

ORIGINAL
ARTICLE

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

Linda Saxe Einbond^{1*}, Morando Soffritti^b, Davide Degli Esposti^b, Taesik Park^a, Erica Cruz^a, Tao Su^a, Hsan-au Wu^a, Xiaomei Wang^a, Yu-Jing Zhang^a, Justin Ham^a, Ira J. Goldberg^a, Fredi Kronenberg^a, Antoaneta Vladimirova^c

^aColumbia University College of Physicians and Surgeons, New York, 10032 NY, USA

^bEuropean Ramazzini Foundation of Oncology and Environmental Sciences, Bologna, Italy

^cIconix/Eucelos 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@columbia.edu

Laboratory of origin: Department of Rehabilitation Medicine, The Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University, HHSC-1509, 701 W. 168th 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 HMGCS1, HMGCR, HSD17B7, NQO1, S100A9; (ii) a progressive increase of BZRP 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. *Cimicifuga racemosa* (L.) Nutt] for centuries to treat a variety of conditions, including rheumatism, arthritis, muscle pain and dysmenorrhoea [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 glycosides actein (Figure 1a) and 23-epi-26-deoxyactein [5] constitute about 6.4% of an n-butanol fraction of black cohosh enriched for triterpene glycosides (27%).

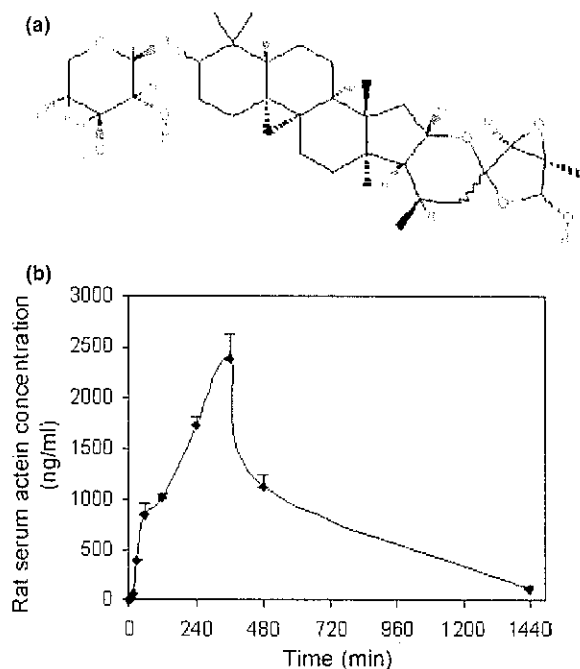


Figure 1 (a) Chemical structure of the triterpene glycoside actein isolated from black cohosh. (b) Concentration of actein in Sprague-Dawley rat serum 0–24 h after treatment with actein at 35.7 mg/kg.

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 (ER⁺, Her2 low) and MDA-MB-453 (ER⁻, 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 ToxPX[®] 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 livers 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 Analytica (Danbury, CT, USA, lot number PA-A-037), purity was over 95% by high pressure liquid chromatography (HPLC; *in vivo* studies) and from ChromaDex (Laguna Hills, CA, USA, lot number 01355-806), purity 89% by HPLC. Actein [Lot no. 01355-805 (P)] and 27-(23-Epi-26-deoxyactein) deoxyactein (A11P) 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, NJ, 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-thiazol)-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 × 25 × 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 Dottori Piccioni). 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/CEE).

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) one-fifth this dose, 7.14 mg/kg, by gastric intubation. A control group of eight female rats was treated by gastric intubation with 1 cc of water. Fresh solution of actein, suspended in water, was prepared just before the treatment.

