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Inhibition of HIV-1 entry by the tricyclic coumarin GUT-70 through the modification of membrane fluidity

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ABSTRACT

Membrane fusion between host cells and HIV-1 is the initial step in HIV-1 infection, and plasma membrane fluidity strongly influences infectivity. In the present study, we demonstrated that GUT-70, a natural product derived from *Calophyllum brasiliense*, stabilized plasma membrane fluidity, inhibited HIV-1 entry, and down-regulated the expression of CD4, CCR5, and CXCR4. Since GUT-70 also had an inhibitory effect on viral replication through the inhibition of NF-κB, it is expected to be used as a dual functional and viral mutation resistant reagent. Thus, these unique properties of GUT-70 enable the development of novel therapeutic agents against HIV-1 infection.

1. Introduction

Membrane fusion between host T cells and human immunodeficiency virus type 1 (HIV-1) is one of the essential steps in HIV-1 infection. HIV-1 enters cells by binding of the HIV-1 envelope protein, gp120 and CD4 on CD4+ T cells or macrophages as a trigger of infection, and gp120 then interacts with the chemokine receptors CXCR4 and CCR5, leading to infection by HIV-1 [1]. HIV-1 destroys CD4+ T cells post-infection through a stage of a relatively long latent period [2–4]. Combination antiretroviral therapy (cART), which combines two reverse transcriptase inhibitors and protease inhibitors or integrase inhibitors, targets the virus life cycle and has successfully reduced the morbidity and mortality caused by HIV-1 infection [5,6]. Although HIV-1 infection can be controlled for prolonged periods, there is currently no fundamental treatment to eradicate the virus from the body. Moreover, the emergence of drug-resistant viruses is also one of the main difficulties associated with the treatment of HIV-1 infection. Novel therapeutic approaches that are distinct from those of currently available anti-retroviral drugs are essential for the continual control of HIV-1 infection.

The genus *Calophyllum* comprises ca. 180 tree species with a pantropical distribution, and dipyrano-tetracyclic coumarins that are active against HIV-1 have been isolated from several *Calophyllum* species. Previous studies on *Calophyllum* species including *Calophyllum brasiliense* demonstrated their inhibiting effects on HIV-1 reverse transcriptase and viral replication [7–9]. The newly discovered anti-tumor agent GUT-70, a natural product derived from the stem bark of *C. brasiliense*, has been characterized as a tricyclic coumarin with the formula 5-methoxy-2, 2-dimethyl-6-(2-methyl-1-oxo-2-butenyl)-10-propyl-2*H*,8*H*-benzo[1,2-*b*;3,4-*b*0] dipyran-8-one($C_{23}H_{26}O_5$). GUT-70 exhibits cytotoxic effects against human leukemia [10] and lymphoma [11]. We also reported that GUT-70 inhibited HIV-1 replication in chronically infected cells via suppression of the NF-κB pathway [12]. Thus, the multiple functions of GUT-70 may be applied to the treatment of both infectious diseases and malignancies.

Viral entry is known to be influenced by cell membrane fluidity [13] and previous studies reported that glycyrrhizin and cepharanthine, derived from natural products, affected HIV-1 infectivity by modifying membrane fluidity [13–15]. Since GUT-70 decreased cell membrane fluidity, we herein focused on the effects of GUT-70 against the HIV-1 entry process. The results obtained clearly demonstrated that GUT-70 inhibited HIV-1 viral entry by reducing cell membrane fluidity.

Abbreviations: HIV-1, human immunodeficiency virus-1; cART, combination antiretroviral therapy; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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2. Materials and methods

2.1. Preparation of GUT-70

GUT-70 used in the present study was synthesized by Nippon Shinyaku Co. (Kyoto, Japan), its structure was confirmed with NMR and an elemental analyzer, and its purity was confirmed with HPLC (purity 97.72%) [10,12].

2.2. Cell lines and culture

The human T cell lines, MOLT-4, $Jurkat_{HXBc2\ (4)}$, and $Jurkat_{522F/Y}$ were obtained through the NIH AIDS Research and Reference Reagent Program (Germantown, MD). PM1-CCR5 cells stably expressing human CCR5 were a gift from Dr. Y. Maeda (Kumamoto Univ., Kumamoto, Japan) [16]. MOLT-4 and PM1-CCR5 cells were maintained in RPMI-1640 medium (Gibco, Gaithersburg, MD) supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml, and 10% fetal bovine serum (HyClone Laboratories, Logan, UT). These cells were cultured in a 5% CO_2 humidified incubator at 37 °C. $Jurkat_{HXBc2\ (4)}$ and $Jurkat_{522F/Y}$ were stably transfected with the Env gene from the $HIV\text{-}1_{HXBc2}$ strain. $Jurkat_{HXBc2\ (4)}$ expresses a functional gp120/gp41 glycoprotein(*Env*), while $Jurkat_{522F/Y}$ contains an F/Y substitution at position 522 in gp41 that prevents fusion [17]. These cell lines were cultured in RPMI-1640 supplemented with 10% FBS (Gibco BRL), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml tetracycline, 200 μg/ml G418, and 200 μg/ml hygromycin. The expression of Env was induced by the removal of tetracycline by washing cells with PBS, and culturing for 3 days in medium without tetracycline before the fusion experiments.

