BIOCHEMICAL PHARMACOLOGY 75 (2008) 2165–2174

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

A cyclopentanediol analogue selectively suppresses the conserved innate immunity pathways, *Drosophila* IMD and TNF-α pathways

Mizuki Sekiya [1], *Kazunori Ueda* [1], *Kaori Okazaki*, *Haruhisa Kikuchi*, *Shoichiro Kurata* [*], *Yoshiteru Oshima* [*]

Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aza-Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan

ARTICLE INFO

Article history:
Received 23 October 2007
Accepted 7 December 2007

Keywords:
Cyclopentanediol analogue
Drosophila
Immune suppressor
Innate immunity
NF-κB
TAK1 kinase

ABSTRACT

Innate immunity comprises evolutionarily conserved self-defense mechanisms against microbial infections. In mammals, innate immunity interacts with adaptive immunity and has a key role in the regulated immune response. Therefore, innate immunity is a pharmaceutical target for the development of immune regulators. Using *Drosophila ex vivo* culture systems, we isolated a cyclopentanediol analogue from *Aspergillus* sp. as an immunosuppressive substance. This compound selectively suppressed activation of the IMD pathway in *Drosophila in vivo* and the target molecules of the compound lie between the Imd adaptor protein and dTAK1 kinase in the IMD pathway. In human cells, the compound suppressed TNF-α, but not IL-1β, stimulation-induced activation of NF-κB, suggesting that its target molecules are upstream of TAK1 in mammalian innate immunity.

1. Introduction

Innate immunity is the front line of self-defense against microbial infections [1,2]. In mammals, innate immunity has an instructive role in adaptive immune responses specific to antigens by inducing co-stimulatory molecules and cytokines, indicating that a concerted and interactive innate and adaptive immune reaction is key for the regulated immune response that is essential for the preservation of homeostasis [1,3]. Therefore, from a pharmaceutical point of view, innate immunity is a good target for the development of immune regulators to suppress unwanted immune responses, such as septic shock, inflammatory diseases, and autoimmunity, and to stimulate protective immune responses to some of the diseases that largely elude the immune system, such as infectious diseases and cancer. *Drosophila melanogaster* is a model organism for genetic and molecular studies of innate immunity because of the striking conservation between the

* *Corresponding authors*. Tel.: +81 22 795 5916; fax: +81 22 795 6802.
E-mail addresses: kurata@mail.pharm.tohoku.ac.jp (S. Kurata), oshima@mail.pharm.tohoku.ac.jp (Y. Oshima).

[1] These authors contributed equally to this study.

Abbreviations: Dif, dorsal related immune factor; Dpt, Diptericin; Drs, Drosomycin; HREIMS, high resolution electron ionization mass spectrometry; HUVECs, human umbilical vein endothelial cells; IκB, inhibitor of κB; IKK, IκB kinase; IL, interleukin; IMD, immune deficiency; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; NF-κB, nuclear factor κB; PGRP, peptidoglycan recognition protein; TAB, TAK1-binding protein; TAK, TGF-β activated kinase; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, TNF receptor associated factor.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.12.020

mechanisms that regulate insect immunity and mammalian innate immunity [2,4].

In *Drosophila*, several antimicrobial peptides are secreted into the hemolymph from the fat body, the functional equivalent of the mammalian liver, by two distinct signaling pathways, immune deficiency (IMD) and Toll pathways, predominantly in response to Gram-negative bacterial infections and to fungal and Gram-positive bacterial infections, respectively [2,4]. The Toll pathway and the IMD pathway are similar to the Toll-like receptor (TLR)/interleukin-1 (IL-1) receptor signaling pathways and tumor necrosis factor (TNF)-α receptor signaling pathway, respectively, in mammals [5,6]. The IMD pathway regulates the synthesis of several antibacterial peptides, such as Diptericin (Dpt), which is mediated by cell surface peptidoglycan recognition protein (PGRP)-LC, Imd adaptor protein, dTAK1 kinase, *Drosophila* IκB kinase (dmIKK) complex, Dredd caspase, and a Rel transcriptional factor, Relish. The TNF-α signaling pathway consists of a receptor-interacting protein, TAK1 kinase, IKK complex, caspase-8, and nuclear factor κB (NF-κB); similar to Imd, dTAK1, dmIKK complex, Dredd, and Relish, respectively, in the *Drosophila* IMD pathway [7]. The Toll pathway regulates the induction of antimicrobial peptides such as Drosomycin (Drs), which is mediated by the Toll receptor, dMyD88 adaptor protein, and Dorsal/Dorsal related immune factor (Dif); similar to TLR, MyD88, and NF-κB in the mammalian TLR/IL-1 signaling pathway [6]. Previously, based on the striking conservation of innate immunity, we established an *ex vivo* whole larval culture system to evaluate the activity of compounds acting on innate immunity [8]. The system is not only useful for identifying compounds that act specifically on innate immunity, including mammalian innate immune responses, but also, when combined with various *Drosophila* mutants, for determining the target molecules affected by the compounds [9]. The insect system, which lacks mammalian-type adaptive immunity, also has other advantages for identifying compounds that act on innate immunity without influencing adaptive immunity.

In this study, we screened secondary metabolites of microorganisms for compounds that suppress or enhance innate immune reactions using the *ex vivo* system and isolated a cyclopentanediol analogue, *c*-4-((*E*)-1-propenyl)-*r*-1,*c*-2-cyclopentanediol, from *Aspergillus* sp. as an innate immune suppressor. This compound selectively suppressed the *Drosophila* IMD pathway and the human TNF-α signaling pathway.

2. Materials and methods

2.1. Isolation of *c*-4-[(*E*)-1-propenyl]cyclopentane-*r*-1,*c*-2-diol

Aspergillus sp. TF-0770 was washed with *n*-BuOH to give an extract (7.0 g), which was then partitioned with EtOAc and H_2O to yield EtOAc solubles (5.5 g). The EtOAc solubles were chromatographed over SiO_2, and the column was eluted with hexane–EtOAc solutions of increasing polarity. The hexane–EtOAc (1:2) eluent (543 mg) was further chromatographed over octadeca silica using H_2O to give crude *c*-4-[(*E*)-1-propenyl]cyclopentane-*r*-1,*c*-2-diol. The crude sample was purified by SiO_2 column chromatography using $CHCl_3$–MeOH (49:1) to give *c*-4-[(*E*)-1-propenyl]cyclopentane-*r*-1,*c*-2-diol (19.6 mg). The data obtained for *c*-4-[(*E*)-1-propenyl]cyclopentane-*r*-1,*c*-2-diol were: colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.33–5.49 (2H, m), 3.97–4.04 (2H, m), 2.48–2.64 (2H, br.s), 2.34–2.42 (1H, m), 2.07–2.15 (2H, m), 1.64 (3H, d, *J* = 5.1 Hz), 1.48 (2H, ddd, *J* = 13.6, 9.6, 6.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 135.4, 123.5, 73.1 (2C), 38.7, 36.8 (2C), 17.8; HREIMS *m*/*z* 142.0983 $[M]^+$ (142.0993 calcd. for $C_8H_{12}O_2$).

