ANALYZING THE EFFECTS OF SINGLE AND MIXTURES OF POTATO GLYCOALKALOIDS ON GENE EXPRESSION IN INTESTINAL EPITHELIAL CELLS

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ANALYZING THE EFFECTS OF SINGLE AND MIXTURES OF POTATO GLYCOALKALOIDS ON GENE EXPRESSION IN INTESTINAL EPITHELIAL CELLS

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The main aims of this dissertation were first, to determine the molecular mechanism(s) underlying glycoalkaloid-induced effects, in particular their membrane disruptive effect in intestinal epithelial cells. Second, to evaluate the usefulness of DNA microarrays in discriminating individual glycoalkaloids and glycoalkaloid mixtures of varying α-chaconine/α-solanine ratios based on their differences in effect severity and potential toxicities. By exploring the application/sensitivity of transcriptomic techniques in identifying early indicators of toxicity and screening between similar class/effect compounds, these studies clarify the potential application of this technique to "whole foods" safety assessments incorporating different models of impaired nutritional states.

The transcriptional effects of individual and mixtures of glycoalkaloids were studied in the intestinal epithelial Caco-2 cell line. The principal finding was the induction of cholesterol biosynthesis genes by non-cytotoxic glycoalkaloid concentrations, which to some extent is in line with their well documented mechanism of membrane disruption. Various genes involved in the MAPK, PI3K/AKT, chemokine and growth signaling, cell cycle and apoptosis pathways also were affected. Confirmatory apoptosis and cell cycle analyses revealed that glycoalkaloids induced necrotic/apoptotic death and disproportionate accumulation of cells in the G_2M phase.

The DNA microarray data were in line with the results from previous studies demonstrating that potato glycoalkaloids have similar mechanisms of action but differ mainly in their adverse effect potency. In addition, this technology could discriminate among the different glycoalkaloid treatments with respect to effect severity, which correlated well with their effects on lactate dehydrogenase membrane leakage.

DNA microarrays are recognized to be useful tools for generating hypotheses and elucidating mechanism of action. Therefore, the effect of α-chaconine on SREBP-2 protein levels and the importance of MAPK and PI3K/AKT pathways in glycoalkaloid-induced transcription of cholesterol biosynthesis genes were determined. α-Chaconine induced proteolytic cleavage of SREBP-2 and phosphorylation of ERK, JNK and AKT kinases. However, the MAPK and PI3K/AKT pathways were not crucial for glycoalkaloid-induced expression of cholesterol biosynthesis genes.

In this dissertation, the usefulness of DNA microarrays in identifying hitherto unknown mechanisms of action, identifying potential toxicity biomarkers, and assessing the effects of simple mixtures of compounds was demonstrated. The studies presented will contribute towards the elucidation of the toxic and potential beneficial effects of potato glycoalkaloids, which may subsequently enhance current efforts to develop transgenic potatoes with altered glycoalkaloid levels/ratios, and the utility of these tools for assessing the safety of whole foods.

BIOGRAPHICAL SKETCH

Tafadzwa Mandimika earned a Ph.D. in 2008 in Human Nutrition from Cornell University, Ithaca, NY, with minors in Developmental biology and Toxicology. She earned a BS in Nutrition from the University of Zimbabwe, Harare, Zimbabwe.

To my parents

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AKT, v-akt murine thymoma viral oncogene homolog

DMEM, Dulbecco's modified Eagle's medium

DMF, dimethyl formamide

DMSO, dimethyl sulphoxide

EGF, epithelial growth factor

EGFR, epithelial growth factor receptor

Erk, extracellular signal related protein kinase

HGF, hepatocyte growth factor

HMGCR, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase

IGF-RI, insulin like growth factor I

JNK, c-jun N terminal protein kinase

LDH, Lactate Dehydrogenase

LDL, low density lipoprotein

LDLR, low density lipoprotein receptor

MIF, migration inhibitor factor

MAPK, mitogen-activated protein kinase

PI3K, phosphatidylinositol 3-kinase

PCA, Principal Component Analysis

SRE, sterol regulatory element

SREBP, sterol regulatory element binding transcription factor

SCAP, sterol regulatory element binding transcription factor cleavage-activating protein

VEGF, vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

A. Literature Review

1. Introduction

Potatoes (*Solanum tubersom L.*) are widely consumed and are a significant source of high quality protein, carbohydrates, vitamins C and B, certain minerals such as potassium, magnesium and phosphorus and antioxidant phytochemicals such as phenolic acids and flavonoids (*1, 2*). However despite this, they also contain naturally occurring toxins called glycoalkaloids, which may have deleterious toxic effects in humans. The two major forms of glycoalkaloids present in potato are α-chaconine and α-solanine, which occur at varying concentrations and ratios, depending on the specific variety of potato (*3*). In combination, they may interact synergistically resulting in a level of toxicity that is more severe than is observed when either glycoalkaloid is administered alone (*3-7*). Most of the reported glycoalkaloid poisoning symptoms are found to be induced by their acetylcholinesterase inhibitory or membrane disruptive activities (*8*). However, while the toxicological effects of potato glycoalkaloids in humans are well described; the mechanisms underlying their effects are poorly understood.

Human consumption of potatoes results in the ingestion of α-chaconine and α-solanine in varying ratios, usually ranging from ~1.2:1 to ~2.4:1 (α-chaconine to α-solanine) (*3*). There is currently research on the development of transgenic potatoes with altered/lowered glycoalkaloid ratios/levels, which may have potentially nutritional/health benefits to consumers (*9*). In order to enhance the development of potentially beneficial potatoes, there is need to investigate the mechanisms underlying the toxic effects or mode of action of individual glycoalkaloids and glycoalkaloid mixtures at varying α-chaconine to α-solanine ratios.

Current conventional food safety assessment methodologies are focused primarily on the evaluation of the toxicity of single chemicals to predict effects of exposures to mixtures of compounds or whole foods. Prediction of adverse effects of whole foods is especially difficult because of the many interactions that may occur among the high number of nutrients and other food substances commonly found in whole foods. Such interactions may alter the degree and possibly the nature of predicted toxic effects of individual food constituents (*10*). In addition, whole foods cannot be tested with the high dose strategy currently used for single chemicals to increase the sensitivity in detecting toxic endpoints, as this may result in nutritional imbalances and decreased palatability. Profiling methods such as DNA microarray technology have been suggested as tools that may facilitate in screening, assessment and/or prediction of putative harmful interactions following exposure to mixtures of substances or whole foods. Changes in gene expression may provide more sensitive, immediate, and comprehensive markers of toxicity than conventional toxicological methods and endpoints (*11*).

The first part of the chapter focuses on potato glycoalkaloids, their known toxic effects, mechanism(s) of action and pharmacokinetics. The second part of the chapter focuses on challenges involved in food safety assessments (whole food or mixtures), and the potential use of DNA microarrays in improving food safety assessments.

2. Nutritional value of potatoes (*Solanum tubersom L.*)

Potatoes represent the secondary staple of many developed countries, but its consumption is less wide spread in developing countries where sweet potatoes, yams and maize are the main staple crops (*12*, *13*). Despite this, the consumption of potatoes in developing countries has been increasing annually by 4.1% (*14*). Potatoes as well as

other root and tuber crops produce large quantities of dietary energy and have stable yields under conditions where other crops may fail (*14*).

Potato tubers besides being a high source of starch (70–90% on dry basis)(*12*), are also an important source of vitamins, minerals, trace elements and high quality protein. The potato protein has higher levels of the essential amino acid lysine and is of higher nutritional value compared to wheat protein, which has insufficient amounts of the two essential amino acids: lysine and threonine (*15*). Potatoes also contain substantial levels of antioxidants such as vitamin C (*2*), vitamin E, carotenoids (lutein)(*16, 17*), phenolic acids (mainly caffeic and chlorogenic acids) (*17-19*) and flavonoids (such as catechin, quercetin)(*17*). Antioxidants protect and prevent cellular damage by efficiently scavenging for superoxides and peroxyl radicals together with endogenous systems of defense limiting oxidative stress. The levels of vitamin C in potatoes are high comparatively to those of rice and wheat, which completely lack this vitamin. The vitamin C levels are about 15 mg/100 g of steamed potato, contributing to 25–30% of the RDA (Recommended Dietary Allowance) (*1, 20*). In Spanish populations, potatoes have also been shown to contribute to the dietary daily intake of potassium, magnesium, protein and fiber in relation to the RDI (recommended daily intakes)(*19*).

They also contain moderate amounts of B vitamins, including thiamin (B1), riboflavin (B2), pyridoxine (B5), and nicotinic acid (B6) (*1*) and are a source of dietary fiber (7% of peeled potato, up to 11% of non-peeled potato). They contain mainly the water-soluble fibers, hemicelluloses and pectins (55%), which together with water insoluble fibers (45%) such as cellulose may have hypocholesterolemic effects (*12, 21*). Besides containing these compounds, which are of significant nutritional value, they also contain naturally occurring toxins called glycoalkaloids that may have toxic effects and are the main focus of this dissertation.

3. Glycoalkaloids

Potatoes belong to the solanaceae plant family, which also includes other members such as capsicum, eggplant, tomato, nightshade and jimson weed seeds (*9*). They all contain secondary metabolites known as glycoalkaloids, which at high levels may be toxic to bacteria, fungi, viruses, insects, animals, and humans. Glycoalkaloids are proposed to function as protection against certain pests and diseases caused by insects and fungi. α-Chaconine and α-solanine account for 95% of the total glycoalkaloids present in potatoes (*22*). They consist of the same aglycone solanidine, but differ with respect to the nature of the carbohydrate side chain attached to the 3-OH group of the aglycone. For α-solanine, the carbohydrate side chain is the branched trisaccharide, β-solatriose (α-L-rhamnopyranosyl-β-D-glucopyranosyl-β-D-galactopyranose) and for α-chaconine is also a branched trisaccharide, β-chacotriose (*bis* α-L-rhamnopyranosyl-β-D-glucopyranose) (Figure 1-1) (*6, 23, 24*). α-Chaconine is more potent and usually present at a slightly higher concentration than α-solanine. The differences in potency has been attributed to their differing carbohydrate side chains (*25, 26*).

α-Chaconine and α-solanine are present in potatoes at varying concentrations. In combination, they may interact synergistically resulting in increased toxicity at lower concentrations than is observed when either glycoalkaloid is administered alone (*3-7*). Consumption of potatoes results in the ingestion of α-chaconine and α-solanine in varying ratios, ranging from ~1.2:1 to ~2.4:1 (α-chaconine to α-solanine), depending on variety (*3*). Assessing the toxic effects, if any of glycoalkaloid mixtures at varying α-chaconine to α-solanine ratios found in some potatoes varieties is essential.

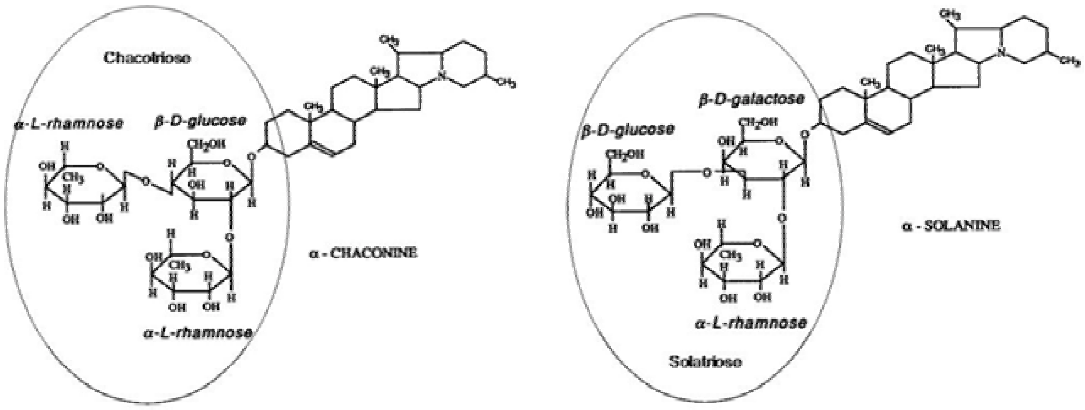

Figure 1-1. The chemical structures of α-chaconine and α-solanine

3.1. Biosynthesis of glycoalkaloids

The biosynthetic pathway of glycoalkaloids has been partially elucidated. Glycoalkaloids are synthesized via the mevalonate/isoprenoid biosynthesis pathway (Figure 1-2), starting with acetyl coenzyme A via the intermediates mevalonate, squalene and cycloartenol through to cholesterol and finally the parent compound solanidine (*27, 28*). Solanidine is then glycosylated by either solanidine UDP-galactose galactosyltransferase (SGT1) or solanidine UDP-glucose glucosyltransferase (SGT2) resulting in either α-solanine or α-chaconine, respectively.

3.2. Distribution and accumulation of glycoalkaloids in potatoes

Glycoalkaloids are produced in all parts of the potato plant including leaves, roots, tubers, and sprouts. The highest concentrations of glycoalkaloids are found in leaves, skin, unripe tubers, flowers and sprouts. Table 1-1, represents the distribution of glycoalkaloids in various parts of the potato. The nature and concentrations of glycoalkaloids depend on the potato variety and the total amounts can be influenced by environmental factors such as soil and climate (*29*) and their physiological state

(*27*). Post harvest exposure of potatoes to sunlight, heat and mechanical damage have been also shown to stimulate glycoalkaloid biosynthesis (*30*).

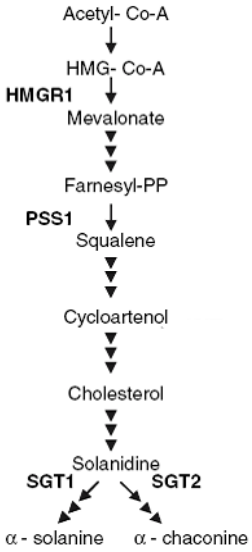

Figure 1-2. Schematic representation of proposed glycoalkaloid biosynthesis. Triple arrowheads represent several enzymatic steps.

Table 1-1. Contents of glycoalkaloids in various parts of the potato

Plant part		mg/kg fresh weight
Sprouts		2000-10000
Flowers		3000-5000
Stems		30-450
Leaves		400-1450
Roots		850
Tubers	whole	20-80
	peel	150-500
	flesh	0-20

Taken from reference (*27*)

4. Bioavailability of Glycoalkaloids (Pharmacokinetics)

Very limited studies have been conducted to study the pharmacokinetics of α-solanine and α-chaconine in man. This presents a huge challenge in the development of toxicity studies. Generally it has been observed that the serum concentrations of both α-solanine and α-chaconine increase steadily after consumption. Peak concentrations are reached after approximately 6 hours of consumption (*31, 32*). There is a linear relationship between dose and peak glycoalkaloid serum concentrations. After reaching peak concentrations, serum concentrations of the glycoalkaloids decrease gradually but do not return to baselines. In addition, α-solanine and α-chaconine have long half-lives, on average 21 and 44 hours respectively (*31*). Therefore, this implies that daily consumption of potato products may cause accumulation of glycoalkaloids, which can consequently lead to adverse health effects.

Sidechains of α-solanine and α-chaconine may be hydrolyzed resulting in their respective β and γ forms. Subsequently, these forms are hydrolyzed into the aglycone solanidine (*32*). Figure 1-3 depicts the hypothesized metabolism of α-solanine and α-chaconine. Potatoes may contain small amounts of the hydrolysis products β and γ forms and solanidine (*9*).

In human serum HPLC chromatograms, only the parent compounds (α-forms) and solanidine could be detected following oral administration of α-solanine and α-chaconine via mashed potatoes (*32*). The mean serum total alkaloid concentration was 2.7 times lower than the solanidine concentration, suggesting that these glycoalkaloids are metabolized to solanidine since the amount of solanidine in potatoes was lower compared to the alkaloids (*31*).

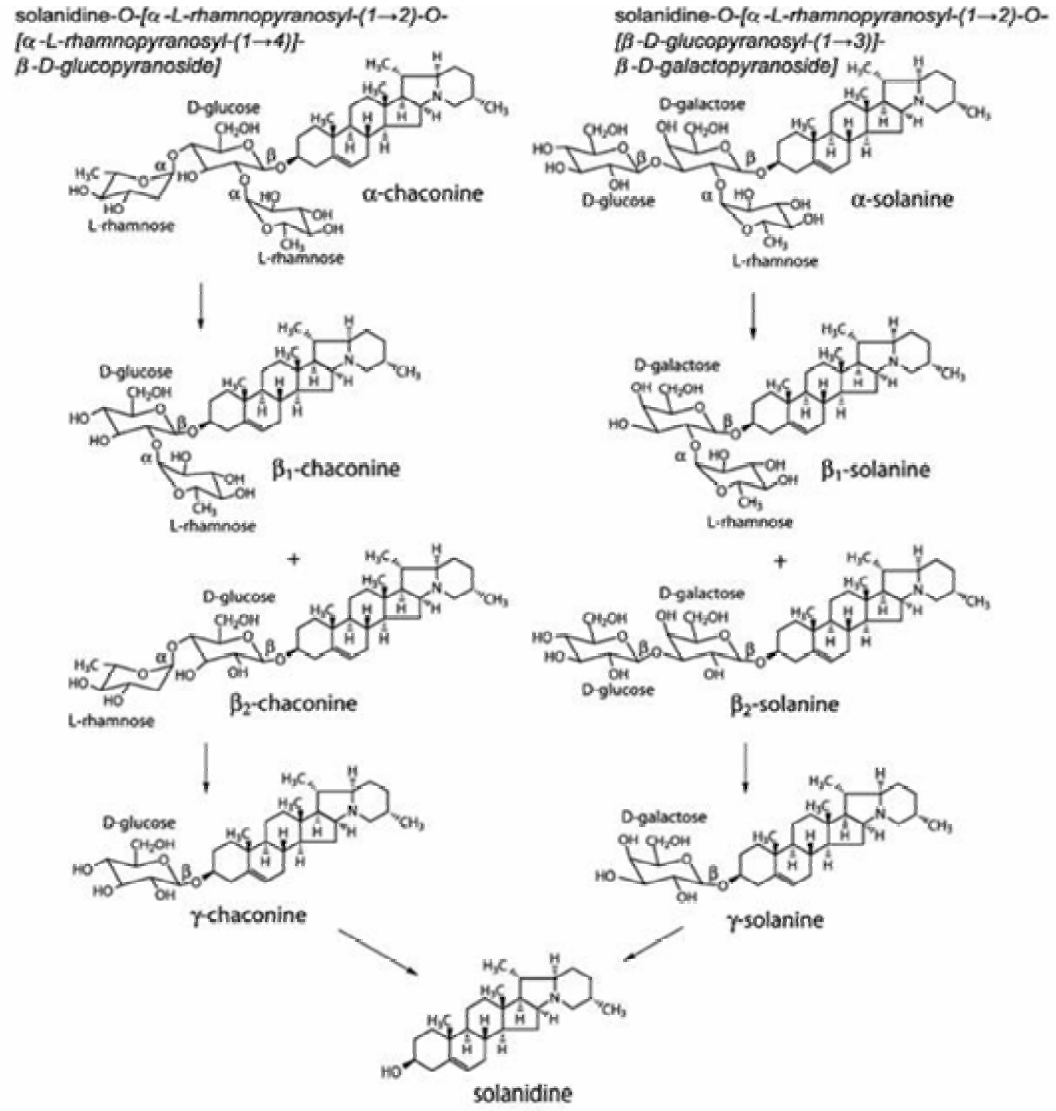

Figure 1-3. Intermediates in the hydrolysis of the trisaccharide side chains of α-solanine and α-chaconine to the glycone solanidine. Taken from reference (*9*)

The site of hydrolysis is unclear, however, it is suggested that hydrolysis may occur in the acid environment of the stomach, or in the gastrointestinal tract at the site of absorption (*33*). Alternatively, the relatively high blood solanidine level could reflect the preferential absorption of the more lipophilic compound.

Solanidine has been shown to accumulate in human livers. Analysis of histologically normal post-mortem livers from human subjects, revealed solanidine to be present in three of the five samples of human livers (*34*) suggesting that it accumulates in the liver.

5. Risks associated with consumption of glycoalkaloids

The potential human toxicity of glycoalkaloids has led to the establishment of safety guidelines limiting the glycoalkaloid content of new cultivars before they are released for commercial use (*9*). The glycoalkaloid concentrations of most commercial potatoes are usually below a safety guideline of 200mg/kg of fresh potatoes (*31*), however, glycoalkaloid concentrations can increase following light exposure or mechanical injury, for example, as a consequence of peeling and slicing (*29*). In addition, glycoalkaloids are relatively heat-stable compounds and are unaffected by food processing (*35*). The estimated highest safe level of total potato glycoalkaloids for human consumption is about 1mg/kg body weight, the acute toxic dose is estimated to be at 1.75 mg/kg body weight, and a lethal dose may be 3-6 mg/kg body weight (*36*). This narrow margin of safety is of concern.

The toxicological effects of glycoalkaloids have been well described in humans, ranging from gastrointestinal disturbances to increased heart-beat, hemolysis and neurotoxic effects (*37*). The toxicities observed are mainly due to the anticholinesterase actvitity of glycoalkaloids on the central nervous system and to disruptions in the cell membranes affecting the digestive system and other organs (*8*). Toxicities induced in other species include hepatoxicity in mice (*38*), induction of hepatic ornithine decarboxylase activity in rats (*39*), craniofacial malformations in hamsters (*40*), and developmental toxicity in frog embryos (*5, 26*).

Toxic Effects

Anticholinesterase activity

α-Solanine and α-chaconine have been shown to inhibit the enzymes acetylcholinesterase (AChE) and butrylcholinesterase (BuChE). Both enzymes are responsible for hydrolyzing the neurotransmitter acetycholine, a key process in nerve impulse conduction across cholinergic synapses (*8*). Inhibition of AChE results in the accumulation of acetylcholine in neuromuscular clefts resulting in neurological damage. The symptoms that are indicative of central nervous system damage attributed to the anticholinesterase effect of glycoalkaloids include rapid and weak pulse, rapid and shallow breathing, delirium and coma (*9*). Both α-solanine and α-chaconine are equally potent with regard to *in vitro* inhibition of bovine and human AChE (*41*). The inhibition of AChE may involve non-covalent competitive binding of the glycoalkaloids to the active site of the enzyme (*9*).

Studies have shown that the structure of the steroid is more important in determining potency rather than the carbohydrate side chain as evidenced by the fact that β_2-chaconine is as effective as α-chaconine (*8*). Structure-inhibitory activity relationship experiments have shown that the unshared electron pair on the ring of the nitrogen of the aglycone may be required for the formation of iminium ions (*42*) and that the nitrogen-containing E/F ring of the aglycone is a more important determinant of anticholinesterase activity than the carbohydrate side chain(*9*). However, the presence of the carbohydrate side chain is necessary for inhibition to occur, indicated by the ineffectiveness of the aglycone solanidine to inhibit AChE.

Furthermore, glycoalkaloids may alter the pharmacokinetics of drugs metabolized by AChE and BuChE, for example, anesthetic drugs such as succinylcholine (*43*). Glycoalkaloids have been also shown to prolong recovery from mivacurium-induced paralysis in rabbits (*44*).

Membrane disruption

Some of the toxicological effects of glycoalkaloids may be due to their membrane disruptive activity. These effects that have been observed in lipid vesicles, membranes of erythrocytes, and various human and mouse cell lines (*7, 24, 45, 46*). The lipophilic moieties of the glycoalkaloids form destabilizing complexes with membrane bound cholesterol resulting in membrane disruption (*45, 47, 48*). This action is thought to be responsible for damaging cells in the gastrointestinal tract and also in other tissues or organs in which these compounds are transported following absorption, for instance, glycoalkaloid-induced hemolysis. The extent of membrane disruption effected by glycoalkaloids has been shown to be dependent on the composition of the glycoalkaloid carbohydrate side chain and the type and content of sterol present in the membrane (*47*). α-Chaconine results in greater membrane disruptive effects than α-solanine (*6, 7, 24*). α-Solanine like α-chaconine binds to cholesterol present in membranes but to a lesser extent (*6*), which may be due to their different carbohydrate side chains. It is well established that synergism between these two glycoalkaloids significantly enhances the membrane-disruptive activity of potato glycoalkaloid mixtures (*6*).

Glycoalkaloid toxicity symptoms at lower doses are mainly gastrointestinal, for example, vomiting, diarrhoea and abdominal pain. The rate of mild glycoalkaloids poisonings is unknown due to the similarity of symptoms to many digestive ailments (*31, 37*). The relative rapidity of symptoms of glycoalkaloid toxicity (0.5-12 hours) suggest that their primary toxic effect may be due to gastrointestinal damage with the secondary occurrence of neurological disorders (*32*). Baker et al., 1991 observed that hamsters fed potato sprouts had gastric necrosis at doses that relatively have a weak effect on AChE activity.

Alteration of membrane potential and transport of calcium and sodium

Glycoalkaloids have been shown to interfere with transepithelial transport of calcium (*49*) and sodium (*50, 51*) ions and alter membrane potential of *Xenopus laevis* frog embryos (*50-52*), which may also influence transport of Na^+ ions. α-Chaconine and also α-solanine but to a lesser extent, were observed to significantly decrease active Na^+ transport. Thus, a possible mechanism of action of glycoalkaloids is that they may affect active transport across cell membranes. Cell culture and experimental animal studies have shown that glycoalkaloids may adversely affect intestinal permeability (*22*).

Developmental toxicity/ teratogenicity in animal studies

Glycoalkaloid-induced craniofacial malformations in mice and multi-organ malformations in frog embryos have been observed at glycoalkaloids levels found in some potato varieties (*40, 53, 54*). Wang et al., 2005 (*55*) also observed that α-solanine and α-chaconine inhibited pre-implantation embryo development in *in vitro* fertilized bovine oocytes, suggesting that these alkaloids may have a negative effect on early embryo development and survival *in vivo* when ingested by man or animals. The developmental toxicity and embryolethality of glycoalkaloids is dependent on the nature and order of attachment of carbohydrate chain side chains (*25, 26*). The outcome of animal studies is cause for concern as humans appear to be more sensitive to glycoalkaloid toxicity than other species (*37*). There is concern that glycoalkaloids may be involved in neural tube defects. Several epidemiologic studies demonstrating an association between neural tube defects and high consumption of severely blighted potatoes have been reported (*56-58*). However, compelling evidence against the potato-terata hypothesis was provided by the birth of children with neural tube defects to mothers on potato avoidance trials (*59*).

