Actein activates stress- and statin-associated responses and is bioavailable in Sprague-Dawley rats

Linda Saxe Einbond\textsuperscript{a}, Morando Soffrilli\textsuperscript{b}, Davide Degli Esposti\textsuperscript{b}, Taesik Park\textsuperscript{a}, Erica Cruz\textsuperscript{a}, Tao Su\textsuperscript{a}, Hsuan-ku Wu\textsuperscript{a}, Xiaomei Wang\textsuperscript{a}, Yu-Jing Zhang\textsuperscript{a}, Justin Ham\textsuperscript{a}, Ira J. Goldberg\textsuperscript{a}, Fredi Kronenberg\textsuperscript{a}, Antoneta Vladimirova\textsuperscript{a}

\textsuperscript{a}Columbia University College of Physicians and Surgeons, New York, 10032 NY, USA
\textsuperscript{b}European Ramazzini Foundation of Oncology and Environmental Sciences, Bologna, Italy
\textsuperscript{c}Borax/Dark Blue Biosciences, Mountain View, CA 94043, USA

---

Keywords
actein.
black cohosh.
microarray.
statin.
triterpene glycoside

Received 24 July 2008; accepted 4 November 2008

*Correspondence and reprints: le2012@cumc.columbia.edu

LABORATORY of origins: Department of Rehabilitation Medicine, The Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University, HSCC-1509, 701 W 188th Street, New York, 10032 NY, USA.

---

ABSTRACT

The purpose of this study was to assess in rats the pharmacological parameters and effects on gene expression in the liver of the triterpene glycoside actein. Actein, an active component from the herb black cohosh, has been shown to inhibit the proliferation of human breast cancer cells. To conduct our assessment, we determined the molecular effects of actein on livers from Sprague-Dawley rats treated with actein at 35.7 mg/kg for 6 and 24 h. Chemogenomic analyses indicated that actein elicited stress and statin-associated responses in the liver: actein altered expression of cholesterol and fatty acid biosynthetic genes, p53 pathway genes, CCND1 and ID3. Real-time RT-PCR validated that actein induced three time-dependent patterns of gene expression in the liver: (i) a decrease followed by a significant increase of HMGS1, HMGR, HSD17B7, NQO1, S100A9; (ii) a progressive increase of BZRPA and CYP7A1 and (iii) a significant increase followed by a decrease of CCND1 and ID3. Consistent with actein's statin- and stress-associated responses, actein reduced free fatty acid and cholesterol content in the liver by 0.6-fold at 24 h and inhibited the growth of human HepG2 liver cancer cells. To determine the bioavailability of actein, we collected serum samples for pharmacokinetic analysis at various times up to 24 h. The serum level of actein peaked at 2.4 μg/mL at 6 h. Actein's ability to alter pathways involved in lipid disorders and carcinogenesis may make it a new agent for preventing and treating these major disorders.

INTRODUCTION

Native Americans have used the North American perennial black cohosh [Actaea racemosa L syn. Cicletaria racemosa (L.) Nutt] for centuries to treat a variety of conditions, including rheumatism, arthritis, muscle pain and dysmenorrhea [1]. In recent years, it has become a popular alternative to hormone replacement therapy to alleviate menopausal symptoms. Recent studies indicate that black cohosh may have chemopreventive and anticancer potential [2,3]. The rhizomes and roots of the plant contain two major classes of secondary metabolites, triterpene glycosides and phenylpropanoids. Of the more than 42 triterpene glycosides present in black cohosh [4], the triterpene glycoside actein (Figure 1a) and 23(24)-epoxyactein [5] constitute about 6.4% of an n-butanol fraction of black cohosh enriched for triterpene glycosides (27%).
Purified triterpene glycosides and aglycones have been shown to selectively inhibit the growth of various types of cancer cells in vitro, including human oral squamous carcinoma cells [6], MCF7 (IR[+], Her2 low) and MDA-MB-453 (IR[+], Her2 overexpressing) breast cancer cells [7] and HepG2 liver cancer cells [8] compared to effects on non-malignant counterparts. Their specificity suggests limited toxicity in vivo. Cimigenol and cimigenol-3,15-dione, from other Cimicifuga species, have been shown to inhibit mouse skin tumour promotion and to have antitumour initiating activity commensurate with the chemopreventive agent epigallocatechin gallate (EGCG) [9].