2.2. Antibodies

Anti-IκB antibody, anti-phospho-IκB-α (Ser32) antibody, anti-IKKβ antibody, anti-phospho-IKKα/β (Ser176/180) antibody, and horseradish peroxidase-conjugated anti-rabbit IgG antibody were purchased from Cell Signaling Technology (Beverly, MA). Anti-actin antibody, anti-p65 antibody, anti-TRAF2 antibody, anti-TAB2, anti-TAK1 antibody, horseradish peroxidase-conjugated anti-mouse IgG antibody, and horseradish peroxidase-conjugated anti-goat IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cy3-conjugated IgG antibody was purchased from Jackson ImmunoResearch (West Grove, PA).

2.3. Fly strains

Drosophila stocks were raised on standard cornmeal/agar medium at 25 °C. The transgenic strains *Dpt-lacZ*, *UAS-PGRP-LCx*, *GS9049*, *UAS-dTAK1*, and *UAS-Relish* were described previously [10–13]. Oregon R (wild-type), *hs-GAL4*, and *UAS-lacZ* transgenic strains were obtained from the Bloomington Stock Center (Bloomington, IN).

2.4. Ex vivo larval culture assay and cytotoxicity assay

The detailed procedure was described previously [8]. Briefly, third-instar larvae were washed with lipopolysaccharide (LPS)-free water and LPS-free saline. The abdominal cavity of the larva was opened using fine pincettes in LPS-free saline. Individual whole larval tissues were cultured in Schneider's *Drosophila* medium (Gibco-BRL, Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (FBS; Valley Biomedical, Winchester, VA) and 1% antibiotics/antimycotics (Gibco-BRL) in each well of a 96-well plate at 25 °C. For each condition, six females were cultured to produce six replicates. The cyclopentanediol analogue was dissolved in DMSO and added to the culture medium. To determine the effects of the cyclopentanediol analogue on the innate immune response, *Dpt-lacZ* larvae were cultured in the presence of 10 μg/mL LPS (Nacalai Tesque, Kyoto, Japan) and this compound at 25 °C for 12 h. The expression of *Dpt-lacZ* is thought to be induced by diaminopimelic acid-containing peptidoglycans contaminating the LPS fraction [14]. To determine the effects of the compound on the heat shock response, it was added to the culture medium of *hs-GAL4/UAS-lacZ* larvae under heat shock conditions (37 °C, 30 min). The cultured individual larvae were sonicated with 200 μL reaction buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgCl_2$) using an Ultrasonic Processor (Misonix, New York, NY). After centrifugation (10,000 × *g*) at 4 °C for 10 min, β-galactosidase activity and total protein amount were determined as pre-

viously described [8]. β-Galactosidase activity was standardized against total protein amount.

Drosophila S2 cells were cultured in Schneider's medium (Gibco-BRL) supplemented with 20% FBS (Valley Biomedical) and 1% antibiotics/antimycotics (Gibco-BRL) at 25 °C. Cytotoxicity was measured using the colorimetric thiazoyl blue conversion assay as described previously [9].

2.5. Infection experiments

Bacterial infections were performed by challenging adult flies with a thin tungsten needle previously dipped into a concentrated culture of either *Erwinia carotovora* (Ecc15) or *Micrococcus luteus* (IFO13867). The wild-type flies were fed a 4% sucrose solution containing 1 mg/mL of the cyclopentanediol analogue according to the protocol indicated in Fig. 3A. Survival experiments were performed with 30 flies at 25 °C. Surviving flies were transferred daily into fresh vials.

2.6. Quantitative RT-PCR

Total RNA was isolated from 30 adult flies 8 h after infection using Trizol reagent (Gibco/BRL) and dissolved in 20 μL of RNase-free water. Total RNA (1 μg) was used in 20 μL of reverse transcription reaction buffer using ReverTraAce reverse transcriptase (Toyobo, Osaka) and oligo (dT) 15 primer (Promega, Madison, WI). The first-strand cDNA (0.5 μL) was used as a template for the quantitative reverse transcription-polymerase chain reaction (PCR). Real-time PCR was performed with a LightCycler (Roche Diagnostics, Somerville, NJ). PCR specificity was confirmed by the molecular weight of the PCR products and melting curve analysis at each data point. The copy numbers of RNA encoding *Diptericin* and *Drosomycin* were standardized against that of the RNA encoding *rp49* in each sample. The following primers were used: *Diptericin*, 5′-GTTCACCATTGCCGTCGCCTTAC-3′, 5′-CCCAAGTGCTGTCCATATCCTCC-3′; *Drosomycin*, 5′-TTGTTCGCCCTCTTCGCTGTCCT-3′, 5′-GCATCCTTCGCACCAGCACTTCA-3′; *rp49*, 5′-AGATCGTGAAGAAGCGCACCAAG-3′, 5′-CACCAGGAACTTCTTGAATCCGG-3′.

2.7. Chemokine assay

The detailed procedure was described previously [9]. Briefly, human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (Walkersville, MA), cultured in 25-cm^2 culture flasks, and maintained in Humedia EB2 medium (Kurabo, Osaka, Japan). The culture medium was supplemented with 2% FBS, 0.1% gentamicin-amphotericin B solution, 10 ng/mL of human epidermal growth factor, 1 μg/mL hydrocortisone, 5 ng/mL human fibroblast growth factor-B, and 10 μg/mL heparin (Kurabo) at 5% CO_2, 37 °C. The cells were incubated in the presence or absence of the compound in a final volume of 100 μL for 3 h at 5% CO_2, 37 °C, and then 11 μL of 10 ng/mL *h*TNF-α or IL-1β was added to each well. After 12 h of incubation at 5% CO_2 and 37 °C, the HUVEC culture supernatants were harvested for enzyme-linked immunosorbent assay. Commercial enzyme-linked immunosorbent assay kits were used for immunologic quantification of *h*IL-8 and MCP-1 (Biosource, Invitrogen).

2.8. Immunocytochemistry

The cells were incubated in the presence or absence of 50 μg/mL of the compound for 3 h on culture slides (Falcon, BD Biosciences, San Jose, CA). After 1 ng/mL *h*TNF-α stimulation, the cells were washed twice with ice-cold 8.1 mM phosphate-buffered saline. For fixation and permeabilization, cells were treated with 4% paraformaldehyde and methanol. The cells were washed and blocked with 10% serum, and incubated with an anti-p65 antibody for 12 h at 4 °C. After washing, the cells were incubated with a Cy3-conjugated secondary antibody for 1 h at 4 °C. Nuclei were then stained with 0.1 μg/mL DAPI.

2.9. Quantification of NF-κB activation

Nuclear extracts were prepared from cells incubated in the presence or absence of 50 μg/mL of the compound for 3 h using a nuclear extract kit (Marligen Biosciences Inc., Ijamsville, MD). NF-κB activity in the nuclear extracts was quantified using an NF-κB Transcription Factor Microplate Assay (Marligen Bioscience Inc.) based on the specific binding of transcription factors to cognate sequences on labeled probes. The assays were performed according to the manufacturer's protocol.

