ABSTRACT

JACKSON, KIMBERLY MARIE PALATINI. Anti-Diabetic Effect of Bitter Botanicals. (Under the direction of Dr. Slavko Komarnytsky.)

Type 2 Diabetes (T2D) affects millions of people worldwide. Although Metformin and similar drugs have arisen as popular treatments in the past few decades, centuries of traditional medicine have utilized botanical remedies. Early diagnosis of T2D allows for dietary and lifestyle interventions to reverse onset and identify the mechanisms through which botanical drugs benefit human health. This dissertation addresses the process of botanical drug development and explores specific botanicals for their potential to alleviate the progression of T2D.

Chapter 1 overviews the drug discovery process and examines the development and approvals of the first two botanical prescription drugs. Veregen (2006) and Fulyzaq (2012) provide the framework for developing new therapies from natural complex mixtures in the US, where the Food and Drug Administration requires botanical products marketed as drugs meet rigorous standards equivalent to non-botanical drugs for quality, safety, and efficacy. The new drug application (NDA) process for botanicals could be shorter, but extensive requirements of botanical drug development are exacerbated by lack of oversight on botanical dietary supplements.

In the interest of botanical drug development, Chapter 2 examines anti-hyperglycemic activity of chicory caffeoylquinic acids. Three di-O-caffeoylquinic acids suppressed hepatic glucose production in H4IIE rat hepatoma cells by reducing expression of key enzymes that regulate hepatic gluconeogenesis. Comparisons between CQAs and metabolites revealed that the caffeic acid moiety accounts for observed effects. Further analysis suggests activation of PI3K and MAPK pathways, and increased mitochondrial respiration, a finding shared between caffeoylquinic and caffeic acids.

Chapter 3 explores acute and persistent effects of dietary chicory supplementation on glucose and lipid metabolism in the polygenic C57BL/6J mouse model of diet-induced obesity. Acute oral administration of aqueous extract at 100- 300 mg/kg resulted in a dose-dependent decrease in fasting blood glucose. However, supplementation with chicory extract at 1% of low- or high-fat diet for 20 weeks did not improve long-term hyperglycemia, but did reduce hepatic triglycerides. A STC-1 cell culture model of intestinal glucose uptake indicates acute anti-hyperglycemic effects of chicory root matrix were primarily mediated by delayed intestinal absorption of carbohydrates and potentiated by bitter sesquiterpene lactones.

The anti-hyperglycemic effect of many bitter botanicals is explored in Chapter 4. Bitter compound size, glycosylation status, and chemical class each had an effect on plasma glucose levels in C57BL/6J mice. The STC-1 model showed decreased glucose absorption and greater Ca^{2+}flux, indicative of G- protein coupled receptor signaling following treatment with bitter compounds. Probenecid, a Type 2 Receptor (T2R) antagonist, restored glucose absorption in both the cell and animal model. Together, these data suggest stimulation of T2R signaling in the gastrointestinal tract by bitter compounds affects hyperglycemia associated with metabolic syndrome.

Chapter 5 compares the hepatoprotective activity of two alternative extracts prepared from bulbs of Easter lily. A hydroethanolic crude bulb extract (CB) and a steroidal glycoside-rich 1-butanol extract (BuOH) were evaluated in a 24-week study in KK.Cg-Ay/J T2D mice. Animals that received CB extract (1%) and BuOH extract (0.1% or 0.2%) in drinking water while on high-fat diet exhibited significantly improved liver function. Oral glucose tolerance tests (OGTT) demonstrate that CB improves glucose metabolism in C57BL/6J mice. These data suggest steroidal glycosides in the hepatoprotective activity of the two BuOH extracts, while the results of the total cholesterol measurements and OGTT implicate other constituents present in the CB extract for its hypocholesterolemic and hypoglycemic activity.

Collectively, these findings support botanical extracts as effective dietary agents to improve health. Bioactive phytochemicals described herein reduced glycemic response through T2R signaling and improved overall liver function in both animal and cellular models of T2D. Thus, this dissertation highlights possible novel targets for future botanical supplement development.

Anti-Diabetic Effects of Bitter Botanicals

by
Kimberly Marie Palatini Jackson

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APPROVED BY

_____	_____
Dr. Slavko Komarnytsky Committee Chair	Dr. Jack Odle Committee Vice Chair
_____	_____
Dr. Joan Eisemann	Dr. Rodolphe Barrangou

_____
Dr. Massimo Iorizzo

DEDICATION

This work is dedicated to all the people I love, for their constant support and encouragement at every step and stumble.

BIOGRAPHY

Kimberly Palatini Jackson was taught to love science at a very early age. Rainy day kitchen chemistry experiments encouraged curiosity and time spent with her mom in the lab only made the love grow. By early high school she was certain she wanted to study biochemistry, and with the support of her parents and some phenomenal teachers, she enrolled at Rutgers University to do just that.

As she progressed through the degree she enjoyed the basic sciences, but she was sure that a career in medicine or pharmacy wasn't her calling. She wanted a path where she could apply her biochemistry degree to a model more relevant than Drosophila. During her junior year she took an Advanced Nutrition course with Dr. Malcom Watford and was hooked. She declared Nutrition as her minor and started planning for graduate school.

At the same time, Kim joined the lab of Dr. Ilya Raskin and Dr. Slavko Komarnytsky, attracted by the idea of early drug discovery from food and plant sources. She started her own project and got to learn so many new skills with the teaching of some wonderful lab-mates. By the end of senior year she completed her senior thesis project, and accepted a position in Dr. Komarnytsky's lab at NCSU's Plants for Human Health Institute to continue that project.

Through her graduate career, Kim has grown to appreciate the importance of nutrition even more as she has worked to understand the beneficial properties of edible plants on the prevention and progression of Type 2 Diabetes. She is excited about the potential for this work and hopes to continue research on the nutritional potential of botanical compounds in the pharmaceutical industry.

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CHAPTER 1: EVIDENCE BASED EVALUATION OF QUALITY, SAFETY, AND EFFICACY OF BOTANICAL DRUGS

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Abstract

The approvals of first two botanical prescription drugs, Veregen (2006) and Fulyzaq (2012), mapped the framework to developing new therapies from natural complex mixtures in the US. To encourage and facilitate botanical drug development, the Food and Drug Administration (FDA) also published industry guidelines in 2004 and revised them in 2015. Under these regulations, botanical products intended to be marketed as drugs in the US are expected to meet the same standards as non-botanical drugs for quality, safety, and efficacy. While this is relatively straightforward for the clinical efficacy evaluation, botanical product quality and safety assurance require additional evidence-based chemistry, manufacturing and controls (CMC) data for raw materials, batch-to-batch consistency, and early adoption of clinically relevant bioassays. Extensive history of prior human use may reduce the early requirements for nonclinical pharmacology and toxicology for the initial (Phase I and II) clinical studies, but these data will be required for the subsequent Phase III and the final marketing approval. As a result, many botanical manufacturers in the US choose to develop and market their products as dietary supplements (i.e. Estrovera launched in 2010), while maintaining strict evidence-based preclinical and clinical programs to evaluate their safety and efficacy. For many botanical drugs, the path to new drug application (NDA) approval has the initial potential to be cheaper and shorter, but the lack of meaningful exclusivity to existing and less costly botanical dietary supplements is a significant disadvantage to rigorous botanical drug development.

1. Introduction

In the US, botanical products with health-related claims can be marketed as conventional foods, dietary supplements, or drugs, depending on the specific claim (Table 1) [1]. These specifications were passed in the Dietary Supplement Health and Education Act (DSHEA) of 1994 and further discussed in the Food and Drug Administration (FDA) industry guidelines of 2004 and 2015 [2]. Under the DSHEA, a botanical product is only considered a drug if it bears a disease claim [3]. This is different from the approaches adopted by European and Canadian regulatory authorities, which put botanical remedies in their own category with unique standard of review. Although manufacturers of botanical products cannot legally make disease claims without the approval of a new drug application (NDA), unsubstantiated medical uses for many botanicals in the US are well known and often promoted in literature and on the Internet. Since many people other than manufacturers can place overt disease claims into the community, it is inevitable that botanical products are often used by self-medicating consumers as drugs without regulatory assurance of efficacy and safety [4].

Historically, botanicals were well-established medicines listed in the US Pharmacopeia (USP) and prescribed by physicians, but this practice ended with strict drug safety and efficacy guidelines issued by FDA in 1962. Only few botanical medicines were grandfathered and allowed to remain on the market as long as their ingredients and labeling remain the same, for example Lydia E. Pinkham's Vegetable Compound was first marketed in 1875 and is still on the market today. The rapid removal of botanical remedies from the USP pushed many of the remaining products to the over-the-counter (OTC) drug marketplace under Category II (not recognized as safe or effective) or Category III (safety and efficiency not yet determined) status. Once FDA completes the ongoing OTC drug review, few botanicals are expected to remain on the OTC active ingredients due to lack of

commercial sponsors and required safety and efficiency data. Since FDA does not consider safety and efficacy of botanical remedies sold in foreign countries as sufficient evidence for the OTC drug candidacy in the US, the modern botanical industry has chosen to develop and market their products as foods (dietary supplements), some of which could be found on the Generally Recognized as Safe and Effective (GRASE) list of 1968.

In order to differentiate and best evaluate drug products derived from botanical sources and aimed at the diagnosis, cure, mitigation, treatment, or prevention of a disease, the Center for Drug Evaluation and Research (CDER) at the FDA developed specific regulatory guidelines that apply only to botanical products intended to be developed and used as "botanical drugs" [2]. This category includes materials derived from plants, algae, macroscopic fungi, and combinations thereof and excludes i) products that contain animals or animal parts and/or minerals, except when these are a minor component in a traditional botanical preparation; ii) materials derived from botanical species that are genetically modified with the intention of producing a single molecular entity; iii) products produced by fermentation of yeast, bacteria, plant cells, or other microscopic organisms, including plants used as substrates, if the objective of the fermentation process is to produce a single molecular entity; and iv) highly purified substances, either derived from a naturally occurring source (e.g. paclitaxel) or chemically modified (e.g., estrogens synthesized from yam extracts) [2].

These guidelines also consider the complex nature of botanical therapies and facilitate the development of new therapies from botanical sources. This is especially important because a conventional drug approved by the FDA has a single, well-characterized active ingredient. In contrast, botanical drug products often have unique features, complex mixtures, and lack of a distinct active ingredient. They are traditionally delivered in multiple forms, including teas, powders, topical gels, and poultices with variation between

individual preparations. It was therefore critical to provide an alternative path for developing these botanical drug products under the strict quality, safety, and efficacy regulations of prescription drugs. Such an integrated approach is best explained and illustrated through the FDA's experience with the first two botanical NDAs (Fulyzaq and Veregen) when compared to a dietary supplement (Estrovera) that was developed following the similar evidence-based preclinical and clinical programs, but has not been subjected to the FDA review and approval (Table 2).

2. Quality, safety, and efficacy of conventional drugs

Before administering an investigational drug to human volunteers, a sponsor must file an investigational new drug (IND) application with the FDA. The IND application includes chemical and manufacturing data, animal test results, and incorporates all pharmacology and safety data gathered during the preclinical stage. These typically include an array of genotoxicity, reproductive toxicity, repeat-dose toxicity in two mammalian species (one rodent), and carcinogenicity (unless exempt for some short-term indications) studies. Toxicokinetic studies to support systemic exposure and safety pharmacology using screens for modes/sites of action are also required. The IND also must explain the rationale for testing a new compound in humans, strategies for protection of human volunteers, and a plan for clinical testing. The FDA has 30 days to interfere before the company can proceed with Phase I testing [4].

Phase I clinical trials include between 20 and 100 individuals, needing both healthy individuals and those with the disease or condition. At this stage, researchers attempt to understand how the drug interacts with the body, effective dosing ranges, and potential side effects associated with different doses. This data provides early information about how effective the new drug is to determine the most appropriate dose, limit risk, and maximize

benefits. Seventy percent of drugs that reach this stage progress to Phase II testing [3,4]. Phase II trials are larger and include several hundred volunteer patients. Despite a greater number of participants, these tests still are not large enough to definitively demonstrate benefits of the drug. Instead, the goal is to gather additional safety data. The new drug can be evaluated for interactions with other medications and on patients with additional complications. Thirty-three percent of drugs that reach this stage progress to Phase III testing [3,4].

Phase III trials include hundreds and often thousands of patients in a study that lasts between one and four years. These trials are large enough to show if a drug will be beneficial to a specific population with the targeted disease or condition. Phase III studies provide safety data that had previously gone unrecorded due to short trial times or small populations. More patients and longer prescriptions allow rare or long-term side effects to present [4]. Often, more than one Phase III trial is required before sponsors can progress to submitting a new drug application (NDA). An NDA is the combined clinical, pharmacological and toxicological data gathered during all three phases of the clinical trial. On average, it takes 3 years from NDA submission before FDA review. In total, bringing a new chemical entity (NCE) to market takes 10 to 15 years, on average, and costs nearly a billion dollars [4].

3. Botanical drug approval: totality of evidence approach

The same FDA staff that oversees approvals of conventional drugs also reviews applications for botanical drugs. However, to ensure consistent implementation of botanical drug guidance, CDER/FDA additionally established the Botanical Review Team (BRT) in 2003. The BRT provides scientific expertise on botanical issues to the reviewing staff, guarantees consistent interpretation and implementation of the Botanical Guidance,

consolidates experiences in regulatory review of botanical applications, and compiles information on the status of botanical drug submissions for agency management [2]. Additionally, the BRT provides assistance to sponsors of botanical applications in the interpretation of the regulations and their interaction with the FDA [1]. The purpose of the BRT review is to provide historical background of the botanical to help the clinical review division better understand the product and search for information that may be relevant to the new use but not submitted in the application.

Biology of the medicinal plants, pharmacology of the botanical product, and prior human experience with the botanical product are very complex and often contradictory issues. Despite their substantial prior human use, the complexity of these treatments makes conventional clinical trials difficult and expensive. To address these challenges, the FDA has developed the "totality of evidence" approach based on knowledge and expertise acquired from the review of botanical IND and NDA submissions. In addition to the conventional chemistry, manufacturing and controls (CMC) data, this integrated approach considers other evidence including raw material control, clinically relevant bioassays and other non-CMC data deemed necessary by the BRT. The degree of reliance on these other data for controlling consistency of quality depends on the extent to which the botanical mixture can be characterized and quantified. For example, early adoption of clinically relevant bioassays provides a measure of overall potency of the botanical product, while demonstration of clinical dose response minimizes concern over batch-to-batch variability (**Table 3**).

4. Unique aspects of botanical drug approval process

Capitalizing on knowledge of botanical products could present an opportunity for a cheaper alternative to the costly process of conventional drug approval. The advantage of botanical drugs lies in their ability to be directly evaluated for clinical efficacy first, rather

than being subjected to pre-clinical testing. Once efficacy is proven, these products can be developed either as standardized heterogeneous mixtures or as purified single-chemical drugs. The novelty of the botanical drug approval process is, therefore, in taking advantage of prior history of human use to jump-start the drug development process. For many botanical preparations, extensive human experience can provide some degree of comfort regarding safety, but these past human experiences have rarely been documented with rigorous scientific standards in mind. They are often abundant but mostly anecdotal and of poor quality. It is therefore challenging to determine how these types of human data can substitute for conventional animal toxicity studies in the safety evaluation.

As the traditional uses of many botanical products are largely based in theory and practice of alternative medicine, interpretation of these experiences has been a problem in designing clinical trials. In standard references, the pharmacology of botanical products is typically complicated because of the complexity of the natural mixtures [2]. The products are often indicated to treat a wide variety of seemingly unrelated symptoms, without reference to the mechanism of action or the effect on the underlying diseases. Furthermore, in alternative medicine, the definitions of diagnoses, symptoms, and treatment-related adverse events are often vague and difficult to understand or correlate with medical terminology [1].

Botanical products derived from multiple or even single plants are complex mixtures of numerous chemical entities. Botanical drugs may contain a single part of one plant, multiple parts of the same plant, or different parts from many plants [5]. Regulations require that the contribution of each component of the fixed combination be shown [2]. Although each of the individual plants in combination may have been used widely, either alone or in combination with other plants, reasons for combining many plants in the specific product are often unclear, and there is rarely good evidence of a contribution to effectiveness. Even

for extensively studied plants, only a small fraction of the constituents have been isolated and identified. Complete characterization of each individual compound in botanical drugs, even from just a single plant, remains a daunting task, resulting in the chemical composition of botanicals being only partially defined [1,5]. Strength and potency of these vaguely defined products are difficult to determine and quantify, especially over time, adding to the difficulties in CMC controls and clinical pharmacology studies.

The crucial question for approval of botanical drugs is whether the future-marketed batches will have the same therapeutic effect as that observed in clinical trials. Batch-to-batch variation is a common problem that can occur for multiple reasons, making the final product differ significantly between producers. Because plant growth and composition can be affected by soil, weather, seasonal variations, geographic location and other agricultural practices, tight controls must be imposed on when and where plants can be grown [1]. Not only does that control for the presence of the active compounds but also the presence of contamination from trace heavy metals, residual pesticides, and infectious micro-organisms [5]. Additionally, the variation in processing methods and their potential influence on the therapeutic effects further complicates the quality of botanical products. For these reasons, a detailed description of the raw materials and processes, not just of the drug substances and the final drug product, is required for all botanical products.

5. Sinecatechins (Veregen): topical formulation of polyphenols extracted from green tea leaves

Veregen, approved in 2006, is the first botanical drug ever approved by the FDA. It is the only botanical drug currently approved for the treatment of external genital and perianal warts (EGWs) in immunocompetent patients 18 years or older. The active ingredients are sinecatechins, extracted from the leaves of *Camellia sinensis* (L.) O Kuntze, and the drug is

prescribed as a topical ointment to be applied 3 times per day (Table 2). Green tea catechins present in Veregen exhibit specific anti-oxidant, anti-viral, anti-tumor and immuno-stimulatory activity, which highly contribute to Veregen's efficacy in treatment of EGWs [6]. Each gram of the Veregen ointment contains 150 mg of sinecatechins in a water-free ointment base consisting of isopropyl myristate, white petroleum, cera alba (white wax), propylene glycol, palmitostearate, and oleyl alcohol [7]. Treatment with Veregen should be continued until complete clearance of all warts, but no longer than 16 weeks [7].

The mode of action of Veregen involved in the clearance of genital and perianal warts is unknown. *In vitro*, sinecatechins inhibited the growth of activated keratinocytes and demonstrated anti-oxidative activity locally, yet the clinical significance of this finding is yet unknown [7]. An *in vitro* study documented the inhibition of an extensive range of enzymes and kinases, including oxygenases, proteases, and protein kinases, involved in the generation of inflammatory mediators with micromolar concentrations of Veregen [8].

5.1. Standard indication: genital warts

Human papillomavirus (HPV) is a widespread infection and the most commonly diagnosed sexually transmitted infection (STI) in several geographic regions of the world [9]. It is estimated that 6.2 million new infections occur annually in individuals aged 14-44 years [10]. EGWs, also known as venereal warts or condylomata acuminata (CA), are benign epithelial mucosal tumors caused by HPV. HPV strains 6 and 11 are responsible for 90 percent of EGW [11]. Generally, these lesions are benign in nature, in contrast to other genital HPV infections caused by high-risk HPV strains 16 and 18 that are associated with cervical cancer [12]. The prevalence of EGW is estimated to be about 1 percent of sexually active individuals in developed countries and is increasing in several regions of the world. However, clinically detectable cases comprise only a small subset of all cases, with 20 million cases between the US and Europe when including sub-clinical HPV [9]. Persisting

genital warts rarely cause severe complaints such as pain, burning, itching, or obstructions, but can be disfiguring and stigmatizing causing fear of cancer or infertility. It is primarily the psychological distress of those affected that represents the main demand for treatment [13].

Infections of HPV are strictly confined to the epithelium. The virus initially infects basal keratinocytes, most likely by small traumas of the stratified epithelium. Subsequent steps of viral genome amplification, expression of capsid proteins, and assembly of virus particles are closely linked to the differentiation of keratinocytes as they migrate through the squamous epithelium [14]. Formation of mature virus particles in the most superficial epithelial layers does not elicit danger signals, since these cells are destined to die. Furthermore, HPVs are able to inhibit type 1 IFN responses, an anti-viral defense mechanism present in all cells, including keratinocytes. As a result, release of pro-inflammatory cytokines is virtually absent. Thus, HPV efficiently evades innate immune responses leaving the adaptive immune system largely ignorant of the infection [15].

Replication of the HPV genome requires S-phase competent cells, providing enzymes necessary for DNA synthesis. Activation of the cell cycle in differentiated keratinocytes is accomplished by the viral E6 and E7 proteins that inhibit tumor suppressors p53 and members of the pRB (retinoblastoma- susceptibility protein) family, respectively [16]. Transcription of these oncogenes is controlled by upstream long control region (LCR), containing the E6/E7 promoters and a number of binding sites for both viral and cellular transcription factors [17]. Inhibition of pRB by E7 results in activation of transcription factor E2F, including expression of genes for DNA replication. In response to unscheduled DNA synthesis in differentiated cells, p53 is usually activated to induce cell cycle arrest or apoptosis. E6 mediated inhibition of p53 prevents apoptosis and permits continuous DNA replication and cell division [17]. These mechanisms are potentially oncogenic, since affected

cells are no longer able to initiate growth arrest or apoptosis in response to DNA damage. Notably, only HR-HPV E6 and E7 can bind to and induce degradation of p53 and pRB [18]. In contrast, LR-HPV E6 and E7 only bind pRB and p53, but fail to induce degradation.[19] Although binding is weaker with HR-HPV oncogenes, interactions of LR-HPV E6 and E7 probably also abrogate pRB and p53 functions. Such interactions of LR-HPV with p53 and pRB members are likely to be important for dysregulation of the cell cycle and survival of infected cells. In addition, LR-HPV may prevent apoptosis independent of p53 via E6-mediated degradation of pro-apoptotic protein Bak [20].

5.2. Chemistry

The primary drug substance in Veregen is sinecatechins, which is a partially purified fraction of the water extract of green tea leaves from *Camellia sinensis* (L.) O Kuntze, and is a mixture of catechins (Figure 1) and other green tea components. Catechins constitute 85 to 95% (by weight) of the total drug substance which includes more than 55% epigallocatechin gallate (EGCg), other catechin derivatives such as epicatechin (EC), Epigallocatechin (EGC), and epicatechin gallate (ECg), and some additional minor catechin derivatives, such as gallocatechin gallate (GCg), gallocatechin (GC) catechin gallate (Cg), and catechin (C). In addition to the known catechin components, it also contains gallic acid, caffeine, and theobromine, which constitute about 2.5% of the drug substance. The remaining amount of the drug substance contains unidentified botanical constituents derived from green tea leaves [21].

5.3. Preclinical characterization

Veregen exhibited no mutagenic markers, being negative in the Ames test, chromosome aberration assay, rat micronucleus assay and UDS test, but positive in the mouse lymphoma assay when used in doses up to 1000 mg/kg/day [21]. When orally administered at doses up to 500 mg/kg/day, Veregen was not associated with either neoplastic or non-neoplastic

lesions in the tissues examined. Veregen was not teratogenic in rats and rabbits [21]. Daily vaginal administration of Veregen to rats during breeding and gestation did not cause adverse effects on mating performance and fertility at an approximate dose of 150 mg/rat/day. Furthermore, there were no adverse effects on embryo-fetal development in rats and rabbits, regardless of administration route [21].

5.4. Pharmacokinetics

Systemic exposure to EGCg, EGC, ECg, and EC were evaluated following either topical application of Veregen to subjects with external genital and perianal warts (250 mg applied 3 times a day for 7 days) or following oral ingestion of green tea beverage (500 ml ingested 3 times a day for 7 days). Following topical application of Veregen, plasma concentrations in all 4 catechins were below the limit of quantification (<5 ng/ml) on day 1. After application of Veregen for 7 days, plasma EGC, ECg, and EC concentrations were below the limit of quantification, while the plasma concentration of EGCg was measureable in 2 out of 20 subjects. The mean maximal plasma concentration of EGCg was 10.1 ng/ml. Oral ingestion of green tea beverage resulted in measurable concentration of EGCg in all subjects both on day 1 and day 7, with mean plasma concentration being 23.0 ng/ml [7].

5.5. Clinical pharmacology

Three randomized, double-blind, placebo-controlled trials were conducted to determine the efficacy and safety of the catechin, Veregen, given as a 10% and 15% ointment. These studies sought to demonstrate the efficacy of each of the two Veregen ointment formulations over placebo with respect to the complete clearance rates of all baseline and new warts [22]. The primary efficacy outcome measure was the response rate defined as the proportion of subjects with complete clinical (visual) clearance of all external genital and perianal warts, baseline and new, by week 16.7 The most affected areas in all studies were the penile shaft and the vulva [20,22–24].

The first multicenter Phase II/Phase III combined study was reported in 2007 and enrolled patients in 28 hospitals and practices in Germany and Russia. Eligible patients were required to be above the age of 18 with between 2 and 30 external angiogenital warts and a total wart area between 12 and 600 mm [2]. Patients were asked to withhold any other treatment of angiogenital warts or Acyclovir/ immunosuppressives 30 days prior to enrollment and have no record of HIV infection [23]. A total of 125 men and 117 women met the inclusion criteria and were randomly assigned to one of three treatment groups. 80 patients (42M/38F) were assigned to receive Veregen (Polyphenon E) 15% ointment, 79 patients (41M/38) assigned to receive Veregen 10% ointment, and 83 patients (42M/41F) were assigned to the placebo group. Treatment was administered topically, 3 times a day for up to 12 weeks or until complete clearance of baseline warts, with a 12-week treatment free follow-up period for complete responders. 90% of patients in all three groups had complete clearance during the 12 week treatment period. Mean time to complete clearance of all baseline warts in all groups was 10.6±2.6 weeks [23].

The next randomized, double-blind, 3 arm parallel group, vehicle-controlled multicenter Phase III trial enrolled 226 women and 277 men from 46 dermatologic, gynecologic, and urologic centers throughout Europe and South Africa. The placebo-controlled trial studied the 15% and 10% Veregen ointments, and the results were reported in 2007 [24]. The median wart area was 51mm^2 and the median wart number was 6. A clearance of greater than 50% was achieved in 77.3, 78.0, and 52.9% of all subjects in the Veregen 15%, 10%, and placebo arms, respectively. Women responded better than men, with 45% of men in both active treatment groups achieving complete clearance of all warts. The median time to complete clearance was estimated at 16.1 weeks for both the 15% and 10% ointments, and 16.7 weeks for placebo ($p<.001$). Adverse events other than mild local reactions that were probably related to study medication were reported by 4 patients, including moderate balanitis,

severe herpes simplex, mild lymphadenitis, and severe phimosis, all in the Veregen 15% ointment group [24].

The third trial randomly assigned 502 subjects (258M/ 244F) from 50 health centers in the United States, Latin Amercia, and Romania to the same 3 treatment arms reported earlier [25]. One hundred and eleven subjects (57.2%) in the 15% ointment arm, 111 subjects (56.3%) in the 10% ointment arm, and 35 subjects (33.7%) in the placebo arm achieved complete clearance of all external genital warts ($p<.001$) [25]. Compared with male patients, the proportion of female patients with complete clearance of all warts was higher in all 3 arms during the 12-week follow-up period. Recurrence occurred in 6.5% to 8.8% of patients in all three treatment groups during the 12-week follow up period. Severe adverse events, comprising lymphadenitis, skin ulcer, vulvitis, and vulvovaginitis, were reported for 5 patients treated with 15% ointment, and for 2 treated with 10% ointment [22,25].

5.6. Totality of evidence

As a naturally occurring mixture in which the active components are not well defined, the identified major and minor chemical components in Veregen need to be monitored and controlled for each marketed product batch. In the absence of data correlating chemical properties and clinical response, the acceptance ranges for these components were primarily established based on their levels observed in the multiple batches tested in clinical studies [26]. Significant variations of catechins and other chemical components have been identified from the tea leaves of different cultivars, and botanical products can have significant bath-to-batch variability (e.g. in total catechins and in ratios of different catechins) [26]. In addition, although the majority of components can be adequately characterized and quantified, there may still be residual uncertainties about the chemical nature of minor components in Veregen. Therefore, to ensure consistent quality for Veregen, FDA considered two important pieces of information from the application, the variability in

product batches due to collection site differences and a multiple dosing scheme [26].

Therefore, the clinical studies performed with Veregen demonstrate good therapeutic efficacy and safety in therapy of genital and perianal warts with complete clearance rates of >50%. Sinecatechins not only reduced baseline warts, but are also active against newly developed warts during treatment. As a possible advantage over Imiquimod and Podophyllotoxin, the recurrence rates after Veregen treatment was lower. Although not yet demonstrated, regression of wart lesions is likely to be caused by activation of cellular immune reactions, induction of cell cycle arrest and apoptosis, as well as inhibition of HPV transcription. This combination of molecular activities may expedite elimination of virus-infected cells of both clinical and sub-clinical lesions [12].

6. Crofelemer (Fulyzaq): oral formulation of purified proanthocyanidin oligomers from the bark latex of the Amazonian tree *Croton lechleri*

Fulyzaq, approved in 2012, is the first orally administered botanical drug approved by the FDA. It is also the first, and so far only, anti-diarrheal for the symptomatic relief of non-infectious diarrhea in adult patients with HIV/AIDS on anti-retroviral therapy. The active ingredients are oligomeric proanthocyanidins extracted from the bark latex of the *Croton lechleri* tree (Table 2), prescribed as a delayed release tablet at a dose of 125 mg taken twice a day. Fulyzaq acts as an anti-secretory agent via its minimal systemic absorption following oral administration, which allows it to act locally in the gastrointestinal tract to block two principal chloride ion channels in the luminal membrane of the enterocyte [27].

The exact mechanism of action for Fulyzaq is unknown, but a number of studies have proposed plausible mechanisms by which Fulyzaq could reduce secretions from the intestinal membrane. One study demonstrated that Fulyzaq inhibited the cyclic adenosine monophosphate (cAMP)-stimulated CFTR chloride channel located on the intestinal apical

membrane, as well as the CaCC located on the intestinal epithelial membrane [28]. Both of these chloride channels regulate chloride and fluid secretion in the intestine and activation of either increases chloride and fluid secretion from the gastrointestinal tract, contributing to secretory diarrhea. Since Fulyzaq inhibits both of these channels, chloride secretion is decreased, causing both stool weight and frequency to be reduced and leading to relief of diarrhea. These results were corroborated in other independent studies investigating the use of Fulyzaq against different types of secretory diarrhea [29–32].

Fulyzaq has also been highly active against diarrhea caused by particular bacterial species because of its inhibition of the CFTR chloride channel. Escherichia coli and Vibrio cholera produce enterotoxins that cause an increase in cAMP production. The elevated levels of cAMP simulate the CFTR chloride channel and increase chloride and fluid secretion, but these are blocked by Fulyzaq's proanthocyanidin oligomers [30]. Fulyzaq also shows anti-viral activity against laboratory identified bacterial strains, including respiratory syncytial virus, influenza A virus, parainfluenza virus, herpes virus 1 and 2 and hepatitis A and B. This activity appears to develop from Fulyzaq's ability to bind to the viral envelope, preventing viral attachment and penetration of the host cell [33,34].

6.1. Standard indication: HIV-associated diarrhea

Secretory diarrhea is commonly comorbid with human immunodeficiency virus (HIV). Diarrhea can affect patients at any stage of illness, with up to 60% of patients with HIV reporting symptoms of diarrhea [35,36]. The type of diarrhea that develops in HIV patients generally has secretory properties. Secretory diarrhea results whenever there is an excess secretion of chloride ions followed by movement of sodium and water into the intestinal lumen. Increased secretion of chloride ions can occur when the cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-activated chloride channels (CaCC) are overstimulated. HIV and various antiretroviral agents can activate these

channels, leading to the development of secretory diarrhea [28]. *Cryptosporidium*, *Isospora belli*, *Microsporidia*, and *Mycobacterium avium-intracellulare*, as well as *Salmonella*, *Shigella*, and *Campylobacter* are all opportunistic pathogens capable of causing diarrhea [36,37]; however, diarrhea caused by opportunistic pathogens was more common before antiretroviral therapy.

The goal of antiretroviral therapy is to completely suppress viral replication via the introduction of highly active antiretroviral therapy (HAART), and HIV patients are experiencing better clinical outcomes with improved survival. Nonetheless, HIV-associated diarrhea remains a common side effect of the HAART, along with HIV enteropathy, autonomic neuropathy, chronic pancreatitis, and exocrine insufficieny [29,38]. HIV enteropathy is a form of diarrhea that can occur during any stage of HIV infection and can last for months. It often arises without a clear infectious cause and it comprises a variety of gastrointestinal illnesses including diarrhea, GI inflammation, increased intestinal permeability, and malabsorption. Especially during the acute phase of HIV infection, gut lymphoid tissue becomes one of the major sites for HIV replication, which can lead to a significant loss of CD4+ T-cells. When the GI tract is infiltrated by lymphocytes, structural changes due to inflammatory processes can occur, like villous atrophy and crypt hyperplasia.

HIV causes a variety of other changes that could be linked to secretory diarrhea. Local activation of immune cells by HIV leads to the release of pro-inflammatory signals like interlukein-1, tumor necrosis factor α, and macrophage inflammatory proteins 1α and 1β in the GI tract [38]. The HIV trans-activating factor protein can stimulate calcium-activated chloride ion secretion in enterocytes and colonic mucosa. This protein may also inhibit the proliferation of enterocytes, leading to induction of apoptosis in these cells. Glycoprotein 120, an HIV envelope protein, causes increased calcium concentration in enterocytes that

leads to tubulin depolymerization and inadequate epithelial ion balance [39]. The HIV protein R has also been shown to possess inflammatory properties. This protein also disrupts barrier function, which contributes to the development of HIV enteropathy. Through these mechanisms, HIV itself can lead to GI dysfunction and possible secretory diarrhea.

6.2. Chemistry

Fulyzaq is a natural compound isolated from the bark of the *Croton lechleri* tree from the Euphorbiaceae family. This tree is commonly found in the western Amazonian region of South America, where the red latex is commonly referred to as "dragons blood" or "sangre de drago"[40]. Fulyzaq is an acid-labile, proanthocyanidin oligomer (Figure 2) with an average molecular weight of 2100 daltons. The monomeric components of the polyphenolic molecule include (+)-catechin, (-)-epichatechin, (+)gallocatechin and (-)galloepicatechin [41].

6.3. Preclinical characterization

Fulyzaq exhibited no mutagenic markers, being negative in the Ames test, chromosome aberration assay at a dose of 600 μg/mL and rat bone marrow micronucleus study at a dose of 50mg/kg [42]. Furthermore, at a dose of 738 mg/kg/day (177 times the recommended human daily dose), Fulyzaq had no effect on fertility or reproductive performance of male or female rats [41,42]. Additionally, Fulyzaq was not teratogenic at this dose [42]. Carcinogenicity studies were not conducted prior to NDA approval by the FDA, but appropriate studies will be completed post-marketing, as agreed in the pre-NDA meeting with the FDA [42].

6.4. Pharmacokinetics

Clinical trials have used a range of doses between 125 and 500 mg every 6 to 12 hours. Recent FDA approval labeling for Fulyzaq recommends using the 125 mg delayed release tablets twice a day for the treatment of symptomatic noninfectious diarrhea in adult

HIV/AIDS patients on antiretroviral therapy [41]. Oral Fulyzaq has little to no systemic absorption. Plasma concentrations are undetectable after ingestion of Fulyzaq and metabolites have not been identified in the blood. Food does not affect the efficiency or absorption of Fulyzaq, as co-administration with a fatty meal did not result in increased systemic exposure. Although *in vitro* studies show that Fulyzaq has the potential to inhibit cytochrome P450 isoenzyme 3A and transporters MRP2 and OATP1A2 at concentrations expected in the gut, due to its minimal absorption Fulyzaq is unlikely to inhibit multiple cytochrome P450 isoenzymes [41]. Despite Fulyzaq's potential, there are no reported clinically relevant drug interactions. Specifically, no drug-drug interactions were found between Fulyzaq and antiretrovirals such as Nelfinavir, Zidovudine, and Lamivudine, although a 20% decrease in Lamivudine exposure was observed in patients receiving Fulyzaq 500mg four times daily, but this was not considered to be clinically important [41].

6.5. Clinical pharmacology

A Phase II, randomized, double-blind, placebo-controlled study was designed to evaluate the safety and efficacy of Fulyzaq for the treatment of HIV-associated diarrhea as assessed by stool weight and frequency. HIV patients between 18 and 60 years old and with chronic diarrhea were included. Chronic diarrhea was defined as having at least 3 soft or watery stools, with stool weight of more than 200 grams per day. Eligible patients were also required to have a diagnosis of AIDS as defined by the CDC and be on appropriate antiretroviral therapy for at least 2 weeks before and throughout the duration of the clinical trial. Study patients discontinued all antidiarrheal agents 24 hours before the study began. Baseline stool weight and frequency were determined for each participant during a 24-hour observation period. A total of 51 patients were included in the study and were randomized to either the treatment or control group. Twenty-six people were assigned 500 mg (two 250 mg tablets) of Fulyzaq every 6 hours for 4 days, and 25 people were assigned two placebo

capsules every 6 hours for 4 days [32].

The mean baseline stool weight assessed during the 24-hour observation period was 914.8 g for the treatment arm and 813.9 g for the placebo arm. The mean baseline stool frequency during the 24- hour observation period was 5.2 stools for both groups [32]. Comparing day 4 results with baseline, patients treated with Fulyzaq had a significantly greater average reduction of stool weight and frequency compared with the placebo group. The mean reduction in stool weight from baseline to day 4 was 451.3 g/day for the treatment group compared to 150.7 g/day in the placebo group. The mean reduction in stool frequency from baseline to day 4 was 3 stools per day in the Fulyzaq group and 2 stools per day in the control group. Patients in the Fulyzaq group had a mean reduction in chloride concentration after 4 days, while the control group had a mean increase [32]. The results of this Phase II trial indicate that Fulyzaq could be useful in reducing stool weight and diarrhea. Another important observation from this study is that 77% of patients were on PI-based HAART. This further supports the role of Fulyzaq in the treatment of diarrhea in HIV patients whose diarrhea has multiple causes.

The ADVENT study was a Phase III, randomized double-blind, placebo-controlled multicenter trial designed to evaluate the efficacy of Fulyzaq in the treatment of secretory diarrhea in HIV-infected patients. For this study, secretory diarrhea was defined as persistently loose stools even with the regular use of antidiarrheal agents, or one or more watery stools per day without the use of antidiarrheal agents. Eligible patients were receiving stable antiretroviral therapy, had a history of diarrhea for at least one month, had CD4+ T cell counts greater than 100 cells/μL, and had no evidence of infection. Patients were excluded if they had a history of GI disease that caused diarrhea [41,43].