Folate status has been implicated as the major environmental factor in the etiology of neural tube defects (NTDs). There is an apparent negative correlation between folate consumption during pregnancy and the occurrence of NTDs in newborns. Studies have shown folate to protect frog embryos against α-chaconine–induced developmental toxicity, mortality and membrane disruption (*23, 60*). Thus implying that folate plays a protective role against glycoalkaloid toxicity. However, a pharmacological rather than a much lower nutritional concentration of folate was required to achieve a protective effect (*60*).

Human clinical and epidemiological studies have demonstrated that maternal use of folic acid in early pregnancy can significantly reduce both the occurrence (*61*), as well as the recurrence (*62*) of NTD-affected pregnancies, and these findings have been further validated by observational studies of women taking daily periconceptional multivitamin supplements containing folic acid (*63, 64*). Whereas the epidemiologic and experimental data support the hypothesis that this apparent reduction in NTD risk may be specifically attributable to folic acid, the mechanisms underlying the protective effects of folic acid are not fully understood.

In epidemiological studies done to assess relationships between glycoalkaloids and the development of neural tube defects, the folate status of the women was not determined (*59*). A possible reason for the absence of an association may have been that the women exposed to glycoalkaloids were of adequate folate nutriture and thus were able to tolerate high levels of glycoalkaloids. An essential question that merits addressing is whether women of compromised folate status when exposed to high concentrations of glycoalkaloids in potatoes are more susceptible to bearing children with neural tube defects.

Other compounds such as glucose-6-phosphate and nicotine adenine dinucleotide phosphate have also been shown to reduce α-chaconine-induced developmental

toxicity in frog embryos (*23*). Taken together, these results may imply that the nutritional status of an individual may play an important role in protecting against glycoalkaloid toxicity.

Induction of ornnithine decarboxylase and estrogenic effects

Intraperitoneal administration of α-solanine, α-chaconine and solanidine was shown to result in markedly elevated induction of hepatic ornithine decarboxylase activity in rats (*39*). Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to putrescine, which is a foundation molecule of polyamines and is known to interact with DNA. In addition, ODC is a marker of liver cell proliferation. The extent of induced ODC activity depends on the nature of the carbohydrate side chain.

The aglycone solanidine and not α-solanine or α-chaconine, was observed to exhibit estrogenic effects in *in vitro* (*36*).

6. Potential beneficial health effects

Although glycoalkaloids are perceived as potentially toxic, they may have beneficial effects, depending on dose and conditions of use (*9*).

6.1. Immune system

Glycoalkaloids have been observed to enhance the immunity of mice against infection by *Salmonella typhimurium* (*65*) Mice injected with low levels of α-chaconine or α-solanine (0.03-0.3 mg/kg of body weight or 0.1-1.0 μg/mouse) were resistant to challenges of lethal doses of *Salmonella typhymurium*. Various organs of treated mice were clear of bacteria (*65*).

6.2. Anti-carcinogenic effects against cancer cell lines

Studies have shown that α-chaconine or α-solanine can inhibit growth of tumor cell lines in vitro (*3, 66*). The antiproliferative effects of many different glycoalkaloids including α-chaconine or α-solanine, were evaluated using a microculture tetrazolium (MTT) assay and all compounds inhibited proliferation. The level of activity was dependent on the chemical structure of the aglycones and the number of carbohydrate groups making up the side chain attached to the aglycones.

7. Food Safety Assessments

Human consumption of potatoes results in the ingestion of α-chaconine and α-solanine in varying ratios, usually ranging from ~1.2:1 to ~2.4:1 (α-chaconine to α-solanine), depending on the specific variety of potato (*3*). Because of the potential risk of increased glycoalkaloid toxicity due to synergism, there is currently an active area of world wide research on the development of transgenic potatoes with altered/lowered glycoalkaloid ratios/levels, which may have potentially nutritional/health benefits to consumers (*9*). Studies have shown that incorporation of an anti-sense transgene encoding either the enzyme solanidine UDP-galactose galactosyltransferase (SGT1) or solanidine UDP-glucose glucosyltransferase (SGT2) in potatoes can result in downregulation of glycoalkaloids biosynthesis, by reducing the levels of either α-solanine or α-chaconine, respectively (*67, 68*). Therefore, from a food safety perspective, assessment of toxic effects of glycoalkaloids; individual and mixtures at varying α-chaconine to α-solanine ratios found in common and possibly transgenic potato varieties, and determination of the molecular mechanism(s) underlying these effects, would be informative for both the development of transgenic potatoes and risk assessment.

7.1. Challenges faced with whole food, nutrient/food mixtures and genetically modified foods assessments

Current conventional food safety assessment methodologies are focused primarily on the evaluation of the toxicity of single chemicals. Predictions of adverse health effects induced by glycoalkaloid and other toxin mixtures are usually based on data obtained from single compound exposures. Unfortunately, observed toxicities commonly deviate from such predictions. Prediction of adverse effects of whole foods is especially difficult because of the many interactions that may occur among the high number of nutrients and other food substances commonly found in whole foods. Such interactions may alter the degree and possibly the nature of predicted toxic effects of individual food constituents (*10*).

Whole food safety assessment remains a difficult proposition because of the various interactions that may occur among nutrients and other substances present in food. In addition, whole foods cannot be tested with the high dose strategy currently used for single (food) chemicals since high dosing of whole foods may be problematic with respect to nutritional inbalances and palatibiliy. In principle, every class of food chemical may exhibit joint similar[1] or dissimilar[2] action, which may lead to non-interactive combined effects. Food chemicals may also interact with one another resulting in stronger effects (synergism, potentiation, supra-addivity) or weaker effects (antagonism, sub-addivity, inhibition). The joint similar or dissimilar actions or interactions of the food chemicals alter the degree and possibly the nature of the potential toxic effects of individual food chemicals (*10*). Possible joint actions or

[1] A non-interactive process whereby each of the chemicals in a mixture have the same mechanism of toxicity and differ only in their potencies: additive effect (*23*)

[2] The modes of action and possibly the nature and site of effect differ among the chemicals in the mixture, which exert their individual effects. They do not modulate each other's toxic effects (*23*)

interactions among food chemicals may result in dose-additivity and synergism, which can lead to increased toxicity.

Nowadays there is a lot of emphasis on production of genetically modified foods with nutritional/health benefits to consumers (second generation genetically modified foods with altered food quality traits). Although there is a steady increase in the production of genetically modified foods, apprehension about the safety of these foods persists. The major concern is whether genetically modified crops are so unique that new safety assessment strategies are required or whether current safety assessments applied to traditionally bred crops can also be applied to genetically modified crops.

The challenges in assessing the safety of genetically modified crops are to characterize the properties of new gene products and potential changes in the levels of endogenous plant constituents, and to identify potential unintended effects due to the genetic modification that may result in adverse impacts on human health and the environment. Thus, concepts were developed to focus the safety assessment of genetically modified crops on any functional and chemical changes that may result from genetic modification (*69, 70*).

The concept of substantial equivalence elaborated by OECD in 1993 has become a key element in the safety assessment of foods derived from genetically modified organisms. This concept is a starting point for the safety assessment of genetically modified crops. The safety assessment is intended to evaluate whether or not the genetically modified crop is as safe as its conventional counterpart. 'Equivalence Criteria' include agronomical and morphological characteristics and chemical composition (including macro- and micro-nutrients, key toxins and anti-nutrients) (*71, 72*). These should allow the identification of significant differences that result from the genetic modification and may potentially affect human health adversely. Subtle unanticipated changes in a plant's composition however may be difficult to detect.

Studies of whole food derived from genetically modified foods appear to be necessary (*73*). As indicated this presents unique problems which, as already mentioned before, are related to nutritional aspects and palatability. To increase the probability of detecting unintended effects, profiling methods such as DNA microarray technology, proteomics and metabolomics have been suggested as tools to characterize not only changes in composition of genetically modified crops but also in assessing the safety and substantial equivalence of genetically modified foods compared to conventional (parental line) foods in animal and *in vitro* studies (*74*). In addition, these tools can be used to identify sensitive and early biomarkers of effect/toxicity at low doses unlike conventional toxicological assays(*11*).

7.2. Issues for consideration in current food safety assessment protocols

7.2.1. Influence of nutritional deficiencies on susceptibility to food toxins

Diet plays an important role on how individuals deal with environmental stressors and toxins to prevent and lessen the impact of disease. Thus it is imperative that during the development or assessment of safety or wholesomeness of conventional or genetically modified foods that one takes into consideration the nutritional status, as well as the consumption patterns of populations for whom particular foods are intended. Current food safety assessment protocols do not take in account such characteristics. The impact of these characteristics on food safety may influence responses to food constituents including natural toxins. In addition, genetically modified foods are promoted, in part, on the basis of their potential health benefits; therefore, their safety should be assessed with target populations in mind.

The bioavailability or toxicity of several compounds has been shown to be strongly influenced by nutritional status. For example, iron-deficient individuals have altered detoxification and antioxidant mechanisms that may affect their susceptibility

to exogenous and endogenous food toxins (*75*). This altered susceptibility may result in further deterioration of health, even when toxins are consumed at levels well tolerated by healthy populations.

In undernourished populations, not only one but several micronutrients are lacking in diets, and as a result, there is high prevalence of simultaneous multiple micronutrient deficiencies. For instance, iron deficiency and anemia is estimated to afflict approximately 1.5 billion people worldwide (WHO, 1992) and usually coexists with other micronutrient deficiencies such as vitamin A and zinc (*76*). In African populations where the diets are mainly cereal based, zinc and iron deficiencies are likely to occur simultaneously (*77, 78*). Cereals have high levels of phytates, which decrease the bioavailability of iron and zinc, and diets that lack or have a small animal based component are usually low in these vital micronutrients (*79*). Concurrent iron and zinc deficiencies have deleterious effects on multiple health outcomes for example the immune system. Thus, of particular concern are the adverse health effects of toxins in individuals with simultaneous multiple micronutrient deficiencies. It is important to note that, food availability alone will not benefit many of those who are at nutritional risk, if there are no corresponding improvements in the nutritional quality and safety of food.

Therefore, it is important to assess potential health consequences of consuming foods in individuals of different nutritional statuses, in particular in individuals who are undernourished. This will enable the development of effective and efficient methods for assessing the nutritional value and safety of foods introduced in developing countries with significant proportions of undernourished populations. This will enhance the efficacy of efforts to increase food availability and its optimal utilization.

7.2.2. Nutrient imbalances: bio-fortification and genetically modified foods with enhanced nutrient levels

The ability to change the overall nutrient profile of a plant product has the potential to improve the nutritional status of individuals. However, in addition to the intended nutrient changes, genetic modification could result in deleterious alterations in the nutrient profiles of the product and thus result in adverse health effects. It is not clear what the nutritional outcome will be, when a single nutrient is added in significant quantities when several nutrients are lacking in diets, for example, malabsorption of other essential nutrients. Iron supplements can interfere with the absorption of zinc and zinc in high doses, with absorption of iron or copper (*80*). Therefore, when enhancing the nutritional quality of crops, it is necessary to determine whether the enhancement will not result in nutrient imbalances.

7.3. Potential of DNA microarrays in food safety assessments

DNA microarrays allow the quantitative simultaneous comparison of the expression of thousands of individual genes in different biological samples. This may facilitate screening, assessment and/or prediction of putative harmful interactions following exposure to mixtures of substances or whole foods. Changes in gene expression may provide more sensitive, immediate, and comprehensive markers of toxicity than conventional toxicological methods and endpoints (*11*). In addition, DNA microarray technology enables detailed analysis of mechanism of toxicity, without the need of *a priori* knowledge on the mode of action, while conventional toxicological methods such as histopathology, clinical chemistry and hematology are usually applied when a preconceived notion exists on the possible mode action of the compound. The value of DNA microarray technology lies in being able to provide

complimentary molecular insight when employed in addition to conventional toxicology tests for food safety as part of a more comprehensive battery of tests.

7.3.1. Effects of whole foods (mixture) and food components:

The classical toxicological approach involves the application of high doses of a single food component to predict effects of exposures to mixtures of compounds, which may not be relevant in real life situations. The genomics approach, has the potential to be used to determine the safety of food components in the context of the diet, at relevant doses of intake and as they occur in the body (*81*). Mechanisms of the combinatorial effects of the different food components can be studied and possibly predicted at the molecular level.

7.3.2. Identification of (early and sensitive) toxicity biomarkers

Subtle changes in gene expression are likely to precede the conventional symptoms of toxicity. Thus, identification of a signature gene expression pattern that changes in a characteristic and reproducible manner can facilitate the identification of toxicity biomarkers (*82*). A gene expression profile as a whole may be used as a biomarker, whereby the patterns of global changes in expression are compared under different conditions. For instance, gene expression profiles have been successfully applied to discriminate samples exposed to different classes of toxicants (*83, 84*). Identification of biomarkers can also lead further to the development of dedicated diagnostic arrays or reporter gene systems for safety assessments. However, it is important to note that in whole foods/mixtures, bioactive food components act simultaneously in constantly changing combinations resulting in different gene expression profiles, which may make the quest to find specific biomarkers a huge challenge.

7.3.3. Studying mechanisms of action

Changes in gene expression play an important role in signal transduction, metabolic pathways and protective responses in cells. The mechanisms leading to overt symptoms of toxicity/effect are recognized through observations of changes in gene or protein expression. DNA microarray technology not only can help to discover novel biomarker genes but also can lead to the exploration of an unknown function of a food component. It can help to define the molecular mechanism(s) by which food components effect their modes of action. Many examples of the use of DNA microarrays for mechanistic toxicology have been described (for recent reviews see (*85, 86*))

7.4. Limitations of DNA microarray studies

Although the application of DNA microaaray technology is highly promising, there are issues that need to be resolved to facilitate the further development of this area. These include the standardization of experimental procedures and systemization of interpretation of results, among others. In addition, DNA microarrays look at just one level at which genes are regulated i.e. transcriptional regulation. Other critical mechanisms that include post-transcriptional effects (RNA stabilization), protein translation and post-translational modifications for example, phosphorylations are not analyzed for. These can be just as profound as mRNA transcription but are not examined by DNA microarrays (*87, 88*). There is a need to apply a systems biology approach by looking at interactions at the gene, protein and metabolome levels (*89*).

The development of a risk profile or identification of early biomarkers of toxicity may be difficult. Clearly different time points, different doses and exposure duration and cell phenotype may give different gene expression profiles. However, selecting the optimal dose and time point at which initial primary responses to a food

component toxicity is induced is essential (*88*), (*90*). Insight into the relationship between genomics-based endpoints and known health endpoints is essential for accurate hazard characterization. Significant changes in gene expression cannot be concluded to represent adverse effects (or a small change to represent its absence) until results are placed in an appropriate biological context (*90-92*). Another challenge is determining whether changes in gene expression at low concentrations are predictive of a pathological outcome or are adaptive effects that are unrelated to the development of the pathologies. Further studies regarding correlations between the changes as indicated by microarray experiments and eventual adverse toxicological effects will provide important information on the establishment of threshold levels below which no adverse effects can be expected. Thus reducing uncertainties in current risk assessments related to high dose to more realistic low dose extrapolations.

B. Objectives and outline of dissertation

The toxicological effects of glycoalkaloids have been well described in humans; however, the molecular mechanisms underlying their toxicity are not fully understood. The dissertation's specific objectives are two. First, to evaluate the usefulness of DNA microarrays (together with in vitro cultures) for screening for (differences in) potential toxicities of individual glycoalkaloids (α-chaconine and α-solanine) and of varying mixtures of the two glycoalkaloids that were tested and second, to gain insights into the molecular mechanism(s) of action of glycoalkaloids in the human intestinal epithelial Caco-2 cell line.

Chapter 2 examines the effect of α-chaconine on gene expression and biological pathways in differentiated Caco-2 cells. This study was an initial step in determining the mechanism of action of glycoalkaloids on their main target of action, i.e. the small intestine.

Chapter 3 evaluates the utility of DNA microarrays to discriminate the severity of effect and potential toxicities of various levels of individual glycoalkaloids and various α-chaconine/α-solanine mixtures. In addition, we examine the transcriptional effects of the different glycoalkaloid treatments in Caco-2 cells.

DNA microarrays serve as useful tools for hypothesis generation and determination of possible mechanisms of action, thus Chapter 4 investigates/elaborates further on the genomics-based hypothesis proposed in Chapter 2. The main objective was to determine via biochemical experiments whether the MAPK and PI3K/AKT signaling pathways are involved in the α-chaconine-induced transcription of cholesterol biosynthesis, as these pathways were found to be affected on basis of the gene expression profiles described in chapter 2.

Chapter 5, comprises a summary of the results and a general discussion.

C. Summary and Conclusions

The utilization of transcriptomics technology has now become an impetus for promoting function and safety analyses of food. There is increasing research employing this technology to analyze the effects of food components; nutrient and non-nutrients at both the cellular and organism levels. Applying a holistic approach, in order to understand the interactions at the protein, gene and metabolome levels is essential so as to obtain understanding of the mechanism(s) of action.

Overall, this dissertation research was designed to enhance understanding of the utility of employing DNA microarray technology in food safety assessments. Studies have shown that microarrays can discriminate different classes of toxicants, and in contrast we have sought to elucidate whether they could be used to discriminate similar classes of toxicants. This is particularly relevant, especially in assessing safety or equivalence of foods with subtle changes in levels or ratios of similar

toxicants/nutrients, for example, genetically modified foods. In this study we focused on non-nutrients, glycoalkaloids present in potatoes, whose underlying mechanisms of action are poorly understood. We anticipated that by employing DNA microarrays, we would be able to unravel some of the mechanisms of action.

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CHAPTER 2

Induction of the cholesterol biosynthesis pathway in differentiated Caco-2 cells by the potato glycoalkaloid α-chaconine[3]

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Abstract

Glycoalkaloids are naturally occurring toxins in potatoes, which at high levels may induce toxic effects in humans, mainly on the gastrointestinal tract by cell membrane disruption. In order to better understand the molecular mechanisms underlying glycoalkaloid toxicity, we examined the effects of α-chaconine on gene expression in the Caco-2 intestinal epithelial cell line using DNA microarrays. Caco-2 cells were exposed for 6 hours to 10μM α-chaconine in three independent experiments (randomized block design). The most prominent finding from our gene expression and pathway analyses was the upregulation of expression of several genes involved in cholesterol biosynthesis. This to some extent is in line with the literature-described mechanism of cell membrane disruption by glycoalkaloids. In addition, various growth factor signaling pathways were found to be significantly upregulated. This study is useful in understanding the mechanism(s) of α-chaconine toxicity, which may be extended to other potato glycoalkaloids more generally.

Keywords: α-chaconine; cell membrane disruption; cholesterol biosynthesis; DNA microarrays; glycoalkaloids; potatoes

1. Introduction

Potatoes (*Solanum tuberosum* L.) contain naturally occurring toxins, α-chaconine and α-solanine, which account for 95% of the total glycoalkaloids (*1*). These glycoalkaloids are thought to be partly responsible for the natural defense against diseases and predation that affect these crops. At high levels these glycoalkaloids may have toxic effects on human health. α-Chaconine and α-solanine are present in potatoes at varying concentrations and in certain ratios they act synergistically resulting in increased toxicity at lower concentrations compared to the individual glycoalkaloids (*2, 3*).

Glycoalkaloid toxicity symptoms at lower concentrations are mainly gastrointestinal and include vomiting, diarrhea and abdominal pain (*4*). Experimental cell culture and animal studies have shown that glycoalkaloids may affect intestinal permeability adversely (*1*). At higher concentrations humans experience severe symptoms such as fever, rapid pulse, low blood pressure, rapid respiration and neurological disorders (*5*). These toxicities are an outcome of the glycoalkaloids' anticholinesterase effects on the central nervous system and disruption of cell membranes. α-Chaconine and α-solanine inhibit the enzyme acetylcholinesterase, which is responsible for the hydrolysis of the neurotransmitter acetylcholine, a key process in regulating nerve impulse conduction across cholinergic synapses (*6*).

Toxicities at levels found in some potato varieties observed in other species include craniofacial malformations in hamsters (*7*) and multi-organ malformations in frog embryos (*2, 8*). α-Chaconine has been shown to induce hepatotoxicity in mice (*9*) and to increase hepatic ornithine decarboxylase activity in rats (*10*).

Studies have shown that α-chaconine and α-solanine cause membrane disruption in lipid vesicles, membranes of erythrocytes, and various human and mouse cell lines (*3, 11-13*). Destabilizing complexes formed between the lipophilic moiety of

glycoalkaloids and membrane bound cholesterol result in membrane disruption (*11, 12, 14*). This action likely accounts for damage to cells lining the gastrointestinal tract and other tissues or organs in which these compounds are transported following absorption.

Despite these and other well documented observations, the molecular mechanisms underlying the effect of glycoalkaloids remain unclear. Transcriptomics and other "omics" technologies are used increasingly in mechanistic toxicological studies. DNA microarrays are transcriptomics tools that offer the opportunity to monitor changes in expression of many genes simultaneously upon exposure of cells to toxic compounds. In particular, when used in concert with established investigative techniques as part of a holistic approach to toxicology, the use of microarrays may contribute significantly to a better understanding of mechanisms of action and the identification of early and sensitive biomarkers of toxicity (*15*).

The main objective of this study was to gain insights of the molecular mechanisms that underlie α-chaconine action. This may result in the identification of sensitive and early biomarkers of α-chaconine toxicity. For that purpose we examined the effect of α-chaconine on gene expression of the human colon carcinoma cell line Caco-2 using DNA microarrays. The Caco-2 cell line was chosen as it is used widely as an *in vitro* model system for the intestinal epithelium, which is one of the first targets of glycoalkaloid toxicity. Although the focus of this work was on α-chaconine toxicity, the outcome of this study may be extended to the mechanism of toxicity of glycoalkaloids more generally.

2. Methods and Materials

2.1. Cell culturing

Human intestinal epithelial Caco-2 cells (ATCC, Manassas, VA) were grown routinely in 75-cm^2 culture flasks at 37°C in air with 5% CO_2 and 100% relative humidity in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Verviers, Belguim) supplemented with $NaHCO_3$ (3.7 g/l, Sigma), non-essential amino acids (1x NEAA; ICN, Zoetermeer, The Netherlands), fetal calf serum (FCS; 10% v/v; Invitrogen, Breda, The Netherlands), penicillin (5,000 U, Sigma), and streptomycin (5 mg/l, Sigma).

2.2. Biochemicals

α-Chaconine was obtained from Sigma-Aldrich (St Louis, MO, USA). A stock solution of α-chaconine was prepared in dimethyl formamide (DMF) (Merck, Germany). The stock solution was diluted with DMEM to the final desired concentrations immediately before use. In every experiment, cells in the control group were treated with an equivalent concentration of DMF (0.005%).

2.3. Lactate dehydrogenase assay

To assess the cytotoxic properties of α-chaconine, a lactate dehydrogenase (LDH) assay was performed. This assay detects the leakage of LDH from impaired cell membranes, which can be used as a measure of cytotoxcity. Caco-2 cells were seeded in 24 well plates (Costar) and grown for 19 days allowing the cells to differentiate. Subsequently, the differentiated cells were exposed for either 6 or 24 hours to 5, 10, 15 and 20μM α-chaconine (n=4). LDH activity was determined using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Benelux b.v., The Netherlands) in accordance with manufacturer instructions.

2.4. Experimental design of the gene expression profiling studies

Caco-2 cells were seeded at a density of 40 000 per cm^2 in 6 well polyester transwell plates (Costar; 0.4 μm pore size, inserts of 24 mm diameter). These were allowed to differentiate by growing the cells for 19 days. Upon differentiation, three types of experiments were performed in which the medium of the upper compartment was replaced with either DMEM containing 0.005% DMF (control exposure) or α-chaconine and the medium of lower compartment was replaced by DMEM only. First, time series experiments were carried out in which differentiated Caco-2 cells (passage 37) were exposed to 10μM α-chaconine (n=4) for either 2, 4, 6 or 24 hours. In a second experiment, the concentration-response relationship was studied by exposing the differentiated Caco-2 cells (passage 44) to 5, 10 and 20μM α-chaconine for 6 hours (n=4). Finally, a randomized block design experiment was performed whereby differentiated Caco-2 cells were grown and exposed to 10μM of α-chaconine for 6 hours (n=4) in three independent experiments (passage numbers 44, 38, 40 respectively). After exposure, medium was removed and both compartments were washed twice with ice-cold phosphate buffered saline. Subsequently, cells in the upper compartment were resuspended in 1ml TriZol (Invitrogen, Breda, The Netherlands) and stored at -80°C until RNA extraction.

2.5. Microarray hybridizations

Total RNA from Caco-2 cell lines was isolated using TriZol reagent according to the manufacturer's directions. RNA clean-up was performed using the RNeasy mini kit (Qiagen, Westburg bv, Leusden, The Netherlands). RNA integrity and purity were verified by gel electrophoresis and UV spectrometry. RNA concentrations were determined by measuring absorbance at 260 and 280nm and purity was estimated by 260/280 nm absorbance ratio. Although each of the exposures were performed in

quadruplicate (n=4), it was decided to pool the four RNAs for hybridization, since pilot microarray experiments with the four individual RNAs within one exposure resulted in a high level of reproducibility (results not shown).

Prior to hybridization to 22K 60-mer oligonucleotide Agilent Human 1A Oligo microarrays V2 (Agilent Technologies, Palo Alto, CA, USA), RNA was amplified using the Agilent low RNA input fluorescent amplification kit protocol (fluorescent cRNA synthesis procedure). 1μg of each of the linearly amplified cRNA preparations (control and α-chaconine treated cells) was labeled by incorporation of Cy5-CTP (PerkinElmer/NEN Life Sciences, Boston, MA, USA). RNAs from control exposures also were used as reference cRNA probes (1μg) and labeled with fluorescent Cy3 dye (Perkin-Elmer/NEN). Each Cy5-labeled experimental cRNA probe was combined with the Cy3-labeled reference probe and hybridized on the 22K microarray following the Agilent 60-mer oligo microarray processing protocol. The microarrays were hybridized for 17 hours at 60˚C in Agilent microarray hybridization chambers (G2534A). Upon hybridization, the microarrays were washed and dried at room temperature following Agilent's instructions.

