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A Novel Conjugable Translocator Protein Ligand Labeled with a Fluorescence Dye for in Vitro Imaging

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A conjugable analogue of the benzodiazepine 4′′-chlorodiazepam (Ro5-4864), C_6Ro5-4864 was synthesized to probe the binding sites of translocator protein (18 kDa; TSPO), previously known as the peripheral benzodiazepine receptor for molecular imaging. The amino group in this analogue allows universal conjugation to signaling molecules. Lissamine−C_6Ro5-4864, synthesized from C_6Ro5-4864 and a lissamine fluorescence dye, was investigated in this study. This imaging agent exhibited micromolar binding affinity ($K_i = 2.6\ \mu$M) to TSPO and was successfully imaged in TSPO rich glioma and breast cancer cell lines. These findings suggest that C_6Ro5-4864 may provide opportunities in imaging disease states where TSPO levels are affected, such as cancer and neurologic diseases.

INTRODUCTION

Molecular imaging (MI) has emerged as an important multidisciplinary area which involves radiology, chemistry, biology, biochemistry, physics, engineering, and medicine. MI combines molecular agents with imaging tools to follow specific molecular pathways in the body and can be widely applied in locating, diagnosing, and treating disease. Synthetic chemistry is essential to MI by providing potent imaging agents.

The translocator protein (18 kDa), TSPO, previously known as the peripheral-type benzodiazepine receptor has become an interesting target in MI (*1*). TSPO is a mitochondrial protein which is associated with a variety of biological activities such as cell proliferation, apoptosis, immunomudulation, steroidogenesis, and transport of porphyrin and heme (*1*−*3*). TSPO is ubiquitously expressed in human body, with high level of expression in non-neoplastic diseases such as ischemia, brain damage induced by toxins, viral encephalitis, hepatic encephalopathy, epilepsy, nerve degeneration, and trauma (*4*). TSPO overexpression has also been observed in various cancers including breast, glioma, prostate, colorectal, esophageal, ovarian, and small cell lung cancer (*4*).

Several selective and potent TSPO ligands have been developed, such as benzodiazepines (Ro5-4864), isoquinoline carboxamides (PK11195), indolacetamide derivatives (FGIN-1−27), pyrisanoindole derivatives (SSR180575), and phenoxylphenyl acetamide derivatives (DAA1106 or DAA1097). The study of TSPO, an important mitochondrial membrane protein is currently limited because only two ligands that exist are conjugable to signaling moieties for tracking and quantitation. An FGIN analogue was labeled with a fluorophore (NBD) which is not ideal for in vitro imaging due to the low molar extinction coefficient (~ 8000 L mol^{-1} cm^{-1}) (*5*, *6*). Our lab developed a PK11195 analogue which has a six-carbon linker and a terminal primary amino group (*7*). Development of conjugable forms of the numerous PBR ligands would facilitate the study of TSPO and possibly allow for it to be used as a target for in vivo imaging.

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A conjugable form of PK11195 has been reported, and the molecule has been coupled to a lanthanide chelate (*7*) and a fluorescence dye (*8*). These TSPO-targeted agents showed good TSPO binding activity, allowing in vitro molecular imaging studies. Ro5-4864 and PK11195 are reversible competitive inhibitors of each other, and they show distinct biological properties in inflammation (*9*), depression (*10*), nociception (*11*), and apoptosis enhancement (*4*). Given these facts, to further study TSPO function in relation to its expression levels, a conjugable form of Ro5-4864 was deemed as a necessity.

We report herein the synthesis of a conjugable form of Ro5-4864 having a six-carbon spacer (C_6Ro5-4864). Through the primary amino group, this molecule can be conjugated to a variety of signaling moieties, thus allowing the use of different imaging modalities. Lissamine−rhodamine B sulfonyl chloride, a fluorescence dye with a high molar extinction coefficient (88 000 L/mol cm in methanol), was selected for conjugation to C_6Ro5-4864. The resulting TSPO-targeted fluorophore, lissamine−C_6Ro5-4864, labels TSPO-expressing cells selectively and shows promise for MI studies in imaging disease states where TSPO levels are affected, either as the cause or the outcome of the disease.

