Identification of HSP70-inducing activity in *Arnica montana* extract and purification and characterization of HSP70-inducers

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The authors have no conflict of interest to declare.

Text word count; 4035

Number of references; 60

Number of figures; 4

Abbreviations: *A. montana*, *Arnica Montana*; DMEM, Dulbecco's modified Eagle's medium; *E. lindleyanum*, *Eupatorium lindleyanum*; FBS, fetal bovine serum; GGA, geranylgeranylacetone; HPLC, High performance liquid chromatography; HSF-1, heat shock factor-1; HSP, heat shock protein; IBMX, 3-isobutyl-1-methylxanthine; MS, mass spectra; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF- κ B, nuclear factor kappa B; NMR, Nuclear Magnetic Resonance; ODS, Octadecylsilane; PBS, phosphate buffered saline; S.D., standard deviation; UV, ultraviolet.

ABSTRACT

Background: The expression of heat shock proteins (HSPs), particularly HSP70, is receiving considerable attention in the field of cosmetics, particularly given our recent report that ultraviolet-induced melanin production, skin damage and wrinkle formation were all suppressed in transgenic mice expressing HSP70.

Objective: In the present study, we searched for HSP70-inducers from a library of herbal extracts that have already been approved as quasi-pharmaceutical products in Japan. We selected an ethanol extract of *Arnica montana* (*A. montana*), based on its high HSP70-inducing activity and low cytotoxicity.

Methods: Cell viability was determined by MTT method and expression of HSP70 was monitored by immunoblotting analysis.

Results: From the extract, we purified and identified eight sesquiterpene lactones (AM1-8) as HSP70-inducers, among which AM-2 (helenalin 2-methylbutyrate) was selected due to its good HSP70-inducing properties and low cytotoxicity. Treatment of cultured mouse melanoma cells with AM-2 or *A. montana* extract up-regulated the expression of HSP70 in a dose-dependent manner. This treatment also activated heat shock factor-1, a transcription factor for *hsp* genes. Furthermore, pre-treatment of cells with AM-2 or *A. montana* extract decreased melanin production and expression and activity of tyrosinase.

Conclusion: These results suggest that AM-2 and *A. montana* extract could be beneficial for use in hypopigmenting cosmetics as a consequence of their stimulatory effects on HSP70 expression.

Key words: heat shock protein 70; *Arnica Montana*; photo-aging; melanogenesis; hypopigmenting cosmetics

1. Introduction

In addition to changes with aging, the skin is damaged by various environmental stressors, particularly solar ultraviolet (UV) radiation (photo-aging) [1]. While UV light induces skin damage (such as erythema, plaque-like thickening, loss of skin tone, deep furrowing and fine wrinkle formation) [2-5], UV-induced skin hyperpigmentation disorders also occur due to abnormal melanin production, leading to clinical and cosmetic problems, for which a number of hypopigmenting drugs and cosmetic agents have been developed [6-8].

The exposure of keratinocytes to UV light stimulates the release of signaling molecules, which in turn induce the expression of tyrosinase at the transcriptional level via an increase in the level of intracellular cAMP [9]. Tyrosinase is a rate-limiting enzyme in melanin synthesis [9]. Therefore, chemicals and natural products that suppress the activity and/or expression of tyrosinase could potentially be pharmaceutically and cosmetically beneficial as hypopigmenting agents. However, because melanin acts as a filter to limit the penetration of UV into the epidermis and dermis, the UV-induced modest production of melanin plays an important role in protecting the skin against UV-dependent damage [10]. Thus, to develop hypopigmenting agents without worsening UV-induced skin damage, it is important to suppress melanin production with simultaneous protection the skin against UV-induced damage.

