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Discovery of Quinic Acid Derivatives as Oral Anti-inflammatory Agents

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Abstract

Quinic acid (QA) esters found in hot water extracts of *Uncaria tomentosa* (a.k.a. Cat's claw) exert anti-inflammatory activity through mechanisms involving inhibition of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB). Herein, we described the synthesis and biological testing of novel QA derivatives. Inhibition of NF-κB was assessed using A549 (Type II alveolar epithelial-like) cells that stably express a secreted alkaline phosphatase (SEAP) reporter driven by an NF-κB response element. A549- NF-κB cells were stimulated with TNF-α (10 ng/mL) in the presence or absence of QA derivative for 18 hours followed by measurement of SEAP activity. Amide substitution at the carboxylic acid position yielded potent inhibitors of NF-κB. A variety of modifications to the amide substitution were tolerated with the N-propyl amide derivative **3** being the most potent. Compound **3** was named as KZ-41. The NF-kB inhibitory potency (IC_{50}) of our most active analog KZ-41 was determined to be 2.83±1.76 mM. Further examination of the structure and activity relationship (SAR) demonstrated that acetylation of the hydroxyl groups reduced NF-κB inhibitory activity. QA amide derivatives lacked anti-oxidant activity and were found to be neither anti-proliferative nor cytotoxic at concentrations up to 100 mM.

The dehydroxyl QA amides **18** and **20** were synthesized to retain anti-inflammatory activity while having enhanced resistance to microbial degradation. They showed NF-kB inhibition at concentration 1 mM. The extent of NF-kB inhibition was close to positive control drug dexamethasone. The dehydroxyl QA amide with a double bond **38** was also synthesized. The QA amide esters **24**, **25**, **29** and **33** were designed and synthesized to retain anti-inflammatory activity with additional antioxidant properties. As expected, compound **25** showed strong anti-oxidant activity. Furthermore, they were hydrolyzed by the microflora, for an example, **25** was hydrolyzed into sinapic acid (SA) and **3**. Both SA and **3** will not be consumed by gut bacteria, and are easily absorbed in animal digestive tract. This was done by other researchers, and not reported here.

To facilitate pre-clinical biopharmaceutic and pharmacokinetic (B/PK) studies of our lead QA amide analog KZ-41, we developed and validated a novel hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC-MS/MS) analytical assay. An analog of KZ-41 was used as internal standard (IS). KZ-41 and the IS were obtained by protein precipitation and separated by HILIC chromatography using acetonitrile and water. A triple quadrupole mass spectrometer operating in the negative electrospray ionization mode with multiple reaction monitoring was used to detect KZ-41 and IS transitions of m/z 232 → 178 and 272 → 218 respectively. The lower limit of quantification (LLOQ) was 0.5 ng/mL in plasma. The method was validated for selectivity, linearity, accuracy and precision in rat plasma. The ion suppression, recovery and stability of the analyte in the biological matrix were also tested. The assay developed

is rapid, sensitive and robust enough to support preclinical B/PK studies of KZ-41.

The study was also conducted to characterize the biopharmaceutics and pre-clinical pharmacokinetics of the lead QA amide analog, KZ-41. Rats (n=6/group) received a dose of either an i.v. or p.o. dose (10 mg/kg) of KZ-41. Pharmacokinetic parameters were determined from concentration - time profiles by non-compartmental analysis. Bacterial stability study was conducted in cultured bacterial *gluconobacter oxydans*. Plasma protein binding and metabolic stability were determined using equilibrium dialysis and rat liver microsomes respectively. Following i.v. administration, KZ-41 demonstrated a medium clearance (15.1±4.8 mL/min/kg), medium volume of distribution (3.3±1.1 L/kg), and a terminal half-life of 2.6±0.4 hrs. KZ-41 was rapidly absorbed with complete oral bioavailability (F≈1), which was consistent with the fact that KZ-41 was not susceptible to degradation in bacterial and liver microsomal studies. KZ-41 binding to plasma proteins was about 30%. These studies demonstrate that KZ-41 is a potential new

orally active anti-inflammatory agent.

In summary, we have discovered a novel series of non-toxic QA amides that potently inhibit NF-κB. The NF-kB inhibitory potency (IC_{50}) of our most active analog KZ-41 was determined as 2.83±1.76 mM. It was rapidly absorbed with complete oral bioavailability (F≈1). It could be a potential new orally active anti-inflammatory agent. Mechanistic studies and pre-clinical efficacy studies of these newly designed compounds in various in vitro and in vivo models are on-going by other researchers, and are not reported in this dissertation.

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DISCOVERY OF QUINIC ACID DERIVATIVES AS ORAL ANTI-INFLAMMATORY AGENTS

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Presented for
The Graduate Studies Council
The University of Tennessee
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In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Kui Zeng
May 2010

DEDICATION

To Tao, my wife; and my daughters.

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ACN	acetonitrile
AUC	area under the plasma concentration-time curve
CA	caffeic acid
CGA	chlorogenic acid
CL	plasma clearance
C_{max}	maximum plasma concentration
CV	coefficient of variation
DMSO	dimethyl sulfoxide
DPPH	diphenyl-1-picrylhydrazyl
DQD	3-dehydroquinate dehydratase
ESI	electrospray ionization
F	bioavailability
FDA	food and drug administration
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HTS	High Throughput Screening
IC_{50}	50 percent inhibition concentration
i.p.	intra-peritoneally
i.v.	intravenous
LC-MS	liquid chromatography-mass spectrometry
LLOQ	lower limit of quantitation
MeOH	methanol
mL	mil liter
MRM	multiple reaction monitor
MS/MS	tandem mass spectrometry
MW	molecular weight
NF-κB	nuclear factor kappa B
PMA	phorbol myristate acetate
PTSA	p-toluenesulfonic acid
QA	quinic acid
QDH	quinate dehydrogenase
SA	sinapic acid
S-ADME	structure – adsorption, distribution, metabolism, elimination
SAR	structure activity relationship
SBDD	structure based drug discovery
SEAP	secreted alkaline phosphatase
TLC	thin layer chromatography
TNF-α	tumor necrosis factor alpha
Vz	volume of distribution based on terminal phase

CHAPTER 1. INTRODUCTION

The interest in development of natural products or modified natural products having anti-inflammatory activity has exploded in recent years. Recently, quinic acid (QA) and QA esters have been identified as active anti-inflammatory ingredients in hot water extracts of the herbal *Uncaria tomentosa* or Cat's claw (e.g., C-MED-100®),[1,2] and shown to enhance immune cell response and DNA repair in humans.[3-5] This chapter will give an overview on the most recent studies on QA and its derivatives in the following fields, such as: biosynthesis and catabolism in nature, synthesis, biological activities, mechanisms of anti-inflammatory activity, etc.

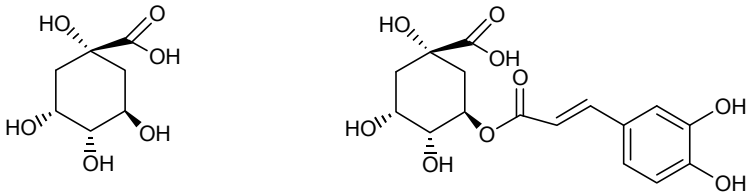
1.1 QA Structure and Its Derivatives

QA, 1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid (Figure 1-1), was first reported in 1790.[6] The structure and the stereochemistry of QA were assigned in 1932 by H. O. L. Fisher and G. Dangschat.[7] QA is a natural compound found widely in plants,[8-10] such as in cinchona bark, particularly in South American barks; also largely in apple, peach, and rose tissue.[11,12] Derivatives of QA, such as chlorogenic acid (CGA) (Figure 1-1), p-coumaryl quinate, etc., are also common in plants.[13-15]

1.2 QA Biosynthesis and Catabolism

1.2.1 The QA Biosynthesis

In nature, QA could be synthesized in one step from dehydroquinate by quinate dehydrogenase or from shikimate by quinate hydrolyase (Figure 1-2).[16] Although the accumulation of QA appears to be restricted to specific plants, the occurrence of chlorogenate and its derivatives is more widespread. In higher plants, QA is the precursor for chlorogenate and may also serve as a storage or transport form of carbon.[17] Chlorogenate is the major soluble phenylpropanoid in tobacco and a preformed protectant against fungal attack.[18] It contributes to general plant health as part of a physical and



Quinic acid Chlorogenic acid

Figure 1-1. Chemical structure of quinic acid and chlorogenic acid.

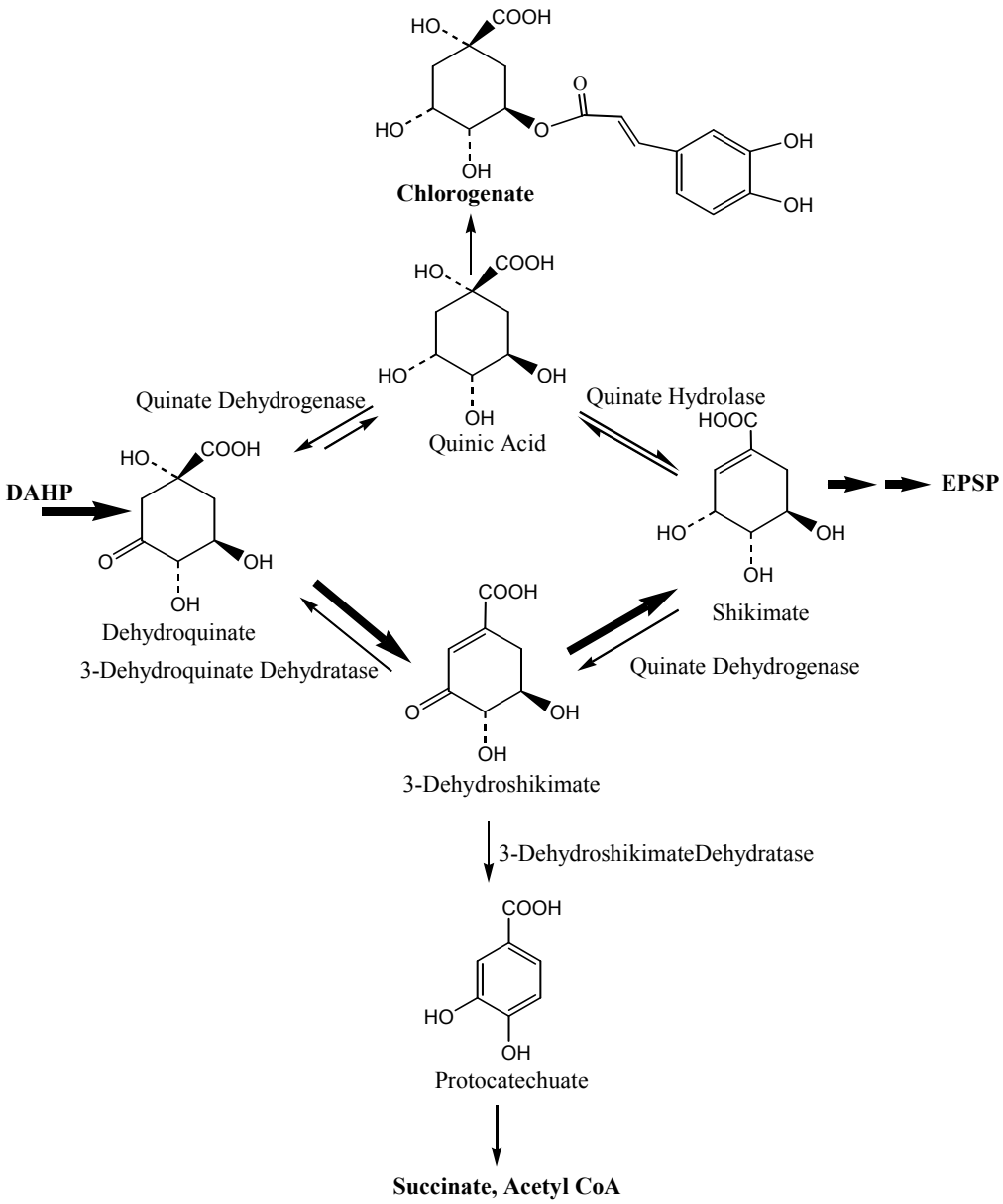


Figure 1-2. QA biosynthesis and biodegradation cycle. The cycle includes three intermediates of the shikimate pathway. DAHP: 3-deoxy-o-arabino-heptulosonate 7-phosphate. EPSP: 5-enolpyruvylshikimate 3-phosphate. The enzymes of the QA cycle are (1) quinate (shikimate) dehydrogenase (QDH); (2) 3-dehydroquinate dehydratase (DQD); and (3) quinate hydrolyase. 3-Dehydroshikimate is aromatized to protocatechuate by Sdehydroshikimate dehydratase (4). Bold arrows indicate reactions of the main trunk of the shikimate pathway.

possibly also a chemical barrier against microbial attack. Once this barrier is broken, the constituents become potential carbon sources for the attacker.

1.2.2 The Shikimate Pathway

The QA biosynthesis and biodegradation is through the shikimate pathway. In Figure 1-2, the bold arrows indicate reactions of the main trunk of the shikimate pathway. There are seven enzymes that catalyze the sequential steps of the shikimate pathway. The first reaction is catalyzed by 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAHP), phosphoenol pyruvate and erythrose-4-phosphate are reacted to form 3-deoxy-darabino-heptulosonate 7-phosphate. The second to last reaction is catalyzed by 5-enolpyruvylshikimate 3-phosphate synthase (EPSP), final to chorismate. The chorismate, the final product of the main trunk of the shikimate pathway, as a substrate for a number of anabolic sequences to primary and secondary compounds. These compounds include the aromatic amino acids phenylalanine, tyrosine, and tryptophan.[19,20] The shikimate pathway has been found only in microorganisms and plants. Bacteria spend >90% of their total metabolic energy on protein biosynthesis. Consequently, the bacterial shikimate pathway serves almost exclusively to synthesize the aromatic amino acids.[21,22] Under normal growth conditions, 20% of the carbon fixed by plants flows through the shikimate pathway.[23]

1.2.3 The QA Degradation

QA has three biotransformation pathways (Figure 1-2).[16] One is the biosynthesis of chlorogenate in plants. The other two pathways are in both microbe and plants. QA, along with shikimate, is readily degraded by fungi and some bacteria, first to protocatechuate and ultimately to succinate and acetyl-C_OA. QA can also be degraded to shikimate, then follow the shikimate pathway to EPSP, and chorismate, and finally to aromatic amino acids phenylalanine, tyrosine, and tryptophan.[19,20]

1.3 QA Degradation by Microorganisms

One of the remarkable features of QA and shikimate pathway is the very different patterns of enzyme organization found in different species.[24] In 1967, a QA oxidizing enzyme produced by acetic acid bacteria was discovered demonstrating that bacteria could catabolize QA.[25] Subsequently, the QA catabolic pathway was described as a result of characterization of fungal mutants with lesions in genes encoding biosynthetic shikimate pathway enzymes.[26] QA, along with shikimate, are degraded to form protocatechuate. The first three steps of QA catabolism are an NAD-dependent oxidation to form dehydroquinate, followed by two successive dehydrations to form dehydroshikimate and protocatechuate. The enzymes responsible for these conversions are quinate dehydrogenase (QDH), 3-dehydroquinate dehydratase (DQD), and 3-dehydroshikimate dehydratase. Both QDH and DQD are located in the cytoplasmic

membrane of bacteria. The QDH can also convert shikimic acid to dehydroshikimate. Bacteria and fungi utilize QA as a growth substrate and thus express an abundance of the aforementioned enzymes involved in QA degradation. High levels of QA catabolizing enzymes in gut bacteria is consistent with the finding that less than 10% of an orally administered dose QA is recovered in rats.[27]

One of the significant enzymes of QA degradation is QDH. QDH has been purified and characterized, and been extensively studied in fields such as catabolism and antibacterials.[28,29] Adachi and coworkers reported QDH screening through several acetic acid bacteria and other bacteria.[28,30] Strong enzyme activity was found in the membrane fraction of *Gluconobacter melanogenus* IFO 329, *Gluconobacter oxydans* IFO 3292, *Gluconobacter oxydans* IFO 3244, and some strains of *Acinetobacter calcoaceticus*. The following section describes the experimental procedure to prepare QDH enzyme from *Gluconobacter oxydans*.

Experiments involving *G. oxydans* was performed as described previously.[31] *G. oxydans* (ATCC, Manassas, VA) was cultured in basal media consisting of glycerol, 3 grams of yeast extract, and 1 gram of polypepton in 1 liter of tap water. In order to induce quinate dehydrogenase (QDH), quinate (2 g) was added and pH adjusted to 7.0. Microorganisms was grown in medium (100 mL) at 30°C. For cell-mass production, a seed culture in 100 mL of medium was carried out overnight and transferred to 5 liters of fresh medium in a table top shaker incubator and cultivated for 12 hr under aeration. Harvested cells were suspended in medium (5 mM potassium phosphate and pH 6.5) and centrifuged to pellet cells. Cells were stored without freezing.