EXPERIMENTAL PROCEDURES

7-Chloro-5-(4-chlorophenyl)-1-methyl-1*H*-benzo[1,2,4]-triazepin-2-one (2). (5-Chloro-2-(methylamino)phenyl)(4-chlorophenyl)methanone **1** (2 g, 7 mmol) and ethyl carbazate (1.5 g, 17 mmol) were stirred at 210 °C for 3.5 h. After the reaction was cooled to room temperature, the product was purified by column chromatography on silica gel by eluting with 1:3 ethyl acetate/hexanes to yield **2** as a yellow solid (774 mg, 34%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38−7.53 (6H, m), 7.15 (1H, d, $J = 8.7$ Hz), 7.05 (1H, d, $J = 2.7$ Hz), 3.29 (3H, s); MS (MALDI)$^+$ m/z 320.3 ([M + H]$^+$, 100%).

(6-Bromohexyloxy)(*tert*-butyl)dimethylsilane (4). To a solution of 6-bromo-1-hexanol (906 mg, 5 mmol) in dichloromethane (25 mL) at 0 °C was added imidazole (1.02 g, 15 mmol). After 5 min, 4-(dimethylamino)pyridine (DMAP) (60 mg, 0.5 mmol) was added, followed by *tert*-butyldimethylsilyl chloride (1.5 g, 10 mmol). The resulting solution was stirred at 0 °C for 2 h, quenched with 30 mL water, and extracted with

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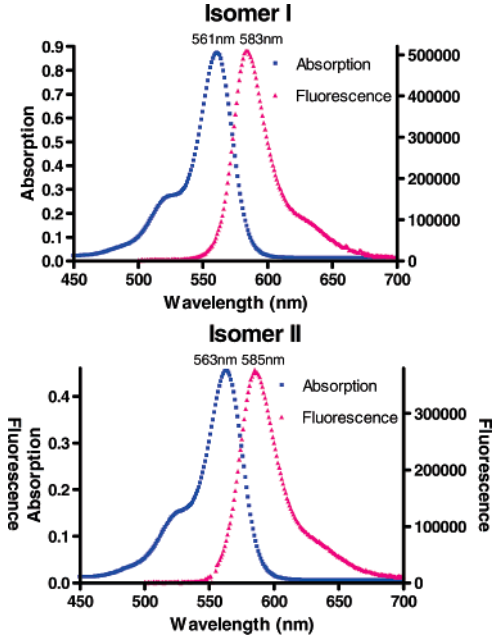


Figure 1. Lissamine−C_6Ro5-4864 isomer I (left) and isomer II (right) absorption and fluorescence curves.

dichloromethane (3 × 30 mL). The organic layers were combined, dried over sodium sulfate, and concentrated by vacuum rotary evaporation. The product was purified by silica gel chromatography using 1:20 ethyl acetate/hexanes as the eluent. Compound **4** was isolated as a colorless liquid (1.3 g, 88%). ^{1}H NMR 300 MHz ($CDCl_3$) δ 3.60 (t, J = 6.3 Hz, 2H), 3.40 (t, J = 6.9 Hz, 2H), 1.86 (q, J = 5.1 Hz, 2H), 1.54−1.35 (m, 6H), 0.89 (s, 9H), 0.04 (s, 6H). MS (GC): 295, 293 [M•].