When cells are exposed to stressors, the expression of heat shock proteins (HSPs) is up-regulated to confer protection against such stressors. The induction of HSP expression by stressors is regulated at the transcriptional level via heat shock factor-1 (HSF-1), a transcription factor for *hsp* genes [11]; this activation is regulated by a

multistep process that includes HSF-1's phosphorylation [12]. Cellular up-regulation of HSP expression, particularly that of HSP70, provides resistance to stressors [13]. In addition to possessing this cytoprotective effect, HSP70 also exerts an anti-inflammatory action via its inhibitory effect on nuclear factor kappa B (NF- κ B) [14-17]. Since stressor-induced tissue damage and inflammation are involved in various diseases, HSPs and HSP-inducers have received much attention for their therapeutic potential [12]. Geranylgeranylacetone (GGA) is a clinically used anti-ulcer drug in Japan that has been described as a non-toxic HSP-inducer [18]. We have shown that administration of GGA could suppress inflammatory bowel disease-related experimental colitis, as well as lesions of the small intestine, Alzheimer's disease-related cognitive dysfunction and pulmonary fibrosis through the up-regulation of HSP expression in mice [18-23].

It is known that HSP70 is constitutively expressed in the skin and that this expression is up-regulated by stressors such as heat-shock and UV radiation [24, 25]. However, the role of this up-regulation had remained unclear until recently. To address this issue, we recently showed that UV radiation-induced skin damage (such as epidermal apoptosis), DNA damage and wrinkle formation were all suppressed in transgenic mice expressing HSP70; these beneficial effects were mediated by various properties of HSP70, including its cytoprotective and anti-inflammatory activities [26, 27]. Furthermore, we reported that melanin production in cultured mouse melanoma (B16) cells was suppressed by the overexpression of HSP70 and that UV radiation-induced melanogenesis in the skin was suppressed in transgenic mice expressing HSP70 [28]. Taken together, these results suggest that HSP70-inducers could be beneficial for use in hypopigmenting cosmetics, wherein they would function by

suppressing melanin production while simultaneously protecting the skin against UV-induced damage.

We recently reported the results of our search for HSP70-inducers from a library of Chinese herbs, where we found that an ethanol extract of *Eupatorium lindleyanum* (*E. lindleyanum*) can up-regulate expression of HSP70 in B16 cells [29]. We also purified the molecules eupalinolide A and B, which are responsible for *E. lindleyanum*'s HSP70-inducing ability [30]. Thus, *E. lindleyanum* extract and eupalinolide A and B may be beneficial for use in hypopigmenting cosmetics. However, in Japan, components of hypopigmenting cosmetics must first be approved for use as quasi-pharmaceutical products, meaning that these materials could not be used immediately in hypopigmenting cosmetics. For this reason, we searched for HSP70-inducers from a library of herbal extracts that have already received approval for use as quasi-pharmaceutical products in Japan. From these we selected an ethanol extract of *Arnica montana* (*A. montana*), a plant that has been used for centuries in Europe for medicinal purposes and its extract and components are sold in over-the-counter drug preparations in Europe and the United States [31, 32]. Ointment containing *A. montana* extract has been used to reduce inflammation and pain caused by sprains, bruises and skin wounds [32]. Furthermore, because of its antiphlogistic and antiseptic properties, components of this plant have been used in many cosmetic preparations [32]. It was reported that the sesquiterpene lactone helenalin, which was purified from *A. montana* extract, has anti-inflammatory activity through direct inhibition of the p65 subunit of NF- κ B [33]. In this study, we purified HSP70-inducers from the ethanol extract of *A. montana*, and identified eight sesquiterpene lactones (AM1-8). Among them, we selected AM-2 (helenalin 2-methylbutyrate). Treatment of B16 cells with AM-2 or *A.*

montana extract not only up-regulated the expression of HSP70 but also activated HSF-1. We also found that pre-treatment of cells with AM-2 or *A. montana* extract suppressed both melanin production and the expression of tyrosinase. These results suggest that AM-2 and *A. montana* extract could be beneficial for use in hypopigmenting cosmetics.

2. Materials and Methods

2.1. *Materials*

Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co (Tokyo, Japan). Fetal bovine serum (FBS), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma-Aldrich Co. (St. Louis, MO). Antibodies against tyrosinase and actin or against HSP70 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) or R&D Systems (Minneapolis, MN), respectively. Antiserum against HSF-1 was kindly provided by Dr. Akira Nakai (Yamaguchi University) [34]. L-DOPA was from Nacalai Tesque (Kyoto, Japan). Octadecylsilane (ODS) columns were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Fuji Silysia Ltd. (Kasugai, Japan). Herbal extracts were obtained from Maruzen Pharmaceuticals (Onomichi, Japan). B16 cells were obtained from RIKEN Cell Bank (Tsukuba, Japan).

