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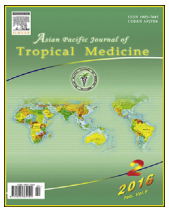

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Evaluation of anti-tubercular activity of linolenic acid and conjugated-linoleic acid as effective inhibitors against *Mycobacterium tuberculosis*

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ABSTRACT

Objective: To evaluate a new pharmacological activity/effect of linolenic acid (α- and γ-form) and conjugated-linoleic acid (CLA) causing antibacterial activity against *Mycobacterium tuberculosis* (Mtb).

Methods: The anti-Mtb activity/effect of linolenic acid and CLA were determined using different anti-Mtb indicator methods such as resazurin microtiter assay (REMA) and MGIT 960 system assay. The Mtb was incubated with various concentrations (12.5–200 μg/mL) of the compounds and anti-Mtb first-line drugs for 5 d in the REMA, and for 3 wk in MGIT 960 system assay.

Results: Linolenic acid and CLA obviously indicated their anti-Mtb activity/effect by strongly inhibiting the growth/proliferation of Mtb in a dose-dependent manner in the REMA and the MGIT 960 system assay. Interestingly, linolenic acid and CLA consistently induced anti-Mtb activity/effect by effectively inhibiting the growth/proliferation of Mtb in MGIT 960 system for 21 d with a single treatment, and their minimum inhibitory concentrations were measured as 200 μg/mL respectively.

Conclusions: These results demonstrate that linolenic acid and CLA not only have effective anti-Mtb activity/properties, but also induce the selective-anti-Mtb effects by strongly inhibiting and blocking the growth/proliferation of Mtb through a new pharmacological activity/action. Therefore, this study provides novel perspectives for the effective use of them and the potential that can be used as potent anti-Mtb candidate drugs, as well as suggests the advantage of reducing the cost and/or time for developing a new/substantive drug by effectively repurposing the existing drugs or compounds as one of new strategies for the global challenge of tuberculosis.

1. Introduction

The rapid emergence of tuberculosis (TB) is still causing a serious global challenge and difficulty including drug-resistant TB despite various global efforts such as hygiene education, environment improvement, and drug development. *Mycobacterium tuberculosis* (Mtb) is a major infectious factor causing the highest human mortality through the co-infection with HIV/AIDS as one of the most dangerous infectious bacteria globally. In 2013, 9.0 million people were estimated as new TB, 1.5 million died from the disease, and 360000 of them were HIV-positive. In addition, 1.1 million of the 9 million people diagnosed as new TB cases in 2013 were HIV-positive. Recently, TB cases co-infected with HIV/AIDS indicated the highest infectious rate in the African region compared with the other countries, particularly, in southern Africa. Furthermore, most of the estimated TB cases in 2013 occurred in Asia (56%) and the African region (29%), and the three countries of the largest number of TB cases were India (2.0

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million–2.3 million), China (0.9 million–1.1 million), and Nigeria (340 000–880 000) [1,2]. For these reasons, various studies for the treatment and/or the inhibition of TB that induces drug-resistance to existing agents have been carried out in the drug discovery field worldwide. Although various anti-TB drugs (from first-line drugs to third-line drugs) were developed for the treatment of TB patients, they have showed the limit in reducing the current TB patients. Recently, compounds such as PA 824, SQ 109, bedaquiline, and linezolid repurposed through chemical remodeling of the existing drugs as well as new compounds such as Q203, TMC 207, pyridomycin, and thiophenes are being tested in Phase I or Phase III trials as novel anti-TB drugs [1,3–7]. Until recently, in spite of the discovery of new targets and/or drugs for treating TB, the rapid emergence of TB, and multi-drug-resistant TB (MDR-TB) or extensively drug-resistant TB has still caused serious concerns in the public health field worldwide. For these reasons, various studies for developing the effective anti-TB drugs with novel mechanisms of action, low cytotoxicity and safety are urgently needed to block and/or to inhibit the TB. In this aspect, this study was carried out to evaluate anti-Mtb activity/effect/ability of linolenic acid (α- and γ-form) and conjugated-linoleic acid (CLA) that effectively act as bioactive substance, and to identify the potential for developing them as novel anti-tubercular drugs.