(*Z*)-3-(6-(*tert*-Butyldimethylsilyloxy)hexyl)-7-chloro-5-(4-chlorophenyl)-1-methyl-1*H*-benzo[*e*][1,2,4]triazepin-2(3*H*)-one (5). Sodium hydride (60% in mineral oil) (20 mg, 0.5 mmol) was added to a stirring solution of compound **2** (256 mg, 0.8 mmol) in dry DMF (4 mL). The mixture was stirred under argon for 30 min. Compound **4** was then added to the mixture, and the resulting mixture was stirred for another 30 min. The reaction solution was poured into 50 mL of a stirring 1 M ammonium chloride solution at 0 °C. The mixture was transferred to a separation funnel and extracted with ethyl ether (3 × 40 mL). The organic layers were combined, dried over sodium sulfate, and concentrated by vacuum rotary evaporation. The crude product was purified by silica gel chromatography using gradient 1:20 to 1:4 ethyl acetate/hexanes as the eluent to give a yellow oil (349 mg, 89%). ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.55−7.53 (m, 2H), 7.46 (dd, J = 8.8, 2.4 Hz, 1H), 7.41−7.39 (m, 2H), 7.12 (d, J = 8.8 Hz, 1H), 7.06 (d, J = 2.4 Hz, 1H), 3.73 (bs, 1H), 3.55 (t, J = 2.4 Hz, 2H), 3.23 (s, 3H), 1.65 (bs, 2H), 1.49−1.43 (m, 2H), 1.33−1.24 (m, 5H), 0.88 (s, 9H), 0.02 (s, 6H). MS (ESI)$^+$: 556.2 Da [M + Na]$^+$.

(*Z*)-7-Chloro-5-(4-chlorophenyl)-3-(6-hydroxyhexyl)-1-methyl-1*H*-benzo[*e*][1,2,4]triazepin-2(3*H*)-one (6). Tetrabutylammonium fluoride (TBAF) (3.5 mL 1 M solution in THF) was added to a stirring solution of compound **5** (377 mg, 0.7 mmol) in THF (2.5 mL) at 0 °C. The reaction solution was stirred at 0 °C for 3 h, quenched with 30 mL of water, and extracted with ethyl ether three times. The organic layers were combined, dried over sodium sulfate, and concentrated by vacuum rotary evaporation. The crude product was purified by column chromatography (silica gel) using 1:2 ethyl acetate/hexanes as eluent. Compound **6** was collected as a pale yellow oil (281 mg, 95%). ^{1}H NMR 300 MHz ($CDCl_3$) δ 7.55−7.53 (m, 2H), 7.46 (dd, J = 9.0, 2.4 Hz, 1H), 7.41−7.38 (m, 2H), 7.12 (d, J = 8.7 Hz, 1H), 7.06 (d, J = 2.4 Hz, 1H), 3.72 (bs, 1H), 3.61−3.56 (m, 3H), 3.23 (s, 3H), 1.69−1.62 (m, 2H), 1.57−1.48 (m, 2H), 1.37−1.24 (m, 5H). ^{13}C NMR 75 MHz ($CDCl_3$) δ 163.6, 161.1, 145.5, 136.5, 134.5, 131.7, 130.3, 129.2, 129.0, 128.8, 128.5, 120.9, 62.8, 51.5, 35.5, 32.6, 27.7, 26.6, 25.4. MS (ESI)$^+$: 442.2 Da [M + Na]$^+$.

(*Z*)-6-(7-Chloro-5-(4-chlorophenyl)-1-methyl-2-oxo-1*H*-benzo[*e*][1,2,4]triazepin-3(2*H*)-yl)hexyl 4-Methylbenzenesulfonate (7). A solution of compound **6** (182 mg, 0.43 mmol) and triethylamine (188 μL, 1.3 mmol) in dry dichloromethane (6 mL) was stirred at 0 °C for 5 min. *p*-Toluenesulfonyl chloride (298 mg, 1.56 mmol) was added slowly to the above solution. The resulting mixture was warmed to room temperature and stirred overnight. The reaction was then quenched with 20 mL of water and extracted with dichloromethane (3 × 20 mL). The combined organic layers were dried over sodium sulfate and concentrated by vacuum rotary evaporation. The crude product was purified by silica gel chromatography using a 1:19 to 2:3 ethyl acetate/hexanes gradient as eluent. Compound **7** was isolated as a yellow oil (238 mg, 96%). ^{1}H NMR 300 MHz ($CDCl_3$) δ 7.77 (dm, J = 8.1 Hz, 2H), 7.54−7.51 (m, 2H), 7.46 (dd, J = 8.7, 2.4 Hz, 1H), 7.52−7.39 (m, 2H), 7.33 (d, J = 7.8 Hz, 2H), 7.12 (d, J = 8.7 Hz, 1H), 7.06 (d, J = 2.4 Hz, 1H), 3.97 (t, J = 6.3 Hz, 2H), 3.70−3.40 (m, 2H), 3.23 (s, 3H), 2.44 (s, 3H), 1.62−1.55 (m, 4H), 1.31−1.21 (m, 4H). MS (ESI)$^+$: 596.3 Da [M + Na]$^+$.

