Development of New Antiviral Agents from Natural Products

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Abstract: The recent great advances in medical treatment and scientific technology include the many antiviral agents that have been developed and are used for treatment of infectious diseases, but such advances have also provoked the appearance of resistant virus strains. Therefore, the development of new antiviral agents with diverse kinds of antiviral actions is required. The search for new antiviral agents focuses on not only synthetic compounds but also natural products such as traditional medicines, dietary supplements, and functional foods, including plants, insects, animal organs, and their components. Natural products have their own metabolites, and some of the metabolites may recognize the differences between viral and host metabolisms, resulting in antiviral activity. In general, they can be obtained cheaply and may be useful resources to develop new antiviral agents with different antiviral actions from those of known antiviral agents. Also, natural products and their components have been demonstrated to modify immunological activities and are candidates for biological response modifiers that are effective in alleviating symptoms and reducing mortality in virus infection. The first half of this chapter introduces natural products and purified compounds that were confirmed by *in vitro* experiments and animal infection models to have direct antiviral activity against herpes simplex virus type 1 (HSV-1) or influenza virus. However, even if a natural product or purified component has strong antiviral activity *in vitro*, if it has no therapeutic efficacy against virus infection in an animal infection model, it is merely an inhibitor and not a medicine. The search for antiviral agents should be based on the demonstration of prophylactic and/or therapeutic efficacy at the proper dosage in animals. In the second half, we introduce a Kampo medicine, Kakkon-to, which is a biological response modifier rather than a direct antiviral agent. It is the most common cold medicine used in traditional therapy and prescribed to about 20 million people annually in Japan. We also introduce the mode of immunomodulating activity of Kakkon-to on influenza virus and HSV-1 infection and its components, which can modulate cytokine production. We hope that this chapter will be useful in verifying the antiviral therapeutic efficacy of natural products against influenza infection and helpful in encouraging development of anti-influenza virus medicines from natural products.

Keywords: Antiviral agents, natural products, influenza virus, herpes simplex virus, immunomodulation.

1. INTRODUCTION

Natural products as crude materials with efficacy against various diseases have been selected by humans over many generations of practical experience. Such experiential evaluation is different from the scientific evaluation of western medicines, and is sometimes underestimated. However many effective medicines, including as aspirin, morphine, atropine, reserpine, ephedrine, and digitoxin, were developed from natural products. The discoveries of these effective compounds warrant the efficacy of using natural products as crude materials, and natural products with biological activity used historically may be good sources for the development of antiviral agents.

Traditional natural remedies have long been administered orally as hot-water extracts like a cup of tea or coffee. Information on their proper use and adverse reactions has been accumulated for a long time. Thus, if the antiviral therapeutic efficacy of natural products can be certified, we can easily convert their conventional use to therapeutic use against viral infection and their daily consumption as a beverage may become prophylactic and/or therapeutic treatment for virus infection. Further, the identification and purification of their effective components may lead to development of new antiviral agents.

Many natural products and their components have been used as traditional medicines, dietary supplements, and functional foods. Recently, their efficacy and safety for humans have been undergoing scientific evaluation worldwide. Thus, verification of antiviral activities of natural products and identification of their active components would demonstrate that they possess biological activity and safety for humans. Such active components may become reliable markers in the quality control of traditional medicines, dietary supplements, and functional foods.

Here, we summarize how the antiviral activities of natural products and their active components against herpes or influenza viruses were evaluated and identified in our studies.

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1876-5181/10

2. NATURAL PRODUCTS AS CRUDE MATERIALS WITH DIRECT ANTIVIRAL ACTIVITY

2.1. Medicinal Herbs with Anti-Herpes Virus Activity

We screened the inhibitory effects of hot-water extracts of more than 140 medicinal herbs on the plaque formation of herpes simplex virus type 1 (HSV-1) using Vero cells [1]. The extracts have been used clinically for many centuries in the treatment of various diseases in Asia, including China, Indonesia, and Japan. We selected 32 extracts that inhibited HSV-1 *in vitro* as candidates for HSV-1-inhibitory medicines. Their therapeutic efficacies were assessed in a murine HSV-1 infection model. This model is very useful to evaluate the therapeutic efficacy of extracts on not only mortality due to herpetic encephalitis but also the development of the herpetic skin lesions seen in humans. The oral dose used for murine infection experiments was deduced from the oral dose used for humans, which is based on the body surface area, assuming the efficacy for humans. Twelve extracts (Table **1**) with therapeutic anti-HSV-1 activities were finally selected using a model of lethal cutaneous HSV-1 infection in mice [1, 2]. All the extracts were significantly effective in delaying the formation of vesicles, erosion, ulceration, and/or zosteriform lesions on the skin of infected mice and/or in prolonging survival times. The efficacies of different lots of extracts were consistent. The amelioration of these herpetic symptoms suggested that the extracts themselves contain effective components that are possibly medicinal for humans. The anti-HSV-1 activity of extracts selected was also found in the serum of guinea pigs administered the extracts orally [3, 4]. This pharmacological serum assay may be useful as a procedure for screening effective extracts of natural products before animal experiments because the anti-HSV-1 activity in the serum was probably due to active compounds absorbed after oral administration.