Cell suspensions were prepared by homogenizing harvested cells at a ratio of 10 g of wet cells per 10 mL of 5 mM potassium phosphate, pH 6.5. Cell suspsensions were passed twice through a French pressure cell press. Intact cells were removed by low speed centrifugation and the cell-free extract will be further centrifuged (68,000 x g for 90 min) to separate the membrane and cytoplasmic soluble fraction. The membrane fraction was washed by homogenizing the precipitate in buffer and centrifugation repeated.

1.4 Biological Activities of QA and Its Derivatives

Uncaria tomentosa commonly known as Una de Gato or Cat's claw has been widely used historically as a natural remedy, and it is currently present in a number of nutritional formulations to treat a large variety of health disorders.[32] Aqueous extracts from the bark or root bark of Cat's claw are used therapeutically in a diversity of diseases including allergy, arthritis, chemotherapy side effects, cancer, bacterial/fungal infections, gastrointestinal inflammation and gastric ulcers.[33-37] The best known commercial preparations of Cat's claw are C-Med-100® and Activar AC-11®.[38-40] The active ingredients of C-MED-100® and Activar AC-11® have been attributed to QA, and QA carboxy alkyl esters, such as CGA.[1] Most recent studies have shown that CGA and QA have anti-hepatitis B virus activity,[41] anti-obesity property,[42] radio-protective effects[43]

and beneficial effects on cardiovascular diseases via suppressing P-selectin expression on platelets.[44] In particular, such extracts have been found to have various anti-inflammatory effects such as inhibition of the production of the inflammatory cytokine TNFα, scavenges free radicals[45] and the activation of the central transcription factor nuclear factor κB (NF-κB).[46-49] This factor regulates the expression of proinflammatory cytokines.[50] In the following two section, we will discuss the anti-inflammatory and anti-oxidation properties of QA and its derivatives, and mechanism of action.

1.4.1 The Anti-inflammatory Activity of QA and CGA through NF-κB Pathway

NF-κB is made up of homodimeric and heterodimeric combinations of subunits which, prior to activation, are located in the cytoplasm bound to an inhibitor, I-κB. The biological system in which NF-κB plays the most important role is the immune system. There are many reviews available for this subject, such as Ghosh et al. in 2004 and 1998,[51,52] Li and Verma in 2002,[53] Bonizzi and Karin in 2004.[54] Careful regulation of the transcriptional responses to many different stimuli is crucial to the proper functioning of the mammalian immune system. The genes regulated by NF-κB include those controlling programmed cell death (apoptosis), cell survival, cell adhesion, proliferation, the innate- and adaptive-immune responses, inflammation, the cellular-stress response and tissue remodeling.[51,55-59] Although the maintenance of appropriate levels of NF-κB activity is a critical factor in achieving normal cellular function, there are many potential applications of inhibition/interference of the NF-κB signal pathway in the treatment of inflammatory disease and cancer.[60-63] Calzado[63] has discussed NF-κB inhibitors used for the treatment of inflammation and cancer. Great efforts have been made for the development of highly specific NF-κB inhibitors, some of them being currently tested in phase II clinical trials.

Chlorogenic acid (3-caffeoyl-D-quinic acid; CGA) is an ester formed between caffeic acid (CA) and QA. CGA and other polyphenolic compounds found in fruits act as potent antioxidants. For example, CGA and CA are both capable of scavenging $NO^{\bullet}$,[64] a pro-inflammatory oxygen radical produced via the L-arginine pathway by infiltrating leucocytes.[65] Interestingly, QA does not possess antioxidant activity suggesting alternative mechanisms underlie its anti-inflammatory activity.[64] One potential mechanism involves inhibition of NF-κB. Using Jurkat T cells transfected with an NF-κB-dependent reporter gene, it was demonstrated that QA was able to inhibit PMA and ionomycin-induced NF-κB activity at concentrations that were neither cytotoxic nor inhibited proliferation.[2] The ability of QA to prevent degradation of IκB-α was shown to be the mechanism by which it inhibited NF-κB activity. Uniquely, QA did not affect the amount of phosphorylated IκB-α. By comparison, the anti-oxidant pyrollidine dithiocarbamate inhibited the phosphorylation, breakdown, and re-synthesis of IκB-α. The authors did not further investigate the mechanism(s) involved in QA's anti-inflammatory potential. To our knowledge, proteasome inhibitors (e.g., bortezomib) are the only approved drugs which inhibit NF-κB without affecting phosphorylation of IκB-α. Bortezomib binds to the chymotryptic site of the 20S b-subunit, thereby blocking the proteasome. As a result IκB will not be degraded into small peptides. It remains bound to the survival protein NF-κB, thereby inhibiting NF-κB action. Inactive NF-κB will keep

the inflammatory gene from transcription and translation to pro-inflammatory molecules, thus inhibiting inflammation. See Figure 1-3 from Vink et al.[66]

Recent work has demonstrated an interaction of QA derivatives with the carbohydrate binding proteins (lectins) involved in key leukocyte-endothelial cell interactions.[67,68] Tetrasaccharide sialyl Lewis x (sLex) ligands found on leukocytes contain sialic acid, L-fucose, and galactose which are essential for calcium-mediated binding of sLex to the carbohydrate binding proteins E, P, and L-selectin. Tetrasaccharide sLex exhibits a poor pharmacokinetic profile and thus cannot be developed as a selectin inhibitor. A search for druggable sLex mimetics led to the substitution of QA for fucose since the hydroxyl groups of QA mimic hydroxyl groups of fucose which make key interactions with protein side chains that bind calcium.[67,68] Selective QA analogs were shown to decrease leukocyte rolling in response to LPS in an intravital microscopy mouse model and to decrease neutrophil influx in a murine thioglycollate-induced peritonitis model.[68] Others have shown that selective QA analogs interact weakly with both P- and E-selectin using HL-60 cells.[67]

1.4.2 The Antioxidant Effect of QA and CGA

Antioxidant plays an important role in the prevention of human diseases. The oxidative damages happen when the reactive oxygen species, e.g. superoxide ($O_2^{\bullet}$) and hydroxyl ($HO^{\bullet}$), attack the lipid in cell membranes, proteins and DNA to cause membrane injury and protein and DNA modification.[69] QA has been confirmed as antioxidant, and extends its mode of action to include a basic nutritional benefit due to the enhanced metabolism of both tryptophan and nicotinamide.[70] But QA was not showing its ability to trap free radicals by DPPH testing method.[71] CGA and CA derivatives possess radical scavenging activity. The antioxidant effects of CGA and its derivatives might be due to three aspects: i) anti lipid-peroxidation; ii) radical scavenging; and iii) antioxidation of low density lipoprotein.[72]

1.5 Bioavailability of QA and Its Derivatives

Although QA and its derivatives showed much good biological activity, including anti-inflammatory activity; unfortunately, QA is utilized by gastrointestinal bacteria as a carbon source for aromatic acid synthesis. Consequently, only a small fraction of QA is absorbed after oral administration of QA. High levels of QA catabolizing enzymes in gut bacteria is consistent with the finding that less than 10% of an orally administered dose QA is recovered in rats.[27] Our goal was to discover and develop stable QA derivatives as orally drugs, and to study the bioavailability of QA and its derivatives.

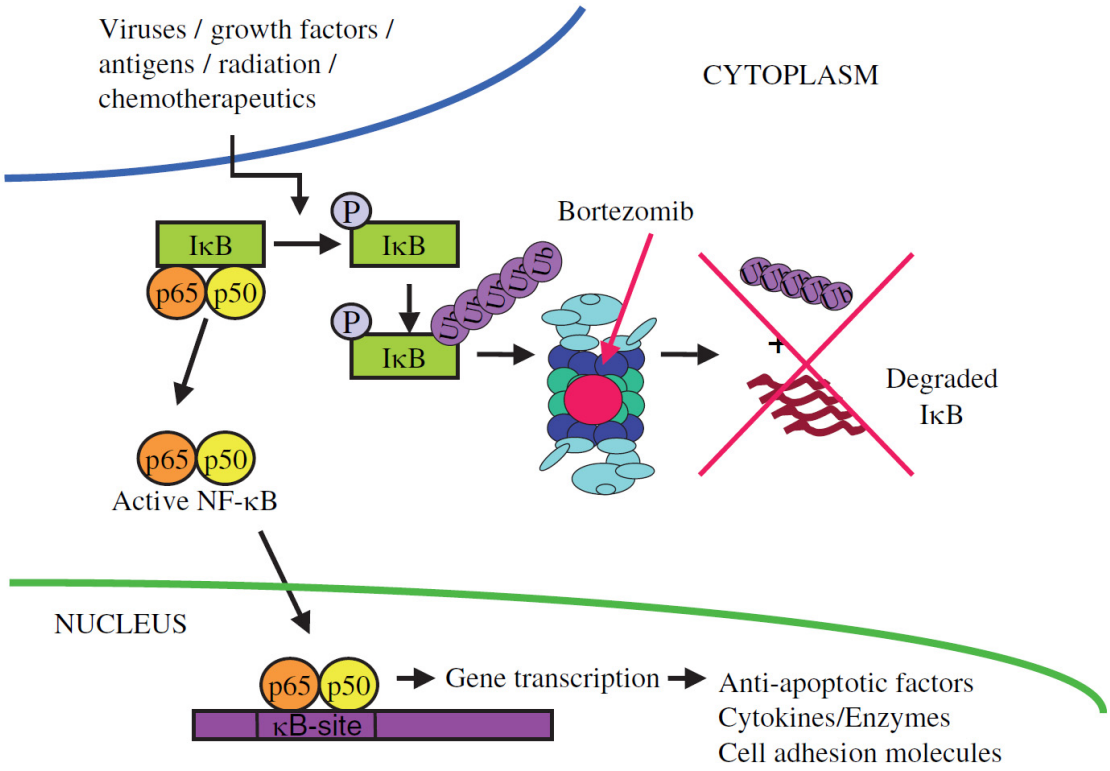


Figure 1-3. Proteasome inhibition affects NF-κB signal pathway. Bortezomib binds to the chymotryptic site of the 20S b-subunit, thereby blocking the proteasome. As a result IκB will not be degraded into small peptides. It remains bound to the survival protein NF-κB, thereby inhibiting NF-κB action. Reprinted with permission. Vink, J.; Cloos, J.; Kaspers, G. J. Proteasome inhibition as novel treatment strategy in leukaemia. *Br J Haematol* **2006,** 134, 253-62.

1.5.1 Bioavailability of CGA

The bioavailability of CGA has been studied by several groups.[73-76] Gonthier and coworkers have studied the bioavailability of CGA and compared it with that of CA and QA in rats fed diets supplemented with pure compounds for 8 days.[73] They found that CGA poorly absorbed through the small intestine, largely reached the cecum of rats where it was hydrolyzed by the microflora, which exhibited esterase activities.[77,78] Caffeic acid (CA) and QA were liberated and further metabolized.[27,79] The proposed CGA metabolism pathway is shown in Figure 1-4. Some of the microbial metabolites still bearing a free phenolic group could act as antioxidants.[80,81] A similar study has done by Azuma and coworkers in 2000, they found CGA was difficult to be absorbed from alimentary tract, although it could easily enter blood vessel after intraperitoneal injection.[74] Unlike CGA, CA was absorbed through digestive tract. The bioavailability of CGA from green coffee extract in humans has also been studied.[75] In this study, CGA was defined as phenolic compounds formed by the esterification of cinnamic acids, such as caffeic, ferulic, and p-coumaric acids, with QA. They were differentially absorbed and/or metabolized throughout the whole gastrointestinal tract.

1.5.2 Bioavailability of QA

Lautemann in 1863 showed that QA was excreted to a large extent as hippuric acid.[82] This has been confirmed by several authors, such as Quick in 1931;[83] Beer in 1951;[84] Cotran in 1960.[85] Adamson in 1970 reported that the aromatization of QA was different between animal species, and gut bacteria played an important role in this process.[27] In man and some old world monkeys, oral quinic acid was extensively aromatized (20-60%) and excreted in the urine as hippuric acid. The aromatization of oral quinic acid was considerably suppressed when gut flora were suppressed by neomycin. In the rat, only a small amount of hippuric acid was found in urine. QA was converted into benzoic acid by gut flora in all species (Figure 1-4).[27,73,86] In summary, bioavailability of QA in animal is very low.

1.6 Synthesis of QA and Its Derivatives

QA total synthesis was reported by Grewe in 1954,[87] and another route was completed by Smissman in 1963.[88] In 1964, Wolinsky developed a method to stereospecifically synthesize QA.[89] Recently, QA applications as a chiral template in natural product synthesis have been rapidly growing. This highly functionalized substrate was used to prepare natural compounds featuring cyclohexane substituted skeletons, and it has also extended to the preparation of open chain building blocks, which could be further transformed into optically active cyclopentane substituted skeletons and nitrogen containing targets. A review prepared by Barco has comprehensively covered QA chemistry literature up to the beginning of 1997.[90] Many new chiral compounds have been synthesized based on inexpensive chiral material QA. More recently, there are many disease driven synthesis based on QA modification. Both Girard and Kaila have

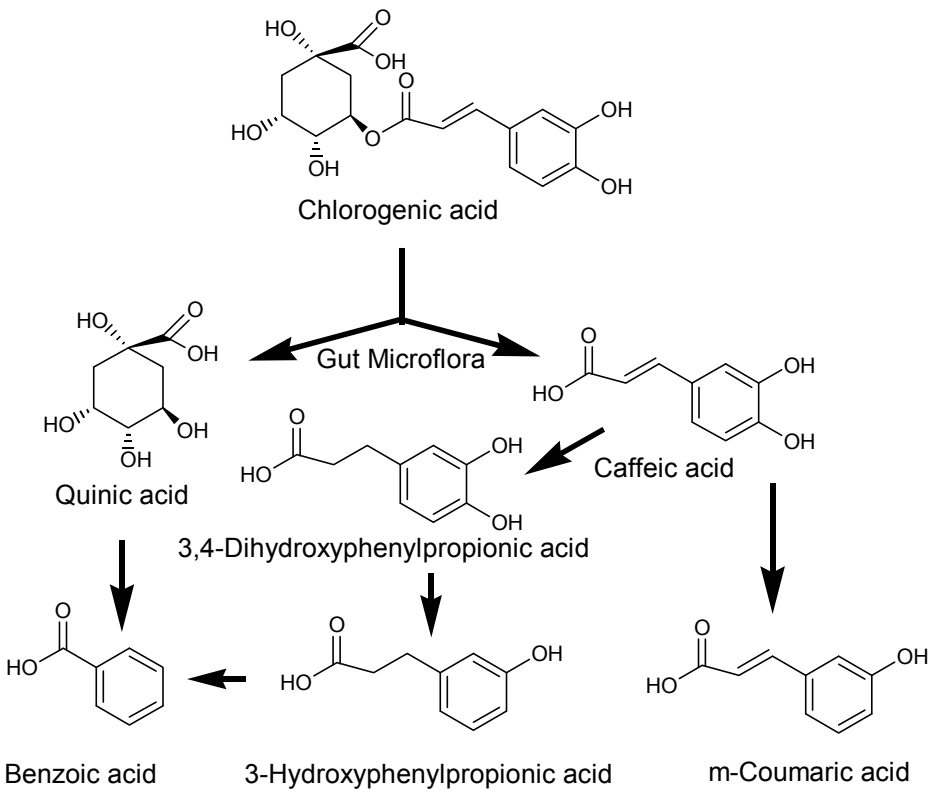


Figure 1-4**.** The general pathway of CGA metabolism by gut microflora in rat. First, the chlorogenic acid is degraded into QA and caffeic acid.

synthesized QA derivatives as sialyl lewis x -mimicking selectin inhibitors in order to target E-selectin.[67,68] Kim made analogues as influenza neuraminidase inhibitors with potent anti-anfluenza activity.[91] Sanchez-Sixto and Metaferia made compounds to kill *Mycobacterium tuberculosis*.[92,93] Payne designed derivatives to act as type II dehydroquinase inhibitors.[94] In 2001, Sefkow has synthesized CGA, 1-, 4-, and 5-Caffeoylquinic Acid as antioxidant.[95,96] Those esters have many other properties such as antibacterial, antimutagenic, antitumor, and antiviral.[97] Metaferia synthesized QA derivatives macrolides that inhibit breast cancer cell migration in vitro.[98] In summary, the interest in development of modified natural products based on QA is high in recent years.

1.7 The Discovery of QA Derivatives as Oral Anti-inflammatory Agents

In this study, we will follow the modern drug discovery procedure to discover and develop the QA derivatives, which possess orally active anti-inflammatory properties.