The ADVENT study consisted of 2 stages. Stage 1 established optimal dosing and stage 2 was used to assess safety and efficacy of Fulyzaq. Each stage included two phases, a 4-week

placebo-controlled phase, and a 5-month treatment-extension phase where all the patients received Fulyzaq. A 10-day screen period during which all patients received placebo preceded the placebo-controlled phase. Randomization to the placebo-controlled phase occurred only if patients experienced one or more watery bowel movements per day on at least 5 of 7 days of the screening period. The primary efficacy endpoint of the study was they proportion of patients who demonstrated a response to Fulyzaq; clinical response was defined as no more than two watery bowel movements per week for at least 2 of the 4 weeks of the placebo-controlled phase [41,43].

A total of 374 patients were enrolled and randomized to each treatment arm: 236 in the Fulyzaq arm and 138 in the placebo arm. The median number of daily watery bowel movements was 2.5 per day. PI based antiretroviral regimens were the most common in the treatment groups (64% in the Fulyzaq 125 mg twice daily group). Among these, 22% of patients in this group were receiving Lopinavir/ Ritonavir [43]. In stage 1, patients were randomized 1:1:1:1 to receive either Fulyzaq 125 mg, 250 mg, 500 mg, or placebo twice daily. Results from stage 1 of the ADVENT study revealed an optimal dosing regimen of Fulyzaq 125 mg twice daily. Patients in this arm experienced a better clinical response than the control group. In stage 2, patients were randomized to receive either 125 mg of Fulyzaq twice daily or the placebo in the first phase. During this phase, 16.3% of patients in the Fulyzaq arm experienced a clinical response, compared with 11.4% in the placebo arm. During the second phase all patients received Fulyzaq. Combined data from both phases showed that 17.6% of patients in the Fulyzaq group demonstrated a significant clinical response versus only 8% in the placebo group. Patients who received placebo during the 4-week phase and then crossed over to Fulyzaq showed considerable improvement after 1 month of use. These patients were also found to have a greater chance of experiencing a clinical response in the remaining 4 months of the treatment period. Treatment response

was consistent among pre-specified subgroups, including duration of diarrhea, baseline number of watery bowel movements, use of PI's, CD4+ count, and age. Fulyzaq was found to be less effective among African Americans when examining treatment effect consistency across race subgroups [41,43]. Results from the ADVENT study provide supportive evidence for FDA approval of Fulyzaq 125mg delayed-release capsules twice daily for the symptomatic relief of noninfectious diarrhea in adults with HIV/AIDS treated with antiretroviral therapy.

6.6. Totality of evidence

Compared with sinecatechins (Veregen), the chemical characterization of Fulyzaq presented an even greater challenge. This drug consists of a mixture of oligomers that vary in composition, sequence and length, which precluded adequate separations and quantification of proanthocyanadin oligomers based on multiple conventional methods of detection. Advanced chromatographic, spectroscopic, spectrometric, and acid hydrolysis methods were needed to provide a comprehensive characterization of Fulyzaq. These analytical methods collectively revealed extensive information on the structural signatures of Fulyzaq but they were ultimately considered insufficient to support the characterization and quality control of this complex botanical mixture [44]. Considering the degree of uncertainty regarding the chemical characterization of Fulyzaq, the FDA concluded that in addition to raw material control and clinical data from multiple doses and product batches, a clinically relevant bioassay to assess the drug product activity was needed before it could be approved [26,28].

7. ERr 731 extract (Estrovera): oral formulation of hydroxystilbenes extracted from rhubarb roots

German clinicians have been recommending a purified, standardized extract of Siberian rhubarb (*Rheum rhaponticum* L.) known as Estrovera/ ERr 731 for long term treatment of menopausal symptoms since 1993 and it was launched in the US market by Metagenics Inc in 2010. The active ingredients are hydroxystilbenes extracted from the root of Siberian rhubarb (*Rheum rhaponticium* L.) (Table 2) [45,46]. Clinical studies have demonstrated that 1 tablet (4 mg) daily offers significantly effective relief versus placebo for the 11 most common menopausal symptoms, including hot flushes. The mechanism of action of ERr 731 is still not completely understood. Preliminary research suggests that Estrovera may act as a selective estrogen receptor modulator (SERM) for estrogen receptor β, which may explain its efficacy and safety profile [47]. Such activation may also alleviate symptoms of depression and anxiety by modulating neurotransmitter release associated with ER signaling [48]. ERr 731 constituents, rhapontigenin and desoxyrhapontigenin, have demonstrated inhibition of monoamine oxidase A with serotonin as a substrate. This effect has been suggested to favorably modulate serotonin and catecholamine metabolism to support a healthy mood and cognitive function [49].

7.1. Standard indication: to relieve menopause symptoms

Menopause is the clinical term used after menstruation has ceased for one year, after which women are considered postmenopausal. Perimenopause is used to describe the time leading up to the final menstruation, which is signaled by irregular menstrual bleeding, erratic hormone levels, and the onset of menopausal symptoms [50]. An estimated 6,000 American women transition to menopause every day, with 75% of women aged 50 to 55 years old assumed to be postmenopausal [51]. Alternatively, induced menopause is the cessation of menstruation caused by suppression of ovarian function either through surgical

removal, pelvic radiation therapy, or chemotherapy. Women with induced menopause may suffer symptoms of greater intensity or frequency. Over 90% of women who undergo surgical removal suffer from hot flushes and other symptoms that can be chronic and severe [52].

The most common symptoms typically fall into 11 categories, with hot flushes being the most common and potentially debilitating. Nearly 80% of women in Western countries suffer from hot flushes, with 30% reporting severe and frequent enough hot flushes to seriously affect quality of life [53]. They can significantly affect daily functioning and sleep, as well as reported state of health. Sleep disturbances are the fourth most frequent menopausal complaint, with up to 60% of women reporting trouble sleeping. Additionally, more than 40% of women experience physical and mental exhaustion and cite forgetfulness as a menopausal symptom [50,51]. Declines in cognitive function have been linked to sleep disturbances during menopause [54].

While the physiology of hot flush vasomotor response is unknown, it appears to be a result of dysfunction of the thermoregulatory centers influenced by the hypothalamus [50,54–56]. Furthermore, estrogen withdrawal, rather than low circulating estrogen, is postulated to be the primary cause of hot flushes. This is supported by reported hot flushes in women who suddenly discontinue hormone therapy [50,54–56]. Norepinephrine is suggested to be the primary neurotransmitter influencing these thermoregulatory changes [55,57]. However, hormonal fluctuations in progesterone, as well as estrogen, are another purported systemic influence for hot flushes [56]. A decline in progesterone, which exerts a sedative and anti-excitatory activity by modulating GABA receptors, may also contribute to anxiety and altered sleep patterns [58]. Furthermore, when ovarian follicles fail to secrete estrogen to provide negative feedback for regular cycling, pituitary gonadotropin increases and leads to increased levels of luteinizing hormone (LH) [47,57]. Though no causal

relationship between the level of circulating LH and hot flushes has been demonstrated, hot flush occurrence has been correlated with pulses in LH levels. These pulses may also involve the thermoregulatory response via their effect on hypothalamic neurons [50].

7.2. Chemistry

ERr 731 is a unique phytoestrogen mixture extracted from the root of Siberian rhubarb (*Rheum rhaponticium* L.) [45,46]. The main active constituents of ERr 731 are the glycoside rhaponticin and desoxyrhaponiticin. The metabolites, rhapontigenin and desoxyrhapontigenin, comprise about 5% of the extract (Figure 3). In plants, these compounds are synthesized to protect against viral and microbial attack, disease, and ultraviolet exposure. These hydroxystilbene compounds are structurally related to resveratrol, which has also demonstrated SERM activity [59,60]. Siberian rhubarb is different than other medicinal rhubarbs from other parts of the world, with fewer anthraquinones (known for their laxative effect) and a higher concentration of hydroxystilbene compounds near the root [45,46].

7.3. Preclinical characterization

Long-term studies in rats have shown no negative effects in bone density or uterine tract changes [61]. Dogs treated with the no-adverse-effect level (NOAEL) of 1000 mg/kg/day for 13 weeks showed no pathological changes to the organs of the uterine tract or changes in uterine weight [62].

7.4. Pharmacokinetics

A dose of 20 mg/kg of R. rhaponticum hydroxystilbenes was administered to mice via an intraperitoneal injection and blood collections were done 5, 10, 30, 45 minutes and 1, 2, 3, 4, and 24 h after administration [63]. In the blood, seven initial trans-hydroxystilbenes and five of their metabolites were identified. Most of the trans-hydroxystilbenes found had peaked in the bloodstream 10 minutes after administration and metabolites peaked

15 minutes after administration [63]. There was no measurable concentration of trans-hydroxystilbenes 3 hours after administration. No bioaccumulation of parent polyphenols or their metabolites was observed in the course of chronic administration [63].

7.5. Clinical pharmacology

Although not approved by the FDA, clinical studies were designed to investigate and minimize factors such as major vasomotor triggers and pre-existing conditions that may influence outcomes. The primary outcome criterion for efficacy used was the change of the Menopause Rating Scale (MRS) between treatment and placebo groups [45]. The exclusion criteria for the following studies are as follows: regular cycles in the last 3 months, Pap smear of class III/IV hyperplasia, BMI below 18 or above 30, or abnormal eating habits [45–47,53]. Subjects were also excluded if they had a history of Type 2 diabetes or prescription corticosteroids, as well as previous or existing thromboembolic disease, insufficiently controlled hypertension, or hypertensive medication. Individuals with a history of smoking, drug or alcohol abuse, previous or existing psychiatric disorders, or high intake of caffeine (>500mg/day) were also excluded [53].

In the first study, 109 symptomatic perimenopausal women received either ERr 731 (n=54) or placebo (n=55) for 12 weeks. The ERr 731 group showed significant improvements in 11 common menopausal symptoms. At 4 weeks there was a significant decrease in the number and severity of hot flushes compared to the placebo group ($p<.0001$), along with significant decrease in Hamilton Anxiety Scale (HAMA) total score for somatic and cerebral anxiety, and a general improvement in total Women's Health Questionnaire Score (WHQ), including measurements in anxiety and poor mood [53]. At 12 weeks, the ERr 731 group demonstrated a significant decrease in total Menopause Rating Scale II (MRS II) score, as well as significant decreases in all 11 individual symptom scores compared to placebo [53]. From week 4, there were improvements in HAMA scores as well as the MHQ score [53].

In another 12-week study, 112 symptomatic perimenopausal women between the age of 45 and 55 were given one tablet per day of Estrovera 4 mg (n=56) or placebo (n=56). At 12 weeks, subjects given Estrovera showed a significant reduction in the number of daily hot flushes, from a median of 12 to 2 [45]. Based on the Hot-Flush-Weekly-Weighted Score (HFWWS), this decrease in hot flushes is comparable to those reported for an ultra-low dose of hormone therapy. Additionally, those with the most severe hot flushes received the most relief from Estrovera intervention. There was also a significant reduction of the MRS total score, from an average of 27 to 12.4 [45]. Estrovera subjects also showed significant reductions in each of the 11 individual MRS scores [45].

In another multi-center clinical trial, 252 women between the ages of 39 and 71 were recruited from 70 gynecological practices in an open observational study to receive ERr 731 at various doses for 6 months [46]. During the first 3 months, 243 participants took 1 tablet (4 mg) daily, 13 took 2 tablets daily, and 1 woman took 4 tablets daily. Over the course of the entire 6-month study, 228 women took 1 tablet daily, and 6 took 2 tablets daily, as recommended. For the majority of women, 1 tablet was enough to significantly relieve menopausal symptoms [46]. Subjects showed a significant decrease in MRSII total score from an average 14.5 to 6.5 points and reported a notable improvement in quality of life most noticeable in women who started with a MRSII score above 18 [46].

Finally, in a 108-week study, 80 subjects from the first 12 weeks study were followed for observational studies along with a placebo group. In the first 48 weeks, subjects received either ERr 731(n=39) or placebo (n=41). In the second 48 weeks, 41 women were given ERr 731 (23 from the last ERr 731 group and 28 from the last placebo group). ERr 731 demonstrated a further decrease or sustained alleviation of menopausal symptoms at 60 and 108 weeks [47]. At 108 weeks, all subjects (now receiving ERr 731, but with varying lengths of treatment) had an average of less than 1.4 slight hot flushes per day [47].

7.6 Totality of evidence

All recommended therapies for menopause (hormonal or non-hormonal) are centered on positive lifestyle changes that can not only ease the degree of symptomology, but also influence the rate of transition. ERr 731 is perhaps the most thoroughly tested phytoestrogen SERM to date that offers a more natural approach to relieving menopausal symptoms, including hot flushes. Published toxicology and clinical studies suggest reliable efficacy and predicted long-term safety with no associated serious adverse events reported to date. When ERr 731 is used in conjunction with a patient-centered approach to menopausal relief, it may offer positive clinical outcomes for women in various stages of menopausal transition [47,50,52].

Despite the clinical data already collected for ERr 731, it has not been submitted as an IND or an NDA and can make no claims to treat a disease. Instead, Metagenics, the company responsible for the production of ERr 731, has created a dietary supplement and must rely directly on the menopausal population and health practitioners to advertise its effectiveness. Thus, foregoing FDA endorsements and allowing personal recommendation and popular knowledge to drive sales while maintaining strict standards of evidence-based preclinical and clinical programs to evaluate botanical product safety and efficacy seems like a less-costly and therefore more attractive strategy to bring botanical products to the market.

8. Bitter gourd- oral formulation extracted from the fruit of *Momoridca charantia*

Traditional herbalists in China, India, South and Central America, Africa and Australia have been recommending the consumption of bitter gourd (*Momoridca charantia*) fruit and extracts, most commonly to treat complications of metabolic syndrome and Type 2 Diabetes (T2D), among other ailments [64]. The active ingredients are a mixture glycosides, saponins, alkaloids, triterpenes, and steroids extracted from all parts of the plant, including fruit pulp,

seeds, and leaves [65]. Clinical studies have demonstrated that a tablet containing 50 grams of dried fruit pulp significantly reduced blood glucose in 9 patients with T2D [66]. The mechanism of action of bitter gourd extract is unknown and further clinical work needs to be done before any extract can reach the market.

8.1. Standard indication: to treat hyperglycemia associated with Metabolic Syndrome

Metabolic syndrome is identified as a collection of symptoms including visceral obesity, insulin resistance, hypertension, and hyperglycemia. The association of several of these symptoms has been known for nearly a century; however, public interest increased in 1988 when Reaven identified syndrome X and the role of insulin resistance and hyperinsulinemia in the etiology of T2D, hypertension, and coronary artery disease [67]. While the ability of metabolic syndrome to predict the onset of T2D may not be greater than the sum of its components [68], it has become a tool intended to alert patients before the necessity of prescription drugs for weight-loss or hyperglycemia. There are both genetic and lifestyle considerations, but lifestyle management at this stage can reverse hyperglycemia, visceral obesity, and other associated symptoms [69].

8.2. Phytochemistry

The hypoglycemic activity of *Momoridica charantia* fruit extract has been attributed to a mixture of steroidal saponins, insulin-like peptides, and alkaloids [65]. Fractionation of bitter gourd has identified two kinds of hypoglycemic activity: a fast acting aqueous fraction that acts within 30 minutes and a fraction with slow hypoglycemic activity [65].

8.3. Preclinical characterization

Momoridica charantia extracts have shown no signs of toxicity in the liver or kidneys, and had no effect on food consumption or organ weights of experimental animals when ingested in low doses for as long as 2 months [70]. However, large quantites of bitter gourd extract induced testicular lesions in dogs, and 800mg/kg/day of Bitter gourd seed extracts for 42

days caused infertility in male rats [71,72]. Bitter gourd seed extracts given to female Albino rats at a dose level of 25 mg/100 g body weight for 30 days has antiovulatory activities [73].

8.4 Clinical pharmacology

Animal studies

A fructose rich diet in rats has been shown to induce metabolic syndrome as it was defined by Reaven et al. in 1988 [67]. Oral administration of aqueous bitter gourd extract at a dose of 400 mg/day for 15 days in rats fed a high fructose diet prevented hyperglycemia and hyperinsulinemia compared to fructose-fed control rats [74]. Virdi et al. demonstrated that a water extract powder of *Momordica charantia* at doses as low as 20 mg/kg body weight can reverse alloxan-induced hyperglycemia in rats with no toxicity to liver and kidneys up to a period of 4 weeks [70]. Uebansco et al. observed that a 600mg/kg methanolic fraction inhibited plasma glucose levels in oral sucrose tolerance tests, whereas aqueous and methanolic extracts inhibited sucrase and maltase activity in Sprague Dawley rat intestinal mucosa [75]. Kar et al. observed that rats treated with an ethanolic extract of *Momordica charantia* fruit (250mg/kg) once daily for 2 weeks had nearly returned to normal blood sugar levels at the end of the treatment period. Meanwhile, chronic treatment with aqueous bitter melon extract caused a significant fall in plasma glucose levels after 4 months of treatment [76].

Human studies

In a study by Leatherdale et al., consumption of 50 mL of an aqueous bitter gourd extract before a 50g oral glucose challenge reduced glucose concentrations of Asian male diabetic patients within 30 minutes of administration, and effects persisted for 2 hours [66]. A similar improvement in glycemic control occurred after 8-11 weeks of daily consumption of fried bitter gourd [66].

The first randomized control trial to compare the hypoglycemic effect of dried bitter

gourd pulp powder with Metformin was done in 2011 by Fuangchan et al. [77]. They performed a 4-week, multicenter, randomized, double-blind, active-control trial in 4 paralleled treatment groups. Diabetic patients meeting the inclusion criteria were randomized into groups to receive bitter gourd 500 mg/day, 1000 mg/day, or 2000 mg/day or metformin 1000 mg/day. All patients were followed for 4 weeks. Roasted rice powder was used as a placebo for bitter gourd and lactose was used as a placebo for Metformin [77]. Bitter gourd and Metformin were well tolerated, there were no serious clinical adverse events, and there were no differences in overall adverse event rates between treatment and placebo groups [77]. Bitter gourd had a modest hypoglycemic effect and significantly reduced fructosamine levels from baseline among patients with T2D who received 2000 mg/day. However, the hypoglycemic effect of bitter gourd was less than Metformin 1000 mg/day [77].

In 2012, a three-month exploratory efficacy, un-blinded, single arm study was run with 42 eligible adult patients diagnosed with metabolic syndrome. Each patient was supplemented with 5 grams (1% w/w diet) of bitter gourd extract daily for three months [78]. The extract was well tolerated and all patients were checked monthly for specific parameters during the study and the subsequent 3 months. Researchers observed significant improvement in metabolic syndrome parameters in the third month of the study, as well as in the first month of follow-up. Waist circumference also significantly decreased following supplementation [78].

8.5 Totality of Evidence

Much more work is needed to bring any purified compounds or consistent extract to market; however, a large body of work on bitter gourd's efficacy and safety is already underway. With the collected information, consumption of *Momordica charantia* fruit can be encouraged in developing countries for the prevention and treatment of hyperglycemia and Type 2 Diabetes.

9. Conclusions

Drawing conclusions on the prospects of botanical drugs in general is still premature for a number of reasons, including variable complex mixtures, multiple plant combinations, extensive previous human use, and availability as dietary supplements before approval as drugs. In fact, of the 282 pre-INDs and INDs submitted between 1999 and 2007, only 36% were multi-plant combinations, reflecting the difficulties in working with more complex preparations [1]. Moreover, as of 2012, the strong interest in developing botanical drugs resulted in more than 500 pending FDA applications, but progress in developing new drugs from botanicals has been slow, with only two botanical NDAs to date. While extensive history of prior human use may reduce the early requirements for nonclinical pharmacology and toxicology for the initial (Phase I and II) clinical studies during the development of the botanical drug, these data will be required for the subsequent Phase III and the final marketing approval.

In this situation, the opportunities for sponsors with limited R&D capabilities and reduced promotional budgets are few: more effective resources management (flexibility, opportunistic approach, focus on niches) but also finding less conventional regulatory approaches and exploring alternative pathways to market. In fact, to bypass FDA regulation of the safety of functional substances, it is thus in a manufacturer's interest to try to market a botanical product as a dietary supplement rather than as a conventional food with added ingredients or a botanical drug. By marketing botanical products as dietary supplements, sponsors can avoid having to prove that added ingredients are GRASE and when questions arise, such products can stay on the market until the FDA proves in court that they may be harmful. Implementation of the strict evidence-based preclinical and clinical programs to evaluate safety and efficacy of the botanical products in either category, however, seems to highlight a prominent unifying trend that will drive the development of future botanical products.

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11. Tables and Figures

Table 1. Common classes of botanical products in the US.		
Form	**Definition**	**Examples**
<u>Food Additives</u>	**<u>Intend to become a component of food</u>**	
Food Colors Not GRASE FDA approval	Federal Food, Drug, and Cosmetic Act (21 U.S.C. §321(s)(t)): *Any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food … is capable (alone or through reaction with other substance) of imparting color thereto.*	Annatto extract Beet powder Carrot oil Turmeric
<u>Functional Foods</u>* <u>Nutraceuticals</u>*	**<u>Intend to add further nutritional value to a diet</u>**	
Foods for Special Dietary Use Same as food	Code of Federal Regulations (21 C.F.R. §105.3): *For supplying particular dietary needs which exist by reason of a physical, physiological, pathological or other condition, including but not limited to the conditions of diseases, convalescence, pregnancy, lactation, allergic hypersensitivity to food, underweight, and overweight; … by reason of age; … for supplementing or fortifying the ordinary or usual diet with any vitamin, mineral, or other dietary property.*	Hypoallergenic foods Infant foods
Dietary Supplements Same as food Structure/function only	Federal Food, Drug, and Cosmetic Act (21 U.S.C. §321(ff)): *A product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract, or combination of any.*	Omega-3 fatty acids Phytosterols

Table 1 Cont. Common classes of botanical products in the US.		
	Intend to diagnose, cure, mitigate, treat, or prevent a disease	
Medical Foods Same as food Medical supervision	Orphan Drug Act (21 U.S.C. 360ee(b)(3)): *A food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation.*	Axona Lofenalac
Patent Medicines* Grandfathered	As long as ingredients and labeling remain the same	Pinkham's Vegetable Absorbine Jr.
Botanical Drugs Same as drug	Federal Food, Drug, and Cosmetic Act (21 U.S.C. 355(b)): *A product that is used as a drug and that contains as ingredients vegetable materials, which may include plant materials, algae, macroscopic fungi, or combinations thereof.*	Fulyzaq Veregen
Pharmaceuticals*	**Intend to diagnose, cure, mitigate, treat or prevent a disease**	
OTC Drugs Same as drug GRASE (old) NDA (new) FTC regulations	As long as the active ingredients are listed in the OTC drug monograph	Aspirin Echinacea
Prescription Drugs IND/NDA FDA approval CMC requirements	Federal Food, Drug, and Cosmetic Act (21 U.S.C. 201(g)(1)(b)): *An article intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; and an article (other than food) intended to affect the structure or any function of the body of man or other animals.*	Morphine Taxol

* Terms not specifically defined by law

Table 2. Comparative analysis of botanical interventions described in this study.				
Form	**Plant**	**Source**	**Putative actives**	**Formulation**
Botanical Drug				
Veregen (Sinecatechins)	Common tea *Camellia sinesis*	Leaf extract	Catechins	Ointment, 15% (topical) 112.5 mg
Fulyzaq (Crofelemer)	Dragon's Blood *Croton lechleri*	Latex extract	Proanthocyanidins	Tablet (oral) 125 mg
Nutraceutical				
Estrovera	Siberian rhubarb *Rheum rhaponticum*	Root extract	Hydroxystilbenes	Tablet (oral) 4 mg

Table 3. Quality, safety, and efficacy requirements for development and approval of botanical drugs.	
Requirements	**Botanical Drugs**
Overall	Same as drug*
Quality	
Botanical raw material	Identification by trained personnel Certificate of authenticity List of all growers and/or suppliers
Botanical drug substance	Qualitative and quantitative description Chemical constituents may not be always defined Active constituents may not be identified Name and address of manufacturer Description of manufacturing process Quality control tests performed Chromatogram fingerprint (presence, not amount) Biological assay (dose-dependent) Description of container/closure system Available stability data Container label
Botanical drug product	Same as above (but for a finished product), plus Placebo and labeling Environmental assessment or claim of categorical exclusion
Safety	
Prior history of human use	Documented daily human consumption > proposed trial dose* Documented duration of human use > proposed trial duration* Equivalency of amount used in raw form > proposed trial dose* Volume of sales (ex-US market)*
Preclinical data	Drug substance, active and known constituents if feasible* Data from toxicological databases (RTECS, Toxline, TOMES)* Extensive literature search (Medline)* Standard nonclinical toxicology tests for Phase III and NDA Genotoxicity Reproductive toxicity Repeat-dose toxicity in two mammalian species (one rodent) Carcinogenicity not required for some short-term indications Toxicokinetic studies to support systemic exposure Safety pharmacology using screens for modes/sites of action
Efficacy	Controlled efficacy trials (Phase I-III)

* May have reduced CMC requirements for initial (Phase I and II) clinical studies

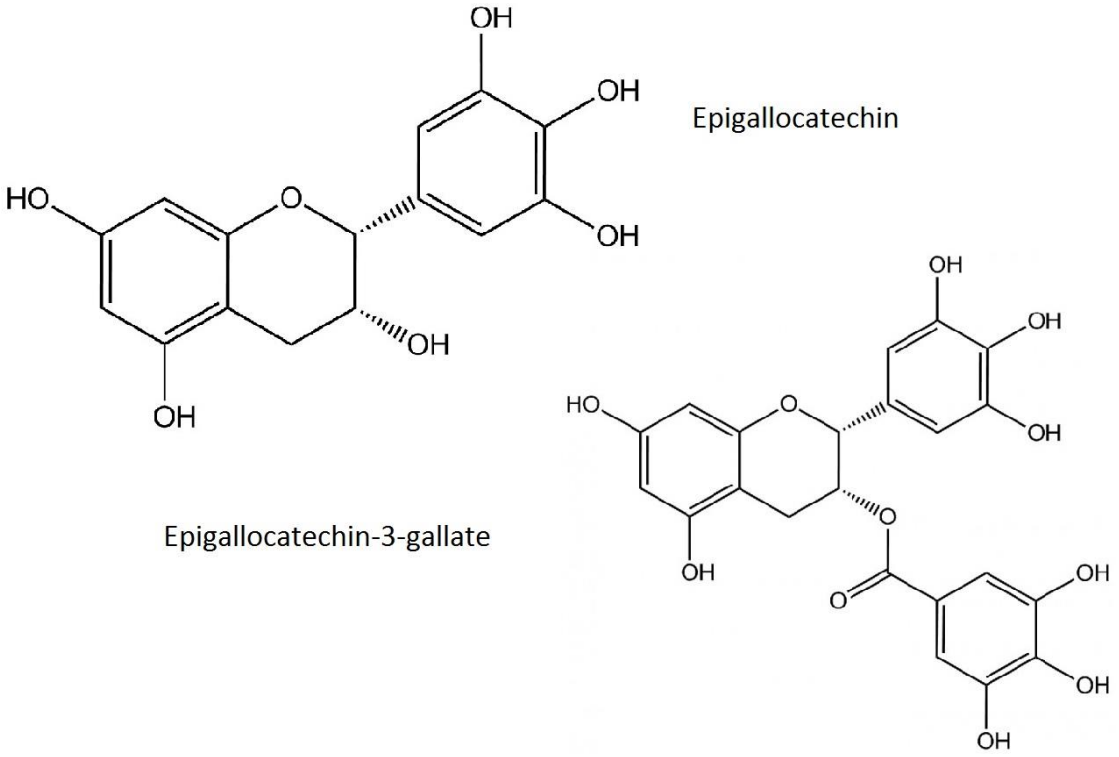

Figure 1. Catechin structure: major bioactive principles in Veregen. Veregen (Sinecatechins) is a botanical drug approved for the topical treatment of external genital and perianal warts.

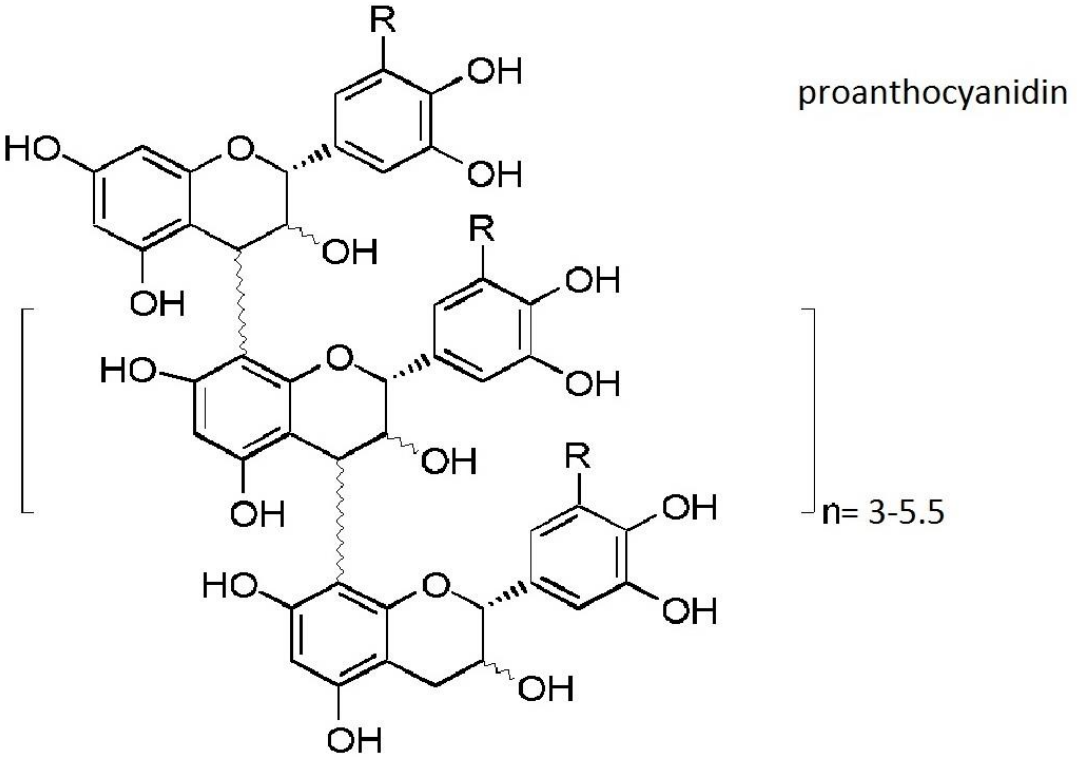

Figure 2. Proanthocyanidin structure: major bioactive principles in Fulyzaq. Fulyzaq (Crofelemer) is a botanical drug approved for relieving symptoms of diarrhea in HIV/AIDS patients taking antiretroviral therapy.

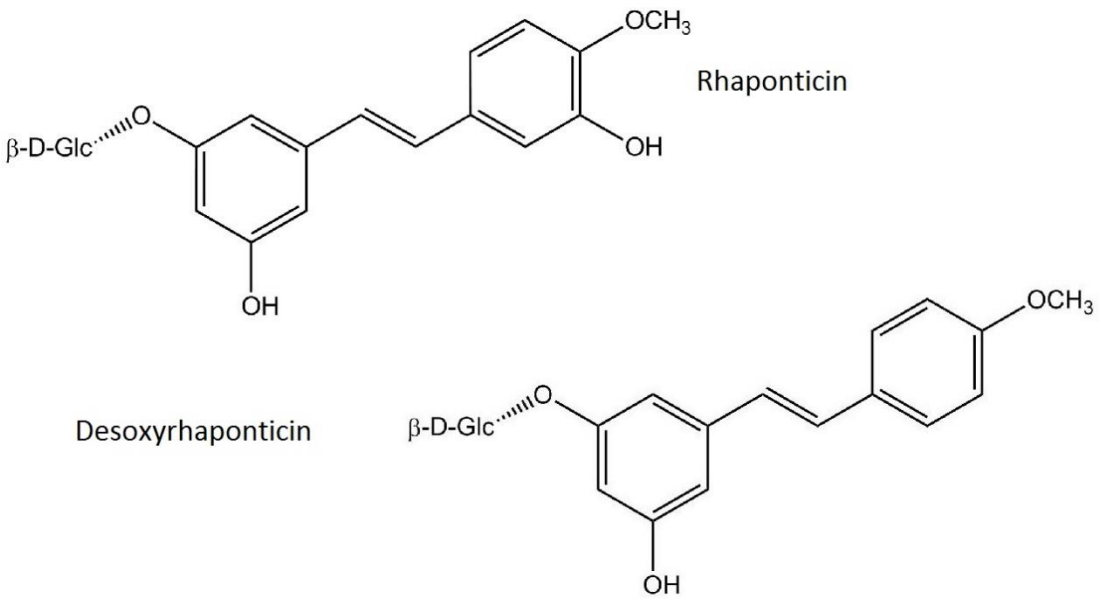

Figure 3. Hydroxystilbene structure: major chemical constituents in Estrovera Estrovera is a nutraceutical product marketed to relieve common symptoms of hot flashes and night sweat associated with the menopause.

CHAPTER 2: STRUCTURAL CONSTRAINTS AND IMPORTANCE OF CAFFEIC ACID MOIETY FOR ANTI-HYPERGLYCEMIC EFFECTS OF CAFFEOYLQUINIC ACIDS FROM CHICORY

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Abstract

Scope: Chicory (*Cichorium intybus* L.) is a perennial herb often consumed as a vegetable, whereas the ground and roasted roots are blended as a coffee substitute. Caffeoylquinic or chlorogenic acids (CQA), the abundant intermediates of lignin biosynthesis in chicory, have been reported to improve glucose metabolism in humans, but the functional group in their structure responsible for this effect has not been yet characterized.

Methods and Results: Here, we showed that three di-O-caffeoylquinic acids suppressed hepatic glucose production in H4IIE rat hepatoma cells by reducing expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), two key enzymes that regulate hepatic gluconeogenesis. Direct comparisons between CQAs and their metabolites (3-caffeoylquinic, caffeic, and quinic acids) revealed the caffeic acid moiety alone was responsible for the observed effects. Further analysis suggested the activation of PI3K and MAPK pathways as a mechanism controlling gene expression and that was shared between caffeoylquinic and caffeic acids. These compounds promoted increased mitochondrial respiration and cellular metabolism, in part by inducing oxidative phosphorylation and proton leak.

Conclusions: We concluded that the caffeic acid moiety was important for suppression of hepatic gluconeogenesis and hyperglycemia, ultimately strengthening the link between dietary interventions based on caffeic acid-containing plant foods and healthy glucose metabolism.

Keywords: blood glucose; cell bioenergetics; diabetes; metabolic syndrome; dietary supplementation

1. Introduction

Metabolic syndrome precedes type II diabetes in many patients and is associated with a group of risk factors that include obesity, hypertension, insulin resistance, and inflammation [1–3]. Organ-specific consequences to altered energy homeostasis include reduced skeletal muscle glucose uptake and increased hepatic gluconeogenesis [4]. Additionally, increased abdominal fat mass yields high circulating free fatty acids (FFA), which drive hepatic FFA uptake and oxidation [5]. This further stimulates gluconeogenesis in the liver, thus increasing total hepatic glucose output when glycogen stores are depleted or the regulation of glycogenolysis is abrogated [6].

Two key enzymes that control gluconeogenesis, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), are transcriptionally regulated by hormones such as insulin, glucagon, incretins, and glucocorticoids via a stimulatory cyclic-AMP (cAMP)- dependent pathway or inhibitory phosphoinositol-3 kinase (PI3K/Akt) pathway [7]. Insulin-resistant hepatocytes are unable to effectively perceive and respond to these signals, leading to increased G6Pase and PEPCK mRNA levels and persistent gluconeogenesis, despite high blood glucose concentration [8]. Several bioactive plant constituents, including guanidine from *Galega officinalis* (French lilac) and its less toxic synthetic derivative metformin, are used as effective anti-hyperglycemic agents for treatment of diabetes [9]. A number of polyphenols from different plant sources, such as *Syzygium aromaticum* (clove) [10] and *Artemisia dracunculus* (tarragon) [11] were also shown to suppress gluconeogenesis by reducing expression levels of gluconeogenic enzymes *in vitro*. Caffeoylquinic or chlorogenic acids (CQA), found in large quantities in *Coffea arabica* (coffee beans) [4] and *Cichorium intybus* (chicory) [5], were shown to reduce glucose absorption and metabolism, in part by inhibiting enzymatic activity of G6Pase [12] and its mRNA levels in the liver [13]. However, in plants, CQAs exist in multiple derivatives,

classified according to the number and position of the caffeoyl groups attached to the quinic acid. Both monoCQA, diCQA, and triCQA derivatives have been reported from nature and synthesized in the lab (8)], but the structure-activity relationship and the principal moiety responsible for their anti-hyperglycemic effect is unknown.

Chicory is a perennial herb in the *Asteraceae* (Compositae) family edible as a vegetable crop and animal forage [10,14]. Contemporary applications for chicory leaves include salad greens, whereas root grounds are added to flavor coffee [10,11]. Inulin content [15–17] has also made chicory root a common soluble fiber additive in food industry. The Generally Regarded as Safe (GRAS) status from the Food and Drug Administration [18] makes chicory root an excellent plant food source of bioactive CQAs [5].

Due to the complex mixture and innate differences in the bioavailability and other pharmacokinetic properties of CQAs [4,19], it is essential to better understand the benefits of individual CQAs and their metabolites on glucose metabolism. In this study, we isolated three diCQAs from chicory root and, together with their metabolites (3-caffeoylquinic, caffeic, and quinic acids), evaluated them for structure-activity relationship on modulating glucose production, gene expression profiles, and bioenergetics parameters of cellular metabolism to suppress gluconeogenesis in H4IIE rat hepatoma cells.

2. Materials and methods

2.1 Plant material and reagents

Solvents were of HPLC grade and purchased from VWR (Radnor, PA), all other chemicals, including chlorogenic (3-caffeoylquinic), caffeic, and quinic acids were from Sigma (Saint Louis, MO), unless specified otherwise.

Chicory cv. Sacson dry roots (200 g) from Leroux (Lille-Valenciennes, France) were extracted and purified as described previously [20]. Briefly, the methanolic extract was

partitioned with ethyl acetate and fractionated into 10 FCPC fractions (Kromaton, Pittsfield, MA) using 3 phase solvent system (hexane: methyl acetate: acetonitrile: water, 4:4:3:4) according to absorbance at 258 nm (Figure 1A) [21]. Bioactive fraction 7 (200 mg) was further purified by reverse phase HPLC (Waters RP-8 300 x 19.0mm, 7μm, 20 – 95% methanol in water containing 0.1% TFA over 60 min, flow rate 10 ml/min, UV detector at 258nm) to yield 95% UV 1,5-dicaffeoylquinic acid (5.2 mg), 3,4-dicaffeoylquinic acid (6.4 mg), and 3,5-dicaffeoylquinic acid (30.0 mg). All isolated compounds were confirmed and matched by ESI-TOF-MS (Agilent G1312B-1200 series Infinity Quaternary HPLC system coupled with Agilent 6530A Accurate-Mass Quadrupole Time-of-Flight MS with Agilent Jet Stream source, drying gas temperature 300 °C, drying gas flow 7 L/min, nebulizer pressure 40 psi, sheath gas temperature 350 °C, sheath gas flow 10 L/min, capillary voltage 3500 V, nozzle voltage 500 V, fragmentor voltage 150 V, skimmer voltage 65 V, octopole RF peak voltage 750 V), 1H NMR and 13C NMR spectral analyses (Bruker Avance-III 600 MHz, Billerica, MA) to earlier identified di-CQAs [22,23].