C_6Ro5-4864 (8). A sealed tube with a solution of compound **7** (25 mg, 44 μmol) in methanol (1 mL) was cooled to −78 °C and flushed with ammonia gas. After about 2.5 mL liquid ammonia was condensed, the tube was sealed and warmed to room temperature. The reaction was stirred under room temperature overnight. The mixture was concentrated by nitrogen flow, redissolved by dichloromethane, and treated with a saturated sodium bicarbonate solution. The mixture was extracted with dichloromethane three times. The organic layers were combined, dried over sodium sulfate, and concentrated by vacuum rotary evaporation. The crude product was purified by column chromatography (silica gel) using a gradient with dichloromethane and dichloromethane/methanol/ammonia (6:1:0.1) as the eluent. C_6Ro5-4864 was collected as a pale yellow oil (14 mg, 77%). ^{1}H NMR 300 MHz ($CDCl_3$) δ 7.55−7.51 (m, 2H), 7.45 (dd, J = 8.7, 2.4 Hz, 1H), 7.41−7.37 (m, 2H), 7.12 (d, J = 8.7 Hz, 1H), 7.06 (d, J = 2.4 Hz, 1H), 3.80−3.45 (m, 2H), 3.23 (s, 3H), 2.62 (bs, 2H), 1.65 (bs, 2H), 1.49−1.38−1.24 (m, 8H). ^{13}C NMR 75 MHz ($CDCl_3$) δ 163.6, 161.1, 145.5, 136.4, 134.5, 131.7, 130.2, 129.2, 129.0, 128.8, 128.5, 120.9, 51.5, 42.1, 35.5, 33.7, 27.7, 26.6, 26.5. MS (ESI)$^+$: 419.3 Da [M + H]$^+$.

Lissamine−C_6Ro5-4864. A mixture of lissamine rhodamine B sulfonyl chloride (mixed isomers) (10 mg, 17 μmol), C_6-Ro5-4864 (8 mg, 19 μmol), and triethylamine (15 μL) in dry dichloromethane (1.6 mL) was stirred at room temperature under argon flow for 2 h. The product was purified through column chromatography (silica gel) using a mixture of 24:1 dichloromethane:methanol as the eluent. Lissamine−C_6Ro5-4864 was isolated as a pink solid (Isomer I, 3.2 mg, 19%; Isomer II, 2.2 mg, 13%). ^{1}H NMR 500 MHz ($CDCl_3$) **Isomer I**: δ 8.81 (d, J = 2.0 Hz, 1H), 7.96 (dd, J = 8.0, 1.5 Hz, 1H), 7.55−7.53 (m, 2H), 7.47 (dd, J = 8.5, 2.5 Hz, 1H), 7.41−7.39 (m, 2H), 7.29 (d, J = 9.5 Hz, 2H), 7.20 (d, J = 8.0 Hz, 1H), 7.15 (d, J = 9.0 Hz, 1H), 7.07 (d, J = 2.0 Hz, 1H), 6.81 (dd, J = 9.5, 2.5 Hz, 2H), 6.66 (d, J = 2.5 Hz, 2H), 4.99 (t, J = 6.5 Hz, 1H), 3.50−3.59 (m, 8H), 3.23 (s, 3H), 3.01 (q, J = 7.0 Hz, 2H), 1.69−1.58 (m, 6H), 1.55−1.50 (m, 2H), 1.29 (t, J = 7.5 Hz, 14H). **Isomer II**: δ 8.66 (s, 1H), 8.38 (dd, J = 8.0, 1.0 Hz, 1H), 7.52−7.50 (m, 2H), 7.46 (dd, J = 8.5, 2.5 Hz, 1H), 7.38−7.36 (m, 2H), 7.23 (d, J = 7.5 Hz, 1H), 7.17 (d, J = 9.5 Hz, 1H), 7.14 (d, J = 9.0 Hz, 1H), 7.04 (d, J = 2.5 Hz, 1H), 6.85 (dd,

