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Novel synthetic compounds with endoperoxide structure damage juvenile stage of *Schistosoma mansoni* by targeting lysosome-like organelles

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ABSTRACT

The new synthetic compound 1,2,6,7-tetraoxaspiro[7.11]nonadecan (N-89), a novel anti-malaria drug candidate, is also a promising drug candidate against schistosomiasis with killing effects against juvenile stage of *S. mansoni*. In order to investigate how N-89 kills schistosomes, we used a derivative of N-89, 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol (N-251), which enables us to conjugate with fluorescent reagents. Firstly, N-251 showed strong killing effects to larvae of *S. mansoni in vitro*. Ultrastructural analysis showed the disruptions of the lysosome-like organelles or the acetabular glands, followed by cytoplasmic lysis inside the worm body in N-251-treated group under electron microscopy. For rhodamine-conjugated N-251 and organelle markers, we observed that N-251 accumulated in acidic organelle. In addition, LysoTracker signals in these acidic organelles disappeared in N-251-treated group over time. Finally, we observed that the activity of cathepsin B, a lysosome-specific enzyme, was also decreased together with alternation of acidic organelle marker signal by N-251-treated group. These results suggested that our synthesized compounds induced the dysfunction or the disruption of acidic lysosome-like organelles and finally led to worm death.

1. Introduction

Human schistosomiasis is one of the Neglected Tropical Diseases (NTDs) caused by trematodes of genus *Schistosoma* such as *Schistosoma mansoni*, *S. japonicum*, *S. haematobium* and at least two other species, and spreads in Africa, South-America and Asia [1–3]. Currently, 779 million people are at risk of infection and at least 258 million people require preventive treatment for schistosomiasis [4,5]. Schistosome larvae penetrate human skin, followed by transformation to juvenile stage worms (schistosomula). Thereafter, they migrate through the circulatory system and finally reach the portal vein of the liver for *S. mansoni* or the vesicle venous plexus for *S. hematobium*, where they lay eggs after mating. These eggs are pathogenic to human host through induction of granulomatous inflammation and subsequent organ fibrosis [1,6]. Praziquantel (PZQ) has been the drug of choice for schistosomiasis in these past decades [7]. PZQ is effective mainly to adult stage of parasite, but less effective to juvenile stage [8,9], therefore, new drugs with high efficacy against juvenile stage parasites are long overdue. Previously, several researchers have shown the emergence of reduced susceptibility or resistance of schistosome to PZQ from field surveillance [10–14]. The possibility to induce resistant *S. mansoni* strains against PZQ under laboratory conditions has been suggested [15–17], thus, the development of new alternative drugs are indispensable.

On the other hand, many researchers have shown that artemisinin and its derivatives, the current drug of choice for malaria, have schistosomicidal effect against not only the adult stage but also the juvenile stage of schistosome [18–22], suggesting that artemisinin derivatives could have inhibition for the disease onset because parasites are killed before the start of egg-laying in the host. However, there are difficulties in the provision of artemisinin and its derivatives on large scale basis and at lower price, because they are purified from extract of natural plant, *Artemisia annua* [23].

N-89 and N-251 are newly developed synthetic compounds with simple endoperoxide structures in their chemical structures [24,25]. As was expected, N-89 and N-251 had high efficacies against malaria parasites *in vitro* as well as *in vivo* [24–26]. Moreover, N-89 also exhibited high efficacy against schistosome parasites *in vitro* and *in vivo*

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[27]. In brief, N-89 has high schistosomicidal effect to the juvenile stage of *S. mansoni* and repressive effect on egg production of female adult worm.

In this study, to investigate the drug action mechanisms of the synthetic compounds for their anti-schistosome activity, N-251 was used, which is a derivative of N-89 with hydroxyl residue in the chemical structure. By the use of N-251 conjugated with fluorescence dye, we observed the distribution of N-251 together with organelle markers. Finally, the direct effects of N-251 against the acidic organelles were observed based on ultrastructural (TEM) and fluorescence signal (LysoTracker Green and Magic Red cathepsin B) analysis.

2. Materials & methods

2.1. Parasites

S. mansoni (Puerto Rican strain) was maintained in our laboratory using ICR mice and *Biomphalaria glabrata*, intermediate host snails. For the preparation of schistosomula, cercariae were shed from infected intermediate host snails. Briefly, they were passed through a 20-gauge needle and their tails mechanically separated from the whole body to transform into schistosomula (mechanically transformed schistsomula) [28]. Obtained larvae were cultured in conditioned medium consisting of RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS (Gibco, USA), penicillin, streptomycin, L-glutamine (Gibco, USA), amphotericin B (Sigma-Aldrich, MO, USA) and gentamicin (Wako, Osaka, Japan) in 5% CO_2 incubator at 37 °C for 1 day before further experimental procedures.