Arrays were scanned using a Scanner Array Express HT microarray scanner (PerkinElmer Life Sciences, Boston, MA, USA). The fluorescent readings from the scanner were converted to quantitative files using Array Vision Software (Imaging Research, Ontario, Canada). Quality check of the arrays was done using macros in Microsoft Excel 2000 (Microsoft Corporation, USA) and software package LimmaGUI in R version 2.3.1 (http://bioinf.wehi.edu.au/limmaGUI/index.html). Single spots or areas on the array with obvious blemishes were flagged. The non-flagged fluorescence signals quantified using Array Vision software were exported to GeneMaths XT software (Applied Maths, St Martens-Latem, Belgium) for further

analyses. Array elements for which the fluorescent intensity in each channel was less than 1.5 times the background were excluded from subsequent analyses.

2.6. Data Analysis

As a first step in the microarray data analyses, data were log transformed and normalized as described by Pellis (*16*). In short, first, the Cy5 values were corrected using values of the Cy3-labeled internal standard to correct for possible differences in hybridization conditions between slides. Subsequently, the median of the adjusted Cy5 signals was used to correct for possible differences between experiments with respect to the efficiency of probe labeling and amount of probe labeled.

2.7. Statistical Analysis

Differentially expressed genes were identified by using both an unpaired student's t-test with p-value ≤ 0 .01 and a fold change criterion of $\geq$1.5. For the time series and concentration effect experiments, only fold change criteria $\geq$1.5 were used since RNA was pooled for each treatment group in those experiments.

Principal Component Analysis (PCA) and clustering analyses were performed using GeneMaths XT software. Pearson correlation and Unweighted Pair Group Method with Arithmetic Means were used to determine the clustering of experimental groups.

An online software suite MetaCore™ Version 3.2.1 (GeneGo Inc., St. Joseph, MI, USA) was used to identify statistically significant pathways responding to α-chaconine treatment in the Caco-2 cell lines. For this purpose, only the data of genes found to be significantly differentially expressed ($p \leq 0.01$ and fold change ≥ 1.5) were imported into the MetaCore program. For the time series and concentration response experiments only genes with a fold change of ≥ 1.5 (either up or down) were imported

into the MetaCore program. MetaCore analyses result in lists of maps ranked according to the most significantly affected map (lowest p-values). The p-values are calculated by the program as a hypergeometric distribution whereby the p-value represents the probability of particular mapping arising by chance, given the numbers of genes in the set of all genes on maps/networks/processes, genes on a particular map/network/process and genes in the experiment (*17*).

3. Results

3.1. LDH leakage from the cell

First, in view of the presumed disruptive effects of glycoalkloids on the cell membrane and to define appropriate concentrations of α-chaconine for gene expression profiling studies, membrane integrity was evaluated by measuring leakage of the cytosolic enzyme lactate dehydrogenase (LDH) from the cells into the medium. Exposure of differentiated Caco-2 cells to increasing concentrations of α-chaconine for 6 or 24 hours resulted in concentration-dependent LDH leakage at both time points (Figure 2-1).

At both 6 and 24 hours of exposure, 20μM α-chaconine resulted in LDH leakage of more than 20%, which is frequently used as a lower level of cytotoxicity. Furthermore, microscopic examination of the monolayer revealed that irrespective of exposure time, some cells were detached from the well surfaces and appeared dead at 20μM α-chaconine only (results not shown). Thus, these results suggest that α-chaconine is cytotoxic at 20μM but not at the lower concentrations.

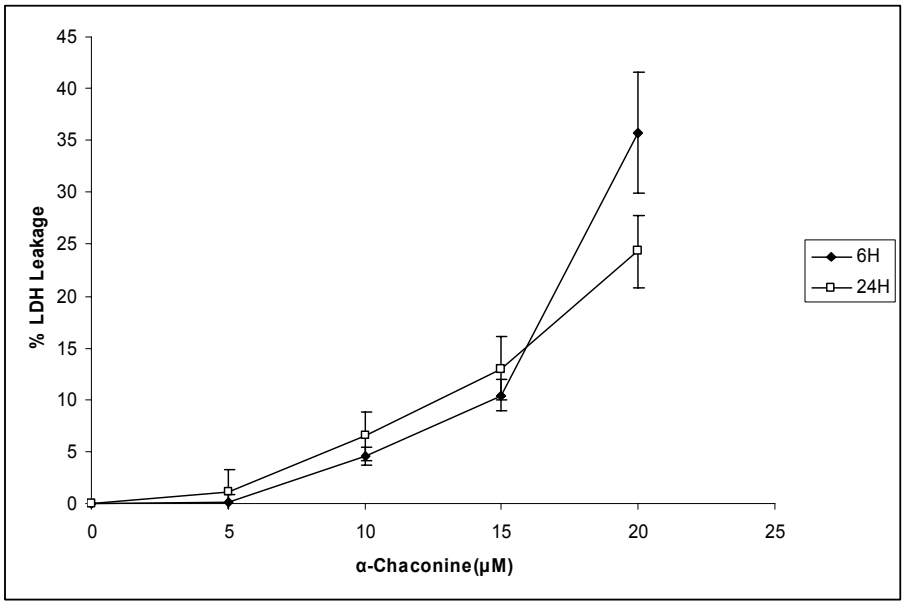


Figure 2-1. Concentration dependency of α-chaconine induced LDH leakage from differentiated Caco-2 cells. Each point represents the average of 4 replicates with a standard deviation of less than 5%.

3.2. Gene expression studies: time series and concentration response

In order to determine the optimal exposure time for further gene expression studies, differentiated Caco-2 cells were exposed for 2, 4, 6, and 24 hours to 10μM α-chaconine. In the LDH assay experiment this was the lowest concentration at which the membrane disruptive effect of α-chaconine was observed both after 6 and 24 hours exposure. Upon hybridization of the RNAs to 22K human oligomicroarrays, the number of differentially expressed genes was determined. Data analyses revealed that the number of genes differentially expressed (fold change $\geq$ 1.5) after 6 hours of exposure (1597 genes) was approximately twice the number obtained after 2 hours (857), 4 hours (815) and 24 hours (855) of exposure.

Subsequently, to analyze the effect of increasing concentrations of α-chaconine on gene expression, differentiated Caco-2 cells were exposed for 6 hours to 5, 10 and

20μM α-chaconine. The hybridization data were subjected to univariate analysis, PCA, hierarchical clustering and pathway analysis.

Univariate analysis of the data showed that the number of genes differentially expressed (fold change ≥ 1.5) at 5, 10 and 20μM α-chaconine were 748, 1062 and 1117 respectively. The Venn diagram below illustrates the overlap of differentially expressed genes among the treatments (Figure 2-2C).

In general, PCA and hierarchical clustering analyses revealed that the gene expression profiles of Caco-2 cells exposed to 5 and 10μM α-chaconine were very similar (Figure 2-2A and B). There is distinctive separation of control cells from treated cells. The PCA results indicate that 92.6% of the variation in the data was explained by the first two components(X: 73.1%; Y: 19.5%). Closer analysis of the components revealed that the gene expression profiles of 10 and 20μM α-chaconine are similar in the X component (component explaining most of the variation in the gene expression data). From the Venn diagram (Figure 2-2C), it is evident that there is a lot of overlap between genes differentially expressed at 10μM α-chaconine and at 5 or 20μM α-chaconine. Thus to some extent cells exposed to 10μM α-chaconine exhibit a similar gene expression profile to cells exposed to either 5 or 20μM.

All genes differentially regulated 1.5-fold or more by either of the α-chaconine concentrations were analyzed using MetaCore. At 5 and 10μM, the most significantly differentially regulated pathways were cholesterol biosynthesis followed by transcription regulation of amino acid metabolism, GTPase-mediated cell signaling and cell adhesion. The most significantly affected pathways at 20μM α-chaconine were mainly pathways involving apoptosis regulation and mitogen-activated protein kinase (MAPK) cascades (Table 2-1). The cholesterol biosynthesis pathway also was regulated significantly at 20μM but not to the same extent as at 5 and 10μM α-chaconine.

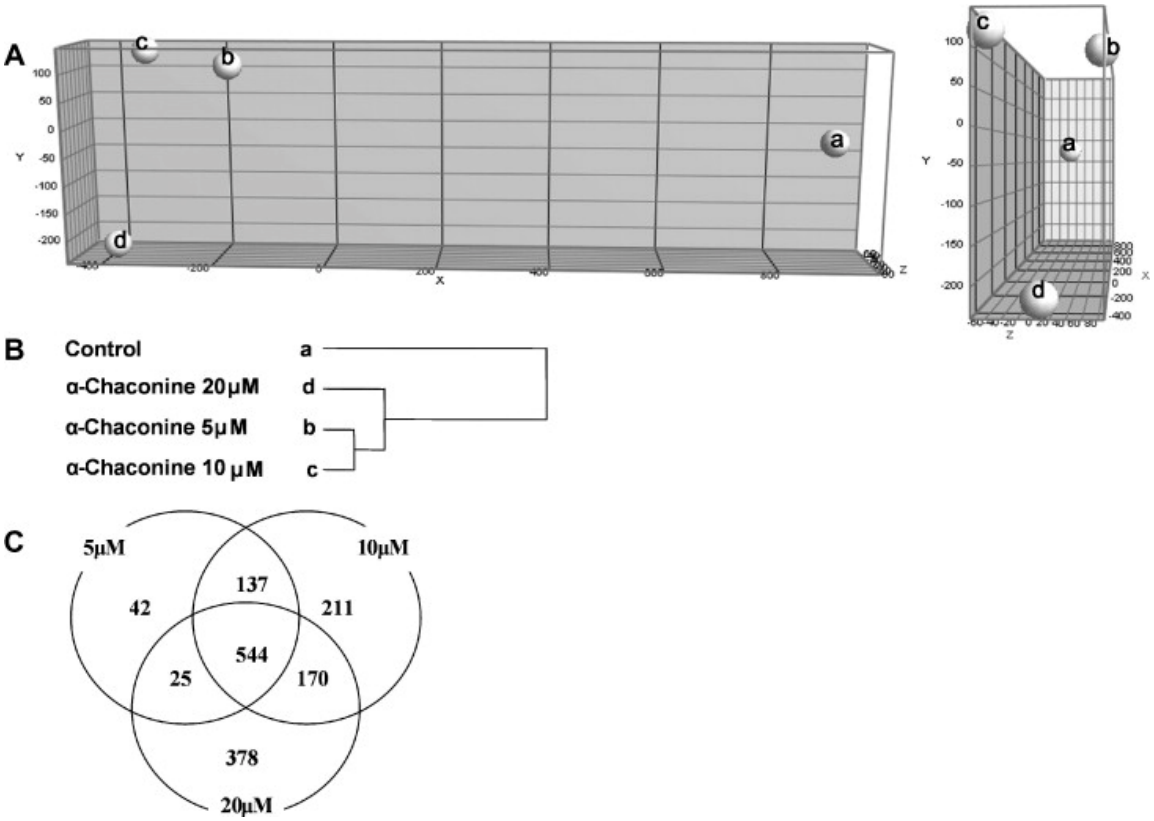


Figure 2-2. (A) PCA mapping and (B) hierarchical clustering of the three different concentrations. a, control cells; b, cells exposed to 5μM; c, cells exposed to 10μM and e, cells exposed to 20μM α-chaconine. (C) Venn diagram showing overlap of differentially regulated genes (fold change ≥ 1.5) across the treatments.

3.3. Reproducibility study with 10μM α-chaconine (3 independent experiments)

To examine the reproducibility of the results, three independent experiments were performed in which different batches of Caco-2 cells were grown and exposed to 10μM α-chaconine for 6 hours. These experimental conditions were chosen based on the outcome of the time series and concentration effect experiments.

Table 2-1. Top 10 pathways differentially regulated following exposure of Caco-2 cells to 5, 10 and 20μM α-chaconine

#	α-Chaconine 5μM	α-Chaconine 10μM	α-Chaconine 20μM
1.	Cholesterol biosynthesis [1(11/21),1(11/21),19(6/21)]	Cholesterol biosynthesis [1(11/21),1(11/21),19(6/21)]	TRAF proteins signaling network [166*(2/35),166*(3/35),1(11/35)]
2.	Transcription regulation of amino acid metabolism [2(9/42),2(12/42),3(11/42)]	Transcription regulation of amino acid metabolism [2(9/42),2(12/42),3(11/42)]	AP1 activation by TRAF protein signaling pathway [77*(3/29),46*(5/29),2(10/29)]
3.	Regulation of actin cytoskeleton by Rho GTPases [3(11/61),7(11/61),171*(5/61)]	EGFR signaling via small GTPases [6(7/39),3(11/39),5(10/39)]	Transcription regulation of amino acid metabolism [2(9/42),2(12/42),3(11/42)]
4.	Receptor mediated axon growth repulsion [4(11/77),4(14/77),175*(6/77)]	Receptor-mediated axon growth repulsion [4(11/77),4(14/77),175*(6/77)]	AP1 activation by MADD protein and c-FLIP/c-RAF-1 related pathway [61*(3/25),68*(4/25),4(8/25)]
5.	Reverse signaling by ephrin B [5(11/84),19(12/84),67(9/84)]	MIF in innate immunity response [11(8/57),5(11/57),8(12/57)]	EGFR signaling via small GTPases [6(7/39),3(11/39),5(10/39)]
6.	EGFR signaling via small GTPases [6(7/39),3(11/39),5(10/39)]	Cytoskeleton remodeling [15(16/178),6(22/178),166*(13/178)]	TRADD interaction with MAPK cascade [155*(2/32),106*(4/32),6(9/32)]
7.	Fibronectin-binding integrins in cell motility [7(8/52),8(10/52),195*(4/52)]	Regulation of actin cytoskeleton by Rho GTPases [3(11/61),7(11/61),171*(5/61)]	Anti-apoptotic function of TRADD/TRAF2 complex [156*(2/32),57*(5/32),7(9/32)]
8.	Integrin outside-in signaling [8(10/79),14(12/79),126(7/79)]	Fibronectin-binding integrins in cell motility [7(8/52),8(10/52),195*(4/52)]	MIF in innate immunity response [11(8/57),5(11/57),8(12/57)]
9.	Integrin mediated cell adhesion [9(11/94),23(12/94),181*(7/94)]	Ras family GTPases in kinase cascades (scheme) [22(5/28),9(7/28),10(8/28)]	MAPK cascade, Part IV, Map 3. Nuclear function of p38-MAPK [17(7/49),12(9/49),9(11/49)]
10.	Spindle assembly and chromosome separation [10(10/83),18(12/83),199*(6/83)]	EGF signaling pathway [21(8/64),10(11/64),20(11/64)]	Ras family GTPases in kinase cascades (scheme) [22(5/28),9(7/28),10(8/28)]

*[5μM, 10μM, 20μM] = order of treatments, [R (n/N),...,...], where R = ranking of the pathway, n = number of regulated genes in the pathway, N = total number of genes in the pathway, *represents pathways which are not significant with p > 0.01*

Using a fold change criterion of ≥ 1.5 and a p-value ≤ 0.01, 446 genes were found to be significantly differentially expressed. Of these 446 differentially expressed genes, 363 genes were upregulated and 83 genes were downregulated. MetaCore analysis of these differentially expressed genes revealed that the pathway affected most significantly by 10μM α-chaconine was cholesterol biosynthesis (Table 2-2), with 9 out of the 21 genes in this pathway being differentially expressed (i.e. upregulated).

Table 2-2. Top 10 pathways differentially regulated by α-chaconine 10μM after 6 hours

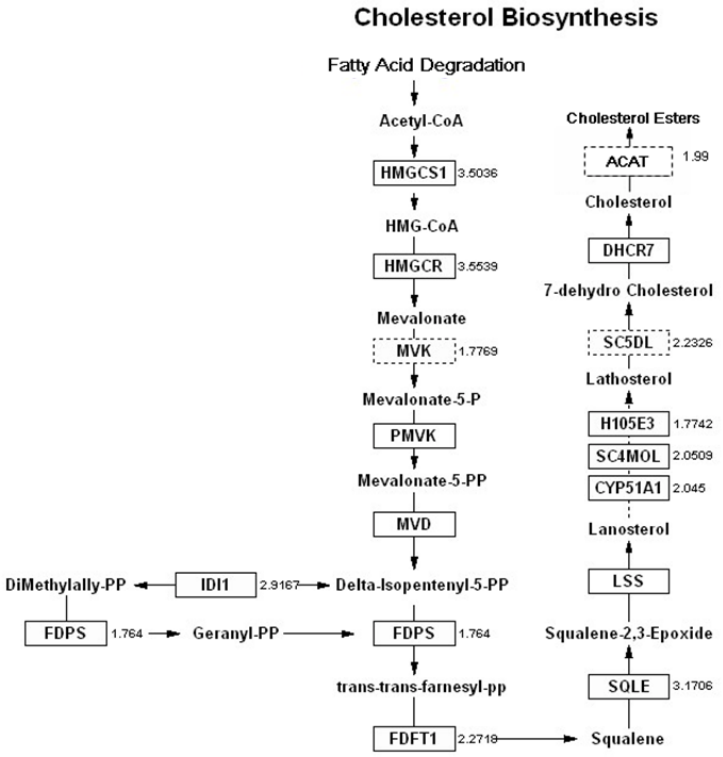
#	Map name	p-value	Expressed	All
1.	Cholesterol biosynthesis	8.439^{e-10}	9	21
2.	Transcription regulation of amino acid metabolism	8.509^{e-06}	8	42
3.	EGF signaling pathway	3.045^{e-05}	9	64
4.	EGFR signaling via small GTPases	4.737^{e-05}	7	39
5.	VEGF signaling via VEGFR2-generic cascades	9.102^{e-05}	7	43
6.	IGF-RI signaling	4.478^{e-04}	8	72
7.	MIF in innate immunity	5.522^{e-04}	7	57
8.	AKT signaling	5.522^{e-04}	7	57
9.	Erk Interactions: Activation and selected Erk targets	5.522^{e-04}	7	57
10.	HGF signaling pathway	6.906^{e-04}	6	43

Upregulation of the cholesterol biosynthesis pathway by 10μM α-chaconine in Caco-2 cells is consistent with the results obtained in our concentration response pilot study. Most of the genes in this pathway (12 out of 21) responded to α-chaconine treatment when the selection criteria were set at $p \leq 0.05$ and fold change ≥ 1.5 (Figure 2-3). Among the genes differentially expressed is 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) a known rate limiting enzyme in cholesterol

biosynthesis. Also significantly upregulated is the low density lipoprotein receptor (LDLR) gene (p-value 0.00046, fold change 3.66), which together with HMGCR, play a central role in maintaining cholesterol homeostasis in the cell. A cholesterol biosynthesis pathway map indicating the genes differentially expressed in the α-chaconine exposed Caco-2 cells is shown in Figure 2-3A. Figure 2-3B shows the corresponding p-values and fold changes of all genes involved in this pathway.

Other pathways responding to α-chaconine treatment, mainly by upregulation of gene expression, were related to the transcription regulation of amino acid metabolism, epidermal growth factor (EGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), insulin like growth factor receptor I (IGF-RI), and AKT signaling pathways, Erk (extracellular signal related protein kinase) activation, and macrophage migration inhibitor factor (MIF) in innate immunity (Table 2-2). Table 2-3 lists all the significant genes in these pathways (excluding those involved in cholesterol biosynthesis). These pathways induce similar signal transduction cascades hence there is overlap of the genes involved in these pathways (Table 2-3).

A



B

Gene Symbol	GenBank ID	Description	Fold Change	p value
CYP51A1	NM_000786	Cytochrome P450, family 51, subfamily A, polypeptide 1	2.05	0.000366
HMGCR	NM_000859	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	3.55	0.000649
SC4MOL	NM_006745	Sterol-C4-methyl oxidase	2.05	0.000656
SQLE	NM_003129	Squalene epoxidase	3.17	0.000822
FDFT1	NM_004462	Farnesyl-diphosphate farnesyltransferase 1	2.27	0.001041
SC5DL	BC012333	Sterol-C5-desaturase (ERG3 delta-5-desaturase homolog)	2.57	0.001183
HMGCS1	NM_002130	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	3.50	0.0013
FDPS	NM_002004	Farnesyl diphosphate synthase	1.76	0.002102
MVK	NM_000431	Mevalonate kinase	1.73	0.002936
SC5DL	NM_006918	Sterol-C5-desaturase (ERG3 delta-5-desaturase homolog)	2.23	0.008359
NSDHL/H105E3	NM_015922	NAD(P) dependent steroid dehydrogenase-like	1.77	0.014452
ACAT2	BC000408	Acetyl-Coenzyme A acetyltransferase 2	1.99	0.022043
MVK	X75311	Hepatitis B virus fusion mRNA for mevalonate kinase	1.78	0.032263
HMGCS1	NM_002130	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	1.86	0.056928
DHCR7	NM_001360	7-dehydrocholesterol reductase	1.55	0.076107
MVD	NM_002461	Mevalonate (diphospho) decarboxylase	1.51	0.084052
LSS	NM_001001438	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	1.20	0.155521
PMVK	NM_006556	Phosphomevalonate kinase	0.85	0.18313
EBP	NM_006579	Emopamil binding protein (sterol isomerase)	1.12	0.504465
ACAT1	NM_000019	Acetyl-Coenzyme A acetyltransferase 1	1.02	0.569719
DHCR24	NM_014762	24-dehydrocholesterol reductase	0.96	0.862203

Figure 2-3. Induction of cholesterol biosynthesis by α-chaconine 10μM in Caco-2 cells. (A) Cholesterol biosynthesis pathway map modified from GenMAPP (http://www.genmapp.org). Genes with cut off fold change ≥ 1.5 and p≤0.05 are indicated. Note dashed box indicates presence of more than one transcript of a particular gene on the microarray. (B) Corresponding p-values and fold changes of all the cholesterol biosynthesis genes.

Table 2-3. Differentially regulated genes (fold change $\geq$ 1.5 and p $\leq$ 0.01) involved in EGF, HGF, VEGF, EGFR via small GTPases, Akt, and IGF-RI signaling, Erk activation, transcription regulation of amino acid metabolism and MIF in innate immunity.

Gene Symbol	GenBank ID	Description	Fold Change	p-value	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
MAFG	NM_032711	V-maf musculoaponeurotic fibrosarcoma oncogene homolog G	2.32	0.001431									x
MAFG	NM_002359	V-maf musculoaponeurotic fibrosarcoma oncogene homolog G	2.07	0.000059									x
MAFK	AK056767	cDNA FLJ32205 fis	1.75	0.009315									x
JUN	NM_002228	V-jun sarcoma virus 17 oncogene homolog	4.84	0.000078	x		x	x		x	x		x
JUND	NM_005354	Jun D proto-oncogene	1.73	0.004551				x		x	x		x
JUNB	NM_002229	Jun B proto-oncogene	2.90	0.000434				x		x	x		x
THC2024073	THC2024073	FRA1_HUMAN (P15407) Fos-related antigen 1	2.15	0.003508	x	x	x	x		x	x		x
FOS	NM_005252	V-fos FBJ murine osteosarcoma viral oncogene homolog	3.54	0.000732	x	x	x	x		x	x		x
AREG	NM_001657	Amphiregulin (schwannoma-derived growth factor)	7.48	0.000341	x								
DUSP1	NM_004417	Dual specificity phosphatase 1	5.18	0.002166	x								
NFKBIA	NM_020529	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor	2.18	0.004109	x	x			x	x	x	x	
RAC1	NM_198829	Ras-related C3 botulinum toxin substrate 1	1.56	0.003821	x	x		x				x	
RAC1	NM_198829	Ras-related C3 botulinum toxin substrate 1	1.66	0.000758	x	x		x				x	
RELB	NM_006509	V-rel reticuloendotheliosis viral oncogene homolog B	2.01	0.002897	x	x			x	x	x	x	x
MYC	NM_002467	V-myc myelocytomatosis viral oncogene homolog (avian)	2.02	0.006219	x	x	x		x				
PIK3CA	NM_006218	Phosphoinositide-3-kinase	1.57	0.001523	x	x		x	x			x	
EPS8	NM_004447	Epidermal growth factor receptor pathway substrate 8	1.64	0.007051		x							
KRAS	NM_033360	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	2.27	0.000853		x							
RAB5A	BC018288	RAB5A	1.70	0.001393		x							
FOXO3A	NM_001455	Forkhead box O3A	1.54	0.003221					x			x	
SFN	NM_006142	Stratifin	1.55	0.001835					x				
YWHAH	NM_003405	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	1.87	0.00925					x				
YWHAZ	NM_145690	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	2.01	0.000184					x				
IPLA2(GAMMA)	NM_015723	Intracellular membrane-associated calcium-independent phospholipase A2 gamma	1.61	0.002617			x			x			
ETS2	NM_005239	V-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	3.29	0.000791			x						
EGR1	NM_001964	Early growth response 1	4.75	0.005134			x						
PPP2CB	NM_001009552	Protein phosphatase 2 (formerly 2A)	2.44	0.000571			x					x	
PPP2CB	NM_004156	Protein phosphatase 2 (formerly 2A)	2.06	0.000681			x					x	
CDKN1A	NM_000389	Cyclin-dependent kinase inhibitor 1A	2.60	0.001069								x	
NFE2L2	NM_006164	Nuclear factor (erythroid-derived 2)-like 2	2.28	0.005308									x

4. Discussion and conclusion

The effects of α-chaconine on the transcriptome of the human intestinal epithelial cell line Caco-2 were determined in a sequentially performed set of experiments. LDH leakage experiments and initial microarray experiments indicated that 20μM, and not 10μM α-chaconine was cytotoxic to the cells. The outcome of these initial experiments directed the design of a final study in which differentiated Caco-2 cells were exposed for 6 hours to 10μM α-chaconine in three independent experiments. Gene expression profiling showed that treatment of the cells with this putative non-cytotoxic concentration, α-chaconine significantly affected several metabolic and signal transduction pathways. Among these pathways, those involving growth factor receptor signaling, MIF, transcription regulation of amino acid metabolism and cholesterol biosynthesis were affected.

The most prominent finding from our microarray studies was the upregulation of several genes involved in cholesterol biosynthesis following exposure of the Caco-2 cells to α-chaconine. Cholesterol is an abundant component of the plasma in eukaryotic cells and plays an important role in maintaining membrane integrity and fluidity. The cholesterol level of cell membranes is known to be tightly regulated and changes in this level have major effects, both direct and indirect, on a wide array of biological functions (*18, 19*).