Triterpene glycosides from black cohosh have been shown to induce cell-cycle arrest at G1 [7]. Gene expression analysis indicates that actein's growth inhibition of breast cancer cells is associated with activation of stress response pathways [10]. Actein induced two phases of the integrated stress response, the survival or apoptotic phase, depending on the dose and duration of treatment. Although these results indicate that actein can induce a complex array of cellular stress responses, they do not reveal its primary cellular targets. The putative targets may play a role in cellular processes involving calcium, as actein altered the expression of several genes involved in calcium homeostasis or lipids, because actein altered the transcription of genes involved in lipid metabolism [10,11].

Little is known about the pharmacokinetics and metabolites of extracts of black cohosh and actein. The catechols do not appear to be absorbed across the intestinal epithelium, whereas the triterpenoids are absorbed [12]. Isolated reports [13] have associated black cohosh use with severe hepatitis. Some studies have indicated that an extract of black cohosh increased lipid levels in clinical trials [14,15].

To clarify actein's mode of action and potential adverse effects, we used the Iconix/Entelos ToxRX® Analysis Suite (Entelos Inc., Foster City, CA, USA), which uses raw gene expression data from a given organ to conduct a comprehensive analysis of the toxicity, safety and mechanism of action of a component in relation to over 630 reference compounds found in Iconix's database DrugMatrix® (Entelos Inc., Foster City, CA, USA). Subtle gene expression changes identified in the liver can be used to predict pathological events occurring in that and other tissues even before toxicological and pathological effects can be detected [16,17].

The purpose of this study was to determine the serum pharmacokinetics of actein after oral administration to rats and cellular and molecular effects in the liver of the treated rats, to assess its potential to treat cancer and other diseases. To confirm the effects on specific physiological parameters, we tested the effect on lipid levels in the rat liver and on the growth of HepG2 human liver cancer cells.

**MATERIALS AND METHODS**

**Chemicals and reagents**

All solvents and reagents were reagent grade; water was distilled and deionized. Actein was obtained from Planta Analytics (Danbury, CT, USA, lot number PA-A-037), purity was over 95% by high pressure liquid chromatography (HPLC; in vivo studies) and from Chromalyx (Laguna Hills, CA, USA, lot number 01355-806), purity 89% by HPLC. Actein [Lot no. 01355-8105 (P)] and 27-(23,26-deoxyactein) deoxyactein (DAHP) were employed for pharmacokinetic and urine analysis. HPLC grade acetonitrile (Part no: A998-4), chloroform (Part no: C298-4) and HPLC grade Water (Part no: W5-4) were obtained from Fisher (Fair Lawn, N.J., USA). Drug-free rat serum (Part no: 40363472) was obtained from Innovative Research Inc. (Southfield, MI, USA).
Cell cultures
HepG2 (p53 positive) human liver cancer cells were obtained from the ATCC (Manassas, VA, USA). Cells were grown in Dulbecco’s Modified Eagle’s medium (Gibco BRL Life Technologies, Inc., Rockville, MD, USA) containing 10% (v/v) foetal bovine serum (Gibco BRL) at 37 °C, 5% CO₂.

Proliferation assay
The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide assay (MTT) assay was used to determine the sensitivity of HepG2 p53 positive human liver cancer cells to actein, as previously described [10].

Animal treatment and data collection
Female Sprague-Dawley rats, 56-week old, were distributed into three groups of eight and randomized to minimize the number of animals from each litter in the same group. The rats were housed individually in makrolon cages (41 x 25 x 15 cm) with a stainless steel wire top and a shallow layer of white wood shavings as bedding. All the animals used in the experiment were kept in a single room at 23 ± 3 °C and at 40-60% relative humidity. Lighting was natural or artificial and the light/dark cycle of 12 h was maintained. The animals were supplied with a pellet diet (Corticella type, Laboratory Diet, B. Piccolini). All animal treatment and data collection was conducted at the Cesare Maltoni Cancer Research Center of the European Foundation of Oncology and Environmental Sciences B. Ramazzini (CMCRC/ERF) of Bologna, Italy, according to Italian Law regulating the use of animals for scientific purposes (Decreto Legislativo 116 1992/EU Directive 86/609/CE).