2.10. Immunoprecipitation and immunoblotting

Cells were incubated in the presence or absence of 50 mg/mL the cyclopentanediol analogue for 3 h in 21-cm^2 Primaria™ cell culture dishes (Falcon, Becton Dickinson AG, Basel, Switzerland). After stimulation with 1 ng/mL *h*TNF-α, the cells were washed twice with ice-cold 8.1-mM phosphate-buffered saline (PBS) and lysed in 0.2 mL 2% sodium dodecyl sulfate sample buffer containing 10% glycerol, 62.5 mM Tris–HCl (pH 6.8), 2% 2-mercaptoethanol, and 0.01% bromophenol blue. Cellular debris was removed by centrifugation at 10,000 × *g* for 10 min. For immunoprecipitation studies, the cells were lysed in 20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Immunoprecipitation was performed using Catch and Release™ kits (Upstate, Lake Placid, NY) as described by the manufacturer. Rabbit anti-TAK1 antibody was added to cell lysates and incubated for 40 min at room temperature. The beads were washed three times and resuspended in SDS sample buffer. For immunoblotting, samples were resolved on SDS-PAGE and transferred to Hybond-P membranes (Amersham Biosciences UK Ltd.). The membranes were immunoblotted with the appropriate antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (Amersham Biosciences).

3. Results

3.1. Identification of a cyclopentanediol analogue as an innate immune suppressor

As microorganisms produce a variety of small molecules with unique structures, they are thought to be ideal sources of

novel bioactive substances. Some microorganisms produce compounds that regulate innate immunity to protect themselves from host defense systems. In this study, we searched among microbial secondary metabolites for compounds that suppress or enhance innate immunity using a *Drosophila ex vivo* system. In the *ex vivo* culture, the IMD pathway-dependent response is monitored by a reporter gene, *Dpt-lacZ*, constructed with the promoter regions of *Dpt* and *lacZ* encoding β-galactosidase. We screened 692 microorganism extracts, 4 of which selectively suppressed LPS fraction-mediated *Dpt-lacZ* activation. No enhancing activity was detected in the screening of these extracts. A bioactive *n*-BuOH extract of *Aspergillus* sp., TF-0770, was partitioned between H_2O and EtOAc, and the resulting EtOAc-soluble fraction had suppressor activity. The active compound was purified by bioassay-guided fractionation using SiO_2 and octadeca silica column chromatographies. The compound was identified as *c*-4-[(*E*)-1-propenyl]cyclopentane-*r*-1,*c*-2-diol by comparison of its spectroscopic data to those of the described molecule [15] (Fig. 1A). The cyclopentanediol analogue suppressed the LPS fraction-mediated activation of *Dpt-lacZ* in a dose-dependent manner, but did not suppress heat shock-mediated activation of *lacZ* or *Drosophila* S2 cell viability (Fig. 1B). Pretreatment of larvae with the cyclopentanediol analogue for 3 h before the stimulation enhanced suppression of the LPS-fraction mediated activation of *Dpt-lacZ* (Fig. 1C). The heat shock-mediated activation of *lacZ* was not affected by pretreatment with the cyclopentanediol analogue. These results indicated that the cyclopentanediol analogue selectively suppressed *Drosophila* innate immunity in the *ex vivo* culture. The cyclopentanediol analogue was synthesized to produce a sufficient quantity of sample for biologic evaluation (the synthesis will be described elsewhere). The synthetic cyclopentanediol analogue was identified by comparing its physicochemical data (^{1}H NMR, ^{13}C NMR, and HREIMS) with data of the isolated and previously reported cyclopentanediol analogues [15]. Moreover, the synthetic compound had the same bioactivity as that of the cyclopentanediol analogue isolated from *Aspergillus* sp. (IC50: synthetic compound, 7.1 μg/mL; natural compound, 6.5 μg/mL). The synthetic compound was used for the following experiments. The cyclopentanediol was detected by TLC analysis of the chloroform-extracted larval tissue fraction following three washes with PBS (data not shown).

3.2. *Identification of targets of the cyclopentanediol analogue using the ex vivo system with transgenic Drosophila larvae*

Previously, we demonstrated that the established system is useful for identifying the target molecules of compounds by targeted activation of the IMD pathway [9] (Fig. 2A). In the absence of microbial infections, *Dpt* expression is induced by the overexpression of components of the IMD pathway, such as PGRP-LC, Imd, dTAK1, and Relish in *Drosophila* [9–12,16]. In addition to the LPS fraction-mediated activation of *Dpt-lacZ*, the activation of *Dpt-lacZ* induced by the overexpression of *PGRP-LCx* and *imd* coding factors upstream of dTAK1 was inhibited by the cyclopentanediol analogue in a dose-dependent manner; the activation of *Dpt-lacZ* induced by the overexpression of *dTAK1* and *Relish*, encoding a transcriptional factor downstream of dTAK1, however, was not inhibited by the cyclopentanediol analogue (Fig. 2B). These results indicated that the target molecules of the cyclopentanediol analogue lie between the Imd adaptor protein and dTAK1 kinase in the *Drosophila* IMD pathway.

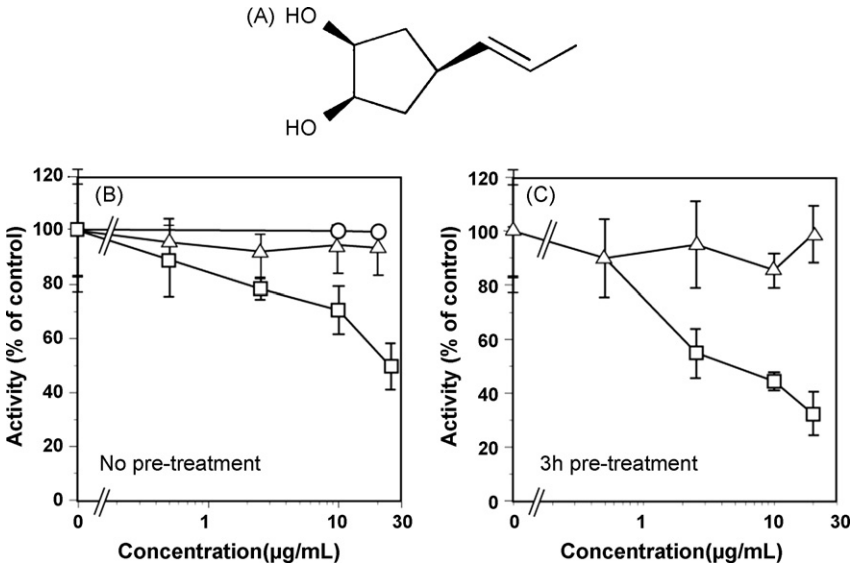

Fig. 1 – *Drosophila*-based screen for small molecules and the identification of a cyclopentanediol analogue that affects innate immunity. (A) The structure of *c*-4-[(*E*)-1-propenyl]cyclopentane-*r*-1,*c*-2-diol. (B, C) Suppression of the LPS fraction-mediated activation of *Dpt-lacZ* by the cyclopentanediol analogue. The cyclopentanediol analogue was added to the culture media at the time of LPS and heat shock stimulation (B) or 3 h before LPS and heat shock stimulation (C). LPS fraction-mediated activation of *Dpt-lacZ* (□), heat shock-mediated activation of *lacZ* (△) in *ex vivo* culture, and S2 cell viability (○) are represented as percent of control (DMSO). Bars indicate standard errors of four independent measurements.

