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T-cell activation-inhibitory assay: a proposed novel method for screening caloric restriction mimetics

Shu Iura[1], Yuusei Ojima[1], Yoshiaki Amakura[2], Morio Yoshimura[2], Atsushi Sawamoto[1], Satoshi Okuyama[1], Yoshiko Furukawa[1], and Mitsunari Nakajima[1]

[1] Department of Pharmaceutical Pharmacology, College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan and [2] Department of Pharmacognosy, College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan

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ABSTRACT

Caloric restriction (CR) is a major contributor to good health and longevity. CR mimetics (CRMs) are a group of plant-derived compounds capable of inducing the benefits of CR. Since a longevity gene, SIRT1, inhibits T-cell activation and SIRT1 loss results in increased T-cell activation, we hypothesized that compounds capable of activating SIRT1 signaling can inhibit T-cell activation and function as CRMs. Thus we propose, in the present study, the application of a T-cell activation-inhibitory assay to screen candidate CRMs. Well-known CRMs, such as resveratrol, butein, and fisetin, suppressed the anti-CD3/CD28 antibody-induced activation of mouse spleen T-cells. We next randomly assessed 68 plant-derived compounds for screening novel candidate CRMs using this bioassay and found that all four compounds showing IC_{50} values <5 μM, such as curcumin, α-mangostin, nobiletin, and heptamethoxyflavone, have beneficial functions for health such as anti-inflammatory effect. These results suggest that the T-cell activation-inhibitory assay can be used to screen candidate CRMs.

A caloric restriction (CR) value of 30–60% has been shown to effectively extend the life span of a variety of organisms by inducing good health (32). However, long-term CR remains a challenge to humans. A practical approach to overcoming this dilemma is developing CR mimetics (CRMs) capable of providing CR benefits without performing the actual CR. The concept of CRMs was first described by Lane *et al.* in 1998 (26). Ingram *et al.* (23) later argued that candidate CRMs should: (i) mimic the metabolic, hormonal, and physiological effects of CR; (ii) significantly reduce long-term food intake; (iii) activate CR-like stress response pathways and provide protection against a variety of stressors; and (iv) produce CR-like effects on longevity, reduction of age-related diseases, and function maintenance. Several studies using microarray-based gene expression analysis have revealed that several medications modulating glucose and lipid metabolism and inflammation, could be potential candidates for CRMs (10, 14).

In genetic studies, Sir2 family genes of NAD^+-dependent deacylases, sirtuins, were extensively studied as longevity genes in yeast (3, 22). Likewise, Sir2 and its orthologs have been proven essential for CR-mediated longevity in several organisms such as worms (51) and flies (44). Specifically, the mammalian Sir2 ortholog SIRT1 was studied in mice and found to regulate metabolic responses to nutritional cues (13, 46). For instance, SIRT1-deficient mice were metabolically unable to adapt to CR (4), indicating the importance of SIRT1 in the physiological response to CR. SIRT1 is expressed in various tissues

Address correspondence to: Dr Mitsunari Nakajima, Matsuyama University, Department of Pharmaceutical Pharmacology, College of Pharmaceutical Sciences, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan
Tel: +81-89-926-7069, Fax: +81-89-926-7162
E-mail: mnakajim@g.matsuyama-u.ac.jp

of animals such as the brain, visceral fat pad, kidney, liver, and muscle (5, 9). Conversely, SIRT1-overexpressing mice showed significant protection against high-fat diet-induced adverse effects on glucose metabolism (1, 42), suggesting that SIRT1 improves metabolism regulation in mice.

Given that sirtuins affect the metabolic responses to CR and longevity, sirtuin-activating compounds were screened to identify candidate CRMs. Howitz *et al.* (21) and Milne *et al.* (33) developed *in vitro* screening systems for SIRT1-activating compounds and identified possible candidate CRMs including resveratrol and SRT1720. However, Pacholec *et al.* (37) reported that these compounds did not activate SIRT1 through native protein substrates. Another study has shown that these SIRT1 activators interact directly with SIRT1, activating it through an allosteric mechanism (12). Hence, to overcome these controversies, alternative screening systems with different concepts are required.