2.2 Cell culture

The rat hepatoma cell line H4IIE (CRL-1600) that maintains active gluconeogenesis pathway was obtained from ATCC (Manassas, VA). Cells were routinely passaged every 3-4 days and maintained in high glucose DMEM containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA), and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37 °C and 5% CO2. Cells were sub-cultured into 24-well plates and, once confluent, changed to induction medium that contained glucose-free DMEM supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate. Gluconeogenesis was induced with 0.5 μM dexamethasone and 10 mM 8-CTP-cAMP (Dex-cAMP) for 8 h and suppressed with 10 nM insulin [24]. H4IIE cell line is uniquely suited for this analysis because it maintains the physiological regulation of gluconeogenesis in response to hormones *in vitro* [25].

2.3 Glucose production

Glucose production was quantified in 50 μl aliquots of cell culture medium from negative controls (vehicle, 0.1% DMSO), induced controls (Dex-cAMP), positive controls (Dex-cAMP with 10 nM insulin), and different treatments (Dex-cAMP with 10 μg/ml fractions or 1-10 μM individual CQAs) in triplicate. Amplex Red Glucose/Glucose Oxidase kit (Life Technologies) detected the conversion of secreted glucose to gluconolactone and hydrogen peroxide using a fluorescence microplate reader at 530/590 nm (BioTek Synergy H1, Sunnyvale, CA), following the manufacturer's protocol. All results were expressed as fold change from the induced Dex-cAMP controls.

2.4 RNA extraction and qPCR

The total RNA was isolated from cells using TRIzol reagent (Life Technologies). RNA was quantified using Synergy H1/Take 3 plate setup (BioTek). The cDNAs were synthesized using 2 μg of RNA for each sample using high-capacity cDNA Reverse Transcription kit (Life Technologies) on an ABI GeneAMP 9700 (Life Technologies), following the manufacturer's protocol.

The resulting cDNA was amplified in duplicate by real-time quantitative PCR (qPCR) using SYBR green PCR Master Mix (Life Technologies). To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) as follows: ß-actin (housekeeping gene), forward primer: 5'-GGG AAA TCG TGC GTG ACA TT-3', reverse primer: 5'-GCG GCA GTG GCC ATC TC-3'; G6Pase, forward primer: 5'-TCT ACC TTG CGG CTC ACT TTC-3', reverse primer: 5'-GAA AGT TTC AGC CAC AGC AAT-3'; PEPCK, forward primer: 5'-GCA GAG CAT AAG GGC AAG GT-3', reverse primer: 5'-TTG CCG AAG TTG TAG CCA AA-3'. qPCR amplifications were performed on an ABI 7500 Fast real time PCR (Life Technologies) using 1 cycle at 50 °C for 2 minutes and 1 cycle of 95 °C

for 10 minutes, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. The dissociation curve was completed with 1 cycle of 1 minute at 95 °C, 30 s at 55 °C, and 30 seconds at 95 °C. mRNA expression was analyzed using the ΔΔCT method [26] and normalized with respect to the expression of the β-actin using ABI 7500 Fast System SDS Software v1.3.0 (Life Technologies). Amplification of specific transcripts was further confirmed by obtaining melting curve profiles. All results were expressed as fold change from the induced Dex-cAMP controls [27].

2.5 qPCR array transcription profiling

Pharmacogenomic profiling of 84 genes related to insulin-responsive pathways, including insulin signaling, carbohydrate, lipid, and protein metabolism, PI3K and MAPK pathways, and transcriptional factors and regulators of cell growth and differentiation, was performed with RT² Profiler PCR Array PARN-030Z (Qiagen, Hilden, Germany), following the manufacturer's protocol. We profiled pooled treatments (3 wells in triplicate) of crude chicory extract (50 μg/ml), 3,5-dicaffeoylquinic, 3-caffeoylquinic, caffeic, and quinic acids (10 μM) applied to Dex-cAMP induced H4IIE cells for 8 hours. RNA isolation, template cDNA and qPCRs were performed according to the manufacturer's instruction and the relative expression levels were normalized to 5 housekeeping genes (beta-actin, beta-2-microglobulin, hypoxanthine phosphoribosyltransferase 1, lactate dehydrogenase A, and large ribosomal protein P1). RT² Profiler PCR Array Data Analysis Tool (ver. 3.5, default settings) was used to generate clustergrams to identify co-regulated genes across treatments and volcano plots to determine significant gene expression changes for individual gene targets.

2.6 Cellular bioenergetics

Changes in mitochondrial respiration and glycolysis in response to CQAs and their metabolites were quantified using Seahorse XF24 analyzer (Agilent Technologies, Santa

Clara, CA). $5x10^4$ H4IIE Dex-cAMP induced cells were subcultured in the 24-well XF assay plates overnight, treated with respective CQAs at 10 μM for 24 h, and subjected to real-time measurements of oxygen consumption (OCR) and extracellular acidification (ECAR). OCR and ECAR were automatically recorded by Seahorse XF24 software v1.8. Cells were rinsed once, changed to 500 μl of XF assay medium (DMEM without NaHCO3, 10 mM glucose, 2 mM pyruvate, 2 mM GlutaMAX, pH 7.4), and equilibrated at non-CO2 incubator and 37°C for 1 h. Following 18 basal measurements of OCR and ECAR, mitochondrial complex inhibitors were injected sequentially in the following order: oligomycin (1 μM, oligomycin-coupled OCR), FCCP (0.75 μM, maximal OCR), antimycin A/rotenone (1 μM each, non-mitochondrial OCR), and 3 readings were taken after each inhibitor. ATP-coupled respiration was measured by subtracting oligomycin-coupled OCR from basal OCR. Proton leak was calculated by subtracting non-mitochondrial OCR from the oligomycin-coupled OCR reads [28].

2.7 Statistical analysis

Statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA) and expressed as mean ± SEM. Two tailed t-test or one-way ANOVA were applied at a significance level of $P < 0.05$. Post-hoc analyses of differences between individual experimental groups were made using the Tukey's multiple comparison test.

3. Results

3.1 Bioactivity-guided isolation of di-CQAs from chicory root

Ten chicory root fractions (Figure 1A) were tested for their ability to inhibit hepatic glucose production in the H4IIE liver cells. Fraction 7, when tested at 30 μg/ml concentration, had the greatest effect on reduction of gluconeogenesis stimulated with Dex-cAMP; some effects were also observed in the fraction 6 (Figure 1B). The predominant

compounds present in fraction 7 were diCQAs, which were further purified for the structure-activity studies. 3,5-dicaffeoylquinic acid was the major diCQA present in the chicory root (4.4 mg/g FW), approximately 5 times higher than 3,4-dicaffeoylquinic and 1,5-dicaffeoylquinic acids found in equal molar ratios (0.8 mg/g FW). Additionally, 3-caffeoylquinic, caffeic, and quinic acids were included in this study as the early phase products of the diCQAs hydrolysis in humans (Figure 2) [29].

3.2 Structure-activity relationship of di-CQAs on gluconeogenesis

When tested in Dex-cAMP stimulated H4IIE liver cells, all diCQAs dose-dependently suppressed hepatic glucose production in the range of 3-30 μM (Figure 3A-C). The 3,5-dicaffeoylquinic acid showed slightly higher biological activity compared to the other diCQAs. This observation was further confirmed by direct inhibition of G6Pase and PEPCK mRNA levels in H4IIE cells by all three diCQAs to a very similar extent (Figure 3D-F). The suppression was strongest for G6Pase, suggesting that this is a primary gluconeogenic target for diCQAs.

3.3 Caffeic acid moiety is responsible for the gluconeogenic effects

The high degree of similarity between the effects of the three diCQAs on hepatic glucose production prompted further analysis of the gluconeogenic-suppressing effects of the early phase products of the diCQAs hydrolysis in humans. Both 3-caffeoylquinic and caffeic, but not quinic acid, decreased glucose production in the liver cells over a lower dose range of 1-10 μM (Figure 4A-C). Similar to the parent diCQAs, 3-caffeoylquinic and caffeic acids showed a greater suppression of G6Pase expression, and this effect was absent in cells treated with quinic acid (Figure 4D-F), suggesting that caffeic acid moiety is responsible for glucose-lowering effects of CQAs.

3.4 Pharmacogenomic comparison of di-CQA and its metabolites

To further elucidate the involvement of 3,5-dicaffeoylquinic acid and its early phase metabolites on glucose-signaling pathways in liver cells, pooled RNA samples were used to measure gene expression changes in insulin signaling, carbohydrate, lipid, and protein metabolism, PI3K and MAPK signaling, and transcriptional factors and regulators of cell growth and differentiation in response to CQAs. The RT² Profiler clustergram (Figure 5A) showed moderate effects of the crude chicory root extract on gene expression levels in the H4IIE cells, potentiated by pure CQAs or their metabolites. The diCQAs, 3-caffeoylquinic, and caffeic acids all modulated nearly identical sets of genes, suggesting that they all contain the structural (caffeic) moiety that is responsible for the observed effects. Quinic acid clustered together with 3-caffeoylquinic acid, with which it shares an exposed quinic acid moiety, and therefore, shares some bioactivity that sets it apart from the rest of the treatments. A Volcano plot was constructed from the RT Profiler data (Figure 5B). A single group of four genes was upregulated by chicory extract and all CQA metabolites with the exception of quinic acid: Akt2 and Akt3 (serine/threonine-protein kinases 2 and 3), Pi3kr2 (PI3-kinase regulatory subunit beta), and FAS (fatty acid synthase), suggesting an increased hepatic PI3K/Akt signaling following the treatment with CQAs. An increase in PI3K/Akt signaling was not mediated by insulin receptor signaling (Irs1-2, Dok1-3, Insr) or insulin-like growth factor signaling (Igf1r, Igf2, Igfbp1) genes; instead, they were suppressed by CQAs. Another group of prominently downregulated genes included components from the MAPK signaling pathway that regulates cell growth and differentiation (Araf, Eif2b1, Hras, Map2k1, Mapk1, Raf1, Rps6ka1, Rras2). The last group of suppressed genes contained targets for carbohydrate/glucose metabolism, including fructose-bisphosphatase 1 (Fbp1), glucose-6-phosphatase catalytic subunit (G6pc), glucokinase (Gck), glycerol-3-phosphate dehydrogenase 1 (Gpd1), phosphoenolpyruvate carboxykinase 2 (Pck2), pyruvate kinase

(Pklr), glucose transporters GLUT1 (Slc2a1) and GLUT4 (Slc2a4), and a serine/threonine-protein kinase (Gsk3b). GSK3B acts as a negative regulator of glucose homeostasis by phosphorylating and inactivating glycogen synthase [30], thus suggesting that a concomitant increase in glycogen synthesis has occurred in the H4IIE liver cells exposed to CQAs.

3.5 Bioenergetic characterization of CQA's effects on metabolism

To further determine whether the suppression of gluconeogenesis by downregulation of the carbohydrate metabolism genes leads to changes in energy metabolism (mitochondrial respiration and glycolysis), we performed cellular bioenergetics measurements in H4IIE hepatocytes using XF24 Extracellular Flux Analyzers (Figure 6). The basal oxygen consumption rate (OCR) of liver cells was 184±17 pmol/min, which was increased to 233±11 pmol/minute by 10 μM 3,5-diCQA ($P<0.05$), while the reference treatment 10 nM insulin had no direct effect on OCR (Figure 6A). Injection of CQA metabolites 3-CQA and caffeic acid at the same concentration resulted in comparable increases in OCR, with the highest response recorded for the caffeic acid alone (244±16 pmol/min, $P<0.05$) (Figure 6B). Neither diCQA nor its metabolites that contain caffeic moiety affected extracellular acidification rate (ECAR), an indirect measure of glycolysis. ECAR, however, was increased both by insulin (9.18±0.45 mpH/min, Figure 6C) and quinic acid (9.73±0.99 mpH/min, Figure 6D) as compared to the basal ECAR rates (6.78±0.44 mpH/min). Further calculations revealed that the suppression of gluconeogenesis with 3,5-diCQA, 3-CQA, and caffeic acid increased maximal respiration by 20-23% (Figure 6E), ATP-generating OCR by 25-38% (Figure 6F), and uncoupled OCR from the proton leak by 27-119% (Figure 6G). Taken together, these results showed that liver cells significantly increased oxidative phosphorylation and ATP production in response to CQAs.

4. Discussion

Chicory root has a long history of human use, both as a food and as a dietary supplement to control high blood sugar levels. This activity is attributed to high amounts of the inulin-type fructans that slow down carbohydrate digestion and glucose absorption in the gastrointestinal tract [31]. Chicory plants also produce a variety of other bioactive secondary metabolites, including caffeoylquinic acids and caffeic acid derivatives as major phenolic constituents [29]. CQAs are not unique to chicory and are also available in high concentration from coffee [32] and artichoke [33]. Both mono and diCQAs have been reported to improve glycemia in animal models through their effects in the liver and muscle tissues [34]. While multiple structural isomers of mono- and diCQAs have been described [29], their biological potencies, the structure-activity relationships, and the principal moiety responsible for their anti-hyperglycemic effects have not been investigated.

DiCQAs previously isolated from chicory root had been identified as the 1,3-, 1,4-, 3,4- and 3,5- isoforms, and some data had already been collected on their function [17,35,36]. New in this study was the bioactivity-guided confirmation of CQAs as major chicory constituents responsible for its anti-hyperglycemic effects (Figure 1), the isolation of novel 1,5-diCQA, and the preliminary suggestion that its function is very similar to the other forms (Figures 2-3). The 1,3- and 1,4-diCQA isoforms reported for chicory cultivars Chioggia, Treviso, and Verona [29] were absent from the chicory cultivar Sacson used in this study, suggesting high metabolic or environmental diversity of chicory plants to accumulate different CQA isoforms. To further complicate the multiplicity of CQA structures (Figure 2), these isoforms have different bioavailability and hydrolyze into early (chlorogenic, caffeic, and ferulic acids) and late (isoferulic, dihydrocaffeic and dihydroferulic acids) plasma metabolites, indicating two different metabolic pathways for CQAs [33]. There is also no general agreement about peak plasma concentration of these metabolites, with multiple

studies reporting the maximum concentrations in the very wide range of 0.3-30 μM [4]. Comparison of urinary metabolites indicated that colonic microflora may be one of the main metabolism sites of CQAs and caffeic acid in both rodents and humans [37]. This complexity, however, did not translate to major differences in the anti-hyperglycemic effects of CQA isoforms in liver cells (Figure 3). Instead, the degree of biopharmacological similarity between the three diCQA isomers used in this study precluded steriochemical effects of the larger compounds and suggested putative activity by the smaller metabolite liberated after hydrolysis of the parent structures.

Direct comparisons between diCQAs and their metabolites in equimolar concentrations (3-caffeoylquinic, caffeic, and quinic acids) revealed that caffeic acid moiety alone was responsible for the suppression of hepatic glucose production in the H4IIE rat hepatoma cells (Figures 3-4). These findings were further supported by nearly identical pharmacogenomic profiles of diCQA and all metabolites that contain caffeic moiety, including free caffeic acid and the original chicory extract from which we isolated diCQAs (Figure 5). Exposure to quinic acid, on the contrary, resulted in a different gene expression profile, which is likely explained by its different chemical structure unrelated to hydroxycinnamic acids. The array data showed a greater than 2-fold upregulation of genes involved in the PI3K signaling pathway (Akt2/3, Pi3kr2, and FAS). Previously, many studies have demonstrated a direct link between the PI3K/Akt activity and FAS upregulation (38)]. This effect was not mediated by insulin signaling, since most of insulin receptor-associated protein or growth factors were not affected or downregulated at the same time. A parallel prominent suppression of genes that regulated glucose metabolism (Fbp1, G6pc, Gck, Gpd1, Pck2, Pklr, Gsk3b) and carbohydrate transport (Slc2a1 and Slc2a4) in the liver cells suggested an alternative signaling, possibly via AMP-activated protein kinase (AMPK) pathway as has been suggested previously for chlorogenic acid [13]. Simultaneous

downregulation of MAPK signaling demonstrated improved cellular homeostasis and opened an attractive possibility to use dietary or supplementary CQAs for normalization of PI3K/Akt, MAPK and AMPK signaling controls to effectively improve glucose homeostasis and acquired insulin resistance in pre-diabetic and diabetic states [7].

At the cellular level, the disruption of glucose homeostasis is caused in part by mitochondrial dysfunction. Upon activation under conditions of metabolic stress, AMPK triggers cytoprotective programs for acutely upregulating ATP production and downregulating non-essential energy expenditure [6]. Indeed, we observed increased maximal and ATP-coupled respiration in response to 10 μM 3,5-diCQA, 3-CQA, and caffeic acid, with the latter molecule inducing the strongest response when applied in equimolar concentration (Figure 6). These findings agreed with earlier observations of increased ATP production in human neuroblastoma SH-SY5Y cells exposed to di-and tri-CQAs. Dissipation of the mitochondrial proton gradient (proton leak) was conferred only when caffeic acid moiety was present, confirming a previous study that reported complete mitochondrial uncoupling effects of caffeic acid derivatives at 50 μM [6]. The exact mechanism or therapeutic target for this effect is unknown.

Abrogating the glucose release in pre-diabetic patients to manage hyperglycemia can be a powerful tool in preventing the onset of insulin resistance and associated metabolic disease risks. Direct inhibition of hepatic gluconeogenesis by dietary CQAs or caffeic acid derivatives via multiple mechanisms, including enzymatic inhibition, PI3K/MAPK/AMPK signaling, and changes in mitochondrial bioenergetics, is a very attractive approach to diet- or lifestyle-based modification of metabolic diseases. However, it is not clear whether dietary CQAs induce both short-lived and long-term effects on glucose metabolism due to rapid absorption and metabolism (i.e. glucuronidation) of CQAs and their metabolites in humans. This question warrants further investigation of dietary CQAs to understand best

dosing and regimen strategies to achieve maximal beneficial effects.

5. References

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6. Figures

Figure 1

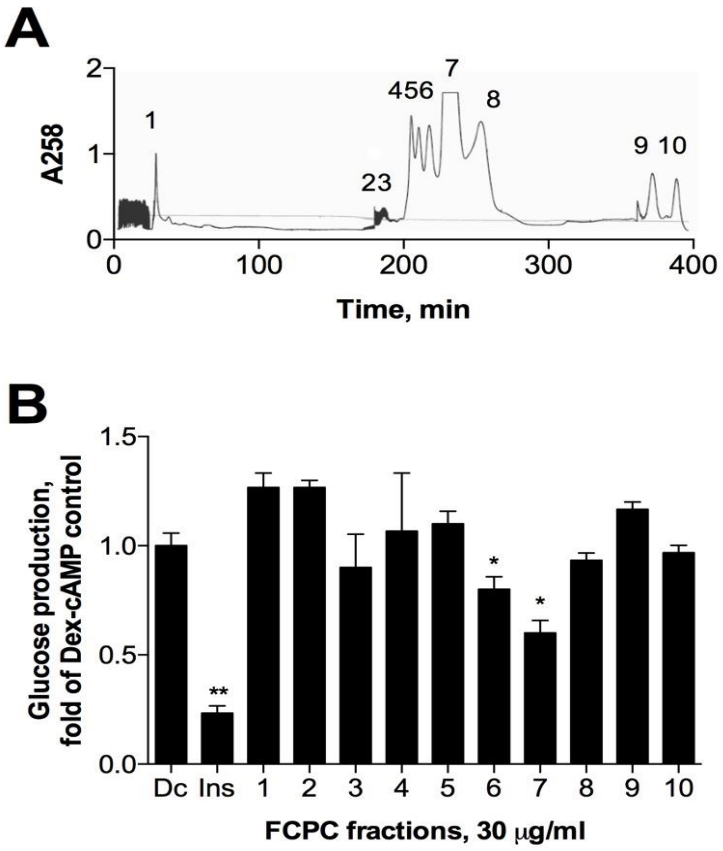


Figure 1. Bioactivity-guided fractionation of chicory root extract. (A) FCPC separation profile yielding 10 fractions. (B) Fraction 7 contained bioactive constituents that suppressed hepatic glucose production *in vitro*. Gluconeogenesis was induced in H4IIE rat hepatoma cells for 8 h and glucose production was quantified in presence of 10 ◎g/ml fractions. All results were expressed as fold change from the Dex-cAMP controls (n=3, means±SEM, *P<0.05).

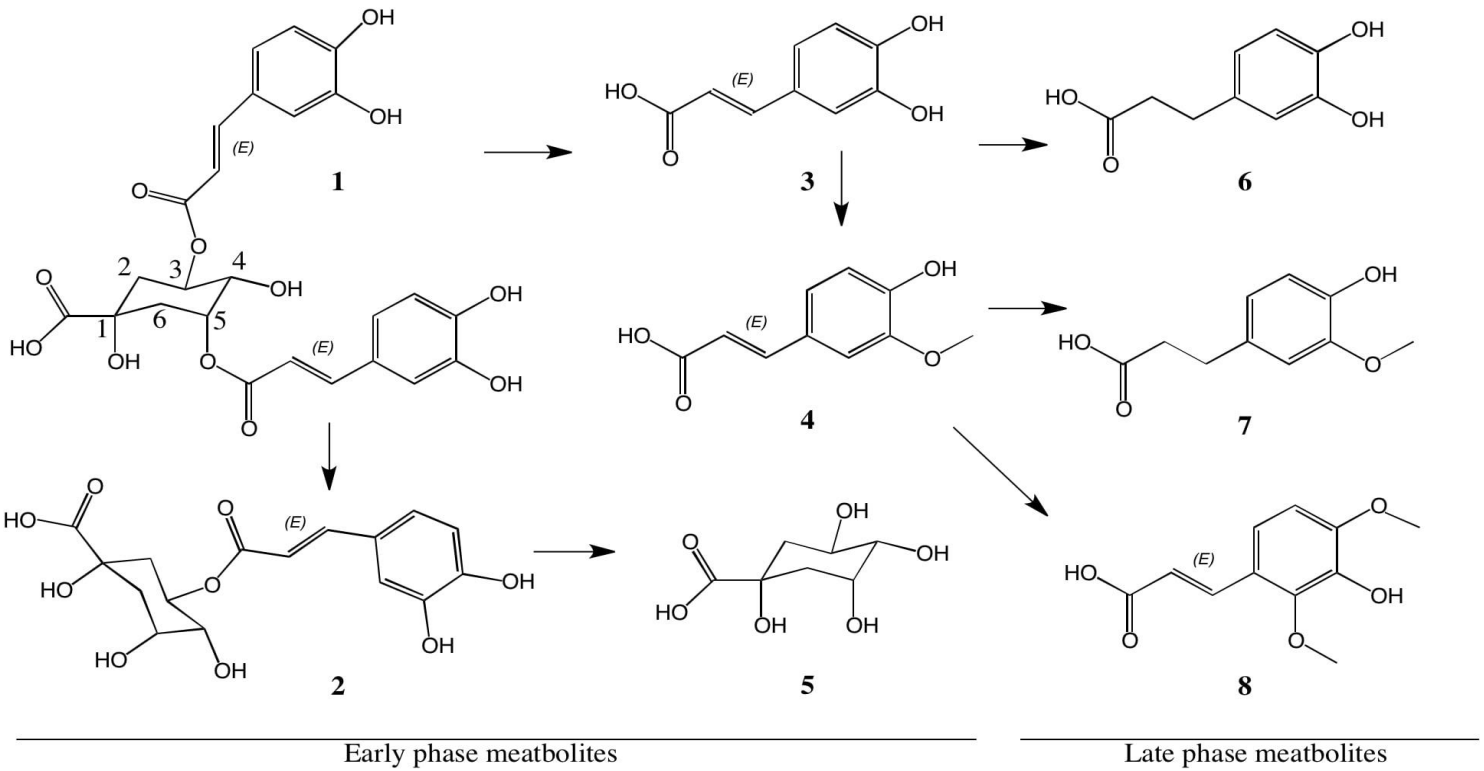


Figure 2. Schematic pathway of CQA hydrolysis and two-phase metabolism, modified after [7]].
(1) 3,5-diCQA, (2) 3-CQA, (3) Caffeic acid, (4) Ferulic acid, (5) Quinic acid, (6) Dihydrocaffeic acid,
(7) Dihydroferulic acid, (8) Sinapic acid.

Figure 3

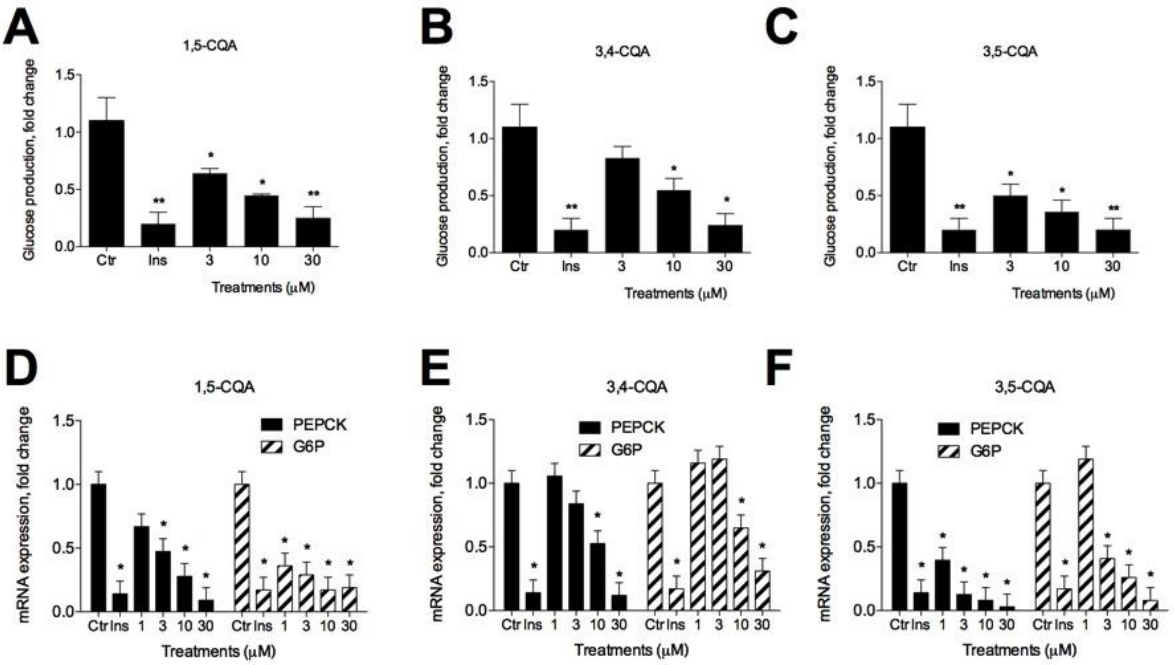


Figure 3. Effect of 3 diCQA from chicory roots on liver glucose production *in vitro*. Dose-dependent suppression of gluconeogenesis in H4IIE cells by (A) 1,5-diCQA, (B) 3,4-diCQA, (C) 3,5-diCQA applied at 3-30 μM. Downregulation of expression of two key enzymes that regulate hepatic gluconeogenesis, G6Pase and PEPCK by (D) 1,5-diCQA, (E) 3,4-diCQA, (F) 3,5-diCQA applied at 1-30 μM. All results were expressed as fold change from the Dex-cAMP controls (n=3, means±SEM, *P<0.05). Insulin at 10 nM was used as a positive control. mRNA expression was analyzed by qPCR and normalized with respect to the expression of the β-actin gene.

Figure 4

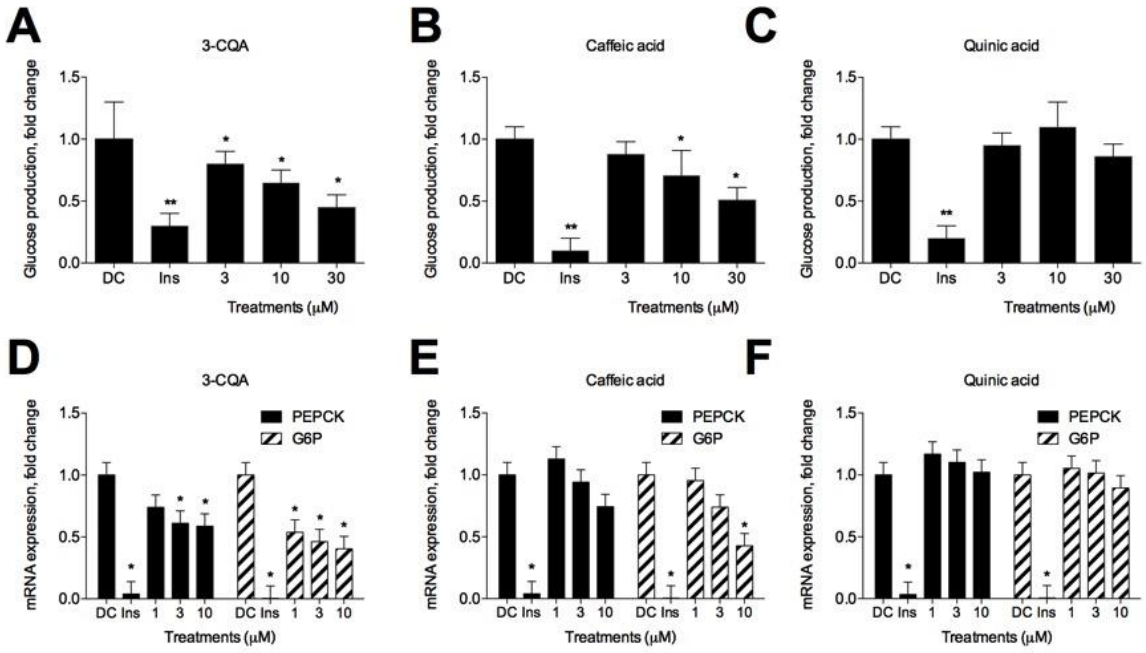


Figure 4. Effect of early phase diCQA metabolites from chicory root on liver glucose production *in vitro*. Dose-dependent suppression of gluconeogenesis in H4IIE cells by (A) 3-CQA, (B) Caffeic acid, (C) Quinic acid applied at 3-30 μM. Downregulation of G6Pase and PEPCK expression by (D) 3-CQA, (E) Caffeic acid, (F) Quinic acid applied at 1-30 μM. All results were expressed as fold change from the Dex-cAMP controls (n=3, means±SEM, *P<0.05). Insulin at 10 nM was used as a positive control. mRNA expression was analyzed by qPCR and normalized with respect to the expression of the β-actin gene.

Figure 5

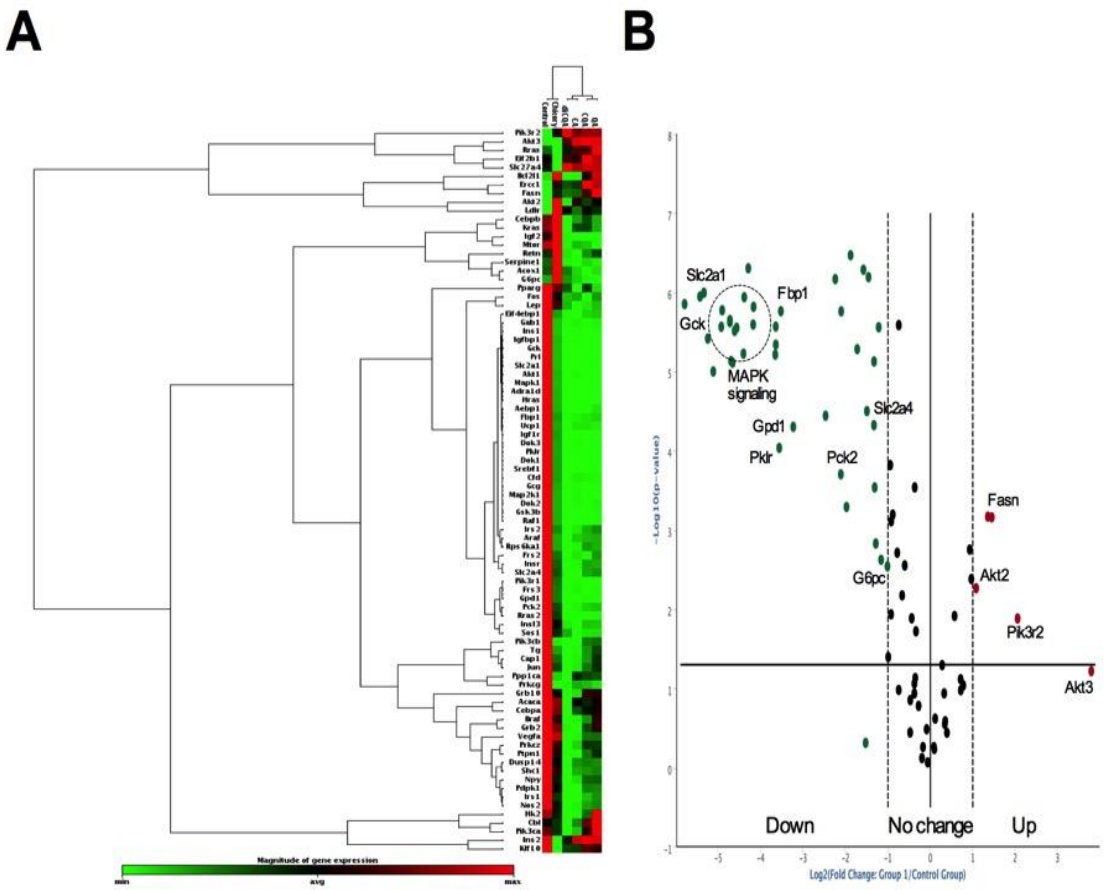
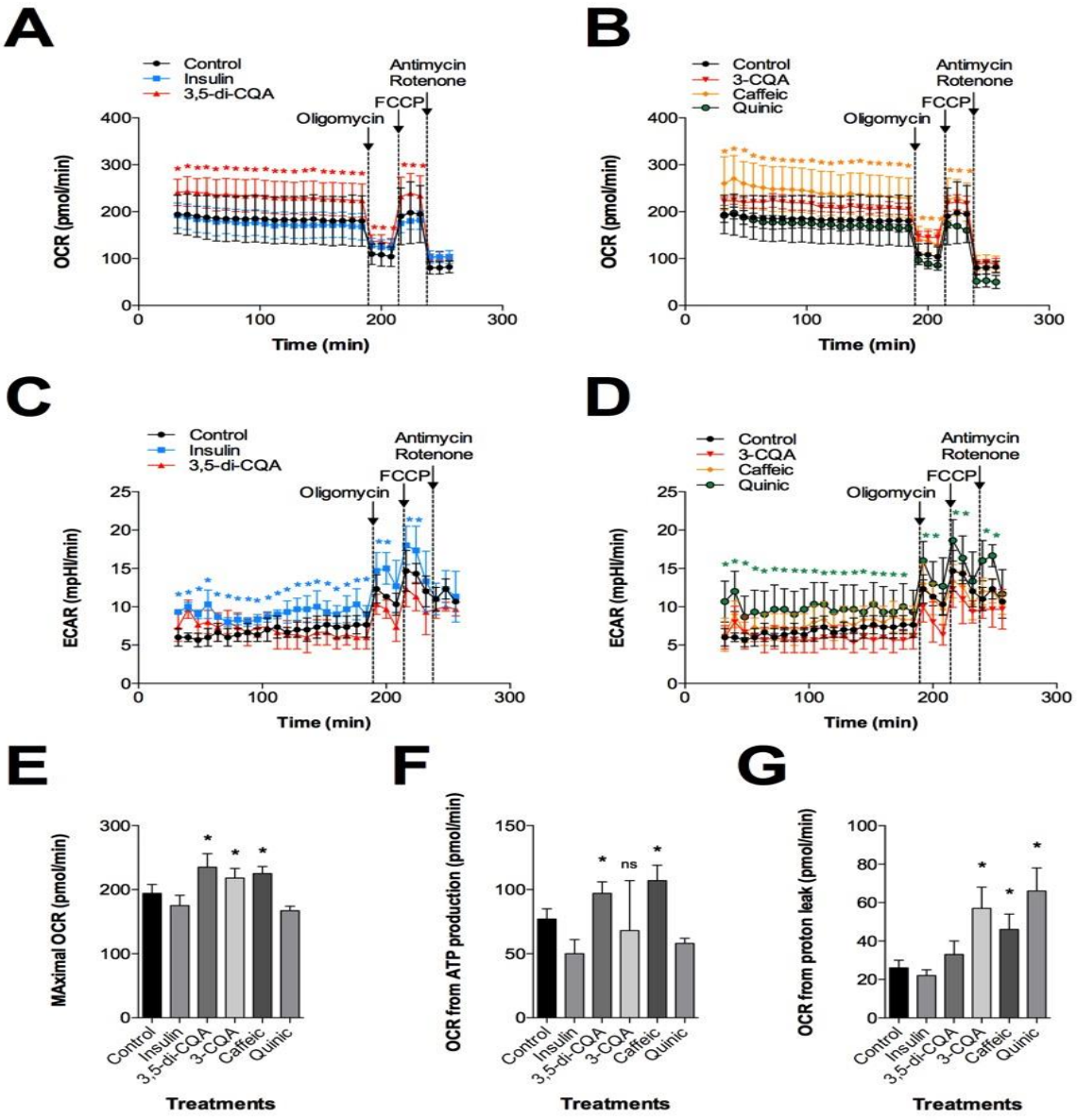


Figure 5. Pharmacogenomic suppression of carbohydrate metabolism and MAPK signaling genes by chicory bioactives. (A) Clastergram analysis of qPCR array gene expression profiles in response to 8 h treatment of Dex-cAMP stimulated H4IIE cells with 30 μg/ml chicory extract or 10 μM 3,5-diCQA and its metabolites. (B) Volcano plot between the combined qPCR array gene expression profile of CQA and metabolites (with the exception of quinic acid) and Dex-cAMP stimulated H4IIE cells.

Figure 6. DiCQA and metabolites enhanced mitochondrial respiration and proton leak in H4IIE liver cells. Change in (A) oxydative phsphorylation and (C) glycolysis rates in response to 10 μM 3,5-diCQA as compared to 10 ng/ml insulin treatment as a reference drug. Respective changes in (B) oxydative phsphorylation and (D) glycolysis rates in response to 10 μM 3-CQA, caffeic and quinic acids. Cells were treated in Seahorse XF assay plates for 8 h and subjected to 20 basal bioenergetics readings. Next, mitochondrial complex inhibitors were injected to all treatments sequentially and 3 readings were taken after each inhibitor. (E) Maximal OCR, (F) OCR from ATP production, and (G) OCR from proton leak were calculated and expressed as means±SEM, *$P<0.05$ (n=3).