1.7.1 Drug Discovery

Drug discovery is very complicated, and is very important in human health, and plays an important role in science and technology. The Science magazine presented several special issues on this topic in 2000, 2004, and 2005.[99-101] Driven by chemistry but increasingly guided by pharmacology and the clinical sciences, drug research has contributed more to the progress of medicine during the past century than any other scientific factor. The advent of molecular biology and, in particular, of genomic sciences is having a deep impact on drug discovery.[102] In 2000, Uppenbrink predicted that drug discovery is poised to revolutionized because of the soon-to-be-completed Human Genome Project, combinatorial chemistry, and other scientific advances.[99] This made the introduction of target-based drug discovery, different from old physiology-based drug discovery.[102-104] In physiology-based drug discovery, drug was tested with animal. But in target-based drug discovery, drug was tested with target. A target is usually a single gene, gene product or molecular mechanism that has been identified on the basis of genetic analysis or biological observations.[105-107] After several years large increases in target-based drug discovery R&D investment, there has been a steady decline in the number of new molecules and biologicals that enter clinical development and finally reach the market.[108,109] This perceived failure of current drug discovery has generated widespread concern, and several divergent opinions about the problem and its potential solutions.[110] In 2008, Ho reported "Systems Biology: An Evolving Approach in Drug Discovery and Development."[111] Butcher asked "Can cell systems biology rescue drug discovery?"[110] Broadly defined, systems biology is the study of the interactions between components of a biological and how these integrate to produce a phenotype. This contrasts with the traditional approach that emphasized identification of the individual components of a system.[111]

No matter what strategies to take, there is a practical approach in drug discovery as shown in Figure 1-5. First, chemical compounds are made based on literature search,

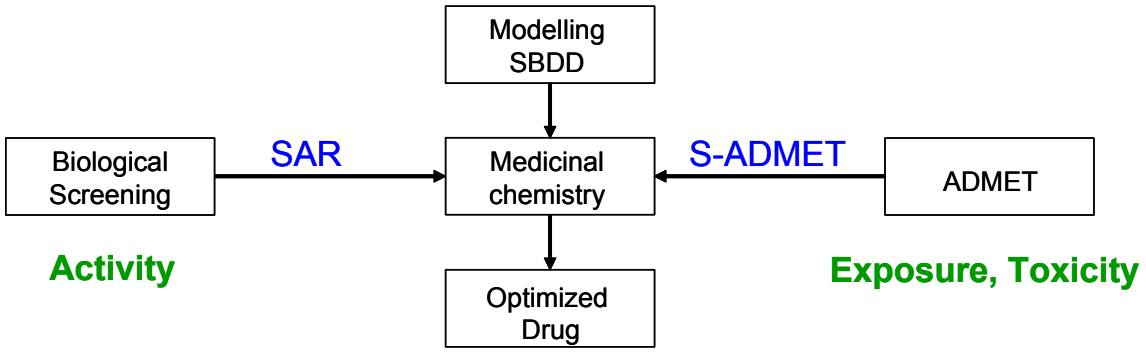


Figure 1-5. Current trends in drug discovery. SAR: Structure Activity Relationship; S-ADMET: Structure – Absorption, Distribution, Metabolism, Elimination, Toxicology; SBDD: Structure Based Drug Discovery.

natural products, or clinical observations. Then biological activity is tested by biological screening model. Structure activity relationship (SAR) is used to optimize the chemical structure. Lead compounds are further tested to optimize their biopharmaceutical, pharmacokinetic, and pharmacotoxic properties. That is structure – adsorption, distribution, metabolism, elimination, toxicology (S-ADMET) study. Computer model is also applied to assist structure based drug discovery (SBDD).

1.7.2 The Study Goals and Hypothesis

Our hypothesis states that it will be possible to convert QA into a derivative that will retain potent anti-inflammatory activity and be resistant to microbial degradation. This will ultimately provide orally active QA analogs. A major current concern with QA is the fact that it is significantly modified by intestinal bacteria that prevent it from showing good oral activity.

1.7.2.1 Synthesis of QA amide analogs

We initially focused on substitution at the carboxylic acid position, as previous work has shown that modifications of the carboxylic acid position modulated interaction of QA analogs with members of the selectin family (E- and P-selectin).[67,68] These same authors showed that the presence of a carboxylic acid functional group was necessary for interaction with the selectins.[67,68] A seriels of QA amides will be prepared, then go to biological screening against our NF-κB activity HTS System. QA amide leads will form a new template for anti-inflammatory agents. Further examination of the SAR and S-ADME will be conducted to optimize the leads. We hypothesize that further modifications to the amide series including substitution of the 3-alcholic functional group of QA will yield more potent analogs with enhanced resistance to microbial degradation.

1.7.2.2 Synthesis of dehydroquinate amide analogs

We are also going to investigate to see if the OHs on the cyclohexane ring are needed for anti-inflammatory activity. We plan to investigate the importance of the hydroxyl groups on the cyclohexane ring by preparing the dehydroquinate amide analogs. We hypothesize these modifications will retain anti-inflammatory activity with enhanced resistance to microbial degradation. Because the first three steps of QA catabolism by bacteria are to form dehydroquinate, followed by two successive dehydrations to form dehydroshikimate and shikimate.[16] We assume the bacteria degradation will not take place when the new substrates lack of hydroxyl groups and/or having a double bond.

1.7.2.3 Synthesis of QA amide ester analogs

Based on the QA amide lead which serves as a new template for anti-inflammatory agents, we are now planning to investigate to see if antioxidant groups are added for having both anti-inflammatory and antioxidant activity. We will prepare a serial of QA amide ester analogs with antioxidant groups such as caffeic acid, sinapic acid. We hypothesize these modifications will retain anti-inflammatory activity with additional antioxidant property. Furthermore, they will be hydrolyzed by the microflora, for an example, CGA is hydrolyzed into Caffeic acid and QA.[77,78] Both caffeic acid and QA amide will not be consumed by gut bacteria, and easily be absorbed in animal digestive tract.

1.7.2.4 QA derivatives anti-inflammatory activity

Comprehensive SAR studies will identify those functional groups which further optimize activity and stability. Anti-inflammatory activity will be assessed using A549 cells containing an NF-κB secreted alkaline phosphatase reporter. To serve this purpose, we developed a cell-based high-throughput screening (HTS) system comprising A549 cells stably transfected with a plasmid containing a secreted alkaline phosphatase reporter gene driven by an NF-κB response element (Figure 1-6). Upon TNF-α stimulation, NF-κB translocates to the nucleus and binds the κB response element causing transcriptional activation. This activation drives production of the SEAP reporter, which can be measured in the cell culture supernatant as a surrogate marker for NF-κB activity. Cytotoxicity will be assessed using an MTS assay. Mechanistic studies and pre-clinical efficacy studies of our lead molecules in various inflammatory animal models are studied by other researchers, and will not be reported in this dissertation.

1.7.2.5 Lead compounds S-ADME study

Rapid degradation by gut flora potentially limits the oral effectiveness of current QA preparations. The resistance to microbial degradation of QA analog lead compound will be determined using bacterial *Gluconobacter oxydans*. Further biopharmaceutical

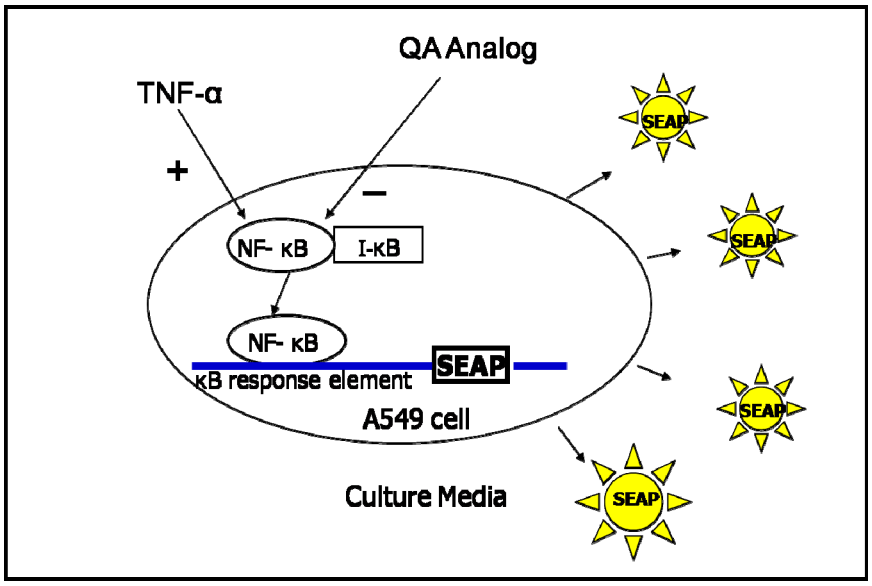


Figure 1-6. NF-κB activity HTS System. A549 cells stably transfected with an NF-κB SEAP reporter were treated with TNF-α (10 ng/mL) and QA analog (1 μM) for 18 hours.

and pharmaceutics studies will also be done. These studies include microsomal stability, metabolite identification, plasma protein binding, pharmacokinetics and bioavailability in rat. In summary, The S-ADME study will investigate the bacterial stability, and absorption, distribution, metabolism and elimination of lead compounds in rat after intravenous and oral administration.

CHAPTER 2. SYNTHESIS AND BIOLOGICAL EVALUATION OF QUINIC ACID AMIDES*

2.1 Introduction

The woody plant *Uncaria* is widely dispersed in tropical regions, including Southeast Asia, Africa, and South America. *Uncaria* genus plants have provided numerous structurally diverse medicinal natural products (e.g., alkaloids, terpenes, quinovic acid glycosides, flavonoids, and coumarins).[112] The species with the most compounds identified (≈50; 15 of which are reported as novel) is the Peruvian *Uncaria tomentosa* commonly known as Uno de Gato or Cat's claw. Most commercial Cat's claw preparations are based on oxindole alkaloid content as alkaloids represent the most abundant class of compounds found in *Uncaria*.[40]

Hot water Cat's claw extracts (e.g., C-Med 100) have very low alkaloid content (<0.5%) and yet retain significant immune enhancing activity. For example, the extract significantly accelerated recovery from doxorubicin-induced leukopenia in rats.[38] Moreover, elevated leukocyte numbers were noted in humans, mice, and rats receiving repeat doses of the extract.[3,113] Enhanced leukocyte counts correlated with prolonged lymphocyte survival, thus providing a potential mechanistic basis for the immune enhancing properties of the extract.[113] Prolonged cell survival has been linked to enhanced DNA repair.[3-5] Recently, quinic acid (QA) esters have been identified as biologically active components in the extract.[1]

The mechanism by which QA and its esters exert their anti-inflammatory activity is unclear, but appears to be related to inhibition of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB). For example, QA inhibited phorbol myristate acetate (PMA) and ionomycin-induced NF-κB activation in Jurkat T cells at concentrations that neither induced cell death nor inhibited proliferation.[2] Moreover, QA prevented degradation of the NF-κB inhibitor protein IκB-α without affecting levels of the phosphorylated IκB-α protein.[2] Base hydrolysis of the extract dramatically reduces biological activity. For example, the lactone ester of QA (QAL) inhibits proliferation of mitogen-stimulated mouse lymphocytes, whereas QA does not affect proliferation.[2] Further, base hydrolysis of the extract dramatically reduces its anti-proliferative effect against HL-60 and human mononuclear cells.[1] Together, these data suggest the ester derivatives of QA found in the extract comprise a significant fraction of the biological activity.

QA is utilized by gastrointestinal bacteria as a carbon source for aromatic acid synthesis. Consequently, only a small fraction of QA is absorbed after oral administration of either QA or chlorogenic acid (CGA), which is a caffeic acid (CA)-containing ester of

*This chapter adapted with permission. Zeng, K.; Thompson, K.E.; Yates, C. R.; Miller, D. D. Synthesis and biological evaluation of quinic acid derivatives as anti-inflammatory agents. *Bioorg Med Chem Lett*, **2009**, 19, 5458-60.

QA.[27] Our group has focused on the discovery and development of stable QA derivatives. Toward this end, we have identified water soluble amide analogs of QA (1,3,4,5-tetrahydroxy-1-cyclohexanecarboxylic acid) which possess potent anti-inflammatory activity.

2.2 Results and Discussion

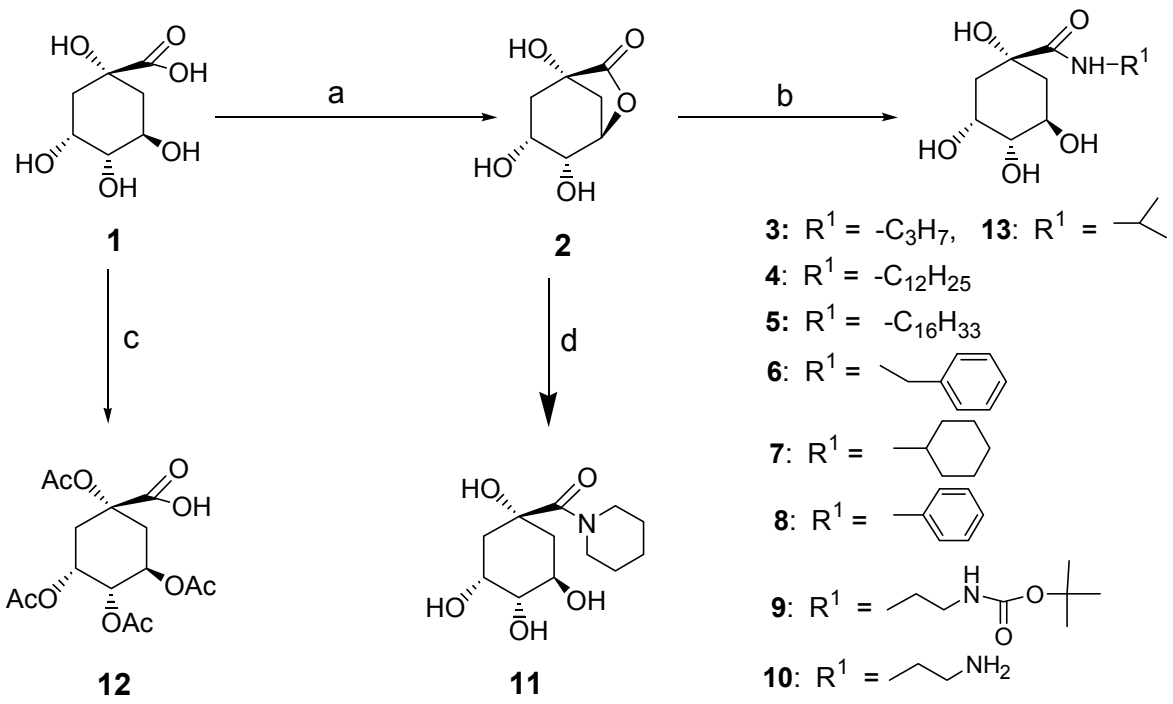
2.2.1 Synthesis of Quinic Acid Amides

Synthesis of the QA analogs was carried out using chemistry previously described.[67,68,114] The synthesis of the lactone **2** was carried out using PTSA in refluxing benzene and DMF according to the procedure of Neelu Kaila et al. with minor revision. The lactone was then allowed to react with N-propyl amine with acetic acid at 85°C and the resulting amide **3** was purified using flash chromatography.[114] The amides **4-11,** and **13** were formed by using different amines with lactone **2** in a manner similar to what was described for compound **3**. QA, **1**, was treated with acetic anhydride /pyridine to give compound **12**.[68] Each compound was characterized with Mass Spectroscopy, NMR, and elemental analysis. The general synthesis of QA analogs is shown in Scheme 2-1.

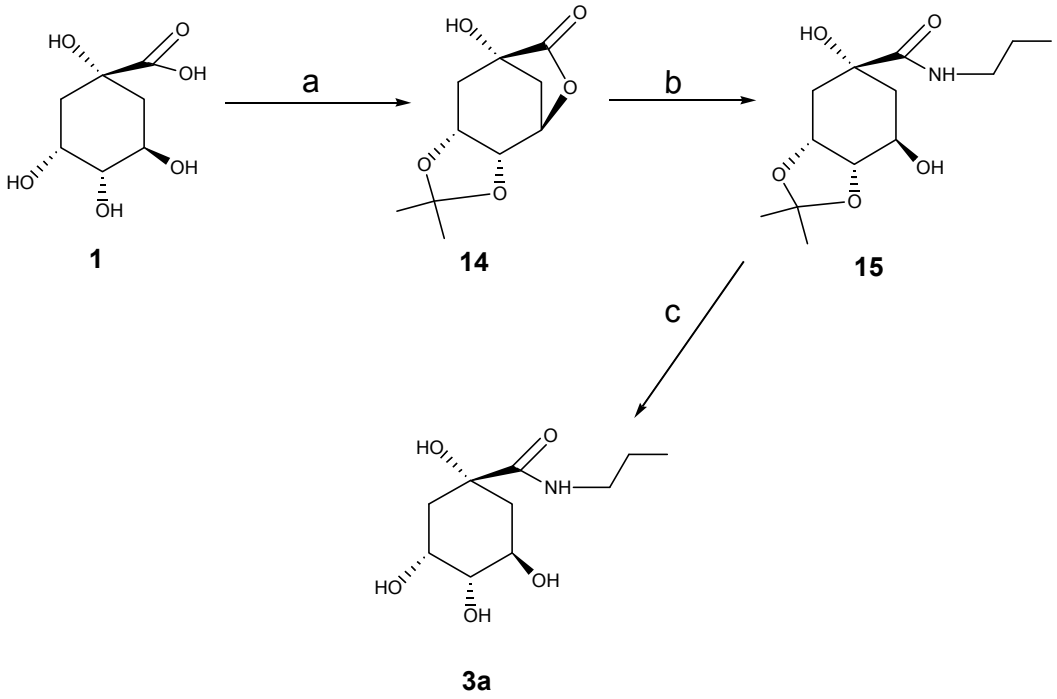
We found that the N-propyl amide derivative **3** had the greatest extent of NF-κB inhibition following derivative in vitro A549 cells screening study.[71] After that, compound **3** was moved up into in vivo study, such as animal biological activity and pharmacokinetics study. Large amount of compound **3** was needed. The above synthesis method used flash chromatography for purification in both steps. Compound **2** is very sticky on silica gel column, only about 4g could be collected after each column. It is highly water soluble, can't be purified by liquid-liquid separation to remove PTSA. The optimized pathway to synthesis of amide **3** is shown in Scheme 2-2. The basic idea is to make intermediates less polar, so aqueous wash and separation are possible during compound purification. In Scheme 2-2, intermediate **14** was obtained in one step with high yield (>80%),[115] it can be purified just by liquid-liquid separation followed by crystallization.