Blood and urine collection

To determine 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 contusion of the retrobulbar plexus with a siliconated 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 anticoagulating 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 Chromadex to determine the presence and quantity of actein in serum and urine samples from Sprague-Dawley rats treated 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 300 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™ (Waters Corporation, Milford, MA, USA) using a BEH C18 column (1.7 µm, 2.1 × 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 Iconix/Entelos ToxFX 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 toxicogenomic report was produced using the ToxFX Analysis Suite. ToxFX analysis uses the Iconix/Entelos database Drug Matrix to match patterns of gene expression changes elicited 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

Log₁₀ ratio data for the actein array data set was compared to the Iconix collection of gene expression biomarkers (Drug Signatures). The degree to which the gene expression profile of actein matched a Drug Signature was reported in ToxFX as a posterior probability score (PPS). PPS >0.9 were considered highly significant. 0.5 < PPS < 0.899 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 processes 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 informatics 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 Trizol (Invitrogen, CA, USA), and purified with the RNeasy Kit (Qiagen, Valencia, CA, USA). mRNA sequences were obtained from the public GeneBank database (<http://www.ncbi.nlm.nih.gov>), and primers were designed using Primer3 software from The Massachusetts Institute of Technology (http://www.frodo.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 101.74 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.

Lipid analysis of rat liver tissue

When we examined the effect of actein (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

Haematoxylin and eosin stained slides of rat livers obtained at 24 h after administration of actein showed hepatotoxicity (Figure 2). 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 actein (35.7 mg/kg) for 6 or 24 h effected 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 (-0.05 , $P < 0.01$) (Table 1a). At 24 h, significant gene alterations included upregulations of IPP (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).

Actein also upregulated genes involved in the acute phase response (A2M), Hypoxia and Hif signalling (TFRC, FLT1), NRF2 mediated Ox stress receptor (PSMB10, NQO1) and p53 signalling (TNFRSF6, FAS). Actein downregulated the expression of genes involved in cell-cycle control (CCND1) and hepatic toxicity: origin of cholestasis (ME1).

Transcriptional pattern matching with drug signatures

We compared expression pattern changes induced by actein to gene expression patterns from compounds in DrugMatrix and found the following three Drug Signatures as having the highest probability matches to actein'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 (PPS = 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 (PPS = 0.56) and (iii) a weak match to cholesterol biosynthesis inhibitor signature at 24 h (PPS = 0.54).