Treatment
In previous experiments, we determined the maximum tolerated dose of an extract of black cohosh enriched for triterpene glycosides (27%) to be 35.7 mg/kg. The extract of black cohosh contained 27% triterpene glycosides, of which 3.4% was actein. In this study, we treated two groups of eight female rats each with: (i) 35.7 mg/kg of actein and (ii) an equal volume of embedding media. After the pre-conditions, the control group of eight female rats was treated by gastric intubation with 1 ml of water. Blood and urine collection

For determination the serum concentration of actein at different times during 24-h, blood was sequentially collected from four animals of the group treated with 35.7 mg/kg actein at intervals of 0, 5, 15, 30, 60 min and 2, 4, 6, 8 and 24 h after the administration of actein. For the 24-h period, animals were starved with free access to water. Blood samples (0.5 mL) were collected through cannulation of the retrobulbular plexus with a silanized glass Pasteur pipette after anaesthetization with ethyl ether. Immediately after sacrifice, blood was drawn from the portal vein with a sterile syringe into vials without anticoagulant agents. Ten minutes after each sampling (the time necessary for the formation of the clot), blood was centrifuged at 1500 g for 10 min. Then, serum was stored in cryogenic screw cap vials at −70 °C.

From the same animals, urine was collected 24 h following administration of compound. Urine was shaken to prevent formation of deposits, after which two samples of 500 µL were collected and stored in cryogenic screw cap vials at −70 °C.

Gene expression samples
Six and 24 h after dosing, four rats from the group treated with 35.7 mg/kg actein and four rats from the control group were sacrificed, and four portions of about 100 mg each were collected from the main lobe of the liver for analysis. Each portion was individually retained in a cryovial, snap frozen in liquid nitrogen and stored at −70 °C until use for array data generation.

Histology
Livers were embedded in optimal cutting temperature compound (to enable cryosectioning of the sample) and stained with haematoxylin and eosin (H&E). All samples were visualized with a Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Thornwood, NY, USA) and images were obtained with a Nikon Coolpix 5000 (Nikon Instruments, Melville, NY, USA) camera.

Lipid analysis
Hepatic lipids were extracted by homogenization of the liver from the 24-h group, followed by addition of chloroform : methanol (2 : 1). After vortexing and centrifugation for 10 min, the organic phase was collected and dried under nitrogen. The dried lipids were dissolved in 1% Triton X-100 in water and sonicated. Extracted hepatic lipids and plasma lipids were measured by cholesterol and triglyceride enzymatic assay kits from Infinity (Louisville, CO, USA), according to the manufacturer’s instructions. Free fatty acids were measured by Enzymatic assay using NEFA C kit from Wako Chemicals (Richmond, VA, USA). Tissue lipids were normalized by protein concentration.
Pharmacokinetic analysis
High pressure liquid chromatography–mass spectrometry (MS) analysis was performed, in duplicate, by Chroma-
dex to determine the presence and quantity of actein in
serum and urine samples from Sprague-Dawley rats
reared with 35.7 mg/kg actein.

Serum preparation
A 100 µL aliquot of rat serum and a 10 µL aliquot
of internal standard solution (deoxyactein, 23-epi-
26-deoxyactein) were placed in eppendorf micro-
tubes with 200 µL of acetonitrile to precipitate
proteins.

Urine preparation
The urine sample was extracted three times with 500 µL
chloroform. The residue was constituted in 150 µL of
acetonitrile.

Analysis of serum and urine samples
Chromatographic separation of the compounds was
performed on a Waters Acquity UPLC T (Waters
Corporation, Milford, MA, USA) using a BEH C18
column (1.7 µm, 2.1 x 50 mm). The mobile phase
consisted of acetonitrile: water (80:20).

The MS instrumentation consisted of a Waters Micro-
mass Quattro Micro™ triple-quadrupole system
(Manchester, UK). Urine analysis by ultra-performance
liquid chromatography compared atmospheric pressure
chemical ionization mode and electrospray ionization
mode.

Chemogenomic analysis
We used Ionom/Entelos ToxPFX analysis to determine
the effects of actein at a dose of 35.7 mg/kg at time
points 6 and 24 h on gene expression patterns in
rat liver. Following standard Affymetrix® (Affymetrix,
Santa Clara, CA, USA) protocols, labelled cDNA was
generated from liver tissue from each study animal and
hybridized to Affymetrix RG230-2.0 rat whole genome
arrays, which are comprised of more than 31 000
probe sets.