2.2.2 Biology Test of Quinic Acid Amides

The results of QA derivative anti-inflammatory high throughput screening are presented in Figure 2-1. The N-propyl amide derivative (**3**) was found to have the greatest extent of NF-κB inhibition following derivative screening. We initially focused on substitution at the carboxylic acid position, as previous work has shown that QA ester derivatives have greater biological activity compared to QA.[1,2] The carboxylic acid substituent appears critical to the interaction of QA derivatives with E- and P-selectin, key mediators of leukocyte endothelial cell interactions during inflammation.[68] Interestingly, our most potent QA analog **3** demonstrates anti-inflammatory activity despite the fact that it lacks a carboxylic acid functional group. Further examination of



Scheme 2-1. Synthetic scheme of QA amides. Reagents and conditions: (a) PTSA, DMF, C6H6, Dean-Stark, reflux, 78%; (b) R1-NH2, AcOH, oil bath 85°C; (c) (Ac)2O; (d) R-NH2, AcOH, oil bath 85°C.



Scheme 2-2**.** Optimized synthetic scheme of compound **3**. Reagents and conditions: (a) PTSA, 2,2-DMP, Acetone, reflux, 80%; (b) C_3H_7-NH_2, AcOH, oil bath 85°C; (c) 1N HCl.

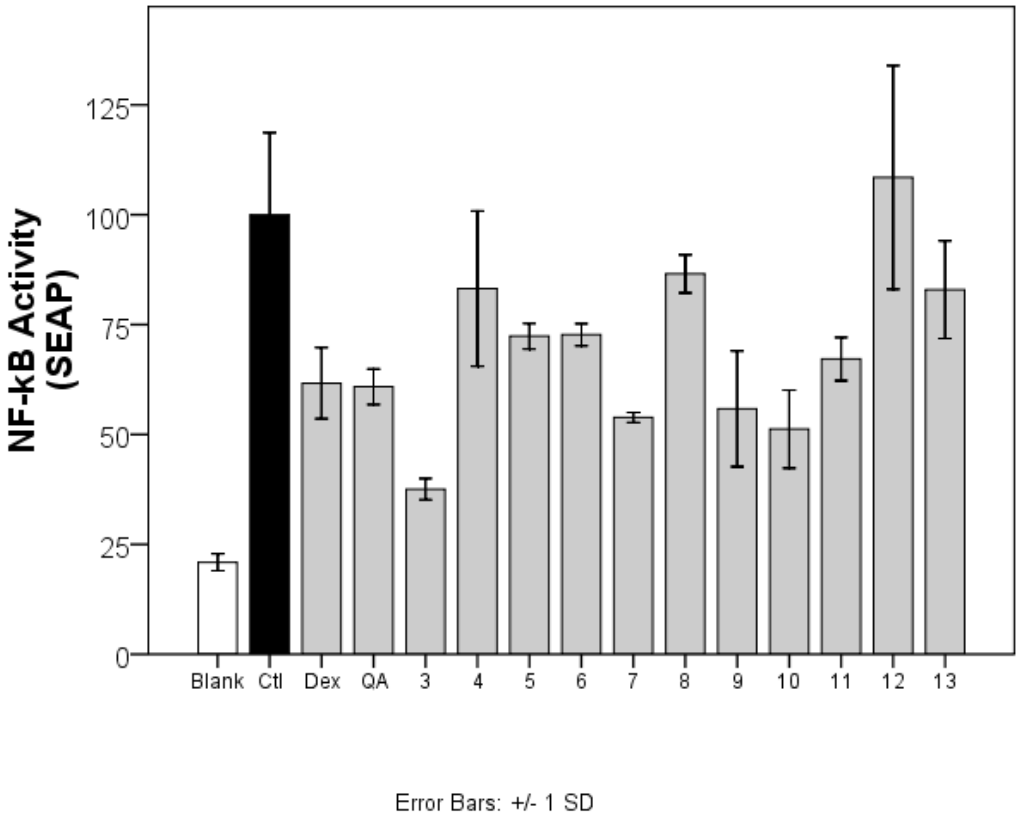


Figure 2-1. NF-κB inhibition by QA and analogs using A549 cells stably transfected with a secreted alkaline phosphatase (SEAP) reporter. NF-κB activity was measured 18 hours after addition of TNF-α (10 ng/mL) and either QA or synthesized derivatives (3-13; 1 μM). Dexamethasone (Dex; 1 μM) was used as a positive control. Data are presented as percent (%) inhibition relative to control (ctl; TNF-α alone) and represent % mean inhibition ±standard deviation (n=3). Blank: media only.

the SAR demonstrated that acetylation of the hydroxyl groups, which yields compound **12**, leads to reduced NF-κB inhibitory activity. A variety of modifications to the amide substitution of **3** were tolerated, e.g., **9**, **10** and **13**, a N-isopropyl amide, but no substitution was better than the N-propyl amide. The NF-κB inhibitory potency (IC_{50}) of our most active analog **3** was determined as 2.83±1.76 μM.

Next, we determined the anti-oxidant potential of QA derivatives using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method.[116] The dietary polyphenol CGA, an ester of QA and CA, possesses potent anti-oxidant activity, which is attributed to the presence of CA. We thus compared the anti-oxidant potential of CGA, CA, QA and **3** to determine if the anti-inflammatory activity of QA and **3** was attributable to anti-oxidant activity. Ascorbic acid 6-palmitate was included as a structurally-distinct molecule with described anti-oxidant activity. As expected, Figure 2-2 demonstrates the anti-oxidant activity of CGA, CA, and ascorbic acid. However, QA and **3** have no such anti-oxidant activity. The fact that QA does not possess anti-oxidant activity is consistent with a previous report.[64] Therefore, the NF-κB inhibitory activity of QA derivatives is attributed to mechanisms unrelated to anti-oxidant activity.

QA esters potently inhibit proliferation of mitogen-stimulated mouse lymphocytes without increasing cytotoxicity.[2] We thus determined the anti-proliferative potential of QA and **3** against A549 cells using the MTS assay. Neither QA nor **3** exhibited cytotoxic activity toward A549 cells at concentrations up to 100 μM (Table 2-1). Thus, it appears that inhibition of NF-κB by QA and **3** is related not to anti-proliferative or cytotoxic activity, but rather to a yet to be determined mechanism.

Figure 2-3 shows the activities of drugs in carrageenan induced paw edema animal model. Positive control dexamethasone significantly reduced the volume of swelled rat paw. QA and **3** had not demonstrated significantly effect on reducing paw edema volume. Therefore, the anti-inflammatory activity of QA derivatives is attributed to mechanisms different from carrageenan induced paw edema.

2.3 Conclusions

We have synthesized novel QA analogs that potently inhibit NF-κB activity in TNF-α-stimulated human alveolar Type II-like epithelial cells (A549). We have demonstrated that the QA analogs presented in this work do not exert their activity via anti-oxidant, cytotoxic, or carrageenan induced paw edema mechanisms. Mechanistic studies and pre-clinical efficacy studies of our lead molecule **3** in various inflammatory animal models are on-going.

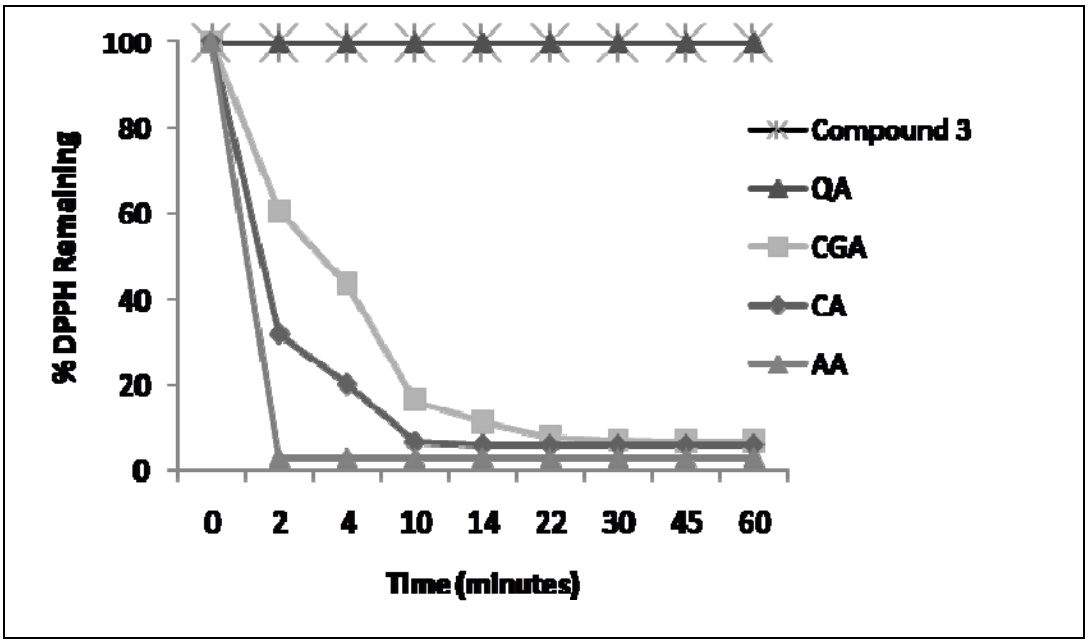
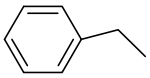
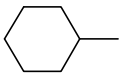
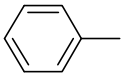
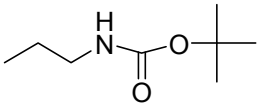
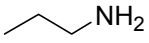
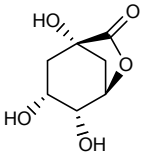
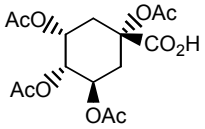
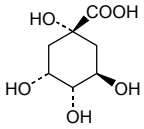


Figure 2-2**.** Reaction kinetics of DPPH for determining antioxidant activity. Caffeic acid (CA), chlorogenic acid (CGA), ascorbic acid (AA), QA and Compound **3** were prepared in 50% acetone. The conventional colorimetric DPPH scavenging capacity was determined by UV absorption measured at 515 nm. CA, CGA and AA showed strong anti-oxidant activity, while QA and **3** had no anti-oxidant activity.

Table 2-1. Cytotoxicity test of quinic acid analogs on A549SN cell line by MTS method.

Compound name	R_1 structure	A549SN cell viability (C=100 μM)
4	$-C_{12}H_{25}$	no observed toxicity
5	$-C_{16}H_{33}$	no observed toxicity
6		no observed toxicity
7		no observed toxicity
3	$-C_3H_7$	no observed toxicity
8		no observed toxicity
11		no observed toxicity
9		no observed toxicity
10		no observed toxicity
2		no observed toxicity
12		no observed toxicity
QA		no observed toxicity

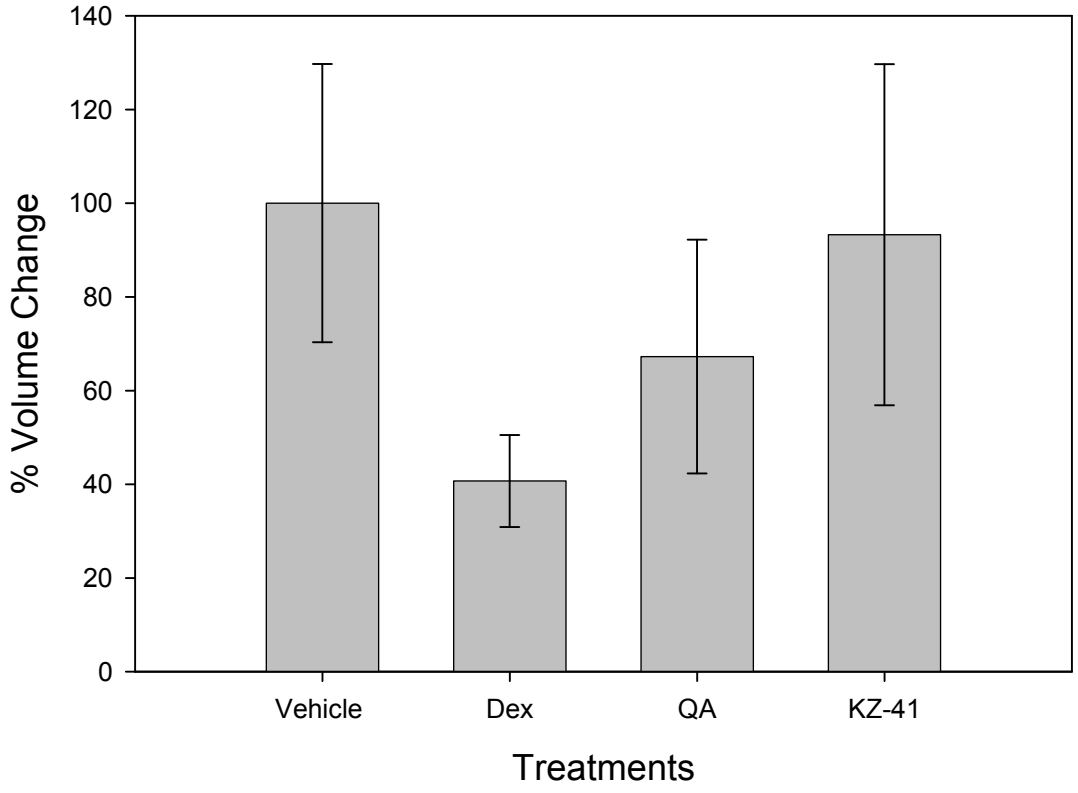


Figure 2-3**.** Rat paw edema percent volume change. (Normalized to Vehicle) (n=6). Treatment (50mg/kg) and 1% Carrageenan (10mg/kg).

2.4 Experimental

2.4.1 Chemistry

All reagents and solvents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO), Fischer Scientific (Pittsburgh, PA), and TCI (Portland, OR), and were used without any further purification. Thin layer chromatography (TLC) was performed on silica gel chromatogram plates purchased from Analtech, Inc. Flash chromatography was performed either on silica gel (60 Å, 200 -425 mesh) or on pre-packed silica gel columns by using a Horizon HPFC system (Biotage, Charlottesville, VA). Molecular masses were collected with electron spray ionization mass spectra (ESI-MS) on a Bruker/Hewlett Packard Esquire LC/MS instrument. Nuclear magnetic resonance (NMR) spectra for ^{1}H NMR and ^{13}C NMR were recorded on a Bruker ARX 300 instrument and/or a Varian Inova-500 MHz instrument. The chemical shifts value (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and coupling constants (J) were reported in hertz. Peaks are abbreviated as were indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Elemental analyses (C, H, N) were performed by Atlantic Microlab Inc. (Norcross, GA), and are accepted within ±0.4 of the theoretical values. High performance liquid chromatography (HPLC) separation was performed on a Hewlett Packard LC instrument with model 1100 system.

The following is the general procedure for the synthesis of quinic acid amides. The synthesis of the lactone **2** was carried out using PTSA in refluxing benzene and DMF according to the procedure of Neelu Kaila et al. with minor revision. The lactone was then allowed to react with N-propyl amine with acetic acid at 85°C and the resulting amide **3** was purified using flash chromatography.[114] The amides **4-11,** and **13** were formed by using different amines with lactone **2** in a manner similar to what was described for compound **3**. QA, **1**, was treated with acetic anhydride /pyridine to give compound **12**.[68] Each compound was characterized with Mass Spectroscopy, NMR, and elemental analysis. The general synthesis of QA analogs is shown in Scheme 2-1.