Further, among the twelve extracts with therapeutic HSV-1 activity in mice, extracts of four plants (*Geum japonicum* Thunb., *Rhus javanica* L., *Syzygium aromaticum* (L.) Merr. et Perry, and *Terminalia chebula* Retzus) showed stronger anti-HSV-1 activity in combination with acyclovir (ACV, a known anti-HSV-1 agent) than the other extracts *in vitro* [2]. Each of the four combinations significantly limited the development of skin lesions and/or prolonged survival times of infected mice compared with both ACV or the extract alone, and reduced virus titers in the brain and skin more strongly than ACV alone [2]. The combinations exhibited stronger anti-HSV-1 activity in the brain than in the skin and were demonstrated to be more effective in reducing the growth of HSV-1 in the central nervous system than ACV alone [2]. In combination, the doses of the extracts for mice, which were deduced from experience in humans, were not toxic in mice. We expect the extracts to be beneficial in humans for prevention of the central nervous system complications caused by HSV infection. The four extracts may be applicable for conventional use in humans as anti-HSV-1 medicines to augment the therapeutic anti-HSV-1 efficacy of ACV, which has been successfully used for the treatment of HSV infection, in humans. Thus, the extracts were suggested to be useful to supplement the efficacy of ACV in humans.

Table 1. Natural Products with Antiviral Activity in Animals

Direct Antiviral Activity	Immunomodulatory Activity
Anti-HSV activity	
Alpinia officinarum [1, 2]	Kakkon-to [35]
Caesalpinia sappan [1]	Kumi-binlo-to [36]
Geum japonicum [1, 2, 3, 6, 7]	Shishi-hakuhi-to [36]
Paeonia suffruticosa [1, 2]	Syo-saiko-to [36]
Phellodendron amurense [1, 2]	Gimgyo-san [36]
Polygala tenuifolia [1, 2]	Lactoferrin [38]
Polygonum cuspidatum [1, 2]	
Punica granatum [1]	
Rhus javanica [1, 2, 6, 7]	
Syzygium aromaticum [1, 2, 6, 7]	
Terminalia arjuna [1, 2]	
Terminalia chebula [1, 2, 6, 7]	
Ayurvedic and Panamanian medical plants [10]	
Indonesian medicinal plants [11]	
Thai medicinal plants [15]	
Marine alga (*Symphyocladia latiuscula*) [16]	
Traditional Chinese medicines [17]	
Anti-cytomegalovirus activity	
Geum japonicum [8]	
Syzygium aromaticum [8]	
Terminalia chebula [8]	
Anti-influenza virus activity	
Propolis (AF-08) [9]	Kakkon-to [29, 33]
	Gimgyo-san [31]
	Lactoferrin [37]
	Onpi-to [39]
	Cinnamomu cassia [40]

Numbers in brackets indicate references.

When the modes of anti-HSV-1 action of the four herbal extracts with anti-HSV-1 activity in mice were assessed, they were shown to be effective in inhibiting the growth of wild-type HSV-1, wild-type HSV-2, and ACV-phosphonoacetic acid (PAA)-resistant (AP^r) HSV-1 as well as viral DNA synthesis in infected cells [5]. PAA inhibits the synthesis of HSV DNA but permits the synthesis of early HSV proteins before viral DNA synthesis. The pattern of viral protein synthesis in infected cells exposed to the extracts was similar to that in the cells exposed to PAA, and

the extracts had no effect on host protein synthesis. Thus the mode of anti-HSV action of extracts was the inhibition of viral DNA synthesis, and it was different from those of ACV and PAA. When the extracts were administered orally to mice infected cutaneously with HSV-2 or AP^r HSV-1, the virus yields in the brain were much lower than those in the control and ACV-treated mice. Thus, different modes of antiviral action of the extracts from ACV and their therapeutic efficacy were confirmed in a murine HSV infection model, suggesting that the extracts contain components with modes of anti-HSV-1 action different from ACV and PAA.

In general, medicinal herbs are cheap and easily obtained. Long-term oral administration of herbal extracts has been utilized in traditional therapy for the treatment of chronic diseases such as chronic pharyngolaryngitis, gastric and duodenal ulcer, and empyema. Their long-term administration is expected to be safe when cautiously managed based on the information accumulated historically. Recurrent herpes such as genital herpes frequently causes episodes of skin lesions for several days, sometimes 7 to 10 days, approximately once in a month are a major cause of morbidity in humans. The daily consumption of an extract for a long period may be useful for the prophylactic treatment of recurrent genital herpes. From these points of view, we examined the prophylactic efficacy of the four herbal extracts (*Geum japonicum*, *Rhus javanica*, *Syzygium aromaticum*, and *Terminalia chebula*) against recurrent HSV-1 disease in mice and demonstrated the prophylactic effectiveness on HSV reactivation in the trigeminal ganglia [6] (Table **1**). Further, we showed that the extract of *Rhus javanica* prophylactically alleviated spontaneously recurrent HSV-2 genital disease in guinea pigs, as a model of recurrent genital herpes in humans [7] (Table **1**). The herbal extracts were verified to be prophylactically useful against recurrent HSV infection.