2.2. *Cell culture*

B16 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO₂ at 37°C. Cell viability was determined by the MTT method [35]. After evaporation of ethanol, the ethanol extract was dissolved in 100% DMSO and the cell culture

experiments were done in the presence of 1% DMSO. We have confirmed that DMSO at this concentration did not affect the expression of HSP70.

2.3 Immunoblotting analysis

Whole cell extracts were prepared as described previously [36]. The protein concentration of each sample was determined by the Bradford method [37]. Samples were applied to polyacrylamide SDS gels and subjected to electrophoresis, after which proteins were immunoblotted with each antibody.

2.4 Extraction, fractionation and identification of AMs

The whole plant of *A. montana* (1.0 kg) was extracted 3 times with ethanol by refluxing for 6 h and the extract was concentrated *in vacuo* to afford residues (39.84 g). This extract was partitioned with ethyl acetate (EtOAc) and 40% methanol (MeOH) to give two fractions (EtOAc fraction: 31.05 g, 40% MeOH fraction: 8.79 g). Most of the HSP70-inducing activity (as determined by immunoblotting analysis) was recovered in the EtOAc fraction, which was loaded onto an SiO₂ column (ϕ 50 × 360 mm) and eluted by stepwise elution with an increasing polarity of solvent (hexane (Hex): EtOAc, from (10 : 1) to (1 : 2); CHCl₃-MeOH, from (4 : 1) to (0 : 1)) to give fractions A-F [A (Hex : EtOAc (10 : 1)) 9.69 g; B (Hex : EtOAc (5 : 1)) 2.29 g; C (Hex : EtOAc (1 : 1)) 5.98 g; D (Hex : EtOAc (1 : 2)) 1.78 g; E (CHCl₃ : MeOH (4 : 1)) 9.64 g; F (CHCl₃ : MeOH (0 : 1)) 4.43 g]. Fraction C (5.98 g), which was eluted by Hex : EtOAc (1 : 1), had the most HSP70-inducing activity (supplementary Fig. 1). This fraction was further

separated using an ODS column (Wako gel, ϕ 50 $\times$ 340 mm) eluted by 40% MeOH, 60% MeOH, 80% MeOH, MeOH and Acetone to afford fractions C-1 to C-13 [C-1 (40% MeOH) 80 mg; C-2 (40% MeOH) 74 mg; C-3 (60% MeOH) 19 mg; C-4 (60% MeOH) 32 mg; C-5 (60% MeOH) 44 mg; C-6 (60% MeOH) 68 mg; C-7 (60% MeOH) 378 mg; C-8 (80% MeOH) 158 mg; C-9 (80% MeOH) 104 mg; C-10 (MeOH) 250 mg; C-11 (MeOH) 1.15 g; C-12 (MeOH) 1.19 g; C-13 (Acetone) 2.35 g]. The active fraction for HSP70-inducing activity, C-7 (378 mg) eluted by 60% MeOH (supplementary Fig. 1), was further separated by ODS column (Chromatorex, ϕ 33 $\times$ 165 mm) eluted by 50% MeOH into fractions C-7-1 to C-7-4 [C-7-1 (47 mg), C-7-2 (275 mg), C-7-3 (27 mg), C-7-4 (10 mg)]. A part of the active fraction for HSP70-inducing activity (C-7-2 (86.9 mg)) (supplementary Fig. 1), which was a sesquiterpene lactone group, was further separated using a preparative high performance liquid chromatography (HPLC) [column: Cosmosil 5C18-MS-II (ϕ 20 $\times$ 250 mm); solvent: 50% MeOH; flow rate: 6.0 mL/min; column temperature: 40°C; detection: UV 254 nm] to yield AM-1 (5.3 mg, t_R = 51.7 min), AM-7 (3.8 mg, t_R = 54.8 min), AM-8 (5.3 mg, t_R = 60.6 min), AM-4 (8.2 mg, t_R = 67.3 min), AM-5 (9.8 mg, t_R = 75.0 min), sub-fraction 1 including AM-6 (11.8 mg, t_R = 77.3 min), sub-fraction 2 including AM-2 (14.5 mg, t_R = 83.9 min), and sub-fraction 3 including AM-3 (3.3 mg, t_R = 89.4 min). The sub-fractions 1, 2 and 3 were finally purified with HPLC [column: Cosmosil 5C18-AR-II (ϕ 10 $\times$ 250 mm); solvent: 55% MeOH; flow rate: 2.0 mL/min; column temperature: 40°C; detection: UV 254 nm] to yield AM-6 (5.7 mg, t_R = 34.3 min), AM-2 (4.8 mg, t_R = 37.0 min), and AM-3 (2.9 mg, t_R = 38.9 min), respectively. The structures of molecules in these fractions were identified on the basis of their nuclear magnetic resonance (NMR) spectra and mass spectra (MS) data that have been described previously [38-40].

