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Synthesis of fluorescent derivatives of praziquantel: cell-imaging and interaction with *Schistosoma japonicum* cercariae†

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Schistosomiasis is one of the most burdensome of the neglected tropical diseases. Praziquantel is a recommended drug for treatment against all forms of schistosomiasis. To investigate the interaction between praziquantel and *Schistosoma japonicum* cercariae, two praziquantel derivatives (PZQ-2 and PZQ-3) and one praziquantel fluorescent derivative (PZQ-5) have been synthesized and characterized using nuclear magnetic resonance spectroscopy (NMR) and MS spectra. The cytotoxicity of PZQ-2, PZQ-3 and PZQ-5 was measured by performing the methyl thiazolyl tetrazolium (MTT) assay. The cell viability for them shows that the three compounds exhibit low cytotoxicity to HeLa cells. Cell imaging experiments demonstrate that PZQ-5 is biocompatible and cell-permeable, which indicates that PZQ-5 is suitable for studying their interaction. Confocal fluorescence microscopy revealed that PZQ-5 is mainly located at the cercarial tegument, which leads to the death of cercariae with the increase in time.

1. Introduction

Schistosomiasis is one of the most burdensome of the neglected tropical diseases.[1,2] The WHO estimates that at least 243 million people required treatment for schistosomiasis in 2011. Over 240 million people require treatment for schistosomiasis yearly. The number of people infected with schistosomiasis increased from 12.4 million in 2006 to 35 million in 2012.[3]

Praziquantel is a recommended drug for treatment against all forms of schistosomiasis. It is effective, safe and low-cost. The WHO strategy for controlling schistosomiasis focuses on reducing disease through periodic, targeted treatment with praziquantel.[4]

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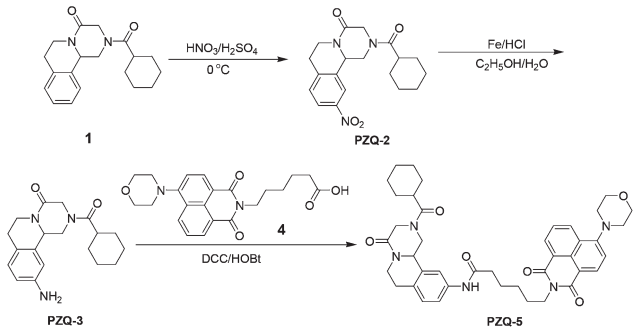
The cercarial stage of the schistosome life-cycle is the only infectious period. The cercarial stage is also the most fragile stage of the life cycle of the schistosome.[5] However, many reports focus mainly on the mechanism of praziquantel action with *S. japonicum* juvenile worm and adult worm, while few papers focus on the mechanism of praziquantel interaction with *S. japonicum* cercaria.[6–9] Our intention is to use fluorescence imaging technology to elucidate the preliminary interaction between praziquantel derivatives and *S. japonicum* cercariae.

Fluorescence imaging has been used as a powerful tool for the study of medicine and bioscience, including parasitology,[10–12] especially for studying the interaction between drugs and parasites *in vitro* and *in vivo*.[13] The abundance of fluorescent probes has promoted the development of fluorescent imaging technology. Currently, the available fluorescent dyes are as follows:[14–20] (1) organic fluorescent dyes, (2) phosphorescent metal complexes,[16] (3) quantum dots,[17] (4) fluorescent protein,[18,19] and (5) up-converting rare-earth nanophosphors.[20] Charlemagne Gnoula *et al.*[10] used 5(6)-carboxyfluorescein diacetate to label nematodes. The result has shown that only dead nematodes could be labeled. Devon B. Keeney *et al.*[11] examined the utility of fluorescent fatty acid analog dyes for labeling larval trematodes to use in experimental infections. The 4,5-diaminofluorescein-2 diacetate (DAF-2 DA) was used by Andrea B. Kohn *et al.*[21] to detect NO in living schistosomes. These reports indicated that fluorescence imaging technology is suitable for the investigated interaction between the drug and *S. japonicum* cercariae.