Cytomegalovirus frequently causes opportunistic infection in immunodeficient patients with diseases such as AIDS and organ transplants and is a major cause of morbidity in humans. Extracts of *Geum japonicum*, *Rhus javanica*, *Syzygium aromaticum*, and *Terminalia chebula*, which have demonstrated anti-HSV-1 activity, were also shown to have anti-cytomegalovirus activity *in vitro* [8]. Among them, *Geum japonicum*, *Syzygium aromaticum*, and *Terminalia chebula* were confirmed to have prophylactic efficacy in a cytomegalovirus infection model in mice (Table **1**), and the activity of *Geum japonicum* was equivalent to 1 mg/kg of ganciclovir [8]. Thus, the prophylactic and therapeutic anti-cytomegalovirus efficacy of herbal extracts that we had selected by *in vitro* screening was verified in animal infection models, suggesting that the herbal extracts are possibly effective in reducing opportunistic infection by cytomegalovirus.

2.2. Propolis with Anti-Influenza Virus Activity

Propolis contains many kinds of components and has been used worldwide as a traditional dietary supplement to maintain and improve human health. We screened the anti-influenza virus activity of 13 ethanol extracts of propolis by a plaque reduction assay and in a murine influenza virus infection model [9]. Among the 13 ethanol extracts, four showed anti-influenza virus activity against A/PR/8/34 (H1N1) virus *in vitro*. In an intranasal influenza virus infection model in mice, however, only one of the four ethanol extracts, AF-08, was found to be effective against influenza infection *in vitro* and *in vivo*. In the murine infection model, the virus yield in the bronchoalveolar lavage fluid (BALF) of infected mice after oral administration AF-08 at 10 mg/kg (Table **1**) was similar to the yield in infected mice after administration of oseltamivir phosphate at 1 mg/kg. The anti-influenza virus activity of AF-08 was confirmed to be effective against influenza virus B and oseltamivir-resistant A/H1N1 viruses as well as influenza A/H1N1 and H3N2 viruses *in vitro*. AF-08 did not inhibit virus adsorption to cells *in vitro*. Thus, AF-08, an ethanol extract of Brazilian propolis, was verified to exhibit anti-influenza activity *in vitro* and *in vivo* and is a possible candidate for an anti-influenza dietary supplement. However, there are many kinds of propolises depending on the species of bee and kind of plants that the bees prefer. Therefore, we cannot assume that all kinds of propolises have anti-influenza virus activity. Nonetheless, it was suggested that AF-08 contains anti-influenza virus compounds and is different from other propolises.

2.3. Utility of Antiviral Natural Products as Crude Materials

We revealed that natural products as crude materials have prophylactic and/or therapeutic antiviral activity in animal infection models, as described above. In addition, we previously showed that many kinds of natural products, including ayurvedic, Panamanian, Indonesian, kampo, Kenyan, and Thai medicinal plants and marine alga (*Symphyocladia latiuscula*) are effective against HSV [10-17], hepatitis B virus [18], or HIV [19, 20] infection *in vitro* and/or *in vivo* (Table **1**). The verification of their antiviral activities suggests a new use for these natural products. Thus, drinking the extracts of natural products as a daily beverage may be useful for prophylaxis and/or therapy of diseases caused by viral infection and contribute to improvement of the quality of life. Studies evaluating toxicity *in vivo* are also important to provide scientific evidence of biological activities of traditional medicines, dietary supplements, and functional foods. If biologically active compounds are identified and isolated from natural products, they may be used as markers in quality control of the natural products.

3. COMPOUNDS WITH DIRECT ANTIVIRAL ACTIVITY PREPARED FROM NATURAL PRODUCTS

3.1. Eugeniin with Anti-HSV Activity (Table 2)

An aim of our research is the development of new antiviral agents having various kinds of antiviral actions from natural products. Many western drugs have been developed from natural sources, and novel antiviral compounds may also be developed from natural products. In our previous studies, antiviral compounds were isolated from herbal extracts that had been verified to exhibit therapeutic antiviral activity in mice using different chromatographic fractionations guided by antiviral activity. Finally, eugeniin (Fig. (**1a**), 1,2,3-trigalloyl 4,6-hexahydroxydiphenoyl β-D-glucopyranose, molecular weight: 938 daltons), a light yellow amorphous solid showing a quasi molecular ion peak at m/z: 939 $[M+H]^+$; $[\alpha]_D$+64.5° (c=0.067, acetone) and soluble in water, was purified as an anti-HSV compound from two

different species of plants, *Geum japonicum* and *Syzygium aromaticum*, of the four herbal extracts with therapeutic anti-HSV activity [21]. This was surprising because the two herbs are different plant species. In addition, this confirmed the validity, accuracy, and feasibility of our screening and assay systems to identify the active compounds from natural products.

Table 2. Compounds with Antiviral Activity in Animals

Direct Antiviral Activity	Immunomodulatory Activity
Anti-HSV activity	
Eugeniin [21, 22]	
Moronic acid [23]	
Anti-influenza virus activity	
Diarylheptanoides	7-Hydroxycoumarin (7HC) [40, 42]
	4-Allylanisole [40]
	Cinnamic acid ethylester [40]
	Acetic acid cinnamylester [40]
	2'-Hydroxyacetophenone [40]
	2-Hydroxycinnamic acid [40]

Numbers in brackets indicate references.