Figure 6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CHAPTER 3: GLYCEMIC CONTROL STATUS AFTER ACUTE AND CHRONIC SUPPLEMENTATION WITH CHICORY ROOT BIOACTIVES IN DIABETIC MICE

Kimberly Marie Palatini Jackson, Pier-Jean Durand, Mickey Wilson, Thirumurugan Rathinasabapathy, Xu Li, Slavko Komarnytsky

Abstract

Chicory (*Chicorium intybus* L.) is a perennial herb commonly consumed as a vegetable, while the ground and roasted roots are often ingested in the form of hot water extract (a coffee substitute). A mixture of soluble fructans, caffeoylquinic acids, and sesquiterpene lactones represents the bulk of water-soluble chicory root matrix that is responsible for a characteristic bitter taste of chicory root. As chicory was traditionally used to control hyperglycemia associated with metabolic disorders, the aim of this study was to evaluate the acute and long-term effects of dietary chicory supplementation on glucose and lipid metabolism in the polygenic C57BL/6J mouse model of diet-induced obesity and diabetes. Acute oral administration of hot water extract at 100-300 mg/kg resulted in a dose-dependent decrease in fasting blood glucose. However, supplementation with chicory extract at 1% of low- or high-fat diet for 20 weeks did not improve long-term hyperglycemia, while still reducing hepatic triglycerides in these animals. Acute antihyperglycemic effect of chicory root matrix was primarily mediated by delayed intestinal absorption of carbohydrates and was potentiated by bitter sesquiterpene lactones, as demonstrated in the STC-1 cell culture model of intestinal glucose uptake. These results strengthened the case for the development of novel functional ingredients from chicory roots that combine multiple soluble and bitter tasting constituents to achieve immediate improved control over acute postprandial hyperglycemia.

1. Introduction

Type 2 diabetes (T2D) is a complex metabolic disorder affecting more than 300 million people worldwide [1,2]. It is characterized by elevated levels of fasting blood glucose and high postprandial glucose spikes due to the severely diminished first-phase insulin response [3]. After-meal blood glucose is more reliable in predicting poor glycemic control [4] and correlates better with glycated hemoglobin HbA1C levels [5]. Therefore, acute treatments that act quickly and for a short time are generally viewed as a better choice for managing the after-meal glucose spikes than ones that work slowly over a long period [6]. While clinicians are presented with multiple oral antidiabetic agents including acarbose, glibenclamide, metformin, miglitol, rosiglitazone and voglibose, most of these interventions have undesirable side effects, develop tolerance after continued administration, and/or are too expensive for patients from the developing world [7]. Acarbose, a widely used α-glucosidase inhibitor to delay digestion and absorption of intestinal carbohydrate is associated with hepatotoxicity, gastrointestinal discomfort, and a rapid adaptive response within the gastrointestinal intestinal tract that greatly diminishes its pharmacological activity over time [8].

Traditional herbal medicine manages T2D with multiple bitter herbal preparations [9]. Bitter chicory root (*Chicorium intybus, Asteraceae)* has a long history of therapeutic use to control hyperglycemia associated with T2D [10,11]. The chicory root matrix contains a bulk of soluble fructans, caffeoylquinic acids, and sesquiterpene lactones that are easily co-extracted as an aqueous decoction [12–15]. A mixture of these bioactive constituents has a high potential to synergistically modulate glucose and lipid metabolism due to multiple modes of action: prebiotic effects of inulin fructans [16], reduction of hepatic glucose output by caffeoylquinic acids [17], and anti-inflammatory effects of sesquiterpene lactones that suppress immunological components of insulin resistance [18]. Even though chicory extract

is Generally Regarded as Safe (GRAS) by the FDA [19], toxicological and clinical data on its use is very limited. Chicory coffee reduced the blood and plasma viscosity and platelet aggregation in 27 healthy subjects consuming 20 g roasted chicory powder in 300 ml of hot water for 1 week [20]. Anti-inflammatory effects of chicory root extracts (600-1,800 mg/day for 1 month) were documented in 40 patients with osteoarthritis of the hip or knee [21].

Anti-hyperglycemic effects of chicory root preparations were reported only from animal studies. For example, 80% ethanolic extract was effective at 125 mg/kg/day for 14 days for reduction of blood glucose, cholesterol, and triglycerides independent of serum insulin levels [22], while perfusion with aqueous extract of chicory root reduced glucose absorption in the rat jejunum [23]. This effect was generally attributed to caffeoylquinic acids and their metabolites, chlorogenic, caffeic and ferulic acids; however, their relative bioavailability and efficacy remained under investigation. While both caffeic and ferulic acids were effectively absorbed in small intestine within 1-2 hours of administration, complex caffeoylquinic acids were primarily metabolized by gut microflora before absorption in the large intestine [24], but these findings were questioned by later studies using green coffee food matrix [25].

The objective of this study was therefore to differentiate the acute and long-term effects of dietary chicory supplementation on glucose and lipid metabolism in the polygenic C57BL/6J mouse model of diet-induced obesity. Individual contributions of major hydroxycinnamic acids naturally present in chicory root matrix were further evaluated in the STC-1 mouse cell model of intestinal glucose absorption.

2. Materials and methods

2.1 Reagents and extraction procedures

All chemicals were obtained from Sigma (St. Louis, MO), cell culture materials from Life Technologies (Carlsbad, CA), and HPLC-grade solvents from VWR (Radnor, PA), unless

specified otherwise. Raw organic chicory root powder was from Starwest Botanicals (Sacramento, CA).

Aqueous (100 g powder in 1L of 80°C water) and ethanolic (100 g powder in 1L of absolute ethanol) extractions were carried for 1 hour, the resulting liquids were vacuum-filtered to remove solids and the ethanol extract was further evaporated using Buchi R210 rotavapor (Flawil, Switzerland). Liquid extracts were lyophilized to dryness in Labconco FreeZone 18 freeze dry system (Kansas City, MS) and stored at -20°C until further use.

2.2 Analytical measurements

Phytochemical composition of the chicory extracts was analyzed in triplicate by LC-MS in positive mode (Agilent G1312B-1200 series Infinity Quaternary HPLC system coupled with Agilent 6530A Accurate-Mass Quadrupole Time-of-Flight MS with Agilent Jet Stream source). Dry samples were reconstituted with 500 µl 80% methanol and 5 µl was injected onto an Agilent ZORBAX Eclipse Plus C18 column (3 x 100mm, 1.8 µm). A gradient from 30% acetonitrile in 0.1% formic acid to 90% acetonitrile in 0.1% formic acid was used for HPLC separation. Mass data were acquired with the following parameters: drying gas temperature 300 °C, drying gas flow 7 L/min, nebulizer pressure 40 psi, sheath gas temperature 350 °C, sheath gas flow 10 L/min, capillary voltage 3500 V, nozzle voltage 500 V, fragmentor voltage 150 V, skimmer voltage 65 V, octopole RF peak voltage 750 V. Standard curves for compounds of interest were obtained in a linear dynamic range of 10-50,000 pg and used for quantitative analysis. Peak area was determined by Agilent MassHunter Qualitative Analysis (version B.05.00) software.

Total fructans including inulin were measured in chicory extract using Fructan Assay Kit according to the manufacturer's protocol (Megazyme, Chicago, IL).

2.3 Animal studies and diets

All animal experiments were performed according to procedures approved by the NC Research Campus Institutional Animal Care and Use Committee in the David H. Murdock Research Institute, the AAALAC accredited animal care facility. Male, 6 week old C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed four animals per cage under controlled temperature (24 ± 2 °C) and light (12 h light-dark cycle, lights on at 0700 h). Immediately upon arrival, animals were allowed to adapt to new conditions for 7 days and handling the animals was performed daily during this time to reduce the stress of physical manipulation. Mice (n=8) were then randomized into ad lib access to a low-fat diet (LFD) containing 10% fat-derived calories (D12450B; Research Diets, New Brunswick, NJ), a low-fat diet supplemented with 1% aqueous chicory root extract (LFDC), a high fat diet (HFD) containing 60% fat-derived calories (D12492, Research Diets), or a high fat diet supplemented with 1% aqueous chicory root extract (HFDC) for 20 weeks. Since a typical 24 h food intake for a C57BL/6 mouse is 2.5-3.0 g or 10-12 kcal/g body weight [26], the animals were expected to consume an average of 25 mg/day of aqueous chicory extract that contained fructans, caffeoylquinic acids, and sesquiterpene lactones (Table 1). Animal weight and food intake (accounting for spillage) were recorded weekly for the duration of the study. All animal diets were kept at -80°C for long-term storage and stability, and freshly thawed food was dispensed to animals every 3-4 days to limit phytochemical degradation in food matrix. For long-term fasting blood glucose levels, mice were tested after 16 h overnight fast on weeks 8, 12, 16, and 20 of the study. At the end of the study, blood was collected by heart puncture after CO_2 inhalation. Gastrointestinal (stomach, duodenum, jejunum, ileum, cecum and colon) and metabolic (liver, gastrocnemius muscle, epididymal fat) tissues were collected and stored at -80°C to determine the temporal sequence and signaling events that are responsible for the observed

changes in physiology and metabolism.

2.4 Oral glucose tolerance tests

A separate cohort of C57BL/6J mice (n=16) were maintained on HFD diet for 7 weeks, and acute oral glucose tolerance tests were performed with orally administered 300 mg/kg of chicory extract or bioactive compounds 30 minutes prior to the glucose gavage. Mice were fasted overnight (16 h) and received oral gavage of D-glucose (1.5 g/kg body weight, Sigma). Blood glucose concentrations were measured at 0, 15, 30, 60 and 120 minutes after glucose challenge in blood samples obtained from tail-tip bleedings, using a glucometer (True Result, Trivida, FL).

2.5 Lipid measurements

Total lipids were extracted from pre-weighed sections of murine liver using the Folch method [27]. The tissue was homogenized with chloroform/methanol (2/1) to a final volume 20 times the volume of the tissue sample and incubated at room temperature for 2 hours. Samples were centrifuged and the liquid phase was mixed with a 0.9% NaCl solution. After centrifuging again, the upper phase was removed and the lower phase was dried in a Rotovap for 6 hours. Liver triglycerides were quantified from remaining lipid extraction using L-Type Triglyceride M kit (Wako Diagnostics, Mountain View, CA).

2.6 Cell culture and glucose uptake measurements

The mouse neuroendocrine intestinal cell line STC-1 (CRL-3254) that acts as a model for glucose absorption and hormone secretion was obtained from ATCC (Manassas, VA). Cells were routinely passaged every 3-4 days and maintained in high glucose DMEM containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37 °C and 5% CO_2. Cells were sub-cultured into 24-well plates and, once confluent, changed to induction medium that contained glucose-free DMEM supplemented with 2 mM sodium pyruvate to induce fluorescent glucose uptake.

Treatment was administered for 2 hours (with and without 50 μM probenecid, a bitter taste receptor blocker [28] before cells were exposed to 10 μM 2-NBDG for 20 minutes, washed with 1xPBS, and imaged using EVOS FL Cell Imaging System (Life Technologies) before analysis by flow cytometry (BD Accuri C6, San Jose, CA). Cells were re-suspended in 1xHBSS and incubated for 20 minutes with propidium iodide to differentiate dead and living cells [29]. Absolute glucose fluorescence intensity values were normalized to the total cell concentration.

2.7 RNA extraction and cDNA synthesis

Total RNA was extracted from cells or liquid nitrogen-preserved murine liver tissues using Trizol reagent (Thermo Fisher, Waltham, MA) following manufacturer's instructions. RNA was quantified spectrophotometrically by absorbance measurements at 260 and 280 nm using the Synergy H1 Take 3 system (Biotek, Wilmington, DE). The cDNAs were synthesized using 2 μg of RNA for each sample with high capacity cDNA Reverse Transcriptase (Thermo Fisher) following manufacturer's instructions.

2.8 qPCR analysis

The qRT-PCR amplifications were carried out in triplicate on an ABI 7300 Real-Time Detection System in a total volume of 20 μl containing 10 μl of SYBR Green 2× Supermix (Applied Biosystems), 5 μl of the 1:50 diluted cDNA, 1 μl of each specific primer, and 3 μl of PCR-grade water. The qRT-PCR program was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. The corresponding primers were selected using the Primer Express version 2.0 software as follows: β-actin (NM_007393.3), forward primer: 5′-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse primer: 5′-CAC AGC CTG GAT GGC TAC GT-3′; G6Pase (NM_008061.3), forward primer 5′-GAA AAA GCC AAC GTA TGG ATT CC-3′, reverse primer 5′-CAG CAA GGT AGA TCC GGG A-3′; FXR (NM_009108), forward primer 5′-CCA CCG GCT GTC AGG ATT T-3′, reverse primer 5′-GCA TAC CTT

TAG CTG GCT TCA CA-3′; FAS (NM_007988.3), forward primer 5′- GGC ATC ATT GGG CAC TCC TT-3′, reverse primer 5′-GCT GCA AGC ACA GCC TCT CT-3′; SREB (NM_008158), forward primer 5′- GAT GTG CGA ACT GGA CAC AC-3′, reverse primer 5′-CAT AGG GGG CGT CAA ACA G-3′. qRT-PCR data were analyzed with a 7300 System SDS Software v1.3.0 (Applied Biosystems) to estimate transcript copy numbers for each sample. Target mRNA expression was analyzed using the ΔΔCT method [30] and normalized with respect to the expression of the β-actin housekeeping gene. The LFD control group served as the calibration sample in this study, and the target gene expression of the calibration sample was assigned a value of 1.0.

2.9 Statistical analysis

Statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA) and expressed as means ± SEM. Two tailed t-test, one-way ANOVA, or two-way ANOVA were applied at a significance level of $p<0.05$. Post-hoc analyses of differences between individual experimental groups were made using the Tukey's multiple comparison test.

3. Results

3.1 Comparison of chicory root extracts

To directly compare aqueous and ethanolic extracts of raw ground chicory root powder, we performed enzymatic analysis of fructan content and LC-MS analysis of hydroxycinnamic acid derivatives and a major sesquiterpene lactone (lactucopicrin). Compounds were identified by comparing their retention times and fragmentation patterns with those of the standard compounds. While fructans were generally absent from the ethanolic extract, it contained on average 2-fold higher amounts of caffeoylquinic acids and lactucopicrin (Table 1). No significant amounts of free caffeic, ferulic, and quinic acids were

detected in either extract. Since only aqueous extract contained a combination of 3 bioactive constituents with potential multiplicity of modes of action including prebiotic effects of inulin fructans, reduction of hepatic glucose output by caffeoylquinic acids, and anti-inflammatory effects of sesquiterpene lactones that suppress immunological components of insulin resistance, was chosen for all subsequent studies.

3.2 Acute anti-hyperglycemic effect of chicory root extract

Obesity and hyperglycemia were induced by feeding a HFD to C57BL/6J mice for 7 weeks before the animals were tested for oral glucose tolerance (Figure 1A). During this time, HFD mice developed significant body weight gain (12.49±1.468 g vs. 6.71±1.042g) and fasting hyperglycemia (162±8.591 mg/dl vs. 84± 4.136 mg/dl) as compared to LFD controls. Chicory root extract showed moderate efficacy at lowering blood glucose levels in C57BL/6J mice following the acute single-dose treatment at 100 and 300 mg/kg doses (Figure 2A). Comparable amounts of hydroxycinnamic acid derivatives such as 3,5-caffeoylquinic acid, chlorogenic acid, and caffeic acid also improved glucose tolerance, although with less efficacy, suggesting presence of an additional bioactive constituent in the chicory extract with anti-hyperglycemic properties (Figure 2B).

3.3 Long-term supplementation with chicory extract

Both LFDC and HFDC animals received LFD and HFD diets supplemented with 1% aqueous chicory root extract for 20 weeks. The final body weights did not differ between the LFD groups, and were slightly higher in HFDC animals compared to HFD controls (Figure 1A). No significant changes in food intake were noted (Figure 1B). Fasting blood glucose was monitored every 4 weeks throughout the feeding study and showed small significant differences between treatments and their respective controls during the first 12 weeks of treatment only (Figure 3), suggesting that long-term chicory supplementation did not improve glucose control in these animals due to an unknown compensatory mechanism.

As previous reports indicated liver as a target tissue for supplementation with bioactive constituents from chicory root, we next performed lipid analysis and analyzed gene expression profiles of key genes responsible for regulation of glucose and lipid metabolism in the liver. Both LFDC and HFDC chicory-supplemented animals showed reduced levels of triglycerides in the liver as compared to the un-supplemented controls (Figure 4A). We observed mixed results on the expression of the key gluconeogenic gene glucose-6-phosphatase (G6P), which is regulated on the transcriptional level. mRNA levels of G6P were slightly reduced (Figure 4B), thus providing a partial explanation for lack of long-term effects of chicory supplementation on fasting blood glucose levels. Transcriptional factors farnesoid X receptor (FXR) and sterol regulatory binding element 1 (SREB) that regulate various aspects of glucose and lipid metabolism showed reduced levels of mRNA expression in the liver, resulting in decreased fatty acid synthase (FAS) expression and reduced levels of hepatic triglycerides (Figure 4B).

3.4 Glucose absorption in STC-1 intestinal cell model

Acute, but not long-term improvement of glucose tolerance in DIO mice suggested a direct effect of bioactive constituents from aqueous chicory extract on glucose absorption in the gastrointestinal tract. To confirm these observations, a quantitative analysis of glucose in STC-1 mouse intestinal cell model was performed to determine which components are most effective at modulating this process. Aqueous chicory root extract at 50 μg/ml showed no cell toxicity (Figure 5A) and was very effective at suppressing glucose uptake in STC-1 cells (Figure 5B). Decreased glucose import was clearly evident from emission intensity images of fluorescent glucose analog 2-NBDG accumulation in STC-1 cells (Figure 5C). Among bioactive constituents tested, all caffeoylquinic acid derivatives showed some degree of suppression of intestinal glucose intake; however, all of them were less effective than the parent extract. To account for this discrepancy, we hypothesized that bitter-tasting

sesquiterpene lactone (lactucopicrin) present in chicory extract contributed to suppression of glucose intake by activating gastrointestinal bitter taste receptors (TAS2Rs). Indeed, co-treatment of STC-1 cells with probenecid, an allosteric inhibitor of TAS2Rs, significantly decreased efficacy of caffeoylquinic acids and bitter lactucopicrin on glucose uptake in STC-1 cells (Figure 5D).

4. Discussion

Chicory roots contain a variety of bioactive carbohydrates (soluble fructans) and secondary metabolites (caffeoylquinic acids, sesquiterpene lactones) with glucose-lowering and anti-inflammatory properties. Traditionally known in many cultures as a dietary intervention to manage symptoms and improve lifestyle of patients with diabetes [31], chicory-based supplements have not been tested in clinical and toxicological studies, with few exceptions [16,20,21]. Chicory coffee is still widely consumed in certain parts of Europe, notably France (19% of women from a 69,532 cohort study, compared to 59% women consuming tea and 85% women consuming coffee) [32]. Only one herbal medicinal product containing chicory as the only ingredient has been registered in the European Union (Poland) in the past 20 years, and there have been several herbal combination products on Polish and German markets for over 30 years, traditionally in support of the gastrointestinal system [33]. This study, therefore, was designed to develop and perform preclinical characterization of a food-grade chicory root extract that combines multiple bioactives targeting glucose metabolism.

Direct comparison between aqueous and ethanolic extracts from chicory roots showed that ethanol-based extracts contained higher levels of caffeoylquinic acids and sesquiterpene lactones but small amounts of fructans. A similar concept for a multi-component chicory root extract that combined fructans and phenols in 75% aqueous ethanol extract was

proposed previously; however, it was never tested in cell culture or pre-clinically to characterize its efficacy [34]. Fructan content of the aqueous extract used in this study was 64.2% and caffeoylquinic acids content was 4.7% of dry matter. The extract was also characterized by a prominent bitter taste due to presence of sesquiterpene lactones that survived the hot water extraction process (0.7% of dry matter). When tested in animals, oral supplementation with 100 and 300 mg/kg/day chicory root extract acutely improved oral glucose tolerance in DIO mice by 19.8%. This treatment was equivalent to consuming 8-24 mg/kg/day or 480-1,440 mg/day of chicory extract for an average adult [35]. However, continuous supplementation with chicory root extract at 1% of dietary intake did not improve long-term fasting blood glucose in this study. Our previous work [17] suggested that the degree of biopharmacological similarity between the caffeoylquinic acids precluded steriochemical effects of the larger compounds and suggested putative activity by a common metabolite (caffeic acid) liberated after hydrolysis of the parent structures. However, most of the ingested caffeic acid was absorbed from the small intestine [36], while additional hydroxycinnamic acid metabolites was released from caffeoylquinic acids by gastrointestinal microbiota and absorbed in the colon [24]. While reduction of hepatic neoglucogenesis by caffeic acid was suggested previously [37] and we observed decreases in G6P mRNA levels in livers of animals supplemented with chicory extract, this reduction was clearly not sufficient to lower long-term fasting blood glucose levels in this study. We also observed decreased levels of hepatic triglycerides and reduced transcription of FXR, SREB-1, and FAS genes in liver tissue of both healthy and obese animals supplemented with chicory root extract, in agreement with previously described hepatoprotective effects of chicory supplementation in animals [38].

The acute onset of glucose-lowering effect (OGTT at 30 min) therefore pointed to another immediate interaction between bioactive constituents in chicory root matrix and

carbohydrate metabolism in the gastrointestinal tract. We envisaged two possible mechanisms for this effect: i) direct inhibition of glucose transporters and ii) activation of the gastrointestinal bitter receptors TAS2Rs that subsequently suppress glucose absorption in the gut. To differentiate between these two outcomes, we studied absorption of 2-NBDG (a fluorescent glucose analog) in the STC-1 cell culture model of intestinal glucose uptake treated with caffeoylquinic acid metabolites and lactucopicrin, a major bitter sesquiterpene in chicory root, with and without probenecid, an allosteric inhibitor of TAS2Rs [28]. Inhibition of TAS2R signaling by probenecid restored glucose absorption in STC-1 cells, thus indicating a cross-talk between bitter taste receptor signaling and glucose uptake in the intestinal cells. Although there is no consensus on how bitter perception in the gastrointestinal tract affected glucose absorption, downstream signaling via the α-subunit of gustducin of the G protein-coupled bitter receptors could lead to greater intracellular calcium ion concentrations, affecting the transport and function of glucose transporters SGLT-1 and SGLT-2 in the enterocyte membranes [39,40].

In conclusion, we confirmed acute hypoglycemic effects of aqueous chicory root extract enriched with soluble fructans, caffeoylquinic acids, and sesquiterpene lactones. Stimulatory effect of chicory root bioactive on glucose metabolism developed quickly and for a short time, and did not translate into a long-term whole body insulin-sensitizing effect. These results strengthened the case for the development of novel functional ingredients from chicory roots that combine multiple soluble and bitter tasting constituents as an alternative choice for managing the acute postprandial hyperglycemia.

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6. Tables and Figures

Table 1. Average of major phytochemical constituents in chicory root extracts. Values are given as mean ± SEM (mg/g dry matter).

Extraction	Fructans	3,5-Caffeoylquinic acid	Chlorogenic acid	Lactucopicrin
Aqueous	642.3±31.6	46.93±13.35	3.90±0.79	7.14±0.38
Ethanolic	Nd	88.29±14.52	5.83±0.24	11.77±0.34

Nd, not detected

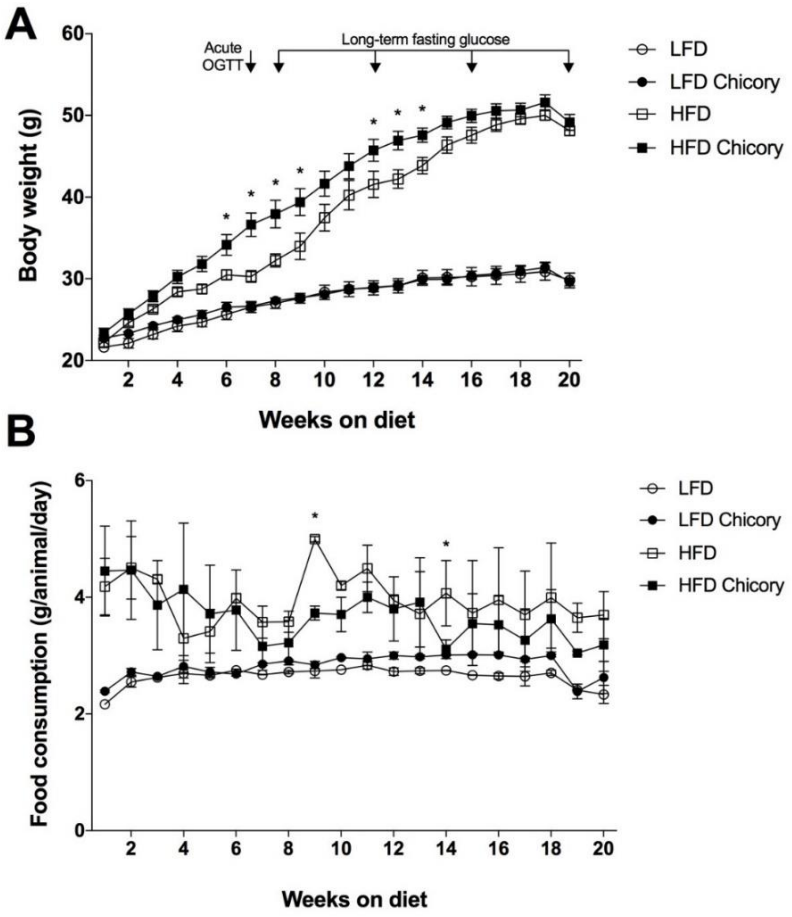


Figure 1. Effect of dietary 1% aqueous chicory root extract on (A) body weight and (B) food intake of LFD and HDF-fed C57Bl/6J mice**.** Animals (n=8) were kept on the respective diets for 20 weeks and acute oral glucose tolerance was measured on week 7 of the HFD diet, while changes in the long-term fasting blood glucose levels were monitored on weeks 8, 12, 16, and 20. Values are means ± SEM, *$p<0.05$ when compared to respective LFD or HFD controls by two-way ANOVA with a post-hoc Bonferroni multiple comparison test.

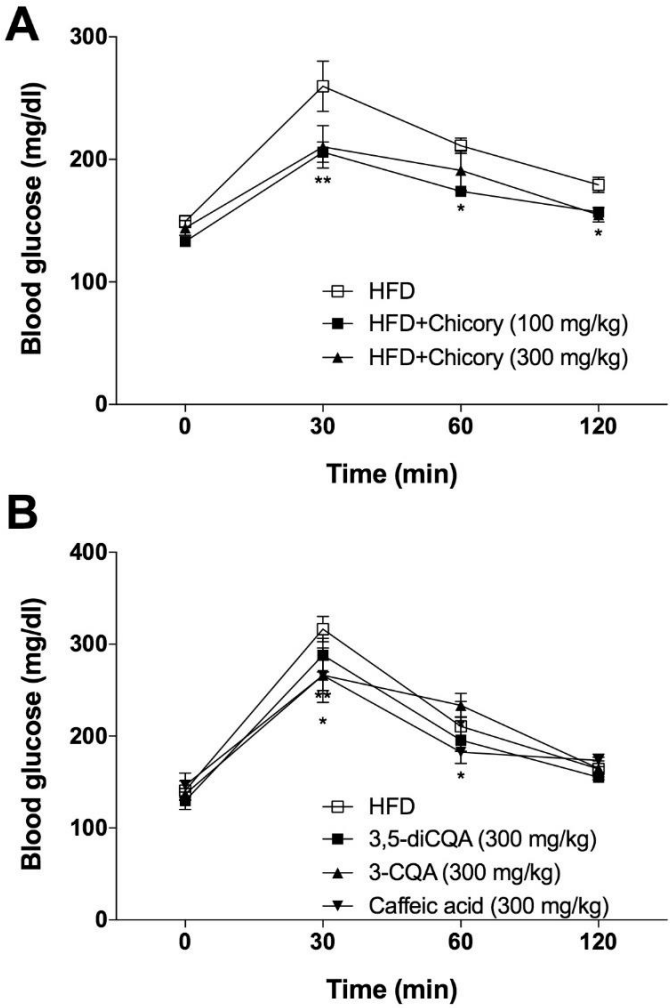


Figure 2. Effect of chicory root bioactives on glucose metabolism in C57Bl/6J mice**.** Acute oral glucose tolerance tests were performed in HFD-fed mice (7 weeks) with a single dose gavage of 100 or 300 mg/kg/day aqueous chicory extract (A), or 100 mg/kg/day dosing of purified caffeoylquinic acids (B). Values are means ± SEM, *p<0.05, **p<0.01 when compared to HFD controls by one-way ANOVA with a post-hoc Tukey's multiple comparison test.

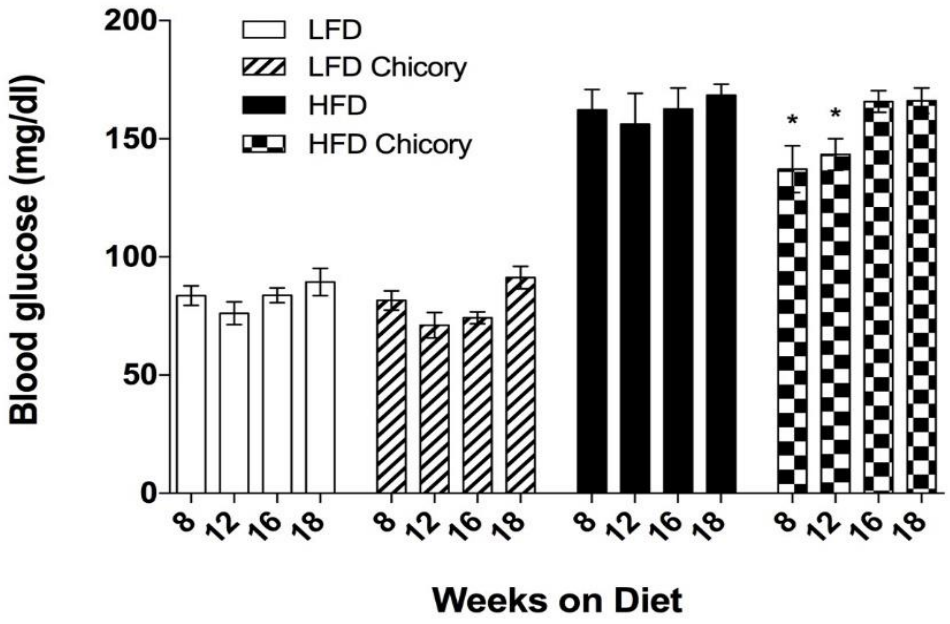


Figure 3. Effect of dietary 1% aqueous chicory root extract on long-term fasting blood glucose levels of LFD and HDF-fed C57Bl/6J mice. Animals (n=8) were kept on the respective diets for 20 weeks and blood glucose was measured after an overnight fast on weeks 8, 12, 16, and 20. Values are means ± SEM, *$p<0.05$ when compared to respective LFD or HFD controls by two-way ANOVA with a post-hoc Bonferroni multiple comparison test.

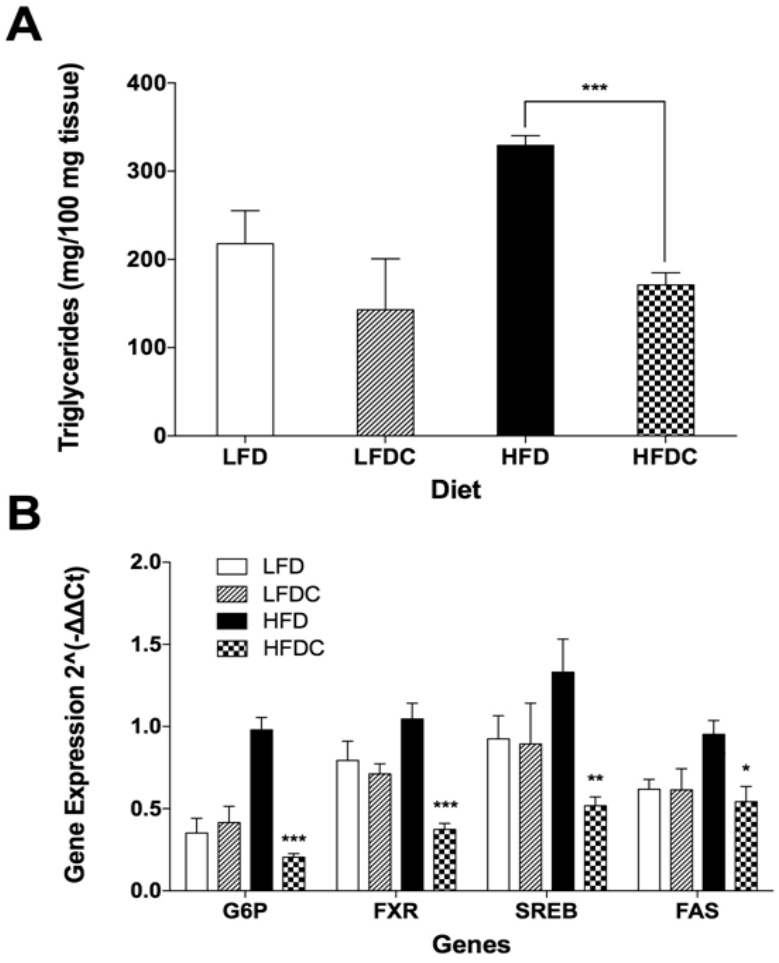
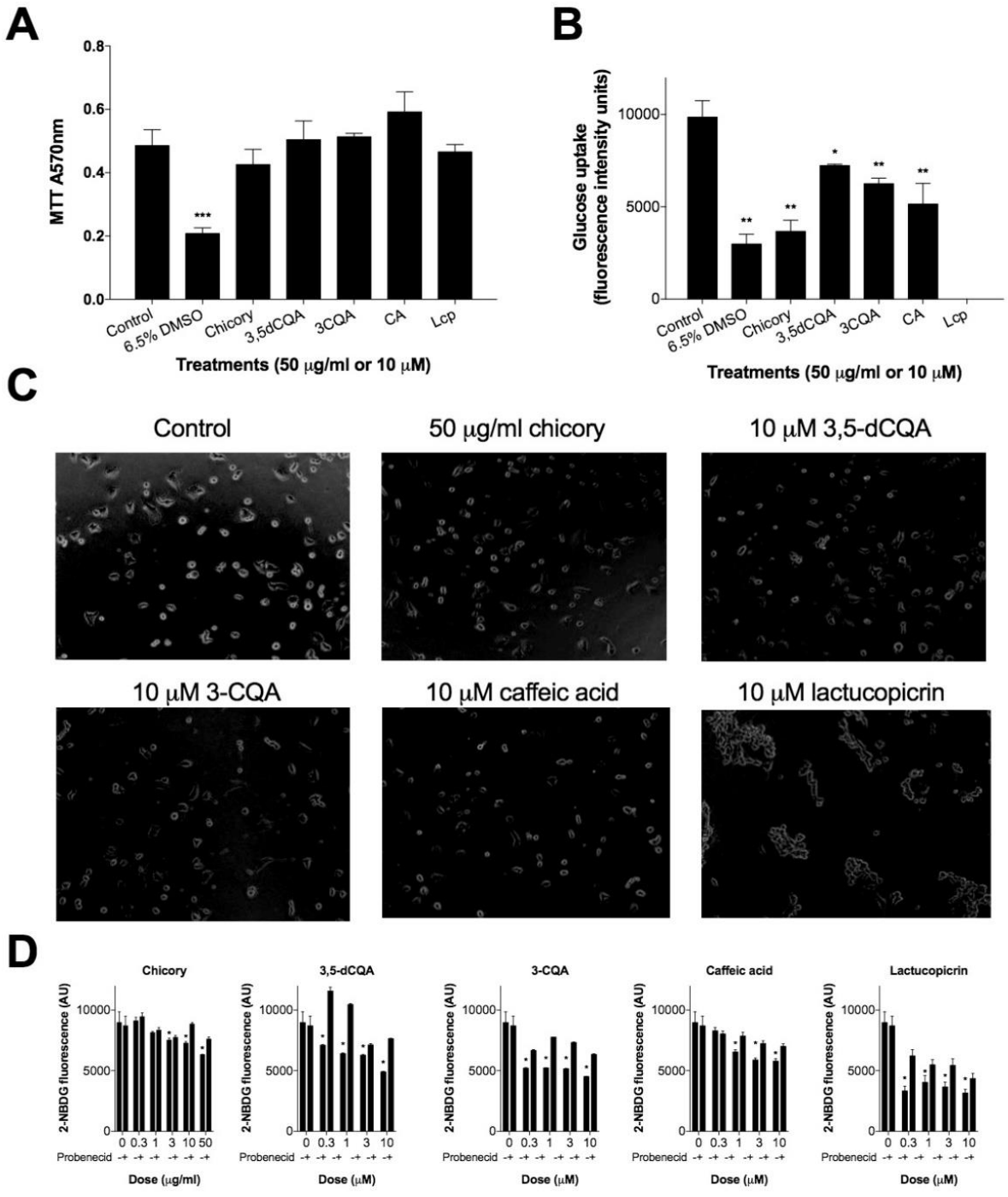


Figure 4. Hepatic lipid accumulation and expression of key glucose and lipid metabolism genes. (A) Total triglycerides as measured by L-Type Triglyceride M assay, (B) qPCR quantification of mRNA levels of gluconeogenesis (G6P) and lipid metabolism (FXR, SREB-1, FAS) genes normalized to β-actin mRNA. Values are means ± SEM (n=8), *p<0.05, **p<0.01, ***<0.001 when compared to respective LFD or HFD controls by two-way ANOVA with a post-hoc Tukey's multiple comparison test.

Figure 5. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cell model following treatment with chicory root bioactives**.** (A) STC-1 cell viability was measured after 24 h exposure to 50 μg/ml chicory extract or 10 μM purified bioactive constituents, with 6.5% DMSO serving as a toxic control, using MTT assay and spectrophotometrically quantified at 570 nm. (B) Exposure to aqueous chicory extract and bioactive compounds reduced fluorescent glucose absorption in the intestinal cells. Cells were incubated with treatments for 2 hours, presented with 2-NBDG for 15 min, and fluorescence was quantified at EX/EM of 465/540 nm. (C) Fluorescent imaging of 2-NBDG glucose intake in STC-1 cells (green) treated with chicory extract and its major bioactive compounds. (D) Dose-dependent effects of chicory bioactives on 2-NBDG glucose absorptions were inhibited by co-exposure to an allosteric inhibitor of bitter taste receptors probenecid (10 μM). Values are means ± SEM (n=3), *$p<0.05$, **$p<0.01$, ***<0.001 when compared to vehicle-treated controls by two-way ANOVA with a post-hoc Tukey's multiple comparison test.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CHAPTER 4: BITTER TASTING BOTANICALS INHIBIT GLUCOSE ABSORPTION AND AFFECT GPCR SIGNALING CASCADE THROUGH TYPE 2 RECEPTOR BINDING

Palatini Jackson Kimberly M., Wilson Mickey, Komarnytsky Slavko

Abstract

Sensitivity to bitter taste developed to discourage the consumption of potentially toxic foods. However, the discovery of Type 2 Bitter Receptors (T2R) in non-gustatory tissues suggests they play a chemosensory role to impart a whole-body response to bitter sensation. T2Rs in the gastrointestinal tract respond to naturally bitter plant compounds to affect digestion and absorption of carbohydrates. This response could explain why many bitter botanical compounds have been used in traditional medicine as treatments for hyperglycemia and Type 2 Diabetes. The goal of this study was to determine how the consumption of many structurally diverse bitter compounds could affect glucose metabolism following carbohydrate consumption. When tested in a C57BL/6J mouse model, bitter compound size, glycosylation status, and chemical class each had an effect on plasma glucose levels following a glucose load. However, bitter compounds administered as gavage or mouthwash both had a significant effect on plasma glucose and GLP-1 mRNA expression. When probenecid, a T2R antagonist was administered prior to bitter treatment, all anti-hyperglycemic activity of bitter treatment *in vivo* was lost. Using a STC-1 murine intestinal cell model, we observed decreased glucose absorption and greater Ca^{2+}flux indicative of G-protein coupled receptor signaling following treatment with bitter compounds *in vitro.* Probenecid also restored glucose absorption in the STC-1 model in the presence of Denatonium Benzoate. Together, these data suggest T2R signaling in the gastrointestinal tract may control hyperglycemia associated with metabolic syndrome by preventing glucose absorption and stimulating satiety hormone secretion following carbohydrate consumption.

