ベンゼン-ジエチルエーテル (9:1), ジエチルエーテルと順次極性を上げて分取し，8分画 (L1~L8) を得た．トリテルペノイドアセテート分画 (L3, 3.1 g) を 20%硝酸銀シリカゲルカラムクロマトグラフィーに付し，ヘキサン-ベンゼン (8:2) で溶出し 3 分画 (L3-1~L3-3) を得た．分画 L3-1 (1.3 g) をアセトンで再結晶をくり返すことにより，β-amyrin acetate (425 mg), α-amyrin acetate (418 mg), bauerenyl acetate (95 mg), germanicyl acetate (46 mg) を得た．ろ液に残った成分を逆相 HPLC により分離し，lactucenyl acetate (19 mg) を得た．分画 L3-2 および L3-3 をアセトンあるいはメタノールで再結晶を行い，各々の分画から taraxasteryl acetate (135 mg) および lupenyl acetate (141 mg) を得た．

C　研究結果

アキノノゲシ新鮮根のヘキサンエキスから分離したトリテルペノイドアセテート分画の成分を精査し，新規化合物 1 種 (lactucenyl acetate, 図), 既知化合物 6 種 (β-amyrin acetate, α-amyrin acetate, bauerenyl acetate, germanicyl acetate, taraxasteryl acetate, lupenyl acetate) の計 7 種を単離同定あるいは構造決定した．新規化合物の構造は各種 NMR (^{1}H, ^{13}C, DEPT135, HSQC, HMBC, ^{1}H-^{1}H COSY, NOESY), EI-MS, IR を解析することにより決定した．

新規化合物の構造解析を行っている際に，我々が 1981 年にカンサイタンポポから新規化合物として単離，構造決定した tarolupenyl acetate の構造式が誤っていることに気がついた．そこで，tarolupenyl acetate の構造を詳細に再解析し，その構造を lup-19(21)-en-3β-yl acetate (図 1) であることを明らかにした．

D　考察

アキノノゲシ新鮮根のトリテルペノイドアセテート分画にはルパン，オレアナン，ウルサン系化合物が含有されることが明らかとなった．ルパン系トリテルペノイドは比較的分布が限られており，植物界に広く分布するオレアナン系トリテルペノイドとは対照的である．特に，転位型ルパンは報告例が少なくキク科の 2 属 (*Lactuca* 属 (本研究), *Taraxacum* 属) 以外に 3 科 4 属に分布するのみである．キク科タンポポ亜科植物の中には転位型ルパン系トリテルペノイド生産能を有する植物が存在することが明らかとなったが，*Ixeris* 属や *Picris* 属からは検出されていない．今後，研究を継続することにより，系統分類と二次代謝産物生産能の関係を明らかにしていく必要があると思われる．

E　結論

キク科タンポポ亜科植物トリテルペノイド成分探索の一環として，アキノノゲシ *Lactuca indica* L. のトリテルペノイド成分の精査を行い，新規転位型ルパン系トリテルペノイド lactucenyl acetate を単離，同定した．

キク科タンポポ亜科植物から転位型ルパ

ン系化合物が単離されたのは *Taraxacum* 属植物につづき 2 例目である．分布が非常に限られている転位型ルパン系化合物が複数のタンポポ亜科植物から検出されたことは，本植物群の顕著な特徴であり，当該骨格を有する化合物群の貴重な供給源になることが期待される．

F　健康危機情報

なし

G　研究発表

1. 論文発表

なし

2. 学会発表

なし

H　知的所有権の取得情報

なし

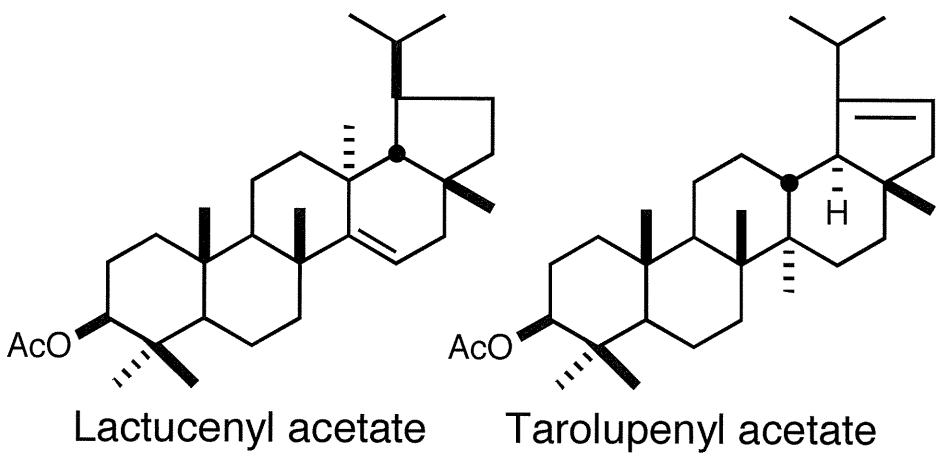

図 1　新規化合物 Lactucenyl acetate の構造式及び Tarolupenyl acetate の改訂構造式

平成２３年度労働厚生省科学研究補助金（地域医療基盤開発推進研究事業）
分担研究年次終了報告書

日本産オウレン属植物の分子系統学的検討

研究分担者：篠崎　淳一　　昭和薬科大学 助教

研究要旨　生薬オウレンの基原植物である *Coptis japonica*, および近縁植物に関して, 葉緑体 DNA の *matK* および核リボソーム DNA の ITS 領域の塩基配列情報を利用して分子系統学的検討を行なった.

A　研究目的

古くからの重要な薬物資源であるオウレンは, 北陸から山陰地方を中心に栽培されてきた. 近年, 国内生産量は減少しており, 中国からの輸入品が中心となっている. このような状況を鑑み, 生薬オウレンは日本特産の *Coptis japonica* に加え, 中国産の *C. chinensis*, *C. deltoidea*, *C. teeta* を基原植物としている.