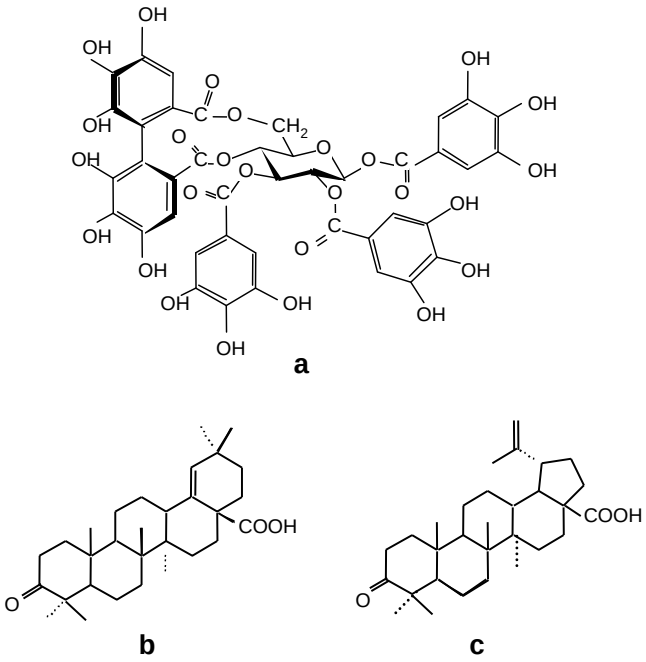


Fig. (1). Structure of eugeniin (**a**) prepared from *Geum japonicum* and *Syzygium aromaticum*, and moronic acid (**b**) and betulonic acid (**c**) prepared from *Rhus javanica*.

Eugeniin inhibited viral DNA and late protein syntheses in Vero cells infected with HSV-1 as well as PAA did but did not inhibit cellular protein synthesis at inhibitory concentrations. The activity of purified HSV-1 DNA polymerase was non-competitively inhibited by eugeniin with respect to dTTP. The apparent K_i value for euginiin was 8.2- and 5.8-fold lower than those of purified human DNA polymerases α and β, respectively [21]. Eugeniin was demonstrated to inhibit HSV-1 DNA synthesis selectively by interfering non-competitively with HSV-1 DNA polymerase activity [21]. Thus, one of the major target sites of the inhibitory action of eugeniin was characterized to be viral DNA synthesis, and the inhibition of viral DNA polymerase activity was novel as compared with anti-HSV nucleoside analogs.

As expected, eugeniin inhibited not only the growth of wild type HSV-1 but also thymidine kinase-deficient (ACV-resistant) HSV-1 and PAA-resistant HSV-1 and HSV-2 [21]. Isobologram analysis demonstrated that eugeniin enhanced the anti-HSV-1 activity of acyclovir but was suggested to be antagonistic to that of PAA [22]. The interaction of eugeniin and PAA on the activity of partially purified HSV-1 DNA polymerase suggested that eugeniin interacted with the polymerase in the vicinity of the PAA-binding site. Thus, eugeniin showed a different anti-HSV-1 action from the therapeutic anti-HSV-1 activities of ACV and PAA in mice.

Oral and intraperitoneal administration of eugeniin at 0.3 mg/kg showed similar therapeutic efficacy in retarding the development of skin lesions of HSV-1-infected mice [22]. Both routes of administration significantly prolonged mean survival times and/or reduced mortality without toxicity. The therapeutic efficacy of oral administration at the various doses of eugeniin was similar to that of intraperitoneal administration, suggesting that the oral bioavailability of eugeniin was high with respect to absorption. The oral administration of eugeniin as well as the extract of *Geum japonicum* reduced virus yields in the skin and brain of infected mice [2, 22]. Eugeniin possibly represented the therapeutic efficacy of the extract and was beneficial in preventing central nervous system complications following HSV infection. Thus, we characterized the anti-HSV activity of eugeniin as a major anti-HSV compound in *Geum japonicum* and *Syzygium aromaticum* in cutaneously HSV-1-infected mice and its interaction with HSV-1 DNA polymerase [21, 22].

3.2. Anti-HSV Activity of Moronic Acid and Betulonic Acid

We also purified and identified two anti-HSV compounds, moronic acid (Fig. **1b**) and betulonic acid (Fig. **1c**), from *Rhus javanica* L. These extracts not only exhibited significant therapeutic anti-HSV-1 efficacy in a primary HSV-1 infection in mice but also alleviated spontaneous and ultraviolet-induced recurrent HSV genital disease in guinea pigs [6, 7]. The effective concentrations of moronic acid and betulonic acid for 50% plaque reduction of wild HSV-1 were 3.9 and 2.6 μg/ml, respectively, but the therapeutic index (50% cytotoxic concentration/50% plaque reduction concentration) of moronic acid was higher than that of betulonic acid [23]. Moronic acid was quantitatively a major anti-HSV compound in the EtOAc soluble fraction of the extract. Moronic acid, like eugeniin, exhibited therapeutic anti-HSV-1 activity in a cutaneous HSV infection model in mice [23], where it suppressed virus yields in the brain more efficiently than those in the skin. This was consistent with the prolongation of mean survival times. Therefore, moronic acid was purified as a major anti-HSV compound from the herbal extract of *Rhus javanica*. The susceptibilities of ACV- and