CRMs, such as resveratrol, butein, and fisetin, are well-known sirtuin-activating compounds and show growth-suppressive effects on various types of T-cells (24, 49, 60). On the other hand, SIRT1 inhibits T-cell activation and SIRT1 loss results in increased T-cell activation (57). These evidences are indicative that compounds capable of activating SIRT1 or upstream/downstream SIRT1 signaling machineries can inhibit T-cell activation and function as CRMs. In the present study, we propose the application of a T-cell activation-inhibitory assay to screen candidate CRMs. We adopt a culture system for this bioassay using anti-CD3/CD28 antibody-induced T-cell activation (19). Initially, we examined the T-cell activation-inhibitory effect of already well-known CRMs, such as resveratrol, and then screened randomly 68 plant-derived compounds for possible CRMs.

MATERIALS AND METHODS

Animals. Female BALB/c mice were purchased (SLC, Shizuoka, Japan) at 6-weeks old, maintained under controlled temperature and light (23°C and 12-h light/dark, respectively) and used at 7–8-weeks old, while receiving food and water *ad libitum*. All experimental procedures followed the Guidelines for Animal Experimentation of the Animal Care and Use Committee of Matsuyama University (Matsuyama, Japan). The female mice were 6–9 weeks old at the start of the experiments.

Spleen cell culture. To examine T-cell growth, single-cell suspensions were prepared by mincing mice spleen tissue, and passing them through a 40 μm nylon mesh (Falcon, Corning, NY, USA). The cell suspensions were then treated with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA) for 5 min and red blood cells were removed from the spleen cell suspensions. After washing with Hank's balanced salt solution (Thermo Fisher Scientific, Waltham, MA, USA), the resulting pellets were used as spleen leukocytes, including a few percent of T-cells. For the T-cell growth-inhibitory assay, the spleen leukocytes were stimulated with 1 μg/mL each of coated anti-CD3 (BioLegend, San Diego, CA, USA) and soluble anti-CD28 (BioLegend) antibodies for 48 h in the presence of assay samples. The assay was performed using culture medium containing indicated concentrations (0–100 μM) of samples and/or 0.1% dimethyl sulfoxide (DMSO), and incubations were done at 37°C, in a humidified atmosphere containing 5% CO_2. The culture medium contained 45 mL of RPMI 1640 (Thermo Fisher Scientific), 50 μL of 2-mercaptoethanol (Thermo Fisher Scientific), 0.5 mL of penicillin/streptomycin/glutamine (Thermo Fisher Scientific), and 5 mL of heat-inactivated fetal calf serum (FCS) (Thermo Fisher Scientific). To assess T-cell growth, we used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation kit (Roche, Basel, Switzerland), following the manufacturer's instructions. MTT is reduced to form purple formazan crystals in metabolically active cells. Thus, the appearance of formazan crystals suggests the existence of anti-CD3/CD28 antibody-activated T-cells in the spleen leukocyte cultures. The formazan crystals were solubilized with 78% DMSO-containing medium and the OD value (570/655) was measured. We defined IC_{50} as concentrations of compounds to inhibit anti-CD3/CD28 antibody-induced T-cell activation by 50% on the OD value.

Mouse monocyte macrophage RAW264.7 culture. Mouse monocyte macrophage RAW264.7 cells were purchased from DS Pharma Biomedical (Osaka, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Nacalai tesque, Kyoto, Japan) supplemented with 10% FCS and 0.1% penicillin/streptomycin/glutamine (Thermo Fisher Scientific, San Diego, CA, USA).