1. Introduction

Type 2 Diabetes (T2D) is often preceded by a group of symptoms including obesity, insulin resistance, hypertension, and hyperglycemia, collectively identified as Metabolic Syndrome [1,2]. Early diagnosis of these symptoms is intended to give a patient options to reverse the onset of T2D via dietary and lifestyle changes before requiring a prescription drug such as metformin, rosiglitazone, or glibicamide [1]. Whereas pharmaceutical options are highly effective at managing hyperglycemia and other symptoms of metabolic syndrome, they can be costly, are associated with side effects, and risk a developed tolerance [3]. Individuals interested in alternative treatment strategies are turning to nutritional supplements and botanical drugs [4]. Although the use of plant extracts is no longer an aspect of Western medical care, partially due to controversy over effectiveness, it is still extremely popular in Asia and parts of Europe [5].

Centuries of traditional herbal medicine have managed T2D using multiple herbal preparations from a variety of plants, such as *Chicorium intybys L.* (chicory), *Eugenia jambolana L.* (black plum), *Gentiana scabra* (Japanese gentian) , *Tinospora cordifolia* (guduchi), and *Momordica charantia* (bitter melon) [6–9]. The anti-diabetic activity of these medicinal plant extracts is often attributed to their high polyphenol, alkaloid, anthocyanin, or flavanol content, ranging from 10-1000 mg/kg [10]. Extracts of *M. charantia*, rich in cucurbitane-type triterpenoids, steroidal saponins, and alkaloids [11] are believed to exert their antidiabetic effects through a variety of pharmacological, biochemical, and physiological roles, including inhibition of intestinal glucose uptake [12,13]. A flavonoid-rich extract of *E. jambolana* (300mg/kg) significantly improved glucose uptake and insulin release in diabetic mice [14], while an alkaloid rich fraction of *T. cordifolia* (50mg/kg), as well as purified alkaloids palmatine, jatrorrhizine, and magnoflorine (10mg/kg) significantly reduced plasma glucose levels in normal rats (15). We have demonstrated that a sesquiterpene lactone and

chlorogenic acid-rich extract of *Chicorum intybus* (300mg/kg) significantly reduced plasma glucose levels in obese C57BL/6J mice (Chapter 3). The wide variety of aforementioned compounds have very few structural similarities, but strikingly, each of these compounds has a reported bitter taste [8,11,15,16].

Type 2 Receptors (T2Rs) are part of the G-Protein Coupled Receptor (GPCR) family and are responsible for all bitter sensation in mammalian species [17,18]. This broad class of 25 receptors in humans and 30 in mice is responsible for identifying the ingestion of any bitter, potentially toxic compound [19,20], but T2R function goes beyond taste sensation. T2Rs are found in cell membranes throughout the body, including in the lungs, heart, kidney, and throughout the gastrointestinal (GI) tract from the tongue to the colon [19]. Bitter receptors co-localize with enteroendocrine cells in the stomach and GI tract, suggesting a whole-body response to bitter ingestion [21,22]. The control over gastrointestinal endocrine and paracrine signaling allows T2Rs to work as chemosensors even when located in non-gustatory tissues [23].

The objective of this study was to determine the anti-hyperglycemic potential of plant metabolites in a C57BL/6J mouse model of diet-induced obesity by considering each compound's ability to stimulate T2Rs and control postprandial plasma glucose levels. We further examined the structure-activity relationship of plant metabolites using a STC-1 mouse cell model of intestinal glucose absorption to determine the effect of T2R activation on glucose absorption, GPCR signaling, gastrointestinal hormone synthesis, and the bioenergetic parameters of cellular metabolism.

2. Materials and Methods

2.1 Reagents

All chemicals were obtained from Sigma (St. Louis, MO) and cell culture materials from Life Technologies (Carlsbad, CA), unless otherwise specified.

2.2 Animal studies and diets

All animal experiments were performed according to procedures approved by the NC Research Campus Institutional Animal Care and Use Committee in the David H. Murdock Research Institute, an AAALAC accredited animal care facility. Male, 6-week-old C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed four animals per cage under controlled temperature (24 ± 2 °C) and light (12 h light-dark cycle, lights on at 0700 h). Immediately upon arrival, animals were allowed to adapt to new conditions for 7 days, and daily animal handling was performed during this time to reduce the stress of physical manipulation. Mice (n=32) were then randomized into a low-fat diet (LFD) (n=16) containing 10% fat-derived calories (D12450B; Research Diets, New Brunswick, NJ) or a high fat diet (HFD) (n=16) containing 60% fat-derived calories (D12492, Research Diets) for 9 weeks. Mice had *ad libitum* access to food and water.

Animal weight and food intake (accounting for spillage) were recorded weekly for the duration of the study. All animal diets were kept at -80°C for long-term storage and stability, and freshly thawed food was dispensed to animals every 3-4 days to limit phytochemical degradation in food matrix. For long-term fasting blood glucose levels, mice were tested after 16 h overnight fast on weeks 4 and 6 of the study. At the end of the study, blood was collected by cardiac puncture after CO_2 inhalation. Gastrointestinal (stomach, duodenum, jejunum, ileum, cecum, and colon) and metabolic (liver, gastrocnemius muscle, and epididymal fat) tissues were collected and stored at -80°C to determine the temporal sequence and signaling events responsible for the observed changes in physiology and metabolism.

2.3 Oral glucose tolerance tests with diverse bitter compounds

Acute oral glucose tolerance tests were performed on a randomized subset of HFD animals with orally administered bitter compounds (30-300mg/kg) 30 minutes prior to glucose gavage. Mice were fasted overnight (16 h) and received oral gavage of D-glucose (1.5 g/kg body weight, Sigma). Blood glucose concentrations were measured at 0, 15, 30, 60, and 120 minutes after glucose challenge in blood samples obtained from tail-tip bleedings using a glucometer (True Result, Trivida, FL).

2.4 Oral glucose tolerance tests with Denatonium Benzoate (DB)

We performed two additional glucose tolerance tests in C57BL/6J mice (n=16). The first was performed following either orally administered gavage or a mouthwash of DB (3mg/kg). The second oral glucose tolerance test was performed following either orally administered or intraperitoneally injected DB (3mg/kg) in the presence or absence of an allosteric inhibitor T2R (Probenecid, 100mg/kg). Treatments were administered 30 minutes prior to the glucose gavage (1.5g/kg body weight). Blood glucose concentrations were measured at 0, 15, 30, 60, and 120 minutes after glucose challenge in blood samples obtained from later tail vein nick using a glucometer (True Result, Trivida FL).

2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from liquid nitrogen-preserved murine duodenum and ileum, using Trizol reagent (Thermo Fisher, Waltham, MA) and following manufacturer's instructions. RNA was quantified spectrophotometrically by absorbance measurements at 260 and 280 nm using the Synergy H1 Take 3 system (Biotek, Wilmington, DE). The cDNAs were synthesized using 2 μg of RNA for each sample with high capacity cDNA Reverse Transcriptase (Thermo Fisher) and following manufacturer's instructions.

2.6 qPCR analysis

The qRT-PCR amplifications were carried out in triplicate on an ABI 7300 Real-Time Detection System in a total volume of 20 μL containing 10 μL of SYBR Green 2× Supermix (Applied Biosystems), 5 μL of the 1:50 diluted cDNA, 1 μL of each specific primer, and 3 μL of PCR-grade water. The qRT-PCR program was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The corresponding primers were selected using the Primer Express version 2.0 software as follows: β-actin (NM_007393.3), forward primer: 5′-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse primer: 5′-CAC AGC CTG GAT GGC TAC GT-3′; T2R108 (NM_020502.1), forward primer 5′- GGT CAA CAG TCG CAG AAT TGC -3′, reverse primer 5′- TGT CCT GGA GGG TAA GCA GC -3′; Proglucagon (NM_008100.4), forward primer 5′- TGA AGA CCA TTT ACT TTG TGG CT -3′, reverse primer 5′- CCA AGT GAC TGG CAC GAG AT -3′; GLP-1 Receptor (NM_021332.2), forward primer 5′- CAG GGC TTG ATG GTG GCT ATC -3′, reverse primer 5′- CGC TCC CAG CAT TTC CG -3′; CCK (NM_031161), forward primer 5′- CAC GAC CCC TCG CTT CTA A -3′, reverse primer 5′-GGC TGC ATT GCA CAC TCT GA-3′. qRT-PCR data were analyzed with a 7300 System SDS Software v1.3.0 (Applied Biosystems) to estimate transcript copy numbers for each sample. Target mRNA expression was analyzed using the ΔΔCT method [24] and normalized with respect to the expression of the β-actin housekeeping gene. The LFD control group served as the calibration sample in this study, and the target gene expression of the calibration sample was assigned a value of 1.0. Amplification of specific transcripts was further confirmed by obtaining melting curve profiles.

2.7 Cell culture and glucose uptake measurements

The mouse neuroendocrine intestinal cell line STC-1 (CRL-3254) that acts as a model for glucose absorption and hormone secretion was obtained from ATCC (Manassas, VA). Cells were routinely passaged every 3-4 days and maintained in high glucose DMEM containing

10% fetal bovine serum (Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37 °C and 5% CO_2. Cells were sub-cultured into 24-well plates and, once confluent, changed to induction medium that contained glucose-free DMEM supplemented with 2 mM sodium pyruvate to induce fluorescent glucose uptake. Treatment was administered for 2 hours before cells were exposed to 10 μM 2-NBDG for 20 minutes, washed with 1x PBS, and imaged using EVOS FL Cell Imaging System (Life Technologies) before spectrophotometric analysis with EX/EM at 465/540nm.

2.8 Calcium Influx

STC-1 cells (ATCC CRL-3254) were plated in 96 well plates at a concentration of 20,000 cells/mL and grown overnight. The following day, Fluo-4NW kit (Invitrogen) was prepared following the manufacturer's instructions. Growth medium was removed from the cells and replaced with 100μL of dye-loading solution into each well. Treatments were added simultaneously and the cells were incubated at 37 °C for 30 minutes. The plate was then moved to the benchtop and allowed to incubate at room temperature for another 30 minutes. Fluorescence was measured by spectrophotometry measuring excitation at a wavelength of 480nm and emission at 516nm [25,26].

2.9 Statistical analysis

Statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA) and expressed as means ± SEM. Two tailed t-test or two-way ANOVA were applied at a significance level of $p<0.05$. Post-hoc analyses of differences between individual experimental groups were made using the Bonferroni's or Dunnett's multiple comparison test.

3. Results

3.1 Acute anti-hyperglycemic effect of diverse plant metabolites

Obesity and hyperglycemia were induced by feeding a HFD to C57BL/6J mice for 4 weeks before the animals were tested for oral glucose tolerance. During this time, HFD mice developed significant body weight gain (3.05±1.3g vs. 2.3±1.2 g, $p<0.05$) and fasting hyperglycemia (138±9.17 mg/dL vs 80±2.78 mg/dL, $p<0.05$) as compared to LFD controls. When tested by oral glucose tolerance test, various alkaloids, polyphenols, and benzoic acids showed significant efficiency at lowering blood glucose levels in C57BL/6J mice; however, the effect showed no trend between compound class (Figure 1D-F).

Compound size and glycosylation status were also considered. In agreement with previous work, oligomers had a significant inhibitory effect ($p<0.05$) on glucose absorption (Figure 1B) [27]. However, glycosylated compounds were less effective at lowering blood glucose concentrations than the nascent quercetin compound (Figure 1C).

3.2 Anti-hyperglycemic effect localized to the gastrointestinal tract

Denatonium Benzoate (DB) is an extremely bitter synthetic compound and was used in order to determine the role of bitter sensing in the GI tract on blood glucose levels from other properties of various plant compounds. As expected, there was a significant difference between animals treated with DB compared to control when administered both as a mouthwash and as a gavage (Figure 2A). When probenecid (100mg/kg), an allosteric inhibitor of T2Rs, was administered prior to DB, probenecid-treated animals had no significant change in circulating glucose concentration after being treated with DB (Figure 2B).

To determine if the resulting decrease in circulating blood glucose levels was due to impaired glucose absorption in the GI tract, DB was delivered via intraperitoneal injection. There was no significant reduction in circulating blood glucose levels in these animals

compared to control (Figure 2C). Injection of probenecid to the intraperitoneal cavity also had no effect on restoring plasma glucose levels.

3.3 Anti-hyperglycemic effect correlated to changes in gastrointestinal hormone production

RT-qPCR for selected genes associated with T2R signaling in the GI tract was used to determine the mechanism by which acute effects were seen in the C57BL/6J mouse model (Figure 3). Expression of T2R108, a bitter receptor activated by DB, and downstream proteins were used as markers of bitter signaling in C57BL/6J mice. Expression of T2R108 in the ileum was significantly greater in animals gavaged with DB compared to both HF and LF control animals ($p<0.05$). The addition of probenecid to DB treated animals significantly decreased T2R108 mRNA expression ($p<0.05$); however, there was no difference in expression in probenecid treated control animals. DB mouthwash also significantly affected the expression of T2R108 in the ileum of C57Bl/6J treated mice (Figure 3A), possibly due to a vagal nerve signaling mechanism [28].

mRNA levels of downstream gastrointestinal hormones glucagon like protein-1 (GLP-1), GLP-1 Receptor, and Cholecystokinin (CCK) were also analyzed. These hormones are associated with ileal neuroendocrine cells and control multiple factors associated with postprandial glucose control. Animals exposed to DB by gavage had no elevation in GLP-1 expression following a glucose load. Animals pre-treated with probenecid had significantly greater GLP-1 mRNA expression (Figure 3B). Mouthwash animals showed greater ileal GLP-1 expression than gavaged animals, but these animals still had significantly less GLP-1 mRNA expression than their probenecid treated controls. There were no significant changes in expression of GLP-1 Receptor and CCK between treatment groups (Figure 3C, D).

3.4 Glucose absorption in STC-1 intestinal cell model

Acute improvement of glucose tolerance in diet-induced obesity mice suggests a direct effect of bitter bioactive constituents on glucose absorption in the GI tract. To confirm these observations, a quantitative analysis of glucose in STC-1 mouse intestinal cell model was performed to determine a mechanism of response. DB at a dose of 30μM showed no cell toxicity (Figure 4A) and was effective at suppressing glucose uptake in STC-1 cells (Figure 4B). Decreased glucose import was clearly evident from emission intensity images of fluorescent glucose analog 2-NBDG accumulation in STC-1 cells (Figure 4C,D). Co-treatment of STC-1 cells with probenecid significantly decreased efficacy of DB, and nearly completely restored the glucose uptake in STC-1 cells (Figure 4C). Quinine tested at identical concentrations as denatonium benzoate also showed no toxicity and a trend towards decreased glucose absorption ($p=0.06$) (Figure 4D).

3.5 Type 2 Receptor Signaling in STC-1 intestinal model

Having demonstrated that STC-1 cells express bitter taste receptors and allosterically inhibiting them with probenecid, the next step was to determine the functional response of bitter sensing in these cells. The addition of bitter compounds DB and quinine at increasing concentrations from 1 to 30μM induced a dose-dependent increase in Ca2+ in STC-1 cells (Figure 5).

4. Discussion

Bitterness evolutionarily signaled potentially toxic compounds to the consumer and serves as a plant's defense mechanism against consumption. Although not always adaptive, mammalian species developed a whole-body response to bitter receptor activation to prevent the absorption of toxins [29,30]. Public health actions ensure most humans no longer ingest toxic foods unknowingly, but this system still works in response to bitter plant

metabolites. The best evidence lies in the traditional medicinal treatments worldwide that utilize bitter plant extracts for the management of hyperglycemia [8,11,14,31]. Based on previous work with chicory, caffeoylquinic acids and sesquiterpene lactones demonstrated acute anti-hyperglycemic effects, supporting centuries of traditional medicine using chicory (Chapter 3) and hundreds of other bitter plant extracts. The goal of this study was to determine how effectively bitter compounds inhibit glucose absorption and how this biological response could be used to reverse hyperglycemia associated with metabolic syndrome and T2D.

Although there is no universal scale for bitterness, the extensive work by Meyerhoff et al. has classified human and murine T2Rs and recorded their binding affinity for an expansive list of bitter compounds [10,19]. BitterX database is an open-accessed web server aimed at providing a platform for identifying bitter receptors for small molecule compounds, but it also contains incomplete information on relative bitterness [32]. In oral glucose tolerance tests, we observed varied results in affecting plasma glucose levels in a C57BL/6J model of diet-induced obesity. Despite testing in an STC-1 cell model, there is not a definitive measurement to determine if lack of response was due to concentration, size, glycosylation status, hydrophobicity causing ineffective receptor binding, or lack of receptors present to bind the specific molecule. These differences exist between species as well as between individuals and are influenced by both diet and genetics [33,34].

The acute response (within 30 minutes) in the OGTT suggested bitter compounds were working in the GI tract to inhibit absorption or had some incretin-like response to increase muscle and liver uptake of circulating plasma glucose. However, a lack of change when DB was injected intraperitoneally suggests a direct role of the GI tract with possible downstream modulation of incretin hormones. When *db*/*db* mice were treated with DB at a dose of 1mg/kg, in a glucose tolerance test (5mg/kg), Kim et al. reported that mice had both

lower blood glucose at 20 and 40 minutes after gavage, as well as lower insulin levels and increase GLP-1 in DB treated animals [35]. Although there is no consensus on how bitter perception in the GI tract affects glucose absorption, downstream signaling via the alpha gustducin subunit of the G protein-coupled bitter receptors could affect the transport and function of glucose transporters SGLT-1 and SGLT-2 in the enterocyte membranes [19,20].

T2R108 was chosen as a gene of interest due to the documented activation in response to stimulation by DB [36]. While a single dose gavage wasn't expected to affect gene expression locally, treatment over the course of 8 weeks and possible vagal nerve stimulation [28] may evoke a change in T2R108 expression, whereas no difference in GLP-1 receptor was expected because acute and transient changes in GLP-1 expression are not expected to alter receptor expression [37]. Vagal nerve stimulation could also explain the stimulation of T2R108 and GLP-1 mRNA in the ileum when bitter treatment was only given as a mouthwash to C57BL/6J animals, assuming no ingestion. CCK in enteroendocrine D cells is not directly stimulated by ingested carbohydrates; instead, it is more responsive to differences in dietary lipids which were equivalent in HF control and treatment animals throughout the study [38].

To further identify the mechanism at work in our C57BL/6J animal model, STC-1 cells were chosen as an *in vitro* model. Treatment with both DB a synthetic compound, and quinine as a common natural alkaloid, were chosen. While we originally controlled for maximum final concentration of 10μM of these compounds in cell culture experiments, due to the differences in bitterness from DB, the lack of statistically significant differences in glucose absorption following quinine exposure is not unexpected. At a 30uM concentration, quinine significantly reduced glucose absorption in the same test (Figure 4).

Beyond the inhibition of glucose absorption, activation of bitter taste receptors promotes the synthesis of second messengers leading to the release of Ca^{2+} from intracellular stores or

modulates the gating of ion channels that control Ca^{2+} transport across the cell membrane [39]. Probenecid acts as a standard inhibitor of nonspecific anion transport in the Fluo-4 NW Calcium Assay kit; however, because of the compound's inhibitory activity on G-protein coupled receptors, we tested for differences in calcium flux in the absence of probenecid [40]. As an additional control, DB and quinine were also tested in 3T3-L1 mouse fibroblast cell line that do not possess T2R. The lack of response in fibroblast cells reinforces the notion that the effects of bitter compounds on second messenger production in STC-1 cells are mediated by specific receptors and signal transducers linked to bitter taste and specific to enterocytes.

In conclusion, we confirmed acute hypoglycemic effects of various bitter botanical compounds, including alkaloids, polyphenols, and benzoic acids. Stimulatory effects of all compounds on glucose metabolism were acute, and did not translate into a long-term insulin-sensitizing effects. Together, these data suggest bitter botanicals stimulate T2R signaling in the GI tract to control acute postprandial hyperglycemia associated with metabolic syndrome by preventing glucose absorption and stimulating satiety hormone secretion following carbohydrate consumption. These results strengthen the case for the use of bitter botanical drugs as an alternative choice for managing postprandial hyperglycemia in individuals with metabolic syndrome.

5. References

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6. Figures

Figure 1. Effect of bitter botanical bioactives on glucose metabolism in C57BL/6J mice. Acute oral glucose tolerance tests were performed in HFD-fed mice (5 weeks) (A-F). Single dose gavage were used to measure the effect of reference compounds (A), compound size (B), glycosylation status (C), and diverse classes of plant compounds; alkaloids (D), polyphenols (E), and benzoic acids (F). Values are means±SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ when compared to HFD controls by one-way ANOVA with a post-hoc Dunnett's multiple comparison test.

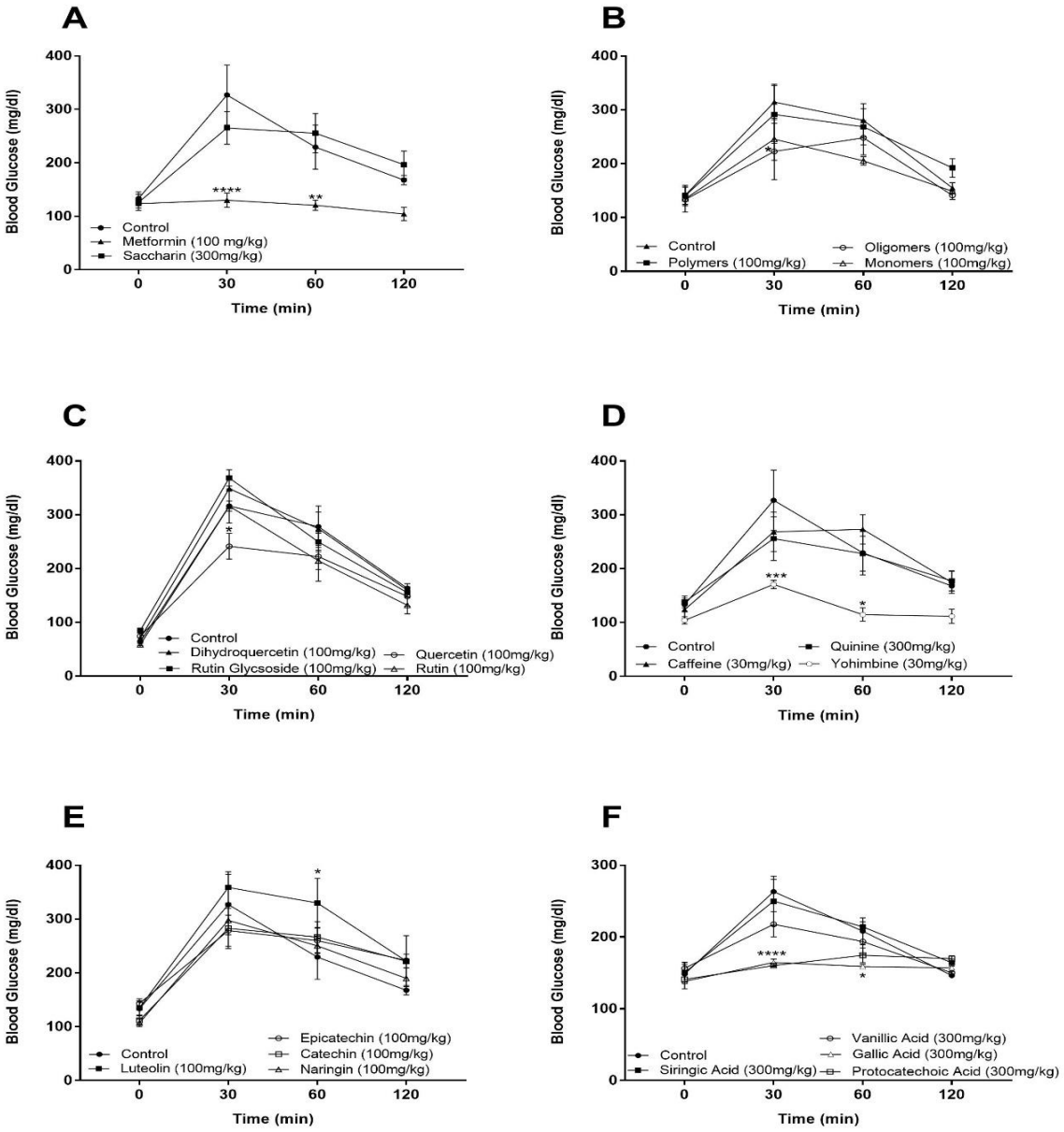
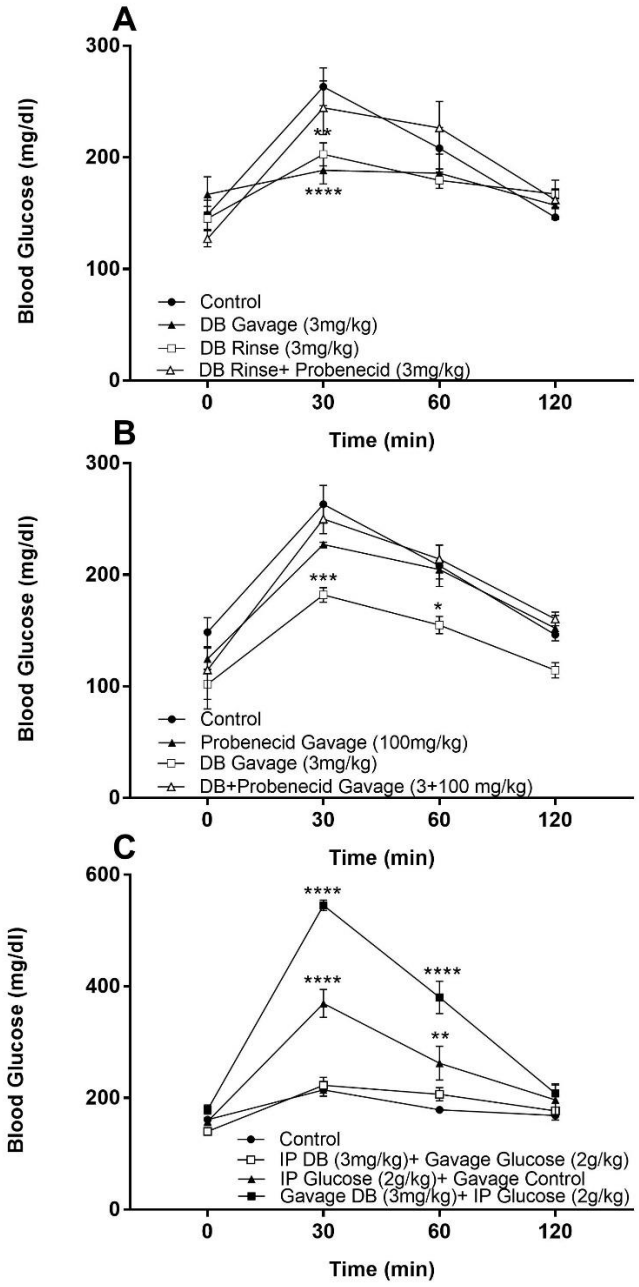

A
Blood Glucose (mg/dl)
Time (min)
Control
Metformin (100 mg/kg)
Saccharin (300mg/kg)
B
Control
Polymers (100mg/kg)
Oligomers (100mg/kg)
Monomers (100mg/kg)
C
Control
Dihydroquercetin (100mg/kg)
Rutin Glycsoside (100mg/kg)
Quercetin (100mg/kg)
Rutin (100mg/kg)
D
Control
Caffeine (30mg/kg)
Quinine (300mg/kg)
Yohimbine (30mg/kg)
E
Control
Luteolin (100mg/kg)
Epicatechin (100mg/kg)
Catechin (100mg/kg)
Naringin (100mg/kg)
F
Control
Siringic Acid (300mg/kg)
Vanillic Acid (300mg/kg)
Gallic Acid (300mg/kg)
Protocatechoic Acid (300mg/kg)

Figure 2. Effect of bitter Denatonium Benzoate on glucose metabolism in C57BL/6J mice**.** Acute oral glucose tolerance tests were performed in HFD-fed mice (n=16) with a single dose gavage or mouthwash of DB (3mg/kg) in the presence or absence of probenecid (100mg/kg). (A) a single dose gavage in the presence or absence of probenecid (B) or intraperitoneal injection DB (C) in the presence and absence of probenecid (100mg/kg). Values are means±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 when compared to HFD controls by one-way ANOVA with a post-hoc Dunnett's multiple comparison test.


A
Blood Glucose (mg/dl)
300
200
100
0
**
****
Control
DB Gavage (3mg/kg)
DB Rinse (3mg/kg)
DB Rinse+ Probenecid (3mg/kg)
0
30
60
120
Time (min)
B
Blood Glucose (mg/dl)
300
200
100
0
***
*
Control
Probenecid Gavage (100mg/kg)
DB Gavage (3mg/kg)
DB+Probenecid Gavage (3+100 mg/kg)
0
30
60
120
Time (min)
C
Blood Glucose (mg/dl)
600
400
200
0
****
****
****
**
Control
IP DB (3mg/kg)+ Gavage Glucose (2g/kg)
IP Glucose (2g/kg)+ Gavage Control
Gavage DB (3mg/kg)+ IP Glucose (2g/kg)
0
30
60
120
Time (min)

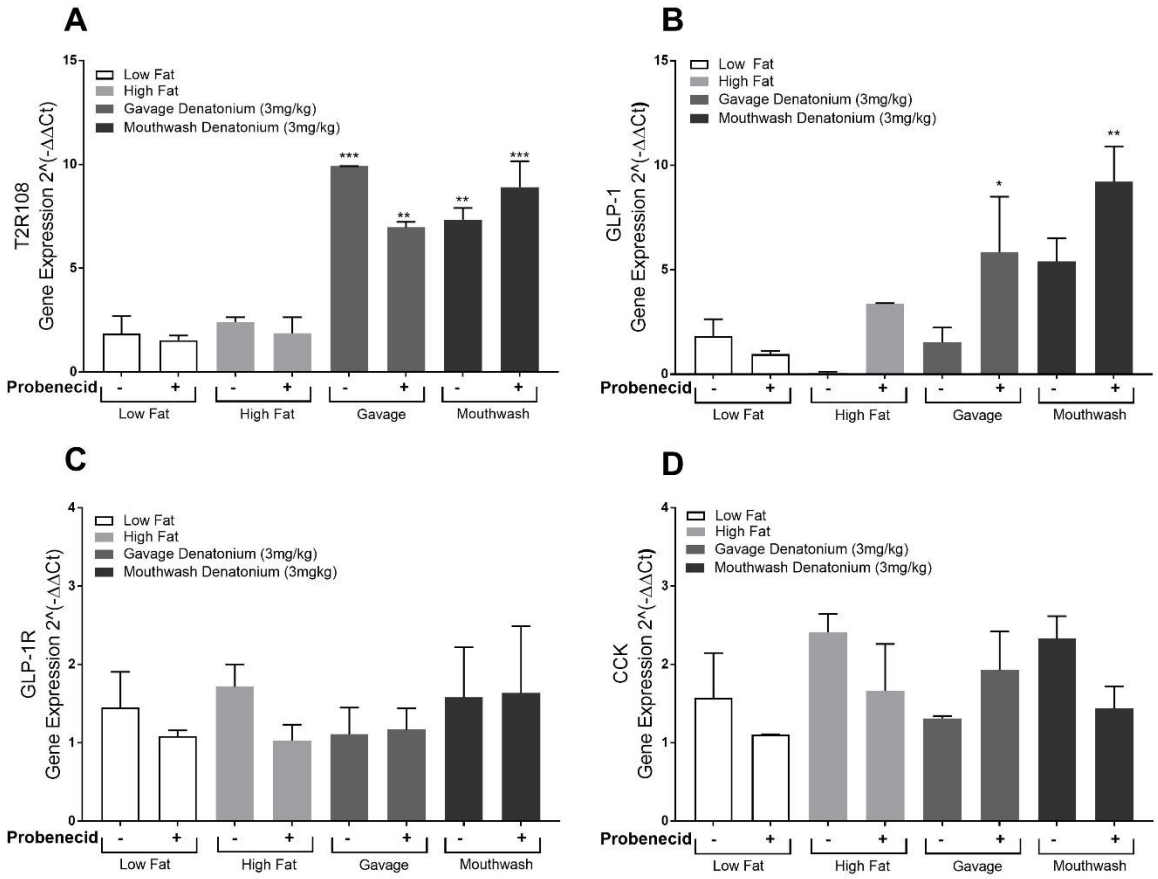


Figure 3. Gastrointestinal expression of Type 2 Receptor 108 and key satiety hormones. qPCR quantification of mRNA levels of (A) T2R108 and satiety hormone (B) GLP-1, (C) GLP-1R and (D) CCK genes normalized to β-actin mRNA isolated from the ileum of C57Bl/6J mice. Values are means±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 when compared to HFD controls by two-way ANOVA with a post-hoc Bonferroni's multiple comparison test.

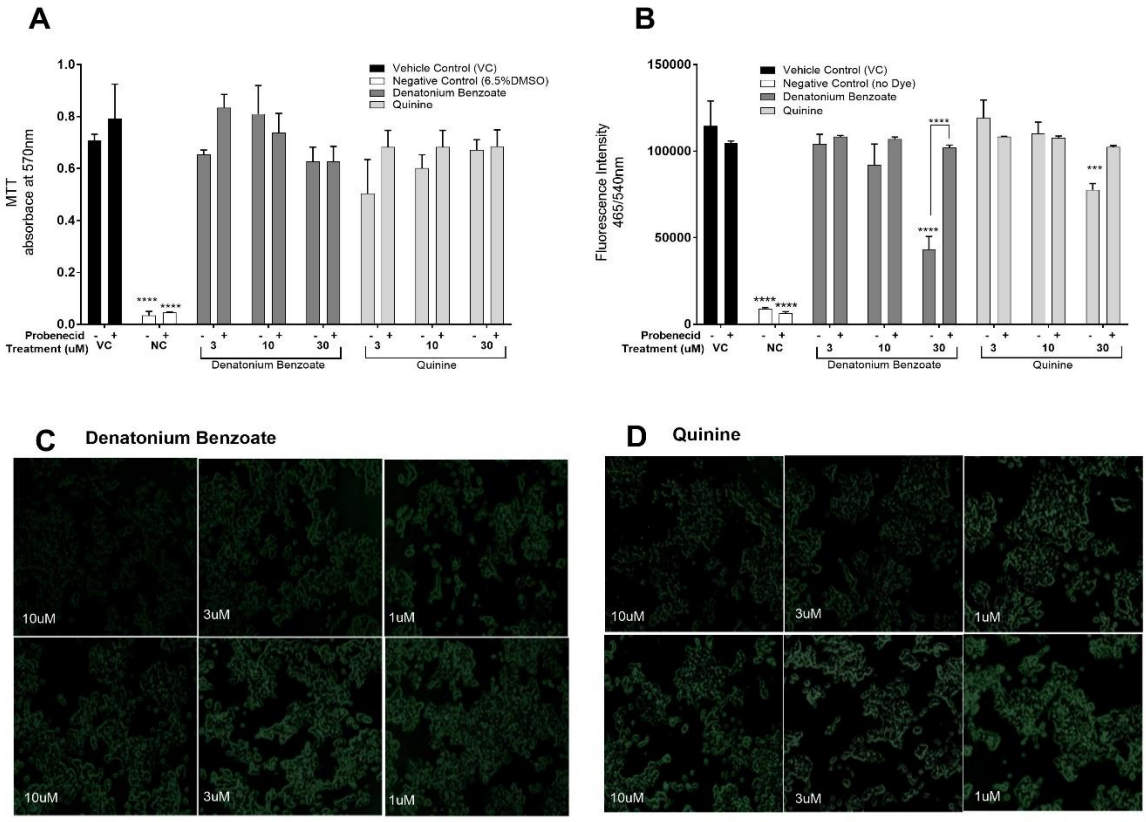


Figure 4. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cell model following treatment with Denatonium Benzoate and Quinine. (A) STC-1 cell viability was measured after 24h exposure to 10μM purified compounds, with 6.5% DMSO serving as a toxic control, using MTT assay and spectrophotometrically quantified at 570nm. (B) Exposure to increasing doses of pure bitter compounds reduced fluorescent glucose absorption in the intestinal cells. Cells were incubated with treatments for 2 hours, presented with 2-NBDG, and fluorescence was quantified at EX/EM of 465/540nm. (C-F) Fluorescent imaging of 2-NBDG glucose intake in STC-1 cells (green) treated with (C) Denatonium and (D) Quinine alone (top) and with the allosteric inhibitor of T2R, probenecid (10μM) (bottom). Values are means±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 when compared to HFD controls by two-way ANOVA with a post-hoc Bonferroni's multiple comparison test.