日本には薬用に用いられる *C. japonica* の他にも, ミツバオウレン *C. trifolia* やバイカオウレン *C. quinquefolia* など数種が知られている.

近年, 塩基配列情報を利用した分子系統学的分類が植物においても広く行なわれるようになってきた. また, 第十五改正日本薬局方第一追補から参考情報「遺伝子情報を利用する生薬の純度試験」として朮類の ITS(internal transcribed spacer) 領域を標的とした識別法が収載されている.

今年度は, 分子系統学的な検討がなされていない日本産オウレン属植物の塩基配列情報からみた類縁関係を検証することを目的とした.

B　研究方法

7 種(*C. japonica* の 3 変種を含む), 28 個体の *Coptis* 属植物の新鮮葉から DNA を抽出した. PCR 法により, 葉緑体 DNA の *matK* (maturase K)遺伝子および核リボソーム DNA の ITS 領域を増幅した. 増幅産物は精製後, 塩基配列解析に供した.

matK 遺伝子および ITS 領域の塩基配列は Clustal W を用いて多重整列を行なった. 分子系統解析は, PHYLIP (ver. 3.69)プログラムを用いて, 近隣結合法(NJ 法), 最大節約法(MP 法), 最尤法(ML 法)を使って系統樹を作成した.

C　研究結果

matK 遺伝子配列の比較から *C. japonica* の 3 変種とウスギオウレンは一つのグループを形成し, このグループはさらに 4

つのサブグループに細分化できることが明らかとなった. サブグループ 2~4 は鳥取県産およびタンバオウレン, サブグループ 1 は鳥取県以外のオウレンで構成されていた.

ITS 領域の塩基配列の比較から, *C. japonica* の 3 変種とウスギオウレンは 2 つに大別できることが明らかとなった. すなわち, 617bp の ITS 領域をもつグループと, 617bp と 618bp の ITS 領域をもつグループに分けられる. 後者は鳥取県産およびタンバオウレン, 前者は鳥取県以外のオウレンで構成されていた.

D　考察

系統分類上, *C. japonica* はウスギオウレンとともにオウレン節に分類されている. *C. japonica* は小葉の形態により, 3 変種に細分類されている. しかし, 中間的な形態の小葉を有する
個体も存在している.

塩基配列の比較から, オウレン節植物は鳥取県産・タンバオウレンとその他の地域の 2 つに大別されることが明らかとなった. 分子系統学的分類と形態分類との相違は, 1) 比較的最近になり, 種分化がおこった, あるいは 2) 未同定の形態的, 組織学的な違いが存在することを示唆していると推察される.

E　結論

日本産オウレン属植物の分子系統学的検討から, オウレン節植物には異なる遺伝子背景を有する 2 群に分類されることが明らかとなった. この分類では, 形態ではなく, 分布域による相違を反映している傾向にある.

このことは, 比較する塩基配列領域の選定を種々検討することにより, 種および産地毎に識別できる可能性を示唆するものである.

F　健康危機情報

なし

G　研究発表

1. 論文発表

なし

2. 学会発表

日本生薬学会第 58 回年会

H　知的所有権の取得情報

なし

厚生労働省科学研究補助金（地域医療基盤開発推進研究事業）

分担研究報告書

漢方処方配合生薬の安定供給及び持続的品質保持における国際標準化に関する研究

分担研究課題：新潟県内におけるオウレン属植物の自生地調査

研究分担者　高野　昭人　昭和薬科大学　准教授

研究要旨　国内の生薬資源の潜在量に関する調査の一環として，生薬黄連の基原植物の一つであるオウレン属植物について新潟県内 4 か所でフィールド調査を行った．その結果，オウレン属植物 3 種（キクバオウレン、ミツバオウレン、コシジオウレン）が自生していることを確認した．また，弥彦山系の国上山には非常に多くのキクバオウレンが自生し，一方，新潟県内山間部の標高の高い地域には，キクバオウレンとミツバオウレンが同時に分布していることを確認した．今後，生薬資源としての品質等を評価し，資源の有効活用に向けた検討が望まれる．

A　研究目的

生薬黄連は，ベルベリンなどのアルカロイドを含有し，黄連解毒湯をはじめとする多くの漢方処方に配合される重要な生薬である．第十六改正日本薬局方には，その基原植物として，日本に自生するキンポウゲ科のオウレン *Coptis japonica* Makino のほか，中国産の *C. chinensis* Franchet, *C. deltoidea* C.Y.Cheng et Hsiao, *C. teeta* Wallich が収載されている．

現在，市場品の生薬黄連の多くは中国産のものであるが，かつては福井県，兵庫県，鳥取県などで盛んに栽培されていた歴史を有する．また，江戸時代に品質がよいとして知られていたのが加賀黄連で日本に自生するキクバオウレン *Coptis japonica* var. *japonica* の根茎に由来するとされる．

御影らは，国産の薬用資源の重要性を考え，キクバオウレン（加賀黄連）と，かつて福井県や兵庫県で栽培されていたセリバオウレン *Coptis japonica* var. *dissecta* Nakai ex Satake について，成分含量や形態について比較し，その結果，両者の成分含量には差がなく，キクバオウレンの方が根茎は大型であることを報告している．

キクバオウレンは，北海道の南西部と本州（日本海側）の温帯から亜寒帯に分布するとされているが，その分布状態に関する詳細な調査報告はなく，資源としての潜在量は不明である．インターネット上でキクバオウレンを検索すると，新潟県の角田山や国上山など日本海側に近い低山が生育地