From the microarray data, a complete toxogenomic
report was produced using the ToxPFX Analysis Suite.
ToxPFX analysis uses the Ionom/Entelos database Drug
Matrix to match patterns of gene expression changes
clicted by actein to those of other compounds (Drug Signatures®; Entelos Inc., Foster City, CA, USA) and to identify perturbed biochemical pathways
[16–18].

Drug signatures
Log_{10} ratio data for the actein array data set was
compared to the Ionimx collection of gene expression
biomarkers (Drug Signatures). The degree to which
the gene expression profile of actein matched a Drug
Signature was reported in ToxPFX as a posterior prob-
ability score (PPS). PPS >0.9 were considered highly
significant, 0.5 < PPS < 0.89 were considered to be of
interest and viewed in the context of pathway matches,
clinical signs and other data.

Pathway analysis
Using the 135 curated pathways within DrugMatrix,
pathway analysis identified particular biological pro-
cesses perturbed by exposure to actein. Fisher's exact test
calculated the statistical likelihood that the same number
of expression changes observed in pathway genes would
be observed against the same number of randomly-
chosen array probe sets.

AffyLimma analysis
To identify individual alterations in gene expression
induced by treatment, an unbiased informatic analysis
was performed using the AffyLimmaGUI package in the
open-source Bioconductor suite, as previously described
[10] (Table S1).

Real-time RT-PCR analysis
Real-time quantitative RT-PCR methods were used to
confirm selected actein-induced changes in gene
expression detected by microarray analysis, as previously described [10]. Total RNA was isolated
using TriReol (Invitrogen, CA, USA), and purified
with the RNeasy Kit (Qiagen, Valencia, CA, USA).
miRNA sequences were obtained from the public
and primers were designed using Primer3 software
from The Massachusetts Institute of Technology
(http://www.fgenome.wi.mit.edu/cgi-bin/primer3/primer3-
www.cgi) (Table S2).

RESULTS
Pharmacokinetic analysis of actein in rat serum
By HPLC analysis, the level of actein (Figure 1b) in
the serum increased up to a peak value of
2395.47 ng/L at 6 h and then decreased to
103.74 ng/mL at 24 h after treatment with actein at
35.7 mg/kg. The level of actein in the urine at 24 h
was 777.07 ng/mL.

© 2009 The Authors. Journal compilation © 2009 Société Française de Pharmacologie et de Thérapeutique
Lipid analysis of rat liver tissue
When we examined the effect of actin (35.7 mg/kg) on lipid levels in the rat livers, we found a 0.6-fold decrease in the free fatty acid (P = 0.012) and cholesterol (P = 0.018) levels and no change in triglyceride content of the treated livers compared to the controls.

Histology of rat liver tissue
Hematoxylin and eosin stained slides of rat livers obtained at 24 h after administration of actin showed hepatotoxicity (Figure 2b). Both displayed vacuolar degeneration. Aggregated lymphocytes were seen in the centrilobular (Figure 2b) and non-centrilobular (Figure 2c) areas, indicating inflammation.

ToxFX analysis
Treatment with actin (35.7 mg/kg) for 6 or 24 h affected a statistically significant change (P < 0.05) in the transcription levels of 297 or 1335 genes, respectively, relative to the control. The significant effects on gene expression at 6 h included downregulation of erythropoietin (~0.22, P < 0.001), CYP2C (~0.26, P < 0.01) and ATP synthase (~0.15, P < 0.01) (Table 1a). At 24 h, significant gene alterations included upregulations of IP3 (0.80, P < 0.01), HMGCS (0.36, P < 0.001), FDPS (0.34, P < 0.01), S100A9 (0.79, P < 0.01), CXCL1 (0.44, P < 0.01), C4BP (0.17, P < 0.01), and CYP7A1 (0.53, P < 0.01), and downregulation of SCD1 (~1.28, P < 0.01) (Table 1b).

Actin also upregulated genes involved in the acute phase response (A2M), Hypoxia and HIF signaling (TEF, PEP1), NRF2 mediated Ox stress receptor (PSMB10, NQ01) and p53 signaling (TNFRSF6, EAS). Actin downregulated the expression of genes involved in cell cycle control (CCND1) and hepatic toxicity: origin of cholestasis (MR1).