The main fluorophores of organic fluorescent dyes are fluorescein, naphthalimide, acridine, rhodamine, coumarins, *etc.* *N*-Hexanoic acid-4-morpholin-1,8-naphthalimide (compound **4**) is a good candidate for fluorescent probes with a large "push–pull" electronic system, good light stability and chemical stability. Its fluorescence emission wavelength is moderate (≈520 nm), the fluorescence quantum yield is relatively high, and the Stokes shift is larger.

Herein, novel derivatives of praziquantel (Scheme 1, PZQ-2 and PZQ-3) were synthesized by means of nitration and



Scheme 1 Synthetic routines of PZQ-5.

reduction reaction. To investigate the interaction between the drug and *S. japonicum* cercariae, a novel fluorescent compound of PZQ-5 was further synthesized by coupling *N*-hexanoic acid-4-morpholin-1,8-naphthalimide (compound **4**, Scheme 1) to the praziquantel derivative PZQ-3. Cell imaging and cell toxicity have been further investigated.

2. Experimental section

2.1. Materials and instrumentations

All of the starting materials (reagents and solvents) were obtained from commercial suppliers and used as received. Praziquantel was purchased from Zhejiang Top Medicine Co., Ltd. (China). The KB cell lines were provided by the Institute of Biochemistry and Cell Biology (China). Infected *Oncomelania hupensis* snails were supplied by the Hunan Institute of Parasitic Diseases (WHO collaborating center for schistosomiasis control in lakes).

^{1}H NMR and ^{13}C NMR spectra were recorded using a Mercuryplus spectrometer at 400 MHz and 100 MHz, respectively. Electrospray ionization mass spectra (ESI-MS) were measured using a Bruker APEX II FT-ICRMS 4.7 T system. UV-visible spectra were recorded using a Shimadzu UV-2550 spectrometer. Fluorescence spectra were measured using an Edinburgh LFS920 fluorescence spectrophotometer. Fourier transform infrared (FT-IR) spectra were measured using a Nicolet Nexus 470 spectrometer with KBr pellets. Fluorescence imaging experiments were performed using an OLYMPUS FV1000 IX81 confocal fluorescence microscope equipped with a 40× oil-immersion objective lens, excitation at 405 nm was carried out with a semiconductor laser and emission was collected at 480 to 580 nm. The MTT assay was measured by means of a Tecan Infinite M200 monochromator-based multifunction microplate reader.

2.2. Synthesis

2.2.1. Synthesis of PZQ-2. PZQ-2 was synthesized as described previously.[22] ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 8.21 (s, 1H), 8.10–8.12 (d, 1H), 7.37–7.39 (d, 1H), 5.25–5.28 (d, 1H), 4.87–4.90 (t, 2H), 4.49–4.53 (d, 1H), 4.08–4.12 (d, 1H), 3.04–3.11 (m, 1H), 2.84–2.94 (m, 3H), 2.45–2.50 (t, 1H), 1.73 (m, 5H), 1.53 (m, 2H), 1.28 (m, 3H).

2.2.2. Synthesis of PZQ-3. PZQ-2 (0.36 g), ethanol (4 mL), acetic acid (4 mL), Fe powder (0.26 g) and distilled water (4 mL) were added to a 100 mL three-neck flask, and finally a drop of concentrated HCl was added. The ensuing mixture was heated under reflux for 5 h, and the solvent was removed under reduced pressure. The water phase was extracted with CH_2Cl_2 (20 mL × 3). The combined organic layer was washed with aqueous sodium bicarbonate and distilled water and dried over anhydrous Na_2SO_4, respectively. The solvent was removed under reduced pressure and the residue was subjected to column chromatography on silica gel. The product was separated with EA–PE (v/v, 9 : 1), yielding a slightly white solid. ^{1}H NMR ($CDCl_3$), δ (ppm): 7.00 (d, 1H), 6.75 (s, 1H), 6.70 (d, 1H), 5.04 (d, 1H), 4.70 (m, 2H), 4.42 (d, 1H), 4.05 (d, 1H), 2.84 (m, 3H), 2.66 (d, 1H), 2.46 (m, 1H), 1.72 (m, 5H), 1.52 (m, 2H), 1.27 (m, 3H).