として検索され，筆者はかつて，弥彦山でその自生を確認している．

そこで今回，国内に自生する生薬資源として，新潟県内におけるオウレン属植物の分布状況を確認することを目的にフィールド調査を実施した．なお，日本の高山帯に分布するオウレン属植物であるミツバオウレン *Coptis trifolia* (L.) Salisb.とコシジオウレン *Coptis trifoliolata* Makino についても同時に調査することとした．

B　研究方法

『日本の野生植物』(平凡社,1982),『植物の世界』（朝日新聞社, 1997）などの書籍やインターネット情報などを利用して，キクバオウレン，ミツバオウレン，コシジオウレンの分布域に関する情報を入手し，国上山，守門岳（大岳），浅草岳，苗場山の 4 ヵ所を調査地として選択した．

Sony 製の GPS を携行して，調査地の散策路または登山道を歩き，植物を観察して写真撮影を行った．撮影した写真に GPS データ(緯度，経度)を保存し，緯度経度データから標高を入手した．

C　研究結果

Ⅰ．国上山

国上山は新潟県燕市にあり，角田山，弥彦山などからなる弥彦山脈の南端に位置する．良寛和尚が滞在したとされる国上寺がある．国上寺の境内から頂上に向かう散策路でみられる植物については,『国上山の植物』（西蒲・燕科学教育センター, 2000）に紹介されており，コシノカンアオイやコシノコバイモなどとともに，キクバオウレンの存在も記録されている．今回の調査では，散策路の入り口から頂上まで，散策路沿いのほとんどすべてでキクバオウレンを確認し，山全体がキクバオウレンで覆われていると思われるほどの量が確認された．

写真　キクバオウレン（国上山）

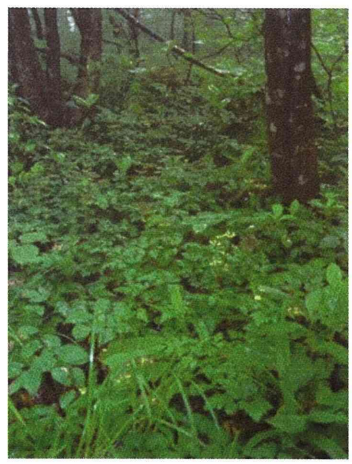

写真　一面にキクバオウレン（国上山）

Ⅱ．守門岳（大岳）

守門岳は新潟県魚沼市，三条市，長岡市にまたがる山群で，大岳，青雲岳，袴岳の 3 つのピークを有し，総称して守門岳という．今回，保久礼コース登山道を大岳頂上まで調査した．

標高 1200m 前後でキクバオウレンが出現し，次いで，1280m ほどでミツバオウレンが出現した．両種は登山道沿いに分布し，標高 1426m の大岳頂上付近まで，間隔をおきながら分布していた．

写真　キクバオウレン（守門岳）

写真　ミツバオウレン（守門岳）

Ⅲ．浅草岳

浅草岳は，新潟県魚沼市，福島県南会津郡只見町にまたがる山で，標高は 1585m．コシジオウレンが分布することが知られている．今回は，ネズミモチ平登山口から前岳を経て浅草岳頂上へ向かうルートを調査した．

まず，キクバオウレンが標高 1389 m 付近で出現した．その後，1424m 付近でミツバオウレンが出現，ミツバオウレンは標高 1538m 付近まで確認された．また，コシジオウレンは，残雪が残る 1520m 付近に分布していた．

写真　キクバオウレン（浅草岳）

写真　ミツバオウレン（浅草岳）

写真　コシジオウレン（浅草岳）

Ⅳ．苗場山

苗場山は，新潟県南部，長野県北東部の県境に位置し，標高は 2145m．ミツバオウレンが分布することが知られている．今回は，新潟県側のかぐらスキー場から神楽ヶ峰を越えて山頂へ至るコースを調査した．

標高 1390m の登山口を出発，標高 1563m 付近でキクバオウレンが出現した．次いで，1594m 付近でミツバオウレンが出現し，ミツバオウレンは標高 1685m 付近の下の芝付近までほぼ連続して観察された．キクバオウレンも標高 1682m 付近でミツバオウレンとともに観察された．

写真　キクバオウレン（苗場山）

写真　キクバオウレン（苗場山）

写真　ミツバオウレン（苗場山）

D　考察

新潟県の日本海側にある弥彦山脈にキクバオウレンが分布していることについては，これまでもよく知られていたが，今回，新潟県内の山間部の標高 1200m から 1700m 付近にもキクバオウレンが自生していることを確認した．また，高山に分布するミツバオウレンとキクバオウレンの分布域が重なることを確認した．

一方，弥彦山系の国上山では，キクバオウレンが山一帯を覆うほど生育していることを確認した．今後，国上山では，その環境調査を行い，年間を通じてキクバオウレンの生育状況を調査し，その特性や品質の評価を行う必要がある．

E　結論

セリバオウレンを中心に国内で栽培されていたオウレンであるが，中国産商品との価格競争に勝てず，現在国内栽培はほとんどなされていない．

今回，新潟県内の海岸に近い低山から山間地の標高の高い地域までキクバオウレンが生育していることを確認し，その資源の

潜在量は多いと考えられる．

これらの潜在する資源を有効に利用するためには，今回確認した各地のサンプルについて，今後，成分含量や根茎の形態を確認し，医薬品原料として利用できるか否か，品質を含めて，その可能性を検討する必要がある．また，生薬黄連の国内栽培を再開し，品質の安定した生薬黄連を供給するためには，さらに，かつて福井県などで行われていた林間栽培方法を超える省力栽培法の検討と開発が必要である．

F　健康危機情報

なし

G　研究発表

1. 論文発表

なし

2. 学会発表

なし

H　知的所有権の取得情報

なし

研究成果の刊行に関する一覧表

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Int. J. Hyperthermia, Month?? 2011; ??(?): 1–8