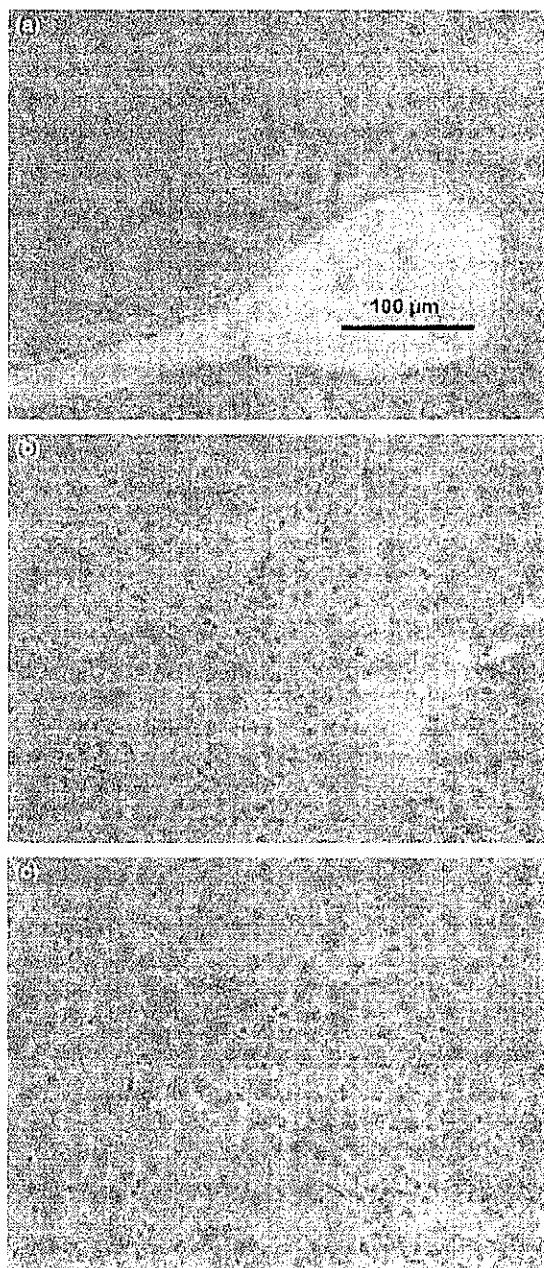


Figure 2 Histology of liver tissue from three different rats. H&E stained sections of control and treated Sprague-Dawley rat livers, obtained 24 h after treatment with or without actein at 35.7 mg/kg, were examined by microscopy, as described in Materials and Methods: (a) control; (b and c) treated with actein 35.7 mg/kg; magnification, $\times 400$.

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.

Pathway category	Affymetrix number	Gene symbol	Gene title	Fold change
(a) Genes significantly altered at 6 h				
LPS and IL-1 inhibit RXR protein function	1387949_at	CYP2C	Cytochrome P450, family 2, subfamily c, polypeptide 22	-0.26*
Xenobiotic metabolism				
Aryl hydrocarbon receptor (AhR) signalling	1387308_at	EPO	Erythropoietin	-0.22**
Hypoxia and HIF signalling				
Mitochondrial oxidative phosphorylation	1387019_at	ATP5I	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit e	-0.05*
(b) Genes significantly altered at 24 h				
Fatty acid biosynthesis and its regulation	1370355_at	SCD1	Stearoyl-coenzyme A desaturase 1	-1.28*
Hepatic toxicity: origin of steatosis				
Cholesterol biosynthesis	1388872_at	IPP	Isopentenyl-diphosphate isomerase	0.80*
	1367932_at	HMGCS1	3-hydroxy-3-methylglutaryl-coenzyme A synthase 1	0.36**
	1367667_at	FDPS	Farnesyl diphosphate synthase	0.34*
Acute phase response	1387125_at	S100A9	S100 calcium binding protein A9 (calgranulin B)	0.79*
	1387316_at	CXCL1	chemokine (C-X-C motif) ligand 1 (also involved in NF-kappa B and TGF-beta signalling)	0.44*
	1383425_at	C5	Complement component 5	0.18*
	1368695_at	C4BP	Complement component 4 binding protein, beta	0.17*
	1369764_at	C4BP	Complement component 4 binding protein, alpha	0.09*
	1387952_a_at	CD44	CD44 antigen	0.08*
	1367804_at	SAP	Serum amyloid P-component	-0.04*
Hepatic toxicity: origin of cholestasis	1368458_at	CYP7A1	Cytochrome P450, family 7, subfamily a, polypeptide 1	0.53*
LPS and IL-1 inhibit RXR protein function				
Mitochondrial oxidative phosphorylation	1387670_at	MG3PDH	Glycerol-3-phosphate dehydrogenase 2, mitochondrial	-0.38*
Beta-oxidation of fatty acid	1367680_at	ACOX1	Acyl-coenzyme A oxidase 1, palmitoyl	-0.17*
TGF-beta signalling				

We used Iconix 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 (log) 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: cholesterol 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; NFE2 mediated oxidative stress gene NQO1; and cholesterol biosynthetic genes HMGCS1, HMGCR and HSD17B7 decreased at 6 h and increased at 24 h; (ii) mRNAs for the cytochrome CYP7A1 and mitochondrial benzodiazepine receptor gene BZRP 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