2.2.3. Synthesis of PZQ-5. **4** was synthesized according to a literature method.[23] Compound **4** (0.099 g) was dissolved in CH_2Cl_2 (10 mL) in a 50 mL three-neck flask. PZQ-3 (0.85 g), DCC (0.052 g) and HOBt (0.034 g) were added with stirring. The ensuing mixture was stirred for 10 h at room temperature. The solvent was removed under reduced pressure and the residue was subjected to column chromatography on silica gel. The product was separated with EA–CH_2Cl_2 (v/v, 4 : 1), yielding a yellow solid. ^{1}H NMR ($CDCl_3$), δ (ppm): 8.58 (d, 1H), 8.53 (d, 1H), 8.43 (d, 1H), 7.71 (t, 1H), 7.64 (s, 1H), 7.26 (m, 2H), 7.13 (d, 1H), 5.05–4.07 (d, 1H), 4.76–4.48 (t, 2H), 4.42–4.46 (d, 1H), 4.15–4.19 (d, 2H), 4.42–4.44 (d, 2H), 3.26–3.28 (d, 2H), 2.89 (m, 3H), 2.72–2.75 (d, 1H), 2.40 (d, 1H), 1.71–1.82 (m, 13H), 1.52 (m, 5H), 1.26 (m, 5H), 0.85–0.88 (m, 3H). ^{13}C NMR ($CDCl_3$), δ (ppm): 24.23, 25.04, 25.73, 26.74, 27.52, 28.40, 28.63, 36.54, 39.25, 40.14, 44.48, 48.41, 52.84, 54.25, 59.82, 66.36, 114.41, 115.99, 116.42, 119.02, 122.61, 125.27, 125.49, 129.13, 129.24, 129.54, 130.61, 132.00, 132.53, 136.69, 155.10, 163.41, 163.85, 166.11, 170.61, 171.24, 174.16. MS calcd For: $C_{41}H_{47}N_5O_6$, 705.84. Found: 728.4 $[M + Na]^+$.

2.3. Toxicity test

The MTT assay was used to detect the cell survival rate of PZQ-5. HeLa cells were plated on a 96 well tissue culture plate under an atmosphere of 5% CO_2, 95% air at 37 °C to adhere for 24 h. The cell pellet was mixed with a PZQ-5 solution (100 μL per well), at the final concentrations of 5, 10, 20, 30, and 40 μM, as the experimental group. The cell pellet was mixed with RPMI 1640 medium containing 0.2% DMSO (100 μL per well), as the reference group. After the cell culture was incubated for 24 h, 20 μL of MTT/PBS (5 mg mL^{-1}) was added to each well and the cell culture was further incubated for 4 h. After removing the culture medium, 100 μL of DMSO was added to each well and the absorbance in each well, including the blanks, was measured at 570 nm using a microtiter plate reader. The reference wavelength was 690 nm. The detailed mathematical description of the cell survival rate is given by the following equation: cell survival rate (%) =

(absorbance of experimental group/absorbance of reference group) × 100%. The experimental data from the three groups of parallel experimental data require obtaining the mean and standard deviation.

2.4. Anti-cercarial activity experiments

The compound PZQ-5 was dissolved in dimethyl sulphoxide (DMSO), and diluted to different concentrations with distilled water as follows: 10 μM, 20 μM, 40 μM, and 60 μM. *S. japonicum* cercariae were collected from the infected *O. hupensis*. The *O. hupensis* were induced to shed cercariae by exposing them to bright light for 2 h, and cercariae were then transferred by metal spatula from the water surface to a plate containing a dilute solution of the compound PZQ-5, and the activity of the cercariae was explored by biological microscopy at 2 h, 4 h, 6 h and 8 h.