2.5 Determination of melanin content of cells

The melanin content of cells was determined as described previously [41-43] with some modifications. Cells were homogenized with 1 N NaOH and the melanin content was determined by measuring the absorbance at 405 nm with a plate reader (Infinite M200 PRO, Tecan Group Ltd, Männedorf, Switzerland).

2.6 Tyrosinase activity assay

Tyrosinase activity was monitored as described previously [43] with some modifications. Cells were washed with phosphate buffered saline (PBS) and homogenized with 0.1% Triton X-100. Tyrosinase activity (as indicated by oxidation of L-DOPA to DOPACHrome) was monitored as follows: cell extract (50 µl) was mixed with 100 µl of freshly prepared substrate solution (0.1% L-DOPA in PBS) and incubated at 37°C. The production of DOPACHrome was monitored by measuring the absorbance at 475 nm with a plate reader (Infinite M200 PRO) and corrected for the auto-oxidation of L-DOPA [43].

2.7 Statistical analysis

All values are expressed as the mean $\pm$ standard deviation (S.D.). One-way analysis of variance followed by the Tukey test was used to evaluate differences, which were

considered to be significant for values of $P < 0.05$.

3. Results

3.1 Screening of herbal extracts for HSP70-inducing activity and identification of HSP70-inducers

B16 cells were used to facilitate the identification of HSP70-inducers from 30 herbal extracts (supplementary Table. 1). We examined the cytotoxicity and HSP70-inducing activity of each extract and subsequently selected three candidate herbs, namely *Salvia officinalis*, *Origanum majorana* and *A. montana*, based on their high HSP70-inducing activity and low cytotoxicity. We then examined the dose-response profiles of extracts of these herbs with respect to their capacity to induce cell death and HSP70 expression (Fig. 1A-C). Treatment of cells with each of these herbal extracts induced HSP70 expression at doses that did not extensively affect cell viability (Fig. 1A-C). To estimate the HSP70-inducing ability of each herbal extract, we determined the concentration of each herbal extract required to double the baseline level of HSP70 expression. This index for *Salvia officinalis*, *Origanum majorana* and *A. montana* was 0.6, 0.3 and 0.1 mg/ml, respectively, for which we selected *A. montana* for further study.

By partition with ethyl acetate and 40% methanol, SiO₂ column chromatography, ODS column chromatography and HPLC, from the ethanol extract of *A. montana*, we purified a series of molecules responsible for the HSP70-inducing activity (Fig. 2A). In the final step of the purification processes, we obtained eight fractions that showed HSP70-inducing activity (Fig. 2A). These fractions were identified by ³H and ¹³C NMR and MS analyses as being sesquiterpene lactones, which we named AM1-8 (Fig. 2B).

The compounds were classified into three groups according to their structure: (1) helenalin type, (2) 11 α , 13-dihydrohelenalin type, and (3) 11 β , 13-dihydrohelenalin type (Fig. 2B).

