.3, 141.1, 128.8, 127.8, 125.3, 122.2, 110.9, 101.7, 51.6, 49.3, 44.8, 33.7, 28.2, 27.2, 26.8, 26.4, 24.6, 20.8; HRMS calcd for $C_{44}H_{56}N_2O_4Cl$, m/z 711.3921; observed m/z 711.3915. λ_{max} absorption and fluorescence wavelengths in methanol were 780 and 801 nm, respectively. *Chloro-3-[2-[1,3-dihydro-3,3-dimethyl-1-(methylhexanoate)-2H-indol-2-ylidene]-ethylidene]-1-cyclo-1-yl]-ethenyl]-3,3-dimethyl-1-(5-carboxypentyl)-3H-indolum bromide (****5****).* Greenish-gold solid, yield 30%, 800 mg, mp 165–167 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 1.53 (m, 4H), 1.72 (s, 14H), 1.87 (t, J_{HH} = 7.4 Hz, 4H), 2.00 (m, 2H), 2.36 (t, J_{HH} = 7.4 Hz, 2H), 2.42 (t, J_{HH} = 7.4 Hz, 2H), 2.71 (m, 4H), 3.66 (s, 3H), 4.13 (m, 4H), 6.14 (d, J_{HH} = 14.0 Hz, 2H), 7.13 (d, J_{HH} = 7.4 Hz, 1H), 7.25 (m, 3H), 7.41 (m, 4H), 8.36 (q, J_{HH} = 14.0 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_C 176.2, 173.8, 172.8, 171.9, 150.7, 144.9, 144.0, 142.2, 142.0, 141.1, 140.9, 129.0, 128.8, 128.6, 127.3, 125.6, 125.2, 122.3, 112.2, 110.7, 101.6, 100.8, 51.6, 49.5, 49.2, 44.7, 44.5, 34.5, 33.9, 33.6, 29.6, 28.5, 28.1, 27.0, 26.5, 26.4, 26.3, 25.5, 24.5, 20.7; HRMS calcd for $C_{43}H_{54}N_2O_4Cl$, m/z 697.3772; observed m/z 697.3763. λ_{max} absorption and fluorescence wavelengths in methanol were 780 and 801 nm, respectively. *Chloro-3-[2-[1,3-dihydro-3,3-dimethyl-1-(5-carboxypentyl)-2H-indol-2-ylidene]-ethylidene]-1-cyclo-1-yl]-ethenyl]-3,3-dimethyl-1-(5-carboxypentyl)-3H-indolum bromide (****6****).* Greenish-gold solid, yield 20%, 520 mg, mp 171–173 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 1.56 (m, 4H), 1.71 (s, 12H), 1.76 (q, J_{HH} = 7.4 Hz, 4H), 2.00 (br t, 2H), 2.46 (t, J_{HH} = 7.4 Hz, 4H), 2.72 (br t, 4H), 4.13 (t, J_{HH} = 7.4 Hz, 4H), 6.20 (d, J_{HH} = 14.0 Hz, 2H), 7.22 (m, 4H), 7.38 (m, 4H), 8.33 (d, J_{HH} = 14.0 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_C 172.2, 148.0, 143.0, 142.0, 141.1, 128.6, 126.2, 125.1, 122.5, 111.5, 101.6, 49.0, 43.7, 33.5, 27.5, 26.7, 25.8, 25.6, 24.2; HRMS calcd for $C_{42}H_{52}N_2O_4Cl$, m/z 683.3616; observed m/z 683.3595. λ_{max} absorption and fluorescence wavelengths in methanol were 780 and 801 nm, respectively.
18. *Cell culture*: PC-3 were grown in RPMI medium, and HeLa and HDF in DMEM medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.
19. *Immunofluorescence microscopy*: The cellular localization of the NIR dyes was imaged using Indocyanine Green (ICG-B) filter Set (Chroma Technology, VT) and 780 nm Collimated LED source (Zeiss, Germany) on an Axio Observer. A1 microscope (Zeiss, Germany). Cells were grown on glass coverslips for immunofluorescence microscopy as described previously.[20] After treatment with drugs, cells were fixed with cold (−20 °C) methanol for 10 min and blocked by incubating with 2% BSA/PBS at 37 8C for 1 h. Alexa Fluor® 555 phalloidin (Invitrogen, Carlsbad, 1:500 dilution) was used to stain for actin and was incubated with the coverslips for 1 h at room temperature. The cells were washed with 2% BSA/PBS for 10 min at room temperature. Cells were mounted with Prolong-Gold antifade reagent that contained DAPI (Invitrogen, Carlsbad).
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