PAA-resistant HSV-1, thymidine kinase-deficient HSV-1, and wild HSV-2 to moronic acid were similar to that of the wild HSV-1, suggesting that mode of the anti-HSV activity was different from that of ACV and PAA. Oral administration of moronic acid showed therapeutic efficacy in HSV-infected mice and possessed novel anti-HSV activity that was consistent with that of the extract. Thus, we verified the therapeutic anti-HSV activity of extract of *Rhus javanica* by identifying the antiviral actions and therapeutic activity of the purified compounds.

3.3. Tannins, Glucosides, and Alkaloids with Anti-HSV Activity

We have also isolated various compounds with anti-HSV activity, such as tannins, glucosides, and alkaloids, from *Geun Japonicum*, *Woodwardis orientalis*, or *Stephania cepharantha* [24-27]. The anti-HSV activities were assessed mainly by *in vitro* assays because many of the antiviral compounds from these natural products had been identified by other researchers. Some of them may be contained in dietary supplements or functional foods, and clarifying their efficacy *in vivo* would be worthwhile.

3.4. Diarylheptanoids with Anti-Influenza Virus Activity

The pandemic influenza H1N1 virus has recently spread worldwide. The appearance of an influenza virus more virulent than the pandemic H1N1 is now predicted. The emergence of viruses resistant to the amantadine and neuraminidase inhibitors zanamivir and oseltamivir has been documented. It is important to develop new types of anti-influenza virus agents with anti-influenza virus actions different from those of the known agents. We found that diarylheptanoids isolated from *Alpinia officinarum* Hance (Fig. **2**) exhibited potential anti-influenza virus activity *in vitro* [28]. Some diarylheptanoids were suggested to have a different anti-influenza virus action than that of oseltamivir and were verified to show anti-influenza activity in a plaque reduction assay and an influenza virus infection model in mice (unpublished data). We are now analyzing the mode of their anti-influenza virus action.

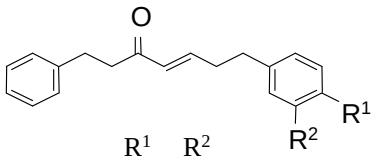
4. NATURAL PRODUCTS AS CRUDE MATERIALS WITH IMMUNOMODULATORY ACTIVITY

Natural products have been demonstrated to modify immunological activity and are candidates for biological response modifiers (BRMs), which are effective in alleviating symptoms and reducing mortality in infectious diseases.

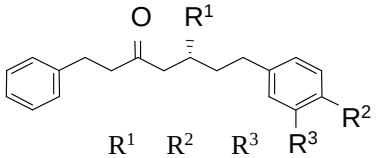
4.1. Kakkon-to, A Medicinal Herb, as BRM Against Influenza Virus Infection (Table 2)

Influenza virus infection causes headache, muscle pains, and malaise, accompanied by high fever after 2-3 days incubation and sometimes is followed by severe complications such as pneumonia and encephalopathy.

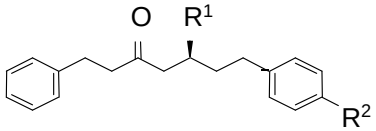
Kakkon-to, a traditional medical prescription used since the days of ancient China, is composed of seven medicinal herbs. In traditional therapy, kakkon-to was used in the early phase of common cold and influenza infections to alleviate symptoms. However, the efficacy of kakkon-to and its pharmacological and biochemical bases of action have not been established.



	R^1	R^2
1	H	H
2	OH	H
3	OH	OCH_3



	R^1	R^2	R^3
4	OH	H	H
5	OH	OH	OCH_3
6	OCH_3	OH	OCH_3



	R^1	R^2
7	OCH_3	H
8	OH	OH
9	OCH_3	OH

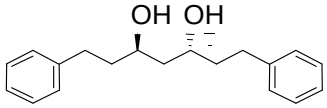


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Fig. (2). Structures of diarylheptanoids prepared from *Alpinia officinarum.*

We evaluated the therapeutic efficacy of kakkon-to in a murine intranasal influenza infection model. Oral administration of kakkon-to was significantly effective in reducing the body-weight loss of infected mice, retarding the development of pneumonia, and decreasing mortality [29]. Kakkon-to administration also suppressed fever 1 to 2 days after infection [29]. Because kakkon-to had no direct anti-influenza virus activity *in vitro*, we focused on its effect on the production of cytokines as a host immune mediator against influenza infection.