The most well-documented mechanism of glycoalkaloid toxicity is the disruption of membrane integrity as a result of the formation of destabilizing complexes between the lipophilic moiety of glycoalkaloids and cholesterol present in the membranes (*11, 12, 14*). The leakage of LDH from the cells as observed in our study may be a reflection of this loss of membrane barrier integrity.

Based upon the known membrane-disruptive properties of α-chaconine and results from the present study, the cholesterol biosynthesis pathway may be induced through

feedback regulation due to depletion of cellular cholesterol by α-chaconine. Cellular cholesterol homeostasis is maintained by endogenous cholesterol synthesis via transcriptional regulation of genes that govern the synthesis of cholesterol or by uptake of exogenous lipoproteins via receptors such as the LDL receptor (*19*). According to this mechanism, depletion of cholesterol in the membrane is sensed by sterol regulatory element binding transcription factor cleavage-activating protein (SCAP), which forms a complex with sterol regulatory element binding transcription factors (SREBPs) and escorts them from the endoplasmic reticulum to the Golgi complex. SCAP facilitates proteolytic cleavage of SREBPs into transcriptionally active segments, which enter the nucleus to activate expression of cholesterol and fatty acid biosynthesis genes (*20*). SREBPs bind to sterol-regulatory elements (SREs) found in the promoter regions of these genes (*21*). In our study, we found several cholesterol biosynthesis genes including the cholesterol biosynthesis rate limiting gene HMGCR to be differentially expressed upon exposure of Caco-2 cells to α-chaconine. Interestingly, the gene encoding the LDL receptor, which maintains both plasma and cellular cholesterol balance by mediating the catabolism of low density lipoprotein (LDL), was also found to be upregulated. The expression of the LDL receptor gene and HMGCR are known to be regulated in a coordinated manner by SREBPs (*19, 21*). Therefore, it may be hypothesized that the sequestering of membrane bound cholesterol by α-chaconine results in disturbance of cholesterol balance. Cells may attempt to counteract this insult by a SREBP-mediated increase of expression of cholesterol biosynthesis and LDLR genes in order to maintain cholesterol homeostasis.

Growth factor signaling pathways mediated by EGF, HGF, VEGF, EGFR and IGF-RI were also found to be differentially regulated by 10μM α-chaconine. These pathways are correlated with a variety of processes and functions, such as cell

survival, proliferation, and differentiation (*22*). Alteration of growth factor mediated signaling results in the induction of either the PI3K-Akt signaling pathway (*23*) or Erk/MAPK pathway (*24*). Studies have shown that activation of the PI3K/Akt pathway induces ER-to-Golgi transport of SREBP and SCAP, which results in the activation of SREBP (*25, 26*). The Erk/MAPK pathway also has been shown to regulate the transcriptional activity of SREBPs (*27, 28*). It is not clear from our present data by which mechanism the growth signaling pathways are being induced and whether induction of these pathways (via PI3K-Akt or Erk/MAPK) resulted in the upregulation of cholesterol biosynthesis genes.

It is interesting to note that these growth signaling pathways have transmembrane receptors associated with lipid raft domains/caveolae (*29-35*). Lipid rafts /caveolae are membrane platforms for signaling molecules (*36*) and it has been observed that alterations in cellular cholesterol levels can result in their disruption (*37, 38*). This can lead to dysregulation of intracellular signaling pathways and cross-talk between different receptor systems (*18*). Thus, a hypothetical mechanism of the effect of α-chaconine on growth signaling pathways could be the formation of glycoalkaloid/sterol complexes in the cell membrane and subsequent lipid raft disruption. Current work is being focused on biochemical (lipid rafts) and functional genomics, i.e. RNA interference, experiments, in order to determine the precise mechanism by which growth signaling pathways are being induced and whether modulation of these growth signaling pathways (via PI3K-Akt or Erk/MAPK) by α-chaconine results in induction of cholesterol biosynthesis genes.

Besides cholesterol biosynthesis and growth factor signaling pathways, also the MIF pathway and regulation of amino acid metabolism were found to be significantly affected by α-chaconine (Table 2-2). Among the differentially expressed genes, which these latter pathways have in common are those encoding the Jun and Fos

transcription factors (Table 2-3). MIF is a pleiotropic cytokine whose functions include mediating inflammation, immune responses, cell proliferation and differentiation (*39*). MIF and growth factor signaling pathways are known to use a common set of signal transduction cascades involving the Erk, MAPK, PI3K, and Akt protein kinases as well as Jun and Fos (*32, 34, 40-42*). With respect to the transcription regulation of amino acid metabolism not only Jun and Fos genes but also the Maf gene was found to be differentially expressed in our α-chaconine exposure experiment (Table 2-3). Fos and Jun proteins are known to accumulate in tissues in response to growth-stimulatory signals and there is evidence that Maf proteins form heterodimers with Fos and Jun which may enable them to exert transcriptional control over expression of various genes including growth-regulatory genes (*43, 44*).

Upregulation of cholesterol biosynthesis genes in Caco-2 cells was observed at the lowest concentration α-chaconine (5μM) and the shortest exposure time (2 hours; results not shown), implying that this pathway is relatively sensitive to α-chaconine treatment and is induced rapidly. Thus, the cholesterol biosynthesis genes found to be differentially expressed in the Caco-2 cells following exposure to 10μM α-chaconine, a concentration at which initial LDH leakage is observed, may serve as possible early and sensitive biomarkers of glycoalkaloid toxicity on cell membranes.

It is important to note that cholesterol biosynthesis and growth factor signaling pathways were induced less significantly in cells exposed to 20μM α-chaconine as compared to 10μM α-chaconine. The most significantly affected pathways at 20μM α-chaconine were those involved in apoptosis regulation. It has been reported that prolonged exposure of the human colon carcinoma HT-29 cell line to α-chaconine induced apoptotic death (*45*). Our LDH leakage experiments showed an increase in LDH release at 20μM α-chaconine, which may indicate that at least some of the cells at this concentration have progressed into a post-apoptotic necrotic phase. These

results together with gene expression data suggest that at low α-chaconine concentrations cells induce rescue mechanisms to counteract the cell membrane damaging effects but that at higher concentrations induce apoptotic processes that eventually lead to necrosis.

In conclusion, the present work is an example of the application of microarray technology and *in vitro* cell culture to get a better insight in the mechanism of action of food toxins and the identification of candidate biomarkers of toxicity. The most prominent finding from our study was the upregulation of cholesterol biosynthesis genes in Caco-2 cells following exposure to α-chaconine, which to some extent is in line with the literature-described mechanism of cell membrane disruption by glycoalkaloids. Cholesterol biosynthesis genes found to be differentially expressed may serve as potential early and sensitive biomarkers of α-chaconine toxicity, which may extend to potato glycoalkaloids in general. Further work is being conducted to determine the mechanism(s) by which α-chaconine induces cholesterol biosynthesis genes and modulates growth factor signaling pathways.

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CHAPTER 3

Differential gene expression in intestinal epithelial cells induced by single and mixtures of potato glycoalkaloids[4]

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Abstract

α-Chaconine and α-solanine are naturally occurring toxins. They account for 95% of the total glycoalkaloids in potatoes (Solanum tuberosum L.). At high levels, these glycoalkaloids may be toxic to humans, mainly by disrupting cell membranes of the gastrointestinal tract. Gene-profiling experiments were performed, whereby Caco-2 cells were exposed to equivalent concentrations (10 μM) of pure α-chaconine or α-solanine or glycoalkaloid mixtures of varying α-chaconine/α-solanine ratios for 6 h. In addition, lactate dehydrogenase, cell cycle, and apoptosis analyses experiments were also conducted to further elucidate the effects of glycoalkaloids. The main aims of the study were to determine the transcriptional effects of these glycoalkaloid treatments on Caco-2 cells and to investigate DNA microarray utility in conjunction with conventional toxicology in screening for potential toxicities and their severity. Gene expression and pathway analyses identified changes related to cholesterol biosynthesis, growth signaling, lipid and amino acid metabolism, mitogen-activated protein kinase (MAPK) and NF-κB cascades, cell cycle, and cell death/apoptosis. To varying extents, DNA microarrays discriminated the severity of the effect among the different glycoalkaloid treatments.

Keywords: α-chaconine; α-solanine; cell membrane disruption; DNA microarrays; glycoalkaloids; potatoes

Introduction

α-Chaconine and α-solanine account for 95% of the total glycoalkaloids present in potatoes (Solanum tuberosum L.) (*1*). These are naturally occurring toxins, which at high levels (3–6 mg/kg body weight) may have toxic effects on human health (*2*). Both consist of the aglycone solanidine but differ in the carbohydrate side chain attached to the aglycone moiety (Figure 3-1). The branched trisaccharides solatriose (α-L-rhamnopyranosyl-β-D-glucopyranosyl-β-D-galactopyranose) and chacotriose (bis-α-L-rhamnopyranosyl-β-D-glucopyranose) are the carbohydrate side chains of α-solanine and α-chaconine, respectively (Figure 3-1) (*3-5*). α-Chaconine is toxicologically more potent and is usually present at slightly higher concentrations in potatoes than α-solanine. Toxicological differences are attributed to the disparate carbohydrate side chains (*6, 7*).

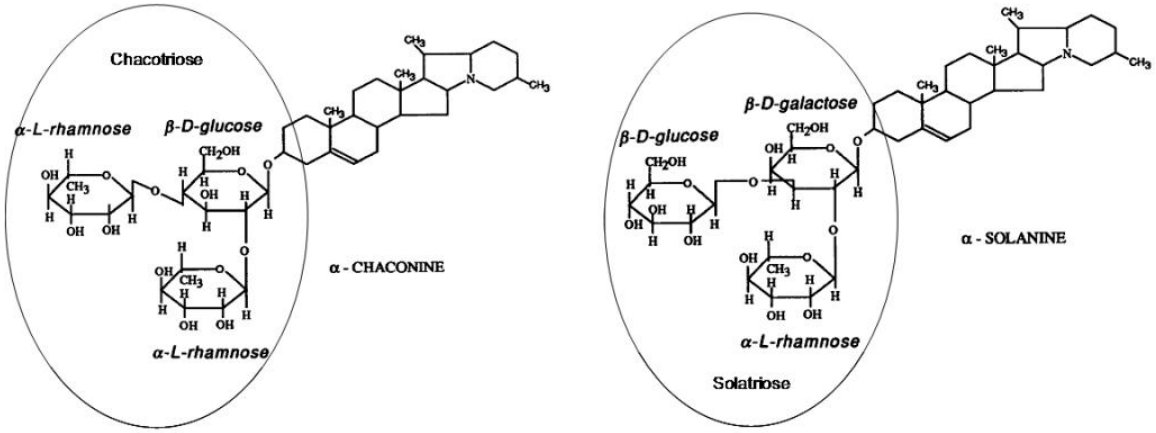

Figure 3-1. Chemical structures of α-chaconine and α-solanine

Toxicological effects of individual glycoalkaloids have been described well in humans. These include gastrointestinal disturbances, increased heart beat, hemolysis, and neurotoxic effects (*8*). Reported toxicities are mainly due to acetylcholinesterase inhibition and cell-membrane disruptive activities that affect digestive and other organs (*9*). Toxicities induced in other species include hepatoxicity in mice (*10*),

increased hepatic ornithine decarboxylase activity in rats (*11*), craniofacial malformations in hamsters (*12*), and developmental toxicity in frog embryos (*6, 13*).

Total glycoalkaloid concentrations of most commercial potatoes are usually below 200 mg/kg of fresh potatoes (*14*) but can increase following light exposure or mechanical injury, for example, from peeling and slicing (*15*). α-Chaconine and α-solanine are present in potatoes at varying concentrations and ratios. In combination, they may interact synergistically, resulting in a level of toxicity that is more severe than is observed when either glycoalkaloid is administered alone (*5, 13, 16-18*). Human consumption of potatoes results in the ingestion of α-chaconine and α-solanine in varying ratios, usually ranging from ~1.2:1 to ~2.4:1 (α-chaconine/α-solanine), depending upon the specific variety of potato (*16*). Studies have shown that incorporation of an anti-sense transgene encoding either the enzyme solanidine UDP-galactose galactosyltransferase (SGT1) or solanidine UDP-glucose glucosyltransferase (SGT2) in potatoes can result in the downregulation of glycoalkaloid biosynthesis, by reducing the levels of either α-solanine or α-chaconine, respectively (*19, 20*). Alteration of glycoalkaloid ratios/levels in potatoes may have potentially nutritional/health benefits to consumers. Therefore, from a food safety perspective, assessment of the toxic effects of glycoalkaloid mixtures at varying α-chaconine/α-solanine ratios found in common and possibly transgenic potato varieties would be informative.

Predictions of adverse health effects induced by glycoalkaloid and other toxin mixtures are usually based on data obtained from single compound exposures. Unfortunately, observed toxicities commonly deviate from such predictions. Prediction of adverse effects of whole foods is especially difficult because of the many interactions that may occur among the high number of nutrients and other food

substances commonly found in whole foods. Such interactions may alter the degree and possibly the nature of predicted toxic effects of individual food constituents (*21*).

DNA microarrays permit the quantitative simultaneous comparison of the expression of thousands of individual genes in different biological samples. This may facilitate screening, assessment, and/or prediction of putative harmful interactions following exposure to mixtures of substances or whole foods. Thus, changes in gene expression may provide more sensitive, immediate, and comprehensive markers of toxicity than conventional toxicological methods and endpoints (*22*).

The present study focused on the detection and possible significance of multiple gene responses induced by equivalent concentrations of pure α-chaconine or α-solanine or glycoalkaloid mixtures of varying α-chaconine/α-solanine ratios in human intestinal epithelial Caco-2 cells. Two α-chaconine/α-solanine ratios were chosen, 1.7:1, a ratio found in some wild-type potato varieties, and 28.8:1, a ratio that can be achieved by genetic modification using a SGT1 anti-sense construct (*20*). The diverse glycoalkaloid treatments resulted in membrane disruptive activities of varying severity as determined by the cellular leakage of lactate dehydrogenase. This outcome was used as an anchor to assess the usefulness of DNA microarrays in screening for potential toxicities and the severity of these compounds alone and as mixtures.

2. Methods and Materials

2.1. Biochemicals

α-Chaconine, α-solanine, and propidium iodide were obtained from Sigma Aldrich (St Louis, MO). Stock solutions of the glycoalkaloids and glycoalkaloid mixtures were prepared in dimethyl formamide (DMF) (Merck, Germany). The stock solutions were diluted with Dulbecco's modified Eagle's medium (DMEM) to the final desired concentrations immediately before use. In every experiment, cells in the

control group were treated with an equivalent concentration of the solvent DMF (0.0005%, v/v).

2.2. Caco-2 Cell Culture

The human intestinal epithelial cell line Caco-2 (ATCC, Manassas, VA) was grown routinely in 75 cm2 culture flasks at 37 °C in air with 5% CO2 and 100% relative humidity in DMEM (BioWhittaker, Verviers, Belgium) supplemented with NaHCO3 (3.7 g/L, Sigma), non-essential amino acids (1× NEAA; ICN, Zoetermeer, The Netherlands), fetal calf serum (FCS; 10%, v/v; Invitrogen, Breda, The Netherlands), penicillin (5000 units, Sigma), and streptomycin (5 mg/L, Sigma).

2.3. Lactate Dehydrogenase (LDH) Assay

A LDH assay was performed to assess the cytotoxic properties of α-chaconine, α-solanine, and two different glycoalkaloid mixtures. This assay detects the leakage of LDH from impaired cell membranes, which can be used as a measure of cytotoxcity. Caco-2 cells were seeded in 24-well plates (Costar) and grown for 19 days, allowing the cells to differentiate. Subsequently, the differentiated cells were exposed in quadruple for either 6 or 24 h to 5, 10, 15, and 20 μM α-chaconine (n = 4), α-solanine (n = 4), α-chaconine/α-solanine (1.7:1) (n = 4), and α-chaconine/α-solanine (28.8:1) (n = 4). LDH activity was determined using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Benelux bv, The Netherlands) in accordance with the instructions of the manufacturer.

2.4. Gene Expression Experiments

In three independent experiments, Caco-2 cells were seeded at a density of 40 000 per cm2 in 6-well polyester Transwell plates (Costar; 0.4 μm pore size, inserts of

24 mm in diameter). The cells were allowed to differentiate by growing them for 19 days. Following differentiation, cells were exposed for 6 h to 10 μM of the following glycoalkaloid preparations: α-chaconine, α-solanine, α-chaconine/α-solanine (1.7:1), and α-chaconine/α-solanine (28.8:1). The exposure time and concentration were based on results from a previous study, in which optimal conditions for studying the effect of α-chaconine on gene expression were determined systematically (23). The media in the upper compartments of the transwells were replaced with DMEM containing 0.01% DMF (control exposure) or one of the glycoalkaloid-containing solutions described above. The media in the lower compartments were replaced with DMEM only, to mimic conditions in the body.

After exposure, media were removed and both compartments were washed twice with ice-cold phosphate-buffered saline (PBS). Cells in the upper compartments were resuspended in 1 mL TriZol (Invitrogen, Breda, The Netherlands) and stored at −80 °C until RNA extraction.

2.5. Microarray Hybridizations

Total RNA from Caco-2 cells was isolated using the TriZol reagent according to the instructions of the manufacturer. RNA purification was performed using the RNeasy kit (Qiagen, Westburg bv, Leusden, The Netherlands). RNA integrity was verified by gel electrophoresis, and RNA concentrations and purity were determined by UV spectrometry by measuring 260/280 and 260/230 nm absorbance ratios, respectively. All RNA samples had OD260/280 ratios between 1.9–2.1 and OD260/230 ratios higher than 1.7.

A control reference design was used to analyze differential gene expression in glycoalkaloid-treated samples versus controls. RNA samples (2 μg each) were amplified and labeled with Cy5- and Cy3-CTP (PerkinElmer/NEN Life Sciences,

Boston, MA) to produce labeled cRNA using Agilent low RNA input fluorescent linear amplification kits following the protocol of the manufacturer. For hybridization, the Agilent 60-mer oligo microarray processing protocol (Rev. 7, SSPE Wash/6-screw hybridization chamber) was followed. Briefly, 1 μg of Cy3-labeled control (reference sample) and 1 μg of Cy5-labeled glycoalkaloid-treatment sample were mixed and hybridized to a 22K 60-mer oligonucleotide Agilent human 1A oligo microarrays V2 (Agilent Technologies, Palo Alto, CA) for 17 h at 60 °C. Upon hybridization, the microarrays were washed and dried at room temperature following instructions by Agilent.

Arrays were scanned using a Scanner Array Express HT microarray scanner (PerkinElmer Life Sciences, Boston, MA). The software package, Array Vision Software 7.0 (Imaging Research, Ontario, Canada), was used to extract data from the scanned images. The quality of the arrays was checked by using Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA) and the software package LimmaGUI in R version 2.3.1 (http://bioinf.wehi.edu.au/limmaGUI/index.html). Single spots or blemished areas on the array were flagged. The non-flagged fluorescence signals that were quantified using Array Vision software were exported to GeneMaths XT software (version 1.5, Applied Maths, St Martens-Latem, Belgium) for further analyses. Array elements for which the fluorescent intensity in each channel was less than 1.5 times the background were excluded, leaving 10 829 transcripts for subsequent analyses. Data normalization was performed with GeneMaths XT, as described previously (*23*).

2.6. Data Analysis

Identification of genes differentially expressed between a glycoalkaloid treatment and control group was performed by using both an unpaired Student's t test with p value < 0.01 or < 0.001 and a fold change criterion > 1.5.

Two complementary methods were applied to relate changes in gene expression to functional changes. First, an online software suite MetaCore version 4.3 (GeneGo, Inc., St. Joseph, MI) was used to identify statistically significant pathways responding to the different glycoalkaloid treatments. For this purpose, only the data of genes found to be significantly differentially expressed ($p < 0.01$ and fold change > 1.5) were imported into the MetaCore program. MetaCore analyses resulted in lists of maps/pathways ranked according to significance (lowest p values), as outlined by Ekins et al. (*24*).

The other approach used was based on over-representation of gene ontology (GO) terms. The software package ErmineJ (*25*), which uses a scoring-based resampling method, was applied to identify significant enrichment or over-representation of biological processes responding to specific glycoalkaloid treatments. All t-test p values from the probe set comparisons across each glycoalkaloid treatment, and control groups were used for these analyses.

A one-way analysis of variance (ANOVA) test ($p < 0.001$) was performed in GeneMaths XT to determine genes that were differentially expressed across the glycoalkaloid treatments. Principal component analysis (PCA) and hierarchical clustering analyses were performed in GeneMaths XT. The gene subset that was identified to be differentially expressed following ANOVA analysis was used for those analyses. Pearson correlation and the unweighted pair group method with arithmetic means (UPGMA) were used to determine the clustering of experimental groups.

2.7. Cell-Cycle Analysis

Cell-cycle analysis was performed on Caco-2 cells grown for 19 days in 6-well polyester Transwell plates exposed to 5, 10, and 20 μM α-chaconine (n = 3) for 6 or 24 h. Caco-2 cells were harvested with trypsin, washed twice with cold PBS, and then resuspended in cold PBS. Cells subsequently were fixed in 70% ethanol for 30 min and stored at 4 °C. Before processing, cells were collected by centrifugation and incubated in RNase (1 mg/mL) for 30 min at 37 °C. Propidium iodide (100 μg/mL) was added, and samples were incubated for 30 min at room temperature. Cell-cycle analysis was performed using a FACSarray flow cytometer (Becton Dickinson, San Jose, CA). In each experiment, a minimum of 20 000 events were evaluated. Cell-cycle distribution was analyzed using FACSdiva software (Becton Dickinson, San Jose, CA). To identify the significant difference between each experimental test condition and control treatment, a Student's t test was performed. A p value < 0.05 was regarded as indicating statistical significance.

2.8. Determination of Apoptosis

The Annexin V assay was conducted on Caco-2 cells grown for 19 days in 6-well polyester Transwell plates exposed to 5, 10, and 20 μM α-chaconine (n = 4) for 6 h. When apoptosis is initiated, the lipid organization of the plasma membrane is altered, exposing phosphatidylserine on the outer membrane surface. Annexin V was used to detect exposure of phosphatidylserine, because it is one of the markers for the early stage of apoptosis (*26*). Cells were harvested with trypsin, washed twice in cold PBS, and resuspended in binding buffer. The cells were washed and subsequently incubated with 2 μL of Annexin V-Fluos (Roche Diagnostics, Penzberg, Germany) in 200 μL of Annexin V buffer according to the protocol of the manufacturer. After an incubation period of 15 min at room temperature, the cells were spun-down and

resuspended in 200 µL of Annexin V buffer and 2 µL of propidium iodide (PI; 1 mg/mL; Sigma). The cells were then immediately analyzed on a FACSArray flow cytometer (Becton Dickinson, San Jose, CA). In each experiment, a minimum of 10 000 events were evaluated. Cell death was analyzed using FACSDiva software (Becton Dickinson, San Jose, CA). A Student's t test was performed to identify the significant difference between each experimental test condition and control treatment. A p value < 0.05 was regarded as indicating statistical significance.

3. Results

3.1. LDH Leakage

Exposure of differentiated Caco-2 cells to increasing concentrations of glycoalkaloid treatments for 6 or 24 h resulted in concentration-dependent leakage of LDH at both time points (Figure 3-2). For all treatments, except α-solanine alone, the higher glycoalkaloid concentration of 20 µM resulted in LDH leakage of more than 20% after 6 and 24 h of exposure. This latter level is used frequently as a lower cutoff for cytotoxicity. With respect to the 10 µM exposures, only the α-chaconine/α-solanine mixture (1.7:1) resulted in a LDH leakage greater than 20% at both exposure times.

The extent of LDH leakage was comparable at 6 and 24 h when cells were treated with α-chaconine alone or the mixture of α-chaconine/α-solanine (28.8:1) (Figure 3-2). α-Solanine, when administered alone for 24 h, caused relatively less membrane disruption, irrespective of the concentration. In general, glycoalkaloid exposures for 24 h induced LDH leakage with the following order of potency: α-chaconine/α-solanine (1.7:1) > α-chaconine/α-solanine (28.8:1) = α-chaconine > α-solanine at all concentrations of exposure. After 6 h of exposure, α-solanine caused less disruption at 20 µM, whereas at concentrations of 15 µM or less, the degree of

LDH leakage was similar to that observed when cells were treated with α-chaconine alone or with α-chaconine/α-solanine (28.8:1).