Transcriptional pattern matching with drug signatures
We compared expression pattern changes induced by actin to gene expression patterns from compounds in DrugMatrix and found the following three Drug Signatures as having the highest probability matches to actin's effects: (i) a weak match to hepatic inflammatory infiltrate, centrilobular signature (clusters of inflammatory cells around or adjacent to the central vein) at 6 h (PES = 0.58); (ii) a weak match to hepatic inflammatory infiltrate, early gene expression signature (clusters of inflammatory cells in the hepatic parenchyma lacking a distinct zonal pattern) at 24 h (PES = 0.36) and (iii) a weak match to cholesterol biosynthesis inhibitor signature at 24 h (PES = 0.54).

Figure 2: Histology of liver tissue from three different rats. H&E stained sections of control and treated Sprague-Dawley rat livers, obtained 24 h after treatment with or without actin at 35.7 mg/kg, were examined by microscopy, as described in Materials and Methods. (a) control; (b) and (c) treated with actin 35.7 mg/kg; magnification ×40 (a).

Pathway responses compared to DrugMatrix
Relative to the 200 compounds in DrugMatrix, ToxFX identified strong transcriptional responses on the
Table 1. Genes significantly altered by treatment with actein, determined by ToxFX analysis.

<table>
<thead>
<tr>
<th>Pathway category</th>
<th>Affymetrix number</th>
<th>Gene symbol</th>
<th>Gene title</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Genes significantly altered at 6 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid and triglyceride metabolism</td>
<td>1367942_at</td>
<td>CYP2C2</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 22</td>
<td>-0.26*</td>
</tr>
<tr>
<td>Aryl hydrocarbon receptor (AhR) signaling</td>
<td>1367306_at</td>
<td>EP3</td>
<td>Synthetase</td>
<td>-0.22**</td>
</tr>
<tr>
<td>Hypoxia and HIF signaling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial oxidative phosphorylation</td>
<td>1367019_at</td>
<td>ATP5I</td>
<td>ATP synthase, H+ transporting, mitochondrial F0 complex, stromal e</td>
<td>-0.05*</td>
</tr>
<tr>
<td>b) Genes significantly altered at 24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acid biosynthesis and its regulation</td>
<td>1367055_at</td>
<td>SCD1</td>
<td>Seryl-Coenzyme A desaturase 1</td>
<td>-1.28*</td>
</tr>
<tr>
<td>Hepatic toxicity: origin of steatosis</td>
<td>1368872_at</td>
<td>IPF</td>
<td>Isopentenyl-diphosphate isomerase</td>
<td>0.90*</td>
</tr>
<tr>
<td>Cholesterol biosynthesis</td>
<td>1367532_at</td>
<td>HMGC5</td>
<td>3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1</td>
<td>0.86*</td>
</tr>
<tr>
<td>Acute phase response</td>
<td>1367667_at</td>
<td>FDX5</td>
<td>Fumaric dehydrogenase synthase</td>
<td>0.34*</td>
</tr>
<tr>
<td></td>
<td>1367125_at</td>
<td>S100A9</td>
<td>S100 calcium binding protein A9 (calgranulin B)</td>
<td>0.75*</td>
</tr>
<tr>
<td></td>
<td>1367318_at</td>
<td>CYCL1</td>
<td>Chorionic (C-X-C motif) ligand 1 (also involved in NF-kappa B and TGF-beta signaling)</td>
<td>0.44*</td>
</tr>
<tr>
<td></td>
<td>1368425_at</td>
<td>C5</td>
<td>Complement component 5</td>
<td>0.18*</td>
</tr>
<tr>
<td></td>
<td>1369695_at</td>
<td>OBP</td>
<td>Complement component 4 binding protein, beta</td>
<td>0.17*</td>
</tr>
<tr>
<td></td>
<td>1369764_at</td>
<td>OBP</td>
<td>Complement component 4 binding protein, alpha</td>
<td>0.09*</td>
</tr>
<tr>
<td>Hepatic toxicity: origin of cholestasis</td>
<td>1367952_at</td>
<td>CD44</td>
<td>CD44 antigen</td>
<td>0.08*</td>
</tr>
<tr>
<td>Lipid and triglyceride metabolism</td>
<td>1367604_at</td>
<td>SAF</td>
<td>Serum amyloid P component</td>
<td>-0.04*</td>
</tr>
<tr>
<td>Mitochondrial oxidative phosphorylation</td>
<td>1350450_at</td>
<td>CYP7A1</td>
<td>Cytochrome P450, family 7, subfamily A, polypeptide 1</td>
<td>0.53*</td>
</tr>
<tr>
<td>Résolution of fatty acid TGF-beta signaling</td>
<td>1367670_at</td>
<td>MGSDFH</td>
<td>Glycol-3-phosphate dehydrogenase Z, mitochondrial</td>
<td>-0.36*</td>
</tr>
<tr>
<td></td>
<td>1367680_at</td>
<td>ACOX1</td>
<td>Arachidonoyl-Coenzyme A oxidase 1, palmitoyl</td>
<td>-0.17*</td>
</tr>
</tbody>
</table>