Western Blot analyses. RAW264.7 cells were treated with/without samples at indicated concentrations. Three hours later, lipopolysaccharide (LPS; 0111E. coli B4, L2630; Sigma-Aldrich, St. Louis, MO, USA) was added to the culture at a concentration of

10 ng/mL, and the cells were harvested 24 h later for Western blotting. Blots were probed with rabbit anti-Cox-2 (D5H5) (Cell Signaling #12282, Tokyo, Japan), rabbit anti-SIRT1 (Cell Signaling #2028), or rabbit anti-acetyl NF-κB p65 (Lys310; D2S3J) antibodies (Cell Signaling #12629); all at 1 : 1000 dilutions, were detected with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Cell Signaling) and re-probed with HRP-conjugated anti-glyceraldehyde 3-phosphate dehydrogenase (G3PDH) antibody (Sigma-Aldrich) or HRP-conjugated anti-α-tubulin (11H10) (Cell Signaling). Data are presented as the fold-change of control mean ± SEM.

Assay samples. 3,5,6,7,8,3',4'-Heptamethoxyflavone (HMF) and nobiletin were generously provided by Ushio ChemiX (Omaezaki, Japan). Resveratrol and rapamycin (Sigma-Aldrich), SRT1720 (AdooQ, Irvine, CA, USA), fisetin (Cayman, Ann Arbor, MI, USA), butein and curcumin (Tokyo Chemical Industry, Tokyo, Japan), and α-mangostin (Wako Pure Chemical Corporation, Osaka, Japan) were commercially obtained.

The 68 randomly screened samples were divided as follows: 18 flanovonoids (including nobiletin and HMF), 14 phenolics, 10 phenylpropanoids, 9 hydrolyzable tannins, 4 coumarins, 3 monoterpenes, 3 diarylheptanoids (including curcumin), 2 xanthones (including α-mangostin), 2 chromones, a lignin, an alkaloid, and a hydroxyl acid.

Statistical analyses. Data are expressed as mean ± standard error of the mean (SEM). The data were analyzed using one-factor analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

To examine the effectiveness of our T-cell activation-inhibitory assay for the screening of candidate CRMs, we first assessed the effects of well-known CRMs, such as resveratrol, which is one of the most studied CRM, on mouse spleen T-cell activation (2, 12, 21, 33, 39, 40, 50). Resveratrol inhibited anti-CD3/CD28 antibody-induced T-cell activation in the mouse spleen cultures (IC_{50} = 12 μM) (Figs. 1, 2, and 3). Other natural CRMs, such as butein and fisetin (11, 21, 27, 38, 48, 53, 58, 59), also inhibited T-cell activation (IC_{50} = 2.8 μM and 5.2 μM, respectively) (Figs. 1 and 3). The synthetic CRM SRT1720 (33, 34) inhibited T-cell activation at a low concentration (IC_{50} = 0.25 μM) (Figs. 1 and 3). Rapamycin, a macrolide antibiotic, is known to inhibit the target of the rapamycin (TOR) signaling pathway and extend the lifespan of genetically heterogeneous mice (20, 47, 52, 54). Rapamycin was highly effective in inhibiting T-cell activation (IC_{50} = 0.16 nM) (Figs. 1 and 3).

Resveratrol, butein, fisetin, and SRT1720 are sirtuin activating compounds which have been reported to act as CRMs by inducing good health and/or longevity in some organisms (15, 21, 25, 53). It is still unclear if rapamycin affects sirtuin signaling. However, cross-reactions between sirtuin and TOR signaling have been reported (18, 56). These results suggest the possibility of the T-cell activation-inhibitory assay to screen candidate CRMs, even in the absence of a direct sirtuin-activating effect.

We equally performed preliminarily screens on candidate CRMs. Sixty-eight natural compounds, most of which were purified from a variety of plants (citrus, curcuma, and garcinia, amongst others), were assessed for their ability to inhibit anti-CD3/CD28 antibody-induced T-cell activation. A considerable number of compounds had substandard inhibitory effects (IC_{50} >50 μM) on T-cell activation. Four compounds had IC_{50} values <5 μM, while the IC_{50} values of some other 4 compounds were intermediate; 5–50 μM (Fig. 3). Three of the 4 active compounds (IC_{50} <5 μM); curcumin (IC_{50} = 2.2 μM), α-mangostin (IC_{50} = 1.2 μM), and nobiletin (IC_{50} = 0.9 μM), are reported to have SIRT1-activating effects (7, 17, 28, 29, 41, 43, 55).