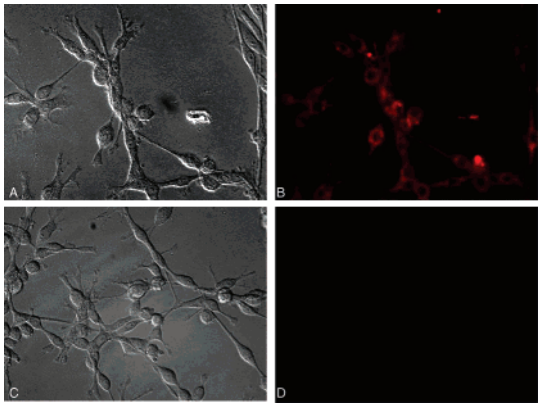


Figure 2. Fluorescence imaging of C6 rat glioma cells: (A) phase contrast microscopy of cells dosed with lissamine−C_6Ro5-4864; (B) fluorescence imaging of cells dosed with 100 nM lissamine−C_6Ro5-4864; (C) phase contrast microscopy of cells dosed with lissamine dye; (D) fluorescence imaging of cells dosed with 100 nM lissamine dye (control).

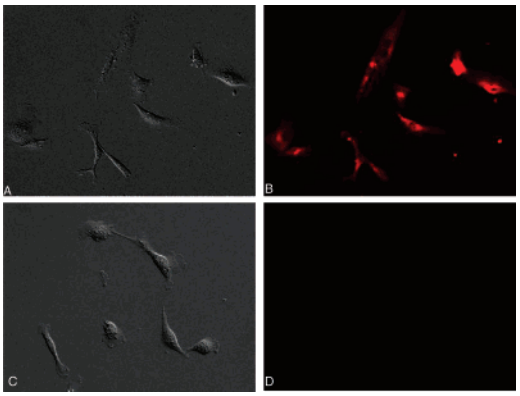


Figure 3. Fluorescence imaging of MDA-MB-231 human breast cancer cells: (A) Phase contrast microscopy of cells dosed with lissamine−C_6Ro5-4864; (B) fluorescence imaging of cells dosed with 100 nM lissamine−C_6Ro5-4864; (C) Phase contrast microscopy of cells dosed with lissamine dye; (D) fluorescence imaging of cells dosed with 100 nM lissamine dye (control).

J = 9.5, 1.5 Hz, 2H), 6.70 (d, J = 2.5 Hz, 2H), 5.24 (bs, 1H), 3.63−3.55 (m, 8H), 3.21 (s, 3H), 2.89 (q, J = 6.5 Hz, 2H), 1.72−1.61 (m, 9H), 1.33 (t, J = 7.5 Hz, 14H). MS $(ESI)^+$: 981.4 Da $[M + Na]^+$. Rf 0.23 (Isomer I), 0.14 (Isomer II) (4% methanol in dichloromethane).

Spectroscopic Characterization. The lissamine−C_6Ro5-4864 absorption and emission spectra (Figure 1) were obtained using a Shimadzu 1700 UV−vis spectrophotometer and ISS PCI spectrofluorometer at room temperature on a 5 μM sample.