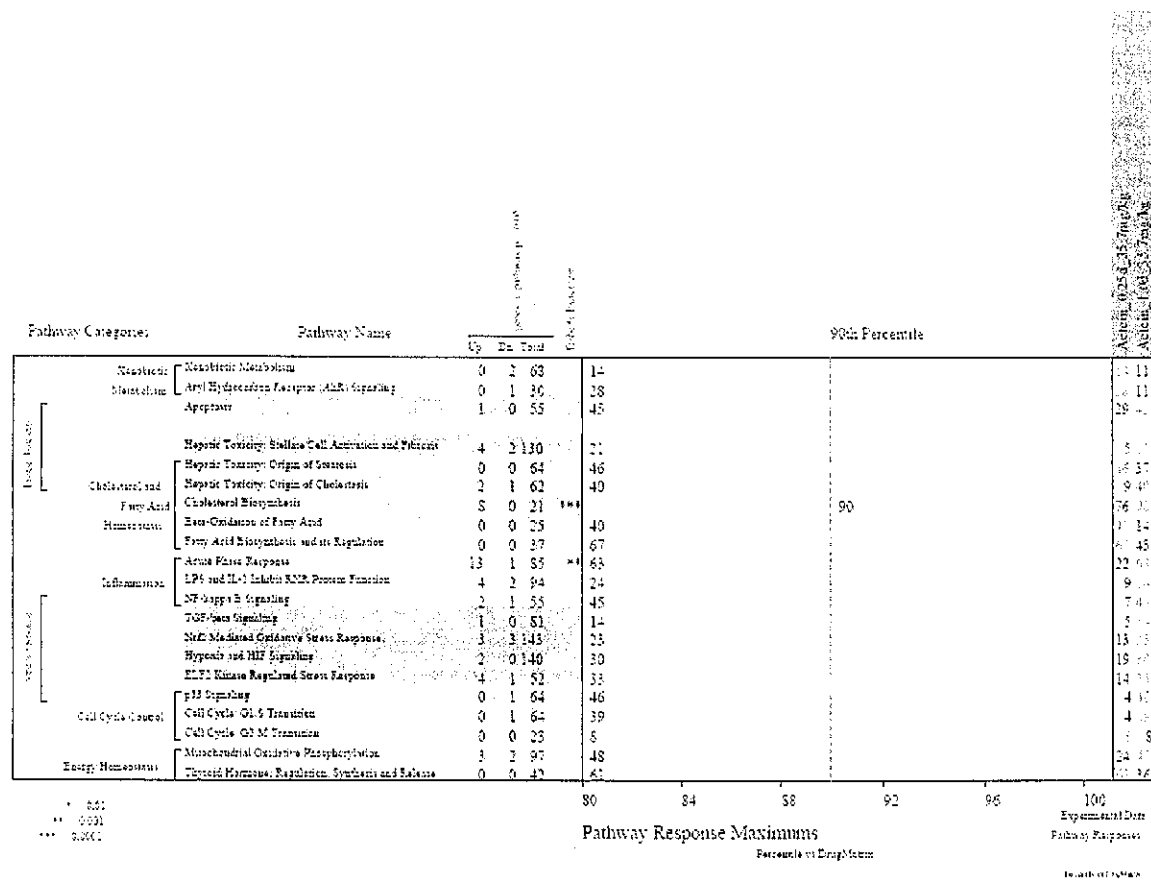


Figure 3 Pathway responses for actein. The effect of the compound on toxicologically important DrugMatrix pathways is displayed based on two different metrics: Maximum pathway impact (using Fisher's exact test), and relative pathway response (using the overall magnitude of pathway gene perturbations). The number of genes in the pathway up or downregulated respectively ($P \leq 0.01$) by the maximally-impacting test compound treatment are reported.

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

Effect of actein on the growth of HepG2 liver cancer cells

Actein inhibited the growth of p53 positive HepG2 liver cancer cells with an IC_{50} value, the concentration that caused 50% inhibition of cell proliferation, of 27 $\mu\text{g}/\text{mL}$ (40 μM).

DISCUSSION

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

associated responses, suggesting that actein 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 TGF β signalling) and C4BP, as well as downregulation of several probes of cJun. 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 erythropoietin, CYP2C and ATP synthase that we observed by ToxFX analysis after treatment with actein for 6 h suggests that