We used ToxFX analysis to determine the effects of actein, at a dose of 35.7 mg/kg and at 6 and 24 h on the gene expression patterns in rat liver. Assays were performed as described in Materials and Methods. Fold change (Log2) is the mean of the ratio of hybridization signals in actein treated vs. control treated cells.

*P < 0.01; **P < 0.001

Following biological pathways after treatment with actein: cholesterole biosynthesis (P < 0.0001); fatty acid biosynthesis and its regulation; acute phase response (P < 0.001); thyroid hormone: regulation, synthesis and release; mitochondrial oxidative phosphorylation; p53 (Figure 3).

**AffyLimma analysis**

AffyLimma gene expression analysis indicated that actein caused a significant alteration in the expression of 0 and 109 genes (B > 0; |M| > 0; ratio up/down: 1.9 : 1) in the rat liver after treatment for 6 and 24 h, respectively (Table S1), when compared with the control.

The effects of actein on expression of specific mRNAs determined by real-time RT-PCR

The RT-PCR results revealed three patterns of gene expression (Figure 4): (i) mRNAs for the stress gene S100A9; NRF2 mediated oxidative stress gene NQO1; and cholesterole biosynthetic genes HMGC5, HMGC1, and cholesterol biosynthetic genes HMGC5. HMGC1, and HMGC2 decreased at 6 h and increased at 24 h; (ii) mRNAs for the cytochrome CYP7A1 and mitochondrial benzodiazepine receptor gene ZRBP progressively increased at 6 and 24 h, whereas (iii) mRNAs for the cell-cycle gene cyclin D1 and the inhibitor of differentiation gene ID3 significantly increased at 6 h and decreased at 24 h. - RT-PCR confirmed the
Figure 1. Pathway responses for acetin. The effect of the compound on toxicologically important DrugMatrix pathways is displayed based on two different metrics: maximum pathway impact (using Fisher's exact test) and relative pathway response (using the overall magnitude of pathway gene perturbations). The number of genes in the pathway up or downregulated respectively (P ≤ 0.01) by the maximally-impacting test compound treatment are reported.

results of AffyLumma microarray analysis, as shown in Table II.

**Effect of acetin on the growth of HepG2 liver cancer cells**

Acetin inhibited the growth of p53 positive HepG2 liver cancer cells with an IC50 value, the concentration that caused 50% inhibition of cell proliferation, of 27 μg/mL (40 μM).

**DISCUSSION**

In this study, we used a chemogenomic approach to elucidate the mode of action of the triterpene glycoside acetin, ToxFX analysis, which reveals the subtle expression signals captured by signatures and pathway analysis, indicated that acetin activated stress- and statin-associated responses, suggesting that acetin may have chemopreventive potential.

Stress-associated responses were indicated by strong transcriptional responses in the acute phase response, p53 stress response, hypoxia and the stress response and mitochondrial oxidative phosphorylation pathways, as determined by ToxFX pathway analysis. The acute phase response pathway was impacted by significant upregulation of genes including CXCL1 (also involved in NF-κB and TGFβ signalling) and C4BP, as well as downregulation of several probes of c-Jun. The p53 stress response pathway included upregulation of FAS and downregulation of CDK6. p53 is a known tumour suppressor protein that is at the nexus of multiple stress response pathways. The downregulations of crythipoietin, CYP2C and ATP synthase that we observed by ToxFX analysis after treatment with acetin for 6 h suggests that
the primary effects of actein may be on hypoxia and the stress response and mitochondrial oxidative phosphorylation. Actein's downregulation of Acox1 at 24 h is consistent with the recent finding that the primary effect of an ethanol extract of black cohosh may be to reduce mitochondrial β-oxidation of isolated rat liver mitochondria [19].