Binding Studies. C6 glioma cells were cultured in Dulbecco's modified Eagle medium (DMEM)-F12 medium (Gibco/Invitrogen) supplemented with 0.5% FBS and 2.5% horse serum (HS) at 3.7% CO_2. Cells were scraped from 150 mm culture dishes into 5 mL of phosphate-buffered saline (PBS), dispersed by trituration, and centrifuged at 500*g* for 15 min. Cell pellets were resuspended in PBS and assayed for protein concentration. [^{3}H]PK11195 binding studies on 30 μg of protein from cell suspensions were performed as previously described (*12*). Displacement studies using increasing concentrations of lissamine−C_6Ro5-4864 were performed in the presence of 15 nM [^{3}H]PK11195. Data were analyzed using PRISM software (vs 4.0, GraphPad, Inc., San Diego, CA).

Cell Imaging. C6 rat glioma and MDA-MB-231 human breast carcinoma cells were plated in MaTek dishes. The growth media was removed and replaced with media containing the contrast agent. C6 and MDA-MB-231 cells were dosed with 100 nM lissamine−C_6Ro5-4864 for 30 min at 37 °C under 5% CO_2. Cells dosed with 100 nM free lissamine dye were imaged as control. At the end of the incubation time, cells were rinsed with PBS three times and imaged under a Nikon Eclipse TE2000-U microscope (Lewisville, TX).

In Vitro Competition. MDA-MB-231 human breast carcinoma cells were plated in MaTek dishes. The growth media was removed and replaced with media containing the contrast agent. Unchallenged MDA-MB-231 cells were dosed with 5 μM lissamine−C_6Ro5-4864. Unchallenged cells were dosed with 5 μM lissamine−C_6Ro5-4864 and PK1195 (500 nM, 5 μM or 50 μM). All cells were incubated for 30 min at 37 °C under 5% CO_2. At the end of the incubation time, cells were rinsed with PBS three times and imaged under a Nikon Eclipse TE2000-U microscope (Lewisville, TX).

RESULTS AND DISCUSSION

Synthesis. The overall goal of this synthetic effort was to construct a conjugable TSPO ligand with a biologically active portion of Ro5-4864. (5-Chloro-2-(methylamino)phenyl)(4-chlorophenyl)methanone **1** was synthesized from commercially available 4-chloro-phenylacetonitrile and 4-chloronitrobenzene as reported (*13*). After several unsuccessful attempts using semicarbazide and high-boiling solvents to induce a ring-closure reaction with **1**, we synthesized the triazepinone **2** by heating a mixture of ethyl semicarbazide and **1** at 210 °C without any solvent (Scheme 1). The *tert*-butyldimethylsilyl (TBS)-protected six-carbon linker **4** was added to **2** by an N-alkylation reaction. It is noteworthy that two other linkers, 1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-hexanoic acid and 6-nitrohexanoic acid were also employed, but we were unsuccessful in achieving the final product. After the TBS group in **5** was removed via TBAF, the hydroxyl group in **6** was converted to a tosyl group and subsequently reacted with ammonia to yield the final product **8**.

Since the commercially available lissamine−rhodamine B sulfonyl chloride is a mixture of two isomers (Scheme 2), the N-sulfonylation reaction between C_6Ro5-4864 and the fluorescence dye produced two isomer products. It was possible to isolate these two products through silica gel column chromatograph (R_f is 0.23 for isomer I and 0.14 for isomer II in 4% methanol in dichloromethane). Isomers I and II have a similar maximum absorption (isomer I: 561 nm; isomer II: 563 nM) and emission (isomer I: 583 nm; isomer II: 585 nm) wavelength to that of the free lissamine dye (Abs = 568 nm and Fluo = 583 nm) and Liss-ConPK11195 (Abs = 571 nm and Fluo = 585 nm) (Figure 1) (*8*). However, the molar extinction coefficients of the produced isomers are significantly different (isomer I: 175 000 L/mol cm; isomer II: 91 000 L/mol cm). Isomer I has much higher ϵ than lissamine−rhodamine B sulfonyl chloride (ϵ = 88 000 L/mol cm), which indicates that C_6Ro5-4864 produces a positive effect on lissamine's absorptive property. The molar extinction coefficient of Liss-ConPK11195 was reported as 350 000 L/mol cm, which is lower than isomer I but higher than isomer II. Isomer I was selected for imaging due to the relative high absorption and fluorescence (Figure 1).