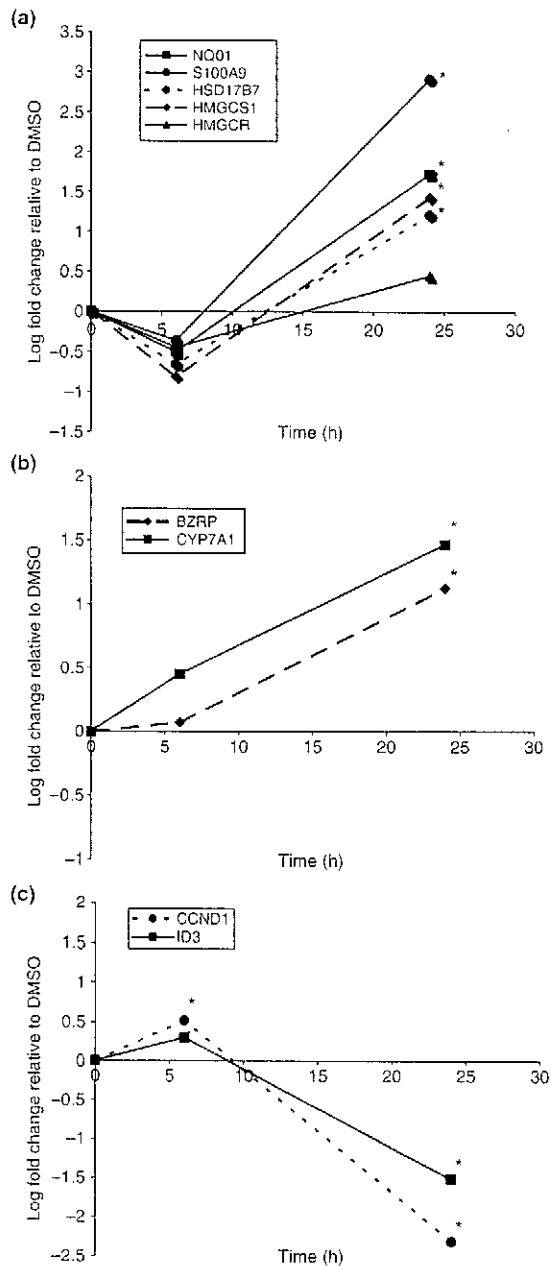


Figure 4 Real-time RT-PCR analysis of RNA obtained from rat liver after treating Sprague-Dawley rats with actein at 35.7 mg/kg for 6 or 24 h. Sprague-Dawley rats were treated with 35.7 mg/kg of actein for 6 or 24 h; extracts were prepared from rat liver tissues and analysed by real-time RT-PCR, as described in Materials and Methods. Fold change indicates relative expression in actein vs. control rat livers. RT-PCR results indicated three patterns of gene expression: (a) a decrease at 6 h, followed by an increase at 24 h; (b) a progressive increase at 6 and 24 h; and (c) an increase at 6 h and decrease at 24 h. **P* values were <0.05; at 6 h: *P* < 0.05 for CCND1 and ID3. At 24 h, all *P* values were <0.05, except HMGCR.

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 ethanolic 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 *BZRP* 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 statins 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 *IPP*, *HMGCS1* and *FDPS*, 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.

Categories	Gene	Affymetrix number	Fold change relative to control fold change (<i>B</i> or <i>P</i> values)							
			Actein treatment (6 h, 35.7 mg/kg)				Actein treatment (24 h, 35.7 mg/kg)			
			RT-PCR		Microarray		RT-PCR		Microarray	
			Fold change	<i>P</i> value	Fold change	<i>B</i> value	Fold change	<i>P</i> value	Fold change	<i>B</i> value
Stress response	S100A9	1387125_at	-0.36	0.30	-0.64	-	2.92	4.34E-06	3.01	3.28
	CYP7A1	1368458_at	0.45	0.15	0.045	-	1.47	7.65E-05	2.04	3.44
	BZRP	1370249_at	0.07	0.63	-0.12	-	1.13	8.93E-04	1.51	1.58
Cell-cycle regulation	CCND1	1371150_at	0.52	0.025	0.57	-	-2.32	1.09E-06	-2.30	0.70
Phase 2	NQO1	1387599_a_at	-0.50	0.31	-0.64	-	1.73	5.58E-05	1.45	-
Transcription regulation	ID3	1387769_a_at	0.30	0.041	0.19	-	-1.52	3.72E-2	-0.58	-
Cholesterol biosynthesis	HMGCS1	1367932_at	-0.82	0.091	-0.66	-	1.44	3.03E-04	1.11	-
	HSD17B7	1387233_at	-0.66	0.18	-0.99	-	1.21	3.85E-3	2.11	-
	HMGCR	1375852_at	-0.45	0.12	-0.61	-	0.45	0.14	1.12	-

- indicates *B* value <0.