Considering the applications for HSP70-inducers to be used in medicines and cosmetics, it is important that they are able to induce the expression of HSP70 without decreasing cell viability. We subsequently compared the HSP70- and cell death-inducing activities of the eight AMs (Fig. 3). To determine beneficial HSP70-inducers, we first selected AM-2, AM-5, AM-6 and AM-8, because they can up-regulate HSP70 expression more than fourfold at concentrations that do not affect cell viability extensively (cell viability, more than 85%). We then determined the concentration of each AM required to quadruple the baseline level of HSP70 expression, which corresponded to 0.6, 4.0, 4.0 and 8.0 μ g/ml for AM-2, AM-5, AM-6 and AM-8, respectively. Based on these results we selected AM-2 for further study. It should be noted that helenalin, which was identified in *A. montana* extract as an active molecule for anti-inflammatory activity, did not clearly up-regulate the expression of HSP70 (Fig. 3I).

3.2 Characterization of HSP70-inducing activities of AM-2 and *A. montana* extract

We next examined the time-course profiles of HSP70 induction by AM-2 or *A. montana* extract after treatment of cells for 3 h. As shown in Fig. 4A, the up-regulation of expression of HSP70 by AM-2 or *A. montana* extract became apparent within 9 h of the cessation of treatment and could still be observed for up to 21 h.

As described in the introduction, the activity of HSF-1 is regulated by its phosphorylation. We therefore examined the phosphorylation of HSF-1 in the presence of AM-2 or *A. montana* extract, which can be detected as an upward band-shift [44, 45]. As shown in Fig. 4B, an upward band-shift of HSF-1 was observed in cells treated with AM-2 or *A. montana* extract (Fig. 4B), suggesting that AM-2 and *A. montana* extract could induce the phosphorylation of HSF-1.

We also examined the effect of AM-2 or *A. montana* extract on mRNA expression of HSP90 β , HSP70, HSP47 and HSP40. As shown in supplementary Fig. 3, the mRNA expression of HSP70 was up-regulated by AM-2 or *A. montana* extract, however, these samples did not affect the mRNA expression of HSP90 β and HSP47 so clearly. AM-2 but not *A. montana* extract up-regulated mRNA expression of HSP40 (supplementary Fig. 3).

3.3 Inhibitory effect of AM-2 and *A. montana* extract on melanogenesis

As the expression of HSP70 suppresses melanin production (see Introduction), we next examined the effect of AM-2 or *A. montana* extract on melanin production in B16 cells. We used IBMX (a cAMP-elevating agent that acts by inhibiting phosphodiesterase) to mimic UV-stimulated melanin production [28]. As shown in Fig. 4c, treatment of cells with IBMX increased the melanin content in cells, while pre-treatment of cells with AM-2 or *A. montana* extract suppressed this increase. We next examined tyrosinase activity in cells and found that treatment of cells with IBMX increased tyrosinase activity, while pre-treatment of cells with AM-2 or *A. montana* extract decreased this activity in the presence of IBMX (Fig. 4D). On the other hand,

when AM-2 or *A. montana* extract was added directly to cell extracts prepared from IBMX-treated B16 cells, the tyrosinase activity was not significantly affected (Fig. 4E). These results suggest that AM-2 or *A. montana* extract alter the expression of tyrosinase. To further test this idea, we examined the effect of AM-2 or *A. montana* extract on the cellular level of tyrosinase and found that pre-treatment of B16 cells with AM-2 or *A. montana* extract suppressed the IBMX-dependent increase in the level of tyrosinase (Fig. 4F).

To examine the relationship between AM-2 or *A. montana* extract-induced expression of HSP70 and the tyrosinase activity (or expression), we examined the dose-response profiles of AM-2 or *A. montana* extract for these phenomena. Plotting expression of HSP70 vs the tyrosinase activity (or expression) yielded an R^2 value more than 0.5 (supplementary Fig. 2), which suggests that the inhibitory effect of AM-2 or *A. montana* extract on tyrosinase activity (or expression) is mediated by the induction of HSP70 expression.

4. Discussion

HSP70 has become a new molecular target of cosmetics, particularly given our recent finding that UV-induced melanin production, skin damage and wrinkle formation were all suppressed in transgenic mice expressing HSP70 [26-28]. This motivated us to search for HSP70-inducers from herbal extracts, as has been done previously by a number of researchers, including ourselves [29, 46], with the defining characteristic feature of this study being that we focused on herbal extracts which have already received approval as quasi-pharmaceutical products in Japan. In this way, any herbal extract identified here could be employed immediately for use in hypopigmenting cosmetics in Japan.