2.5. Photophysical properties

The UV-vis and fluorescence emission spectra of PZQ-5 in a diluted solution of a mixed solvent (PBS–DMSO = 399 : 1) were studied, and fluorescence quantum yields of PZQ-5 were determined using quinine sulfate as the standard (yield = 0.53, in 0.1 M H_2SO_4, λ_{ex}: 365 nm).

2.6. Cell and cercaria imaging experiments

HeLa cells were plated on 18 mm glass coverslips under an atmosphere of 5% CO_2, 95% air at 37 °C to adhere for 24 h. Then the HeLa cells were stained with a 2.5 μM PZQ-5 solution in DMSO–PBS (v/v, 1 : 399) buffer for 15 min.

Cercariae were suspended in a 200 μL 3 μM solution of PZQ-5 on a special plate for imaging. Lastly, fluorescence imaging of cercariae was performed by confocal microscopy at 1 h, 3 h and 4 h, respectively.

3. Results and discussion

3.1. Toxicity test

Two praziquantel derivatives (PZQ-2 and PZQ-3) and one praziquantel fluorescent derivative (PZQ-5) were synthesized. In order to study their cytotoxicity, the MTT assay was used to detect the cell survival rate of PZQ-5. After HeLa cells were incubated with each compound for 24 hours, the cell survival rate is also over 85% in 40 μM PZQ-5. In addition, the cell survival rate is about 90% in 40 μM compound **4**, and the cell survival rate is also over 85% in 40 μM PZQ-2 and PZQ-3 (Fig. S3–5†). Herein, no sub-cellular apoptotic changes or significant cell death was seen in the cells after incubation with working concentrations for imaging. In general, PZQ-5 exhibits low cytotoxicity when used in a certain range of concentrations and within limited time periods of incubation (Fig. 1).

3.2. Anti-cercarial activity experiments

In order to study the anti-cercarial ability of a praziquantel fluorescent derivative, anti-cercarial activity experiments were performed at different concentrations of the compound PZQ-5.

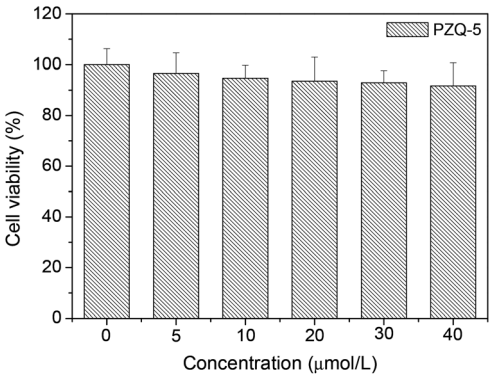


Fig. 1 Cell viability (%) estimated using a MTT proliferation test *versus* incubation concentrations of PZQ-5. HeLa cells were cultured in the presence of 0–40 μmol L^{-1} PZQ-5 at 37 °C for 24 h.

Table 1 The mortality rate of *Schistosoma japonicum* cercariae for the cercaricide PZQ-5 (25 °C)

		Mortality (%)			
Concentration (μM)	Number of cercariae	2 h	4 h	6 h	8 h
Black	19	0	0	0	0
10	19	0	5	21	84
20	18	0	28	56	100
40	13	8	54	77	100
60	16	19	75	100	100

The mortality rate of *S. japonicum* cercariae for the compound PZQ-5 was high, not only at a high concentration of 60 μM but also at a low concentration of 10 μM. At a low concentration of 10 μM the mortality reached 84% at 8 h, which implies that the cercaricide PZQ-5 maintained excellent bioactivity against cercariae even though the PZQ-5 was further synthesized by coupling *N*-hexanoic acid-4-morpholin-1,8-naphthalimide to the praziquantel derivative PZQ-3 (Table 1).

3.3. UV-vis and fluorescence emission spectra

The photophysical properties of PZQ-5 were investigated before PZQ-5 was used to label HeLa cells and cercariae. The UV-vis absorption and fluorescence emission spectra of PZQ-5 in a diluted solution (PBS–DMSO = 399 : 1) were studied and are shown in Fig. 2. The derivative PZQ-5 exhibits a broad UV-vis band and maximal absorbance at a wavelength of 383 nm, corresponding to the π–π* transition of fluorophore **4**. Under excitation at 383 nm, PZQ-5 emits green fluorescence at a maximum wavelength of 510 nm. The fluorescence quantum yield of PZQ-5 in water was measured to be 0.086 using quinine sulfate as the standard.