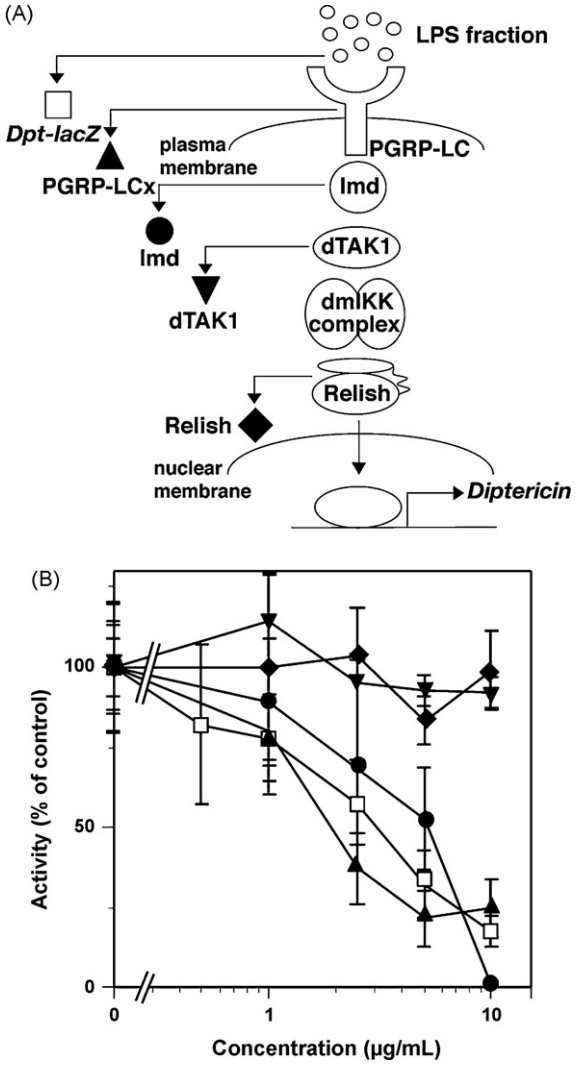

Fig. 2 – Determination of target molecules of the cyclopentanediol analogue in the IMD pathway. (A) Schematic representation of targeted activation of the IMD pathway by the overexpression of *PGRP-LCx*, *imd*, *dTAK1*, and *Relish ex vivo*. (B) Effects of the cyclopentanediol analogue on targeted activation of the IMD pathway by overexpression of *PGRP-LCx* (▲), *imd* (●), *dTAK1* (▼), and *Relish* (◆) *ex vivo*. Effect of the cyclopentanediol analogue on LPS fraction-mediated activation of *Dpt-lacZ* (□). Bars indicate standard errors of four independent measurements.

3.3. *Selective suppression of the IMD pathway-mediated host defense in Drosophila in vivo by the cyclopentanediol analogue*

Drosophila genetic studies have revealed that activation of the IMD pathway and activation of the Toll pathway are crucial for host defense against bacteria with some species specificity [17–19]. For example, *imd* mutant flies are more susceptible to *E. carotovora*, a Gram-negative bacterial infection [18], and mutant flies of *Dif* encoding a transcriptional factor of the Toll pathway are more susceptible to *M. luteus*, a Gram-positive bacterial infection [19]. We examined the effects of administration of the cyclopentanediol analogue on host resistance to *E. carotovora* and *M. luteus* infections *in vivo* (Fig. 3). Wild-type flies were fed a 4% sucrose solution containing 1 mg/mL of the cyclopentanediol analogue before and after *M. luteus* or *E. carotovora* infection using the protocol shown in Fig. 3A. In the control experiments, wild-type flies were resistant to both *E. carotovora* and *M. luteus* infection. Administration of the cyclopentanediol analogue to wild-type flies decreased the resistance against *E. carotovora* infection (Fig. 3B), whereas resistance against *M. luteus* infection was not affected (Fig. 3C). These results suggest that the cyclopentanediol analogue suppresses activation of the IMD pathway, but not that of the Toll pathway, *in vivo*. Consistent with these results, administration of the cyclopentanediol analogue to wild-type flies suppressed the expression of *Dpt*, an IMD pathway inducible gene, in response to *E. carotovora* infection (Fig. 3D), but not the expression of *Drs*, a Toll pathway inducible gene, in response to *M. luteus* infection *in vivo* (Fig. 3E). Administration of the cyclopentanediol analogue had no effect on the survival of uninfected wild-type flies, indicating that the cyclopentanediol analogue is not toxic to flies (Fig. 3B and C).

3.4. *Selective suppression of TNF-α stimulated activation of NF-κB in HUVECs by the cyclopentanediol analogue*

The *Drosophila* IMD pathway is similar to the mammalian TNF-α signaling pathway [2,6]. The TNF-α signaling pathway has a critical role in the inflammatory response, sepsis, and rheumatoid arthritis by the production of co-stimulatory molecules, cytokines, chemokines, and adhesion molecules through the activation of NF-κB [20]. To examine whether the cyclopentanediol analogue suppresses the mammalian TNF-α signaling pathway as well as the *Drosophila* IMD pathway, we investigated the effect of the cyclopentanediol analogue on TNF-α-stimulated activation of NF-κB in human umbilical vein endothelial cells (HUVECs). TNF-α-stimulated activation of NF-κB activity in nuclear extracts was suppressed by treatment with the cyclopentanediol analogue (Fig. 4A). In the TNF-α signaling pathway, NF-κB, including the p65/p50 heterodimer, is activated by translocation of NF-κB to the nucleus, which is caused by the degradation of IκB [21]. Immunocytochemistry revealed that the TNF-α-stimulated nuclear translocation of p65 was suppressed by treatment with the cyclopentanediol analogue (Fig. 4B and C). IκB degradation is regulated by the phosphorylation of IκB through a polyubiquitination-dependent proteasome pathway, which is induced by the phosphorylation of IKK downstream of TAK1 kinase [21,22]. In Western blotting analysis, TNF-α stimulated the phosphorylation of IKKα and IKKβ, and the IκB-α phosphorylation was suppressed by treatment with the cyclopentanediol analogue (Fig. 5C and D). These results suggest that the cyclopentanediol analogue suppresses the mammalian TNF-α signaling pathway upstream of IKK. IKK phosphorylation is mediated by the activation of TAK1 downstream of the adaptor proteins TRAF2/5 and TAB2/3 [23–25]. The cyclopentanediol analogue suppressed the TNF-α stimulated association of TAK1 and TRAF2, but not the association of TAK1 and TAB2 (Fig. 5C and D), suggesting that