2.2. Compounds and reagents

N-89 and N-251were chemically synthesized as described previously [24,25] and artemether was purchased from Sigma-Aldrich, (MO, USA). They were dissolved in dimethyl sulfoxide (DMSO) to 50 mM stock solutions and stored at −20 °C. Rhodamine-conjugated N-251 (N-251-R) was purchased from NARD Institute (Amagasaki, Japan). It was kept as 1 mM stock solution at -20 °C. Following the manufacturer's instruction, LysoTracker Green and MitoTracker Green (Molecular Probes, USA) was stored as 1 mM Stock solution at −20 °C. Magic Red cathepsin B Assay Kit (Immunochemistry Technologies, MN, USA) was dissolved in DMSO as a stock solution, according to the instruction manual. It was kept at −20 °C after aliquoting into small volumes for one time usage. Hoechst 33342 (Invitrogen, USA) was prepared for counter staining of the nucleus. Propidium Iodide (TONBO Biosciences, CA, USA) was prepared for dead worm staining.

2.3. Killing assay for S. mansoni schistosomula

We dissolved 50 mM of stock solution to final concentration of N-89/N-251 (50, 25, 12.5, 6.25, 3.125 and 1.5625 μM) or artemether (500, 250, 125, 62.5, 31.25 and 15.625 μM) in conditioned medium. For each concentration of artemether and N-89/N-251 used, >100 worms were utilized for the killing assay per drug concentration. The worms were cultured in the compounds for the entire 7 days without media change or addition of fresh compound, followed by evaluation of killing effect on the test samples. The number of larvae alive was counted every 24 h. The dead larvae were determined using 3 criteria: mobility, worm thickness change and tegmental damage, called killing criteria (Supplementary Table 1). Mobility was decided by the observation for 5 s under microscopy. No movement was a sign of worm damage. Collapsed worms with tegmental change indicated that the parasite had been injured. Worms that exhibited all the three criteria were defined as dead worms. Killing rate was calculated as number of dead worm for each day divided by the number of total worm alive observed before treatment. The value of EC_{50} in killing assessment was calculated with the R software ver. 2.15.3.

2.4. Uptake and localization of rhodamine-conjugated N-251 in schistosomula

The prepared schistosomula were cultured after treatment with N-251-R (1 μM) for 30 min followed by 3 times washing with conditioned medium. LysoTracker (75 nM) or MitoTracker (500 nM) was added at 2.5 h before observation. The living larvae were observed under LSM510 (Zeiss, Oberkochen, Germany) or SP8 (Leica, Welzlar, Germany) confocal laser microscopy.

2.5. The influence of N-251 on acidic organelle after the treatment

The schistosomula were treated with LysoTracker Green (150 nM) and Magic Red cathepsin B (4-fold diluted working concentration) for 2 h and 15 min before observation respectively. The living larvae were observed under confocal laser microscopy. The larvae treated with N-251 (50 μM) were cultured with LysoTracker Green (75 nM) or Magic Red cathepsin B (working concentration) as same procedure as above. Fluorescence images of parasites were obtained using LAS-AF-Lite software under confocal laser microscopy. Propidium iodide staining and killing criteria (mobility, worm thickness change and tegmental change) were used for determination of worm death. Signal intensity was quantified by the gradation value (0 to 256) and the gross area of parasite was measured by Image J software ver.1.49v. The intensity per 1 pixel (1 pixel intensity) of a worm was calculated with total gradation value inside the worm divided by gross area of parasite. Signal intensity was calculated as the total average value of 1 pixel intensity using >20 worms per group.

2.6. Ultrastructural analysis with transmission electron microscopy (TEM)

Schistosomula treated with 50 μM of N-251 for 2, 6 or 16 h were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4 °C for 24 h. The fixed samples were processed by modified cold dehydration method. This method enables detailed morphological observation of the extracellular matrices and cytoskeletons, as previously reported [29]. In brief, the samples were successively immersed in 0.4% OsO_4 in 0.1 M phosphate buffer for 1 h, 2% low molecular weight tannic acid (Electron Microscopy Sciences, PA, USA) in 0.05 M maleate buffer for 4 h, and 1% uranyl acetate in 0.05 M maleate buffer for 3 h. The samples were then dehydrated in a graded series of cold acetone, and embedded in epoxy resin (Oken Epok 812; Oken-shoji, Tokyo, Japan). Ultrathin silver-gold sections were cut with a diamond knife, and transferred to copper grids (50 mesh) that had been coated with Formvar membrane. After staining with uranyl acetate and lead citrate, the sections were observed with a JEM1230 transmission electron microscope (JEOL, Tokyo, Japan) and a H-7700 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan).

2.7. Statistical analysis

Statistical analysis was performed using the student *t*-test with $^{*}P < 0.05$ and $^{**}P < 0.001$ as the criterion of significance.

3. Results

3.1. Schistosomicidal effect of N-89 and N-251 in vitro

To confirm whether N-89 and N-251 directly affect the survival of schistosomula, we treated them with each compound for 7 days *in vitro*. Light microscopic images showed that there was schistosomicidal activity by the compounds over time and there was no significant difference in worm damage effects at 7 days post-treatment between N-89 and N-251 (Supplementary Fig. 1). Killing effects of both compounds against parasites were in a dose-dependent manner (Fig. 1A and B). All worms were killed by 50 μM