3.4. Cell imaging

In view of its favorable spectroscopic properties, PZQ-5 should be suitable for fluorescence imaging in living cells. As determined using confocal fluorescence microscopy, HeLa cells stained with a 2.5 μM solution of PZQ-5 in DMSO–PBS buffer

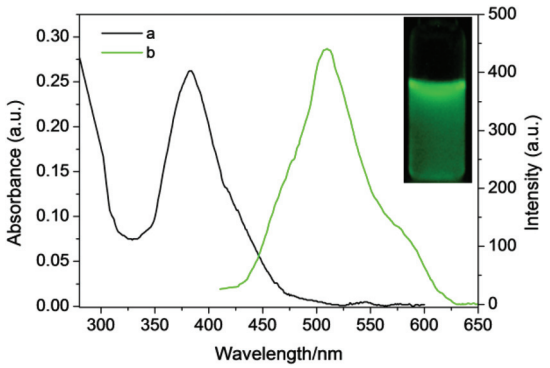


Fig. 2 UV-vis absorption (a) and fluorescent emission spectra (b) of PZQ-5 (3 μM) in a mixed solvent (PBS–DMSO = 399 : 1).

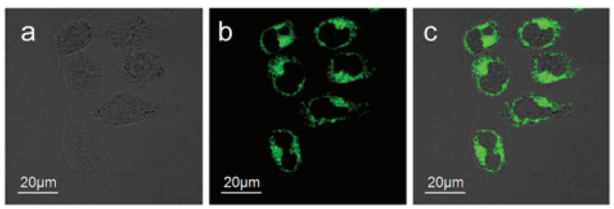


Fig. 3 Confocal bright-field image (a), fluorescence image (b) and overlaid image (c) of HeLa cells incubated with PZQ-5 (2.5 μM) for 15 min at 37 °C. The excitation wavelength was 405 nm, and emission was collected at 480 to 580 nm. Scale bar = 20 mm.

(v/v, 1 : 399) for 15 min showed significant intracellular fluorescence (Fig. 3). The overlay of fluorescence and bright-field images revealed that the fluorescence signal was mainly located in the intracellular region. Moreover, quantization of the fluorescence intensity profile of cells stained with PZQ-5 indicated that an extremely high signal ratio was associated with the cytoplasm of live cells (Fig. S6†). These results indicated that PZQ-5 was biocompatible and cell-permeable. It provides a basis for cercariae imaging.

3.5. Cercaria imaging

Cell imaging experiments demonstrate that PZQ-5 is biocompatible and cell-permeable. The praziquantel derivative was used for labeling cercariae to observe the interaction between PZQ-5 and cercariae. The interaction between fresh cercariae and PZQ-5 (3 μM) was *in situ* observed using confocal fluorescence microscopy, as shown in Fig. 4. The PZQ-5 was taken up by cercariae through the tegument at 1 h, but its fluorescence intensity was very low (Fig. 4a). Then, the fluorescence intensity in the cercarial tegument continually increased during the period from 3 h to 4 h (Fig. 4b and c). Even if the test time is extended, PZQ-5 is still mainly located at the cercarial tegument.

According to the reference mentioned,[24] the pharmacological effects on schistosomes are as follows: excited activity, insect bodies contracture and cortex damage after praziquantel acts on them. Praziquantel not only influences physiological