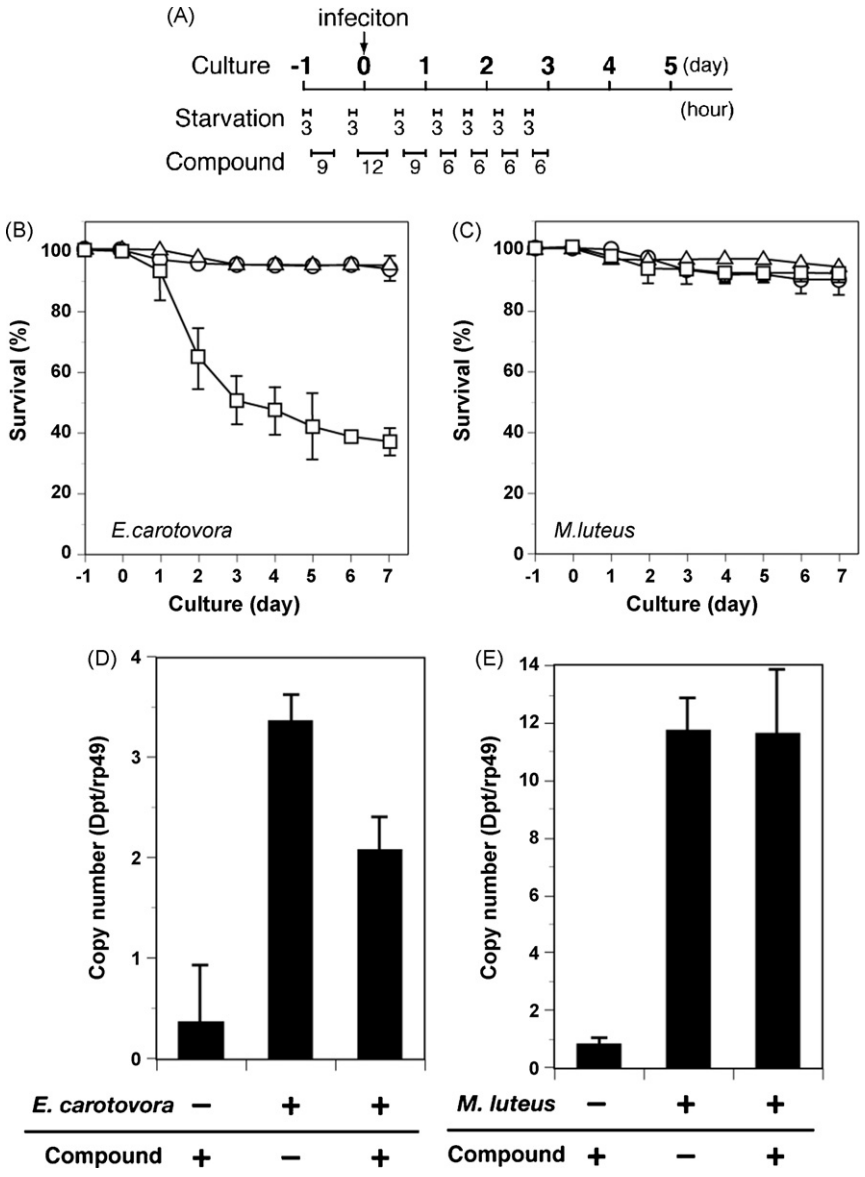

Fig. 3 – Effects of the cyclopentanediol analogue on host survival and induction of antimicrobial peptide genes against Gram-negative and Gram-positive bacterial infections. (A) Schematic representation of the protocol for administration of the cyclopentanediol analogue. Wild-type flies were cultured with paper wetted with a 4% sucrose solution containing 1 mg/mL of the cyclopentanediol analogue for the indicated time after starvation. (B, C) Survival rate of the cyclopentanediol analogue fed flies infected with the indicated bacteria (□) or without infection (○). The survival of wild-type flies against the indicated bacterial infections (no cyclopentanediol analogue control, △). (D, E) Effects of the cyclopentanediol analogue on the induction of antimicrobial peptide genes in response to *Erwinia carotovora* (D) and *Micrococcus luteus* (E) infections. The amount of mRNA of *Diptericin* (D), and *Drosomycin* (E), and *rp49* internal control was quantified by real-time RT-PCR. The survival experiments were performed at 25 °C. Bars indicate standard errors of two independent measurements.

the cyclopentanediol analogue suppresses the TNF-α dependent activation of NF-κB upstream of TAK1.

HUVECs stimulated with TNF-α and IL-1β produce IL-8, a neutrophil chemotactic factor, and MCP-1, a monocyte chemotactic factor. The TNF-α signaling pathway and the IL-1β signaling pathway branch out upstream of TAK1 (Fig. 6A). TNF-α stimulated production of IL-8 and MCP-1 was suppressed by treatment with the cyclopentanediol analogue in a dose-dependent manner, whereas IL-1β stimulated production of IL-8 and MCP-1 was not markedly affected by the cyclopentanediol analogue (Fig. 6B–E). IL-8 production was suppressed by treatment with the cyclopentanediol analogue in various concentrations of TNF-α (Fig. 6G), but not in various concentrations of IL-1β (Fig. 6F). Consistent with the results shown in Fig. 5, these data suggest that the cyclopentanediol analogue selectively suppresses the TNF-α dependent activation of NF-κB upstream of TAK1 in mammalian cells.