B value >0 is significant.

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 acid 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 SREB1 α 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 microvesicular 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, peaking at a value of 2.4 μ g/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 mitogen during chronic inflammation [31], and a Drug Signature match to compounds that cause hepatic centrilobular and non-zonal inflammatory cell infiltrate, 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 effected 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 Hongbao Ma, Maya Panjikaran and Ryota Kashiwazaki for excellent technical help. This research was supported by NIH-NCCAM K01 AT001692-01A2 and The Susan G. Komen Breast Cancer Foundation Grant BCTRO402502 to L. S. E. The contents of this study are solely the responsibility of the authors and do not necessarily reflect the official views of NIH-NCCAM.

REFERENCES

- 1 Foster S. Black cohosh: *Cimicifuga racemosa*. A literature review. *Herbal Gram.* (1999) 45 35–49.
- 2 Rebbeck T.R., Troxel A.B., Norman S. *et al.* A retrospective case-control study of the use of hormone-related supplements and association with breast cancer. *Int. J. Cancer* (2007) 120 1523–1528.
- 3 Zepelin H.H., Meden H., Kostev K., Schröder-Bernhardt D., Stammwitz U., Becher H. Isopropanolic black cohosh extract and recurrence-free survival after breast cancer. *Clin. Pharmacol. Ther.* (2007) 45 143–154.
- 4 Jiang B., Kronenberg P., Nuntanakorn P., Qiu M.H., Kennelly E.J. Evaluation of the botanical authenticity and phytochemical profile of black cohosh products by high-performance liquid chromatography with selected ion monitoring liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* (2006) 54 3242–3253.
- 5 Chen S.N., Li W., Fabricant D.S. *et al.* Isolation, structure elucidation, and absolute configuration of 26-deoxyactein from *Cimicifuga racemosa* and clarification of nomenclature associated with 27-deoxyactein. *J. Nat. Prod.* (2002) 65 601–605.
- 6 Watanabe K., Mimaki Y., Sakagami H., Sashida Y. Cycloartane glycosides from the rhizomes of *Cimicifuga racemosa* and their cytotoxic activities. *Chem. Pharm. Bull. (Tokyo)* (2002) 50 121–125.
- 7 Einbond L.S., Shimizu M., Xiao D. *et al.* Growth inhibitory activity of extracts and purified components of black cohosh on human breast cancer cells. *Breast Cancer Res. Treat.* (2004) 83 221–231.
- 8 Tian Z., Si J., Chang Q. *et al.* Antitumor activity and mechanisms of action of total glycosides from aerial part of *Cimicifuga dahurica* targeted against hepatoma. *BMC Cancer* (2007) 7 237.
- 9 Sakurai N., Kozuka M., Tokuda H. *et al.* Cancer preventive agents. Part I: chemopreventive potential of cimigenol, cimigenol-3,15-dione, and related compounds. *Bioorg. Med. Chem.* (2005) 13 1403–1408.
- 10 Einbond L.S., Su T., Wu H.A. *et al.* The growth inhibitory effect of actein on human breast cancer cells is associated with activation of stress response pathways. *Int. J. Cancer* (2007) 121 2073–2083.
- 11 Einbond L.S., Su T., Wu H.A. *et al.* Gene expression analysis of the mechanisms whereby black cohosh inhibits human breast cancer cell growth. *Anticancer Res.* (2007) 27 697–712.
- 12 Johnson B., Van Breemen R. *In vitro* formation of quinoid metabolites of the dietary supplement *Cimicifuga racemosa* (black cohosh). *Chem. Res. Toxicol.* (2003) 16 838–846.
- 13 Cohen S., O'Connor A., Hart J., Merel N., Te H. Autoimmune hepatitis associated with the use of black cohosh: a case study. *Menopause* (2004) 11 575–577.
- 14 Wuttke W., Gorkow C., Seidlova-Wuttke D. Effects of black cohosh (*Cimicifuga racemosa*) on bone turnover, vaginal mucosa, and various blood parameters in postmenopausal women: a double-blind, placebo-controlled, and conjugated estrogens-controlled study. *Menopause* (2006) 13 185–196.
- 15 Raus K., Brucker C., Gorkow C., Wuttke W. First-time proof of endometrial safety of the special black cohosh extract (Actaea or *Cimicifuga racemosa* extract) CR BNO 1055. *Menopause* (2006) 13 678–691.
- 16 Ganter B., Tugendreich S., Pearson C.I. *et al.* Development of a large-scale chemogenomics database to improve drug candidate selection and to understand mechanisms of chemical toxicity and action. *J. Biotechnol.* (2005) 119 219–244.
- 17 Fielden M.R., Pearson C., Brennan R., Kolaja K.L. Preclinical drug safety analysis by chemogenomic profiling in the liver. *Am. J. Pharmacogenomics* (2005) 5 161–171.
- 18 Natsoulis G., El Ghaoui L., Lanckriet G.R. *et al.* Classification of a large microarray data set: algorithm comparison and analysis of drug signatures. *Genome Res.* (2005) 15 724–736.
- 19 Lude S., Török M., Dieterle S. *et al.* Hepatic effects of *Cimicifuga racemosa* extract *in vivo* and *in vitro*. *Cell Mol. Life Sci.* (2007) 64 2848–2857.
- 20 Deane N., Parker M., Aramandla R. *et al.* Hepatocellular carcinoma results from chronic cyclin D1 overexpression in transgenic mice. *Cancer Res.* (2001) 61 5389–5395.
- 21 Iavarone A., Lasorella A. ID proteins as targets in cancer and tools in neurobiology. *Trends Mol. Med.* (2006) 12 588–594.
- 22 Campbell M.J., Esserman L.J., Zhou Y. *et al.* Breast cancer growth prevention by statins. *Cancer Res.* (2006) 66 8707–8713.
- 23 Gaube F., Wolff S., Pusch L., Kroll T., Hamburger M. Gene expression profiling reveals effects of *Cimicifuga racemosa* (L.) Nutt. (black cohosh) on the estrogen receptor positive