A number of compounds and herbal extracts have been shown to induce the expression of HSP70; however, in most cases the induced expression is accompanied by a simultaneous decrease in cell viability. These cytotoxic effects of HSP70-inducers could result in undesired side effects, resulting in their unsuccessful use in cosmetics. Therefore, we focused our search here not only on the capacity of identified compounds to induce HSP70 expression, but also on their capacity to have low cytotoxicity. Compared to other herbal extracts, *A. montana* extract showed lower cytotoxicity at concentrations that were high enough to induce HSP70 expression. Thus, we selected *A. montana* extract and tried to identify active molecules within this extract that had HSP70-inducing ability. Induction of HSP70 by *A. montana* extract in HaCaT keratinocytes was also reported recently [47]. We identified eight sesquiterpene lactones in fractions that showed HSP70-inducing activity (AM-1-8). Since HPLC analysis suggested that each fraction contained a single species of molecule, we

concluded that it was these molecules that had the HSP70-inducing activity. Although it was reported that *A. montana* contains helenalins and 11 α , 13-dihydrohelenalins [40, 48], this is the first time that 11 β , 13-dihydrohelenalin has been identified in *A. montana* extract. While *A. montana* has been used for centuries in Europe as a traditional medicine to treat skin wounds, bruises and contusions [32, 49], several previous studies identified various molecules in its extract [50-52]. AMs, except for AM-8, were previously identified as major sesquiterpene lactones in *A. montana* extract [40, 48]. However, this is the first observation that these sesquiterpene lactones have HSP70-inducing activity. In addition to sesquiterpene lactones, *A. montana* was reported to contain flavonoid glycosides, triterpene alcohols, coumarins, phenolic acids and a volatile oil; however, only sesquiterpene lactones with γ -butyrolactone side-group were purified as molecules with HSP70-inducing activity. Thus, this structure may be important for the induction of HSP70 activity. Supporting this idea, eupalinolide A and B, which were purified from *E. lindleyanum* extract based on their HSP70-inducing activity are also sesquiterpene lactones with γ -butyrolactone side-group [30].

Based on its high HSP70-inducing activity and low cytotoxicity, we selected AM-2 and examined its HSP70-inducing properties and inhibitory effects on melanogenesis. We suggested that treatment of cells with AM-2 or *A. montana* extract induces HSP70 expression via the activation of HSF-1. As for melanogenesis, we showed that AM-2 or *A. montana* extract suppressed the IBMX-induced increase in melanin production, as well as the increase in tyrosinase activity and the expression of tyrosinase. On the other hand, when AM-2 or *A. montana* extract was added directly to cell extracts prepared from IBMX-treated B16 cells, the tyrosinase activity was not significantly affected. Furthermore, the concentration of AM-2 or *A. montana* extract required to suppress

melanin production was similar to that required to induce HSP70 expression; in line with this we previously reported that the expression of HSP70 suppresses the IBMX-induced melanogenesis and expression of tyrosinase [28]. Thus, the inhibitory effect of AM-2 or *A. montana* extract on melanin production seems to be mediated by the HSP70-dependent suppression of tyrosinase expression. It should be noted that a traxastane-type triterpene was isolated from *A. montana* extract based on its inhibitory effect on melanogenesis [53]. Thus, it is possible that an HSP70-independent action of *A. montana* extract is also involved in its inhibitory effect on melanogenesis.

Since hypopigmenting reagents could serve as important constituents of cosmetics, this has led to the discovery and development of a number of compounds that inhibit the activity and/or expression of tyrosinase. However, most of their cosmetic and pharmaceutical applications have not been successful due to problems associated with skin irritation [54, 55]. This could be due to the fact that UV-induced melanogenesis has a protective role against UV-induced skin damage [56]. Based on the results of the present study, we propose that *A. montana* extract could be beneficial as a hypopigmenting agent in cosmetics given that HSP70 protects against UV-induced skin damage [26].