Another compound, HMF showing IC_{50} = 1.0 μM, is reported to ameliorate brain and bone injuries (30, 31, 35, 36, 45), and recently shown to possess anti-metabolic effects (16). However, HMF-induced SIRT1-related functions are yet to be reported. Thus, we performed detailed analysis concerning the relationship between HMF and SIRT1. The SIRT1 protein is associated with the RelA/p65 subunit of NF-κB. The transcriptional activity of NF-κB is critical for the expression of inflammatory proteins such as cyclooxygenase-2 (Cox-2), and is known to be suppressed by deacetylation at the Lys310 residue of its RelA/p65 subunit, mediated by the SIRT1 protein (6, 8). As shown in Fig. 4 A, HMF decreased LPS-induced Cox-2 expression in RAW264.7 macrophage-cells. We also observed an increase in SIRT1 expression, and the Lys310 residue of the RelA/p65 subunit of NF-κB was deacetylated by HMF in the experimental condition (Fig. 4 B and C). These observations suggest that HMF activates SIRT1 signaling directly or indirectly, which causes suppressed activity of NF-κB by deacetylation of the RelA/p65

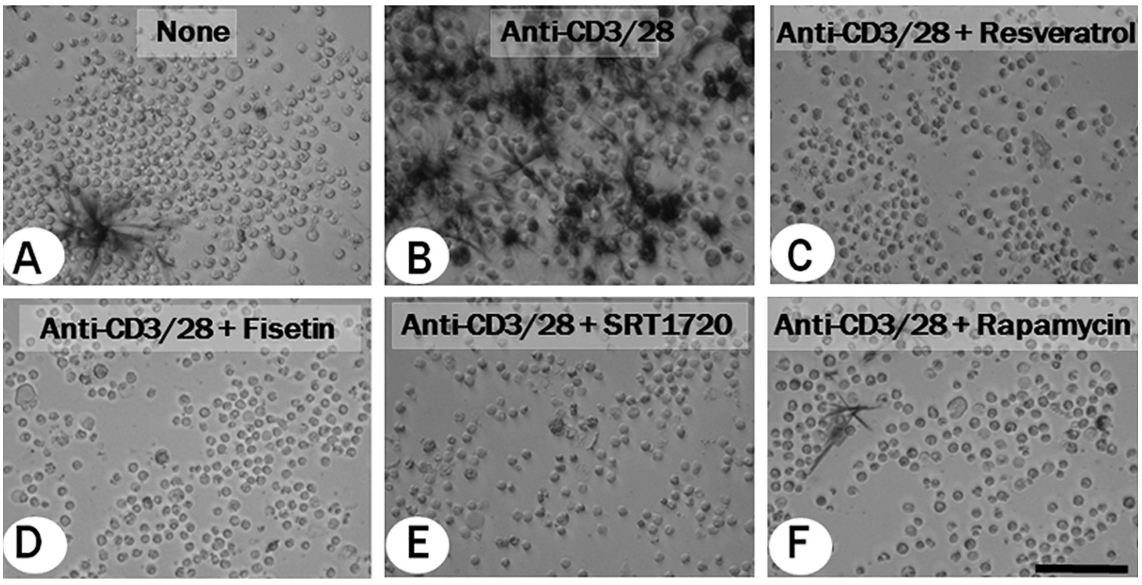

Fig. 1 Photos of the T-cell activation-inhibitory assay culture. Spleen leukocytes were stimulated with anti-CD3/CD28 antibodies for 48 h in the presence of assay samples. MTT was added to the culture and changed to purple formazan crystals in activated T-cells. (**A**) Neither sample nor antibody stimulation. (**B**) Sample absent, but antibody stimulation present. An enormous number of formazan crystals are visible. (**C**) Resveratrol (50 μM) with antibody stimulation. (**D**) Fisetin (50 μM) with antibody stimulation. (**E**) SRT1720 (1 μM) with antibody stimulation. (**F**) Rapamycin (100 nM) with antibody stimulation. Small formazan crystals seen in (A) and (F) were sporadically observed. As these crystals are considered to be formed by macrophages which exist sporadically in the spleen leukocyte cultures, the influence to the assessment of IC_{50} of CRMs could be negligible. Bar = 50 μm.

subunit and reduced expression of Cox-2.