In order to analyze the antipyretic action of kakkon-to in infected mice, we have developed a fever production system using a mouse strain with a high susceptibility to interferon (IFN), which is known to be an endogenous pyrogen and induce fever [30]. Actually in humans IFN treatment of chronic hepatitis causes the influenza-like fever and symptoms seen in patients just after the administration of IFN. We screened seven mouse strains to identify a strain suitable for this analysis, and DBA/2 Cr mice were found to be the most suitable model for analyzing the mechanism of fever production by influenza infection. Using this model, we demonstrated the cascade of fever production in influenza infection shown in Fig. (**3**): influenza virus infection, elevated IFN

activity, IL-1α production, elevated cyclooxygenase activity and prostaglandin E_2 production, and fever induction [30]. Kakkon-to treatment did not affect IFN activity or the levels of IL-2, tumor necrosis factor (TNF)-α, and IFN-α in serum after infection but significantly suppressed the rise of IL-1α level in serum and BALF compared with untreated mice. When the fever abated in infected mice treated with kakkon-to, the IL-1α level also decreased in serum and was maintained at the same level as in uninfected mice. Fever production was significantly suppressed by treatments with anti-IFN-α/β or anti-IL-1α antibody in infected mice, and the former significantly suppressed responsive IL-1α production. A similar antipyretic action was also observed in infected mice administered another herbal medicine, gingyo-san (Yin-Qiao-San) (Table **2**), which has been used for the treatment of the common cold and influenza infection in China [31]. The suppression of responsive IL-1 production by kakkon-to correlated with the reduction of cell infiltration in the lungs and was suggested to alleviate pneumonia. Thus, the alleviation of pneumonia and fever by kakkon-to is possibly based on the suppression of IL-1α production induced by IFN in influenza. Kakkon-to was confirmed to make pneumonia milder and reduce fever in infected mice by its immunomodulatory activity.

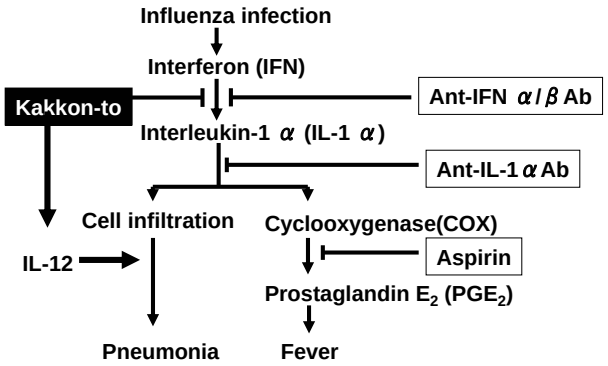

Fig. (3). Cascade of fever production in influenza and due to Kakkon-to.

We also showed that direct instillation of the optimal dose of IL-12 into the noses of influenza virus-infected mice was effective in reducing virus yield in the lungs in the early phase of influenza and might be crucial for recovery from influenza infection [32]. This evidence was obtained from a study of the immunomodulatory activity of clarithromycin in influenza virus-infected mice [32]. Then we examined IL-12 levels in the kakkon-to-treated mice infected with influenza virus [33]. In infected mice treated with kakkon-to, the virus yield in the BALF was significantly lower than that in the control on day 3 after infection. On day 2 after infection, only the level of IL-12, but not IL-4, IL-10, and IFN-γ, in the BALF was increased significantly in kakkon-to-treated mice [33]. Thus, the significant enhancement of IL-12 correlated with the reduction of virus yields in BALF and the alleviation of pneumonia in the early phase of infection. This suggested that IL-12 played a key role in the alleviation of influenza in the kakkon-to–treated mice and confirmed the important action of IL-12 in influenza symptoms [32, 33]. Recently we demonstrated that IL-12 is possibly produced by infiltrating macrophages after infection as the first response cytokine and may direct an early immune defense against influenza infection [34].

Kakkon-to reduced IL-1α to the basal level in influenza virus–infected mice, resulting in the reduction of fever. However, it elevated the IL-12 level in a timely manner after infection, resulting in the increase of early immune defense activity against influenza infection. Thus, we speculate that kakkon-to plays a critical role in the cytokine production as an immune mediator against influenza virus infection. The alleviation of pneumonia and novel antipyretic action by kakkon-to were suggested to originate in the modulation of responsive cytokine production after infection.

4.2. Kakkon-to as BRM Against HSV Infection (Table 2)

Oral administration of kakkon-to at a dose corresponding to the human dose was also significantly effective in reducing mortality in HSV-1–infected mice and localized herpetic skin lesions in a murine HSV-1 infection model, but it did not exhibit anti-HSV-1 activity *in vitro* [35]. In the analysis of the mode of its efficacy, we found that the delayed type hypersensitivity (DTH) response to HSV antigen was significantly stronger in kakkon-to-treated mice than in untreated mice [35]. In the infected mice, natural killer cell activity and the population of T-cell subsets in the spleen cells, interferon induction, anti-HSV antibody production, and cytokine levels such as IL-1α, IL-2, IFN-γ, and TNF-α in the serum were not affected by kakkon-to treatment. Thus, kakkon-to locally induced strong DTH to HSV in the infected mice, and this might have caused localization of skin lesions and reduction of mortality in the treated mice. The efficacy of kakkon-to resulted from the augmentation of DTH to HSV, which is the most important protective immunity in the cutaneous HSV infection model. Using the same HSV-1 infection model in mice, we also found that kumi-binlo-to (Jiu-Wei-Bing-Lang-Tang), shishi-hakuhi-to (Zhi-Zi-Bai-Pi-Tang), syo-saiko-to (Xiao-Chai-Hu-Tang), and gingyo-san (Yin-Qiao-San) were effective in alleviating herpetic skin lesions and in augmenting the DTH reaction [36] (Table **2**). The possibility that these herbal medicines are useful for the treatment of HSV infection in humans was indicated.