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RESEARCH ARTICLE

Chemical inducers of heat shock proteins derived from medicinal plants and cytoprotective genes response

KANWAL AHMED[1,2], YUKIHIRO FURUSAWA[3], YOSHIAKI TABUCHI[4], HEBA F. EMAM[3], JIN-LAN PIAO[3], MARIAME ALI HASSAN[3], TAKESHI YAMAMOTO[1], TAKASHI KONDO[3], & MAKOTO KADOWAKI[1]

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Abstract

Environmental stress induces damage that activates an adaptive response in any organism. The cellular stress response is based on the induction of cytoprotective proteins, the so-called stress or heat shock proteins (HSPs). HSPs are known to function as molecular chaperones which are involved in the therapeutic approach of many diseases. Therefore in the current study we searched nontoxic chaperone inducers in chemical compounds isolated from medicinal plants. Screening of 80 compounds for their Hsp70-inducing activity in human lymphoma U937 cells was performed by western blotting. Five compounds showed significant Hsp70 up-regulation among them shikonin was most potent. Shikonin was able to induce Hsp70 at 0.1 μM after 3 h without activation of heat shock transcription factor 1 (HSF-1). It also induces significant reactive oxygen species generation. The expression level of genes responsive to shikonin was studied using global-scale microarrays and computational gene expression analysis tools. Significant increase in the nuclear factor erythroid 2-related factor 2 (Nrf2, NFEL2L2) mediated oxidative stress response was observed that leads to the activation of HSP. The results of gene chip analysis were further confirmed by real-time qPCR assay. In short, the detailed mechanisms of Hsp70 induction by shikonin is not fully understood, Nrf2 and its target genes might be involved in the Hsp70 up-regulation of U937 cells.

Keywords: *Heat shock proteins, Nrf2, oxidative stress, shikonin*

Introduction

Human exposure to environmental toxicants has been associated with etiology of many diseases including inflammation, cancer, cardiovascular and neurodegenerative disorders. To counteract the detrimental effects of environmental insults, mammalian cells have evolved a hierarchy of sophisticated sensing and signalling mechanisms to turn on or off endogenous antioxidant responses accordingly [1]. The ability of cells to counteract stressful conditions, known as cellular adaptive response, requires the activation of pro-survival pathways and the production of molecules with antioxidant, antiapoptotic and proapoptotic activities [2]. Among the cellular pathways conferring protection against oxidative stress, a key role is played by vitagenes, which include heat shock proteins (HSPs) such as heme oxygenase-1 (HMOX1) and Hsp70, as well as thioredoxin/thioredoxin reductase system [3]. Heat shock or stress response is a cellular adaptive response, which contributes to establishing a cytoprotective state in a wide variety of human diseases. When appropriately

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activated, cellular stress response has the capability to restore cellular homeostasis and rebalance redox equilibrium [4].

Among the many changes in cellular activity and physiology, the most remarkable event in stressed cells is the production of a highly conserved set of proteins, the heat shock or stress proteins (HSPs) [5]. HSPs are also expressed constitutively at normal growth temperatures and play an essential role as molecular chaperones by assisting the correct folding of nascent and stressed accumulated misfolded proteins, preventing their aggregation [6], assembly/disassembly of multi-subunit oligomers, translocation of proteins across intracellular membranes, process of endocytosis, regulation of apoptosis and cytoskeletal organisation [7]. A number of studies have reported that molecular chaperones can confer cellular and tissue stress tolerance and provide beneficial effects on various pathological states, such as stress ulcers and ischaemia-induced injuries, as well as on diseases associated with protein misfolding and aggregation [8–10].

Given the broad cytoprotective properties of the heat shock response, there is now strong interest in discovering and developing pharmacological agents capable of inducing stress responses. Therefore, in the current study we screened some phyto-medicinal compounds for their HSP-inducing activity.

Materials and methods

Chemical compounds

Chemical compounds used in this study were obtained from the Institute of Natural Medicine, University of Toyama, Japan.

Cell culture

A human lymphoma cell line, U937 was obtained from the Human Sciences Research Resource Bank (Japan Human Sciences Foundation, Tokyo, Japan). The cells were maintained in Roswell Park Memorial Intitute (RPMI) 1640 medium (Sigma, St Louis, MO, USA) with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences Lenexa, KS, USA) and incubated in a CO_2 incubator with 5% CO_2 and 95% air at 37°C.

Hyperthermia treatment

Hyperthermia (HT) treatment was used as a positive control for induction of HSPs. Cells were collected and suspended in 2 mL fresh medium in plastic culture tubes, and were exposed to 44°C (±0.05°C) for 15 min in a water bath (NTT-1200, Eyela, Tokyo, Japan). After HT treatment, cells were incubated for desired time at 37°C in humidified air with 5% CO_2.

Assessment of apoptosis

For the detection of early apoptosis and secondary necrosis, Annexin V-FITC kit, purchased from Immunotech (Marseille, France), was utilised according to the manufacturer's recommendations. Briefly, cells were stained simultaneously with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labelled annexin V and assessed with a flow cytometer (Beckman-Coulter EPICS XL^{TM}).

Determination of cell survival by WST-8 assay

Cell survival was determined using a Cell Counting Kit-8. The cells (2×10^4 /well) in 96-well plates were incubated with various concentrations of shikonin for 6 h. After incubation, WST-8 reagents were added to each well. Absorbance at 450 nm was measured using a microplate reader (BioRad, Hercules, CA) after 2 h of incubation with WST-8 reagents. Absorbance is proportionally related to the number of live cells.

Western blot analyses for proteins

Western blot analysis was performed for Hsp70, HSF-1, NRF2 and β-actin by using specific polyclonal or monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as described in previous papers [11, 12]. For the detection of these specific antibodies the chemiluminescence ECL detection reagents were used following the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK).