Cell Imaging and Binding Study. The cellular uptake of lissamine−C_6Ro5-4864 was examined in C6 rat glioma and MDA-MB-231 human mammary adenocarcinoma cells by fluorescence microscopy. Both cell types were previously shown to contain relatively high levels of TSPO (*14*, *15*). The resulting images were compared with those obtained using the same concentrations of the free lissamine dye as control. Cell uptake of lissamine−C_6Ro5-4864 was observed in fluorescence images of both cell lines (Figures 2and 3, panel B) while the control exhibited no significant fluorescence. Images using Liss-ConPK11195 showed similar quality (*8*).

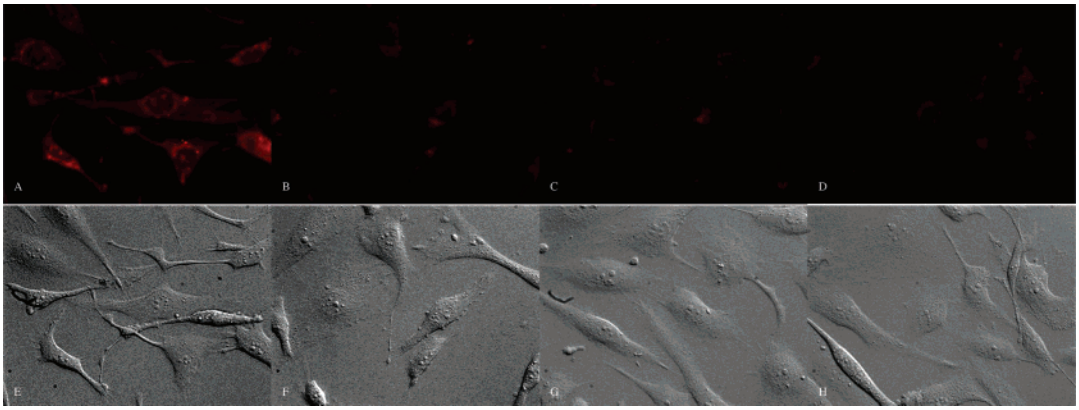
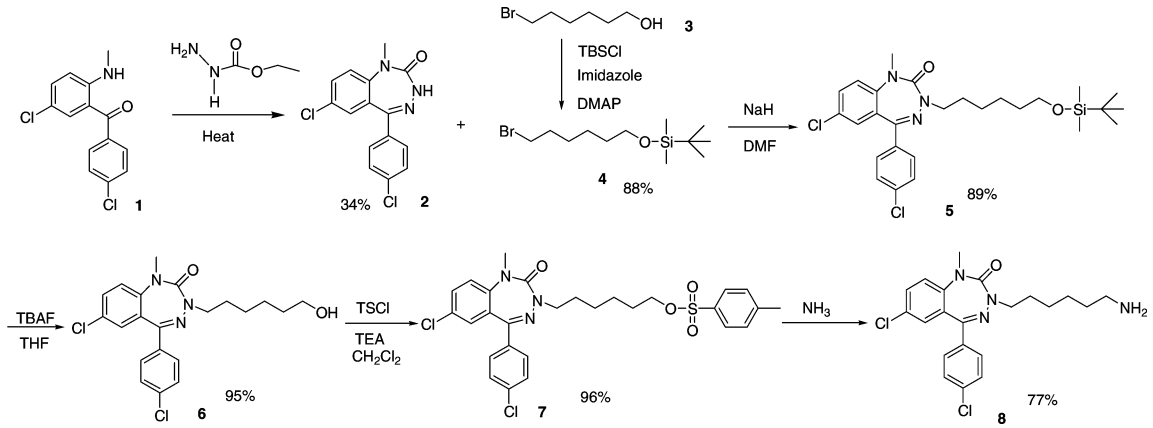
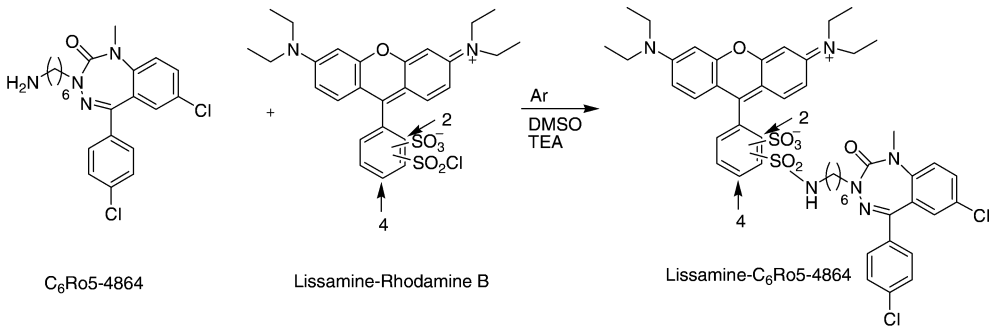