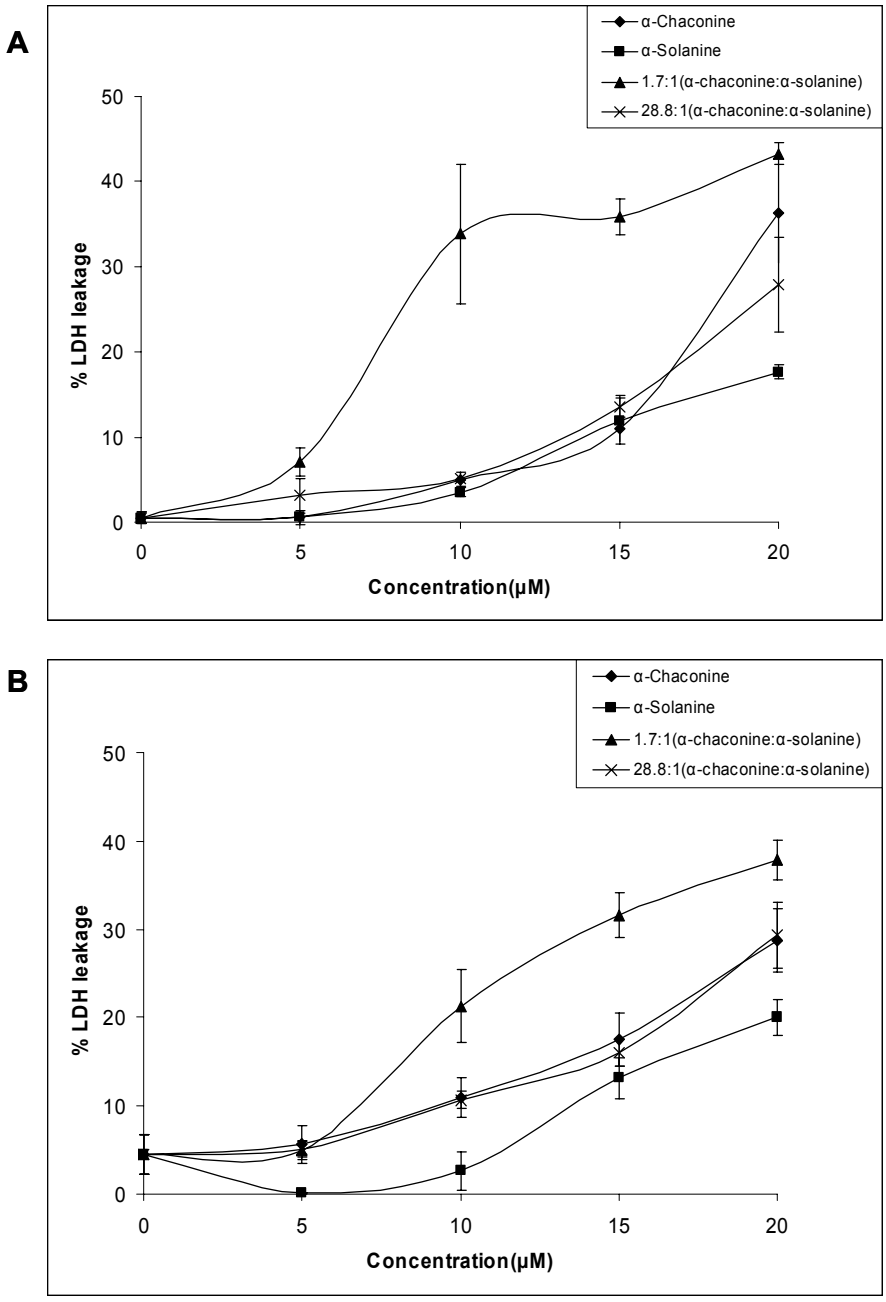

Figure 3-2. Concentration dependent LDH leakage induced by the glycoalkaloid treatments after (A) 6 hours and (B) 24 hours exposures. Each point represents the average of 4 replicates with a standard deviation of less than 5 %.

3.2. Gene Expression Studies

Exposure of Caco-2 Cells to α-Solanine, α-Chaconine, and Glycoalkaloid Mixtures. Gene expression profiles were determined to assess glycoalkaloid-induced transcriptional effects. The number of genes up- or downregulated at significance levels of either $p < 0.01$ or < 0.001 and that exhibited fold changes > 1.5 are shown in Table 3-1. α-Solanine (10 μM) had very little effect on gene expression in the Caco-2 cell line compared to the other treatments that were tested.

Table 3-1. Numbers of significantly up- and down- regulated genes in Caco-2 cells after treatment with glycoalkaloids

Glycoalkaloid Treatment	p≤0.01 (FC ≥ 1.5)			p≤0.001 (FC ≥ 1.5)		
	upregulated	downregulated	total	upregulated	downregulated	total
10 μM -Solanine	2	5	7	-	-	-
10 μM -Chaconine	54	97	451	124	20	144
10 μM -Chaconine: -solanine (28.8:1)	310	204	514	97	27	124
10 μM -Chaconine: -solanine (1.7:1)	264	157	421	50	15	65

FC: Fold Change; p: Student's t-test p-value; -: not significant

PCA and hierarchical clustering analyses were performed using 444 genes that were expressed differentially by at least one of the glycoalkaloid treatments (ANOVA, $p < 0.001$). These results were consistent with a compound-specific response on gene expression (Figure 3-3). Cells exposed to α-solanine alone were clustered closer to control cells. The other glycoalkaloid treatments elicited markedly distinct gene expression profiles from those of the control and α-solanine-only groups. Gene-expression profiles observed following α-chaconine and α-chaconine/α-solanine (28.8:1) treatments were more similar to each other than to the profile observed following α-chaconine/α-solanine (1.7:1) treatment.

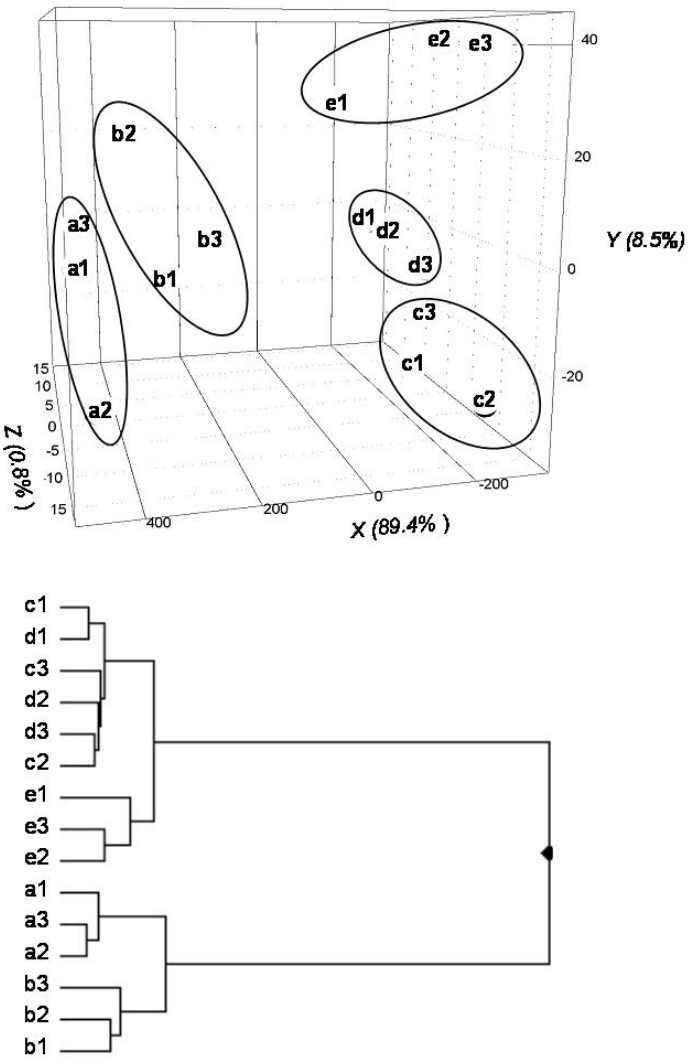

Figure 3-3. PCA mapping and hierarchical clustering of the different glycoalkaloid treatments with genes found to be significantly differentially expressed (ANOVA, $p \leq 0.001$). The axes on the PCA plot show the gene-expression profiles of the different treatment groups in the principal component x, y, and z (which explain the largest part of the variance). a, control cells; b, cells exposed to α-solanine; c, cells exposed to α-chaconine; d, cells exposed to α-chaconine/α-solanine (28.8:1); and e, cells exposed to α-chaconine/α-solanine (1.7:1). The results are based on three (1–3) independent experiments.

Genes differentially expressed following distinct glycoalkaloid treatments ($p < 0.01$ and fold changes > 1.5) were imported into MetaCore to identify pathways that were affected by the respective treatments. Microarray data obtained with α-solanine were not subjected to MetaCore analysis because only seven genes were differentially expressed following this treatment. The 10 most significantly affected pathways by the various treatments tested that were identified by MetaCore are presented in Table 3-2.

Comparisons of treatment effects on Caco-2 cell gene expression revealed that cholesterol biosynthesis was one of the pathways most significantly affected by both α-chaconine administered alone and the α-chaconine/α-solanine (28.8:1) mixture (Table 3-2). These treatments resulted in the upregulation of several genes involved in cholesterol biosynthesis ($p < 0.01$ and fold change > 1.5) (Table 3-4). The α-chaconine/α-solanine (1.7:1) mixture did not significantly affect the cholesterol biosynthesis pathway (Table 3-2). Most importantly, the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), was not significantly induced by α-chaconine/α-solanine (1.7:1) (Table 3-4).

Generally, MetaCore analysis revealed that the glycoalkaloid treatments affected the same pathways and genes. Besides cholesterol biosynthesis, most of the pathways affected appeared to be those involved in growth-related signaling (e.g., EGF), lipid metabolism, transcription regulation of amino acid metabolism, Ras family GTPases cascades, and chemokine- and cytokine-mediated signaling.

Table 3-2. Top 10 Lists of MetaCore Pathways Affected by the Glycoalkaloid Treatments

#	map	*p* value[a]	expressed[b]	all[c]
	10 µM -Chaconine			
1	cholesterol biosynthesis	4.592×10^{-10}	9	21
2	EGF-signaling pathway	1.764×10^{-5}	9	64
3	EGFR signaling via small GTPases	3.037×10^{-5}	7	39
4	transcription regulation of amino acid metabolism	4.263×10^{-5}	7	41
5	oncostatin M signaling via MAPK in mouse cells	5.013×10^{-5}	7	42
6	oncostatin M signaling via MAPK in human cells	5.868×10^{-5}	7	43
7	VEGF signaling via VEGFR2, generic cascades	6.839×10^{-5}	7	44
8	IGF–RI signaling	0.000 280 9	8	72
9	MIF in innate immunity response	0.0003251	7	56
10	AKT signaling	0.0003632	7	57
	10 µM -Chaconine/ -Solanine (28.8:1)			
1	transcription regulation of amino acid metabolism	1.356×10^{-5}	8	41
2	cholesterol biosynthesis	1.632×10^{-5}	6	21
3	oncostatin M signaling via MAPK in mouse cells	0.005757	5	42
4	role of AP-1 in regulation of cellular metabolism	0.00637	5	43
5	oncostatin M signaling via MAPK in human cells	0.00637	5	43
6	Ras family GTPases in kinase cascades (scheme)	0.006946	4	28
7	VEGF signaling via VEGFR2, generic cascades	0.007027	5	44
8	triacylglycerol metabolism p.1	0.007885	4	29
9	EGF signaling pathway	0.00832	6	64
10	IL2 activation and signaling pathway	0.008481	5	46
	10 µM -Chaconine/ -Solanine (1.7:1)			
1	phospholipid metabolism p.2	0.001715	3	11
2	EGF-signaling pathway	0.003392	6	64
3	PPAR regulation of lipid metabolism	0.003588	4	28
4	fatty acid omega oxidation	0.003596	3	14
5	triacylglycerol metabolism p.1	0.004088	4	29
6	proline metabolism	0.00442	3	15
7	methionine–cysteine–glutamate metabolism	0.007534	3	18
8	role of VDR in regulation of genes involved in osteoporosis	0.009688	5	57
9	transcription regulation of amino acid metabolism	0.01407	4	41
10	P53-signaling pathway	0.02075	4	46

[a] For a calculation of *p* values, see the Materials and Methods. [b] The number of differentially expressed genes ($p < 0.01$ and FC > 1.5) in a pathway/map. [c] The total number of genes in a particular pathway/map.

A parallel analysis using ErmineJ was performed to determine the biological processes affected by the different glycoalkaloid treatments. These results were consistent with those obtained by MetaCore (Table 3-3). For all glycoalkaloid treatments, similar processes were found to be affected. Over-represented GO classes included descriptors for lipid metabolism, cytokine- and chemokine-mediated pathways, amino acid metabolism, MAPK and NF-κB cascades, cell death/apoptosis, and the cell cycle. As found with MetaCore analysis, cholesterol biosynthesis was observed to be affected significantly by α-chaconine and α-chaconine/α-solanine (28.8:1) exposure and not by α-chaconine/α-solanine (1.7:1). More cell death/apoptotic and oxidative stress processes were regulated differentially by this latter treatment.

In a dose–response microarray experiment conducted with these glycoalkaloid treatments, induction of cholesterol biosynthesis genes was evident at low concentrations of 5 μM glycoalkaloid (mixtures), except when α-solanine was administered alone (data not shown). Cholesterol biosynthesis genes were only induced at a concentration of 20 μM following this latter treatment. Except for α-solanine administered alone, glycoalkaloid concentrations of 20 μM affected apoptotic/cell death pathways rather than the cholesterol biosynthesis pathway (data not shown). Table 3-4 presents lists of genes classified in selected processes that were affected by the specified treatments.

Table 3-3. Gene Ontology classes overrepresented following glycoalkaloid treatment

number	GO class	GO ID	probes	genes in class	raw score	FDR
		10 μM α-Chaconine				
1	cell-substrate adhesion	GO:0031589	24	23	1.43	5.21×10^{-10}
2	protein targeting	GO:0006605	59	54	1.15	2.61×10^{-10}
3	epidermis development	GO:0008544	32	32	1.49	1.74×10^{-10}
4	protein amino acid dephosphorylation	GO:0006470	66	61	1.19	1.30×10^{-10}
5	cell death	GO:0008219	18	17	1.53	1.04×10^{-10}
6	locomotion	GO:0040011	64	57	1.09	8.68×10^{-11}
7	protein amino acid O-linked glycosylation	GO:0006493	16	15	2.68	7.44×10^{-11}
8	anti-apoptosis	GO:0006916	67	63	1.17	6.51×10^{-11}
9	main pathways of carbohydrate metabolism	GO:0006092	73	66	1.07	5.79×10^{-11}
10	cholesterol biosynthesis	GO:0006695	23	18	2.18	5.21×10^{-11}
11	glycerol metabolism	GO:0006071	9	9	2.87	4.74×10^{-11}
12	actin cytoskeleton organization and biogenesis	GO:0030036	61	55	1.39	4.34×10^{-11}
13	dephosphorylation	GO:0016311	68	63	1.18	4.01×10^{-11}
14	cell migration	GO:0016477	32	29	1.47	3.72×10^{-11}
15	regulation of translation	GO:0006445	59	56	1.34	3.47×10^{-11}
16	positive regulation of I-κB kinase/NF-κB cascade	GO:0043123	51	48	1.66	3.26×10^{-11}
17	microtubule-based movement	GO:0007018	40	38	1.49	3.07×10^{-11}
18	vesicle-mediated transport	GO:0016192	118	110	0.90	2.89×10^{-11}
19	cytokine- and chemokine-mediated signaling pathway	GO:0019221	12	10	3.65	2.74×10^{-11}
20	response to virus	GO:0009615	30	30	1.52	2.61×10^{-11}
		10 μM α-Chaconine/α-Solanine (28.8:1)				
1	epidermis development	GO:0008544	32	32	2.06	5.21×10^{-10}
2	protein amino acid dephosphorylation	GO:0006470	66	61	1.36	2.61×10^{-10}
3	translational elongation	GO:0006414	15	13	1.96	1.74×10^{-10}
4	cell death	GO:0008219	18	17	2.39	1.30×10^{-10}
5	protein amino acid O-linked glycosylation	GO:0006493	16	15	1.78	1.04×10^{-10}
6	anti-apoptosis	GO:0006916	67	63	1.61	8.68×10^{-11}
7	inactivation of MAPK activity	GO:0000188	11	11	2.12	7.44×10^{-11}
8	cholesterol biosynthesis	GO:0006695	23	18	2.68	6.51×10^{-11}
9	glycerol metabolism	GO:0006071	9	9	2.81	5.79×10^{-11}
10	dephosphorylation	GO:0016311	68	63	1.34	5.21×10^{-11}
11	cell migration	GO:0016477	32	29	1.47	4.74×10^{-11}
12	positive regulation of I-κB kinase/NF-κB cascade	GO:0043123	51	48	1.49	4.34×10^{-11}
13	organ development	GO:0048513	108	97	1.10	4.01×10^{-11}
14	microtubule-based movement	GO:0007018	40	38	1.66	3.72×10^{-11}
15	cytokine- and chemokine-mediated signaling pathway	GO:0019221	12	10	2.68	3.47×10^{-11}
16	response to virus	GO:0009615	30	30	1.55	3.26×10^{-11}
17	DNA catabolism	GO:0006308	9	9	2.65	3.07×10^{-11}
18	Golgi vesicle transport	GO:0048193	81	73	1.11	2.89×10^{-11}
19	DNA replication	GO:0006260	89	84	1.32	2.74×10^{-11}
20	hemopoiesis	GO:0030097	12	11	4.76	2.61×10^{-11}
		10 μM α-Chaconine/α-Solanine (1.7:1)				
1	main pathways of carbohydrate metabolism	GO:0006092	73	66	1.19	5.21×10^{-10}
2	integrin-mediated signaling pathway	GO:0007229	30	26	2.01	2.61×10^{-10}
3	negative regulation of programmed cell death	GO:0043069	12	11	2.39	1.74×10^{-10}
4	positive regulation of I-κB kinase/NF-κB cascade	GO:0043123	51	48	1.49	1.30×10^{-10}
5	DNA integrity checkpoint	GO:0031570	9	8	3.02	1.04×10^{-10}
6	positive regulation of MAPK activity	GO:0043406	10	9	2.27	8.68×10^{-11}
7	cytokine- and chemokine-mediated signaling pathway	GO:0019221	12	10	2.37	7.44×10^{-11}
8	DNA catabolism	GO:0006308	9	9	3.91	6.51×10^{-11}
9	positive regulation of signal transduction	GO:0009967	59	53	1.65	5.79×10^{-11}
10	death	GO:0016265	13	12	1.95	5.21×10^{-11}
11	G2/M transition of mitotic cell cycle	GO:0000086	12	9	2.57	4.74×10^{-11}
12	apoptotic nuclear changes	GO:0030262	9	8	2.68	4.34×10^{-11}
13	oxygen and reactive oxygen species metabolism	GO:0006800	37	36	1.40	4.01×10^{-11}
14	physiological interaction between organisms	GO:0051706	17	14	3.12	3.72×10^{-11}
15	DNA damage response, signal transduction	GO:0042770	13	12	2.01	3.47×10^{-11}
16	prostaglandin metabolism	GO:0006693	9	9	2.39	3.26×10^{-11}
17	response to oxidative stress	GO:0006979	34	33	1.54	3.07×10^{-11}
18	cellular lipid metabolism	GO:0044255	85	79	1.24	2.89×10^{-11}
19	cellular macromolecule catabolism	GO:0044265	13	12	1.97	2.74×10^{-11}
20	response to chemical stimulus	GO:0042221	108	104	1.13	2.61×10^{-11}

A scoring-based resampling method was used to identify significantly overrepresentedGOclasses. More than ten thousand t-test *p*-values from the probe set comparisons across the glycoalkaloid treatments were used. The analysis was performed using the tool ErmineJ (23). Only classes for the concept

"biological process" (top 20 with a FDR <0.0001) are shown. For the analysis only classes containing 8 through 125 genes were taken into account.

Table 3-4. Partial List of Differentially Expressed Genes Classified According to Selected Processes Commonly Affected by All Treatments[a]

		10 μM α-solanine		10 μM α-chaconine		10 μM GA mixture (28.8:1)		10 μM GA mixture (1.7:1)	
gene name	gene ID	FC	*p* value	FC	*p* value	FC	*p* value	FC	*p* value
Cholesterol/Sterol Biosynthesis									
3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR)	NM_000859	1.19	0.462 013	3.53	0.000 638	2.46	0.000 747	1.08	0.339 773
3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (soluble) (HMGCS1)	NM_002130	1.61	0.046 436	3.47	0.001 317	3.42	0.000 248	2.02	0.024 735
cytochrome P450, family 51, subfamily A, polypeptide 1 (CYP51A1)	NM_000786	1.29	0.117 001	2.03	0.000 333	1.84	0.000 212	1.21	0.050 832
squalene epoxidase (SQLE)	NM_003129	1.30	0.098 264	3.14	0.000 889	2.91	0.008 428	1.72	0.035 499
sterol-C5-desaturase (ERG3 δ-5-desaturase homologue, fungal)-like (SC5DL)	NM_006918	1.23	0.253 956	2.21	0.007 952	2.10	0.003 434	1.37	0.043 46
sterol-C5-desaturase (ERG3 δ-5-desaturase homologue, fungal)-like, (cDNA clone MGC:2084 MAGE:3537285)	BC012333	1.20	0.191 643	2.55	0.001 105	2.41	0.001 199	1.42	0.026 284
sterol-C4-methyl oxidase-like (SC4MOL)	NM_006745	1.46	0.035 287	2.04	0.000 714	1.90	0.000 229	1.38	0.133 572
mevalonate kinase (mevalonic aciduria) (MVK)	NM_000431	1.07	0.805 267	1.71	0.002 905	1.51	0.038 069	0.73	0.023 069
Homo sapiens /hepatitis B virus fusion for mevalonate kinase	X75311	0.97	0.916 08	1.76	0.0329	1.57	0.105 179	0.85	0.457 067
sterol regulatory element binding transcription factor 1 (SREBF1), transcript variant 2	NM_004176	1.06	0.816 728	1.65	0.001 823	1.48	0.069 865	1.44	0.059 699
sterol regulatory element binding transcription factor 2 (SREBF2)	NM_004599	1.08	0.294 725	1.48	0.003 126	1.28	0.012 238	1.11	0.116 347
farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase) (FDPS)	NM_002004	1.23	0.217 881	1.75	0.002 132	1.42	0.029 285	1.06	0.527 974
farnesyl-diphosphate farnesyltransferase 1 (FDFT1)	NM_004462	1.13	0.535 23	2.25	0.000 949	1.74	0.024 414	1.05	0.554 356
acetyl-coenzyme A acetyltransferase 2 (acetoacetyl coenzyme A thiolase) (ACAT2)	NM_005891	1.08	0.818 932	1.82	0.002 172	1.41	0.032 83	0.99	0.918 362
low-density lipoprotein receptor (familial hypercholesterolemia) (LDLR)	NM_000527	1.44	0.128 684	3.63	0.000 499	3.33	0.007 036	2.30	0.007 428
isopentyl disphosphate isomerase (IDI1)	NM_004508	1.13	0.622 388	2.89	6.40×10^{-5}	2.59	0.000 436	1.44	0.050 39
Lipid Metabolism									
likely orthologue of mouse acyl-coenzyme A thioesterase 2, mitochondrial (ACATE2)	NM_012332	1.12	0.370 984	2.26	6.30×10^{-5}	1.95	0.001 83	1.73	0.000 397
dihydrolipoamide *S*-acetyltransferase (E2 component of pyruvate dehydrogenase complex) (DLAT)	NM_001931	1.01	0.841 188	1.46	0.000 213	1.21	0.013 567	1.06	0.297 008
cytochrome P450, family 3, subfamily A, polypeptide 5 (CYP3A5)	NM_000777	1.16	0.017 193	1.22	0.003 768	1.32	0.000 391	1.37	0.000 301
malonyl-CoA decarboxylase (MLYCD)	NM_012213	0.89	0.197 086	0.59	0.002 579	0.54	0.004 72	0.68	0.007 64
phosphatidylcholine transfer protein (PCTP)	NM_021213	0.91	0.286 463	0.75	0.011 503	0.64	0.001 818	0.63	0.001 254
nuclear receptor subfamily 2, group F, member 2 (NR2F2)	NM_021005	0.89	0.496 072	0.25	0.000 482	0.22	0.000 088	0.39	0.005 142
sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1 (SULT2A1)	NM_003167	0.84	0.151 283	0.62	0.003 309	0.65	0.011 688	0.62	0.006 45
UDP glycosyltransferase 2 family, polypeptide B7 (UGT2B7)	NM_001074	0.74	0.043 648	0.62	0.008 049	0.61	0.018 008	0.58	0.007 79
acetyl-coenzyme A acyltransferase (ACAA1), nuclear gene encoding mitochondrial protein	NM_001607	0.81	0.119 165	0.72	0.102 146	0.60	0.001 572	0.55	0.001 403
choline kinase α (CHKA), transcript variant 1	NM_001277	1.18	0.050 329	1.45	0.042 749	1.54	0.027 971	1.57	0.002 661
fatty acid binding protein 5 (psoriasis-associated) (FABP5)	NM_001444	0.80	0.036 323	0.62	0.007 922	0.66	0.000 014	0.67	0.001 734
ferredoxin reductase (FDXR), nuclear gene encoding mitochondrial protein, transcript variant 2	NM_004110	0.84	0.090 416	0.88	0.213 827	0.77	0.136 108	0.64	0.003 278
monoglyceride lipase (MGLL), transcript variant 1	NM_007283	1.07	0.240 551	2.22	0.004 31	1.95	0.014 279	1.73	0.000 186
platelet-activating factor acetylhydrolase, isoform Ib, α subunit 45 kDa (PAFAH1B1)	NM_000430	1.06	0.410 604	1.60	0.004 101	1.66	0.007 962	1.58	0.003 005
Apoptosis/Programmed Cell Death									
B-cell CLL/lymphoma 10 (BCL10)	NM_003921	1.26	0.106 827	2.34	0.001 278	2.05	0.001 197	1.81	0.009 346
pleckstrin homology-like domain, family A, member 1 (PHLDA1)	NM_007350	1.58	0.034 105	3.30	0.000 334	3.81	0.000 079	4.03	0.002 348
pleckstrin homology-like domain, family A, member 2 (PHLDA2)	NM_003311	1.53	0.115 573	4.69	0.000 916	5.00	0.000 709	6.25	0.013 172
BCL2 binding component 3 (BBC3)	NM_014417	1.30	0.080 683	4.36	0.000 112	3.74	0.006 004	2.00	0.018 965
SH3-domain GRB2-like endophilin B1 (SH3GLB1)	NM_016009	1.12	0.100 532	1.94	0.000 195	1.90	0.000 861	1.54	0.002 113
tribbles homologue 3 (*Drosophila*) (TRIB3)	NM_021158	1.39	0.262 859	4.27	0.001 354	2.98	0.006 729	1.82	0.038 111
tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A)	NM_001065	1.23	0.421 639	2.65	4.10×10^{-5}	2.04	0.001 448	1.86	0.000 36

Table 3-4 (Continued).

gene name	gene ID	10 μM α-solanine FC	10 μM α-solanine *p* value	10 μM α-chaconine FC	10 μM α-chaconine *p* value	10 μM GA mixture (28.8:1) FC	10 μM GA mixture (28.8:1) *p* value	10 μM GA mixture (1.7:1) FC	10 μM GA mixture (1.7:1) *p* value
tumor necrosis factor receptor superfamily, member 12A (TNFRSF12A)	NM_016639	1.46	0.021 69	4.67	0.000 124	5.21	0.000 577	4.95	0.001 486
tumor necrosis factor receptor superfamily, member 21 (TNFRSF21)	NM_014452	1.38	0.041 717	2.38	0.001 824	2.09	0.002 007	1.88	0.011 591
kruppel-like factor 6 (KLF6), transcript variant 2	NM_001300	2.07	0.023 023	10.77	0.000 021	8.87	0.000 134	5.85	0.001 658
myeloid cell leukemia sequence 1 (BCL2-related) (MCL1), transcript variant 1	NM_021960	1.43	0.009 187	3.61	0.000 072	3.70	0.000 091	3.06	0.001 308
programmed cell death 8 (apoptosis-inducing factor) (PDCD8), nuclear gene encoding mitochondrial protein	NM_004208	0.68	0.019 167	0.64	0.014 379	0.59	0.007 481	0.55	0.003 852
extra spindle poles like 1 (*Saccharomyces cerevisiae*) (ESPL1)	NM_012291	0.90	0.306 476	0.63	0.003 176	0.59	0.001 181	0.64	0.005 335
Bcl2 modifying factor (BMF), transcript variant 2	NM_033503	0.90	0.424 475	0.63	0.010 225	0.57	0.008 203	0.52	0.018 726
				Cell Cycle					
cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), transcript variant 1	NM_000389	1.20	0.139 883	2.58	0.001 035	2.27	0.001 779	2.06	0.004 558
cyclin-dependent kinase inhibitor 1C (p57, Kip2) (CDKN1C)	NM_000076	1.31	0.044 667	2.30	0.000 426	2.74	0.000 725	2.41	0.000 595
polo-like kinase 3 (*Drosophila*) (PLK3)	NM_004073	1.42	0.0307	3.29	7.10×10^{-5}	3.64	0.000 259	3.52	0.004 782
polo-like kinase 2 (*Drosophila*) (PLK2)	NM_006622	1.51	0.036 132	2.92	0.006 303	3.08	0.003 052	2.80	0.004 228
protein phosphatase 2 (formerly 2A), catalytic subunit, β isoform (PPP2CB), transcript variant 2	NM_001009552	1.23	0.241 224	2.42	0.000 508	2.04	0.001 555	2.15	0.004 221
putative lymphocyte G0/G1 switch gene (G0S2)	NM_015714	0.77	0.245 573	0.24	0.000 736	0.23	0.000 445	0.26	0.003 552
v-jun sarcoma virus 17 oncogene homologue (avian) (JUN)	NM_002228	1.92	0.008 419	4.80	0.000 07	4.79	0.000 93	2.76	0.004 977
jun B proto-oncogene (JUNB)	NM_002229	1.23	0.236 543	2.88	0.000 496	2.71	0.001 525	2.28	0.014 609
v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue (KRAS), transcript variant a	NM_033360	1.08	0.413 903	2.25	0.000 912	1.94	0.002 58	1.56	0.031 253
v-fos FBJ murine osteosarcoma viral oncogene homologue (FOS)	NM_005252	1.70	0.021 487	3.51	0.000 744	2.68	0.004 253	2.92	0.006 751
v-myc myelocytomatosis viral oncogene homologue (avian) (MYC)	NM_002467	1.26	0.140 158	2.00	0.006 322	2.20	0.012 852	3.32	0.003 831
BTG family, member 3 (BTG3)	NM_006806	1.15	0.119 292	2.05	0.012 971	2.16	0.001 447	1.95	0.000 833
headcase homologue (*Drosophila*) (HECA)	NM_016217	1.19	0.046 632	2.03	0.001 548	1.94	0.003 73	1.74	0.003 227
dual specificity phosphatase 4 (DUSP4), transcript variant 1	NM_001394	1.15	0.028 773	1.42	0.000 564	1.32	0.006 108	1.42	0.001 007
proliferating cell nuclear antigen (PCNA), transcript variant 1	NM_002592	0.77	0.112 291	0.56	0.019 576	0.53	0.007 429	0.58	0.015 66
anaphase promoting complex subunit 5 (ANAPC5)	NM_016237	1.02	0.792 272	0.73	0.028 854	0.74	0.035 319	0.77	0.079 311

[a] FC, fold change; GA, glycoalkaloid.