RNA isolation

Total RNA was extracted from cells using an RNAeasy Total RNA Extraction kit (Qiagen, Valencia, CA) and treated with Dnase I (RNase-free Dnase kit, Qiagen) for 15 min at room temperature to remove genomic DNA.

Assessment of intracellular reactive oxygen species (ROS)

To measure intracellular superoxide (O_2^-) we used 2 μM hydroethidine (HE) (Molecular Probes, Eugene, OR) a dye that is oxidized within the cell and fluoresces when it intercalates into DNA. To measure intracellular peroxides including H_2O_2 5 μM dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) was utilised. DCFH-DA upon entering into the cells de-esterified and then oxidized to dichlorofluorescein (DCF). The fluorescence emission was analysed by flow cytometry [13].

High-density oligonucleotide microarray and computational gene expression analysis

Gene expression was analysed using a GeneChip® system with a Human Genome U133-plus 2.0 array (Affymetrix, Santa Clara, CA) spotted with 54,675 probe sets, more than twice the molecular probes used in our previous studies [14, 15]. Samples for array hybridisation were prepared as described in the Affymetrix GeneChip® Expression Technical Manual. Briefly, 5 g of total RNA was used to synthesise double-stranded cDNA with a GeneChip® Expression 30-Amplification Reagents One-Cycle cDNA Synthesis kit (Affymetrix). Biotin-labelled cRNA was then synthesised from the cDNA using GeneChip® Expression 3′-Amplification Reagents for IVT labelling (Affymetrix). After fragmentation, the biotinylated cRNA was hybridised to the GeneChip array at 45° C for 16 h. The arrays were washed, stained with streptavidin-phycoerythrin, and scanned using a probe array scanner. The scanned chip was analysed using GeneChip Analysis Suite Software (Affymetrix). The obtained hybridisation intensity data were converted into a presence or an absence call for each gene, and changes in gene expression level between experiments were detected by comparative analysis. The data were further analysed using Gene-Spring software (Silicon Genetics, Redwood City, CA) to extract the significant genes [16, 17]. To examine gene ontology, including biological processes, cellular components, molecular functions, and genetic networks, the obtained data were analysed using Ingenuity Pathways Analysis tools (Ingenuity Systems, Mountain View, CA), a web-delivered application that enables the identification, visualisation and exploration of molecular interaction networks in gene expression data. The gene lists identified by GeneSpring containing Affymetrix gene ID and the natural logarithm of normalised expression signal ratios from GeneChip CEL files were uploaded into the Ingenuity Pathways Analysis system. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These so-called focus genes were then used as a starting point for generating biological networks [16, 17].

Real-time quantitative PCR assay

Real-time quantitative PCR (qPCR) assay was performed on a real-time PCR system (Mx3000P, Stratagene, Tokyo, Japan) using SYBR PreMix ExTaq (Takara Bio, Shiga, Japan) or Premix ExTaq (for the use of TaqMan probes; Takara Bio) in accordance with the manufacturer's protocols. Reverse transcriptase reaction (Omniscript Reverse Transcriptase, Qiagen) was carried out with DNase-treated total RNA using an oligo (dT) 16 primer. Real-time qPCR assay was performed using the specific primers listed in Table I. Each mRNA expression level was normalized to the mRNA expression level of GAPDH.

Results

Screening of medicinal compounds for Hsp70 up-regulation

Eighty medicinal compounds shown in Table 1 were examined for their ability to induce Hsp70 up-regulation. The screening procedure was accomplished by treating U937 cells with the compounds 1 to 10 at 0.1 mM for 24 h followed by western blot analysis. Among the tested samples no band of Hsp70 was observed with compounds 3–5 suggesting the toxicity of the dose (data not shown). Therefore, we selected compounds 3–5 and did concentration-dependent screening for Hsp70 up-regulation. U937 cells were treated with 0.1 μM, 1 μM and 10 μM for

Table I. List of 80 chemical compounds that were examined for their Hsp70 inducing ability.

1. Aconitine	17. Bufotalin	33. Epihesperidin	49. Gomisin N	65. Paeoniflorin
2. Albiflorin	18. Capillarisin	34. Ergosterol	50. Hesperidin	66. Paeonol
3. Alisol A	19. Capsaicin	35. beta-Eudesmol	51. Hirsutine	67. Palmatine chloride
4. Alisol B	20. Catalpol	36. (E)-Ferulic acid	52. Honokiol	68. (S)-Perillaldehyde
5. Alkannin	21. (E)-Cinnamic acid	37. Geniposide	53. Hypaconitine	69. Puerarin
6. Amygdalin	22. Cinobufagin	38. Geniposidic acid	54. Icariin	70. Rhynchophylline
7. Arbutin	23. Cinobufotalin	39. Gentiopicroside	55. Isofraxidine	71. Saikosaponin a
8. Astragaloside IV	24. Coptisine chloride	40. [6]-Gingerol	56. (Z)-Ligustilide	72. Saikosaponin b2
9. Atractylenolide III	25. Corydaline	41. Ginsenoside Rb1	57. Limonin	73. Saikosaponin c
10. Aucubin	26. Curcumin	42. Ginsenoside Rc	58. Loganin	74. Schizandrin
11. Baicalein	27. Dehydrocorydaline nitrate	43. Ginsenoside Rd	59. Magnolol	75. Sennoside A
12. Baicalin	28. Dehydrocostuslactone	44. Ginsenoside Re	60. Mesaconitine	76. Shikonin
13. Barbaloin	29. Dihydrocapsaicin	45. Ginsenoside Rg1	61. Naringin	77. [6]-Shogaol
14. Berberine chloride	30. Dimethylesculetin	46. Glabridin	62. Nodakenin	78. Sinomenine
15. Bergenin	31. Eleutheroside B	47. Glycyrrhizic acid	63. Osthole	79. Swertiamarin
16. Bufalin	32. (-)-Epigallocatechin gallate	48. Gomisin A	64. Oxymatrine	80. Wogonin