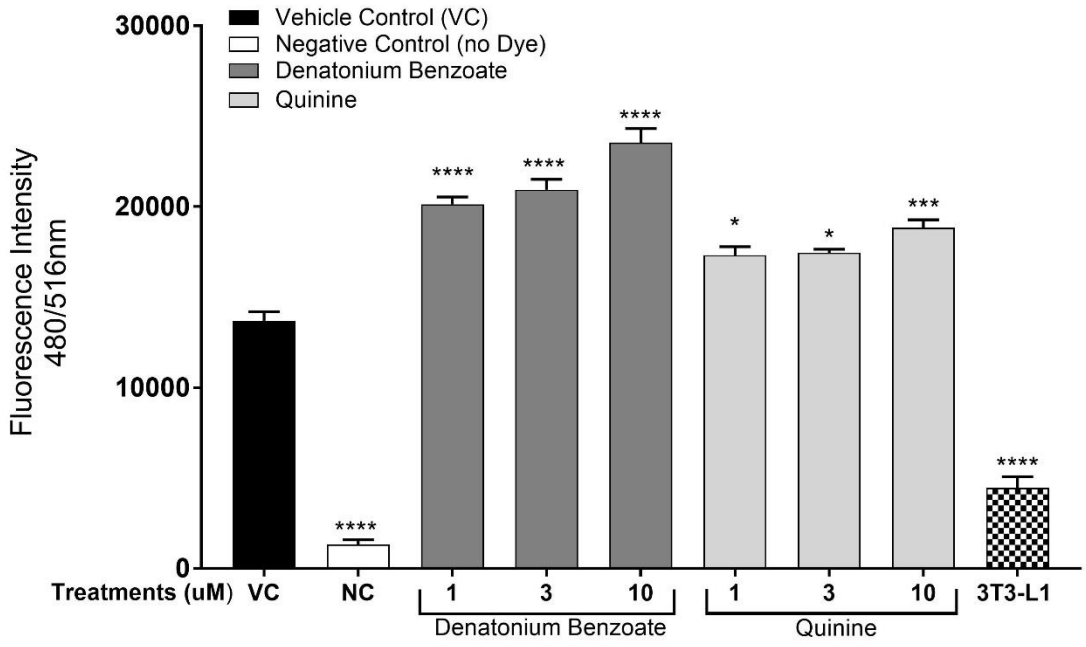


Figure 5. Fluorescent Calcium flux in the STC-1 intestinal model following treatment with Denatonium Benzoate and Quinine**.** Exposure to increasing doses of pure bitter compounds stimulated increased Ca^{2+} flux in the intestinal cells. Cells were incubated with treatments for 1 hour and fluorescence was quantified at EX/EM of 480/516nm. Values are means ± SEM (n=3), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 when compared to vehicle-treated controls by two-way ANOVA with a post-hoc Bonferroni's multiple comparison test.

CHAPTER 5: HEPATOPROTECTIVE ACTIVITY OF EASTER LILY (*LILIUM LONGIFLORUM* THUNB.) BULB EXTRACTS

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Abstract

The hepatoprotective activity of two different extracts, a hydroethanolic crude bulb extract (CB) and a steroidal glycoside-rich 1-butanol extract (BuOH), prepared from the bulbs of Easter lily (*Lilium longiflorum* Thunb.), were evaluated in a 24 week study in the female KK.Cg-Ay/J Type 2 diabetic mouse model. Animals were divided in six groups (n=16): control mice received Easter lily bulb extract-free drinking water together with a low or high fat diet (diabetic control); drinking water for the remaining groups was supplemented with CB extract (1%), BuOH extract (0.1% or 0.2%) and a reference drug Metformin (0.001%, an equivalent of 300 mg/kg/d per treatment), together with a high fat diet. Both CB and BuOH extract treatment groups exhibited significantly improved liver function based on comparisons of triglycerides [diabetic 219 ± 34 mg/dL, CB 131 ± 27 mg/dL, BuOH(0.2%) 114 ± 35 mg/ dL], CB total cholesterol (TC) (diabetic 196 ± 12 mg/dL, CB 159 ± 5 mg/dL), average liver mass [diabetic 2.96 ± 0.13 g, CB 2.58 ± 0.08 g, BuOH(0.1%) 2.48 ± 0.13 g], alanine transferase [diabetic 74 ± 5 units/L, CB 25 ± 1 units/L, BuOH(0.1%) 45 ± 1 units/L], and histological examinations. Glucose metabolism was improved only in CB, which was confirmed by oral glucose tolerance tests (OGTT) in diet-induced obese C57BL/6J mice exposed to CB extract. These data suggest that steroidal glycosides 1 - 5 play a role in the hepatoprotective activity of the two BuOH extracts, while the results of the TC measurements and OGTT study indicate that other constituents present in the CB extract are responsible for its hypocholesterolemic and hypoglycemic activity.

Keywords: Lilium longiflorum Thunb., Liliaceae, Easter lily, steroidal glycoside, hepatoprotective activity, hypoglycemic activity

1. Introduction

Especially in the United States, clinical data on the negative health impacts of diabetes is considerable and continues to accumulate. The number of Americans with diabetes has increased by approximately 15% in the past 2 years and the number worldwide is predicted to increase from 183 to 366 million by 2030 [1]. Two forms of diabetes mellitus (DM), Type 1 and Type 2, are well known [2, 3]. Type 2 diabetes ranges from predominantly insulin resistant with relative insulin deficiency to predominantly insulin secretory defective with insulin resistance. More than 90 percent of all diabetes diagnoses worldwide are Type 2.

It is well established that a degree of hyperglycemia sufficient to cause pathological and functional changes in target tissues, but without indicating clinical symptoms, may be present prior to a clinical diagnosis of diabetes [4]. As a consequence, individuals with Type 2 diabetes commonly have a higher incidence of liver function abnormalities. One prevalent liver function abnormality is characterized by elevated levels of alanine transferase (ALT), which catalyze the transfer of an amino group from L-alanine to α-ketoglutarate. Accordingly, its measurement is employed as a biomarker for the evaluation of hepatocellular injury because elevated ALT levels are indicative of liver-specific damage common in diabetes [5]. Anti-diabetic agents have been shown to decrease ALT levels as normal blood glucose levels are achieved [6]. Changes in blood lipid profiles including triglyceride (TG), total cholesterol (TC), and high-density lipoprotein (HDL) levels are also widespread in diabetics and may occur as a complication of impaired liver function [7]. Also, an abnormality in carbohydrate metabolism can be observed during this asymptomatic period, based on the quantification of plasma glucose levels in the fasting state or after a challenge with an oral glucose load [8].

Diet, representing one of the most important lifestyle factors, can appreciably influence the incidence of diabetes. To date, a great number of supplements, including traditional

Chinese medicine formulas, have been used for the treatment of diabetes and have been approved by the China Food and Drug Administration (CFDA) as anti-diabetic formulations [9-15]. Estimates indicate that more than 200 species of botanicals, including many ordinary plants such as pumpkin, wheat, celery, wax gourd, lotus root and bitter melon exhibit hypoglycemic properties [16]. Pharmacological and clinical evaluations have demonstrated that long-term use of various traditional herbal mixtures may be advantageous over some pharmaceuticals in alleviating chronic secondary diseases and complications associated with diabetes [17]. As a result, in recent years there has been a steadily increasing interest in the identification of biologically active natural products found in traditional botanicals for the prevention and treatment of diabetes and its metabolic complications.

The bulbs of the Easter lily (*Lilium longiflorum* Thunb., family *Liliaceae*), although not well-known as an edible in the United States, are often used as both food and medicine in Asian countries, including China and Japan. In China, the bulbs of various lily species, including *L. longiflorum*, are regular ingredients in stir-fries, soups, and stew-like dishes. Moreover, the bulbs have a long, documented history of such medicinal uses as aiding in sedation, anti-inflammation, and anti-tussive effects [18]. Extensive work has been done on the isolation and characterization of steroidal glycosides, including steroidal saponins and steroidal glycoalkaloids, in the *Lilium* genus [19-22]. Recently, two steroidal glycoalkaloids, (22R, 25R)-spirosol-5-en-3β-yl O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (1), and (22R, 25R)-spirosol-5-en-3 β-yl O-α-L-rhamnopyranosyl-(1→2)-[6-O-acetyl-β-D-glucopyranosyl-(1→4)]- β-D-glucopyranoside (2), as well as three furostanol saponins, (25R)-26-O-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-O-α-L-rhamnopyranosyl-(1→2)- β-D-glucopyranosyl-(1→4)- β-D-glucopyranoside (3), (25R)-26-O-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-

arabinopyranosyl-(1→3)-β-D-glucopyranoside (4) and (25R)-26-O-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-xylopyranosyl-(1→3)-β-D-glucopyranoside (5) were identified and quantified in the various plant organs of *L. longiflorum* [23]. It has been documented that steroidal glycosides from diverse plant species, including *Borassus flabellifer* [24], *Polygonati rhizoma* [25], *Anemarrhena asphodeloides* [26], and *Asparagus officinalis* [27] exhibit hypoglycemic, hypolipidaemic, and hepatoprotective activities. Although Easter lily bulbs are commonly consumed as a food in Asian cuisine, to date, no investigations have been reported on the hepatoprotective effect of the consumption of Easter lily bulb extracts during the onset of diabetes have been reported.

The objective of the present investigation was to quantify the levels of steroidal glycosides 1 - 5 in two Easter lily extracts with differential steroidal glycoside contents and evaluate them for their potential hepatoprotective activity in female KK.Cg-Ay/J Type 2 diabetic mice fed a high fat diet for 24 weeks. The treatments consisted of a crude hydroethanolic bulb extract (CB), and two levels of a partially purified steroidal glycoside-rich 1-butanol extract (BuOH). Hepatoprotective activity was estimated based on comparisons of body and liver mass, blood lipid profiles (TG, TC, and HDL), ALT measurements, and histological examinations of liver sections. In addition, the hypoglycemic effects of the Easter lily extracts were explored by oral glucose tolerance tests (OGTT) in diet-induced obese C57BL/6J mice.

2. Materials and Methods

2.1 Chemicals

All solvents, acetonitrile, 1-butanol, ethyl acetate, ethanol, formic acid, and pentanes were of chromatographic grade and were obtained from Thermo Fisher Scientific Inc. (Fairlawn, NJ). Pyridine-d5 [0.03% v/v TMS] was obtained from Sigma-Aldrich, (St. Louis,

MO). Deionized (DI) water (18 MΩ cm) was generated using a Milli-Q-water purification system (Millipore, Bedford, MA).

2.2 Preparation of Easter Lily Bulb Extracts

L. longiflorum, cultivar 7-4 bulbs were obtained from the lily breeding program at Rutgers University. Lyophilized lily bulbs (100 g) were frozen in liquid nitrogen, ground into a fine powder with a laboratory mill, and extracted on an autoshaker with pentanes (3 x 100 mL) at room temperature for 15 min. After centrifugation (3000g) for 10 min, the solvent was discarded and the residue taken to dryness. The defatted material was subsequently extracted on an autoshaker with a mixture of ethanol and DI water (7:3, v/v; 2 x 150 mL) for 45 minutes at room temperature. After a 10 minutes centrifugation (3000g) followed by vacuum filtration, the supernatant was collected and the residue discarded. The supernatant was then evaporated under reduced pressure (30 °C; 1.0 x 10-3 bar) and lyophilized, yielding a crude bulb extract (CB, 11.9 g). Next, a portion of the lyophilized Bulb extract (CB, 5.0 g) was dissolved in DI water (50 ml) and washed with ethyl acetate (5 x 50 ml) to remove the ethyl acetate soluble constituents and the organic phase was then discarded. The subsequent aqueous phase was extracted with 1-butanol (5 x 50 ml) and the aqueous phase discarded. Finally, the organic phase was evaporated under reduced pressure (30 °C; 1.0 x 10-3 bar) and lyophilized, yielding a steroidal glycoside-rich 1-butanol extract (BuOH, 1.1 g). Both CB and BuOH extracts were stored in a desiccator prior to use.

2.3 Purification and Confirmation of Analytical Standards

Five steroidal glycosides listed below were isolated from lyophilized *L. longiflorum* bulbs following a recently reported procedure [23] (Figure 1). The compounds were obtained as white amorphous powders in high purity > 98%, as determined by liquid chromatography- mass spectroscopy (LC-MS) and nuclear magnetic resonance (NMR). Compound 1, (22R, 25R)-spirosol-5-en-3β-yl O-α-L-rhamnopyranosyl-(1→2)-β-D-

glucopyranosyl-(1→4)-β-D-glucopyranoside. 1H NMR and 13C NMR were consistent with the literature [23]. Compound 2, (22R, 25R)-spirosol-5-en-3 β-yl O-α-L-rhamnopyranosyl-(1→2)-[6-O-acetyl-β-D-glucopyranosyl-(1→4)]- β-D-glucopyranoside. 1H NMR and 13C NMR were consistent with the literature [23]. Compound 3, (25R)-26-O-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-O-α-L-rhamnopyranosyl-(1→2)- β-D-glucopyranosyl-(1→4)- β-D-glucopyranoside. 1H NMR and 13C NMR were consistent with the literature [23]. Compound 4, (25R)-26-O-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranoside. 1H NMR and 13C NMR were consistent with the literature 23. Compound 5, (25R)-26-O-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-xylopyranosyl-(1→3)-β-D-glucopyranoside. 1H NMR and 13C NMR were consistent with the literature [23].

2.4 Nuclear Magnetic Resonance Spectroscopy (NMR).

1H NMR and 13C NMR spectra were acquired on an AMX-400 spectrometer (Bruker, Rheinstetten, Germany). For NMR analysis, all compounds were dissolved in pyridine-d5. Chemical shifts were generated as δ values with reference to tetramethylsilane (TMS).

2.5 Analytical Standard Preparation

Isolated and purified as described above, steroidal glycosides 1-5 were used as analytical standards 23. The standards were accurately weighed into volumetric flasks (10 mL) and partially filled with ethanol and DI water (7:3, v/v; 7 mL each). Solutions were sonicated (5 min) and filled to full volume (10 mL) with ethanol and DI water (7:3, v/v); solutions for calibration curves were prepared by diluting stock solutions. External calibration curves were established over 6 data points covering a concentration range 1 - 300 μg/mL. To establish calibration equations, mean areas (n = 3) generated from standard solutions were plotted against concentration. Standard solutions were stored at 4°C.

2.6 Quantitative Analysis of Steroidal Glycosides 1 – 5 in Lily Bulb Extracts

Following removal from the desiccator, lyophilized CB and BuOH extracts were weighed separately (50 mg each) into volumetric flasks (10 mL) and partially filled with ethanol and DI water (7:3, v/v; 7 mL each). Solutions were sonicated (5 min), filled to full volume (10 mL) with ethanol and DI water (7:3, v/v), and vortexed (3 min). Prior to liquid chromatography-mass spectrometry (LC-MS) analysis, all samples were filtered through a 0.45 μm PTFE syringe filter.

LC-MS analysis was conducted according to a previously published method [23], with some modifications. Quantitative analysis of CB and BuOH extracts was performed in positive ionization mode using an Agilent 1100 series HPLC system (Agilent Technologies Inc., Santa Clara, CA) equipped with auto injector, quaternary pump, column heater, diode array detector, and interfaced to a 1100 series single-quadruple mass selective detector (MSD) equipped with an API-ESI ionization source. Chromatographic separations were made on a Prodigy C18 column (250 x 4.6mm i.d.; 5.0 μm particle size) (Phenomenex, Torrance, CA) operated at a flowrate of 1.0 mL / min, with a column temperature set to 23 ± 2 °C and an injection volume of 10 μL. The binary mobile phase was composed of (A) 0.1% formic acid in DI water and (B) 0.1% formic acid in acetonitrile. Chromatographic separations were executed with a linear gradient of 15 – 34.6% B over 28 minutes and then to 95% B over 5 minutes; thereafter, elution with 95% B was performed (5 min). Re-equilibration time was 10 min. ChemStation software (Version 3.01) was used for data acquisition and analysis. Ionization parameters included: capillary voltage, 3.5 kV; nebulizer pressure, 35 PSI; drying gas flow, 10.0 mL / min; and drying gas temperature, 350° C. MSD signal parameters included: mode, Selected Ion Monitoring (SIM); polarity, positive; fragmentor voltage, 70V; gain, 1.0; dwell time, 144 msec; and percent relative dwell time, 25. Individual steroidal glycosides 1 – 5 were quantified by monitoring the following ions:

1 (m/z 884.5), 2 (m/z 926.5), 3 (m/z 1047.5), 4 (m/z 1017.5), and 5 (m/z 1017.5).

2.7 Preparation of steroidal glycoside-containing drinking solutions

To prepare CB 1% drinking solution, CB extract (CB, 1.0 g) was dissolved in sterile DI water (99.0 g) and allowed to completely dissolve on an autoshaker. To prepare the BuOH 0.1 and 0.2% drinking solutions, BuOH extracts (BuOH, 0.1 and 0.2 g) were dissolved in sterile DI water (99.9 and 99.8g respectively), and allowed to completely dissolve on an autoshaker. Prior to consumption, all drinking solutions were filtered through a 0.45 μm PTFE filter.

2.8 KK.Cg-Ay/J Type 2 Diabetic Mouse Model

Female KK.Cg-Ay/J diabetic mice and normal wildtype non-agouti (a/a) KK mice were purchased from Jackson labs (Bar Harbor, ME), housed in stainless steel wire-bottomed cages, and acclimatized under laboratory conditions (19 - 23 °C, humidity 60%, 12 h light/dark cycle). The high fat diet used in this study, composed of 20 kcal % protein, 20 kcal % carbohydrate, and 60 kcal % fat (from butter), was procured from Research Diets Inc. (New Brunswick, NJ). The control diet was normal chow 5001 from LabDiet (St. Louis, MO) and was composed of 28.5 kcal % protein, 58 kcal % carbohydrate and 13.5 kcal % fat. All extracts were dissolved in drinking water and administered to mice *ad libitum*. Solutions were prepared and replaced every 3 days. Metformin-containing drinking solution was prepared as 0.001% in water and replaced likewise.

Animals were divided into six groups: KK mice as normal controls received steroidal glycoside-free drinking water together with low fat diet (n = 20), and KK.Cg-Ay/J mice as diabetic controls received steroidal glycoside-free drinking water together with a high fat diet (n = 16), while the remaining KK.Cg-Ay/J animals were exposed to drinking water supplemented with CB extract (1%, n = 16), BuOH extract (0.1% or 0.2%, n = 16), and a reference drug Metformin (0.001%, n =16, an equivalent of 300 mg/kg/d treatment) together

with a high fat diet. Body mass, food and water uptake by the mice were measured weekly. Subgroups of 5–8 mice were sacrificed on weeks 3, 16, and 24 for blood biochemistry and tissue histology measurements. Livers were removed, weighed, and kept in 80% formalin for histological examination.

2.9 Alanine transferase (ALT) Activity Measurement

Blood samples were collected at the time of sacrifice and centrifuged at 12,500 rpm for an hour. ALT activity was determined by colorimetric method with a Randox Diagnostic kit No. 146 (Kearneysville, West Virginia).

2.10 Blood Chemistry

Triglycerides (TG), total cholesterol (TC), and high-density lipoprotein (HDL) levels were measured with PTS Lipid Panel test strips (CardioCheck, Indianapolis, IN). Blood glucose was measured with a Contour blood glucose meter (Bayer, Tarrytown, NY) using whole blood from a tail vein.

2.11 Oral Glucose Tolerance Test (OGTT)

Diet-induced obese C57BL/6J mice (12 weeks on 60 kcal% high fat diet) were fasted overnight (16 h) and received oral gavage of vehicle (water), 300 mg/kg CB or BuOH extracts, or Metformin. Thirty minutes later the animals were gavaged with D-glucose (2 g/kg body mass, Sigma). Blood glucose concentrations were measured at 0, 15, 30, 60 and 120 minutes after glucose challenges in samples obtained from tail-tip bleedings, using a TRUEResult blood glucose monitor and TRUEtest strips (Nipro Diagnostics, Fort Lauderdale, FL).

2.12 Histological Evaluations

Livers were removed and examined for gross appearance or color change; tissue sections were preserved for histopathological studies. Tissue samples were placed in 10% phosphate-buffered formalin at room temperature overnight, dehydrated in ascending

concentrations (80, 95, and 100%) of ethanol, cleared in xylene, and embedded in Paraplast Plus (Fisher Scientific, Pittsburgh, PA). Serial sections (4 μm) were deparaffinized, rehydrated with water, and subjected to hematoxylin and eosin (H&E) staining. H&E sections were examined under a light microscope (Nikon Corporation, Tokyo Eclipse E600, Japan).

2.13 Statistical Analysis

All analyses were performed at least 3 times. Results are expressed as means ± SE. Statistical analyses were calculated using one-way analysis of variance followed by Dunnett's multiple-range tests (* denotes a difference statistically significant from Control group, # denotes a difference statistically significant from Diabetic group, $p < 0.05$).

3. Results and Discussion

3.1 Quantitative Analysis of Steroidal Glycosides

To investigate the concentration of five steroidal glycosides in CB and BuOH extracts, compounds 1 - 5 were purified as analytical standards according to the literature [23]. To quantify compounds 1 - 5, CB and BuOH extracts were analyzed by LC-MS operating in SIM mode. Calibration curves were constructed over a range of 6 concentrations and good linearity was achieved over concentration ranges of 0.086-2.75 μg/mL for each compound. The correlation coefficients for compounds 1 - 5 ranged from R2 = 0.9995 - 0.9999. Results of the quantitative analysis are shown in Table 1. The lyophilized CB and BuOH extracts contained 391.2 and 6806.9 μg/g of compounds 1 - 5, respectively, whereas the CB extract contained altogether 49.7 μg/g of steroidal glycoalkloids 1 and 2 and 341.5 μg/g of furostanol saponins 3 – 5. The ratio of steroidal glycoalkaloids to furostanol saponins in the CB extract was 1 / 6.82.

For the feeding study, a 1% solution of CB extract was prepared in DI water, delivering

a final concentration of 3.9 μg/mL of compounds 1 – 5. The BuOH extract contained a total of 1879.8 μg/g of steroidal glycoalkaloids 1 and 2 per gram and 4927.1 μg/g of furostanol saponins 3 – 5 per gram. The ratio of steroidal glycoalkloids to furostanol saponins in the BuOH extract was 1 / 2.62. Two solutions, 0.1 and 0.2 %, of BuOH extract were prepared in DI water for the feeding trial delivering a final concentration of compounds 1-5 of 6.8 and 13.6 μg/mL, respectively. As the adjusted daily water intake in KK mice was reported 6.5 ml/30g body mass 28, the animals were estimated to have received an average, respective daily dose of 1500 and 3000 μg/kg/d steroidal glycosides in drinking water, respectively.

3.2 Effect of Easter lily bulb extract Supplementation on Body Mass

The amount of food and water consumed in groups II-VI fed a high fat diet was not statistically different ver the 24 week study period (data not shown). To examine the effects of CB and BuOH treatments on the body mass of KK.Cg-Ay/J Type 2 diabetic mice, body mass measurements were taken at weeks 3, 16, and 24. At week 3, the average body mass of the diabetic mice was significantly higher than the control group, but no significant differences were observed between any of the treatments and the diabetic group. A similar pattern was observed at week 16, with the exception of the BuOH 0.2% treatment group (43.8 ± 0.73 g), which had a lower average body mass than the diabetic group (47.9 ± 1.16 g). At the end of the study (week 24), the average body mass of the BuOH 0.2% group (47.8 ± 1.28 g) was significantly lower than the average body mass of the diabetic control group (51.9 ± 1.63 g) (Figure 2). This effect was not observed in the CB or BuOH 0.1% treatment groups. This outcome suggests that the BuOH 0.2% (total steroidal glycosides 1 – 5, 13.6 μg/mL) significantly reduced cumulative body weight gain on the KK.Cg-Ay/J Type 2 diabetic mice fed a high fat diet.

3.3 Blood biochemistry of Easter Lily bulb extract treated mice

To examine the effects of the lily bulb preparations on the blood chemistry profiles of KK.Cg-Ay/J diabetic mice fed a high fat diet, 3 blood lipid species (TG, TC, and HDL) were quantified after short-term (3 week) and long-term (24 week) treatments. The effect of the long term treatment of the bulb preparations on blood lipid profile is given in Table 2. The diabetic mice had elevated levels as compared to the control group in all three of the measured blood lipid species (TG, TC, and HDL); however, only TC and HDL were significant ($p < 0.05$). The results of the HDL measurements indicated that the diabetic mice had elevated levels of HDL as compared to the control mice; however, no effects on HDL were observed with any of the treatments. Results of the TG level measurements revealed that 1% CB (130.5 ± 26.6 mg/dL), 0.2% BuOH (114.0 ± 19.2 mg/dL), and the Metformin groups (111.50 ± 5.17 mg/dL) differed significantly (~50% lower) from the diabetic group (219.25 ± 33.76 mg/dL). In terms of TC levels, 1% CB (total steridal glycosides 1 – 5, 3.9 μg/mL) showed a marked lower level of TC (159.25 ± 4.50 mg/dL) than the diabetic group (196.00 ± 11.85 mg/dL). Other treatments were not statistically distinguished from the diabetic or control groups. These results suggest that supplementation with 1% CB lowers TC levels in the blood of KK.Cg-Ay/J diabetic mice fed a high fat diet. The 1% CB solution was the lowest steroidal glycoside-containing treatment employed in this study, with 3.9 ⌾g/mL of 1 – 5. In turn, this suggests that steroidal glycosides 1 – 5 are not likely the active compounds in the CB extract since the solutions (BuOH 0.1 and 0.2 %) containing higher levels of 1 – 5, 6.8 and 13.6 μg/mL, respectively, had increased TC, unlike the 1% CB treatment. Other constituents present in the CB extract appear to cause the hypocholesterolemic activity; however, further studies are needed to support this hypothesis.

3.4 Hepatoprotective activity of Easter lily bulb extracts

The effects of various lily bulb preparations on liver function were estimated by liver appearance, enzyme levels, and histology. Based on visual examination and mass measurement, the diabetic mouse livers were enlarged compared with the normal mice (Figure 3). It is well documented that diabetes causes liver damage and increases in liver mass [29]. All the treatment groups in this study had a markedly higher average liver mass than the control group. However, two treatment groups, 1% CB 1% and 0.1% BuOH, had a lower average liver mass than the diabetic group ($p = 0.03$). In addition, elevated ALT levels were found in the diabetic group compared to the control group (Figure 4). ALT, an enzyme associated with the liver function, is measured as a biomarker for the evaluation of hepatocellular injury; its measurement is thus often employed as a diagnostic for liver health. Accordingly, increased ALT levels in the bloodstream are indicative of liver-specific damage [5]. Interestingly, all treatment groups had distinctly decreased ALT levels as compared to the diabetic group, demonstrating that oral consumption of Easter lily bulb extracts lowered ALT levels in KK.Cg-Ay/J diabetic mice suggesting potential hepatoprotective activity.

Besides liver mass and ALT measurements, liver tissues were sectioned and stained with H & E for morphological evaluations (Figure 5). At 40x magnification, the appearance of liver sections prepared from the diabetic mice were swollen and irregularly shaped as compared to the control group. At 400x magnification, liver histological examinations revealed a greater accumulation of lipid droplets in the cytoplasm of hepatocytes in the diabetic mice than the control hepatocytes. In addition, there was a loss of the usual concentric arrangement of hepatocytes in the diabetic mouse liver sections. Based on visual examination, the lily extract and Metformin treatments differentially ameliorated the damage to the hepatocytes. It is worth noting that microphotographs of liver sections at

400x magnification from diabetic mice treated with 0.2% BuOH resemble normal liver hepatocytes. In addition to the decrease in body mass (0.2% BuOH), liver mass (1% CB and 0.1% BuOH), TG (1% CB and 0.2% BuOH), TC (1% CB), and ALT (1% CB, 0.1 and 0.2% BuOH), the histological examinations of liver sections from the experimental treatments provide further evidence of hepatoprotective activity of lily bulb extracts in the KK.Cg-Ay/J type 2 diabetic mouse model.

3.5 Hypoglycemic activity of Ester lily bulb extract

In a preliminary pilot test study, fasting blood glucose was found to decrease in Easter lily extract treated animals as compared to diabetic controls (data not shown). To probe the potential hypoglycemic activity of lily bulb preparations, aqueous solutions of CB and BuOH extracts (300 mg/kg) were evaluated in a single dose OGTT using DIO C57BL/6J mice with impaired glucose tolerance [30]. Compared with low fat diet control mice, diabetic group blood glucose levels were noticeably higher at 30, 60, and 120 minutes after oral gavage, while the CB extract treatment reversed peak blood glucose concentration at 30 and 60 minute time points (Figure 6). This effect, however, was not observed in BuOH treatments containing higher levels of steroidal glycosides 1 – 5 (Table 1), indicating that steroidal glycosides 1 – 5 are not likely responsible for hypoglycemic properties of Easter lily bulbs. These data suggests that other compounds differentially present in the CB extract are most likely responsible for the hypoglycemic effect; however, further studies are required to support this hypothesis.

Lily bulbs have long been utilized as both food and medicine, but the compounds responsible for their purported medicinal properties remain largely unknown. In the present work, two extracts were prepared from Easter lily bulbs with differential concentrations of steroidal glycosides 1 – 5 and their concentrations quantified by LC-MS. In a 24-week course using a female KK.Cg-Ay/J type 2 diabetic mouse model, it was observed

that CB and BuOH extracts improved liver function and lipid metabolism. Similar to the observed effect of CB treatment on lowering TC levels, OGTT performed in a DIO C57BL/6J mouse model revealed that the CB extract ameliorated glucose intolerance while the BuOH extract did not. Since the observed effects were not proportional to the steroidal glycoside content in the extracts, it appears that other constituents may be playing a role in the hypocholesterolemic and hypoglycemic activities of the extracts. Based on these results, activity-guided fractionation studies of the CB extract are currently underway to identify the compounds responsible for these observed biological activities. The results of this investigation support that lily bulb extracts appear to elicit hepatoprotective activity in the KK.Cg-Ay/J type 2 diabetic mouse model based on measurements of average body and liver mass, blood lipid measurements (TG and TC), ALT levels and histological examinations of liver sections. Although lily bulbs are regularly consumed as both a food and medicine in Asia, the impact that their consumption has on human health, including liver function during the onset of diabetes, is unknown. The results of this investigation suggest that Easter lily bulbs exhibit a hepatoprotective activity in a KK.Cg-Ay/J type 2 diabetic mouse model and show both hypocholesterolemic and hypoglycemic activities; however, more investigations are clearly needed to determine the effect of their consumption on human health and toxicology. The results of this study support the multimodal effects of lily bulb supplementation on body mass, lipid and glucose metabolism and begin to elucidate the putative bioactive compounds present in these extracts.

4. References

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5. Tables and Figures

Table 1. Quantification of five steroidal glycosides in CB and BuOH extracts by LC-MS operation in SIM mode.*

Cmpd**	Solid extract, μg/g		Drinking solution, μg/ml		
	CB	BuOH	CB (1%)	BuOH (0.1%)	BuOH (0.2%)
1	41.2	1331.9	0.4	1.3	2.7
2	8.5	547.9	0.1	0.5	1.1
3	159.1	2774.1	1.6	2.8	5.5
4	114.2	1393.0	1.1	1.4	2.8
5	68.2	760.0	0.7	0.8	1.5
Total 1-5	391.2	6806.9	3.9	6.8	13.6

*The values in the table are an average of duplicate measurements.
** Cmpd= compound number.

Table 2. Effect of CB and BuOH extracts on lipid profile of female KK.Cg-A^{y}/J mice

	TG (mg/dL)*	TC (mg/dL)*	HDL (mg/dL)*
Control	138.00ab ±18.76	152.00a ± 6.68	82.25a ± 3.71
Diabetic	219.25a ± 33.76	196.00b ± 11.85	100.00b ± 0.00
CB (1%)	130.5b ± 26.56	159.25a ± 4.50	91.75b ± 4.80
BuOH (0.1%)	138.00ab ± 20.86	177.50ab ± 12.80	98.75b ± 1.25
BuOH (0.2%)	114.0b ± 34.54	177.75ab ± 17.20	88.5ab ± 2.36
Met	111.5b ± 5.17	188.25ab ± 15.04	95.00b ± 3.72

* TG= Triglyceride levels; TC= Total cholesterol levels; HDL= High Density Lipoprotein levels at week 24. Values with different letters in the same column are significantly different ($p<0.05$)

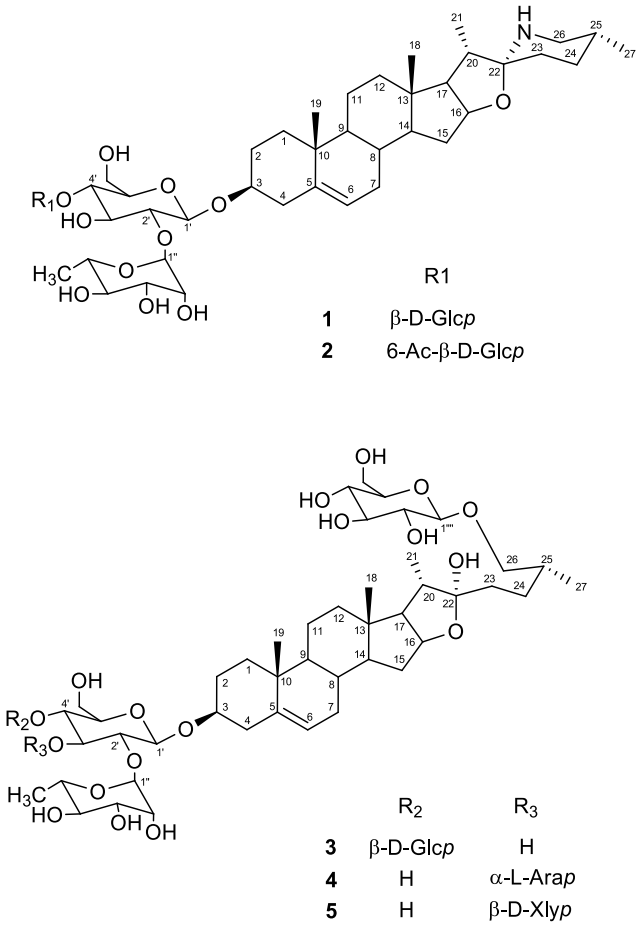

Figure 1. Structures of steroidal glycosides **1 – 5** isolated from Easter lily bulbs. The structures were confirmed by a combination ^{1}H NMR, ^{13}C NMR, ESI^{+}-MS, and comparison of retention times with authentic standards.

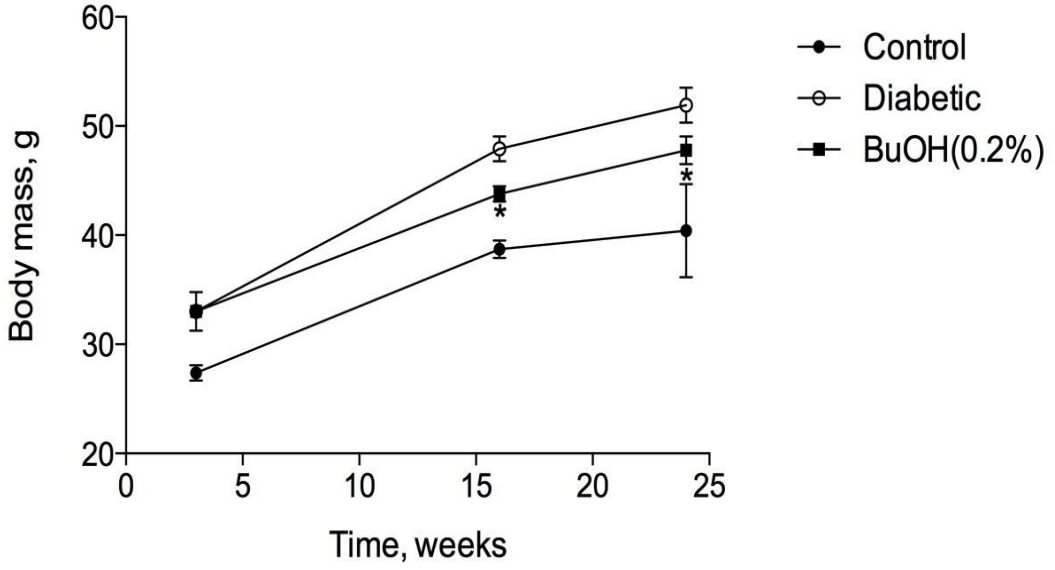

Figure 2. The effect of BuOH (0.2%) extract treatment on the body mass of KK.Cg-A^{y}/J mice over a 24 week period. At weeks 3, 16 and 24, body mass was recorded. For weeks 3 and 16, values are the average of five mice ± SE for KK.Cg-A^{y}/J mice, and six mice ± SE for KK control mice; for week 24, values are an average of six mice ± SE for KK.Cg-A^{y}/J mice, and eight mice ± SE for KK control mice.

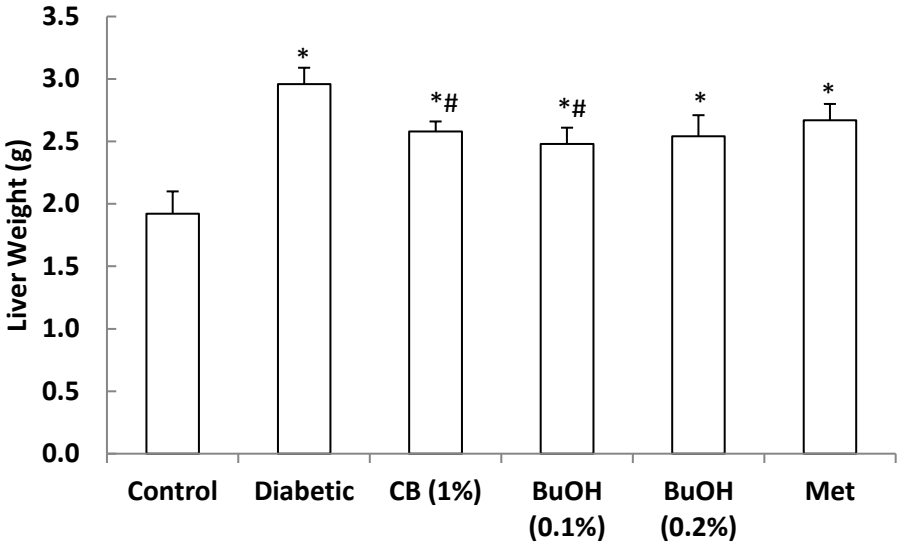

Figure 3. Liver mass following 24 week feeding study in KK.Cg-A^y/J mice. Values are the average of six mice ± SE for KK.Cg-A^y/J mice and eight mice ± SE for KK control mice. * denotes a difference statistically significant from control group ($p < 0.05$). # denotes a difference statistically significant from diabetic group ($p < 0.05$).

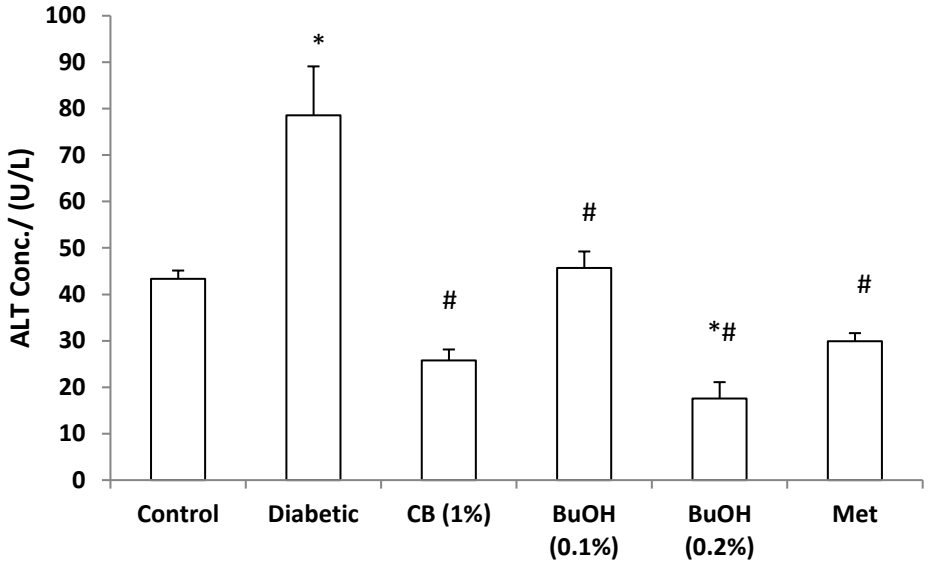

Figure 4. Effect of CB and BuOH extracts on alanine aminotransferase (ALT) levels in the liver. After 24 weeks of treatment KK.Cg-A^y/J mice were sacrificed and liver samples homogenized with homogenate buffer. ALT levels were measured with a commercial kit. Values are the average of six mice ± SE for KK.Cg-A^y/J mice, and eight mice ± SE for KK control mice. * denotes a difference statistically significant from the control group ($p < 0.05$). # denotes a difference statistically significant from the Diabetic group ($p < 0.05$).