Figure 4. Fluorescence imaging of MDA-MB-231 human breast cancer cells: (A) fluorescence imaging of cells dosed with 5 μM lissamine−C_6Ro5-4864; (B) fluorescence imaging of cells dosed with 5 μM lissamine−C_6Ro5-4864 and 500 nM PK11195; (C) fluorescence imaging of cells dosed with 5 μM lissamine−C_6Ro5-4864 and 5 μM PK11195; (D) fluorescence imaging of cells dosed with 5 μM lissamine−C_6Ro5-4864 and 50 μM PK11195; (E−H) corresponding phase contrast microscopy.

Scheme 1 . Synthesis of C_6-Ro5-4864



Scheme 2 . Synthesis of Lissamine−C_6Ro5-4864



Radioligand binding studies further demonstrated specific binding of lissamine−C_6Ro5-4864 to the TSPO protein. In these studies, [^{3}H]PK11195, the well characterized diagnostic TSPO drug ligand, was used as the competitive radioligand. The concentration of lissamine−C_6Ro5-4864 tested varied from 10^{-11} M to 10^{-4} M in the competitive binding assays performed. The K_i value derived from IC_{50} was calculated to be 2.6 ± 1.4 μM, which is similar to Liss-ConPK11195 (1 μM) (*8*). Even though the binding affinity is significantly lower than that of [^{3}H]Ro5-4864 (K_i = 5 nM) (*16*), the selective binding of lissamine−C_6Ro5-4864 to TSPO is clearly shown and visualized. The specificity of lissamine−C_6Ro5-4864 to TSPO is further verified by in vitro competition study. In this experiment, MDA-MB-231 cells were treated with 5 μM lissamine−C_6Ro5-4864 and PK11195 with different concentrations as competitor. Challenged cells showed reduced fluorescence signal compared to unchallenged cells (Figure 4). Taken together these data suggest that lissamine−C_6Ro5-4864 is a promising TSPO targeted MI agent.

CONCLUSIONS

We synthesized and characterized a conjugable form of the benzodiazepine Ro5-4864, C_6Ro5-4864, which can be conjugated to a variety of signaling moieties for TSPO-targeted imaging. This TSPO ligand has been coupled to a fluorescent dye, and the resulting imaging agent, lissamine−C_6Ro5-4864, displays attractive optical properties for fluorescence microscopy imaging. The specific binding of this agent to TSPO was demonstrated by radioligand binding studies and live cell imaging on MDA-MB-231 breast cancer and C6 glioma cells.

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