In addition to its cytoprotective actions, HSP70 also has anti-inflammatory properties [14-17]. Consistent with this finding, *A. montana* extract has been used clinically as an anti-inflammatory agent [31, 49]. Thus, it is possible that the anti-inflammatory activity of *A. montana* extract is primarily a consequence of its HSP70-inducing activity. However, we showed here that helenalin, compound with anti-inflammatory property in *A. montana* extract, did not clearly affect the expression of HSP70. Furthermore, although it was reported that AM-1 has anti-inflammatory

activity, the concentration required to achieve this activity [33] was much higher than that required to up-regulate the expression of HSP70 (this study). Thus, we consider that the anti-inflammatory activity of *A. montana* extract is likely to be mediated by an HSP70-independent mechanism. It should be noted that induction of HSP70 by helenalin in HaCaT keratinocytes was reported recently [47].

The ameliorative effects of HSP70 due its cytoprotective, anti-inflammatory and molecular chaperone (quality control of proteins) properties have been reported for various diseases. For example, we have shown using transgenic mice and GGA that HSP70 protects against irritant-produced lesions in the stomach and small intestine, as well as in inflammatory bowel disease-related experimental colitis, pulmonary fibrosis and in Alzheimer's disease-related phenomena in mice [21, 22, 57-60]. Based on these observations, AMs, especially AM-2, could be therapeutically beneficial for use in combatting these diseases.

Acknowledgements

We thank to Dr. Akira Nakai (Yamaguchi University) for providing antiserum against HSF-1. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan; as well as Center of Innovation Program from Japan Science and Technology Agency; Scientific Technique Research Promotion Program for Agriculture, Forestry and Food Industry; and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Figure legends

Fig. 1. Effects of various herbal extracts on cell viability and HSP70 expression. B16 cells were incubated with indicated concentration of each herbal extract (a, *Salvia officinalis*; b, *Origanum majorana*; c, *A. montana*) for 3 h and then in the absence of the extract for a further 21 h. Cell viability was determined by the MTT method (filled squares). Whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or actin. The intensity of the HSP70 band was determined and expressed relative to the control (open diamonds). Values are given as mean $\pm$ S.D. ($n=3$).

Fig. 2. Purification and structures of AMs. Procedure for the purification of AMs from *A. montana* is outlined in the materials and methods section (A). Structures of AMs are shown (B).

Fig. 3. Effect of AMs or helenalin on cell viability and HSP70 expression. B16 cells were incubated with indicated concentration of each AM or helenalin for 3 h and then in their absence for a further 21 h. Cell viability was determined by the MTT method (filled squares). Whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or actin. The intensity of the HSP70 band was determined and expressed relative to the control (open diamonds). Values are given as mean $\pm$ S.D. ($n=3$).

Fig. 4. Effect of AM-2 or *A. montana* extract on HSP70 expression, phosphorylation of HSF-1, and melanogenesis. B16 cells were incubated with 0.6 $\mu\text{g/ml}$ of AM-2 or 125 $\mu\text{g/ml}$ of *A. montana* extract for 3 h (A-D, F). Cells were further incubated in their

absence for indicated period (A) or 3 h (C, D, F). Cells were then incubated with or without 100 μ M IBMX for 36 h (C) or 24 h (D, F). Indicated concentration of AM-2 or *A. montana* extract was added to cell extracts prepared from IBMX-treated or non-treated B16 cells (E). Whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 (A), HSF-1 (B), actin (A, B, F) or tyrosinase (F). Melanin content (C) and tyrosinase activity (D, E) were determined as described in the materials and methods section and expressed relative to the control. Values are given as mean $\pm$ S.D. ($n=3$). ** $P<0.01$, n.s. not significant.

Vitae

Keiko Usui was born at Nagoya in 1991. In 2009, she graduated from Nazan Girls' senior high school and entered Keio University, School of Pharmacy. She plans on graduating from Keio University in 2015 and entering Ph. D course of Graduate School of Pharmaceutical Science, Keio University.

Fig. 1

Fig. 2

A

B

Helenalin Type

11 α ,13-Dihydrohelenalin Type

11 β ,13-Dihydrohelenalin Type

R

R

R

AM-1

AM-5

AM-8

AM-2

AM-6

AM-3

AM-7

AM-4

Fig. 3

I

Fig. 4