1,3,4-Trihydroxy-6-oxa-bicyclo[3.2.1]octan-7-one (2). In a 200 mL round-bottom flask fitted with a stirring bar, reflux condenser, Dean-Stark trap, and argon inlet, 5 g of quinic acid (**1**,26 mmol) was placed and 10 mL of dry DMF was added via syringe and the slurry stirred at room temperature. Next, benzene 60 mL and p-toluenesulfonic acid 0.5 g were added, and the slurry was heated to reflux for 26h. Check TLC to confirm the completion of reaction. A 1:1 mixture of EtOAc and heptane (100 mL) was added to the cooled reaction mixture. The mixture was stirred for 1 hr at room temperature and filtered. The collected solid was again stirred with a 1:1 mixture of EtOAc and heptane (100 mL) for 1 hr at room temperature and filtered. Tituration was repeated one more time with a 1:1 mixture of EtOAc and heptane (100 mL) and the precipitate collected to give 3.5 g of Lactone **2** (78% yield). mp: 192-193°C, R_f 0.25 (EtOAc); MS: $[M-H]^-$: 171; ^{1}H NMR (300 MHz, DMSO) δ 1.72 (t, J=1.6 Hz, 1H), 1.82-1.88 (m, 1H), 2.07-2.13 (m,

1H), 2.25 (d, J=1.1 Hz, 1H), 3.49 (ddd, J=11.4, 6.3, 5.7 Hz, 1H), 3.82 (t, J=4.5 Hz, 1H), 4.61 (t, J=5.1 Hz, 1H), 4.816 (d, J=6.0 Hz, 1H), 5.23 (d, J=4.5 Hz, 1H), 5.89 (s, 1H). Anal. ($C_7H_{10}O_5$) calculated: C, 48.28; H, 5.79. Found: C, 48.38; H, 5.76.

1,3,4,5-tetrahydroxy-cyclohexane-carboxylic acid propylamide (3). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.196 g Lactone **2** (1.125 mmol), and propylamine (0.83 mL, 0.6g, 10.13 mmol) were combined, then glacial acetic acid (0.19 mL, 0.20 g, 3.36 mmol) was added. The solution was warmed to 85°C in oil bath for 30 min, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. Reaction mixture was purified by flash column with same solvent as TLC. Collect product **3,** 206mg (80% yield). mp: 132-133°C, R_f 0.03 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M-H]^- = 232$; ^1H NMR (300 MHz, DMSO) δ 0.81 (t, J=6.0 Hz, 3H), 1.41 (ddd, J=13.8, 7.5, 6.9 Hz, 2H) 1.66-1.83 (m, 4H), 3.02 (dd, J=6.9, 6.6 Hz, 2H), 3.22 (t, J=6.0 Hz, 1H), 3.75 (ddd, J=13.8, 9.0, 5.7 Hz, 1H), 3.94 (s, 1H), 4.69 (d, J=4.5 Hz, 1H), 4.90 (d, J=5.4 Hz, 1H), 5.19 (d, J=3.6 Hz, 1H), 5. 47 (s, 1H), 7.76 (t, J=5.4 Hz, 1H). Anal. ($C_{10}H_{19}NO_5$) calculated: C, 51.49; H, 8.21; N, 6.00. Found: C, 51.57; H, 8.40; N, 6.01.

1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid dodecylamide (4). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.12 g Lactone **2** (0.706 mmol), and dodecylamine (1.19g, 6.42 mmol) were combined, then glacial acetic acid (0.13 mL, 2.20 mmol) was added. The solution was warmed to 85°C in oil bath for 30 min, at which time TLC (EtOAc) indicated complete consumption of the starting lactone. Reaction mixture was purified by flash column with same solvent as TLC. Collect product **4**, 34 mg. Rf 0.15 (EtOAc); MS: $[M-H]^- = 258$; ^1H NMR (300 MHz, DMSO) 0.85 (t, J=6.0 Hz, 3H), 1.20-1.41 (20 H) 1.66-1.83 (m, 4H), 3.02 (dd, J=6.9, 6.6 Hz, 2H), 3.22 (t, J=6.0 Hz, 1H), 3.75 (ddd, J=13.8, 9.0, 5.7 Hz, 1H), 3.94 (s, 1H), 4.69 (d, J=4.5 Hz, 1H), 4.90 (d, J=5.4 Hz, 1H), 5.19 (d, J=3.9 Hz, 1H), 5. 47 (s, 1H), 7.76 (t, J=5.4 Hz, 1H).

1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid hexadecylamide (5). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.23 g Lactone **2** (1.34 mmol), and hexadecylamine (2.91g, 12.04 mmol) were combined, then glacial acetic acid (0.24 mL, 4.01 mmol) was added. The solution was warmed to 85°C in oil bath for 60 min under argon, at which time TLC (EtOAc: MeOH=20:1) indicated complete consumption of the starting lactone. Reaction mixture was purified by flash column with same solvent as TLC. Collect product **5**, 54 mg. Rf 0.05 (EtOAc: MeOH=20:1); MS: $[M-H]^- = 414$; ^1H NMR (300 MHz, DMSO) 0.85 (t, J=6.0 Hz, 3H), 1.20-1.41 (28 H) 1.66-1.83 (m, 4H), 3.02 (dd, J=6.9, 6.6 Hz, 2H), 3.22 (t, J=6.0 Hz, 1H), 3.75 (ddd, J=13.8, 9.0, 5.7 Hz, 1H), 3.94 (s, 1H), 4.69 (d, J=4.5 Hz, 1H), 4.90 (d, J=5.4 Hz, 1H), 5.19 (d, J=3.9 Hz, 1H), 5. 47 (s, 1H), 7.76 (t, J=5.4 Hz, 1H). Anal. ($C_{23}H_{45}NO_5$) calculated: C, 66.47; H, 10.91; N, 3.37. Found: C, 66.04; H, 10.91; N, 3.37.

1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid benzylamide (6). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.23 g Lactone **2** (1.34 mmol), and benzylamine (1.27g, 11.70 mmol) were combined, then glacial acetic acid

(0.23 mL, 3.90 mmol) was added. The solution was warmed to 85°C in oil bath for 45 min under argon, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. Reaction mixture was purified by flash column with same solvent as TLC. Collect product **6**, 210 mg (80% yield). Rf 0.15 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M-H]^-$ =280; ^1H NMR (300 MHz, DMSO) 1.66-1.87 (m, 4H), 3.02 (dd, J=6.9, 6.6 Hz, 2H), 3.22 (t, J=6.0 Hz, 1H), 3.75 (ddd, J=13.8, 9.0, 5.7 Hz, 1H), 3.96 (s, 1H), 4.27 (d, J=6.0 Hz, 1H), 4.69 (d, J=4.5 Hz, 1H), 4.90 (d, J=5.4 Hz, 1H), 5.22 (d, J=3.6 Hz, 1H), 5. 53 (s, 1H), 7.72 – 7.74 (m, 5H), 8.32 (t, J=6.0 Hz, 1H). Anal. ($C_{14}H_{19}NO_5$) calculated: C, 59.78; H, 6.81; N, 4.98. Found: C, 59.65; H, 6.77; N, 4.89.

1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid cyclohexylamide (7). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.20 g Lactone **2** (1.15 mmol), and cyclohexylamine (1.18 mL, 1.03g, 10.34 mmol) were combined, then glacial acetic acid (0.23 mL, 3.90 mmol) was added. The solution was warmed to 85°C in oil bath for 90 min under argon, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. Reaction mixture was purified by flash column with same solvent as TLC. Collect product **7**, 75 mg (25% yield). Rf 0.05 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M-H]^-$ =272; ^1H NMR (300 MHz, DMSO) 1.15-1.40 (m, 5H), 1.50 -1.80 (m, 8H), 3.22 (ddd, J=13.8, 9.0, 5.7 Hz, 1H), 3.50 (d, 1H), 3.70 - 3.80 (m, 1H), 3.93 (d, 1H), 4.68 (d, J=4.5 Hz, 1H), 4.87 (d, J=6.0 Hz, 1H), 5.18 (d, J=3.9 Hz, 1H), 5. 50 (s, 1H), 7.40 (d, J=8.4 Hz, 1H). Anal. ($C_{13}H_{23}NO_5$) calculated: C, 57.13; H, 8.48; N, 5.12. Found: C, 57.13; H, 8.48; N, 4.49.

1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid phenylamide (8). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.23 g Lactone **2** (1.34 mmol), and phenylamine (1.11 mL, 1.13g, 12.15 mmol) were combined, then glacial acetic acid (0.24 mL, 4.40 mmol) was added. The solution was warmed to 85°C in oil bath for 120 min under argon, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. Reaction mixture was purified by flash column with same solvent as TLC. Collect product **8**, 38 mg (10.1% yield). Rf 0.05 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M-H]^-$ =266; ^1H NMR (300 MHz, DMSO) 1.84 -1.95 (m, 4H), 3.24 - 3.32 (m, 1H) 3.80 - 3.86 (m, 1H), 4.04 (d, J=4.5 Hz, 1H), 4.73 - 4.76 (m, 2H), 5.35 (d, J=3.9 Hz, 1H), 5. 76 (s, 1H), 7.06 (t, J=7.5 Hz, 1H), 7.29 (t, J=7.5 Hz, 2H), 7.70 (t, J=7.5 Hz, 2H), 9.50 (s, 1H). Anal. ($C_{13}H_{17}NO_5.0.5H_2O$) calculated: C, 56.51; H, 6.57; N, 5.07. Found: C, 56.80; H, 6.48; N, 4.72.

2-[(1,3,4,5-Tetrahydroxy-cyclohexanecarbonyl)-amino]-ethyl-carbamic acid tert-butyl ester (9). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.14 g Lactone **2** (0.82 mmol), and (2-Amino-ethyl)-carbamic acid tert-butyl ester (1.07g, 6.24 mmol) were combined, then glacial acetic acid (0.13 mL, 2.10 mmol) was added. The solution was warmed to 85°C in oil bath for 180 min under argon, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. Reaction mixture was purified by flash column with same solvent as TLC. Collect product **9**, 270 mg (97% yield). Rf 0.05 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M+Na]^+$ =357; ^1H NMR (300 MHz, DMSO) 1.38 (s, 9H), 1.78 -1.82

(m, 4H), 3.00 (d, J=3 Hz, 2H), 3.10 (d, J=3 Hz, 2H), 3.19 - 3.32 (m, 1H) 3.72 - 3.94 (m, 1H), 3.94 (d, J=4.5 Hz, 1H), 4.69 (d, J=4.8 Hz, 1H), 4.82 (d, J=6.0 Hz, 1H), 5.21 (d, J=4.2 Hz, 1H), 5. 45 (s, 1H), 6.82 (t, J=5.4 Hz, 2H), 7.82 (t, J=6.0 Hz, 1H). Anal. ($C_{14}H_{26}N_2O_7$. $0.33H_2O$) calculated: C, 49.40; H, 7.90; N, 8.23. Found: C, 49.56; H, 7.90; N, 8.04.

1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid (2-amino-ethyl)-amide (10). In a 50 mL round-bottom flask fitted with a stirring bar, 0.22 g amide **9** (0.65 mmol), then 10 mL solution (TFA : CH_2Cl_2=1:1) was added, stirred for 1 hr under argon. Reaction mixture was evaporated to dry under reduced pressure. Then 10 mL 1.25 M HCl- Methanol solution was added, stirred for 1 hr under argon. Reaction mixture was evaporated to dry under reduced pressure. Collect product **10**, 175 mg (99% yield). MS: $[M\text{-}Cl]^+$ =235; ^{1}H NMR (300 MHz, DMSO) 1.73 -1.81 (m, 4H), 2.85 (dd, J=12, 6 Hz, 2H), 3.22 (dd, J=8.4, 3 Hz, 1H), 3.32 (d, 2H), 3.72 - 3.94 (m, 1H), 3.95 (d, J=4.5 Hz, 1H), 4.02 – 4.52 (4H), 7.91 (s, 3H), 8.09 (t, J=6.0 Hz, 1H). Anal. ($C_9H_{19}ClN_2O_5$. $0.5H_2O$) calculated: C, 37.42; H, 7.33; Cl, 112.28; N, 9.70. Found: C, 37.42; H, 7.39.

Piperidin-1-yl-(1,3,4,5-tetrahydroxy-cyclohexyl)-methanone (11). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.20 g Lactone **2** (1.14 mmol), and piperidine (1.04 mL, 0.89g, 10.49 mmol) were combined, then glacial acetic acid (0.20 mL, 3.50 mmol) was added. The solution was warmed to 85°C in oil bath for 220 min under argon, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. Reaction mixture was purified by flash column with same solvent as TLC. Collect product **11**, 135 mg (44.9% yield). Rf 0.05 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M+Na]^+$ =282; ^{1}H NMR (300 MHz, DMSO) 1.57 -1.72 (m, 7H), 1.88 – 1.96 (m, 3H) 3.20 - 3.26 (m, 1H), 3.4 (m, 2H), 3.69 – 3.83 (m, 4H), 4.62 (d, J=4.5 Hz, 1H), 5.00 (d, J=4.8 Hz, 1H), 5.08 (d, J=5.4 Hz, 1H), 5. 56 (s, 1H). Anal. ($C_{12}H_{21}NO_5$. $0.33H_2O$) calculated: C, 54.33; H, 8.23; N, 5.28. Found: C, 54.66; H, 8.24; N, 5.30.

1,3,4,5-Tetraacetoxy-cyclohexanecarboxylic acid (12). In a 50 mL round-bottom flask fitted with a stirring bar, 1.35 g quinic qcid **1** (7.03 mmol), and 15 mL anhydrous pyridine, were added. Cooled in an ice bath under argon, 15 mL acetic anhydride was added dropwise, warmed to rt slowly, stirred for 24 hr at rt. The solvent was removed under high vacuum to get yellow foam. The residue was purified by flash column with solvent (EtOAc: Hex =1:1 v/v). Collect product **12**, 2.41 g (97% yield). Rf 0.12 (EtOAc: Hex =1:1 v/v); MS: $[M\text{-}H]^-$ =359; ^{1}H NMR (300 MHz, DMSO) 2.07 – 2.25 (m, 16H), 5.09 (m, 1H), 5.23 (m, 1H), 5.37 (m, 1H), 13.24(s, 1H). Anal. ($C_{15}H_{20}O_{10}$) calculated: C, 50.00; H, 5.59. Found: C, 50.61; H, 5,87.

1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid isopropylamide (13). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.19 g Lactone **2** (1.12 mmol), and isopropylamine (0.82 mL, 0.59g, 10.13 mmol) were combined, then glacial acetic acid (0.20 mL, 0.20 g, 3.36 mmol) was added. The solution was warmed to 85°C in oil bath for 30 min, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. The solvent was removed under

reduced pressure. The residue was purified by flash column with same solvent as TLC. Collect product **13,** 170 mg (75% yield). R_f 0.03 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M+H]^+$ =234; ^1H NMR (300 MHz, DMSO) δ 1.02 (d, J=6.6 Hz, 6H), 1.61-1.78 (m, 4H), 3.18 (t, J=3.6 Hz, 1H), 3.71 – 3.85 (m, 2H), 3.94 (s, 1H), 3.90 (s, 1H), 4.64 (d, J=3.9 Hz, 1H), 4.83 (d, J=3.3 Hz, 1H), 5. 15 (s, 1H), 7.38 (d, J=8.1 Hz, 1H). Anal. ($C_{10}H_{19}NO_5$) calculated: C, 51.49; H, 8.21; N, 6.00. Found: C, 51.58; H, 8.15; N, 5.83.

8-Hydroxy-4,4-dimethyl-3,5,10-trioxa-tricyclo[6.2.1.$0^{2,6}$]undecan-9-one (14). A mixture of quinic qcid (**1**) (20g, 104.1 mMol), p-totuenesulfonic acid monohydrate (0.2g), 2,2-dimethoxypropane (38g, 364 mMol), and acetone (100 mL) was heated to reflux for 2 hr. The reaction mixture was cooled down, and solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (100 mL), washed with 5% aqueous sodium bicarbonate (100 mL). The aqueous phase was back-extracted with ethyl acetate (50x 2 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and the solvents were removed in vacuo. The residue was crystallized with hot ethyl acetate, got white solid **14** 16g (80% yield). mp: 147-149°C R_f =0.3 (EtoAC: Hex=3:7). MS: $[M+Na]^+$=237. ^1H NMR ($CDCl_3$) δ 1.37 (s, 3H), 1.55 (s, 3H), 2.21 (dd, J= 14.7, 3.0 Hz, 1H), 2.30-2.44 (m, 2H), 2.68 (d, J=11.7 Hz, 1H), 2.83(s, 1H), 4.32-4.36 (m, 1H), 4.51-4.58 (m, 1H), 4.79 (dd, J=2.6, 6.3 Hz, 1H).