- human breast cancer cell line MCF-7. *BMC Pharmacol.* (2007) 7 11.
- 24 Steiner S., Gatlin C., Lennon J. et al. Proteomics to display lovastatin-induced protein and pathway regulation in rat liver. *Electrophoresis* (2000) 21 2129–2137.
- 25 Mo H., Elson C.E. Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. *Exp. Biol. Med.* (2004) 229 567–585.
- 26 Hartman M.G., Lu D., Kim M.L. et al. Role for activating transcription factor 3 in stress-induced beta-cell apoptosis. *Mol. Cell. Biol.* (2004) 24 5721–5732.
- 27 Kotzka J., 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.
- 28 Einbond L.S., Shimizu M., Nuntanakorn P. et al. Actein and a fraction of black cohosh potentiate antiproliferative effects of chemotherapy agents on human breast cancer cells. *Phanta Med.* (2006) 72 1200–1206.
- 29 Chow H., Cai Y., Hakim I. et al. Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. *Clin. Cancer Res.* (2003) 9 3312–3319.
- 30 Swezey R.R., Aldridge D.E., LeValley S.E., Crowell J.A., Hara Y., Green C.E. Absorption, tissue distribution and elimination of 4-[(3H)-epigallocatechin gallate in beagle dogs. *Int. J. Toxicol.* (2003) 22 187–193.
- 31 Shibata E., Miyama K., Shinoda F., Mizumoto J., Takano K., Nakagawa H. Fibroblast growth-stimulating activity of S100A9 (MRP-14). *Eur. J. Biochem.* (2004) 271 2137–2143.

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 actein 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.