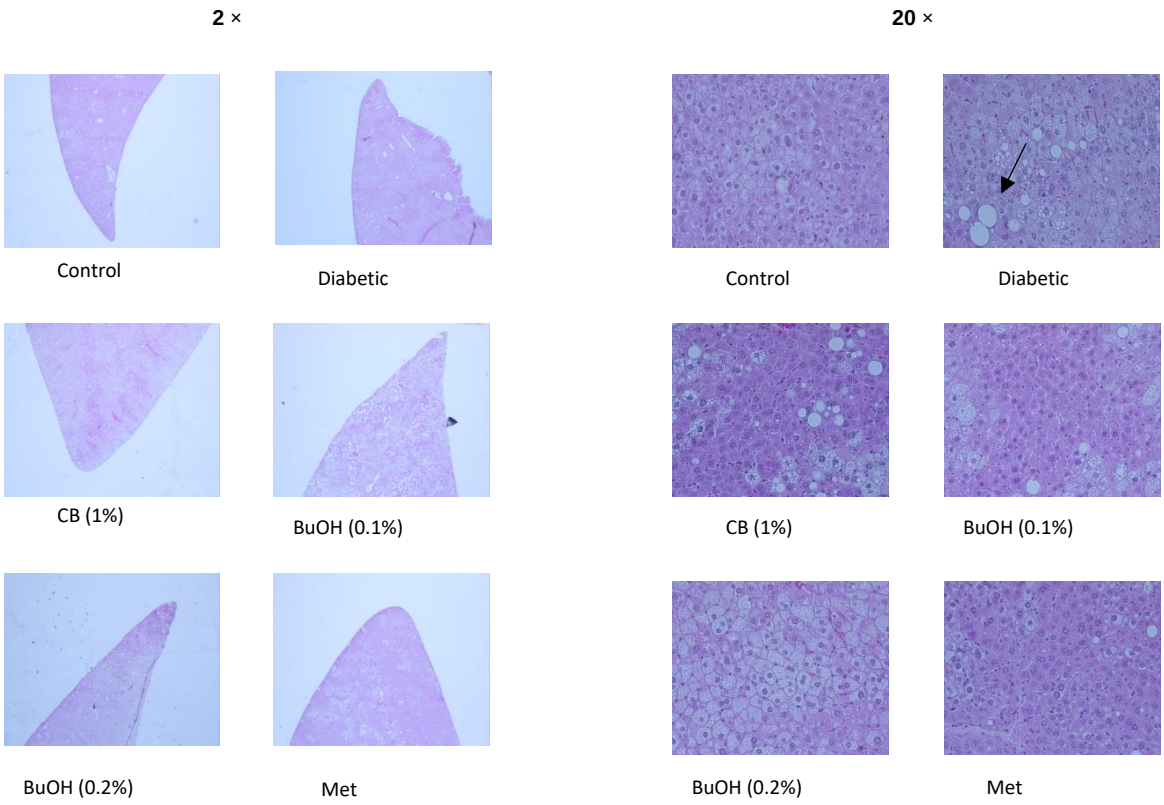

Figure 5. Effect of CB and BuOH extracts on liver morphology after H&E staining. After 24 weeks, KK.Cg-A^{y}/J mice were sacrificed, and liver tissues sectioned and stained with hematoxylin and eosin for morphology. Microscopic images of ×2 and ×20 magnification were acquired and shown. Arrow is pointing to lipid droplets.

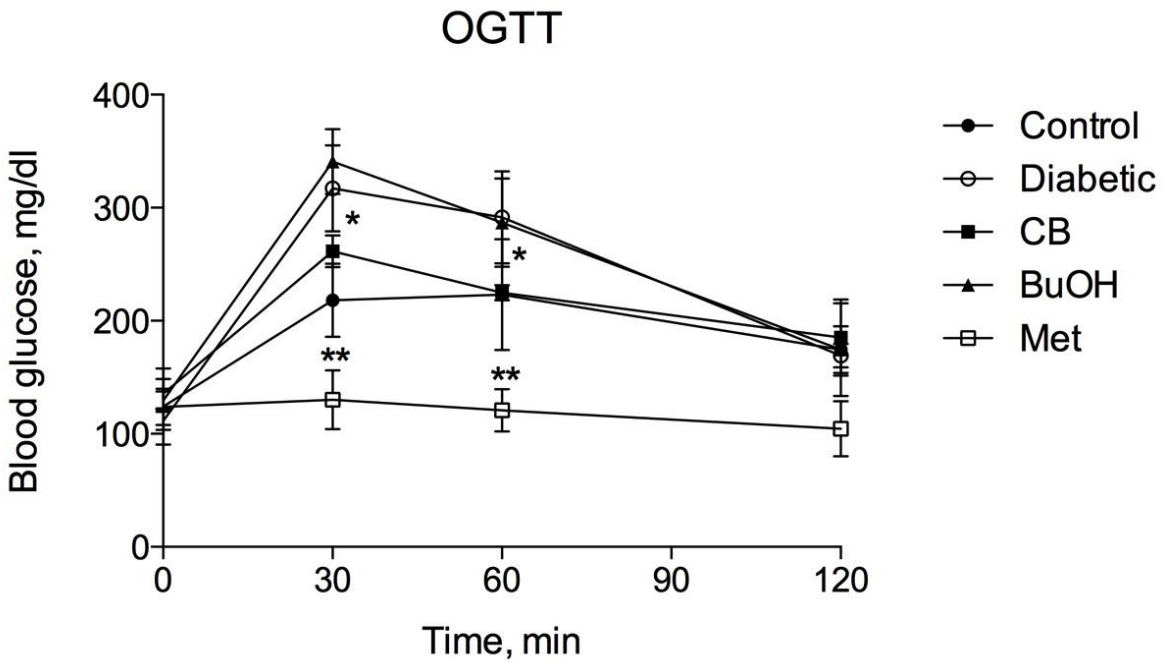

Figure 6. Oral glucose tolerance test (OGTT) in DIO C57BL/6J mice. Mice were treated with a single dose of 300 mg/kg CB, BuOH, or Metformin (reference drug) 30 min prior to glucose challenge. Overnight-fasted mice were administrated 2 g/kg glucose by a gastric gavage. Blood samples were taken at 0, 60, 90 and 120 min. Blood levels of glucose were measured and presented as means ± SEM (n = 8).

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

Kimberly M. Palatini Jackson

1. Conclusions

Type 2 Diabetes (T2D) and associated conditions are worldwide problems that have garnered increasing attention since Reaven described syndrome X, now identified as metabolic syndrome, nearly three decades ago [1,2]. As a result of news and research attention, lifestyle-related chronic conditions are becoming a prominent section of food and nutrition research aiming to inspire positive lifestyle management that could result in the prevention or reversal of T2D. In the interest of better health, patients are becoming increasingly interested in alternative treatments for early diagnoses of metabolic syndrome and hyperglycemia to delay or eliminate the need for common medicines such as metformin, following the advice of Hippocrates, "Let thy food be thy medicine and thy medicine be thy food".

Traditional medicine has been utilizing the natural medicinal properties of edible plants for centuries, and many common drugs were first isolated from botanical sources. More than 400 traditional botanical treatments for diabetes have been recorded, but only a small number have been evaluated to assess efficacy and safety [3]. Metformin (dimethylbiquanide) can be traced back to the use of *Galega officinalis* Linn (French lilac) as herbal medicine in medieval Europe [4]. More recently, *Momorida chantria* (bitter melon) is well documented in South and Central American, Asian, Indian, and Australian cultures to control postprandial glucose levels in adults with T2D [5]. Advances in technology have guided the field of drug discovery toward synthetic chemistry, but have also provided more resources for the generation of new drugs from natural sources, and for safety and efficacy testing for treatments isolated from plant sources [6].

The new drug approval (NDA) process requires stringent guidelines on health and safety be met before any drug is marketed. While the centuries of anecdotal evidence could

relieve some of a burden from Phase I and II trials for pharmacology and toxicology, many considerations still remain before a botanical extract can reach Phase III testing. Many botanical manufacturers do not pursue an NDA, but instead enter the dietary supplement market and still complete rigorous safety and efficacy tests for their own claims. Unfortunately, the supplement market is expansive and suffers from a lack of oversight that often leads to confusion for consumers due to difficulties differentiating safe and well-researched supplements from snake oils and placebos. Despite this, it should not detract from the value of well-researched nutritional supplements. In evidence of this challenge, only two botanical drugs have been approved to date; however, many more, such as Estrovera, are nearly as well researched (Chapter 1).

In the interest of botanical drug development, both *Cichorium intybus* (chicory) and *Lilium longiflorum* (Easter lily) extracts have been examined herein as possible treatments for hyperglycemia and liver dysfunction associated with T2D. The caffeic acid moiety isolated from di-O-caffeoylquinic acids in chicory root material reduced gluconeogenic mRNA expression and promoted PI3K and MAPK pathway signaling when tested in an H4IIE rat hepatoma cell model (Chapter 2). The introduction of chicory extract into the diet of C57BL/6J mice had no long-term effects on hyperglycemia, but had a significant effect on hepatic triglycerides (Chapter 3). Hydroethanolic crude bulb and glycoside-rich 1-butanol extracts of Easter lily significantly improved liver triglycerides and total cholesterol in the KK.Cg-Ay/J Type 2 diabetic mouse model, while the crude bulb extract alone improved glucose metabolism in C57BL/6J mice (Chapter 5).

Unlike Metformin, the effectiveness of many botanical supplements cannot be attributed to a purified compound. The plant matrix is often important to the pharmacological activity of the extract. Yet, underlying the anti-hyperglycemic activity of many botanical remedies may be the activation of Type 2 bitter receptors by bitter phytochemicals. Chicory extract,

rich in bitter lactucopicrin and chlorogenic acids, had an acute effect on postprandial glucose absorption following a 2g/kg glucose load (Chapter 3). Investigation of many other classes of botanical bitter compounds to affect postprandial plasma glucose levels resulted in a similar conclusion (Chapter 4). Furthermore, the administration of bitter compounds and bitter chicory extract to an STC-1 mouse neuroendocrine cell model confirmed that bitter botanicals activate Type 2 bitter receptors in the gastrointestinal tract by stimulating GPCR signaling cascade (Chapter 4).

2. Future Directions

During the course of this project, new questions have arisen. The potential of many medicinal botanicals has yet to be thoroughly investigated, and provides numerous opportunities for safety, efficacy, and toxicity evaluation. The identification of possible biochemical mechanisms lends credibility to nutritional and health claims, and represents positive steps in elucidating nutrition and supplementation science.

The low bioavailability of chlorogenic acids in the upper gastrointestinal tract could explain the lack of long-term response to chicory feeding. Because we have identified bioactivity of chlorogenic acids in the liver at concentrations as low as 3 μM, as demonstrated in the H4IIE cell model, this could indicate these levels were not reached in our animal model and explain our contradictory finding in the bioactivity observed *in vitro*. The development of a more concentrated extract for the use in feeding studies could help answer these questions.

Although bitterness typically makes a product less marketable, there may be a route for increasing the public's acceptance for bitterness if a health connection was established. Bitterness may even be masked in many cases by complexing bitter compounds with larger binding molecules. The determination of efficient binding molecules that allow masking

bitter flavors in the mouth, but liberates active compounds in the stomach, would be extremely useful for a wide variety of new and existing products. While the density of taste receptors is greatest in the mouth, bitterness sensed in the stomach and gastrointestinal tract has also been shown herein to be effective in inhibiting plasma glucose levels.

A study involving human participants would be the next logical step. A placebo-controlled, blinded study could monitor circulating glucose and metabolic markers following the ingestion of bitter treatments. Treatments could be administered as a solution or capsule to determine if the anti-hyperglycemic effect of bitter extracts is conserved in humans. If effective, this trial could provide a lens through which to examine traditional botanical medicine and a route to develop alternative treatments for metabolic syndrome and T2D.

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APPENDICES

APPENDIX 1: THIAZOLOPYRIDINES IMPROVE ADIPOCYTE FUNCTION BY INHIBITING 11BETA-HSD1 OXOREDUCTASE ACTIVITY

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Research Article

Thiazolopyridines Improve Adipocyte Function by Inhibiting 11 Beta-HSD1 Oxoreductase Activity

Thirumurugan Rathinasabapathy,[1] Kimberly Marie Palatini Jackson,[2,3] Yiwen Thor,[3] Ayuba Sunday Buru,[1] Debora Esposito,[2,4] Xu Li,[2,5] Mallikarjuna Rao Pichika,[1] Ahmad Sazali Hamzah,[6] and Slavko Komarnytsky[2,3]

[1]*Department of Pharmaceutical Chemistry, School of Pharmacy, International Medical University, 126, Jalan Jalil Perkasa 19, Bukit Jalil, 57000 Kuala Lumpur, Malaysia*
[2]*Plants for Human Health Institute, North Carolina State University, North Carolina Research Campus, 600 Laureate Way, Kannapolis, NC 28081, USA*
[3]*Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, 400 Dan Allen Drive, Raleigh, NC 27695, USA*
[4]*Department of Animal Science, North Carolina State University, 120 Broughton Drive, Raleigh, NC 27695, USA*
[5]*Department of Plant and Microbial Biology, North Carolina State University, 100 Derieux Place, Raleigh, NC 27695, USA*
[6]*Center for Synthesis and Chemical Biology, Institute of Science, Universiti Teknologi MARA, 40450 Shah Alam, Selangor Darul Ehsan, Malaysia*

Correspondence should be addressed to Slavko Komarnytsky; komarnytsky@ncsu.edu

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Background. Glucocorticoid excess has been linked to clinical observations associated with the pathophysiology of metabolic syndrome. The intracellular glucocorticoid levels are primarily modulated by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme that is highly expressed in key metabolic tissues including fat, liver, and the central nervous system. *Methods*. In this study we synthesized a set of novel tetrahydrothiazolopyridine derivatives, TR-01–4, that specifically target 11β-HSD1 and studied their ability to interfere with the glucocorticoid and lipid metabolism in the 3T3-L1 adipocytes. *Results*. Based on the docking model and structure-activity relationships, tetrahydrothiazolopyridine derivatives TR-02 and TR-04 showed the highest potency against 11β-HSD1 by dose-dependently inhibiting conversion of cortisone to cortisol (IC_{50} values of 1.8 μM and 0.095 μM, resp.). Incubation of fat cells with 0.1–10 μM TR-01–4 significantly decreased cortisone-induced lipid accumulation in adipocytes and suppressed 11β-HSD1 mRNA expression. Observed reduction in adipocyte fat stores could be partially explained by decreased expression levels of adipogenic markers (PPAR-γ, aP2) and key enzymes of lipid metabolism, including fatty acid synthase (FAS), hormone sensitive lipase (HSL), and lipoprotein lipase (LPL). *Conclusions*. The tetrahydrothiazolopyridine moiety served as an active pharmacophore for inhibiting 11β-HSD1 and offered a novel therapeutic strategy to ameliorate metabolic alterations found in obesity and diabetes.

1. Introduction

The worldwide surge in prevalence of obesity and associated type 2 diabetes mellitus increases the need for novel preventive and therapeutic strategies as stated by the World Health Organization [1]. The metabolic disorders are no longer confined to residents of affluent industrialized Western countries (i.e., United States, where about 30% of adults are overweight and another 30% are obese) [2] but become more widespread in the developing world, including Malaysia, where 30% and 15% of adults are overweight and obese, respectively [3].

High levels of circulating glucocorticoids, as seen in Cushing syndrome, promote hyperphagia, central obesity, and insulin resistance [4]. In the majority of obese subjects, however, circulating glucocorticoids do not correlate with obesity, blood glucose, or insulin levels due to increased cortisol clearance [5] and tissue-specific metabolism by

11β-hydroxysteroid dehydrogenases (11β-HSDs) [6]. Intracellular regeneration of active steroids cortisol from cortisone (in humans) and corticosterone from 11-dehydrocorticosterone (in rodents) is achieved through 11β-HSD type 1 (11β-HSD1), a lower-affinity NADP(H)-dependent enzyme that acts as oxoreductase in tissues with high sensitivity to glucocorticoids [7]. The opposing dehydrogenase activity of 11β-HSD1 (conversion of cortisol to cortisone) becomes evident only in cell culture and tissue homogenates or upon purification due to decrease of ER luminal cofactor NADP(H) generated by hexose-6-phosphate dehydrogenase [8].

Several studies have highlighted 11β-HSD1 as a novel therapeutic target in metabolic syndrome. 11β-HSD1 knockout mice had low intracellular glucocorticoid levels and were protected from obesity, diabetes, and dyslipidemia [9, 10]. Conversely, transgenic overexpression of 11β-HSD1 in white adipose tissue resulted in elevated intracellular glucocorticoid levels, central obesity, insulin resistance, hyperglycemia, and dyslipidemia in mice [11]. A number of structural classes of 11β-HSD1 inhibitors have been described in the literature [12], including the benzothiazole class of compounds which are bioisostere to tetrahydrothiazolopyridine [13] and are frequently reported as dipeptidyl peptidase IV inhibitors. In preclinical studies with C57BL/6J mice fed high fat diet, the beneficial effects of 11β-HSD1 inhibition were observed, including reduced body weight, food intake, and fasting glucose and insulin levels [14]. However, phase IIb clinical trials with existing 11β-HSD1 inhibitors resulted in modest improvements of glucose homeostasis in type 2 diabetic subjects [15, 16]. For example, salicylates administered at 50 mg/kg/day for 2 weeks reduced 11β-HSD1 mRNA levels in human subcutaneous fat, and a similar effect was observed in fully differentiated human SGBS adipocytes in the dose range of 10–100 μM [17].

Given the public health significance of metabolic disorders and the observation that past therapeutic approaches have not met with success in addressing this issue, new strategies are clearly needed. We therefore explored a novel class of 11β-HSD1 inhibitors based on the tetrahydrothiazolopyridine skeleton. Previously, several members of this class of compounds were reported to inhibit phosphatidylinositol 3-kinase [18]. Here, we report on the synthesis of four TR1-4 derivatives, their inhibitory effects on 11β-HSD1 activity, adipocyte differentiation, and expression of key adipose genes associated with lipid metabolism and insulin resistance.

2. Materials and Methods

2.1. Chemicals and Analytical Methods. All chemicals and solvents (anhydrous and ACS grade) were purchased from Sigma-Aldrich (Saint Louis, MO) unless specified otherwise. Structures and purity (>95%) of all compounds were confirmed by LC-MS using Bruker (Billerica, MA) Avance Electrospray Ionization quadrupole time-of-flight mass detector (ESI-Q-TOF-MS) with MS/MS (Triple quadrupole) interface, using Agilent Technologies (Santa Clara, CA) Extend C18 reverse phase column (250 mm × 4.6 mm, 5.0 μm). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance 300, 500 MHz NMR stations.

2.2. Synthesis of 2-Amino-5-benzyl-4,5,6,7-tetrahydrothiazolo[5,4-C]pyridine (TR-01). 2-Amino-5-benzyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine was synthesized using a previously reported procedure with some modifications [19]. 1-Benzyl-4-piperidone (1.0 g, 5.3 mM) was dissolved in 30 ml of cyclohexane. To this solution, pyrrolidine (0.4 g, 5.5 mM) and p-toluenesulphonic acid (catalytic quantity) were added and refluxed for 2.5 h using Dean-Stark trap. Then, the reaction mixture was cooled to room temperature and filtered and the filtrate was concentrated to dryness in vacuo. The formed residue was dissolved in 25 ml of dry methanol; sulphur (S_8, 0.17 g, 0.66 mM) was added at once and the reaction mixture was stirred for 10 min at room temperature. Then, the reaction mixture was cooled to 0°C, cyanamide (0.22 g, 5.3 mM) in 5 ml of dry methanol was added slowly in dropwise manner, and the reaction was continued for 5 h at the same temperature. After completion of the reaction, the mixture was filtered and concentrated to obtain a crude compound. TR-01 was chromatographed over silica gel with dichloromethane/methanol (99 : 1) as eluent, yielding 51.2%. The purity of the compound was confirmed by TLC using $CHCl_3/CH_3OH$ [(95 : 5); R_f = 0.78], ESI-Q-TOF-MS: (m/z) 246.1 [M + H] and NMR spectra; ^{1}H NMR (500 MHz, DMSO-d_6) δ = 2.48 (t, 2H, J = 6.0 Hz), 2.70 (t, 2H, J = 6.0 Hz), 3.53 (s, 2H), 3.64 (s, 2H), 6.69 (s, 2H, NH_2), 7.25 (dt, 1H, J = 4.0 Hz), 7.34 (m, 4H); ^{13}C NMR (125 MHz, DMSO-d_6) δ = 26.9, 50.0, 50.3, 61.2, 112.6, 127.4, 128.7, 129.2, 139.0, 143.9, 166.6.

2.3. Synthesis of N-(5-Benzyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine-2-yl)-3-(4-chlorophenyl)-2-cyanoacrylamide (TR-02). The intermediate cyanoester compound was synthesized by Knoevenagel condensation method [20] in which 4-chlorobenzaldehyde (1.0 g, 7.1 mM) was dissolved in 25 ml of methanol. tert-Butyl cyanoacetate (1.0 g, 7.1 mM) and piperidine (5 drops) were added and the mixture was refluxed for 45 min. After the completion of the reaction, 100 ml of ice-cold water was added, and the formed precipitate was collected by filtration, recrystallized from ethanol-water to give tert-butyl-3-(4-chlorophenyl)-2-cyanoacrylate. This ester compound was dissolved in 10 ml of dichloromethane, to which 10 ml of trifluoroacetic acid was added and stirred at room temperature for 1 h. The reaction mixture was evaporated in vacuo; the solid was triturated with water, filtered, and dried to give 3-(4-chlorophenyl)-2-cyanoacrylic acid. The purity of this compound was confirmed by TLC using $CHCl_3/CH_3OH$ [(90 : 10); R_f = 0.4] and melting point (m.p. 192°C). The compound (0.5 g, 2.4 mM) was further dissolved in 30 ml of dichloromethane, EDCI (0.55 g, 2.9 mM), HOBt (0.39 g, 2.9 mM), and DIPEA (0.69 g, 5.3 mM) and stirred for 1 h at room temperature. Then TR-01 (0.59 g, 2.4 mM) was added, and the stirring was continued at same temperature for 24 h. The reaction mixture was washed with water, saturated $NaHCO_3$, and brine and concentrated by drying over anhydrous Na_2SO_4. The crude compound was chromatographed over silica gel using dichloromethane/methanol (95 : 5) as eluent, yielding 47.6% of titled TR-02. The purity of the compound was confirmed by TLC using $CHCl_3/CH_3OH$ [(95 : 5); R_f = 0.65] and NMR

spectra; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.62 (t, 2H, J = 5.1 Hz), 2.79 (t, 2H, J = 5.1 Hz), 3.50 (s, 2H), 3.70 (s, 2H), 7.28 (t, 1H, J = 3.9 Hz, ArH), 7.33 (m, 4H), 7.65 (d, 2H, J = 8.4 Hz), 8.00 (d, 2H, J = 8.4 Hz), 8.34 (s, 1H), 13.19 (s, 1H, NH).

2.4. Synthesis of 2-Amino-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine (TR-03). 2-Amino-4,5,6,7-tetrahydrothia-zolo[5,4-c]pyridine was synthesized using a previously reported procedure [19]. 1-tert-Butoxycarbonyl-4-piperidone (1.0 g, 5.0 mM) was dissolved in 30 ml of cyclohexane. To this solution, pyrrolidine (0.38 g, 5.3 mM) and p-toluenesulphonic acid (catalytic quantity) were added and refluxed for 2.5 h using Dean-Stark trap. Then, the reaction was cooled to room temperature and filtered and the filtrate was concentrated to dryness in vacuo. The formed residue was dissolved in 25 ml of dry methanol; sulphur (S_8, 0.16 g, 0.63 mM) was added at once and the reaction mixture was stirred for 10 min at room temperature. Then, the reaction mixture was cooled to 0°C, cyanamide (0.21 g, 5.0 mM) in 5 ml of dry methanol was added slowly in dropwise manner, and the reaction was continued for 5 h at the same temperature. After completion of the reaction, the mixture was filtered and concentrated to get the crude TR-03a. TR-03a was further chromatographed over silica gel with dichloromethane/methanol (98 : 2) as eluent, yielding 73.4%. The purity of the compound was confirmed by TLC using $CHCl_3/CH_3OH$ [(95 : 5); R_f = 0.72], ESI-Q-TOF-MS: (m/z) 256.3 [M + H], and NMR spectra; ^{1}H NMR (500 MHz, DMSO-d_6) δ = 1.41 (s, 9H), 2.43 (t, 2H, J = 6.0 Hz), 3.56 (t, 2H, J = 5.5 Hz), 4.29 (s, 2H), 6.80 (s, 2H, NH_2).

The confirmed TR-03a was then treated with 1 : 1 ratio of dichloromethane and trifluoroacetic acid and stirred for 1 h at room temperature. The reaction mixture was concentrated and washed with water and diethyl ether. Then the reaction mixture was dissolved in dichloromethane, washed with water and brine, and concentrated by drying over anhydrous Na_2SO_4 to obtain TR-03. The purity of the compound was confirmed by NMR spectra; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.32 (t, 2H, J = 5.4 Hz), 2.86 (t, 2H, J = 5.4 Hz), 3.59 (s, 2H), 6.63 (s, 2H, NH_2).

2.5. Synthesis of 2-Amino-5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine (TR-04). 2-Amino-5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine was synthesized using a previously reported procedure [19]. 1-Methyl-4-piperidone (1.0 g, 8.84 mM) was dissolved in 30 ml of cyclohexane. To this solution, pyrrolidine (0.66 g, 9.3 mM) and p-toluenesulphonic acid (catalytic quantity) were added and refluxed for 2.5 h using Dean-Stark trap. Then, the reaction was cooled to room temperature and filtered, and the filtrate was concentrated to dryness in vacuo. The formed residue was dissolved in 25 ml of dry methanol; sulphur (S_8, 0.28 g, 1.1 mM) was added at once and the reaction mixture was stirred for 10 min at room temperature. Then, the reaction was cooled to 0°C, cyanamide (0.37 g, 8.84 mM) in 5 ml of dry methanol was added slowly in dropwise manner, and the reaction was continued for 5 h at the same temperature. After completion, the reaction mixture was filtered and concentrated to get the crude TR-04. TR-04 was further chromatographed over silica gel with dichloromethane/methanol (98 : 2) as eluent, yielding 67.8%. The purity of the compound was confirmed by TLC using $CHCl_3/CH_3OH$ [(95 : 5); R_f = 0.72] and NMR spectra; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.30 (s, 3H, N-CH_3), 2.43 (t, 2H, J = 5.4 Hz), 2.59 (t, 2H, J = 5.4 Hz), 3.28 (s, 2H), 6.68 (s, 2H, NH_2).

2.6. Molecular Docking Studies. The molecular docking tool, GLIDE (Schrodinger Inc., USA; release 2014-2), was used for 2-amino-5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine (TR4) docking studies into 11β-HSD1 enzyme binding pocket. The crystal structure of 11β-HSD1 was obtained from the RCSB protein data bank (PDB ID: 4K1L) [21]. The protein preparation was carried out using the default parameters of "protein preparation wizard," in Maestro 9.8. The active site of the receptor was defined using the default settings of "receptor grid generation." The chemical structure of TR-04 was drawn using ChemBiodraw Ultra 12.0, imported into Maestro and its 3-D structure was prepared by using the default parameters of "Ligprep." The lowest energy conformation of TR-04 was selected for docking studies. The docking was performed using default parameters of "Ligand Docking wizard" with a standard precision (SP) docking mode. The final evaluation is done with the glide score and the single best pose is generated as the output for TR-04. The docking reliability was evaluated by calculating the root mean square deviation (RMSD) between the pose of cocrystallized ligand and the binding pose predicted by the docking protocol. If the RMSD is below 2 Å [22, 23], the docking protocol was considered reliable for predicting the binding poses of ligands.

2.7. Adipocyte Cell Culture. The mouse embryonic fibroblast cell line 3T3-L1 (CL-173) that undergoes a preadipose to adipose conversion and expresses the major pathways of glucose and lipid metabolism was obtained from ATCC (Manassas, VA). Cells were routinely passaged every 3-4 days and maintained in high glucose DMEM containing 10% fetal bovine serum FBS (Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37°C and 5% CO_2, following an established protocol [24] with some modifications. Cells were subcultured into 6-well dishes at a density of 1×10^5 cells/well (day 0), changed to fresh DMEM/FBS medium once confluent (day 2), and induced to adipose conversion by subsequently changing to D1 medium (DMEM/FBS, 1 μg/ml insulin, 500 μM 3-isobutyl-1-methylxanthine, and 0.25 μM dexamethasone) on day 4 and to D2 medium (DMEM/FBS, 1 μg/ml insulin) on day 6. Mature adipocytes were changed to fresh DMEM/FBS medium on day 8 and used for treatments on the same day.

2.8. Measurement of 11β-HSD1 Activity in Adipocytes. 11β-HSD1 bidirectional oxoreductase (conversion of cortisone to active cortisol) and dehydrogenase (conversion of cortisol to inactive cortisone) activities were measured by incubating 3T3-L1 preadipocytes (days 0 and 4) or mature adipocytes (day 8) in medium with either 1 μM cortisone or cortisol,

respectively, for 20 min at 37°C. After extraction with ethyl acetate and evaporation in vacuo, glucocorticoid concentrations were measured in triplicate by LC-MS in positive mode (Agilent G1312B-1200 series Infinity Quaternary HPLC system coupled with Agilent 6530A Accurate-Mass Quadrupole Time-of-Flight MS with Agilent Jet Stream source). Dry samples were reconstituted with 500 μl 80% methanol, centrifuged, and filtered, and 5 μl was injected onto a Agilent ZORBAX Eclipse Plus C18 column (3 × 100 mm, 1.8 μm). A gradient from 30% acetonitrile in 0.1% formic acid to 90% acetonitrile in 0.1% formic acid was used for HPLC separation. Mass data were acquired with the following parameters: drying gas temperature 300°C, drying gas flow 7 L/min, nebulizer pressure 40 psi, sheath gas temperature 350°C, sheath gas flow 10 L/min, capillary voltage 3500 V, nozzle voltage 500 V, fragmentor voltage 150 V, skimmer voltage 65 V, and octopole RF peak voltage 750 V. Standard curves for compounds of interest were obtained in a linear dynamic range of 10–50,000 pg and used for quantitative analysis. Peak area was determined by Agilent MassHunter Qualitative Analysis (version B.05.00) software. The percent of conversion ratio was calculated as 100 − [(initial concentration − final concentration)/(initial concentration)] ∗ 100% ratio.

TR-01–4 for cell culture studies was dissolved in DMSO as 1000x stocks and stored at −20°C. When indicated, 11β-HSD1 activity was inhibited by exposure of cells for 2 h to vehicle control (0.1% DMSO) or the appropriate TR-01–4 in the dose range of 0.1–100 μM. Cell viability was estimated using the MTT assay [25] by absorbance read at 570 nm on a Synergy H1 microplate spectrophotometer (BioTek, Sunnyvale, CA).

2.9. Adipocyte Differentiation and Lipid Accumulation. The 3T3-L1 preadipocytes were induced to adipose conversion as described above (dexamethasone was omitted) and treated with 1 μM cortisone and 0–10 μM TR-01–4 throughout differentiation. Cells were stained with Oil red O on day 8 to visualize differentiated adipocytes and quantify lipid accumulation following an established protocol [24] with some modifications. After medium removal, cells were washed twice with ice-cold PBS and fixed in 10% neutral buffered formalin at room temperature for 10 min. Cells were then washed twice with ice-cold PBS and stained with 1% Oil red O in isopropanol, diluted 3 : 2 in PBS, for 1 h at room temperature. Following two additional PBS washes, fresh PBS was added to cover the cell surface and prevent dehydration. Cells were photographed at 40x magnification using EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA). Lipid-bound Oil red O stain was eluted by 30 min incubation with isopropanol and quantified by absorbance read at 520 nm on a Synergy H1 spectrophotometer (BioTek).

2.10. RNA Extraction and Gene Expression by qPCR. The total RNA was isolated from cells using TRIzol reagent (Life Technologies) following the manufacturer's instructions. RNA was quantified using Synergy H1/Take 3 plate setup (BioTek). The cDNAs were synthesized using 2 μg of RNA for each sample using high-capacity cDNA Reverse Transcription kit (Life Technologies), following the manufacturer's protocol on an ABI GeneAMP 9700 (Life Technologies).

The resulting cDNA was amplified in duplicate by real-time quantitative PCR (qPCR) using SYBR green PCR Master Mix (Life Technologies). To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) as follows: β-actin (housekeeping gene), forward primer: 5′-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse primer: 5′-CAC AGC CTG GAT GGC TAC GT-3′; adiponectin (AdiQ), forward primer: 5′-TGT TCC TCT TAA TCC TGC CCA-3′, reverse primer: 5′-CCA ACC TGC ACA AGT TCC CTT-3′; leptin (Lep), forward primer: 5′-GAG ACC CCT GTG TCG GTT C-3′, reverse primer: 5′-CTG CGT GTG TGA AAT GTC ATT-3′; glucose transporter type 4 (GLUT4), forward primer: 5′-CAG CTC TCA GGC ATC AAT-3′, reverse primer: 5′-TCT ACT AAG AGC ACC GAG-3′; fatty acid synthase (FAS), forward primer: 5′-GGC ATC ATT GGG CAC TCC TT-3′, reverse primer: 5′-GCT GCA AGC ACA GCC TCT CT-3′; hormone sensitive lipase (HSL), forward primer: 5′-CCA AGT GTG TGA GCG CCT ATT-3′, reverse primer: 5′-CAC GCC CAA TGC CTT CTG-3′; lipoprotein lipase (LPL), forward primer: 5′-CTG AAA GTG AGA ACA TTC CCT TCA-3′, reverse primer: 5′-CCG TGT AAA TCA AGA AGG AGT AGG TT-3′; peroxisome proliferator activated receptor (PPAR-γ), forward primer: 5′-GCC CTT TGG TGA CTT TAT GGA-3′, reverse primer: 5′-GCA GCA GGT TGT CTT GGA TG-3′; and fatty acid binding protein 4 (aP2), forward primer: 5′- TCA CCT GGA AGA CAG CTC CT-3′, reverse primer: 5′- AAT CCC CAT TTA CGC TGA TG′. qPCR amplifications were performed on an ABI 7500 Fast real-time PCR (Life Technologies). Cycle conditions were 50°C for 2 min, 95°C for 10 min, and then 40 cycles at 95°C for 15 s and 60°C for 1 min. mRNA expression was analyzed using the ΔΔCT method [26] and normalized with respect to the expression of the β-actin using ABI 7500 Fast System SDS Software v1.3.0 (Life Technologies). Amplification of specific transcripts was further confirmed by obtaining dissociation (melting) curve profiles with 1 cycle of 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C.

2.11. Statistical Analysis. Statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA). Data were reported as means ± SEM and analyzed by one-way ANOVA or two-tailed Student's t-test, as appropriate. ANOVA post hoc analyses of differences between individual experimental groups were made using Dunnett's multiple comparison tests. Significance was set at $p < 0.05$.