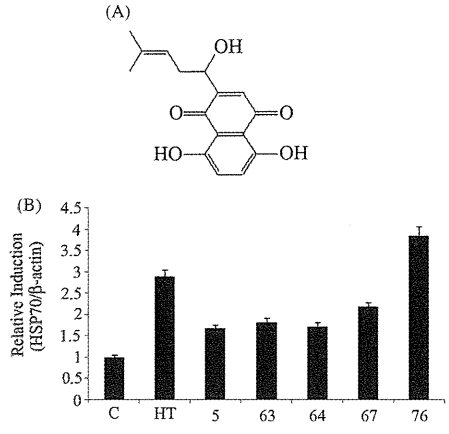

Figure 1. (A) Chemical structure of shikonin. (B) Bands of Hsp70 induced by five active compounds at 1 μM concentration were quantified by densitometry and normalised with β-actin. Hyperthermia (HT) 44°C for 15 min was used as a positive control. Bars indicate standard deviation ($n = 3$).

24 h followed by western blot analysis. Significant Hsp70 up-regulation was observed with compound 5 at 1 μM (densitometric ratio: control: 1.0 ± 0.08, 0.1 μM: 1.25 ± 0.12, 1 μM: 1.50 ± 0.14, 10 μM: 1.12 ± 0.13, mean ± SD, $n = 3$) while compounds 3 and 4 did not show any significant increase at all concentrations as compared to control. From these results we selected 1 μM concentration for the screening of medicinal compounds.

Out of the 80 compounds, five (5, 63, 64, 67 and 76) showed significant Hsp70 up-regulation. Among them compound 76 (shikonin) was the most potent (Figure 1A, 1B).

Effects of shikonin on apoptosis induction in U937 cells

To determine the non-toxic concentration of shikonin, cells were exposed to the drug concentration-dependently for 6 h. This was followed by measurement of early apoptosis and secondary necrosis by annexin V FITC/PI staining using flow cytometry. No apoptosis was observed at 0.01 μM and 0.1 μM, significant apoptosis was observed at 1 μM concentration (Figure 2A).

Effects of shikonin on cell survival

To further confirm the non-toxic concentration, cell survival assay was performed with 0.01, 0.1 and 1 μM concentrations after 6 h incubation. The results also showed the toxicity at 1 μM while no toxicity was observed with 0.01 and 0.1 μM concentrations. (Figure 2B).

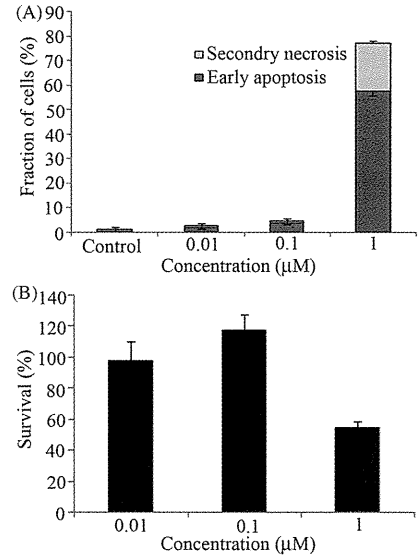

Figure 2. (A) U937 cells were treated with shikonin concentration-dependently for 6 h followed by assessment of early apoptosis (black bar) and secondary necrosis (grey bar) by flow cytometry. Bars indicate standard deviation ($n = 3$). (B) Cell survival assay was performed with 0.01, 0.1 and 1 μM concentrations of shikonin. After 6 h incubation WST-8 reagent was added to each well. Bars indicate standard deviation ($n = 3$).

Effects of shikonin on ROS formation

Previously it has been reported that shikonin can induce ROS formation [18]. Therefore, in the current study we investigated that whether at non-toxic concentration shikonin can induce ROS. Cells were exposed to 0.1 μM shikonin and the levels of DCF and HE fluorescence were monitored time dependently via flow cytometry. Intracellular peroxide level was increased as early as 30 min after treatment (Figure 3A), while no intracellular O_2^- was observed (data not shown).

Time-dependent effects of shikonin on Hsp70 induction

U937 cells were treated with 0.01 μM and 0.1 μM shikonin time-dependently, followed by western blot analysis. Up-regulation of Hsp70 was observed with 0.1 μM after 3 h incubation and continued to increase time-dependently, while no up-regulation of Hsp70 was observed at 0.01 μM shikonin (Figure 3B, 3C)

The transcription of *HSP* genes is regulated by transcription factor HSF, which senses cellular exposure to stress and turns on rapid induction of HSPs [19]. Therefore we examined the effects of shikonin on the activation of HSF1. U937 cells were

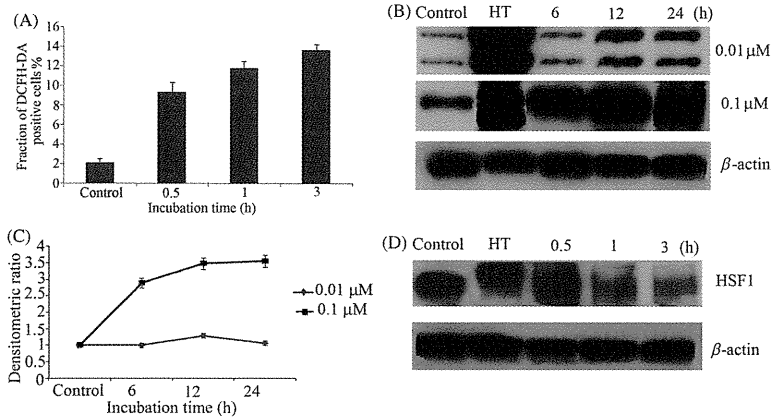

Figure 3. (A) U937 cells were first incubated with DCFH-DA for 30 min and then treated with shikonin in a time-dependent manner. Intracellular peroxide level was measured by flow cytometry. (B) Concentration and time-dependent induction of Hsp70 was measured by western blotting. Hyperthermia (HT) 44°C for 15 min was used as a positive control. (C) Bands were quantified by densitometry and normalised with β-actin. Bars indicate standard deviation ($n = 3$). (D) HSF1 phosphorylation was measured time-dependently by western blotting. Hyperthermia (HT) 44°C for 15 min was used as a positive control.