5,7-Dihydroxy-2,2-dimethyl-hexahydro-benzo[1,3]dioxole-5-carboxylic acid propylamide (15). Lactone (**14**), (10g, 46.7 mMol), propylamine (30 mL), and glacial acetic acid (10 mL) was refluxed under argon with oil bath (85°C) for 45 min. Solvents were removed under reduced pressure. The residue was washed with saturated sodium bicarbonate solution (100 mL), extracted with ethyl acetate (100 x 3 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and the solvents were removed in vacuo. The residue was crystallized with hot ethyl acetate, got white solid **15** 9.7g (76% yield). mp: 85-86°C, R_f=0.15 (EtoAC: Hex=2:1). MS: $[M+Na]^+$=296. ^1H NMR (DMSO) δ 0.83 (t, J=6.0 Hz, 3H), 1.24 (s, 3H), 1.38 (s, 3H), 1.40-1.45 (m, 2H), 1.61-1.64 (m, 2H), 1.72 (dd, J=14.7, 5.7 Hz, 1H), 2.04 (dd, J=14.7, 5.7 Hz, 1H), 3.01 (dd, J=6.6, 7.2 Hz, 2H), 3.80 (ddd, J=9.9, 6.3, 3.6 Hz, 2H), 4.31 (d, J=5.4 Hz, 1H), 5.01 (d, J=5.1 Hz, 1H), 5. 28 (s, 1H), 7.73 (t, J=5.7 Hz, 1H).

1,3,4,5-tetrahydroxy-cyclohexane-carboxylic acid propylamide (3a). The amide (**15)** (5.0g) was dissolved in water (10mL), then added aq. 1M HCl (40 mL) at room temperature. The reaction mixture was stirred at room temperature for 1hr. Solvent was removed under reduced pressure. Residue HCl was removed by adding EtOEt (20 x 3 mL) under vacuo. The residue was further dried under oil pump. Collect product **3a**.26g (100% yield). mp: 132-133°C, R_f 0.03 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M-H]^-$=232; ^1H NMR (300 MHz, DMSO) δ 0.81 (t, J=6.0 Hz, 3H), 1.41 (ddd, J=13.8, 7.5, 6.9 Hz, 2H) 1.66-1.83 (m, 4H), 3.02 (dd, J=6.9, 6.6 Hz, 2H), 3.22 (t, J=6.0 Hz, 1H), 3.75 (ddd, J=13.8, 9.0, 5.7 Hz, 1H), 3.94 (s, 1H), 4.69 (d, J=4.5 Hz, 1H), 4.90 (d, J=5.4 Hz, 1H), 5.19 (d, J=3.6 Hz, 1H), 5. 47 (s, 1H), 7.76 (t, J=5.4 Hz, 1H). Anal. ($C_{10}H_{19}NO_5$) calculated: C, 51.49; H, 8.21; N, 6.00. Found: C, 51.57; H, 8.40; N, 6.01.

2.4.2 Biology

All chemicals and solvents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) if not specified, and were used without any further purification. TNF-alpha was purchased from Invitrogen (Carlsbad, CA). Great EscAPe SEAP Chemilumi Kit was purchase from Clontech Lab, Inc. (Mountain View, CA).

2.4.2.1 A549SN cell line and culture

A549 cells (ATCC), which are a human lung adenocarcinoma cell line representative of alveolar type II epithelium, were maintained in a 5% CO_2 incubator at 37°C using BME (Cellgrow) supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine (Gibco), 100U/mL penicillin-100 μg/mL streptomycin (Gibco). The plasmid NF-κB-SEAP-ntp was cloned.[117] For NF-κB transcription activity testing, A549 cells were transfected with NF-κB-SEAP-ntp plasmid, this is the A549SN cell line.

2.4.2.2 NF-κB activity – A549SN cell SEAP method

Day1: Plate NF-κB cells: (1) Plate NF-κB_SEAP A549 cells, 6.0x104 cells/well in three 24 well plates, 1000 μL DMEM complete medium: 10% heat inactivated FBS, 2mM L-Glu, 100U/mL or 100 μg/mL Penn/Strep, 500 μg/mL G418. (2) Incubate at 37°C incubator with 5% CO_2. Day2: Treat cells with drugs: Cells are all alive, not confluent (~25-40%). Treat cells with QA=1μM, or other compounds @ 1 μM (990 μL 1% FBS DMEM + 10 μl (10/500) PBS diluted compounds from 5mM in DMSO), + 10 ng/mL TNFα, for 24 hrs. Day 3: Measure SEAP activity: 24 h later, take out 50μL medium. Store at –20°C. Use Clontech "Great EscAPe SEAP Chemiluminescence Detection Kit" (Cat# K2041-1) to measure luminescence through microplate luninometer (Packard αHT microplate reader). Measure 1 second for once at RT. Cells left in plate were lysed in 250 μl lysis buffer (10mMK_2HPO_4; 1mMKH_2PO_4; 1%Triton X100; 1mMDTT) for protein quantification (Pierce BCA Protein Assay Kit, Microplate Procedure) and SEAP activity was normalized to the total protein content. Inhibitory potency (IC_{50}) was determined from dose-response curves (n=3, separate experiment).

2.4.2.3 MTS -cytotoxicity studies in cultured human lung adenocarcinoma A549SN cells

A549SN cells were suspended in culture medium at a density of 10×10^4 cells/mL. Then 1×10^4 cells in 200 μL were plated into 96-well flat-bottom plates. Following incubation for 24 hours at 37°C, drugs, vehicles and controls consisting only of medium and cells were dispensed in 200 μL volumes in duplicate into the appropriate wells, and incubated for 18 hours. QA derivatives were tested at concentrations ranging from 0.01 to 100 uM. Cell viability was assessed by the MTS-CellTiter 96® aqueous non-radioactive cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's protocol after 2 hours of culture. The number of living cells in the culture

is directly proportional to the absorbance at 490 nm by a formazan product bioreduced from MTS by living cells. Absorbance was measured using a DTX 880 multimode detector (Beckman Coulter, Fullerton, CA). The absorbance of media-only well was subtracted from reading of control or treated wells.

2.4.2.4 Anti-oxidant activity test method

The DPPH and all standard antioxidant compounds including trolox were dissolved in 50% acetone. The DPPH stock solution at a concentration of 0.625 mM was prepared monthly and kept at 4°C in dark. The 0.208 mM fresh DPPH working solution was made daily by further diluting the stock solution in 50% acetone for each test. Stock solutions of CA, CGA, AA and compound **3** were prepared in 50% acetone at concentrations of 10 mM, respectively, and stored at 4°C. A series of working solutions were made by appropriate dilutions of the above standard phenolic acid stock solutions with 50% acetone.

2.4.2.5 Conventional colorimetric analysis

The conventional colorimetric DPPH scavenging capacity assay was performed according to a previously described laboratory protocol.[116] Briefly, an aliquot of 500 μL of different concentrations of sample extracts in 50% acetone was added to 500 μL of 0.208 mM DPPH solution. The initial concentration was 0.104 mM for DPPH in all reaction mixtures. Each mixture was vortexed for a few seconds and test immediately. The absorbance (*A*) of each reaction mixture at 515 nm was measured against a blank of 50% acetone using a UV-visible spectrometer. The level of DPPH remaining for each reaction time was calculated as: % DPPH remaining=($A_{\text{sample-t}} / A_{\text{control}}$) x 100.

2.4.2.6 Carrageenan induced paw edema

Carrageenan 1% in sterile filtered saline (10 mg/mL) using lysing matrix D tubes, add saline to the tube first, then add Carrageenan, vortex for 2-3 minutes, heat in 60°C water bath for 1 hour, vortex 2-3 min, inject 100 μL into plantar region of right hind paw. Dexamethasone: 0.5 mg/kg in normal saline with 2% EtOH administered intra-peritoneally (i.p.) Recommended volume 10 mL/kg (2-3 mL depending on weight). Should be ~ 200-300 g per rat, will prepare 100 μg/mL solution and calculate the injection volume based on the weight of the rat. Treatment: 50 mg/kg in 90% normal saline, 5% EtOH, 5% stepantex administered i.p. Rat were tattooed to draw line on L and R hind paw below ankle and above plantar region. Take R and L paw measurement before treatment. Inject drug solution or vehicle into the lower right quadrant by i.p. Inject 100 μL 1%Carrageenan saline solution into plantar region of R hind paw. Inject 100 μL Normal Saline into plantar region of L hind paw. Measure volume of both paws at 1, 3, 5 hours.

CHAPTER 3. DESIGN AND SYNTHESIS OF QUINIC ACID DERIVATIVE ESTERS AND COMPOUND WITH DOUBLE BOND

3.1 Introduction

Hot water Cat's claw extracts (e.g., C-Med 100) have very low alkaloid content (<0.5%) and yet retain significant immune enhancing activity. Recently, quinic acid (QA) esters have been identified as biologically active components in the extract.[1] Base hydrolysis of the extract dramatically reduces biological activity. For example, the lactone ester of QA (QAL) inhibits proliferation of mitogen-stimulated mouse lymphocytes, whereas QA does not affect proliferation.[2] Further, base hydrolysis of the extract dramatically reduces its anti-proliferative effect against HL-60 and human mononuclear cells.[1] Together, these data suggest the ester derivatives of QA found in the extract comprise a significant fraction of the biological activity.

The first three steps of QA catabolism are an NAD-dependent oxidation to form dehydroquinate, followed by two successive dehydrations to form dehydroshikimate and shikimate.[16] Several research groups have made QA derivatives to target the QA catabolism enzymes. Kim made analogues as influenza neuraminidase inhibitors with potent snti-anfluenza activity.[91] Sanchez-Sixto and Metaferia made compounds to kill *Mycobacterium tuberculosis*.[92,93] Payne designed derivatives to act as type II dehydroquinase inhibitors.[94] We have identified water soluble QA amide **3** which possess potent anti-inflammatory activity.[118,119] QA amide lead **3** will serve as a new template for anti-inflammatory agents. We plan to investigate the importance of the hydroxyl groups on the cyclohexane ring by preparing the dehydroquinate amide **3** analogs. We also plan to investigate the importance of double bonds on the cyclohexane ring by preparing the dehydroquinate double bond amide **3** analogs. We hypothesize these modifications will retain anti-inflammatory activity with enhanced resistance to microbial degradation. Because the first three steps of QA catabolism by bacteria are to form dehydroquinate, followed by two successive dehydrations to form dehydroshikimate and shikimate.[16] We assume QA degradation by the bacteria will not take place when the new substrates lack of hydroxyl groups and/or having a double bond.

Chlorogenic acid (3-caffeoyl-D-quinic acid; CGA) is an ester formed between caffeic acid (CA) and QA. CGA and other polyphenolic compounds found in fruits act as potent antioxidants. For example, CGA and CA are both capable of scavenging $NO^{\bullet}$,[64] a pro-inflammatory oxygen radical produced via the L-arginine pathway by infiltrating leucocytes.[65] Interestingly, QA does not possess antioxidant activity suggesting alternative mechanisms underlie its anti-inflammatory activity.[64] In 2001, Sefkow has synthesized CGA, 1-, 4-, and 5-Caffeoylquinic Acid as antioxidant.[95,96] Those esters have many other properties such as antibacterial, antimutagenic, antitumor, and antiviral.[97] Metaferia synthesized QA derivatives macrolides that inhibit breast cancer cell migration in vitro.[98] QA amide **3** does not possess antioxidant activity as showed in Chapter 2. Based on QA amide **3** lead which serves as a new template for anti-inflammatory agents, we are now planning to investigate to see if antioxidant groups are

added for having both anti-inflammatory and antioxidant activity. We will prepare a serial of QA amide ester analogs with antioxidant groups such as caffeic acid, sinapic acid. We hypothesize these modifications will retain anti-inflammatory activity with additional antioxidant property. Furthermore, they will be hydrolyzed by the microflora, for an example, CGA is hydrolyzed into Caffeic acid and QA.[77,78] Both caffeic acid and QA amide will not be consumed by gut bacteria, and easily be absorbed in animal digestive tract.