The RT-PCR analysis confirmed transcriptional effects of actein on genes involved in stress response pathways. In particular, we observed a decrease followed by a significant increase of the Nrf2 stress response gene NQO1; a progressive increase of the mitochondrial receptor gene B2RP and cytochrome CYP7A1; and a significant increase followed by a decrease of CD1, which may be a strong oncogene in the liver [20] and ID3, which may play a role in regulating the Rb tumour suppressor gene [21].

In support of actein's effects on the stress response, actein inhibited the growth of HepG2 liver cancer cells. This is consistent with the findings that triterpene glycosides from related Cimicifuga species selectively inhibited the growth of human liver cancer cells compared with liver hepatocytes [8,19] and that lipophilic statins inhibit tumorigenesis in vivo [22]. Gene expression analysis in the present study echoed previous findings that the growth inhibitory effects of actein and an extract of black cohosh on human breast cancer cells can be attributed to the activation of stress response pathways [11,23], depending on the duration of exposure.

Statin-associated responses were indicated by a Drug Signature match to cholesterol biosynthesis inhibitors, in particular, the lipophilic statin simvastatin and cerivastatin. This effect was strongly confirmed by pathway analysis: actein elicited a maximum pathway response for cholesterol biosynthesis in the 90th percentile as well as a strong transcriptional response in the fatty acid biosynthesis and regulation pathway. The cholesterol biosynthesis pathway was significantly impacted by upregulation of genes including HMGCR, IDOS, a precursor to the farnesylated oncoproteins (Table 1b). As has been speculated for lovastatin [24], these upregulations may be a feedback mechanism in response to inhibition of cholesterol biosynthesis. The gene SCD1, part of the fatty acid biosynthesis and regulation pathway, was significantly downregulated. It is possible that actein induces post-transcriptional downregulation of HMGCR, as has been shown for related isoprenes [25].

The dual impact of actein on the cholesterol biosynthesis and stress response pathways is not surprising.
Table II: Comparison of the effects of actein on selected liver genes by real-time PCR and microarray analysis after treating Sprague-Dawley rats with actein at 35.7 mg/kg for 6 or 24 h.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Gene</th>
<th>Affymetrix number</th>
<th>Fold change (6 h, 35.7 mg/kg)</th>
<th>Fold change (24 h, 35.7 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RT-PCR Microarray</td>
<td>RT-PCR Microarray</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fold change P value Fold change P value</td>
<td>Fold change P value Fold change P value</td>
</tr>
<tr>
<td>Stress response</td>
<td>S100A9</td>
<td>1387125_at</td>
<td>-0.36 0.30 -0.64 -</td>
<td>2.97 4.34E-06 3.01 2.28</td>
</tr>
<tr>
<td></td>
<td>CYP7A1</td>
<td>1368458_at</td>
<td>0.43 0.30 0.045 -</td>
<td>1.47 7.65E-05 2.01 3.44</td>
</tr>
<tr>
<td></td>
<td>BSEP</td>
<td>1370249_at</td>
<td>0.07 0.63 -0.12 -</td>
<td>1.13 8.99E-04 1.51 1.58</td>
</tr>
<tr>
<td>Cell-cycle regulation</td>
<td>CNDN1</td>
<td>1371150_at</td>
<td>0.52 0.025 0.57 -</td>
<td>-2.32 1.09E-06 -2.30 0.76</td>
</tr>
<tr>
<td>Phase 2</td>
<td>NQ01</td>
<td>1387559_at</td>
<td>-0.50 0.31 -0.64 -</td>
<td>1.73 5.38E-05 1.45 -</td>
</tr>
<tr>
<td>Transcription 750</td>
<td>FOS</td>
<td>1387759_at</td>
<td>0.39 0.041 0.19 -</td>
<td>-1.52 3.72E-02 -0.58 -</td>
</tr>
<tr>
<td>Cholesterol biosynthesis</td>
<td>HMGC51</td>
<td>1367032_at</td>
<td>-0.82 0.091 -0.66 -</td>
<td>1.44 3.03E-04 1.11 -</td>
</tr>
<tr>
<td></td>
<td>HS2I7R</td>
<td>1387233_at</td>
<td>-0.66 0.18 -0.99 -</td>
<td>1.21 8.86E-3 2.11 -</td>
</tr>
<tr>
<td></td>
<td>HMGCR</td>
<td>1375852_at</td>
<td>-0.45 0.12 -0.61 -</td>
<td>0.45 0.14 1.12 -</td>
</tr>
</tbody>
</table>