3.3. Cell-Cycle Analysis

Gene-expression analyses revealed that cell-cycle genes were also affected significantly by the glycoalkaloid treatments (Table 3-4). Therefore, a cell-cycle analysis was performed to determine which cell-cycle phase(s) was affected. In general, an accumulation of cells in the G2/M phase was noted after 6 or 24 h of exposure to 5, 10, or 20 μM α-chaconine (Figure 3-4).

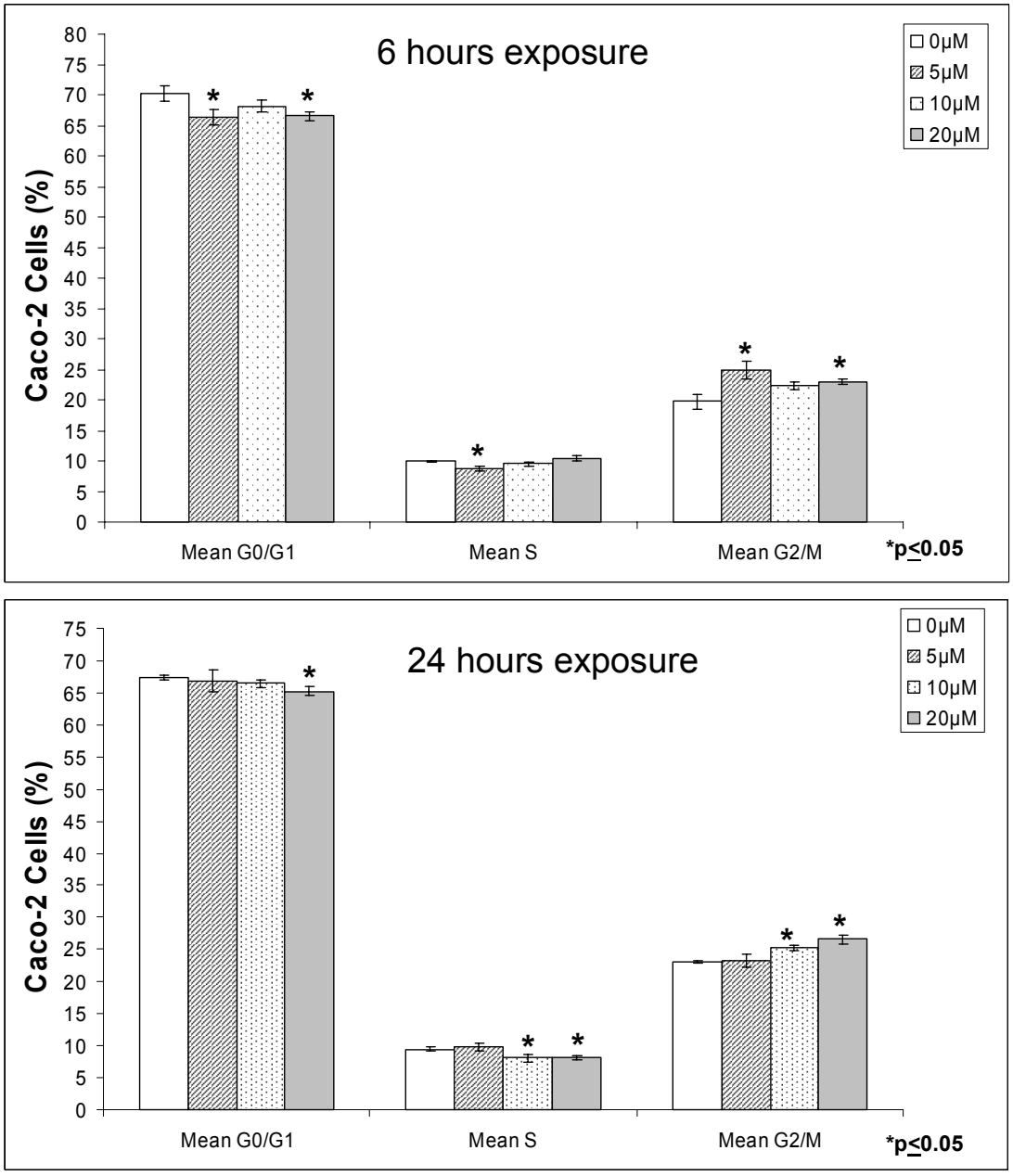

Figure 3-4. Effect of various concentrations of α-chaconine on the cell-cycle phase in Caco-2 cells. Cells were grown for 19 days and then exposed to 5–20 μM α-chaconine for either 6 or 24 h. Cell-cycle phases were identified by propidium iodide flow cytometry. Values are expressed as mean ± standard deviation (SD). (*) p values shown are in comparison to the values of the control group.

3. 4. Determination of Apoptosis

Our data revealed a concentration-dependent increase in a late apoptotic or necrotic phase in Caco-2 cells exposed to 5, 10, and 20 µM -chaconine for 6 h. We did not observe any significant effect on the early apoptotic cells at the concentrations tested (Figure 3-5).

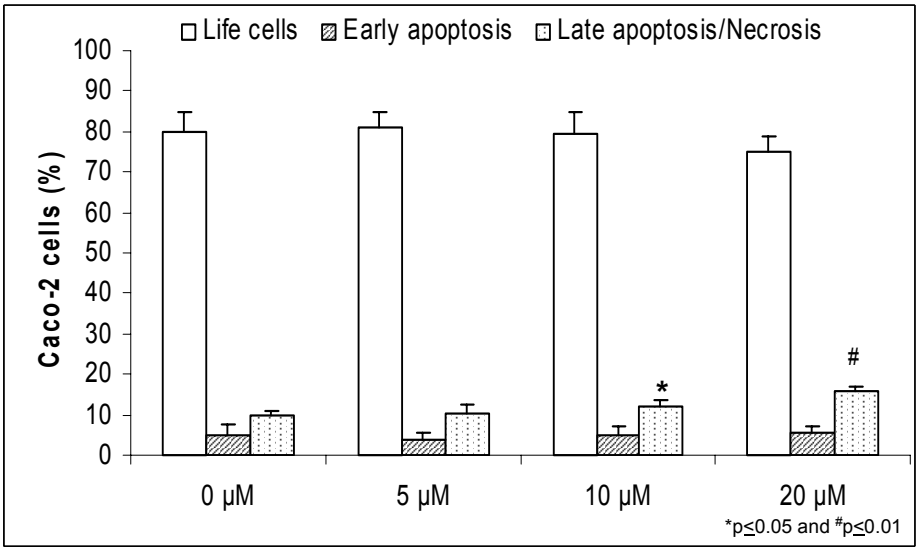

Figure 3-5. Effect of various concentrations of α-chaconine on apoptosis/necrosis in Caco-2 cells. Cells were grown for 19 days and then exposed to 5 µM to 20 µM α-chaconine for 6 hours. Values are expressed as mean ± SD. (P values shown are in comparison to the control group's values.)

Discussion

The toxic effects of single and mixtures of potato glycoalkaloids on gene expression in Caco-2 cells were investigated, and the utility of DNA microarrays in screening for toxic effects, assessing effect severity, and identifying potential mechanisms of toxicity were evaluated in this model system. Differences in the responses to the various glycoalkaloid treatments were mainly due to the differing degrees of potency of the glycoalkaloids, as noted in earlier studies (*4, 5, 17*). DNA

microarrays, to varying extents, discriminated severity of effect/potency among the different glycoalkaloid treatments.

α-Solanine (10 μM) was the least potent of the glycoalkaloid treatments, as observed in both LDH leakage and gene profiling experiments. α-Solanine administered alone caused less LDH leakage compared to equimolar amounts of α-chaconine and the glycoalkaloid mixtures. PCA and hierarchical cluster analyses revealed that the gene expression profile of cells treated with 10 μM α-solanine mostly resembled that of control cells, but expression profiles of cells subjected to the other glycoalkaloid treatments differed significantly from those of the controls. α-Chaconine alone (10 μM) and α-chaconine/α-solanine (28.8:1, 10 μM) had similar gene expression profiles in Caco-2 cells, suggesting common mechanisms of action and/or similar degrees of effect. In addition, these latter treatments resulted in similar LDH leakage. On the other hand, exposure to 10 μM α-chaconine/α-solanine (1.7:1) resulted in a gene expression profile that was distinguishable from those observed following the other treatments. As documented by LDH leakage experiments, 10 μM α-chaconine/α-solanine (1.7:1) was the most potent membrane disrupter. Clearly, glycoalkaloid treatments elicited signature patterns of gene expression that appeared to reflect potency, thereby indicating the usefulness of DNA microarrays to screen for differences in potencies of distinct glycoalkaloid treatments and, as is described below, provide additional valuable information.

To obtain a comprehensive overview of the response of Caco-2 cells to the selected glycoalkaloid treatments that were tested, significant over-representation of differentially expressed genes with roles in specific biological processes and pathways were identified using ErmineJ and MetaCore, respectively. Those analyses revealed that, for the most part, similar biological processes/pathways were affected by the tested treatments. Pathways/biological processes affected by all of the tested

treatments included cytokine- and chemokine-mediated signaling, growth signaling, MAPK and NF-κB cascades, lipid metabolism, the cell cycle, and cell death/apoptosis. The most distinctive observation was the significant induction of the cholesterol biosynthesis pathway in Caco-2 cells by either α-chaconine/α-solanine (28.8:1) or α-chaconine alone but not by the other treatments.

Intestinal membrane disruptive activity is the most well-described toxic mechanism of action of the potato glycoalkaloids. Those effects were attributed to the formation of destabilizing complexes between the lipophilic moieties of glycoalkaloids and membrane-bound cholesterol (*27-29*). In previous work, we observed that α-chaconine induced cholesterol biosynthesis genes in Caco-2 cells prior to other changes reflective of cytotoxicity. This pathway ceased to be important with either prolonged exposure to low α-chaconine concentrations or shorter exposure to higher, cytotoxic concentrations of α-chaconine (*23*). Therefore, it is likely that disturbances in cellular cholesterol levels/homeostasis as a result of the formation of membrane glycoalkaloid/sterol complexes result in the induction of cholesterol biosynthesis to regain homeostasis. We also observed general downregulation of lipid metabolism pathways that use cholesterol (e.g., decreased expression of SULT2A1 and UGT2B7 involved in steroid metabolism, Table 3-4), and as such, influence intracellular cholesterol levels might be explained in this view.

Results of both MetaCore and ErmineJ analyses indicated that non-cytotoxic concentrations (LDH leakage < 20%) of 10 μM of either α-chaconine alone or the α-chaconine/α-solanine (28.8:1) mixture induced genes of the cholesterol biosynthesis pathway. In contrast, α-chaconine/α-solanine (1.7:1) did not significantly affect the cholesterol biosynthesis pathway. The results from the LDH assay suggest that α-chaconine and α-solanine act synergistically when present in the ratio of 1.7:1 and that this mixture is more potent than equivalent concentrations of either α-chaconine or α-

solanine alone. We suggest that the induction of cholesterol biosynthesis is precluded by apoptotic processes that result in cell death. Other studies have also shown that, at certain α-chaconine/α-solanine ratios, mixtures of glycoalkaloids interact synergistically, resulting in increased toxicity (*5, 13, 16-18*).

Exposure to α-chaconine was shown to result in greater membrane disruptive effects than exposure to α-solanine (*4, 5, 17*). The differences in potency between α-solanine and α-chaconine can either be explained by subtle changes in the sugar moiety volume or by the different side chains on the sugar ring structures that influence sugar–sugar intermolecular interactions and, thereby, the formation of stable glycoalkaloid–sterol complexes (*27*). α-Solanine binds to membrane-bound cholesterol but to a lesser extent than α-chaconine, thus resulting in reduced membrane disruption compared to that observed following α-chaconine exposure (*5*). A concentration of 10 μM α-solanine proved to be too low to detect any effects on gene expression. Exposure of Caco-2 cells to 20 μM α-solanine (data not shown) affected the same pathways as did exposure to 5 or 10 μM α-chaconine and to α-chaconine/α-solanine mixtures, indicating similarities in their mechanisms of action. For example, the induction of cholesterol biosynthesis genes by α-solanine was only observed at a higher concentration of 20 μM and not at 10 μM.

Because several cell-cycle genes were affected by the treatments, cell-cycle analysis experiments were conducted using α-chaconine. Yang et al. (*30*) showed that the exposure of intestinal HT29 cells to 5 μg/mL (~5.9 μM) α-chaconine and 10 μg/mL (~11.5 μM) α-solanine for more than 48 h induced accumulation of cells in the sub-G0/G1 phase. However, our data indicated accumulation of Caco-2 cells in the G2/M phase at concentrations of 5–20 μM. We also exposed cells to 100 μM α-chaconine (6/24 h) (data not shown); however, we were unable to measure 20 000 events because of significant cell death. Despite this, it appears that cells tend to

accumulate disproportionately in the G0/G1 phase when exposed to higher glycoalkaloid concentrations. These results imply that the effect of α-chaconine on the cell cycle may depend upon the cell type and/or exposure concentration. Among the cell-cycle genes affected by all of the selected treatments in the current study was polo kinase 3 (Plk3), which is involved in the regulation of cell-cycle progression through the M phase (*31*). Plk3 is a NF-κB downstream target gene (*32*) and induces cell death, possibly by inducing p53-dependent and -independent pathways. Ectopic expression of Plk3 or its mutants perturbs microtubule integrity, resulting in dramatic morphological changes, G2/M arrest, and apoptosis (*31*). Also induced in the present experiments was cyclin-dependent kinase inhibitor 1A (CDKN1A or p21 or Cip1), which inhibits both cyclin-dependent G1 kinases (*33*) and the G2/M-specific cdc2 kinase (*34-36*). Thus, CDKN1A can result in cell-cycle arrest in either the G1 or G2/M phase. Also induced was cyclin-dependent kinase inhibitor 1C (CDKN1C or p57 or Kip2), which, similar to CDKN1A, inhibits DNA replication by binding to the proliferating cell nuclear antigen (PCNA), resulting in antiproliferative effects (*37, 38*). Expression of either Plk3 or CNKN1A can result in either G2/M or G0/G1 arrest; therefore, this may explain the different effects on the cell cycle observed using selected α-chaconine concentrations.

We also observed the differential expression of genes encoding potential regulators/players of proapoptotic/apoptotic or cell-death cascades. Cell death may occur because of either necrotic or apoptotic processes depending upon the cell type and stimulus. Apoptosis is executed mainly by a family of proteases called caspases, which can be activated by two main pathways, i.e., the extrinsic (via cell-surface death receptors) and intrinsic (via perturbation of the mitochondrial membrane) pathways (*39, 40*). Necrosis is characterized by swelling of the cell and its organelles, resulting in cell-membrane disruption and cell lysis. Downstream mediators of the extrinsic

tumor necrosis factor (TNF) pathway were affected by glycoalkaloid treatments in the present study (Table 3-4; TNFRSF1A, TNFRSF11B, TNFRSF12A, and TNFRSF21). TNF was observed to induce, via binding to TNF receptors, either apoptosis or necrosis depending upon cellular context (*41, 42*). However, we also observed the induction of genes that are involved in the intrinsic mitochondria-mediated apoptotic pathways (Table 3-4; BBC3, BMF, SH3GLB1, and PHLDA1). In addition, Yang et al. observed that, in HT29 cells, α-chaconine induced apoptosis. This effect may be mediated through the suppression of Erk1/2 phosphorylation and subsequent activation of caspase 3. The results obtained with the annexin V assay indicated that α-chaconine exposure resulted in late apoptosis and necrosis rather than early apoptosis in differentiated Caco-2 cells. Thus, both apoptosis and necrosis may have occurred simultaneously.

Many of the genes showing alterations in expression were encoding transcription factors, including NF-κB and activating protein 1 (AP-1) dimers (c-Fos, FosB, c-Jun, JunB, and ATF3). NF-κB activity is stimulated by a wide range of stimuli, such as pathogens, stress signals, and pro-inflammatory cytokines (*43*). AP-1 activity is induced by growth factors, cytokines, neurotransmitters, bacterial and viral infections, and a variety of physical and chemical stresses (*44*). NF-κB and AP-1 are key transcription factors that regulate the expression of many genes important for diverse processes, for example, cell growth, development, inflammation, stress responses, immune, cell-cycle progression, and apoptosis (*43-45*). Because we observed the differential expression of several genes/processes involved in growth signaling, cell-cycle regulation, apoptosis, and chemokine and cytokine signaling, which are targets of NF-κB and AP-1, it is conceivable that potato glycoalkaloids affect these processes via these transcription factors.

In conclusion, this study describes changes in gene-expression profiles in response to potato glycoalkaloids. Gene-profiling experiments revealed that the glycoalkaloids, α-chaconine and α-solanine, and their mixtures act by the same mechanisms, with the main difference being the degree of potency. Most importantly, we observed the induction of cholesterol biosynthesis genes by non-cytotoxic glycoalkaloid treatments and the repression of their induction by more severe glycoalkaloid concentrations that result in cytotoxicity. Induction of cholesterol biosynthesis genes may be an early response to glycoalkaloid toxicity, induced perhaps to rescue cells from the progression to death. Furthermore, we conclude that microarrays used in conjunction with classical toxicological tests can be useful in discriminating glycoalkaloid treatments on the basis of potency or degree of effect, thus demonstrating the potential of microarray technology as a tool for detecting subtle differences in toxicities of mixtures and/or possibly also whole foods. Mixture studies, such as those performed in the present work, can be helpful to predict the toxicological consequences of changing relative levels of compounds in food crops, for example, changing α-chaconine/α-solanine ratios in potato by genetic modification.

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CHAPTER 4

The PI3K/AKT, JNK and ERK pathways are not crucial for α-chaconine-induced transcription of cholesterol biosynthesis genes in intestinal epithelial cells

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Abstract

We previously reported that non-cytotoxic concentrations of potato glycoalkaloids induce expression of cholesterol biosynthesis genes in the intestinal epithelial Caco-2 cell line. Genes involved in MAPK and PI3K/AKT pathways and their downstream effectors such as Jun, c-Myc and Fos also were induced. MAPK and PI3K/AKT pathways have been described to regulate the activity of SREBPs and consequently the expression of cholesterol biosynthesis genes. In this study, in order to understand the mechanism of induction of cholesterol biosynthesis by α-chaconine, its effect on SREBP-2 protein levels was investigated. We also examined whether MAPK and PI3K/AKT pathways are required for the observed induction of these genes by α-chaconine. Differentiated Caco-2 cells were pre-treated with LY294002 (PI3K inhibitor), PD98059 (MEK1 inhibitor) or SP600125 (JNK inhibitor) or a combination of all inhibitors for 24 hours prior to co-incubation with 10 μM α-chaconine for 6 hours. Significant increases in precursor and mature protein levels of SREBP-2 were observed following α-chaconine treatment. We also observed that α-chaconine induced significant phosphorylation of AKT, ERK and JNK kinases but not that of p38. In general, the kinase inhibitor experiments revealed that phosphorylation of kinases of PI3K/AKT, ERK and JNK pathways was not crucial for glycoalkaloid-induced expression of cholesterol biosynthesis genes, with the exception of SC5DL. For the SC5DL gene, α-chaconine-induced transcription was reduced when all three pathways were inhibited. Based on these results, it can be postulated that other mechanisms, which may be independent of the MAPK and PI3K/AKT pathways, including possibly post-translational activation of SREBP-2 by α-chaconine may be more pivotal for the induction of cholesterol biosynthesis genes by α-chaconine.

Keywords: glycoalkaloids, α-chaconine, MAPK, PI3K/AKT, cholesterol biosynthesis

Introduction

Potatoes (*Solanum tuberosum L.*) contain the toxins, α-chaconine and α-solanine. These account for 95% of potatoes' total glycoalkaloids (*1*). These glycoalkaloids at high levels (3-6mg/kg body weight) may have toxic effects on human health (*2*). These include gastrointestinal disturbances, increased heart-beat, he molysis and neurotoxic effects (*3*). Reported toxicities are due mainly to acetylcholinesterase inhibition and cell membrane disruption that affect digestive and other organs (*4*). Toxicities induced in other species include hepatoxicity in mice (*5*), increased hepatic ornithine decarboxylase activity in rats (*6*), craniofacial malformations in hamsters (*7*) and anatomical developmental toxicities in frog embryos (*8, 9*).

The most well documented mechanism of glycoalkaloid toxicity is the disruption of membrane integrity, which is caused by the formation of destabilizing complexes between the lipophilic moiety of glycoalkaloids and cholesterol present in membranes (*10-12*). At certain sterol threshold concentrations, glycoalkaloids can form irreversibly glycoalkaloid/sterol complexes in cell membranes resulting in rapid loss of membrane barrier integrity (*10, 12*). In previous studies, we observed increased lactate dehydrogenase leakage in Caco-2 cells following exposure to increasing concentrations of glycoalkaloids, an indicator of increased membrane disruption (*13, 14*). In addition, we observed that α-chaconine induced cholesterol biosynthesis genes in Caco-2 cells prior to changes reflective of cytotoxicity. Prolonged exposure to low α-chaconine concentrations or shorter exposure to higher, cytotoxic concentrations of α-chaconine resulted in reduced induction of those genes (*13*). These observations were interpreted to suggest that disturbances in cellular cholesterol levels due to the formation of glycoalkaloid/sterol complexes may have resulted in the homeostatic induction of cholesterol biosynthesis genes.

Cholesterol is an important component of cellular membranes, thus levels of cholesterol are tightly regulated. Cholesterol biosynthesis genes are regulated at the transcriptional level and their transcriptional regulation is controlled by membrane-bound transcription factors, sterol regulatory element binding proteins (SREBPs)(*15*). SREBPs bind to sterol-regulatory elements (SREs) found in the promoter regions of many cholesterol and fatty acid biosynthesis genes inducing their transcription.

The regulation of SREBP activity occurs at the transcriptional and post-translational levels (*16, 17*). The post-translational regulation of SREBP activity involves sterol-mediated suppression of SREBP proteolytic cleavage (*18*). Whereas, regulation at the transcriptional level is more complex, one mechanism involves feed-forward regulation, whereby the SREBPs regulate the transcription of their own genes via SRE in the enhancer or promoter region of each gene (*16, 19*). Other factors such as liver X-activated receptors (LXRs), insulin and glucagon have been shown to regulate SREBP transcription (*16*).