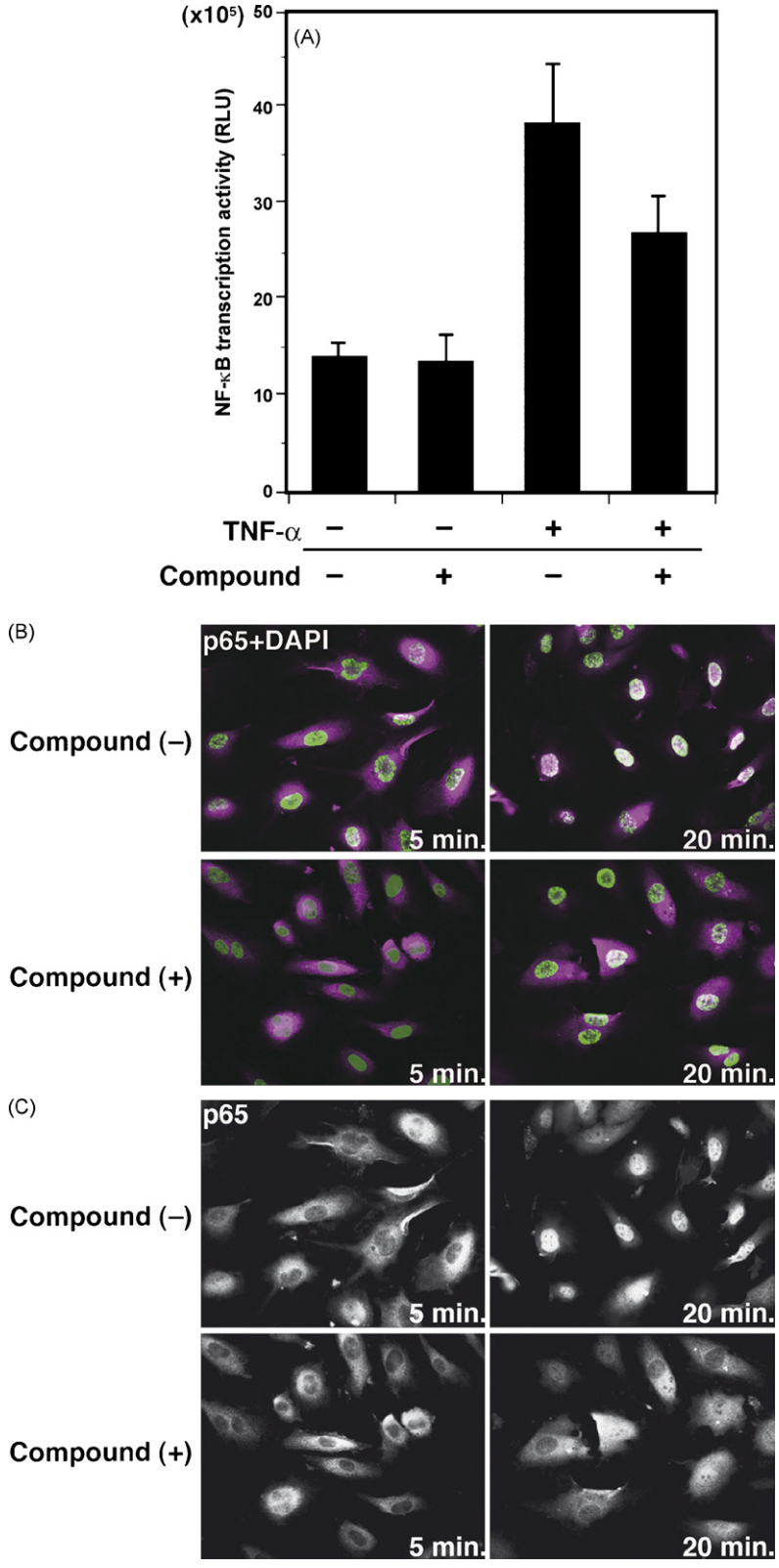

Fig. 4 – Effects of the cyclopentanediol analogue on TNF-α stimulated activation of NF-κB in HUVECs. (A) Suppression of the TNF-α-mediated activation of NF-κB by the cyclopentanediol analogue in HUVECs. HUVECs were treated for 3 h with the cyclopentanediol analogue or DMSO (control) and then stimulated with TNF-α (1 ng/mL) for 30 min. Nuclear extracts were prepared and the DNA binding activity of activated NF-κB was measured with p65 DNA binding sequences. Bars indicate the variance of duplicate measurements. (B, C) Suppression of nuclear translocation of NF-κB by the cyclopentanediol analogue. Nuclear import of p65 was monitored by staining with an anti-p65 antibody after the indicated duration of TNF-α (1 ng/mL) stimulation (magenta in B and white in C in web version). Nuclei were stained with DAPI (green in B in web version).

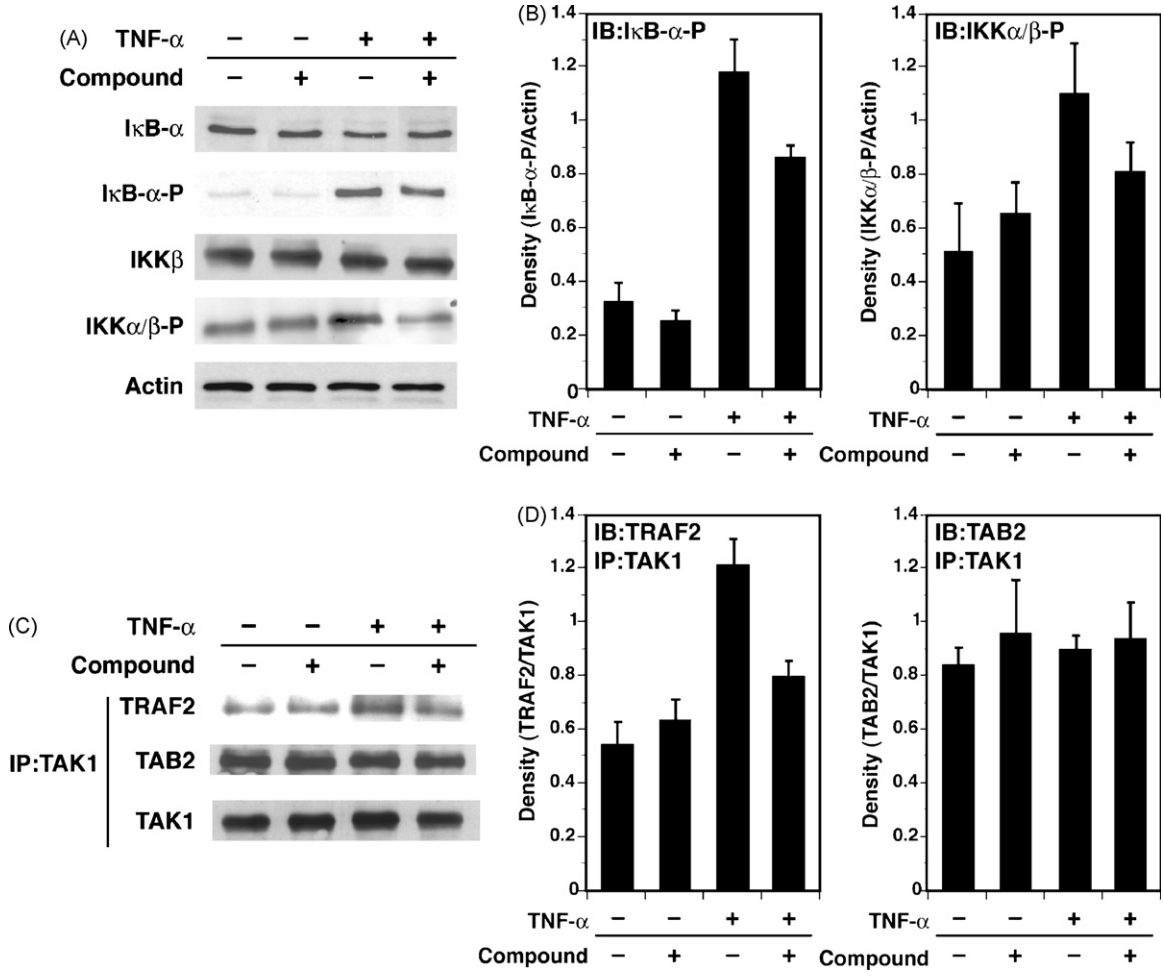

Fig. 5 – Effects of the cyclopentanediol analogue on TNF-α stimulated phosphorylation of IκB and IKK and the association between TAK1 and TRAF2, and between TAK1 and TAB2. (A, B) Suppression of IκB and IKK phosphorylation by the cyclopentanediol analogue. HUVECs were treated for 3 h with the cyclopentanediol analogue or DMSO prior to stimulation with TNF-α (1 ng/mL). Cells were harvested at 10 min after stimulation, and total cell extracts were prepared for Western blotting (A). The Western blot band intensity was quantified using the NIH Image program (B). (C, D) Selective suppression of the TNF-α dependent association of TAK1 and TRAF2 by the cyclopentanediol analogue. Cells were harvested at 15 min after stimulation and the TAK1 association complex was immunoprecipitated with TAK1 antibody (IP) and analyzed by immunoblotting using antibodies against TRAF2 and TAB2 (IB). The Western blot band intensity was quantified using the NIH Image program (D). Bars indicate standard errors of three (B) and four (D) independent measurements.