2. Materials and methods

2.1. Materials

Various materials used in this study, rifampicin, isoniazid, linolenic acid (α- and γ-form), CLA [conjugated(9Z,11E)-linoleic acid, conjugated(9E,11E)-linoleic acid, and conjugated(10E,12Z)-linoleic acid], resazurin powder and DMSO, were purchased from Sigma–Aldrich Chemical, Co., Ltd. (St, Louis, MO, USA), and MGIT™ 960 system indicator 7 mL growth media tubes with BACTEC™ MGIT™ 960 supplement kit were purchased from Becton–Dickinson and Company (Sparks, MD, USA). All other chemicals and reagents were purchased from Merck Chemical Co., Ltd. (Darmstadt, Germany) and Sigma–Aldrich Chemical Co., Ltd. (St. Louis, MO, USA).

2.2. Preparation of anti-Mtb drugs

The anti-Mtb first-line drug, isoniazid, was dissolved in sterile distilled water, and rifampicin was dissolved in DMSO, to a concentration of 50 mg/mL according to the manufacturer's instruction. The anti-Mtb first-line drugs were used as reference standard drugs. All compounds were filtered using 0.2 μm membrane syringe filter (Roshi Kaisha, Ltd., Tokyo, Japan) before use and stored at −80 °C deep-freezer until use.

2.3. Preparation and growth conditions of Mtb

Mtb $H37R_V$ (ATCC 27294) used in this study was purchased from American Type Culture Collection (Manassas, VA, USA). Mtb $H37R_V$ was grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% (V/V) oleic acid/albumin/dextrose/catalase enrichment (Becton–Dickinson and Company, Sparks, MD, USA) and 0.05% (v/v) Tween 80 (Sigma–Aldrich Chemical, St, Louis, MO, USA) to the log phase at 37 °C for 4–5 wk on shaking incubation of 100 rpm.

2.4. Drug susceptibility testing of Mtb

The *in vitro* anti-Mtb activity of linolenic acid (α- and γ-form) and CLA [conjugated(9Z,11E)-linoleic acid, conjugated(9E,11E)-linoleic acid, and conjugated(10E,12Z)-linoleic acid] against Mtb was confirmed by resazurin microtiter assay (REMA) using a 96-well micro-plate. Briefly, Mtb was grown in fresh Middlebrook 7H9 broth supplemented with 10% (v/v) oleic acid/albumin/dextrose/catalase enrichment and 0.05% (v/v) Tween 80 until the culture reached a turbidity equal to that of 1.0 McFarland standard (3.0×10^8 CFU/mL) at 37 °C. The bacteria were adjusted to a density of 2×10^6 CFU/mL in fresh culture broth. Finally, the bacterial suspensions were inoculated into all wells of a 96 well microtiter plate containing final concentrations (12.5–200 μg/mL) of linolenic acid (α- and γ-form), CLA [conjugated(9Z,11E)-linoleic acid, conjugated(9E,11E)-linoleic acid, and conjugated(10E,12Z)-linoleic acid] and anti-TB first-line drugs (1.25–5 μg/mL), and Mtb growth controls containing no anti-Mtb first-line drugs and blank controls without inoculation were also included. The 96 well-plates, covered with lids, placed in a plastic bag, were incubated at 37 °C for 5 d. After incubation, 20 μL of freshly prepared 0.05% (w/v) resazurin solution was added to all wells of a 96 well microtiter plate, and the plates were re-incubated at 37 °C for 36 h. A change in color from blue to pink indicating bacterial growth was observed after 36 h of incubation. The minimum inhibitory concentration (MIC) was expressed as the lowest concentration of the drug that inhibited Mtb growth or prevented change in color of the resazurin from blue to pink based on a REMA.

2.5. Evaluation of drug susceptibility of Mtb by MGIT 960 system assay

To evaluate anti-Mtb effects of linolenic acid (α- and γ-form) and CLA [conjugated(9Z,11E)-linoleic acid, conjugated(9E,11E)-linoleic acid, and conjugated(10E,12Z)-linoleic acid] against the growth/proliferation of Mtb, the drug susceptibility testing of the strain was performed using the BACTEC™ MGIT 960 system (Becton Dickinson and Company, Sparks, MD, USA.). In brief, 100 μL of a suspension of Mtb culture, adjusted to 9.6×10^6 CFU/mL, was inoculated in an MGIT growth media tube with BACTEC™ MGIT 960 growth supplement (Becton Dickinson and Company), which were incubated with different concentrations (12.5–200 μg/mL) of the tested compounds and anti-Mtb first-line drugs (10 μg/mL), and Mtb growth controls containing no anti-Mtb first-line drugs were also included. They were incubated into the BACTEC™ MGIT 960 system device for 3 wk for determination of Mtb drug susceptibility.

2.6. Statistical analysis

All results were expressed as mean ± standard deviation of three independent experiments. Statistical analysis of the data was performed using the Student's *t*-test and one-way analysis of variance.