Novel Fluorescent Derivative of Praziquantel Interaction with Clonorchis sinensis Cercariae

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Abstract
Clonorchiasis is a common infection of dogs and other fish-eating carnivores (reservoir final hosts). Praziquantel is the only medicine, which has been recommended by WHO for treatment of clonorchiasis. To investigate the interaction between praziquantel and Clonorchis sinensis cercariae, two praziquantel derivatives (PZQ-2 and PZQ-3) and one praziquantel fluorescent derivative (PZQ-5) were have been synthesized and characterized by nuclear magnetic resonance spectroscopy (NMR) and MS spectra. Confocal fluorescence microscopy revealed that PZQ-5 is mainly located at cercarial tegument, which leads to the death of cercariae with the time increasing.

Keywords: Clonorchiasis; Cholangiocarcinoma; Parasitology; C. sinensis cercariae

Introduction
Clonorchiasis caused by Clonorchis sinensis is a fish-borne trematode infection, which is endemic in China, Korea, Japan, and other Southeast Asian countries, with approximately 35 million people being infected globally, of whom approximately 13 million were in China [1]. Epidemiologic and experimental studies have revealed that C. sinensis infections can induce biliary epithelial hyperplasia, periductal fibrosis and cystic changes in the ducts, and promote the development of cholangiocarcinoma [2,3]. Due to a high correlation between clonorchiasis and cholangiocarcinoma, C. sinensis was classified as a class 1 carcinogen at the meeting for the International Agency for Research on Cancer (IARC) in 2009 [4].

Praziquantel is the only medicine recommended by WHO for treatment of clonorchiasis. It is effective, safe and low-cost. WHO recommends carrying out community diagnosis at the district level, and implementing preventive chemotherapy with praziquantel [5].

The cercarial stage of the C. sinensis life-cycle is the important infectious stage. The cercarial stage is also the most fragile stage in the life cycle of C. sinensis. However, few papers focus on mechanism of praziquantel interaction with C. sinensis cercariae. In our intention paper, we will use fluorescence imaging technology to elucidate the preliminary interaction between praziquantel derivatives and C. sinensis cercariae.

Fluorescence imaging has been used as a powerful tool in the study of medicine and bioscience, including parasitology [6-8], especially in studying the interaction between drugs and parasites in vitro and in vivo [9]. Because the abounding of fluorescent probes promoted the development of fluorescent imaging technology. Currently, the available fluorescent dyes have been [10-16]: 1) organic fluorescent dyes, 2) phosphorescent metal complexes [12], 3) quantum dots [13], 4) fluorescent protein [14-15], 5) up-converting rare-earth nanophosphors [16]. Charlemagne Gnoula et al. [6] used 5(6)-carboxyfluorescein diacetate to label nematodes. The result has shown that only death nematodes could be labeled. Devon B. Keeney et al. [7] 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. [17] to detect NO in living schistosomes. These reports indicated fluorescence imaging technology is suitable for investigated interaction between drug and C. sinensis cercariae.

Herein, novel derivatives of praziquantel (Scheme 1, PZQ-2 and PZQ-3) were synthesized by means of nitrization and reduction reaction. To investigate the interaction between drug and C. sinensis 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 praziquantel derivative PZQ-3. The cell imaging have been further investigated.

**Experimental Section**

**Materials and instrumentation**

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

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

**Synthesis**

**Synthesis of PZQ-2:** PZQ-2 was synthesized as described previously [18,19]. 1H NMR (400 MHz, CDCl3), δ (ppm): 8.21 (s, 1H), 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). IR (KBr) cm⁻¹: 3352, 2927, 2854, 1648, 1509, 1422, 784, 748. MS: m/z 327.19.

**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 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/PE (v/v, 9:1), yielding a slightly white solid. 1H NMR (CDCl3), 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). IR (KBr) cm⁻¹: 3352, 2927, 2854, 1648, 1509, 1422, 784, 748. MS: m/z 327.19.

**Synthesis of PZQ-5:** 4 was synthesized according to the literature.2019 Compound 4 (0.099 g) was dissolved in CH2Cl2 (20 mL×3). The combined organic layer was washed with aqueous sodium bicarbonate, distilled water and dried over anhydrous Na2SO4, λex: 365 nm). Determined using quinine sulfate as a standard (yield=0.53, in 0.1 M H2SO4, λex: 365 nm).

**Cercaria imaging experiments**

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

**Results and Discussion**

**UV-vis and fluorescence emission spectra**

The photophysical properties of PZQ-5 were investigated before PZQ-5 was used to labeled cercariae. The UV-vis absorption and fluorescence emission spectra of PZQ-5 in a diluted solution of CH2Cl2 were studied and are shown in Figure 1. Derivative PZQ-5 exhibits a broad UV-vis band and maximal absorbance at wavelengths of 400 nm, corresponding to the π-π* transition of fluorophore [4]. Under excitation at 400 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 a standard.

**Toxicity test**

In order to study their cytotoxicity, the MTT assay was used to detect cell survival rate of PZQ-5. The result of MTT can be seen in supporting information. Herein, no sub-cellular apoptotic changes or significant cell death was seen in 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.

**Cercaria imaging**

In view of its favorable spectroscopic properties, PZQ-5 should...
be suitable for fluorescence imaging in living cells. The praziquantel derivative was used for labeling cercariae to observe the interaction between PZQ-5 and cercariae. The process of fresh cercariae labelled with PZQ-5 (5 μM) was in-situ observed using confocal fluorescence microscopy, as shown Figure 2 The PZQ-5 was taken up by cercariae through the tegument at 1 h, but its fluorescence intensity was very low (Figure 2a). Then, fluorescence intensity in the cercarial tegument continually increased in the period from 2 h to 3 h (Figure 2b and 2c). Even if the test time is extended, PZQ-5 is still mainly located at cercarial tegument.

From the reference mentioned [20] mentioned [21], the pharmacological effects of C. sinensis are cortex damage after praziquantel acting on them. It not only influences physiological function, but also affects some biochemical metabolism of C. sinensis. Pang H.L. group has reported the behavior of praziquantel acting on C. sinensis. 1 h after administration the glycogen content showed a slight decrease which became prominent 24h later and almost disappeared at 48h post-medication. There was an increase in protein content in the parenchymal tissues of worms 1 h after treatment, especially in the reproductive organ 24 h after treatment. RNA content was decreased 1 h post administration and continued decreasing gradually so that very little could be seen 48 h after. An increase in the activities of SDH, MDH and Ca-ATP was seen at the beginning and became marked 24 h and cholinesterase variation, we can't obtain same conclusions above here yet.

Conclusion
In summary, UV-vis and fluorescence emission spectra experiments proved that PZQ-5 have favorable spectroscopic properties. Fluorescence imaging experiments reveal that PZQ-5 is mainly located at cercarial tegument. It is concluded that the praziquantel can influence or demolish the cercarial tegument, which may lead to a series of change of morphology and biological metabolism for cercariae.

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