4. Discussion

The use of low-background, high-throughput phenotype-based screening, and rapid analysis of the mechanism of action has led to recent discoveries or target identifications of bioactive small molecules using non-mammalian model organisms, such as zebrafish, yeast, and nematode [26–28]. *Drosophila* are also a useful system for identifying immune regulators, because *Drosophila* have a highly sophisticated innate immune system that is conserved throughout the animal kingdom, but lack the mammalian-type adaptive immune system that, when combined with innate immunity, makes a complex immune system that is more difficult to study. The powerful genetics and molecular techniques used for studying *Drosophila* are also quite useful for analysis of the modes of action of potentially therapeutic compounds. Previously, based on the striking conservation of innate immunity between species, we established a *Drosophila ex vivo* culture system in which compounds acting on innate immunity and their target molecules can be identified [9]. In the present study, we screened microbial secondary metabolites for compounds that suppress or enhance innate immunity using the *ex vivo* system and identified a cyclopentanediol analogue from *Aspergillus* sp. Activation of the *Drosophila* IMD pathway was suppressed by treatment with the cyclopentanediol analogue *ex vivo* and *in vivo* and, as expected, TNF-α stimulated activation of NF-κB was also suppressed by the cyclopentanediol analogue in human cells. These results demonstrated that the *ex vivo* system is highly useful for identifying immune regulators acting on human innate immunity. A remarkable feature of the cyclopentanediol analogue is its ability to selectively suppress TNF-α stimulated

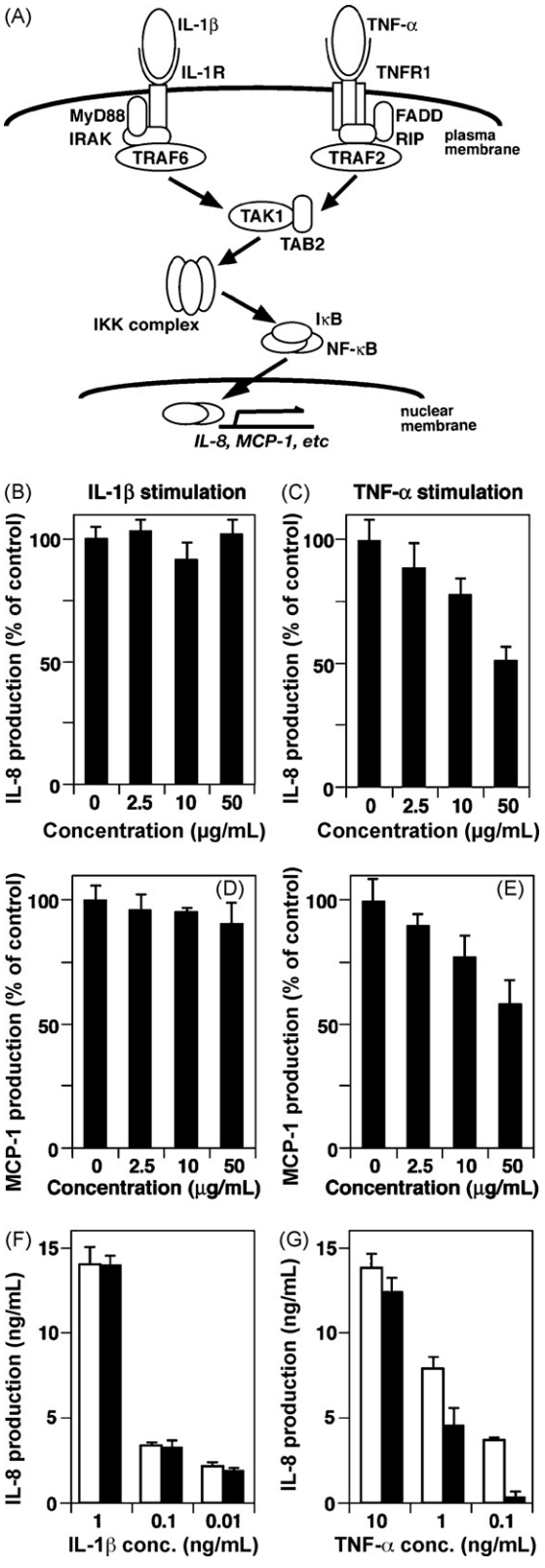

Fig. 6 – Effects of the cyclopentanediol analogue on IL-8 and MCP-1 production induced by TNF-α and IL-1β in HUVECs. (A) Schematic representation of the human IL-1 signaling pathway and TNF-α signaling pathway. (B–G) Effects of the cyclopentanediol analogue on the production of IL-8 (B, C, F, G) and MCP-1 (D, E) induced by stimulation with IL-1β (B, D, F) and TNF-α (C, E, G). HUVECs were treated with the indicated concentrations of the cyclopentanediol analogue for 3 h prior to stimulation with IL-1β (1 ng/mL) and TNF-α (1 ng/mL) (B–E). Cells were stimulated with different concentrations of IL-1β (F) and TNF-α (G) in the presence (50 μg/mL, black column) or absence (white column) of the cyclopentanediol analogue. Bars indicate standard errors of two (B–E) and four (F and G) independent measurements.

activation of NF-κB, but not IL-1β stimulated activation of NF-κB. From a pharmaceutical point of view, the effect of the cyclopentanediol analogue on mammalian innate immune responses, however, is relatively weak, and therefore structural optimization of the cyclopentanediol analogue is required for the development of lead compounds for immune suppressors to be used in human beings. A more extensive search for compounds that regulate innate immunity using the *Drosophila* system might lead to the identification of novel, potent, and selective immune regulators of innate immunity from natural resources.

Administration of the cyclopentanediol analogue had no effect on the survival of uninfected wild-type flies, whereas severe susceptibility to Gram-negative bacterial infection was induced in wild-type flies, suggesting that the cyclopentanediol analogue might be developed as a new type of insecticide to enhance the effects of microbial insecticides, and to control insect vector-born diseases caused by protozoa, such as malaria and sleeping sickness. Recent studies demonstrated that the IMD pathway of *Anopheles* and *Glossina* is involved in regulating infection by the parasites *Plasmodium* and *Trypanosoma*, which cause malaria and sleeping sickness in humans, respectively [29–31]. Insecticides affecting only the vectors carrying the parasites produce less selective pressure to the vectors, which might solve drug resistance problems.