Previously, we observed that α-chaconine induced genes involved in PI3K/AKT, MAPK and growth (which are mediated by either AKT or MAPK) signaling pathways and downstream effectors of these pathways, such as Jun, Fos and c-Myc (*13, 14*). The MAPK family consists of at least three different sub-groups that include ERK1/2 (extracellular signal related kinase), JNK (c-jun N terminal protein kinase, also referred to as stress-activated protein kinase, SAPK), and p38. MAP kinases play a pivotal role in orchestrating intracellular events essential for cell functioning, growth, and apoptosis (*20*). On the other hand, PI3K and its substrate, i.e. AKT kinases, play a central role in diverse signaling cascades that regulate cell proliferation and survival, cell size and response to nutrient availability, glucose metabolism, cell invasiveness, genome stability and angiogenesis (*21, 22*). Once activated, these kinases can phosphorylate and activate transcription factors which

regulate gene expression. In particular, studies demonstrate that activation of the PI3K/AKT pathway induces SREBP activity at the transcriptional and post-translational levels. AKT activation results in upregulation of SREBP (*23*) or alternatively induces ER-to-Golgi transport of SREBP and SCAP, resulting in proteolytic cleavage of SREBP (*24*). On the other hand, the MAPK pathway was shown to regulate the transcriptional activity of SREBPs via phosphorylation of ERK (*25, 26*).

In an attempt to further understand the mechanism of action of potato glycoalkaloids on the induction of cholesterol biosynthesis genes, in the present study we investigated the effect of α-chaconine on SREBP-2 protein levels and determined whether the PI3K/AKT or MAPK signaling pathways are necessary for α-chaconine-induced transcription of these genes.

2. Methods

2.1. Reagents

α-Chaconine was obtained from Sigma Aldrich (St Louis, MO, USA). Chemical inhibitors LY294002 (PI3K inhibitor), PD98059 (MEK1 inhibitor) and SP600125 (JNK inhibitor) were purchased from Calbiochem (Darmstadt, Germany). Stock solutions of α-chaconine and the chemical inhibitors were prepared in dimethyl sulfoxide (DMSO) (Merck, Germany). The stock solutions were diluted with DMEM to the final desired concentrations immediately before use. In every experiment, cells in the control group were treated with an equivalent concentration of the solvent (0.01 v/v % DMSO). Rabbit polyclonal antibodies against Total p44/42 (ERK), phopho-p44/42 (ERK 1/2) (Thr 202/Tyr 204), phopho-AKT (Ser 473), Total AKT, phopho-SAPK/JNK 1/2 (Thr 183/ Tyr 185), Total SAPK/JNK and Total p38 were obtained from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibody against

phospho-p38 (Thr 180/Tyr 182) and goat polyclonal antibody against Actin (C-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas the rabbit polyclonal antibody against SREBP-2 was obtained from Abcam (Cambridge, UK). The horseradish peroxidase-conjugated anti-rabbit and donkey anti-goat antibodies were obtained from Promega (Madison, WI, USA). Reagents for electrophoresis and Western blotting were obtained from Amersham Bioscience (Arlington Heights, IL, USA). Detergent compatible (DC) protein assay for protein quantification was obtained from BioRad (Hercules, CA, USA).

2.2. Caco-2 cell culture

The human intestinal epithelial cell line Caco-2 (ATCC, Manassas, VA) was grown routinely in 75-cm^2 culture flasks at 37°C in air with 5% CO_2 and 100% relative humidity in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Verviers, Belguim) supplemented with $NaHCO_3$ (3.7 g/l, Sigma), non-essential amino acids (1x NEAA; ICN, Zoetermeer, The Netherlands), fetal calf serum (FCS; 10% v/v; Invitrogen, Breda, The Netherlands), penicillin (5,000 U, Sigma), and streptomycin (5 mg/l, Sigma).

2.3. Treatment with α-Chaconine and Chemical Inhibitors

Caco-2 cells were seeded at a density of 40 000 per cm^2 in 6-well polyester Transwell plates (Costar; 0.4 μm pore size, inserts of 24 mm diameter). The cells were allowed to differentiate by growing them for 19 days. Following differentiation, cells were exposed for 6 hours to 10 μM α-chaconine. The exposure time and concentration were based on results from a previous study in which optimal conditions for studying the effect of α-chaconine on gene expression were determined systematically (*13*). When chemical inhibitors were used, cells were pre-treated for 24 hours with either

LY294002 (60 μM) or PD98059 (50 μM) or SP600125 (60 μM) or a combination of all inhibitors, prior to co-incubation with 10 μM α-chaconine for 6 hours. As stock solutions were dissolved in DMSO, an equal volume of DMSO (final concentration 0.01%, v/v) was added to the control cells. The media in the upper compartments of the transwells were replaced with DMEM containing 0.01% (v/v) DMSO (i.e. control exposure (with or without inhibitor(s)) or 10 μM α-chaconine (with or without inhibitor(s)). The media in the lower compartments were replaced with DMEM only.

2.4. Western blotting

Isolation of SREBP-2 proteins

Following treatment of Caco-2 cells for 6 hours with and without 10 μM α-chaconine, nuclear and membrane fractions were prepared by a modification of the procedure described by Field et al (*27*). The precursor form of SREBP-2 is predominantly in the membrane fraction, as it is normally bound to membranes of the endoplasmic reticulum and nuclear envelope. After proteolytic cleavage the mature form is released and enters the nucleus which is the site of action (*27*). Briefly, following treatment, cells were washed twice with PBS and resuspended in ice-cold buffer A [10 mM HEPES-NaOH (pH 7.4), 1.5 mM MgCl2, 10 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycoltetraacetic acid (EGTA)]. They were allowed to swell for 30 minutes. Subsequently, the cells were homogenized by passage through a 22-gauge needle 15 times. First, the homogenate was centrifuged at 1,500 rpm for 5 minutes to obtain a nuclear pellet. The supernatant was centrifuged at 40,000 rpm for 45 minutes to isolate a membrane fraction. Nuclear proteins were extracted from the nuclear pellet with 0.1 mL of buffer C [20 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl2, 25% glycerol, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA]. Membrane proteins were extracted from the membrane fraction with

0.15 mL of buffer B [125 mM Tris (pH 6.0), 160 mM NaCl, 1% Triton X-100]. Both fractions were sonicated for 10 seconds followed by centrifugation in a microcentrifuge for 30 minutes at 14,000 rpm. Protease inhibitors N-acetyl-leucyl-leucyl-norleucinal (50 μg/ml), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin A (5 μg/ml), leupeptin (10 μg/ml), 1 mM Pefabloc, 10 mM DTT, and aprotinin (2 μg/ml) were added to all buffers used for preparing the cell fractions. All protein isolation procedures were performed at 4°C. Protein concentrations were determined by the BioRad DC protein assay and the samples were stored at -80ºC until further use.

Isolation of MAPK kinase proteins

Upon treatment, Caco-2 cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in 0.5 ml lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM PMSF). Cell debris was removed by centrifugation at 14,000 rpm for 10 minutes at 4ºC. Protein concentrations in the supernatants were determined by the BioRad DC protein assay and the samples were stored at -80ºC.

For Western blot analyses, 30 μg (MAPK/AKT) or 80 μg (SREBP-2) of protein were subjected to sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% polyacrylamide) before transferring onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The PVDF membrane was blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T), pH 7.4 for 1 hr at room temperature, probed with rabbit antibodies against total or phospho-p38, total or phospho-ERK1/2,

total or phospho-JNK, total or phospho-AKT, SREBP-2, and the goat antibody against actin (1:1,000 dilution) at 4°C overnight. The membrane was washed and incubated with secondary anti-rabbit (Promega) or donkey anti-goat antibodies (Promega) (1:10,000 dilution) coupled to horseradish peroxidase for 1 hr at room temperature. Antibody–antigen complexes were then detected using the ECL Plus™ chemiluminescent detection system according to the manufacturer's instructions (GE Healthcare Bio-Sciences). Band intensities were quantified using the software program Quantity One 1-D analysis software version 4.6.1 (Bio-Rad).

Reprobing the immunoblots

The immunoblots were soaked in stripping buffer (0.7% 2-mercaptoethanol, 2% SDS, 62.5 mM Tris pH 6.8) and incubated at 50°C for 30 minutes with gentle shaking. After stripping, the membranes were washed 4 times for 5 minutes with TBS-T, then blocked in 5% skimmed milk in TBS-T, pH 7.4 for 1 hr at room temperature, followed by probing with the primary and secondary antibodies of interest.

2.5. Real time RT-PCR of cholesterol biosynthesis genes

Total RNA from Caco-2 cells was isolated using TriZol reagent (Invitrogen, Breda, The Netherlands) as specified in the manufacturer's instructions. To remove any genomic DNA contaminants, RNA samples were treated with DNAse-I RNase free (Promega) followed by phenol/chloroform/isoamylalcohol (25:24:1;v:v:v) and chloroform/isoamylalcohol (24:1;v:v) purification steps. RNA concentration and purity were determined by measurement of absorbance at 260 and 280 nm using a Nanodrop (Isogen Life Science). Complementary DNA (cDNA) was synthesized from 1 μg total RNA for each sample using the iScript cDNA synthesis kit, following instructions of the manufacturer (Bio-Rad). Primers for SYBR Green probes were

designed with Beacon Designer 5.0 (Premier Biosoft International, Palo Alto, CA) and are shown in Table 4-1. After primer design, all primers were run through the National Center for Biotechnology Information (NCBI) Blast database in order to check for specificity. PCR amplification and detection were performed with the iQ SYBR Green Supermix and the MyIQ single color real time PCR detection system (Bio-Rad). Standard curves were constructed for each amplified gene sequence using serial dilutions of a reference sample from the cDNA samples known to induce selected genes significantly. The level of mRNA for each gene was normalized using Ribosomal protein L12 (RPL-12) as a reference gene, which was chosen on the basis of microarray data (data not shown) since it showed similar expression levels for control and α-chaconine-treated groups. To verify the RPL12-normalized results, data also were normalized using a well accepted reference gene Hypoxanthine phosphoribosyltransferase 1 (HPRT1). The outcome of that analysis was similar (data not shown).

2.6. Statistical analysis

Results are expressed as means ± standard deviation (S.D.). Comparisons of changes in protein levels among treatment groups were analyzed statistically by the unpaired Student's t-test (two-tailed). RT-PCR gene analysis was conducted using a two way ANOVA with the factors α-chaconine treatment (2 levels: with or without α-chaconine) and inhibitor(s) pre-treatment (5 levels: no inhibitor(s), ERK pathway inhibitor (PD98059), JNK pathway inhibitor (SP600125), PI3K/AKT pathway inhibitor (LY294002) and combination of all inhibitors). Ratios of the signal intensities of the gene of interest versus the reference gene were calculated and these ratio values were log transformed to stabilize the variance. The main effects of α-chaconine treatment, inhibitor(s) pre-treatment and interaction of α-chaconine and

inhibitor(s) on gene expression were determined**.** The effect of inhibitor(s) pre-treatment was split up in 4 contrasts between the level of 'No inhibitor(s)' versus each of the other 4 levels. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of α-chaconine on SREBP-2 protein expression and cleavage

To better understand the mechanism by which α-chaconine regulates the expression of cholesterol biosynthesis genes in Caco-2 cells, we determined its possible effect on expression of SREBP-2 at the (post)-translational level. Upon exposure of differentiated Caco-2 cells to 10 μM α-chaconine for 6 hours, nuclear and membrane fractions were isolated from the cell lysate and Western blot analysis was conducted using a specific antibody against SREBP-2. Western blotting demonstrated that the level of precursor SREBP-2 in the membrane fraction was increased significantly after treatment with α-chaconine, however there was no significant increase in the levels of the mature protein (Figure 4-1A). With respect to the abundance of SREBP-2 in the nuclear fraction, no precursor SREBP-2 could be detected (data not shown) but treatment of Caco-2 cells with α-chaconine resulted in a up to 3-fold increase in mature SREBP-2 protein levels (Figure 4-1B). However, this increase was of marginal statistical significance ($p=0.07$) due to large variation among the α-chaconine-treated samples (S.D. = $\pm$ 1.78).

Table 4-1. Primers used for quantitative real time PCR. All primers were intron-spanning.

Gene Name only	Gene Symbol	Sequence ID	Forward Primer (5′ – 3′)	Reverse Primer (5′ – 3′)
Cytochrome P450, family 51	CYP51A1	NM_000786	CAACTCAATGAAAAGGTAGCACAGC	TCTGCGTTTCTGGATTGCCTTATAG
3-hydroxy-3-methyl-Coenzyme A reductase	HMGCR	NM_000859	TTCCAGAGCAAGCACATTAGCAAAG	GCCAAAGCAGCACATAATTTCAAGC
3-hydroxy-3-methyl-Coenzyme A synthase	HMGCS1	NM_002130	ACCGCTGCTATTCTGTCTACTGC	TCTCTATTCTGGTCATTAAGGAAGTCATTC
Sterol-C5-desaturase	SC5DL	NM_006918	GTTGGTGCTTACATCCTTTATTTCTTCTG	GCCTGGACAGTAAACTTAATCTCTCG
Squalene epoxidase	SQLE	NM_003129	ACGAAGAGCCAGTATCAGAAGAGTATC	AAAGTATGTGAAGCCAAGTTGTATAGGG
Sterol regulatory element binding transcription factor 2	SREBP-2	NM_004599	AGCACCACTCCGCAGACG	GCTTTGGACTTGAGGCTGAAGG
Ribosomal protein L12	RPL12	NM_000976	GACACCAGCCGCCTCCAC	TCCAGTCACCCGTTGCCTTG

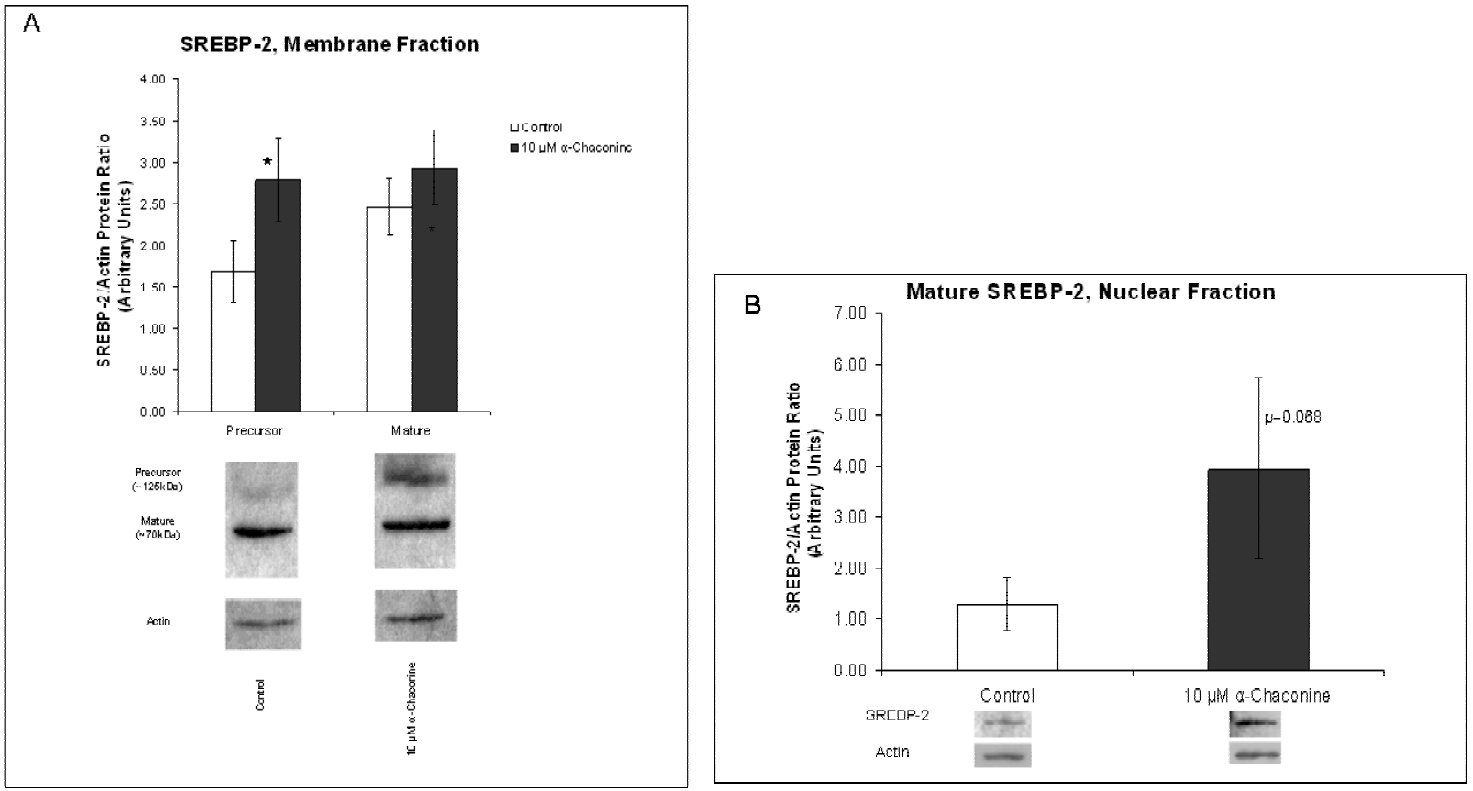

Figure 4-1. Effect of α-chaconine on SREBP-2 protein expression and cleavage. Caco-2 cells were exposed to 10 µM of α-chaconine for 6 hours. Membrane (A) and nuclear fractions (B) (corresponding to 80 µg of protein) were analyzed by Western blotting with an anti-SREBP-2 antibody. The corresponding blots were stripped and reprobed with an actin antibody for assessment of equal loading. Integrated densitometric data from 3 independent experiments are shown as a bar graph and the illustrated data represent one of the three independent experiments. Results are presented as means $\pm$ S.D. (n=3). * Significant difference from solvent control, $p<0.05$.

3.2. Effect of α-chaconine on phosphorylation of MAP and AKT kinases

Since analysis of data from previous microarray experiments indicated that several genes involved in MAPK and AKT pathways also were affected, we examined by Western blotting whether α-chaconine treatment resulted in activation (i.e. phosphorylation) of AKT and the MAP kinases ERK, JNK and p38. Exposure of differentiated Caco-2 cells to 10 μM α-chaconine for 6 hours resulted in a significant increase in phosphorylation of JNK, ERK and AKT but not p38 (Figure 4-2). Since p38 was not phosphorylated and thus might not be involved in induction of cholesterol biosynthesis genes by α-chaconine, it was not included in the subsequent kinase inhibitor experiments.

3.3. Effects of LY294002, PD98059 and SP600125 on α-chaconine-induced expression of cholesterol biosynthesis genes

In order to examine whether, and if so, which of the α-chaconine-induced phosphorylations in the MAPK and PI3K/AKT pathways are crucial for the upregulation of cholesterol biosynthesis genes, we determined the effects of cell permeable inhibitors of MEK1 (PD98059), JNK (SP600125) and PI3K (LY294002) on the expression of a number of representative genes by real-time RT-PCR (Table 4-2). LY294002 was used as PI3K is an upstream regulator of AKT (*28, 29*), PD98059 inhibits MEK1, which is a dual-specificity kinase that phosphorylates ERK1/2 (*30*), and SP600125 directly inhibits JNK. Caco-2 cells were pre-treated with either PD98059 (50 μM) or SP600125 (60 μM) or LY294002 (60 μM) or a combination of all inhibitors for 24 hours prior to co-incubation with 10 μM α-chaconine for 6 hours. As shown in Figure 4-3, each inhibitor was able to block the phophorylation of its respective kinase and α-chaconine could not overcome this inhibition (Figure 4-3).

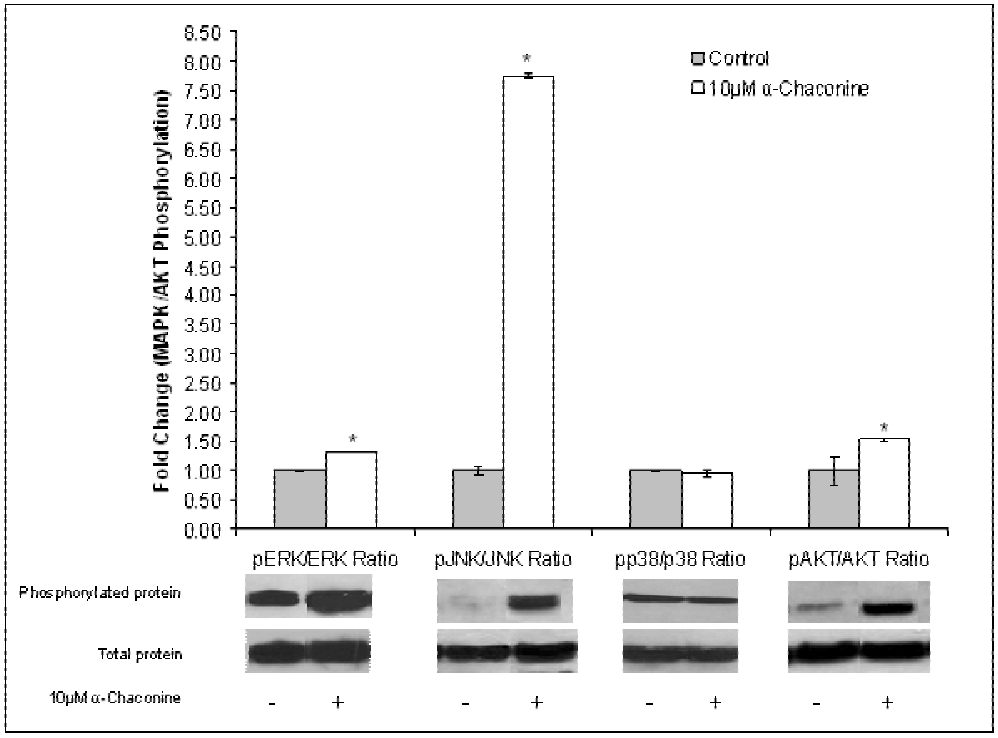

Figure 4-2. Effect of α-chaconine on ERK, JNK, p38 and AKT phosphorylation. Caco-2 cells were exposed to 10 μM of α-chaconine for 6 hours. Total cell lysates (30 μg of protein) were analyzed by western blotting for phosphorylated and total ERK, JNK, p38 and AKT. First, blots were probed for the phosphorylated forms, and then they were stripped and reprobed with a corresponding antibody against the total protein form for assessment of equal loading. Integrated densitometric data from 3 independent experiments are shown as a bar graph and the illustrated data represent one of the three independent experiments. Results are presented as means ± S.D. (n=3). * Significant difference from solvent control, $p<0.05$.

Two way ANOVA and contrast analysis of the RT-PCR data allowed the determination of the effects of α-chaconine or the inhibitors and possible interaction between α-chaconine and the inhibitors on the transcription of cholesterol biosynthesis genes (Table 4-2, Figure 4-4). In particular, determining the presence of an interaction between α-chaconine and the kinase inhibitors would reveal the possible role of the MAPK and PI3K/AKT pathways in α-chaconine-induced expression of cholesterol

biosynthesis genes. Our data showed a distinct effect of α-chaconine or inhibitor(s) on the level of cholesterol biosynthesis gene expression, with no significant interaction observed between α-chaconine treatment and inhibitor(s) pre-treatment, with the exception of the gene SC5DL (Table 4-2, Figure 4-4). Figure 4 shows that the expression of most cholesterol biosynthesis genes was increased when cells were exposed to α-chaconine in the absence as well as in the presence of inhibitors (Table 4-2, $p<0.05$). For the SC5DL gene, a statistically significant interaction effect was observed between α-chaconine and inhibitor, when all three kinase inhibitors (ERK, JNK and AKT) were simultaneously applied. A decrease in expression of this gene was observed suggesting that blocking all pathways affected the induction of this gene by α-chaconine (Table 4-2, p-value of 0.02). Kinase inhibitors that were tested, either alone or in combination, however, did not prevent the induction of the other cholesterol biosynthesis genes following α-chaconine exposure. Using the exposures without inhibitors (with or without α-chaconine) as a basis of comparison, pre-treatment of the cells with PI3K/AKT pathway inhibitor LY294002 alone or a cocktail of all inhibitors, reduced the expression levels of cholesterol biosynthesis genes significantly (Table 4-2, Figure 4-4). On the other hand, pre-treatment with the ERK inhibitor PD98059 alone, resulted in increased transcription of CYP51A1 (Figure 4-4, Table 4-2, $p<0.04$). Taken together, the overall outcome of the inhibition experiments indicate that, although particularly the PI3K/AKT signaling pathway is important for basal cholesterol biosynthesis gene expression, neither this pathway nor JNK and ERK pathways are mediating the α-chaconine-induced expression of these genes.

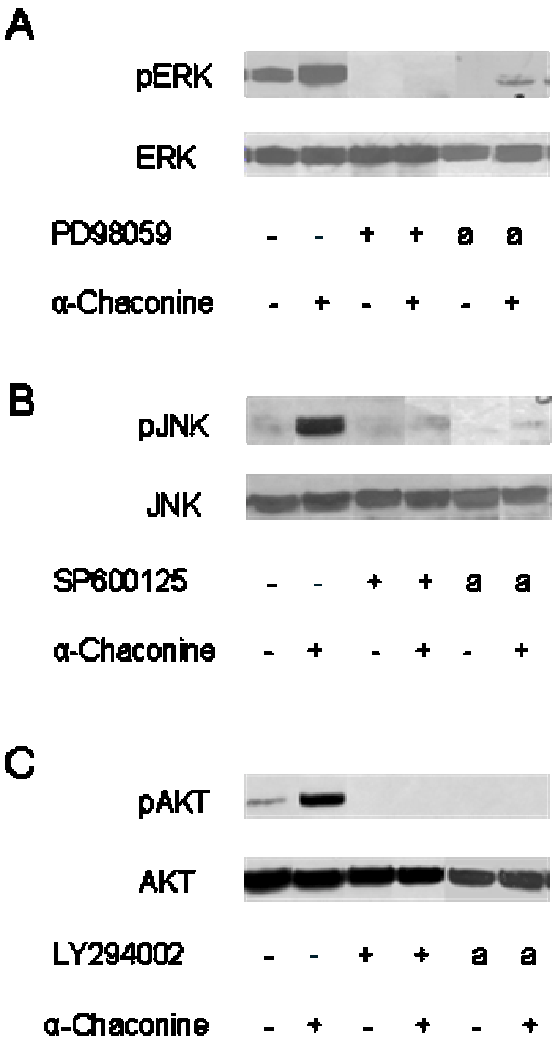

Figure 4-3. Confirmation of the inhibition of phophorylation of ERK, JNK and AKT kinases by specific chemical inhibitors. Caco-2 cells were incubated with PD98059 (50 μM; Panel A) or SP600125 (60 μM; Panel B) or LY294002 (60 μM; Panel C) or all chemical inhibitors (indicated with "**a**" in Panels A-C) for 24 hours prior to exposure to 10 μM of α-chaconine for 6 hours. Total cell lysates (30 μg of protein) were analyzed by western blotting for phosphorylated and total ERK, JNK and AKT. First, blots were probed for the phosphorylated forms, and then they were stripped and reprobed with a corresponding antibody against the all forms for assessment of equal loading. The illustrated data represent one of the three independent experiments.