treated with 0.1 μM shikonin in a time-dependent manner followed by western blot analysis.

Phosphorylation of HSF1 is usually detected as an upward band shift [20]. No upward band shift was observed after shikonin treatment till 3 h, while very clear upward shift was observed in HT-treated cells. This result indicates that HSF1 is not playing a role in shikonin-induced HSP up-regulation (Figure 3D).

Identification of genes responsive to shikonin treatment

As shikonin treatment did not show the activation of HSF1, we carried out gene chip analysis of cells treated with or without shikonin after 3 h incubation. Many probe sets were differentially expressed by >2-fold in cells treated with the compound, 277 up-regulated and 262 down-regulated in comparison to control. The complete list of genes from all samples is available on the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24743). The biologically relevant functions and networks of the up-regulated probe sets obtained from the gene chip analysis were identified using Ingenuity Pathways Knowledge Base. Of 277 up-regulated probe sets analysed, 77 genes were functionally annotated. On the basis of significance, the top three molecular functions were cellular compromise (p value: 3.73E-12 to 2.68E-02), cellular function and maintenance (3.73E-12 to 4.65E-02), and post-transcriptional modification (1.36E-7 to 3.34E-02). As shown in Figure 4, a significant gene network contained HSP-related genes such as HSPA1A (heat shock 70 kDa protein 1A), HSPA6 (heat shock 70 kDa protein 6) and DNAJA1 (DnaJ (Hsp40) homologue, subfamily A, member 1) and Nrf2(NFE2L2)-target genes such as HMOX1 (heme oxygenase (decycling) 1), NQO1 (NAD(P)H dehydrogenase, quinone 1) and SQSTM1 (sequestosome 1). To confirm the results of gene chip analysis, a real-time qPCR assay was performed for four selected genes in the network. As we expected, the expression levels of these genes were significantly increased by the treatment (Figure 5).

Discussion

Toward the goal of developing novel cytoprotective agents we did screening of 80 chemical compounds isolated from medicinal plants for their HSP-inducing activities. Among the five active Hsp70 inducers, shikonin was most potent. Shikonin is a chemical compound isolated from the root of a plant *Lithospermum erythrorhizon*. It is known to have antibacterial [21], antifungal [21], anti-human immunodeficiency virus [22], anticancer [23] and anti-inflammatory activity [23]. But to our knowledge there is no report on its ability to induce HSP up-regulation.

Several chemical compounds are known to induce HSP through the activation of HSF1 [11]. In this study, after shikonin treatment significant Hsp70 up-regulation was observed without the activation

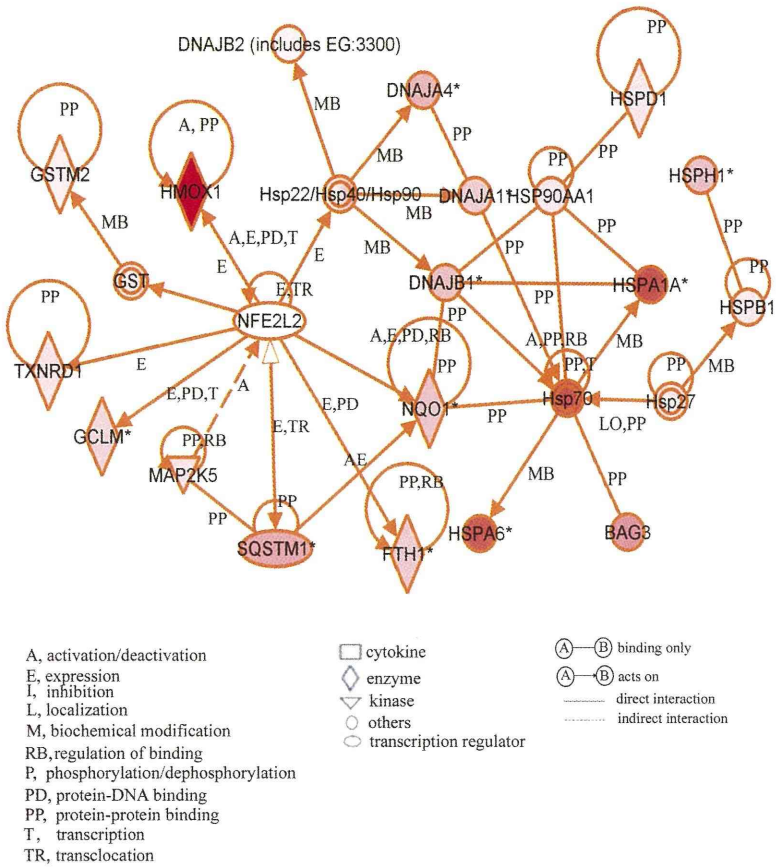

Figure 4. A gene network including up-regulated genes. Cells were treated with the compound and cultured at 37°C for 3 h. Gene chip analysis was performed. Genes that were up-regulated were analysed using Ingenuity Pathways Analysis tools. The network is displayed graphically as nodes (genes or protein group) and edges (the biological relationships between the nodes). The node colour of genes indicated the expression level of genes. Nodes and edges are displayed in various shapes and labels that present the functional class of genes and the nature of the relationship between the nodes, respectively.