- indicates P value <0.05

because the sterol regulatory pathway is known to share components with stress pathways [26]. While low doses of lovastatin have been shown to elicit an effect on the cholesterol biosynthesis pathway in the rat liver, high doses of lovastatin are shown to induce a complex set of stress response proteins involved in cytoskeletal structure, calcium homeostasis, protease inhibition, nucleic and amino acid metabolism and cell signalling [24]. The findings that extracellular signal related kinases (ERK 1/2) control gene transcription mediated by sterols in HepG2 liver cancer cells and that ERK1/2 appears to phosphorylate SREB1a and -2 in vitro [27] may link the cholesterol and stress responses that we observed.

An assessment of physiological parameters supported actein’s pharmacological utility. First, actein reduced free fatty acid and cholesterol content in hepatocytes by 0.6-fold at 24 h. The microvascular steatosis [19] and increases in lipid levels [14,15] that have been associated with the administration of black cohosh extracts may therefore be due to components other than actein or related triterpene glycosides. Secondly, actein was bioavailable in the rats, reaping at a value of 2.4 μg/mL in the serum. Our previous report suggests that this value is sufficient to synergize with chemotherapy agents [28]. Prolonged administration may lead to a concentration of actein in target tissues and hence require a lower effective dose, as has been shown for green tea extracts [29,30].

We observed a few untoward transcriptional effects elicited by actein: an upregulation of the acute phase response gene S100A9, which stimulates proliferation of fibroblast cells and may act as a mediator during chronic inflammation [31], and a Drug Signature match to compounds that cause hepatic cholestasis and non-alcoholic inflammatory cell infiltration, which we confirmed by microscopy. These results are consistent with reports of idiosyncratic hepatotoxicity associated with the use of black cohosh [13].

Treatment with actein for 6 or 24 h elicited a statistically significant change in the levels of 297 or 1335 genes respectively. Because the median response for all compounds in DrugMatrix is 3783 [16], this is considered a weak response. This could be due to low compound concentration, short exposure time, poor pharmacokinetic or pharmacodynamic properties in the organ. The weak response could also be related to the fact that these results were obtained after treating older (56-week old) female rats, while the data in DrugMatrix were generated using juvenile (8-10 week old) male rats [16]. A cause for concern is also that the stress response could be a result of treatment with high doses of actein.

**CONCLUSIONS**

The individual and contextual transcriptional effects of actein that we observed in the rat liver predict a significant impact of this natural compound on stress and cholesterol biosynthesis pathways. We confirmed these alterations using biological assays: actein inhibited the proliferation of HepG2 human liver cancer cells and
reduced the levels of free fatty acid and cholesterol in the rat liver. Furthermore, actein was bioavailable in the serum at levels sufficient for synergy with chemotherapy agents. Chemogenomic analysis also offered insights as to potential safety concerns that may arise with the use of actein. These results demonstrate the utility of chemogenomic analysis in providing a biologically relevant overview of a compound’s effects and direction for future research. Based on our findings, we can conclude that actein may be useful to prevent and treat cancer and lipid disorders and is worthy of further study.

ACKNOWLEDGEMENTS

We thank Dr I. Bernard Weinstein, Dr Donald Halbert and Dr Richard Brennan for guidance; Dr Richard Friedman and Dr Rong Cheng for assistance in bioinformatics analysis; and Dr Hongma Ma, Mayu Panikutam and Ryota Kashiyawazi for excellent technical help. This research was supported by NIH-NCCAM RO1 AT01 092-01-A2 and The Susan G. Komen Breast Cancer Foundation Grant BCTR0402502 to L. S. R. The contents of this study are solely the responsibility of the authors and do not necessarily reflect the official views of NIH-NCCAM.

REFERENCES

23 Gaudet E., Wotton S., Pack I., Kroll T., Hambarger M. Gene expression profiling reveals unique effects of *Cimicifuga racemosa* (L.) NÜPP (black cohosh) on the estrogen receptor positive.

27 Koda H., Müller-Wieland D., Roth G. et al. Sterol regulatory element binding proteins (SREBP)-1a and SREBP-2 are linked to the MAP-kinase cascade. J. Lipid Res. (2000) 41 99–108.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Processed liver gene expression data after treating Sprague-Dawley rats with acetin at 35.7 mg/kg for 24 h.

Table S2. Designed primer sequences used in RT-PCR.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.