Analysis of the mechanism of action suggests that the cyclopentanediol analogue targets molecules that are between Imd and dTAK1 in *Drosophila* and upstream of TAK1 in HUVECs. In *Drosophila*, the mechanism of dTAK1 activation through Imd is not clear, but some ubiquitin-conjugating enzymes are essential for dTAK1 activation [32]. In mammalian cells, the ubiquitination or protein–protein interactions of RIP, TRAF2/5, and TAB2/3 are required for TNF-α induced TAK1 activation [33]. The cyclopentanediol analogue suppresses the TNF-α stimulated association of TAK1 and TRAF2, but not the association of TAK1 and TAB2, Therefore, the cyclopentanediol analogue or some derivatives might serve as useful molecular probes for investigating the activation of the IMD pathway in *Drosophila* and TNF-α mediated signaling.

Acknowledgements

We are grateful to K. Anderson, D. Hultmark, J.A. Hoffmann, B. Lemaitre, M. Nakamura, the Bloomington Stock Center, *Drosophila* Genetic Resource Center at the Kyoto Institute of Technology, and the Genetic Strain Research Center of the National Institute of Genetics for the fly stocks. This work was supported by Takeda Science Foundation; Grants-in-Aid for Scientific Research from the Ministry of Education, Culture,

Sports, Science, and Technology of Japan; the Japan Society for the Promotion of Science; the Program for the Promotion of Basic Research Activities for Innovative Biosciences; and the Naito Foundation.

REFERENCES

[1] Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17:1–14.
[2] Hoffmann JA, Reichhart JM. *Drosophila* innate immunity: an evolutionary perspective. Nat Immunol 2002;3:121–6.
[3] Janeway Jr CA. How the immune system works to protect the host from infection: a personal view. Proc Natl Acad Sci USA 2001;98:7461–8.
[4] Hultmark D. *Drosophila* immunity: paths and patterns. Curr Opin Immunol 2003;15:12–9.
[5] Ferrandon D, Imler JL, Hoffmann JA. Sensing infection in *Drosophila*: Toll and beyond. Semin Immunol 2004;16:43–53.
[6] Silverman N, Maniatis T. NF-kappaB signaling pathways in mammalian and insect innate immunity. Genes Dev 2001;15:2321–42.
[7] Royet J, Reichhart JM, Hoffmann JA. Sensing and signaling during infection in *Drosophila*. Curr Opin Immunol 2005;17:11–7.
[8] Yajima M, Takada M, Takahashi N, Kikuchi H, Natori S, Oshima Y, et al. A newly established in vitro culture using transgenic *Drosophila* reveals functional coupling between the phospholipase A2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (imd) pathway in insect immunity. Biochem J 2003;371:205–10.
[9] Sekiya M, Ueda K, Fujita T, Kitayama M, Kikuchi H, Oshima Y, et al. Establishment of ex vivo systems to identify compounds acting on innate immune responses and to determine their target molecules using transgenic *Drosophila*. Life Sci 2006;80:113–9.
[10] Choe KM, Werner T, Stoven S, Hultmark D, Anderson KV. Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in *Drosophila*. Science 2002;296:359–62.
[11] Georgel P, Naitza S, Kappler C, Ferrandon D, Zachary D, Swimmer C, et al. *Drosophila* immune deficiency (IMD) s a death domain protein that activates antibacterial defense and can promote apoptosis. Dev Cell 2001; 1:503–14.
[12] Khush RS, Cornwell WD, Uram JN, Lemaitre B. A ubiquitin-proteasome pathway represses the *Drosophila* immune deficiency signaling cascade. Curr Biol 2002;12:1728–37.
[13] Takatsu Y, Nakamura M, Stapleton M, Danos MC, Matsumoto K, O'Connor MB, et al. TAK1 participates in c-Jun N-terminal kinase signaling during *Drosophila* development. Mol Cell Biol 2000;20:3015–26.
[14] Kaneko T, Goldman WE, Mellroth P, Steiner H, Fukase K, Kusumoto S, et al. Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the *Drosophila* IMD pathway. Immunity 2004;20:637–49.
[15] Ghisalberti EL, Narbey MJ, Rowland CY. Metabolites of Aspergillus-Terreus antagonistic towards the take-all fungus. J Nat Prod 1990;53:520–2.
[16] Hedengren M, Asling B, Dushay MS, Ando I, Ekengren S, Wihlborg M, et al. Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. Mol Cell 1999;4:827–37.
[17] Leulier F, Parquet C, Pili-Floury S, Ryu JH, Caroff M, Lee WJ, et al. The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. Nat Immunol 2003;4:478–84.
[18] Takehana A, Yano T, Mita S, Kotani A, Oshima Y, Kurata S. Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity. EMBO J 2004;23:4690–700.
[19] Ramet M, Manfruelli P, Pearson A, Mathey-Prevot B, Ezekowitz RA. Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for *E. coli*. Nature 2002;416:644–8.
[20] Hehlgans T, Pfeffer K. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology 2005;115:1–20.
[21] Karin M, Yamamoto Y, Wang QM. The IKK NF-kappa B system: a treasure trove for drug development. Nat Rev Drug Discov 2004;3:17–26.
[22] Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 2001;412:346–51.
[23] Tada K, Okazaki T, Sakon S, Kobarai T, Kurosawa K, Yamaoka S, et al. Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. J Biol Chem 2001;276:36530–4.
[24] Takaesu G, Surabhi RM, Park KJ, Ninomiya-Tsuji J, Matsumoto K, Gaynor RB. TAK1 is critical for I kappa B kinase-mediated activation of the NF-kappa B pathway. J Mol Biol 2003;326:105–15.
[25] Ishitani T, Takaesu G, Ninomiya-Tsuji J, Shibuya H, Gaynor RB, Matsumoto K. Role of the TAB2-related protein TAB3 in IL-1 and TNF signalling. EMBO J 2003;22:6277–88.
[26] MacRae CA, Peterson RT. Zebrafish-based small molecule discovery. Chem Biol 2003;10:901–8.
[27] Parsons AB, Lopez A, Givoni IE, Williams DE, Gray CA, Porter J, et al. Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast. Cell 2006;126:611–25.
[28] Kwok TC, Ricker N, Fraser R, Chan AW, Burns A, Stanley EF, et al. A small-molecule screen in *C. elegans* yields a new calcium channel antagonist. Nature 2006;441:91–5.
[29] Frolet C, Thoma M, Blandin S, Hoffmann JA, Levashina EA. Boosting NF-kappaB-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. Immunity 2006;25:677–85.
[30] Meister S, Kanzok SM, Zheng XL, Luna C, Li TR, Hoa NT, et al. Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*. Proc Natl Acad Sci USA 2005;102:11420–5.
[31] Hu C, Aksoy S. Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. Mol Microbiol 2006;60:1194–204.
[32] Zhou R, Silverman N, Hong M, Liao DS, Chung Y, Chen ZJ, et al. The role of ubiquitination in *Drosophila* innate immunity. J Biol Chem 2005;280:34048–55.
[33] Adhikari A, Xu M, Chen ZJ. Ubiquitin-mediated activation of TAK1 and IKK. Oncogene 2007;26:3214–26.