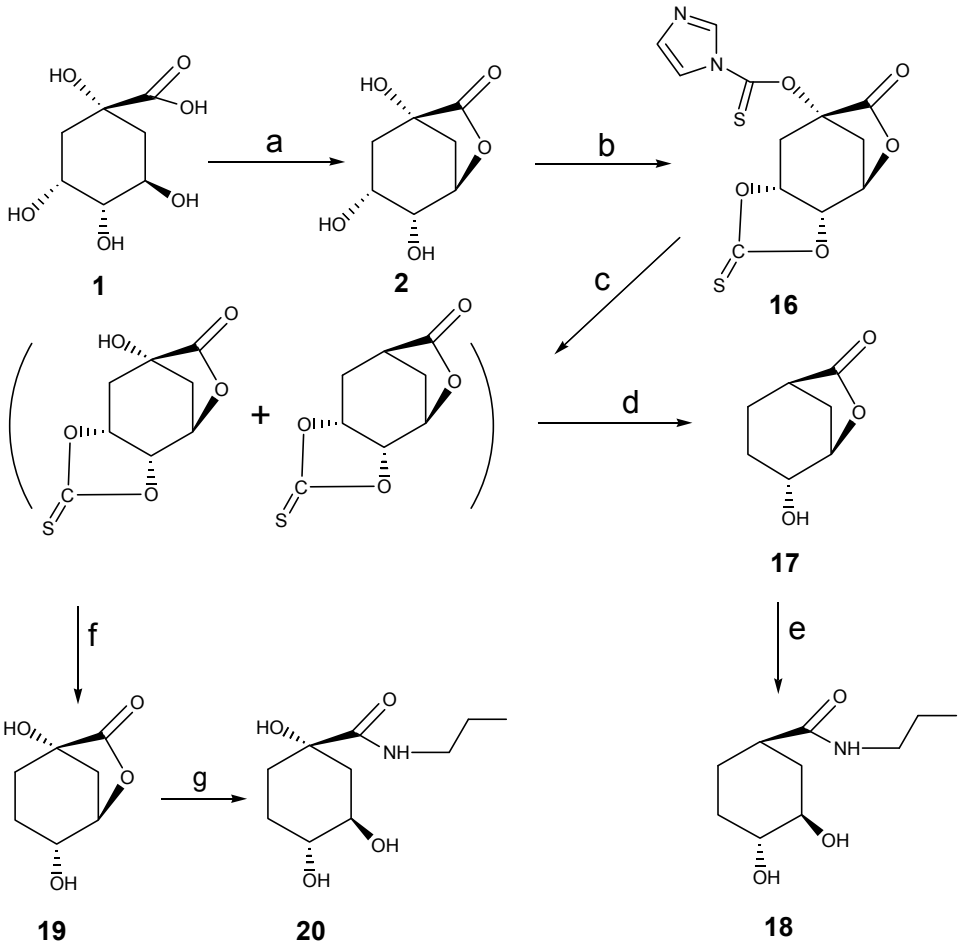
3.2 Results and Discussion

3.2.1 Synthesis of Quinic Acid Derivatives

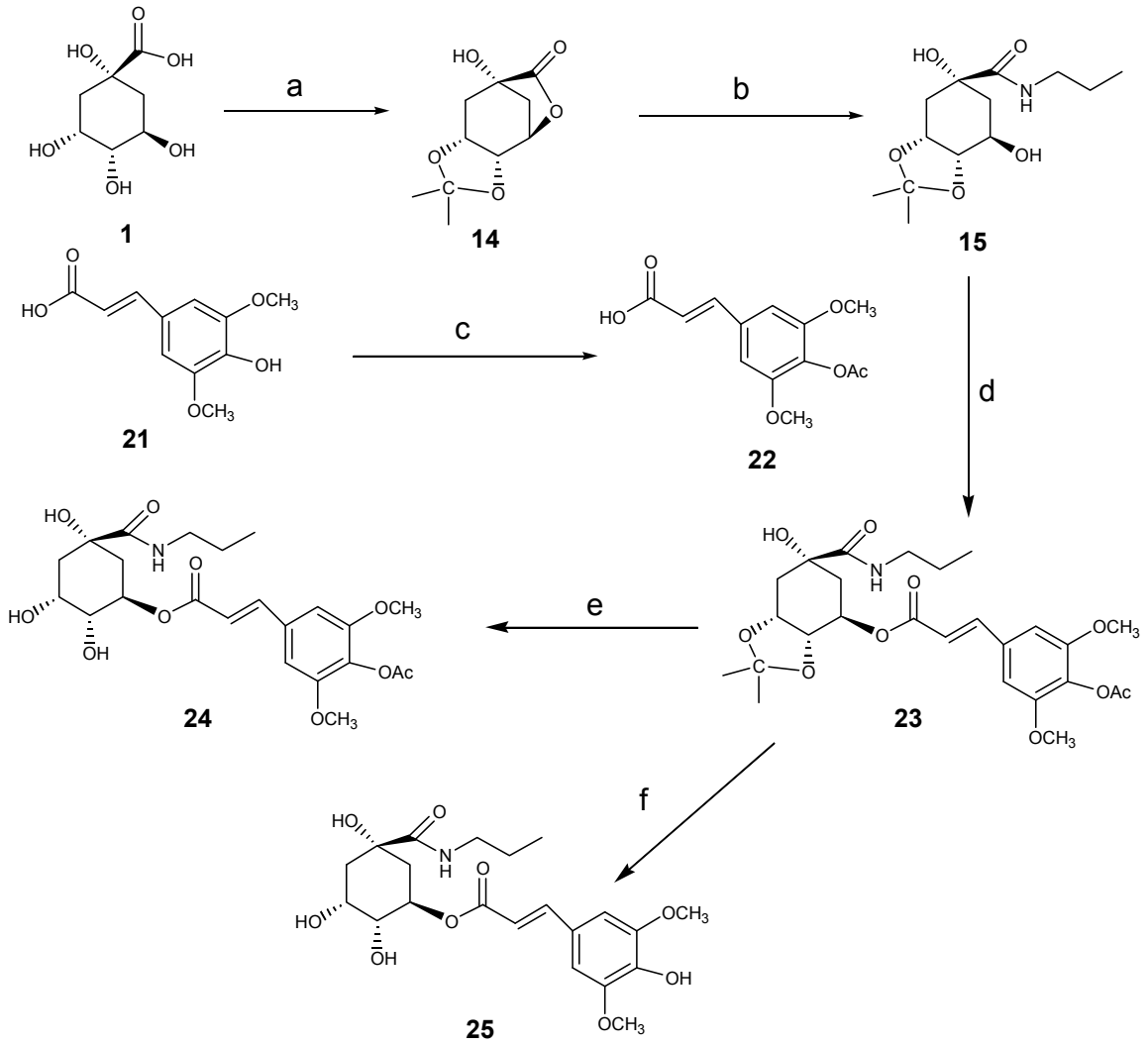
Synthesis of 3, 4-Dihydroxy-cyclohexanecarboxylic acid propylamide **18** and 1,3,4-Trihydroxy-cyclohexanecarboxylic acid propylamide **20** were carried out as shown in Scheme 3-1.[67, 68, 114, 120] The synthesis of the lactone **2** was carried out using quinic acid **1** and PTSA in refluxing benzene and DMF according to the procedure of Neelu Kaila et al. with minor revision. The lactone **2** was then allowed to refluxed with ClC(S)Cl and imidazole in $ClCH_2CH_2Cl$, and then resulting imidazole-1-carbothioic acid O-(9-oxo-4-thioxo-3,5,10-trioxa tricyclo[6.2.1.02,6] undec-8-yl) ester **16.** Compound **16** was treated with Bu_3SnH/xylene, then Bu_3SnH and AIBN/xylene to give compounds 4-Hydroxy-6-oxa-bicyclo[3.2.1]octan-7-one **17** and 1,4-Dihydroxy-6-oxa-bicyclo[3.2.1]octan-7-one **19.** The lactones **17** and **19** were then allowed to react with N-propyl amine with acetic acid at 85°C, and then resulting amides **18,** and **20** respectively**.** Amides **18,** and **20** were purified using flash chromatography.[114]

Synthesis of 3-(4-Hydroxy-3, 5-dimethoxy-phenyl)-acrylic acid 2,3,5-trihydroxy-5-propylcarbamoyl-cyclohexyl ester **25** was carried out as shown in Scheme 3-2.[96,115,121] The synthesis of the lactone **14** was carried out using quinic acid **1** and PTSA in refluxing 2,2-DMP and acetone. The lactone **14** was then allowed to to react with N-propyl amine with acetic acid at 85°C, and then resulting amide **15.** The synthesis of compound **22** was carried out using sinapic acid **21** treated with Ac_2O in pyridine, DMAP, at room temperature; Compounds **15** and **22** were mixed in CH_2Cl_2, treated with DMAP, DIC, at room temperature, and then resulting 3-(4-Acetoxy-3,5-dimethoxy-phenyl)-acrylic acid 6-hydroxy-2,2-dimethyl-6-propylcarbamoyl-hexahydro-benzo[1,3]dioxol-4-yl ester **23.** Ester **23** was treated with 1N HCl for 20 min, obtained compound **24**; ester **23** was treated with 1N HCl for 7 days, obtained compound **25**. Compounds **23**, **24**, and **25** were purified using flash chromatography.

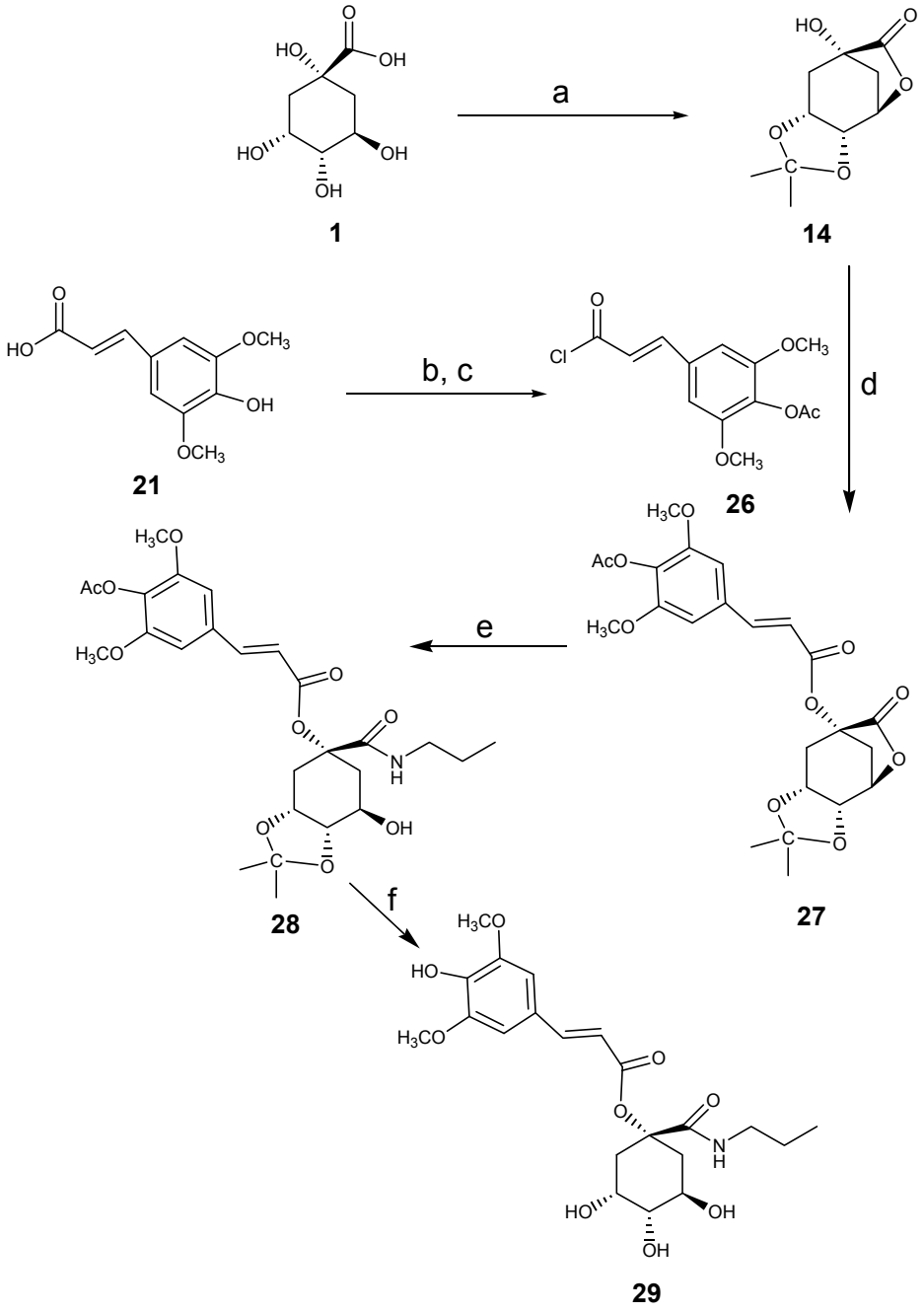
3-(4-Hydroxy-3, 5-dimethoxy-phenyl)-acrylic acid 3,4,5-trihydroxy-1-propylcarbamoyl-cyclohexyl ester **29** was synthesized as shown in Scheme 3-3.[96,115,121] The synthesis of the lactone **14** was carried out using quinic acid **1** and PTSA in refluxing 2,2-DMP and acetone.The synthesis of compound **26** was carried out in two steps, first using sinapic acid **21** treated with Ac_2O in pyridine, DMAP, at room temperature; then the product was refluxed in $SOCl_2$ and CH_2Cl_2. Compounds **14** and **26** were mixed in



Scheme 3-1**.** Synthetic scheme of compounds **18** and **20**. Reagents and conditions: (a) PTSA, DMF, C_6H_6, Dean-Stark, reflux, 78%; (b) ClC(S)Cl, imid, $ClCH_2CH_2Cl$, reflux; (c) Bu_3SnH, xylene, reflux; (d) Bu_3SnH, AIBN, xylene, reflux; (e) C_3H_7-NH_2, AcOH, oil bath 85°C; (f) Bu_3SnH, AIBN, xylene, reflux; (g) C_3H_7-NH_2, AcOH, oil bath 85°C.



Scheme 3-2**.** Synthetic scheme of compound **25**. Reagents and conditions: (a) PTSA, 2,2-DMP, Acetone, reflux, 80%; (b) C_3H_7-NH_2, AcOH, oil bath 85°C; (c) Ac_2O, Pyridine, DMAP, rt; (d) CH_2Cl_2, DMAP, DIC, rt; (e) 1N HCl, rt, 20 min; (f) 1N HCl, rt, 7 days.



Scheme 3-3**.** Synthetic scheme of compound **29**. Reagents and conditions: (a) PTSA, 2,2-DMP, Acetone, reflux, 80%; (b) Ac_2O, Pyridine, DMAP, rt; (c)$SOCl_2$, CH_2Cl_2, reflux; (d) CH_2Cl_2, Pyridine, DMAP, rt; (e) C_3H_7-NH_2, AcOH, oil bath 85°C; (f) 1N HCl, rt, 5 days.

CH_2Cl_2, treated with DMAP, Pyridine, at room temperature, and then resulting ester **27.** Ester **27** was allowed to react with N-propyl amine with acetic acid at 85°C, and then resulting ester **28**; Compound **29** was carried out using ester **28** treated with 1N HCl for 5 days. Compounds **29** were purified using flash chromatography.

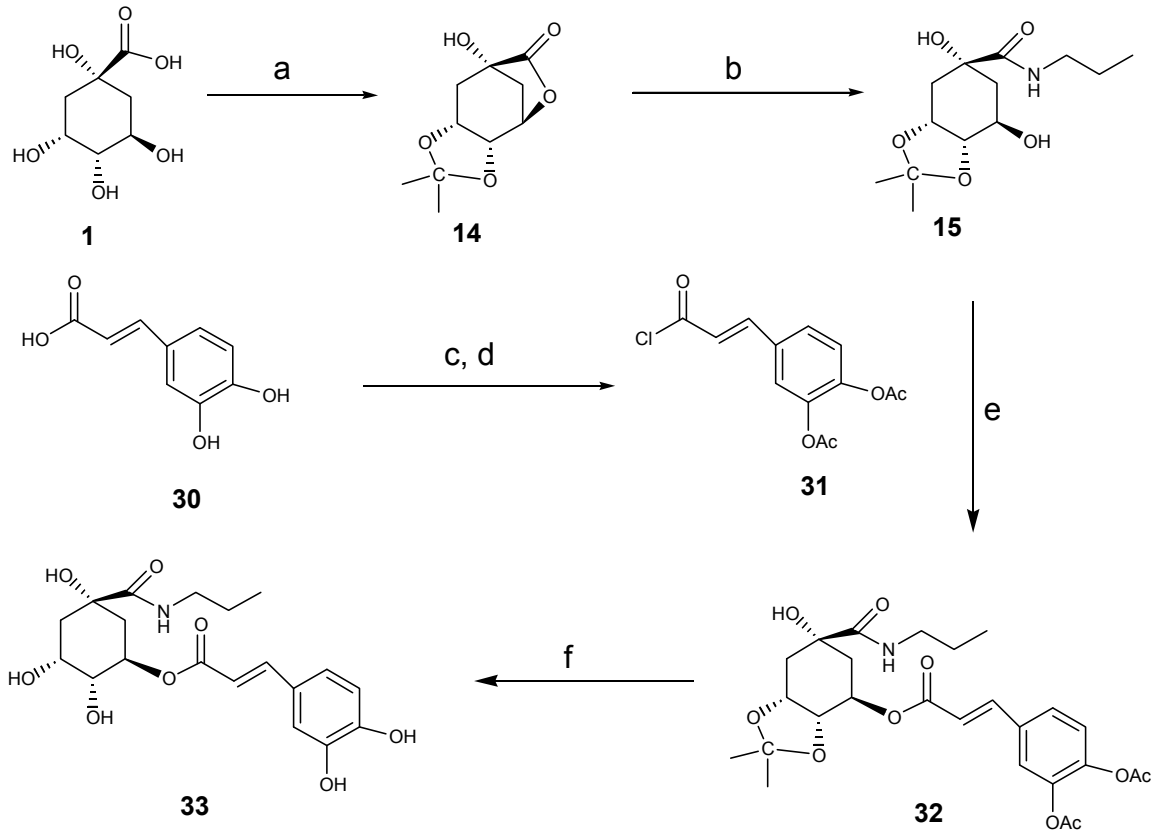
3-(4-Hydroxy-3,5-dimethoxy-phenyl)-acrylic acid 2,3,5-trihydroxy-5-propylcarbamoyl-cyclohexyl ester **33** was synthesized as shown in Scheme 3-4.[96,115,121] The synthesis of the lactone **14** was carried out using quinic acid **1** and PTSA in refluxing 2,2-DMP and acetone. lactone **14** was allowed to react with N-propyl amine with acetic acid at 85°C, and then resulting ester **15**; The synthesis of compound **31** was carried out in two steps, first using caffeic acid **30** treated with Ac_2O in pyridine, DMAP, at room temperature; then the product was refluxed in $SOCl_2$ and CH_2Cl_2. Compounds **15** and **31** were mixed in CH_2Cl_2, treated with DMAP, Pyridine, at room temperature, and then resulting ester **32.** Compound **33** was carried out using ester **32** treated with 1N HCl for 5 days. Compounds **33** were purified using flash chromatography.

The synthesis of 1,4,5-Trihydroxy-cyclohex-2-enecarboxylic acid propylamide **38** was shown in Scheme 3-5.[122-124] Compound **34** was synthesized by using quinic acid **1** and PTSA in refluxing benzaldehyde and benzene. lactone **34** was allowed to react with NBS, AIBN, reluxed in benzene, and then resulting ester **35**; The synthesis of compound **36** was carried out using compound **35** treated with DBU, *tert*-butyldimethylsilyl chloride in acetonitrile, refluxed. Compounds **36** was allowed to react with N-propyl amine with acetic acid at 85°C, and then resulting amide **37.** Compound **38** was carried out using amide **37** treated with 1M TBAF solution. Compounds **38** were purified using flash chromatography.

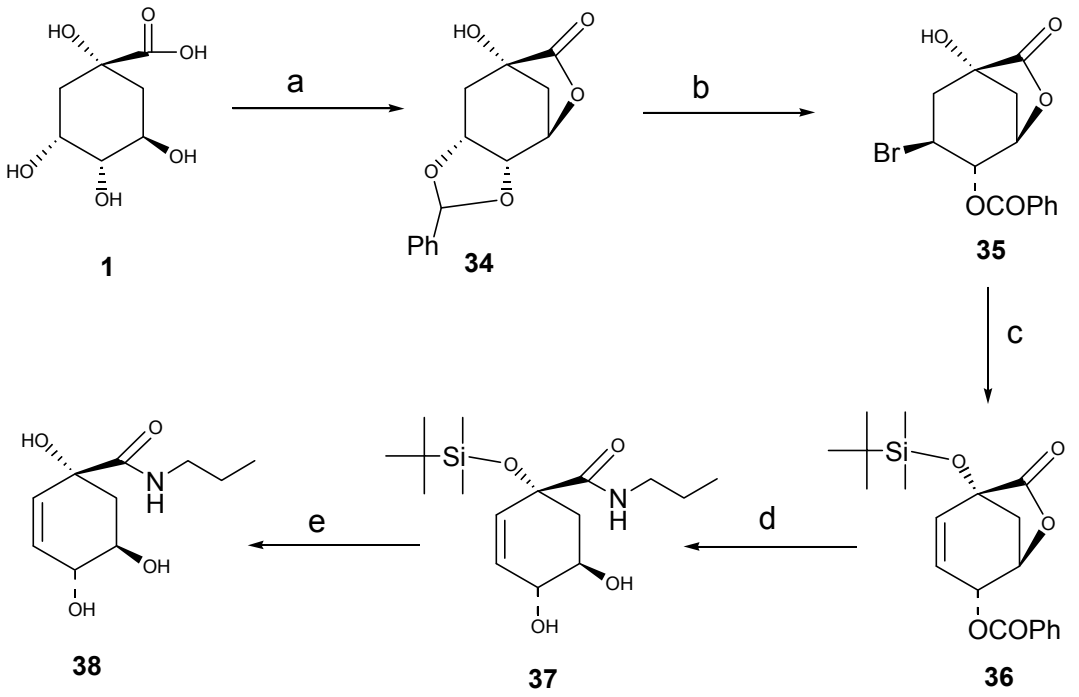
3.2.2 Biology Testing of Dehydroxyl QA Amides and Esters

The results of dehydroxyl QA amides anti-inflammatory high throughput screening are presented in Figure 3-1. Both compounds **18** and **20** showed NF-κB inhibition at concentration 1 μM. The extent of NF-κB inhibition was close to positive control drug dexamethasone. The compounds **18**, **20** and **38** were designed to retain anti-inflammatory activity while having enhanced resistance to microbial degradation. Their biological activities were tested by other researchers. The compounds **25**, **29** and **33** were designed to retain anti-inflammatory activity with additional antioxidant properties. Furthermore, they were hydrolyzed by the microflora, for an example, **25** was hydrolyzed into SA and **3**. Both SA and **3** will not be consumed by gut bacteria, and easily be absorbed in animal digestive tract. This was done by other researchers, and not reported here. Mechanistic studies and pre-clinical efficacy studies of these newly designed compounds in various in vitro and in vivo models are on-going by other researchers, and are not reported in this dissertation.