This study was prompted by research articles which indicate that well-known CRMs such as resveratrol, butein, and fisetin, are sirtuin-activating compounds and show growth-suppressive effects on a variety of T-cells (24, 49, 60). Secondly another article indicated that SIRT1 was essential for maintaining T-cell tolerance (57). Overall, these articles are indicative of the fact that compounds that enhance SIRT1-signaling may suppress T-cell growth, though with poorly understood mechanisms. This implies that compounds directly activating SIRT1 or its upstream/downstream signaling machineries, may be screened as candidate CRMs by the T-cell activation-inhibitory assay.

The T-cell activation-inhibitory assay is a unique method for routine CRM screening. The assay *1)* is very simple to perform, *2)* can screen both direct SIRT1-activating compounds and indirect ones, *3)* is sensitive and needs only minute sample quantities (0.1 mg) for the screening procedure, and *4)* is a bioassay, as opposed to being an *in vitro* assay. This assay's specificity might be weak in some cases because it also detects compounds that suppress T-cell growth by modulating intracellular signaling pathways other than the SIRT1 signaling pathway. For example, cyclosporine, which impedes the calcineurin pathway, inhibits T-cell activation (19). Thus, additional steps, such as testing anti-inflammatory and/or anti-metabolic functions, could be used to exclude such mismatching compounds from the list of candidate CRMs. After getting the present study, we found an additional function of HMF in the early adipogenesis, which would be published elsewhere.

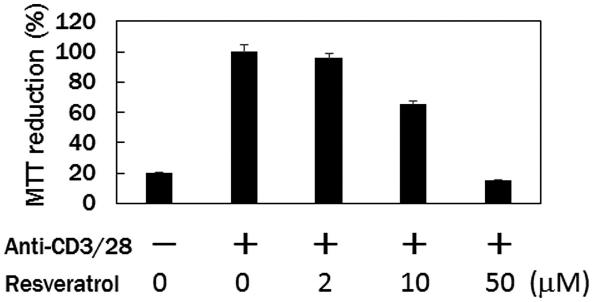

Fig. 2 Resveratrol inhibits anti-CD3/CD28 antibody-induced T-cell activation dose dependently. Spleen leukocytes were stimulated with anti-CD3/CD28 antibodies for 48 h in the presence of resveratrol at indicated concentrations, and the MTT assay performed.