Kakkon-to showed different immunomodulatory activities in HSV infection and influenza virus infection, but it was protective against both virus infections. This suggests that the immunomodulatory activities of kakkon-to as a crude material are effective against different virus infections, and suggests that natural products that have shown biological activity historically are useful sources of BRMs in viral infection.

4.3. Lactoferrin as BRM Against Influenza Virus and HSV Infection

Immunomodulatory activity in influenza virus–infected mice was observed after the oral administration of bovine lactoferrin or lactoperoxidase [37]. In infected mice, the decrease of serum IL-6 level correlated with the reduction of the number of infiltrated leukocytes and amelioration of lung consolidation [37].

Lactoferrin feeding of mice with cutaneous HSV-1 infection inhibited the appearance of herpetic skin lesions and prevented body weight loss and the decrease of splenocyte number associated with HSV-1 infection [38] (Table **2**). Lactoferrin increased the serum IL-18 level and splenocyte production of IFN-γ and IL-12 in the infected mice [38]. Lactoferrin feeding was suggested to contribute to the maintenance of homeostasis and concomitant increases of cytokine responses during HSV-1 infection.

Lactoferrin and kakkon-to are BRMs that exhibit immunomodulatory activity in both HSV infection and influenza virus infection. The production of different cytokines was modulated in these diseases. This difference is probably due to a difference in the major immunological host defense systems against HSV-1 and influenza virus. It is possible that a natural product as a crude mixture works as a BRM against different virus infections.

4.4. Onpi-to (Wen-Pi-Tang), an Herbal Extract, as BRM Against Influenza Virus Infection (Table 2)

Further, the oral administration of onpi-to extract (Table **2**) reduced the elevation of xanthine oxidase activity in the lungs due to influenza virus infection in mice [39]. Analysis of catalase activity suggested that onpi-to extract prevented the generation of highly toxic hydroxyl radicals in the lungs of infected mice. These results correlated with a reduction of the consolidation score of the lungs of infected mice. Onpi-to extract was suggested to ameliorate pathological conditions of the lungs induced by influenza virus infection.

5. COMPOUNDS AS BRMS ORIGINATED FROM NATURAL PRODUCTS

5.1. Cinnamyl Derivatives and Related Compounds with Antipyretic Activity Based on Cytokine-Modulatory Activity

We identified compounds with antipyretic and IL-1α-modulatory activities in kakkon-to, which is composed of seven medicinal herbs, by using a murine influenza virus infection model [40]. Among the seven herbs, the organic solvent-extractable fractions of *Cinnamomum cassia* showed both antipyretic activity and IL-1α-modulatory activity in serum. Because many cinnamyl derivatives and related compounds have been isolated and identified from the organic fractions of *Cinnamomum cassia*, we screened the antipyretic activity of 48 such compounds in infected mice and six of them (7-hydroxycoumarin (7HC), 4-allylanisole, cinnamic acid ethylester, acetic acid cinnamylester, 2'-hydroxyacetophenone, and 2-hydroxycinnamic acid, Fig. **4**) were found to show antipyretic activity and/or IL-1α-modulatory activity [40]. In particular, 7HC, 4-allylanisole, cinnamic acid ethylester, and acetic acid cinnamylester simultaneously exhibited antipyretic and interleukin-1α-modulatory activities with significant correlations in influenza virus-infected mice. The antipyretic action of the four did not inhibit the fever induced by injection of IL-1α into mice in contrast to aspirin, which inhibits cyclooxygenase activity (Fig. **3**). Thus, the antipyretic action of kakkon-to, which is based on the suppression of IL-1α production induced by IFN in influenza virus–infected mice as described above, could be explained by the activities of these four compounds.

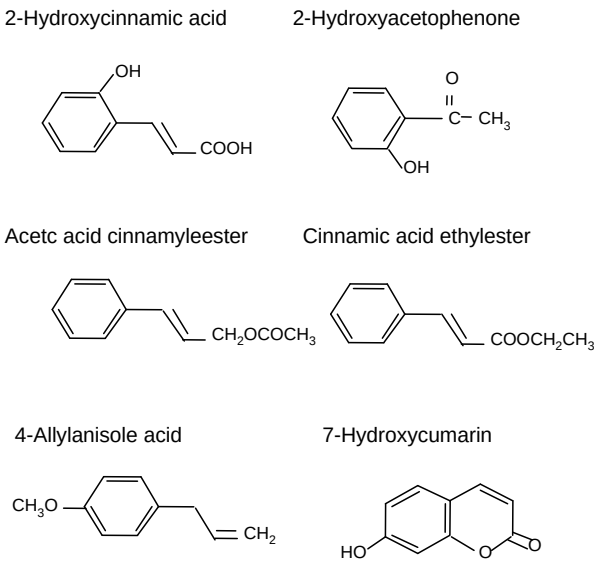

Fig. (4). Cinnamyl derivatives and related compounds with immunomodulatory activity.