of HSF1. With the high-density oligonucleotide microarrays and computational gene expression tools we identified a unique gene network containing HSPs and Nrf2-target genes. Nrf2 is an antioxidant transcription master regulator and belongs to the cap 'n' collar family of transcription factors. Small Maf proteins (MafF, MafG and MafK) possess the region-leucine zipper (L-Zip) domain that is required for homodimer or heterodimer complex formation with other basic L-Zip transcription factors such as Nrf2 [24]. Nrf2 is sequestered in cytoplasm by its repressor Keap1, released and translocated into the nucleus under oxidative stress [25]. In the nucleus, the heterodimer complex of Nrf2 and small Maf proteins binds to the antioxidant-responsive element (ARE) sequence leading to transcriptional activation of downstream genes encoding phase II detoxifying enzymes, antioxidants [26] and chaperone proteins [27].

Almeida et al. [28] recently indicated that Hsp70 expression is regulated by an ARE/EpRE sequence in a Zebrafish model. In many studies, Nrf2 have been reported to induce different Hsps [27, 29–31] and in one study, Hsp70 was specifically up-regulated in mouse liver in wild-type but not knockout mice [29]. Furthermore, it has also been reported that activation of Nrf2 increases expression of Hsp40 [27]. In addition, several studies reported a concomitant induction of Hsp70 and Nrf2-regulated gene HMOX1 by electrophiles [32].

Although the detailed mechanisms by which shikonin induces protein expression of Hsp70 are not fully understood, Nrf2 and its target genes may have participated in the up-regulation of Hsp70 in

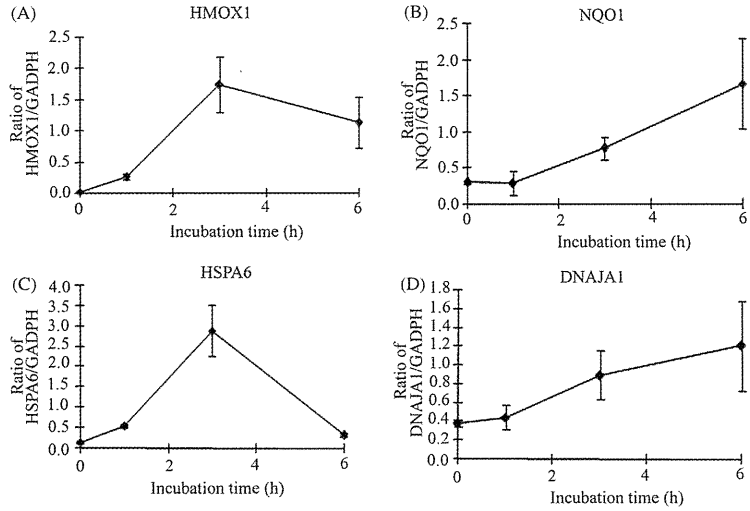

Figure 5. Verification of microarray results by qPCR assay. Cells were treated with 0.1 μM shikonin time-dependently and then real-time qPCR assay was performed. (A) HMOX1 (heme oxygenase (decycling) 1) (B) NQO1 (NAD(P)H dehydrogenase, quinone 1) (C) HSPA6 (heat shock 70 kDa protein 6) (D) DNAJA1(DnaJ (Hsp40) homologue, subfamily A, member 1). Each mRNA expression level was normalised with GADPH. Data are presented as mean ± SD ($n = 3$).

U937 cells. The elucidation of the molecular mechanism to induce Hsp70 by shikonin remains for further investigation in the future.

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Chemical and biological characterization of four new linear cationic α-helical peptides from the venoms of two solitary eumenine wasps

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ABSTRACT

Four novel peptides were isolated from the venoms of the solitary eumenine wasps *Eumenes rubrofemoratus* and *Eumenes fraterculus*. Their sequences were determined by MALDI-TOF/TOF (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) analysis, Edman degradation and solid-phase synthesis. Two of them, eumenitin-R (LNLKGLIKKVASLLN) and eumenitin-F (LNLKGLFKKVASLLT), are highly homologous to eumenitin, an antimicrobial peptide from a solitary eumenine wasp, whereas the other two, EMP-ER (FDIMGLIKKVAGAL-NH_2) and EMP-EF (FDVMGIIKKIAGAL-NH_2), are similar to eumenine mastoparan-AF (EMP-AF), a mast cell degranulating peptide from a solitary eumenine wasp. These sequences have the characteristic features of linear cationic cytolytic peptides; rich in hydrophobic and basic amino acids with no disulfide bond, and accordingly, they can be predicted to adopt an amphipathic α-helix secondary structure. In fact, the CD (circular dichroism) spectra of these peptides showed significant α-helical conformation content in the presence of TFE (trifluoroethanol), SDS (sodium dodecylsulfate) and asolectin vesicles. In the biological evaluation, all the peptides exhibited a significant broad-spectrum antimicrobial activity, and moderate mast cell degranulation and leishmanicidal activities, but showed virtually no hemolytic activity.

1. Introduction

Solitary wasps are known to inject their venoms into insects or spiders, paralyzing the prey in order to feed their larvae. Therefore, the solitary wasp venoms should contain a variety of neurotoxins acting on nervous systems. In fact,

Abbreviations: MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; CD, circular dichroism; TFE, trifluoroethanol; SDS, sodium dodecylsulfate; PC, L- -phosphatidylcholine; PG, L- -phosphatidyl-DL-glycerol; HEPES, (N-[2-hydroxyethyl]piperazine-N -[2-ethanesulfdonic acid]; PBS, phosphate buffered saline.

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