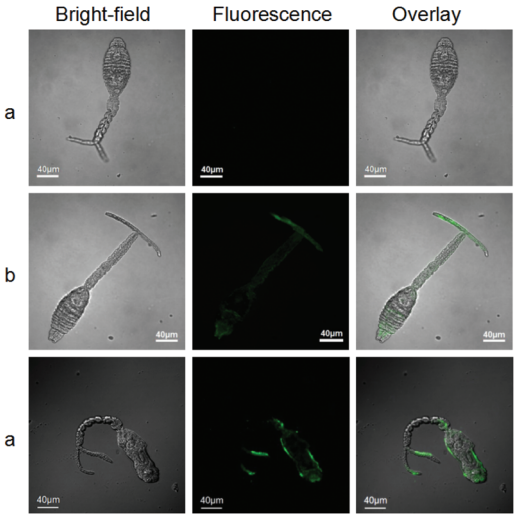


Fig. 4 Confocal bright-field, fluorescence and overlay images of cercariae incubated with PZQ-5 (3 μM) in PBS buffer at room temperature for 1 h (a), 3 h (b) and 4 h (c). All excitation wavelengths were 405 nm, and emission was collected at 480 to 580 nm. Scale bars = 40 μm.

functions, but also affects biochemical metabolism of schistosomes. The S. H. Xiao group has reported on the behavior of praziquantel acting on cercariae. The cercarial tegument had swelled 30 min after adding the drug. With extending the acting time to 2 h, the insect bodies would irregularly shrink and swell, and local erosion on the cortex also appeared. The TEM images showed that the cercarial tegument changed after a half-hour praziquantel treatment. The glycocalyx on the insect cercarial tegument had also greatly decreased. Two hours later, the glycocalyx even totally disappeared. The parenchyma cells on the cercarial body surfaces significantly changed as well. Some articles showed that praziquantel mainly destroyed the glycocalyx of the cercarial tegument, thus affecting the osmotic pressure of insects, making the insects unable to adapt to lack of isosmotic water.[25]

As a result, no obvious fluorescence signal was detected in the initial one hour, but the test time was extended, and a more significant fluorescence signal appeared in 3 h, especially in the head and tail. With extending the time to 4 h, the fluorescence of the cercariae tegument continued to increase. Like the changes mentioned above, we think that in 1 h the accumulation of drugs in the tegument was less as the drug action time was short, so the fluorescence signal could not be detected. With an increase of reaction time, the drug in the cercariae tegument gathered more, and the fluorescence signal also increased. According to all the results above, fluorescence signals focus mainly on the insects' epidermis; we could conclude that praziquantel possibly mainly acts on the cercarial tegument, thereby causing different morphological and biological metabolism transformations. Even though praziquantel has several effects on schistosomes such as on glycometabolism, ATP suppression and cholinesterase variation, we cannot obtain the same conclusions as described above yet.

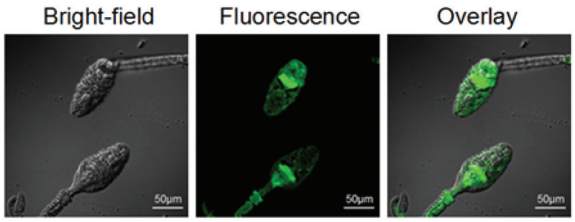


Fig. 5 Confocal bright-field, fluorescence and overlay images of cercariae incubated with compound **4** (5 μM) in PBS buffer at room temperature for 2 h. All excitation wavelengths were 405 nm and emission was collected at 480 to 580 nm. Scale bars = 50 μm.

To further understand the drug's action in cercariae, we also carried out a control experiment using fluorophore **4**. We selected images stained by compound **4** after 2 h (Fig. 5). The fluorescence signal of compound **4** was localized around the ventral sucker or the pre-acetabular gland and post-acetabular gland, but the fluorescence signal was remarkably weak in other parts (Fig. 5c). It is concluded that there is a big difference between the imaging of PZQ-5 and that of compound **4**.

4. Conclusion

In summary, the cytotoxicities of the novel derivatives of praziquantel PZQ-2, PZQ-3 and PZQ-5 are relatively low. PZQ-5 maintained excellent bioactivity against cercariae. Cell imaging experiments proved that PZQ-5 is biocompatible and cell-permeable. Fluorescence imaging experiments revealed that PZQ-5 is mainly located at the cercarial tegument. It is concluded that the praziquantel can influence or demolish the cercarial tegument, which may lead to a series of changes in morphological and biological metabolism for cercariae.

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