Table 4-2. Effects of kinase inhibitors LY294002, PD98059 and SP600125 on α-chaconine-induced cholesterol biosynthesis gene transcription

		p-values for each gene					
Source of Variation		CYP51A1	HMGCR	HMGCS1	SC5DL	SQLE	SREBP-2
Effect of α-Chaconine (-/+ inhibitors)		0.007	<0.001	<0.001	0.014	<0.001	0.307
Effect of Inhibitors (-/+ α-Chaconine)							
	Control (No Inhibitors) vs ERK pathway Inhibitor (PD98059)	0.039	0.117	0.059	0.231	0.252	0.509
	Control (No Inhibitors) vs JNK pathway Inhibitor (SP600125)	0.718	0.672	0.918	0.105	0.836	0.509
	Control (No Inhibitors) vs AKT pathway Inhibitor (LY294002)	0.020	<0.001	<0.001	<0.001	<0.001	0.002
	Control (No Inhibitors) vs All pathways Inhibitors	0.036	<0.001	<0.001	<0.001	<0.001	<0.001
Interaction Effect : Inhibitor.α-Chaconine							
	Control (No Inhibitors).α-Chaconine vs ERK pathway Inhibitor.α-Chaconine	0.608	0.720	0.814	0.066	0.563	0.110
	Control (No Inhibitors).α-Chaconine vs JNK pathway Inhibitor.α-Chaconine	0.319	0.905	0.854	0.319	0.871	0.774
	Control (No Inhibitors).α-Chaconine vs AKT pathway Inhibitor.α-Chaconine	0.629	0.717	0.262	0.096	0.641	0.818
	Control (No Inhibitors).α-Chaconine vs All pathways Inhibitors.α-Chaconine	0.663	0.591	0.538	0.016	0.792	0.443

Caco-2 cells were incubated with PD98059 (50 μM) or SP600125 (60 μM) or LY294002 (60 μM) or all chemical inhibitors for 24 hours prior to exposure to 10 μM of α-chaconine for 6 hours. Effects of the chemical inhibitors on cholesterol biosynthesis gene transcription were analyzed by real-time RT-PCR. RPL12 was used as an internal reference. A Two way ANOVA and contrast analysis was conducted to determine significance of the effects of α-chaconine and the inhibitors on gene expression.

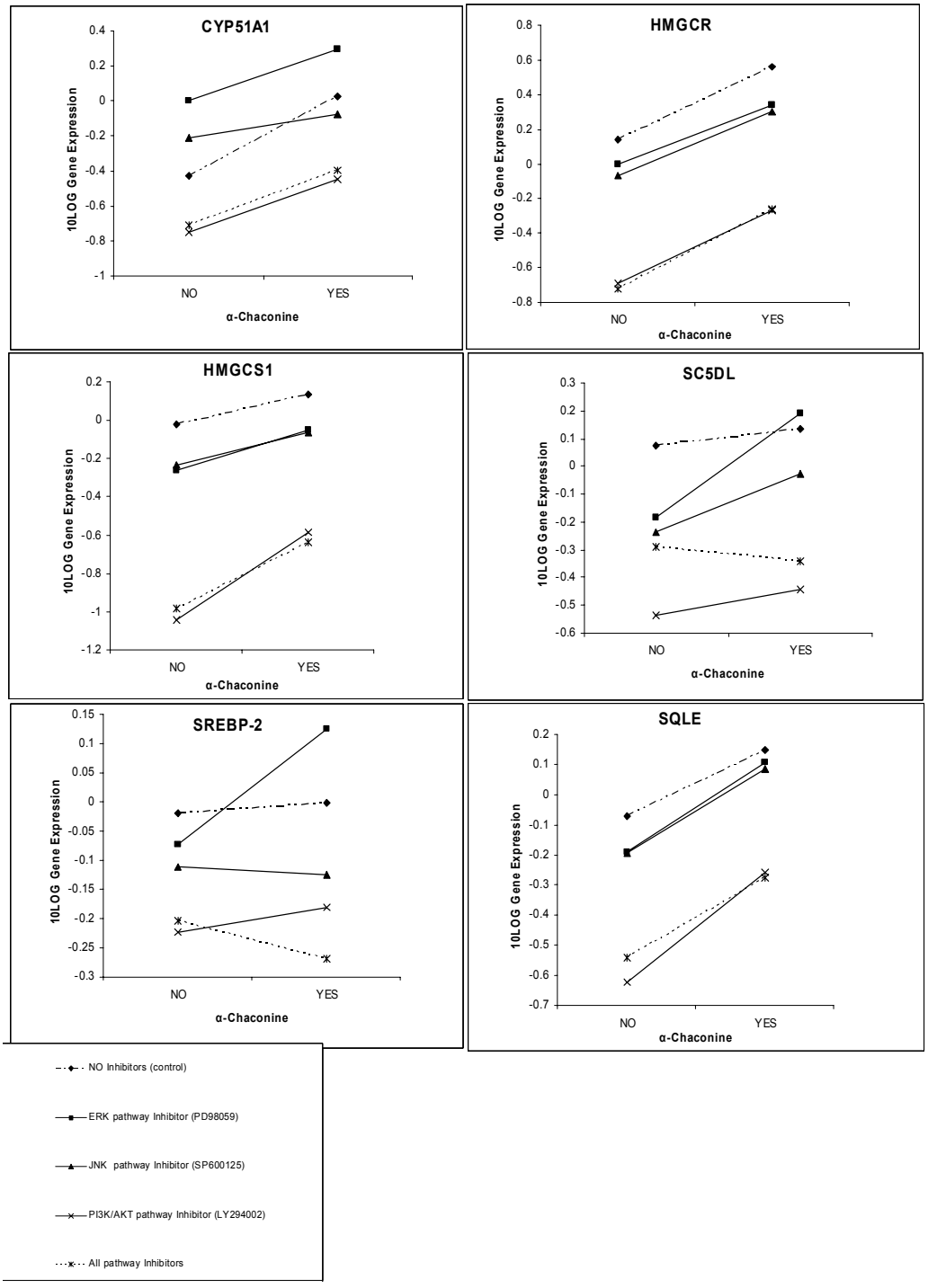

Figure 4-4. Two Way ANOVA to examine possible interaction of α-chaconine and inhibitors (ERK pathway inhibitor (PD98059), JNK pathway inhibitor (SP600125), PI3K/AKT pathway inhibitor (LY294002) and combination of all inhibitors) on expression of selected cholesterol biosynthesis gene.

Discussion

Our previous microarray studies showed that α-chaconine, α-solanine and varying mixtures of these two glycoalkaloids induce cholesterol biosynthesis genes at non-cytotoxic concentrations in intestinal epithelial cells (*13, 14*). We also found that genes in the PI3K/AKT and MAPK pathways and their downstream effectors such as Jun, Myc and Fos were upregulated. To understand the mechanisms underlying the induction of cholesterol biosynthesis genes by glycoalkaloids, the effect of α-chaconine on the expression of SREBP-2, the main regulator of cholesterol biosynthesis, was investigated. Since the MAPK and PI3K/AKT pathways have been shown to regulate the activity of SREBP-2 and consequently cholesterol biosynthesis gene transcription (*23-26*), the importance of PI3K/AKT and MAPK pathways in the induction of cholesterol biosynthesis genes by glycoalkaloids also was determined.

Our data indicate that α-chaconine regulates SREBP-2 expression at the (post-) translational level. A significant increase in SREBP-2 precursor protein levels in the membrane fraction of the Caco-2 cell lysates was observed. Although, this increase in precursor levels could be a reflection of the increase in SREBP-2 gene expression by α-chaconine, it could also be due to increased SREBP-2 protein stabilization, as normally these proteins are rapidly ubiquitinated and degraded by 26S proteosome (*31*). In addition, we also observed a 3-fold increase in mature SREBP-2 protein levels in the nuclear fraction of α-chaconine-treated cells. This increase of cleaved SREBP-2 in the nucleus is correlated to increased precursor protein levels in the membrane fraction of the cell lysates. However, other explanations for this increase can be envisaged. It is possible that the increase is (at least partially) attributed to a stimulatory effect of α-chaconine on translocation of the mature SREBP-2 fragment into the nucleus, where it activates transcription of cholesterol biosynthesis genes. The initial trigger for this stimulatory effect may be due to depletion of cholesterol in

membranes caused by the formation of glycoalkaloid/sterol complexes (*12*). Decreased membrane cholesterol, may promote a sterol mediated proteolytic cleavage that increases levels of mature SREBP-2 protein (*18*).

In the current study, Western blot analyses revealed that levels of phosphorylated AKT, JNK and ERK proteins increased following treatment of Caco-2 cells with α-chaconine. Subsequently, we determined whether the ERK, JNK or PI3K/AKT pathways were necessary for the induction of cholesterol biosynthesis genes by α-chaconine using specific kinase inhibitors. We observed a significant increase in expression of cholesterol biosynthesis genes in Caco-2 cells following α-chaconine treatment, which confirmed our previous studies (*13, 14*). Inhibiting the signaling pathways, particularly the PI3K/AKT pathway by LY294002, reduced the (basal) expression levels of cholesterol biosynthesis genes significantly, both in the absence and presence of α-chaconine. This is in agreement with findings from other studies (*23*) that indicate a role of the PI3K/AKT pathway in cholesterol biosynthesis gene regulation.

In spite of these inhibition effects, two-way ANOVA analysis revealed no significant interactions between α-chaconine and the inhibitors of ERK, JNK or PI3K/AKT pathways, indicating that the inhibitors and α-chaconine did not influence each other's effect on the transcription of cholesterol biosynthesis genes, except for SC5DL. Inhibition of all pathways reduced α-chaconine-induced transcription of SC5DL. Given that five out of the six genes analyzed did not show a significant interaction effect between inhibitors and α-chaconine, we concluded that activation of these pathways is not crucial for glycoalkaloid-induced transcription of cholesterol biosynthesis genes but may have a modulatory role (i.e. enhancer effect). The results suggest that α-chaconine induces cholesterol biosynthesis gene expression through other more pivotal mechanisms, which may be independent of the MAPK and

PI3K/AKT pathways. We observed that α-chaconine treatment resulted in an increase of precursor and mature SREBP-2 in the membrane and nuclear fractions of Caco-2 cell lysates, respectively. It still remains to be determined whether these changes in the level of SREBP-2 are linked to a mechanism that is crucial for α-chaconine-induced expression and acts independently of the MAPK and PI3K/AKT pathways.

Thus far, the precise mechanisms underlying the toxicological and potential beneficial effects of glycoalkaloids are understood poorly. As indicated in the present study, the PI3K/AKT, JNK and ERK signaling pathways were affected by α-chaconine and may play a role in some of the observed effects. It is likely that the biological processes affected by these pathways are dependent on the cell type, glycoalkaloid concentration, and the status of other signal transduction pathways. For instance, Yang and co-workers (*32*) observed that in HT29 cells, α-chaconine induced apoptosis, which may be mediated through the suppression of ERK1/2 phosphorylation and subsequent activation of caspase 3, whereas we have observed increased phosphorylation of ERK1/2.

In conclusion, this study indicates that α-chaconine induces phosphorylation of AKT, ERK and JNK in intestinal epithelial cells. However, these phosphorylation events are not necessary for the induction of cholesterol biosynthesis genes by α-chaconine. Since these signaling pathways play a central role in many diverse cellular processes and possibly influence glycoalkaloid toxic outcomes, further studies of their roles in other glycoalkaloid-induced effects, e.g., apoptosis and cell cycle effects (*13, 14*) would be informative. The present work contributes to the knowledge base of the molecular mechanisms of action of glycoalkaloids and may lead to a better understanding of their toxic and possible beneficial effects.

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CHAPTER 5

CONCLUSION

The specific aims of this dissertation were first, to determine the molecular mechanism(s) underlying glycoalkaloid-induced effects, in particular their membrane disruptive effect in intestinal epithelial cells. Second, to evaluate the usefulness of DNA microarrays in discriminating individual glycoalkaloids and glycoalkaloid mixtures of varying α-chaconine/α-solanine ratios based on their differences in effect severity and potential toxicities. We demonstrated that DNA microarrays can be useful in identifying hitherto unknown mechanisms of action, identifying potential toxicity biomarkers and assessing the effects of mixtures of compounds. These studies contribute towards a better insight into the toxic and potential beneficial effects of potato glycoalkaloids. This understanding may enhance current efforts to develop transgenic potatoes with altered glycoalkaloid levels and/or ratios. In addition, by exploring the application/sensitivity of transcriptomic techniques in identifying early indicators of toxicity and screening between similar class/effect compounds, these studies clarify the potential application of this technique to "whole foods" assessments incorporating different models of impaired nutritional states.

This chapter summarizes the dissertation's main findings, implications and future perspectives.

A. Main findings

i. Detection of glycoalkaloid-induced transcriptional effects in Caco-2 cells

Analysis of data of the gene expression profiling studies revealed that in differentiated Caco-2 cells, potato glycoalkaloids affect several genes and processes involved in cholesterol biosynthesis, apoptosis, cell cycle, lipid metabolism, amino

acid, growth, chemokine and MAPK and PI3K/AKT signaling. The interpretation of these data was strengthened by anchoring observations of transcriptional effects with functional endpoints. Conventional toxicological or molecular assays, i.e., LDH assays, apoptosis and cell cycle analyses experiments, RT-PCR and Western blot analyses were conducted to interpret and confirm observed effects at the gene expression level.

The most prominent and consistent finding was the induction of transcription of cholesterol biosynthesis genes in intestinal epithelial cells by potato glycoalkaloids. Chapters 2 and 3, examine the induction of these genes by non-cytotoxic glycoalkaloid treatments and the repression of their induction by higher, cytotoxic concentrations. This was explained, in part, by glycoalkaloids' well described mechanism of membrane disruption (*1-3*). It was concluded that early induction of cholesterol biosynthesis genes likely reflects a "rescue" response to glycoalkaloid toxicity that prevents cells from dying.

Several genes involved in cell cycle progression and apoptosis also responded to glycoalkaloid treatments. Chapter 3 describes cell cycle and apoptosis analyses experiments designed on the basis of those observations. 5-20μM α-chaconine resulted in disproportionate accumulation of Caco-2 cells in the G_2/M phase. On the other hand, a more cytotoxic concentration of 100 μM resulted in cell accumulation in the G_0/G_1 phase, which was in agreement with a previous study that showed that prolonged exposure of HT29 cells to 6 μM α-chaconine resulted in accumulation of cells in the sub G_0/G_1(*4*). Hence, the glycoalkaloid-induced effects on the cell cycle appeared to depend on the cell type, exposure duration and glycoalkaloid concentration. Annexin V assays confirmed gene expression data and revealed that glycoalkaloid exposure results in late-apoptotic or necrotic death of Caco-2 cells, which was in agreement with observed LDH assay results.

ii. Elucidation of mechanism(s) of action/effect

DNA microarray technology serves as a useful tool for generating and/or focusing hypotheses. In Chapter 2, we proposed that non-cytotoxic glycoalkaloid concentrations induce the formation of glycoalkaloid/sterol complexes in cellular membranes that, in turn, result in the depletion of cellular cholesterol resulting in disruption of lipid rafts. We also proposed that lipid raft disruption also affects several growth, MAPK and PI3K/AKT pathways, subsequently inducing cholesterol biosynthesis gene expression. Chapter 4 describes experiments designed to test the role of MAPK or PI3K/AKT pathways in α-chaconine-induced transcription of cholesterol biosynthesis genes in Caco-2 cells. Western blot analyses revealed that although α-chaconine induced the phophorylation of JNK, ERK and AKT proteins, these pathways are not crucial for α-chaconine-induced transcription of cholesterol biosynthesis genes. Therefore, it can be postulated that other mechanisms, which may be independent of the MAPK and PI3K/AKT pathways, including possibly (post)-translational effects of α-chaconine on the expression of SREBP-2 may be more pivotal for the observed induction of cholesterol biosynthesis genes (see below). Also in Chapter 4, the possible effect of α-chaconine on expression and processing of the transcription factor SREBP-2, which is known to function as key regulator of cholesterol biosynthesis genes (*5*), was investigated. We observed that α-chaconine treatment resulted in an increase of precursor and mature SREBP-2 in the membrane and nuclear fractions of Caco-2 cell lysates, respectively. It still remains to be determined whether these changes in the level of SREBP-2 are linked to a mechanism that is crucial for α-chaconine-induced expression and acts independently of the MAPK and PI3K/AKT pathways (see also Biii).

Although we observed that the MAPK and PI3K/AKT pathways were not required for α-chaconine-induced transcription of cholesterol biosynthesis genes, they

play a central role in many diverse cellular processes, for example the cell cycle, apoptosis and cell differentiation, and therefore can influence toxic outcomes. It is likely that the toxicological outcomes/effects of glycoalkaloids may depend on the status of these signaling pathways, cell phenotype and the concentrations of glycoalkaloid exposure.

iii. Detection of signature expression patterns and subtle effect differences

Gene expression profiles potentially identify signature patterns that reflect cellular responses to treatments/exposures. Chapter 3 compared transcriptional responses of Caco-2 cells to treatments with equimolar concentrations of the two main potato glycoalkaloids, α-chaconine and α-solanine, and their mixtures thereof. Principal component and hierarchical analyses revealed clustering or separation of the gene expression profiles of the different glycoalkaloid treatments, which correlated well with their severities of effect on lactate dehydrogenase membrane leakage in Caco-2 cells. Gene expression analyses based on the pathway analysis tools, MetaCore and ErmineJ revealed that glycoalkaloid treatments affected similar pathways and processes. Thus, we concluded that observed differences in gene expression profiles reflected differences in effect intensity. This confirmed previous studies (*6-12*) demonstrating that potato glycoalkaloids act by the same mechanism of action but differ with regard to potency. α-Solanine was the least potent of the two glycoalkaloids, whereas for the glycoalkaloid mixtures, confirmation that at certain α-chaconine/α-solanine ratios, there is increased toxicity/potency due to synergism. To varying extents, DNA microarrays used in conjunction with LDH assays discriminated among the glycoalkaloid treatments on the basis of effect severity, thus demonstrating DNA microarray technology's potential for detecting subtle differences in the toxicities of individual compounds, mixtures, and/or possibly of whole foods.

These studies also observed overlaps in the gene expression profiles of the various glycoalkaloid treatments. Several genes involved in the cell cycle, apoptosis, growth signaling, MAPK and PI3K/AKT signaling and lipid metabolism were particularly common in these expression profiles. Such overlapping gene lists potentially provide a basis for reliably identifying (specific) biomarkers of glycoalkaloid toxicity, however further studies are required to fully explore this possibility.

B. Implications and Future perspectives

i. Systems biology approach

Understanding the transcriptional effects of glycoalkaloids is an initial step in determining their mechanism(s) of action, however, a 'systems biology approach' (*13*) is necessary for a more complete insight. As shown in Chapter 4, some of the effects of glycoalkaloids may occur at the protein level, for example phophorylation of JNK, ERK and AKT proteins and possibly proteolytic cleavage of SREBP-2, or at the metabolome level (interactions of glycoalkaloids with (and the generation of) other metabolites). Therefore, to obtain a holistic picture of the mechanisms of action of glycoalkaloids, and generally of other toxins, studying effects at the genome, metabolome and (phospho) proteome levels is essential.

Chapter 3 demonstrates the potential use of microarrays in combination with classical toxicological tests in testing compounds with similar chemical structures, mechanism(s) of action and present in different food/chemical component ratios. As this technology has the potency to provide early and sensitive effect indicators, it may be used to screen for subtle differences of whole foods, for example the assessment of the substantial equivalence of conventionally bred and genetically modified crops. However, in whole foods/mixtures and diets, food components act simultaneously in

constantly changing combinations resulting in different gene expression profiles, which may make the quest to identify signature gene expression patterns and to translate these to safety of food components difficult. In spite of this challenge, application of a systems biology approach whereby the effects of glycoalkaloids are assessed in the context of the diet or the whole potato and at relevant doses of intake (*13*) is essential and likely will provide a fuller understanding of the effects of dietary mixtures.

Another likely important determinant of toxicological outcomes is the nutritional status of the consumer. For instance, studies on potato glycoalkaloids have shown that folate, glucose-6-phosphate and nicotine adenine dinucleotide phosphate reduce glycoalkaloid-induced developmental toxicity in frog embryos (*14, 15*). Given that potatoes represent a major nutritional part of the diet of many individuals, further studies determining the effect of nutritional status on glycoalkaloid-induced toxicities are recommended. In this respect, in particular the effect of folate nutriture on glycoalkaloid-induced toxicities provides an interesting model for examining such relationships.

ii. Identification of glycoalkaloid specific effects

Gene expression profiling experiments generally produce many data. Reducing these data to useful information remains a challenge, for example distinguishing adaptive/reversible responses from early specific glycoalkaloid-induced toxicities. In order to define the conditions resulting in reproducible and specific gene responses (i.e responses not due to cytotoxicity), in Chapter 2 Caco-2 cells were exposed in three independent experiments to various concentrations of α-chaconine for 2, 4, 6 and 24 hours, which were conditions representing various degrees of membrane disruption. Based on the outcome of these experiments, Chapters 3 and 4 mainly focused on a

concentration (10 μM) and exposure duration (6 hour) at which minimal membrane disruption and maximal relevant gene expression effects were observed.

Studies have shown that gene expression profiles also vary depending on tissue or cell type, for instance, hexachlorobenzene elicited different gene expression profiles in various organs in Brown Norway rats (*16*). Although the intestine is the main target of glycoalkaloids, determination of their effects in other tissues and cell lines may be necessary to identify cell or tissue specific responses. Comparisons of glycoalkaloid-induced gene effects with those of other known chemical class toxicants (particularly membrane disruptors; see below) also are essential. These will help to better assess the significance and/or specificity of glycoalkaloid-induced effects. Determining cell- , tissue- and glycoalkaloid-specific effects will help elucidate more fully mechanism(s) of effect, identify previously unknown functions (beneficial or deleterious) and assist in the identification of glycoalkaloid toxicity biomarkers.

iii. Confirmation of hypotheses

Chapter 2 described the hypothesis that glycoalkaloids deplete cellular cholesterol subsequently leading to lipid raft disruption and activation of various signaling pathways. Unfortunately, time constraints and technical problems, precluded examining glycoalkaloid effects on lipid rafts. Nevertheless, we compared the gene expression effects of α-chaconine in Caco-2 cells with those of other well known membrane disrupters and detergents (saponin, Triton X100, SDS, filipin and methyl-β-cyclodextrin). We observed that α-chaconine and methyl-β-cyclodextrin, which is a well known cholesterol-depleting agent that results in lipid raft disruption (*17-22*), exhibited similar gene expression profiles (data not shown). Lipid raft isolation experiments using sucrose gradient centrifugation and confocal microscopic visualization and analysis of lipid raft domains by fluorescence tagging with for

example FITC-CTx, which selectively binds to the lipid raft marker ganglioside GM1 (*23*), could be carried out to assess effects of glycoalkaloids on lipid rafts.

In addition, as already mentioned before under Aii, the precise mechanism of α-chaconine-induced transcription of cholesterol biosynthesis genes remains unclear. Further elucidation of the mechanism of proteolytic cleavage and possibly protein stabilization of the SREBP-2 are necessary, for example by analysis of SREBP-2 processing by SCAP-insig1-binding assay and effect of α-chaconine in SCAP-deficient cell lines. Also 26S proteosome inhibition experiments would be informative.

iv. Extrapolation of *in vitro* results to *in vivo*

This dissertation explored the effects of glycoalkaloids *in vitro*; these studies should be followed by assessments of their *in vivo* relevance. Though not included in this dissertation, we in collaboration with the Danish National Food Institute conducted *in vivo* experiments, whereby the transcriptional effects of 33.3mg of total glycoalkaloid in α-solanine/α-chaconine ratios of either 1:70 or 1:3.7 on epithelial cells from the small intestine of Syrian hamsters were determined. The treatments were administered by gavage for 28 days. In this hamster study, and in contrast to the *in vitro* studies, we did not observe an increase in expression of cholesterol biosynthesis genes. This discrepancy may have been a consequence of cross hybridization of hamster RNA on to human arrays (hamster-specific arrays were not available). However, a down regulation of genes involved in lipid metabolism and bile acid biosynthesis pathways was observed and can be explained in the light of depletion of cholesterol from cell membranes due to the formation of glycoalkaloid-sterol complexes. Moreover, significant down regulation of several genes involved in sphingolipid metabolism was identified by MetaCore software. Other studies have

shown parallel increases in sphingomyelin and cholesterol content in various models including Caco-2 cells (*24*). Sphingomyelin, one of the main forms of sphingolipids and a main constituent of lipid rafts (*25*), was shown to have a high affinity for cholesterol and may be a major player in maintaining cholesterol in plasma membrane compartments (*26*). In particular, sphingomyelin phosphodiesterase 3 (SMPD3) (sphingomyelinase), a gene that initiates sphingomyelin catabolism (*27*) was significantly down regulated in our hamster study. Thus, intestinal cells may preserve sphingomyelin levels by reducing sphingomyelin catabolism. However, further studies are necessary to allow extrapolation of observed *in vitro* effects to the *in vivo* situation.

D. Conclusions

This dissertation demonstrates the potential application of DNA microarray technology in food safety assessments, i.e. to identify known and thus far unknown effects of single or multiple-mixture compounds and to elucidate mechanism(s) of action. Although, the results obtained did not indicate that this technology was more sensitive than current assays, for example cell leakage, they demonstrated an added advantage of detecting subtle toxic differences. This advantage is particularly relevant to whole food testing for which conventional toxicology tests are frequently insufficient. This dissertation also illustrated that DNA microarray experiments allow the definition of hypotheses on candidate markers of toxicity and mechanisms of action, which can be further addressed using a systems biology approach in which various other aspects and techniques are incorporated. With respect to safety assessment of food, such an approach will be particularly attractive when for example the nutritional status of consumers is suspected to play a role.

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