3. Results

3.1. Synthesis of Tetrahydrothiazolopyridine Derivatives and Molecular Docking Simulations. The molecular docking studies were performed to understand the molecular interactions between TR-04 and 11β-HSD1 enzyme. First, the reliability of the docking protocol was determined by RMSD calculation between the poses of cocrystallized ligand and its docked conformation. The superimposition of cocrystallized ligand

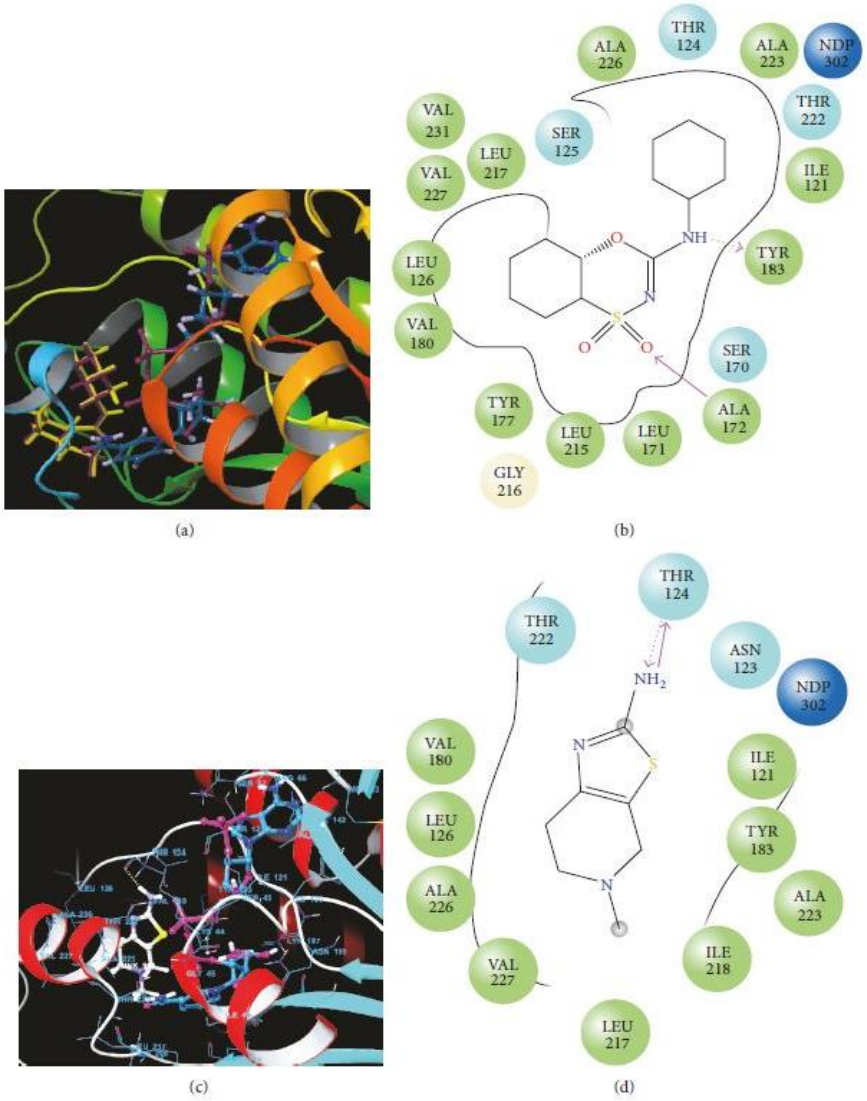

FIGURE 1: Binding modes of native ligand and TR-04 in the X-ray crystal structure of 11β-HSD1 (PDB ID: 4K1L). (a) Superimposition of the poses of cocrystallized native ligand (shown in yellow colored tube representation) with its binding conformation (shown in maroon colored tube representation). (b) The interactions between inbound ligand and amino acid residues of binding pocket (4K1L). (c) The pose of TR-04 (shown in ball-and-stick representation). (d) The interactions between TR-04 and amino acid residues of binding pocket (4K1L). Green colored circles indicate hydrophobic amino acid residues; blue colored circles indicate polar amino acid residues.

and its docked pose along with its interactions with receptor is shown in Figure 1. The RMSD was found to be 0.158 indicating the docking protocol used in this study is appropriate for reliable prediction of docking pose of TR-04. Its molecular interactions with amino acid residues of the binding pocket (PDB ID: 4K1L) were similar to those of inbound ligand. The binding pose of TR-04 and its molecular interactions with amino acid residues of binding pocket, facilitating its

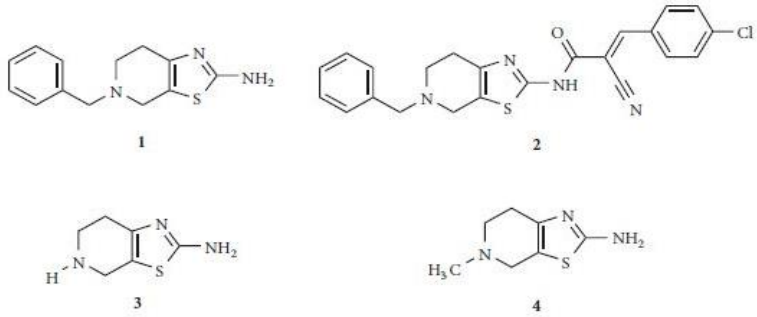

FIGURE 2: Chemical structure of tetrahydrothiazolo[5,4-c]pyridine-2-amine (TR-01) and its derivatives (TR-02–4) used in this study.

binding (docking score = −5.924 Kcal/mol; glide score = −6.398 Kcal/mol), is shown in Figure 1. The –NH2 group of TR-04 forms hydrogen bonding with THR-124 while tetrahydrothiazolopyridine ring occupies the hydrophobic pocket containing ILE-121, THR-183, ALA-223, ILE-218, LEU-217, VAL-227, ALA-226, LEU-126, and VAL-180. The docking results demonstrated possible broad and tight interactions between TR-04 and amino acid residues of 4K1L binding pocket and this may result in potent activity of TR-04. The docking studies provided insights into further modification of TR-04 to develop analogues with more potent activity. Based on these observations, we synthesized N-unsubstituted and N-substituted tetrahydrothiazolopyridine derivatives TR-01–4 (Figure 2). The substitution is based on the requirement of secondary amine (NH) group and/or primary amine (NH2) group for preserving biological activity as well as pharmacokinetic stability of these analogues. The detailed synthetic routes to each compound and their characterization are summarized in Supplementary Material, available online at https://doi.org/10.1155/2017/3182129.

3.2. 11β-HSD1 Activity in 3T3-L1 Adipocytes. Undifferentiated confluent 3T3-L1 cells showed a small amount of the 11β-HSD1 oxoreductase activity (8% of cortisone to cortisol conversion in 2 h), while the 11β-HSD1 dehydrogenase activity (i.e., cortisol to cortisone conversion) was negligible in preadipocytes. As adipocyte differentiation progressed from day 0 to day 4, we observed a steady increase in 11β-HSD1 activity. Near-complete adipogenic differentiation (day 8) was associated with a significant increase in the oxoreductase activity with up to 48% cortisone to cortisol conversion after mature adipocytes were incubated with 1 μM of steroid for 2 h (Figure 3, black bars). The 11β-HSD1 dehydrogenase activity remained relatively low throughout the differentiation process and reached maximum 6% cortisol to cortisone oxidation on day 8 following the induction of adipogenesis (Figure 3, open bars). As 11β-HSD1 dehydrogenase activity was minimal in these cells, the next set of experiments focused on 11β-HSD1 oxoreductase activity as the main measure of thiazolopyridine-induced inhibition of 11β-HSD1.

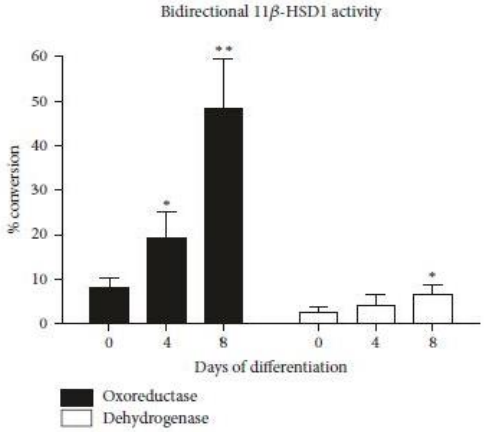

FIGURE 3: Bidirectional oxoreductase (conversion of cortisone to active cortisol, black bars) and dehydrogenase (conversion of cortisol to inactive cortisone, open bars) activity of the 11β-HSD1 enzyme in 3T3-L1 adipocytes. Cells were grown to confluence and differentiated into adipocytes by day 8 of the treatment. Cells were incubated in DMEM containing cortisone or cortisol (1 μM) for 20 min and the respective metabolites/conversion ratios were measured by LC-MS. Data are the mean ± SEM ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle by Dunnett's test subsequent to one-way ANOVA.

3.3. Tetrahydrothiazolopyridine Derivatives Inhibit 11β-HSD1. 11β-HSD1 oxoreductase assays in mature adipocyte cells supplemented with 1 μM cortisone for 2 h showed significant reduction of the oxoreductase activity following incubation with 0.1–100 μM of TR-01–4. Parent pharmacophore TR-01 showed moderate efficacy at inhibiting 11β-HSD1 oxoreductase in a dose-dependent manner (Figure 4(a)). Addition of benzyl group at 5th position and 4-chloro-α-cinnamoyl moiety at 2nd position in TR-02 increased 11β-HSD1 potency as compared to the original molecule TR-01. Unsubstituted TR-03, on the other hand, exhibited decreased inhibitory activity

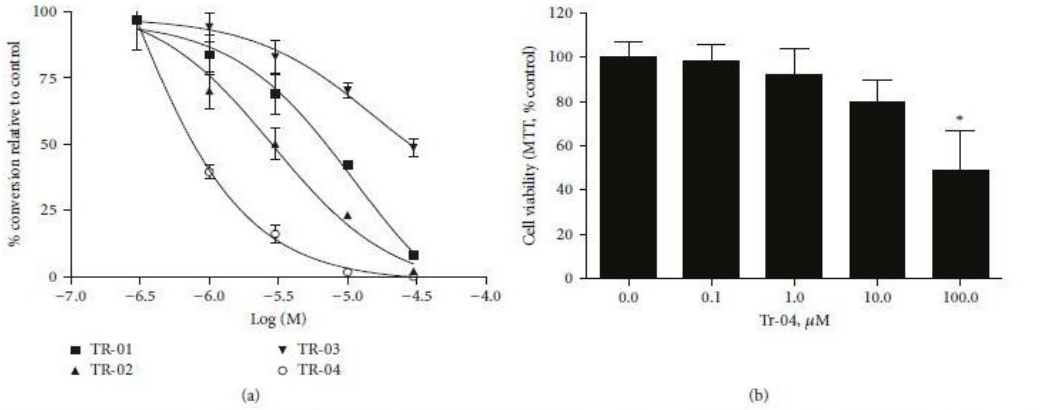

FIGURE 4: Tetrahydrothiazolopyridine derivatives TR-01–4 inhibited 11β-HSD1 oxoreductase activity in the mature 3T3-L1 adipocytes. Cells were treated for 2 h with 0–100 μM of the test compounds and incubated in DMEM containing cortisone (1 μM) for 20 min. The resulting metabolite/conversion ratios were measured by LC-MS. Data are the mean ± SEM ($n = 3$). $^*P < 0.05$ versus vehicle by Dunnett's test subsequent to one-way ANOVA.

for 11β-HSD1. In order to understand the disparity between TR-02 and TR-03, a methyl analogue TR-04 was synthesized. Treatment with TR-04 resulted in a total inhibition of 11β-HSD1 oxoreductase activity when tested at the concentrations of >10 μM with a corresponding IC_{50} value of 0.095 μM. No cytotoxicity was observed in MTT assay at concentrations lower than 10 μM for TR-04 (Figure 4(b)).

3.4. Adipogenesis in 3T3-L1 Cells Incubated with Tetrahydrothiazolopyridine Derivatives. 3T3-L1 cells differentiated for 8 days with 1 μM cortisone (instead of 0.25 μM dexamethasone used in the standard adipogenesis protocols) showed increased 11β-HSD1 reductase activity of 22.4 ± 9.4% cortisone to cortisol conversion, a 2-fold decrease over the standard differentiation protocol (Figure 5(a)). Similarly, cortisone-induced adipocytes expressed higher levels of 11β-HSD1 mRNA (11.1-fold versus control, $p < 0.01$) that also did not reach 11β-HSD1 mRNA levels observed after dexamethasone-stimulated differentiation (20.2-fold versus control, $p < 0.001$, Figure 5(b)). The change in adipogenesis and lipid accumulation was quantified through staining the cells with Oil red O after 8 days of differentiation. A marked increase in the number of red-stained cells was observed in samples differentiated with dexamethasone (39.0-fold versus control, $p < 0.001$) or cortisone (13.3-fold versus control, $p < 0.01$) (Figure 5(c)).

Coincubation with 0.1–10 μM compounds TR-01–4 abolished these effects to a various extent. 3T3-L1 adipocytes differentiated with cortisone and TR-04 at doses as low as 100 nM had significantly lower 11β-HSD1 mRNA levels (0.6-fold versus cortisone alone, $p < 0.05$, Figure 5(d)) and lipid content (54.8% of cortisone alone, $p < 0.05$, Figure 5(e)). At highest concentration tested, compound 4 nearly abolished 11β-HSD1 oxoreductase activity (Figure 5(d)) and suppressed adipogenesis and lipid accumulation in cortisone-stimulated adipocytes (Figure 5(d) and 5(e)). Size and degree of lipid droplets accumulation in the presence of the different concentrations of TR-04 (0–10 μM) indicated dose-dependent suppression of adipogenesis in response to treatment with thiazolopyridine derivatives (Figure 5(f)).

3.5. Gene Expression of Adipogenic and Insulin Sensitivity Markers. 3T3-L1 cells differentiated for 8 days in the presence of cortisone showed increased expression of markers of lipid metabolism (FAS, HSL, and LPL), insulin signaling (GLUT4), adipocyte function (Lep), and differentiation (PPAR-γ, aP2); however, the levels of adiponectin (AdiQ) cytokine that regulates energy metabolism and insulin sensitivity were drastically reduced. Coincubation of 3T3-L1 cells with 100 nM TR-04 normalized mRNA levels of biomarkers of lipid metabolism and adiponectin (1.6-fold versus 0.2-fold in cortisone-treated cells, $p < 0.01$) but had little effect on expression levels of insulin-inducible GLUT4 transporter (Figure 6). Reduced mRNA levels of HSL and LPL were also observed. This is in agreement with previous studies that showed that HSL knockout mice had lower weight gains and adiposity, likely due to compensatory decrease in the reesterification of fatty acids that results in decreased resynthesis of TAG and increased liberation of fatty acids to the vasculature [27]. LPL expression followed the same pattern, although its connection to adipogenesis was not clear and warrants further investigation.

4. Discussion

The present study demonstrated a potent inhibition of the 11β-HSD1 oxoreductase activity (conversion of inactive cortisone to active cortisol) by tetrahydrothiazolopyridine derivatives in the 3T3-L1 adipocyte cell culture. 11β-HSD1 acts as a tissue-specific regulator of cortisol exposure in obesity and

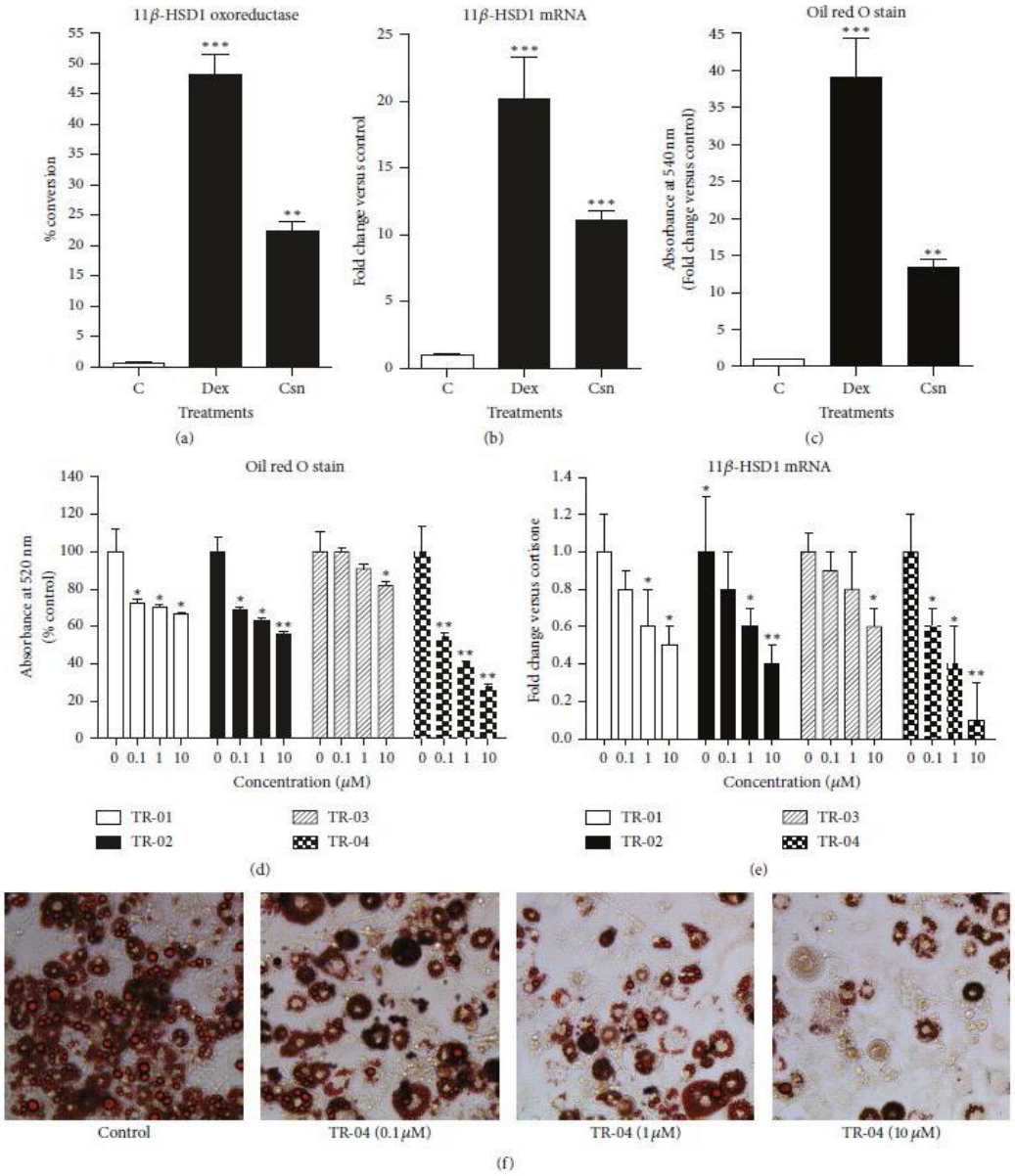

FIGURE 5: Tetrahydrothiazolopyridine derivatives TR-01–4 reduced adipogenesis in the 3T3-L1 adipocytes in a dose-dependent manner. Adipocyte differentiation in the presence of 1 μM cortisone (Csn) was half less efficient than standard 0.25 μM dexamethasone (Dex) induction as evident from induction of the (a) 11β-HSD1 activity, (b) 11β-HSD1 expression, or (c) lipid accumulation. Dosing of cortisone-treated adipocytes with 0–10 μM of TR-01–4 further decreased (d) lipid accumulation as quantified with Oil red O staining and (e) 11β-HSD1 mRNA levels as measured by qPCR. (f) Oil red O staining of adipocytes differentiated with different concentrations of TR-04 for 8 days. Data are the mean ± SEM ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus vehicle control by Dunnett's test subsequent to one-way ANOVA.

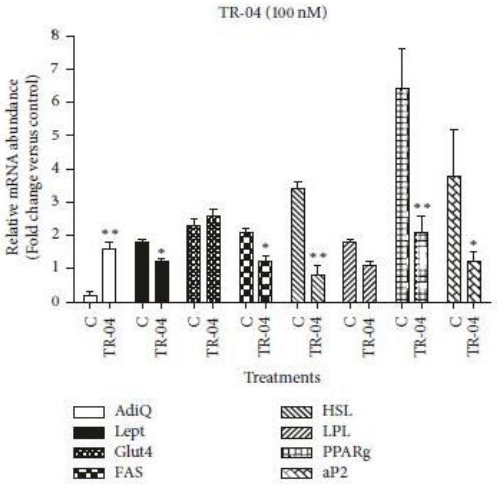

FIGURE 6: Effect of tetrahydrothiazolopyridine derivative TR-04 on the expression levels of adipocyte genes associated with lipid metabolism and insulin resistance. Adipocytes were differentiated in the presence of 1 μM cortisone and 100 nM of TR-04 and extracted with TRIzol for mRNA measurements by qPCR. Data are the mean ± SEM ($n = 3$), normalized with respect to the expression of the β-actin of undifferentiated control cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle control by two-tailed Student's t-test.

type 2 diabetes and presents an attractive therapeutic strategy to alleviate multiple complications in patients with metabolic syndrome and excess weight. As expected, undifferentiated 3T3-L1 preadipocytes showed low levels of both 11β-HSD1 oxoreductase and dehydrogenase activity due to bidirectional nature of the 11β-HSD1 enzyme. 11β-HSD1 is a lower-affinity NADP(H)-dependent enzyme that acts as oxoreductase that regenerates active glucocorticoids from their inactive keto-glucocorticoid precursors in key metabolic tissues [7]. The opposing dehydrogenase activity of 11β-HSD1 (conversion of cortisol to cortisone) becomes evident only in cell culture and tissue homogenates or upon purification due to decrease of ER luminal cofactor NADP(H) generated by hexose-6-phosphate dehydrogenase [8]. Mature 3T3-L1 adipocytes exhibited an expected switch predominantly to 11β-HSD1 oxoreductase activity [28] by day 8 of differentiation.

TR-02 and TR-04 inhibited 11β-HSD1 oxoreductase activity with IC_{50} values of 1.8 μM and 0.095 μM, respectively. Incubation of cells with 0.1 μM–10 μM significantly decreased cortisone-induced lipid accumulation in adipocytes and suppressed 11β-HSD1 mRNA expression in these cells. In rodents, an application of selective 11β-HSD1 inhibitor adamantyl triazole improved lipid metabolism and reduced body weight and the progression of atherosclerosis [21]. In humans, the situation is less clear as 11β-HSD1 overexpression was described only in subcutaneous, but not omental adipose tissue [22]. It is not clear if tetrahydrothiazolopyridine derivatives TR-02 and TR-04 used in this study reduced adipogenesis across all adipose tissue depots, or this effect was limited to the cutaneous lineages similar to the 3T3-L1 adipocytes used in this study. Further preclinical studies are needed to explore this regulation and its application to metabolic phenotypes. However, pharmacological activity of TR-04 in vitro compared favorably to salicylates that reduced 11β-HSD1 mRNA levels in fully differentiated human SGBS adipocytes in the dose range of 10–100 μM [17].

We also demonstrated that reduced adipogenesis data was consistent with changes in expression of key adipogenic genes associated with lipid metabolism and insulin resistance. Simultaneous increase in adiponectin and decrease in leptin expression suggested that treatment with TR-02 and TR-04 might be effective at improving regulation of insulin resistance and energy homeostasis in obese subjects [23]. At the same time, the observed reduction in the levels of FAS, HSL, and LPL in tetrahydrothiazolopyridine-treated adipocytes indicated reduced deposition of fatty acids in adipose cells. This finding was in a total agreement with Oil red O staining that quantifies the presence of fat or lipids in adipocytes.

5. Conclusions

Taken together, our findings suggested that tetrahydrothiazolopyridine derivatives TR-02 and TR-04 are the strong 11β-HSD1 inhibitors and attractive preclinical candidates for the treatment of obesity and type 2 diabetes mellitus.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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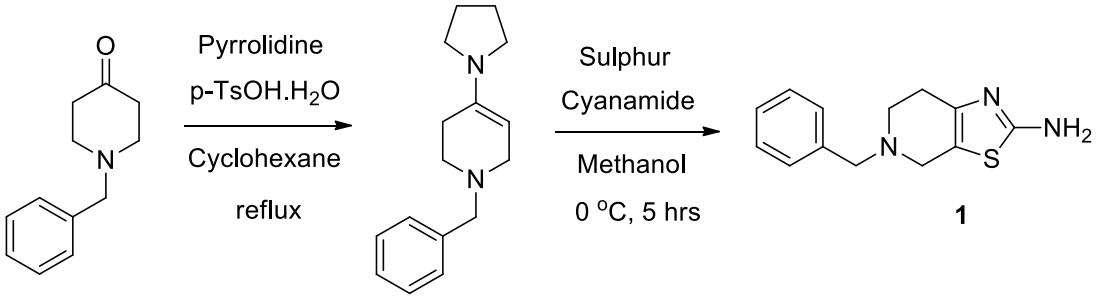
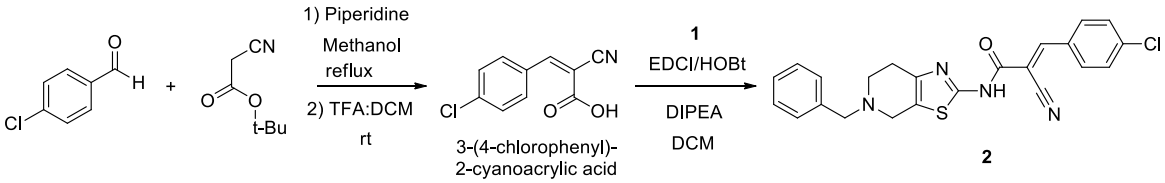
7. Supporting Information

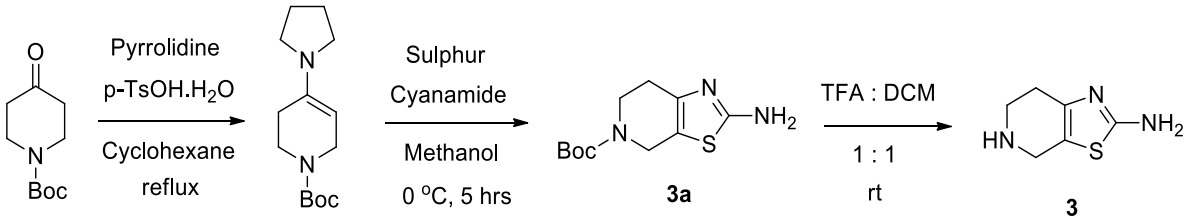
1) Schematic synthesis of 2-amino-5-benzyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine (TR-01):



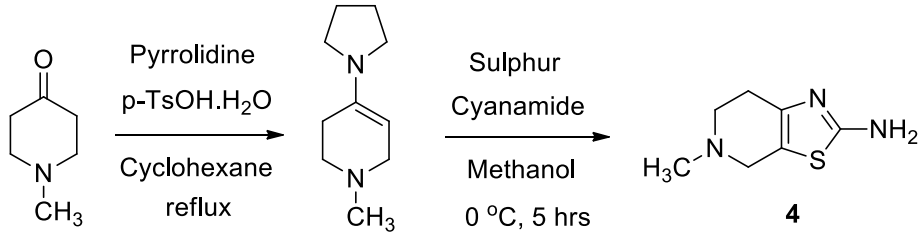
2) Schematic synthesis of N-(5-benzyl)-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine-2-yl)-3-(4-chlorophenyl)-2-cyanoacrylamide (TR-02):



3) Schematic synthesis of 2-amino-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine (TR-03):



4) **Schematic synthesis of 2-amino-5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine (TR-04)**:



4

APPENDIX 2: OTHER PUBLISHED WORK

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Hepatoprotective Activity of Easter Lily (*Lilium longiflorum* Thunb.) Bulb Extracts

Wenping Tang,[†] John P. Munafo, Jr.,[‡] Kimberly Palatini,[∥,⊥] Debora Esposito,[∥,⊥] Mou-Tuan Huang,[§] Slavko Komarnytsky,[∥,⊥] Chi-Tang Ho,[†] and Thomas J. Gianfagna*[,‡]

[†]Department of Food Science, Rutgers-The State University of New Jersey, 65 Dudley Road, New Brunswick, New Jersey 08901, United States

[‡]Department of Plant Biology, Rutgers-The State University of New Jersey, 59 Dudley Road, New Brunswick, New Jersey 08901, United States

[§]Department of Chemical Biology, Rutgers-The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, New Jersey 08854, United States

[∥]Plants for Human Health Institute, North Carolina State University, North Carolina Research Campus, 600 Laureate Way, Kannapolis, North Carolina 28081, United States

[⊥]Department of Food, Bioprocessing & Nutrition Sciences, North Carolina State University, 400 Dan Allen Drive, Raleigh, North Carolina 27695, United States

ABSTRACT: The hepatoprotective activities of two different extracts, a hydroethanolic crude bulb extract (CB) and a steroidal glycoside-rich 1-butanol extract (BuOH), prepared from the bulbs of Easter lily (*Lilium longiflorum* Thunb.), were evaluated in a 24 week study in the female KK.Cg-A^y/J Type 2 diabetic mouse model. Animals were divided into six groups (n = 16): control mice received Easter lily bulb extract-free drinking water together with a low- or high-fat diet (diabetic control); drinking water for the remaining groups was supplemented with CB extract (1%), BuOH extract (0.1 or 0.2%), and reference drug Metformin (0.001%), together with a high-fat diet. Both CB and BuOH extract treatment groups exhibited significantly improved liver function based on comparisons of triglycerides [diabetic 219 ± 34 mg/dL, CB 131 ± 27 mg/dL, BuOH(0.2%) 114 ± 35 mg/dL], CB total cholesterol (TC) (diabetic 196 ± 12 mg/dL, CB 159 ± 5 mg/dL), average liver mass [diabetic 2.96 ± 0.13 g, CB 2.58 ± 0.08 g, BuOH(0.1%) 2.48 ± 0.13 g], alanine transferase [diabetic 74 ± 5 units/L, CB 25 ± 1 units/L, BuOH(0.1%) 45 ± 1 units/L], and histological examinations. Glucose metabolism was improved only in CB, which was confirmed by oral glucose tolerance tests (OGTT) in diet-induced obese C57BL/6J mice exposed to CB extract. These data suggest that steroidal glycosides **1**−**5** might play a role in the hepatoprotective activity of the BuOH extracts, while the results of the TC measurements and OGTT study indicate that other constituents present in the CB extract are responsible for its hypocholesterolemic and hypoglycemic activity.

KEYWORDS: *Lilium longiflorum Thunb., Easter lily, steroidal glycoside, hepatoprotective activity, hypoglycemic activity*

INTRODUCTION

Especially in the United States, clinical data on the negative health impacts of diabetes are considerable and continue to accumulate. The number of Americans with diabetes has increased by approximately 15% in the past 2 years, and the number worldwide is predicted to increase from 183 to 366 million by 2030.[1] Two forms of diabetes mellitus (DM), Type 1 and Type 2, are well-known.[2,3] Type 2 diabetes ranges from predominantly insulin resistant with relative insulin deficiency to predominantly insulin secretory defective with insulin resistance. More than 90% of all diabetes diagnoses worldwide are Type 2.

It is well established that a degree of hyperglycemia sufficient to cause pathological and functional changes in target tissues, but without indicating clinical symptoms, may be present prior to a clinical diagnosis of diabetes.[4] As a consequence, individuals with Type 2 diabetes commonly have a higher incidence of liver function abnormalities. One prevalent liver function abnormality is characterized by elevated levels of alanine transferase (ALT), which catalyzes the transfer of an amino group from L-alanine to α-ketoglutarate. Accordingly, its measurement is employed as a biomarker for the evaluation of hepatocellular injury because elevated ALT levels are indicative of liver-specific damage common in diabetes.[5] Anti-diabetic agents have been shown to decrease ALT levels as normal blood glucose levels are achieved.[6] Changes in blood lipid profiles, including triglyceride (TG), total cholesterol (TC), and high-density lipoprotein (HDL) levels, are also widespread in diabetics and may occur as a complication of impaired liver function.[7] Also, an abnormality in carbohydrate metabolism can be observed during this asymptomatic period, based on the quantification of plasma glucose levels in the fasting state or after a challenge with an oral glucose load.[8]

Diet, representing one of the most important lifestyle factors, can significantly influence the incidence of diabetes. To date, a

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RESEARCH ARTICLE

Structural constraints and importance of caffeic acid moiety for anti-hyperglycemic effects of caffeoylquinic acids from chicory

Kimberly M. Palatini Jackson[1,2], Thirumurugan Rathinasabapathy[1,3], Debora Esposito[1,4] and Slavko Komarnytsky[1,2]

[1] Plants for Human Health Institute, North Carolina State University, Kannapolis, NC, USA
[2] Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Raleigh, NC, USA
[3] Department of Pharmaceutical Chemistry, School of Pharmacy, International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia
[4] Department of Animal Science, North Carolina State University, Raleigh, NC, USA

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Scope: Chicory (*Cichorium intybus* L.) is a perennial herb often consumed as a vegetable, whereas the ground and roasted roots are blended as a coffee substitute. Caffeoylquinic or chlorogenic acids (CQA), the abundant intermediates of lignin biosynthesis in chicory, have been reported to improve glucose metabolism in humans, but the functional group in their structure responsible for this effect has not been yet characterized.

Methods and results: Here, we showed that three di-O-caffeoylquinic acids suppressed hepatic glucose production in H4IIE rat hepatoma cells by reducing expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), two key enzymes that regulate hepatic gluconeogenesis. Direct comparisons between CQAs and their metabolites (3-caffeoylquinic, caffeic, and quinic acids) revealed the caffeic acid moiety alone was responsible for the observed effects. Further analysis suggested the activation of PI3K and MAPK pathways as a method of controlling gene expression was shared between caffeoylquinic and caffeic acids. These compounds promoted increased mitochondrial respiration and cellular metabolism, in part by inducing oxidative phosphorylation and proton leak.

Conclusion: We concluded that the caffeic acid moiety was important for suppression of hepatic gluconeogenesis and hyperglycemia, ultimately strengthening the link between dietary interventions based on caffeic acid-containing plant foods and healthy glucose metabolism.

Keywords:
Blood glucose / Cell bioenergetics / Diabetes / Dietary supplementation / Metabolic syndrome

1 Introduction

Metabolic syndrome precedes type II diabetes in many patients and is associated with a group of risk factors that include obesity, hypertension, insulin resistance, and inflammation [1–3]. Organ-specific consequences to altered energy homeostasis include reduced skeletal muscle glucose uptake and increased hepatic gluconeogenesis [4]. Additionally, increased abdominal fat mass yields high circulating free fatty acids (FFA), which drive hepatic FFA uptake and oxidation [5]. This further stimulates gluconeogenesis in the liver, thus increasing total hepatic glucose output when glycogen stores are depleted or the regulation of glycogenolysis is abrogated [6].

Two key enzymes that control gluconeogenesis, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), are transcriptionally regulated by hormones such as insulin, glucagon, incretins, and glucocorticoids via a

Correspondence: Dr. Slavko Komarnytsky
E-mail: komarnytsky@ncsu.edu

Abbreviations: **CQA**, caffeoylquinic acids; **G6Pase**, glucose-6-phosphatase; **PEPCK**, phosphoenolpyruvate carboxykinase; **PI3K**, phosphoinositide 3-kinase; **MAPK**, mitogen-activated protein kinase; **AMPK**, 5′ AMP-activated protein kinase; **FFA**, free fatty acids; **Dex-cAMP**, dexamethasone and cyclic AMP; **OCR**, oxygen consumption rate; **ECAR**, extracellular acidification rate

Colour Online: See the article online to view Figs. 5 and 6 in colour.

APPENDIX 3: ORIGIONAL FIGURES

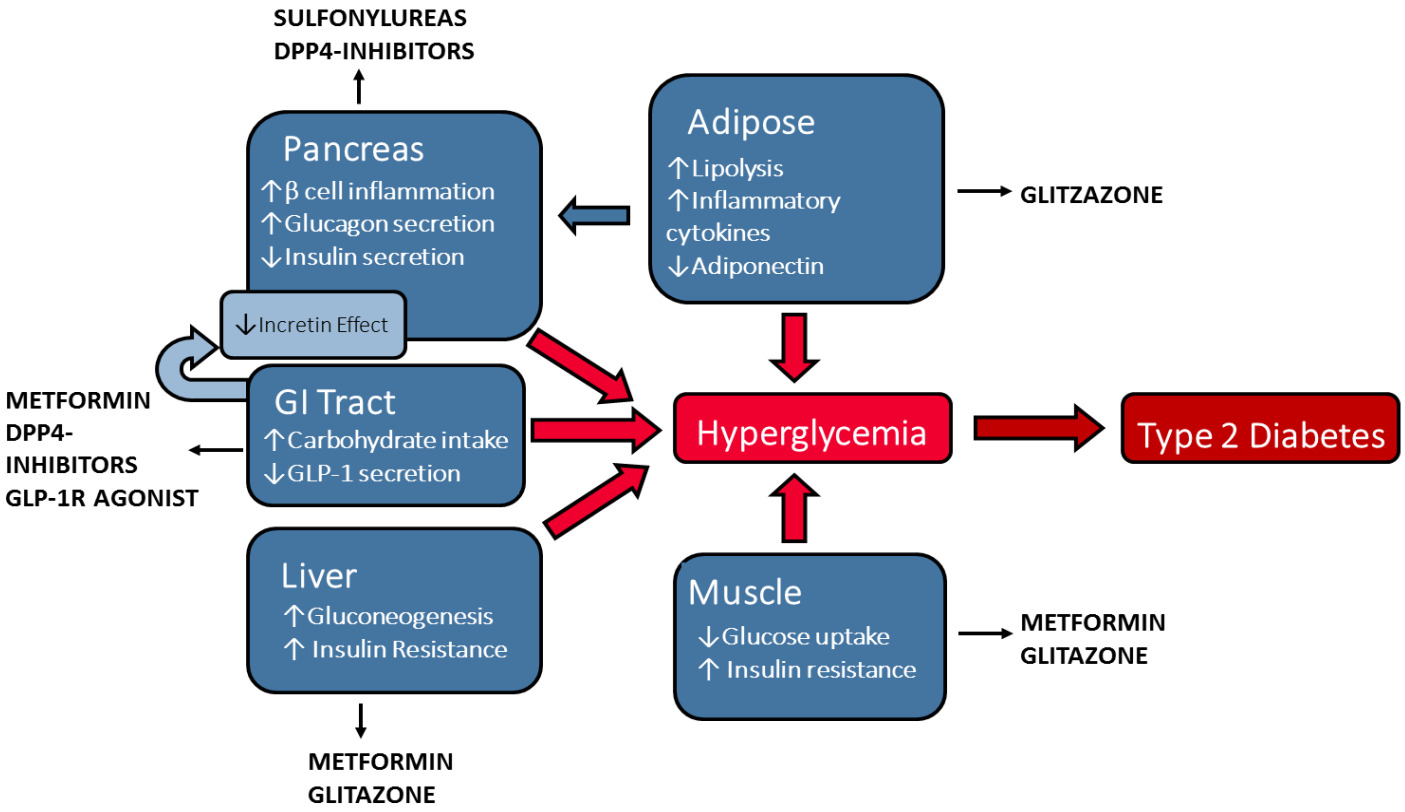

Figure 1. Type 2 Diabetes Pathways and Drug Options.

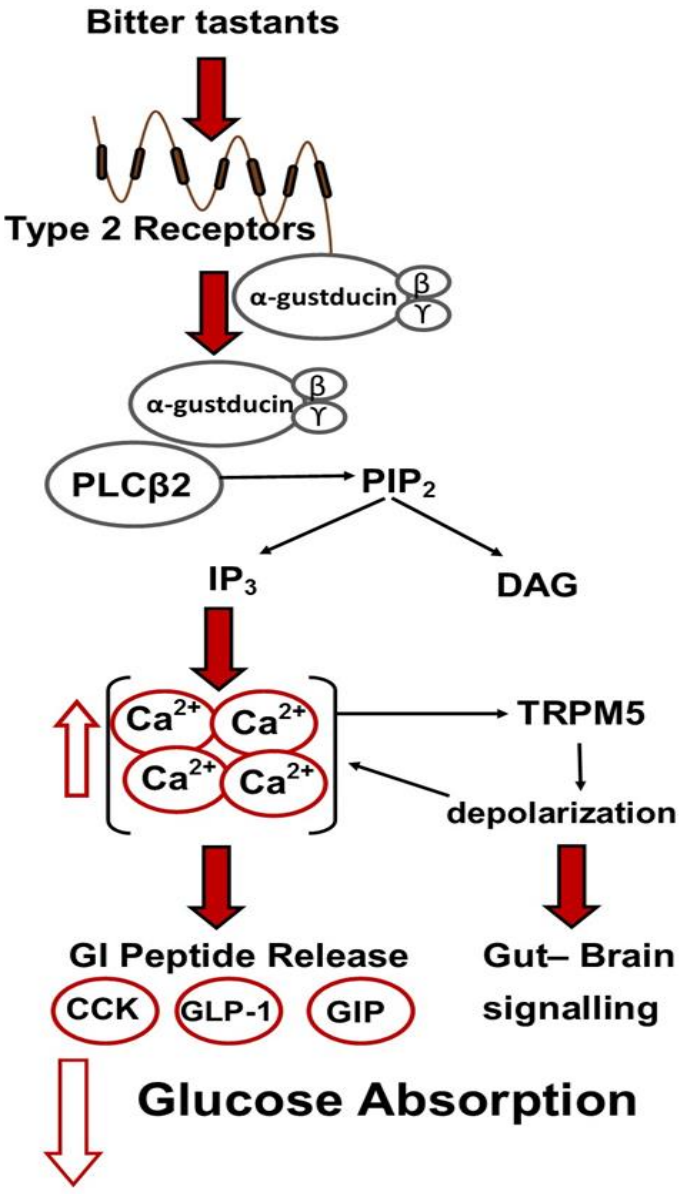

Figure 2. Type 2 Receptor Signalling Cascade.