2.3. Measurement of cell membrane fluidity

Cell membrane fluidity was measured using a spectrophotofluorometer (F4500, HITACHI, Japan) on the basis of the fluorescent depolarization method [18,19]. Briefly, MOLT-4 and PM1-CCR5 T cell lines (2.5×10^6 cells) were labeled with DPH (1,6-diphenyl-1,3,5-hexatriene, Wako, Japan) at a final concentration of 2×10^{-6} M and were incubated at 37 °C under light-shielded conditions for 30 min. After labeling with DPH, cells were washed with PBS and 2.5×10^5 cells/ml was prepared using PBS. GUT-70 was added to cells at final concentrations of 0, 10, 50, and 100 μM. The fluorescence polarization degree (P value) was measured for 5 min following the addition of GUT-70.

2.4. Cell–cell fusion inhibition assay

Env-expressing cell lines ($Jurkat_{HXBc2\ (4)}$ and $Jurkat_{522F/Y}$) and the MOLT-4 T cell line were labeled with the PKH67 Green Fluorescent Cell Linker Kit (Sigma, St. Louis, MO) and PKH26 Red Fluorescent Cell Linker Kit (Sigma), respectively. MOLT4 cells were treated with GUT-70 at final concentrations of 0–10 μM for 1 h. The two cell populations were then co-cultured at a ratio of 1:1 for 24 h. Cells were analyzed using an LSR II flow cytometer (BD Bioscience, San Jose, CA). PKH67 and PKH26 double positive cells were defined as fused cells. Data were analyzed with FlowJo (Tree Star, San Carlos, CA) software. Cells were also observed using fluorescent microscopy (Biozero, KEYENCE, Japan) after 48 h.

2.5. Virus infection and flow cytometric detection of HIV-1 infected cells

293T cells were maintained in DMEM containing 10% FCS. The virus was produced from 293T cells by transfection with the proviral plasmid $HIV\text{-}1_{NL4\text{-}3}$ (NIH AIDS Research and Reference Reagent Program) or JR-FL (kindly provided by Prof. Y. Koyanagi, Kyoto Univ., Kyoto Japan) using Hily Max (Dojin Chemical, Kumamoto, Japan). The culture supernatant was collected, the p24 value was measured using the p24 antigen ELISA kit (ZeptoMetrix Corp., Buffalo, NY), and it was then stored at −80 °C until use.

2.6. Virus infection and flow cytometric detection of HIV-1 infected cells

The PM1-CCR5 T cell line (5×10^5 cells/ml) was treated with GUT-70 at final concentrations of 0–100 μM and was then incubated for 10 min at 37 °C. Cells were infected with $HIV\text{-}1_{NL4\text{-}3}$ (X4 tropic) (p24 concentration; 25 ng/ml) or $HIV\text{-}1_{JR\text{-}FL}$ (R5 tropic) (p24 concentration; 50 ng/ml) for 1 h, washed twice with PBS, and then cultured for 48 h. HIV-1 infected cells (intracellular p24 positive) were detected by flow cytometry [20]. Briefly, cells were stained with anti-human CD4-APC mAb (clone: RPA-T4) (BioLegend, San Diego, CA). After 30 min of being incubated on ice, cells were washed twice with washing medium, fixed with 1% paraformaldehyde/PBS for 20 min in the dark, and permeabilized with 0.1% saponin/PBS. After 10 min of being incubated on ice, cells were stained with anti-HIV-1 Gag p24-FITC mAb (Beckman Coulter, Fullerton, CA) for 30 min on ice. Cells were then analyzed on an LSR II flow cytometer. Data were analyzed with FlowJo software.

2.7. Quantification of HIV-1 p24 in cell culture supernatants

The amount of the p24 antigen in cell culture supernatants was determined using an HIV-1 p24 antigen ELISA kit [20].

2.8. Quantitative viral DNA analysis

Total DNA was extracted to detect HIV-1 proviral DNA by quantitative-PCR [21,22]. Quantitative PCR analyses for HIV-1 Gag and internal controls human GAPDH were performed using the SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and StepOne real-time PCR system (Applied Biosystems). Primer sequences were as follows: HIV Gag: 5′-AGTGGGGGGA-CATCAAGCAGCCATGCAAAT, 5′-TACTAGTAGTTCCTGCTATGT-CACTTCC; hGAPDH: 5′-CGGGAAGCTTGTGATCAATGG, 5′-GGCAGTGATGGCATGGACTG.

2.9. Entry assay

The entry process of virions into cells was quantitatively analyzed by Gag p24 ELISA, as previously described [23,24]. Briefly, PM-1 CCR5 T cell lines (5×10^5 cells/ml) were treated with GUT-70 for 10 min, infected with $HIV\text{-}1_{NL\text{-}43}$ and $HIV\text{-}1_{JR\text{-}FL}$ for 1 h at 37 °C, trypsinized for 5 min, washed extensively, lysed with NP-40, and the internalized virion HIV-1 Gag p24 protein was then measured using a HIV-1 p24 antigen ELISA kit (ZeptoMetrix Corp., Buffalo, NY).

2.10. Flow cytometric analysis of receptor expression

PM1-CCR5 T cell lines (5×10^5 cells/ml) were treated with GUT-70 at final concentrations of 0, 50, and 100 μM and were incubated for 1 h at 37 °C. Cells were washed twice with washing medium for extracellular or intracellular staining. The extracellular antigens were stained with anti-human CD4-Pacific Blue mAb (clone: RPA-T4), anti-human CXCR4-APC mAb (clone: 12G5) (BioLegend), and anti-human CCR5-PE mAb (clone: HEK/1/85a) (BioLegend) for 30 min on ice. Cells were fixed with 1% paraformaldehyde/PBS for 20 min in the dark, and permeabilized with 0.1% saponin/PBS. After