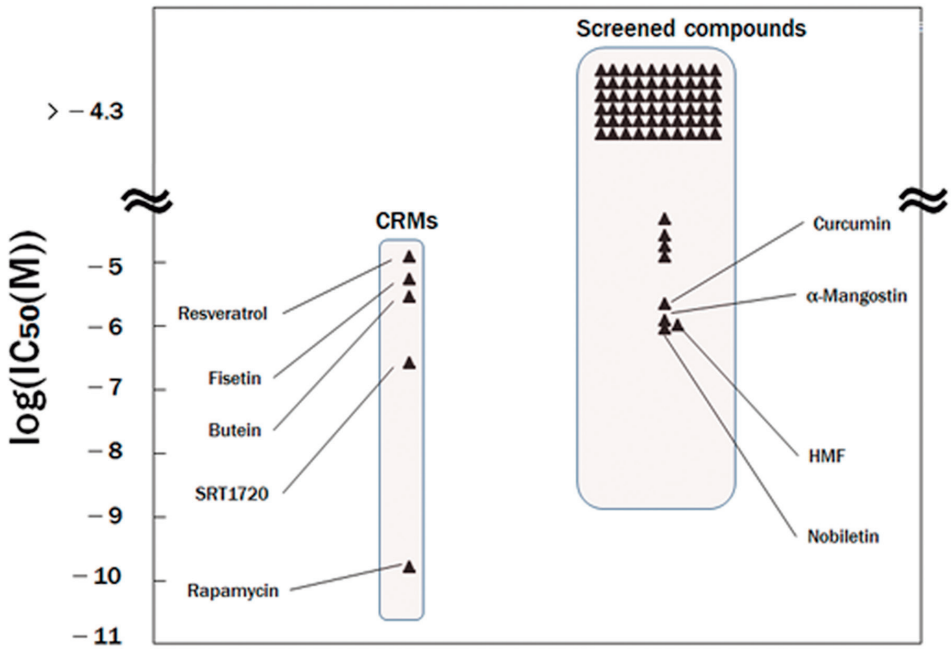

Fig. 3 Summary of the T-cell activation-inhibitory assay. Spleen leukocytes were stimulated with anti-CD3/CD28 antibodies for 48 h in the presence of a variety of samples, and the MTT assay was performed. The IC_{50} of the most studied CRMs: resveratrol, fisetin, butein, SRT1720, and rapamycin, are plotted on the left, and those of the randomly screened 68 compounds on the right.

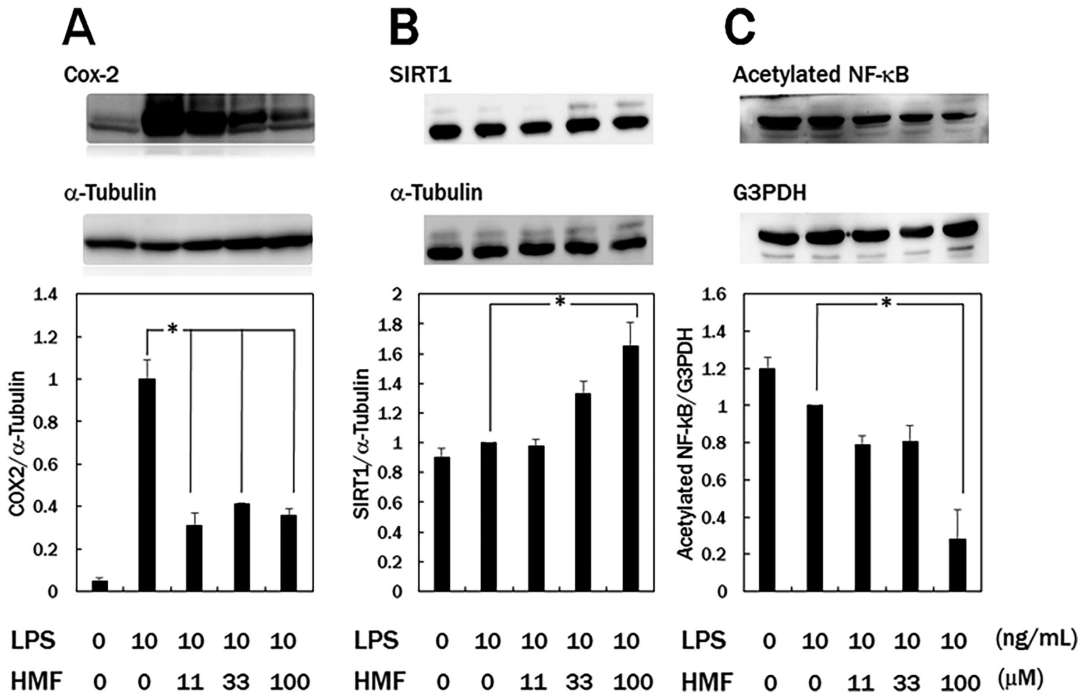

Fig. 4 HMF reduces Cox-2 expression, upregulates SIRT1 expression, and promotes deacetylation of the SIRT1 target, the RelA/p65 subunit of NF-κB. The expression of Cox-2, SIRT1, and the acetylated RelA/p65 subunit of NF-κB in RAW264.7 macrophage cells were analyzed using Western blotting. (**A**) LPS-induced Cox-2 expression was reduced by HMF. (**B**) SIRT1 expression in the presence of LPS was upregulated by HMF. (**C**) Acetylation of the RelA/p65 subunit of NF-κB in the presence of LPS was downregulated by HMF. Significant differences, compared to the HMF-untreated control are indicated with an asterisk.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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