5.2. 7HC with Cytokine-Modulatory Activity

The 7HC in kakkon-to was also demonstrated to be effective in reducing proinflammatory cytokine production in lipopolysaccharide (LPS)-exposed macrophage-like P388D1 cells [41]. This is consistent with the IL-1α–modulatory activity leading the reduction of fever in influenza virus–infected mice [40]. 7HC was confirmed to suppress the secretion of proinflammatory cytokines and production of their mRNAs in P388D1 cells due to influenza virus infection [42]. In a murine influenza virus infection model, the rise in the production of proinflammatory cytokines (TNF-α and IL-6) in the BALF was significantly suppressed by 7HC in the early phase of infection, and this suppression correlated with the reduction of virus titers and alleviation of lung consolidation in infected mice [42]. 7HC was suggested to alleviate pneumonia in influenza virus–infected mice through suppression of the cytokine production induced by infection. The cytokine-modulatory activity of 7HC was verified *in vitro* and *in vivo*. We have confirmed the pharmacological basis of anti-influenza virus action of kakkon-to in mice using components of kakkon-to.

5.3. 7-Amino-4-Methylcoumarin (AMC) with Cytokine-Modulatory Activity

Cinnamyl derivatives and related compounds were indicated to contribute to the modification of cytokine production and the alleviation of influenza virus infection in our previous study. Such modulation of proinflammatory cytokine production was observed in LPS-exposed P388D1 cells, and we further demonstrated the modulatory activity in an endotoxin shock model in mice [41]. Among the cinnamyl derivatives and related compounds, we found that AMC suppressed the production of pro-inflammatory cytokines (IL-1α, IL-6, and TNF-α) and their LPS-induced mRNAs in P388D1 cells through suppression of their transcription by reducing the DNA-binding amounts of NF-κB and activator

protein (AP)-1. Oral administration of AMC (30 mg/kg) as well as dexamethasone and anti-TNF-α and anti-IL-1α antibodies significantly prevented death due to endotoxic shock in mice. We verified that the rise of systemic pro-inflammatory cytokine levels, especially TNF-α, in AMC-treated mice was suppressed.

The cytokine-modulatory activity of AMC was also confirmed in a collagen-induced arthritis (CIA) model in mice. AMC reduced the incidence and severity of CIA prophylactically and therapeutically, and suppressed the rise of systemic pro-inflammatory cytokine levels to the basal levels in the early phase of CIA. The cytokine-modulatory activity of AMC was verified *in vitro* and in different animal models (unpublished data).

5.4. Glycyrrhizin Modulates Viral Surface Antigen

Glycyrrhizin is a major component of a legume (licorice) and has been used intravenously for the treatment of chronic hepatitis B in Japan. Although this compound improves liver function with occasional complete recovery from hepatitis, the pharmacological basis for its effectiveness was not clear. We showed that glycyrrhizin binds to hepatocytes to modify the expression of HBV-related antigens and suppress sialylation of the HBV surface antigen (HbsAg) [43]. Based on this study, more effective glycyrrhizin derivatives are being sought.

6. CURRENT & FUTURE DEVELOPMENTS

Natural products are potential sources of a wide range of chemicals with remarkable diversity and accessibility in nature. In the recent development of anti-influenza virus agents, oseltamivir, a neuraminidase inhibitor, is produced from shikimic acid from Chinese star anise. Shikimic acid is an important biochemical intermediate in plants and microorganisms. This confirms that natural products are useful sources of materials to develop anti-influenza virus agents. In fact, we have clarified the potential of natural antiviral products and their components using animal infection models. In future, rational chemical designs based on the structures of effective antiviral compounds identified from natural products will probably lead to the synthesis of new antiviral agents. We hope that the extensive studies of natural products with antiviral activity can contribute to the development of novel antiviral agents that inhibit virus replication.

In addition to the evaluation of direct antiviral actions of natural products, the involvement of host immune responses has been suggested in the pathogenesis of virus infection. Cytokines are known to be produced locally and systemically in animals during influenza virus infection [44-49]. Recently, proinflammatory cytokines have been reported to be markedly elevated in human cells and mice during infection with the highly pathogenic H5N1 influenza virus [50-53]. The occurrence of a 'cytokine storm' has been proposed to contribute to the increased severity of the disease caused by the highly pathogenic virus [54-56]. It was also demonstrated that the recruitment of TNF and inducible NOS-producing dendritic cells correlated with the severity of inflammatory responses induced by influenza virus [57]. Such advances in understanding the pathogenesis of influenza virus infection and information on the viral genome as well as in other virus infections are indispensable to development of better BRMs that alleviate the symptoms of virus infection. As we showed in this review, natural products are effective against different types of virus infection, and information about natural products that are effective against other types of infections will be useful to develop more efficient and available BRMs against virus infection.

ACKNOWLEDGEMENTS

We thank Mr. Yoshida, Ms. Hama, Ms. T. Okuda, and Ms. A. Hino for their excellent technical assistance. We also thank Ms. Katherine Ono for her editorial assistance.

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Received: January 17, 2010 Revised: April 11, 2010 Accepted: April 12, 2010