Next, we determined the anti-oxidant potential of QA derivatives using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method.[116] As expected, caffeic acid (CA), sinapic acid (SA) and compound **25** showed strong anti-oxidant



Scheme 3-4**.** Synthetic scheme of compound **33**. Reagents and conditions: (a) PTSA, 2,2-DMP, Acetone, reflux, 80%; (b) C_3H_7-NH_2, AcOH, oil bath 85°C; (c) Ac_2O, Pyridine, DMAP, rt; (d)$SOCl_2$, CH_2Cl_2, reflux; (e) CH_2Cl_2, Pyridine, DMAP, rt; (f) 1N HCl, rt, 5 days.



Scheme 3-5**.** Synthetic scheme of compound **38**. Reagents and conditions: (a) PTSA, Benzaldehyde, Benzene, reflux; (b) NBS, AIBN, Benzene, reflux; (c) DBU, *tert*-butyldimethylsilyl chloride, Acetonitrile, reflux; (d) C_3H_7-NH_2, AcOH, oil bath 85°C; (e) TBAF, THF.

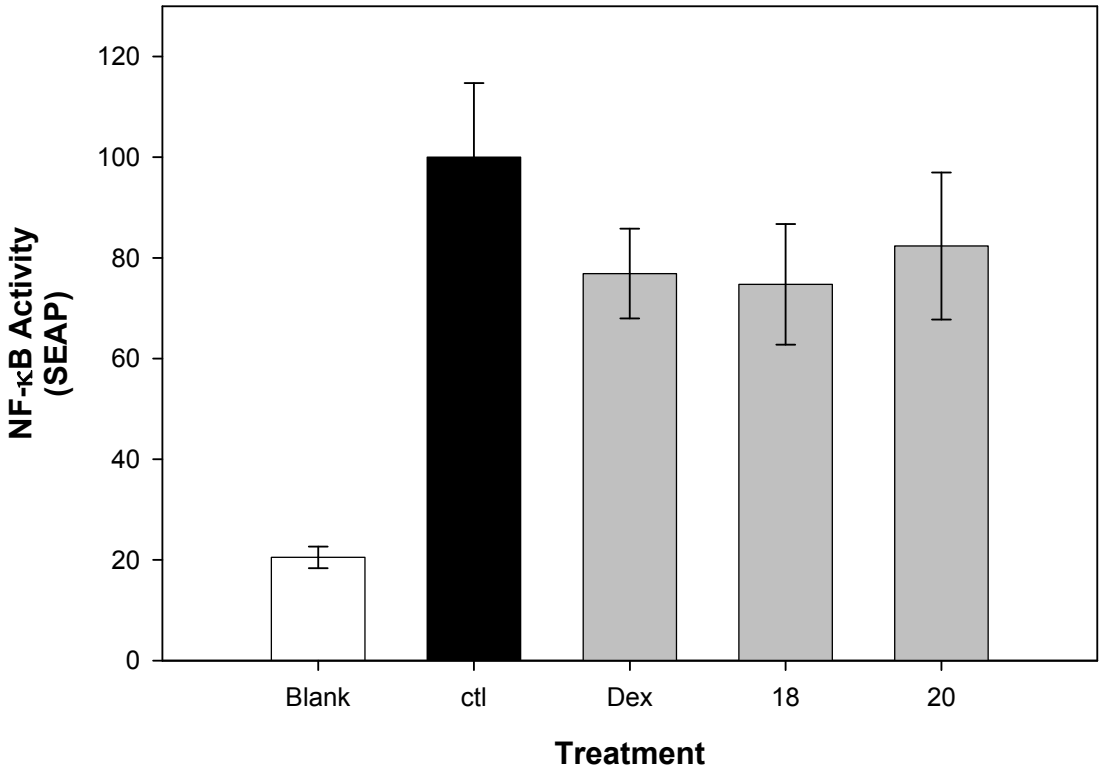


Figure 3-1. NF-κB inhibition by KZ-41 derivatives using A549 cells stably transfected with a secreted alkaline phosphatase (SEAP) reporter. NF-κB activity was measured 18 hours after addition of TNF-α (10 ng/mL) and QA synthesized derivatives (**18**, **20**; 1 μM). Dexamethasone (Dex; 1 μM) was used as a positive control. Data are presented as percent (%) inhibition relative to control (ctl; TNF-α alone) and represent mean % inhibition ±standard deviation (n=3). Blank: media only.

activity, while **24** had no anti-oxidant activity (Figure 3-2). The fact that **24** does not possess anti-oxidant activity is consistent with a previous report.[64] Because it does not bear a free phenolic group could act as antioxidants.[80, 81]

3.3 Conclusions

The dehydroxyl QA amides **18** and **20** were synthesized. They showed NF-κB inhibition at concentration 1 μM. The extent of NF-κB inhibition was close to positive control drug dexamethasone. The dehydroxyl QA amide with a double bond **38** was also synthesized. The QA amide esters **24**, **25**, **29** and **33** were designed and synthesized. As expected, compound **25** showed strong anti-oxidant activity. Furthermore, they were hydrolyzed by the microflora, for an example, **25** was hydrolyzed into SA and **3**. Both SA and **3** will not be consumed by gut bacteria, and easily be absorbed in animal digestive tract. Mechanistic studies and pre-clinical efficacy studies of these newly designed compounds in various in vitro and in vivo models are on-going by other researchers, and are not reported in this dissertation.

3.4 Experimental

3.4.1 Chemistry

All reagents and solvents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO), Fischer Scientific (Pittsburgh, PA), and TCI (Portland, OR), and were used without any further purification. Thin layer chromatography (TLC) was performed on silica gel chromatogram plates purchased from Analtech, Inc. Flash chromatography was performed either on silica gel (60 Å, 200 -425 mesh) or on pre-packed silica gel columns by using a Horizon HPFC system (Biotage, Charlottesville, VA). Molecular masses were collected with electron spray ionization mass spectra (ESI-MS) on a Bruker/Hewlett Packard Esquire LC/MS instrument. Nuclear magnetic resonance (NMR) spectra for ^{1}H NMR and ^{13}C NMR were recorded on a Bruker ARX 300 instrument and/or a Varian Inova-500 MHz instrument. The chemical shifts value (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and coupling constants (J) were reported in hertz. Peaks are abbreviated as were indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Elemental analyses (C, H, N) were performed by Atlantic Microlab Inc. (Norcross, GA), and are accepted within ±0.4 of the theoretical values. High performance liquid chromatography (HPLC) separation was performed on a Hewlett Packard LC instrument with model 1100 system.

Imidazole-1-carbothioic acid O-(9-oxo-4-thioxo-3, 5, 10-trioxa tricycle [6.2.1.02, 6] undec-8-yl) ester (16). In a 250 mL round-bottom flask fitted with a stirring bar, reflux condenser, and argon inlet, 7.0 mL of thiophosgene (90 mmol) was

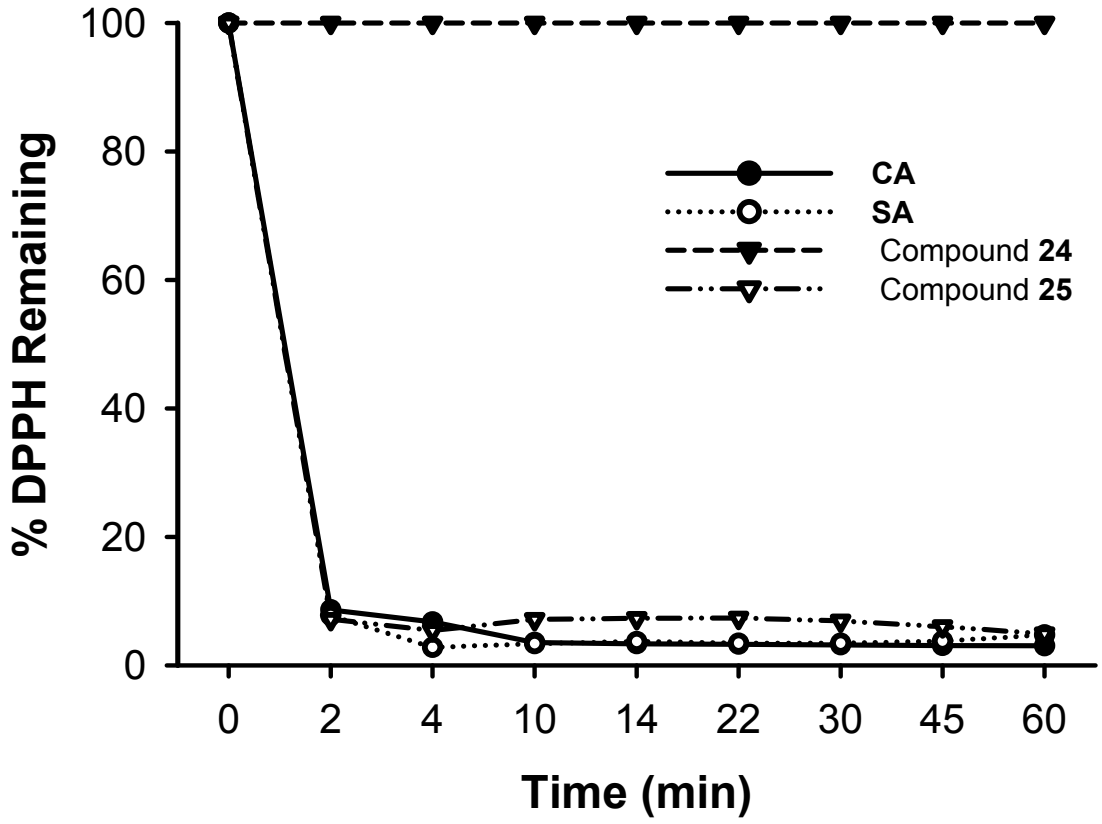


Figure 3-2**.** Determining antioxidant activity by reaction kinetics of DPPH. Caffeic acid (CA), sinapic acid (SA), compound **24** and **25** were prepared in 50% acetone. The conventional colorimetric DPPH scavenging capacity was determined by UV absorption measured at 515 nm. CA, SA and compound **25** showed strong anti-oxidant activity, while **24** had no anti-oxidant activity.

added dropwise under argon to rapidly stirred suspension of imidazole (25g) in dry $ClCH_2CH_2Cl$ (150 mL). Following completion of the addition, the mixture was agitated 30 min. Then added lactone (**1**, 5g, 30 mMol) in one portion, refluxed 3 hr, cooled to rt. The solvent was removed immediately under high vacuum to get dry brown oil. Added cold water (100 mL x 2), washed the residue carefully, then carefully washed the residue with acetone (5 mL x 3), filtered, collect white solid **16**, 4.61 g (47% yield). MS: $[M+Na]^+$: 349; ^{1}H NMR (300 MHz, DMSO) δ 2.40 (d, J=12 Hz, 1H), 2.79 -2.84 (m, 1H), 3.03 (dd, J=15.0, 3.0 Hz, 1H), 3.80 – 3.94 (m, 1H), 5.33-5.40 (m, 2H), 5.63 – 5.70 (m, 1H), 7.12 (d, J=1.0 Hz, 1H), 7.84 (d, J=1 Hz, 1H), 8.54 (s, 1H).

4-Hydroxy-6-oxa-bicyclo[3.2.1]octan-7-one (17). In a 200 mL round-bottom flask fitted with a stirring bar, reflux condenser, 3.26 g Ester **16** (10 mmol), was suspended in 80 mL dry xylene, refluxed under argon. Treated with 2.51 mL TBTH first, after 30 min, added 2.51 mL TBTH more, after another 1 hr, added 4.0 mL TBTH and 0.02 g AIBN, refluxed for 2.5 hr. The solvent was removed under high vacuum. Reaction residue was purified by flash column (Hex : EtOAc =1:1). Collect product **17,** 110 mg (8% yield). R_f 0.13 (Hex : EtOAc =1:1); MS: $[M+Na]^+$: 165; ^{1}H NMR (300 MHz, DMSO) 1.66-1.83 (m, 5H), 2.10 -2.20 (m, 1H), 2.24 (d, J=7.8 Hz, 1H), 2.24 (s, 1H), 3.89 (m, J=4.5 Hz, 1H), 4.54 (t, J=5.4 Hz, 1H), 5.21 (d, J=3.3 Hz, 1H).

3,4-Dihydroxy-cyclohexanecarboxylic acid propylamide (18). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.25g Lactone **17** (1.24 mmol), and propylamine (0.83 mL, 0.6g, 10.13 mmol) were combined, then glacial acetic acid (1.1 mL, 0.75 g, 12.71 mmol) was added. The solution was warmed to 85°C in oil bath for 30 min, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. The solvent was removed under reduced pressure. The residue was purified by flash column with same solvent as TLC. Collect product **17,** 256mg (64% yield). mp: 105-107°C, R_f 0.15 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M+Na]^+$ =224; ^{1}H NMR (300 MHz, DMSO) δ 0.81 (t, J=6.0 Hz, 3H), 1.41-1.61 (m, 5H), 1.76 (d, J=3.0 Hz, 1H), 1.81 (d, J=3.0 Hz, 2H), 2.12 (t, J=1 Hz, 1H), 2.96 (dd, J=6.3, 12.6 Hz, 2H), 3.08 (dd, J=4.5, 9.0 Hz, 2H), 4.55 (d, J=3.0 Hz, 1H), 4.60 (d, J=3.0 Hz, 1H), 7.67 (t, J=5.4 Hz, 1H). Anal. ($C_{10}H_{19}NO_3$) calculated: C, 59.68; H, 9.52; N, 6.96. Found: C, 59.25; H, 9.46; N, 6.91.

1,4-Dihydroxy-6-oxa-bicyclo[3.2.1]octan-7-one (19). In a 200 mL round-bottom flask fitted with a stirring bar, reflux condenser, 3.26 g Ester **16** (10 mmol), was suspended in 80 mL dry xylene, refluxed under argon. Treated with 2.51 mL TBTH first, after 30 min, added 2.51 mL TBTH more, after another 1 hr, added 4.0 mL TBTH and 0.02 g AIBN, refluxed for 2.5 hr. The solvent was removed under high vacuum. Reaction residue was purified by flash column (Hex : EtOAc =1:1). Collect product **19,** 220 mg (14% yield). R_f 0.08 (Hex : EtOAc =1:1); MS: $[M+Na]^+$: 181; ^{1}H NMR (300 MHz, DMSO) 1.66-1.83 (m, 4H), 2.10 -2.20 (m, 1H), 2.24 (d, J=7.8 Hz, 1H), 2.24 (s, 1H), 3.89 (m, J=4.5 Hz, 1H), 4.54 (t, J=5.4 Hz, 1H), 5.21 (d, J=3.3 Hz, 1H), 5.84 (s, 1H).

1,3,4-Trihydroxy-cyclohexanecarboxylic acid propylamide (20). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.30g Lactone **19** (1.90

mmol), and propylamine (0.83 mL, 0.6g, 10.13 mmol) were combined, then glacial acetic acid (1.3 mL, 0.94 g, 15.93 mmol) was added. The solution was warmed to 85°C in oil bath for 30 min, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. The solvent was removed under reduced pressure. The residue was purified by flash column with same solvent as TLC. Collect product **20,** 240 mg (58% yield). mp: 151-154°C, R_f 0.12 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M+Na]^+$ =240; ^1H NMR (300 MHz, DMSO) δ 0.81 (t, J=4.5 Hz, 3H), 1.37-1.44 (m, 3H), 1.51-1.71 (m, 5H), 3.00 (q, J=6.6 Hz, 2H), 3.12 (m, 1H), 3.45 (m, 1H), 4.49 (d, J=1.8 Hz, 1H), 4.52 (d, J=3.0 Hz, 1H), 5.17 (s, 1 H), 7.67 (t, J=5.4 Hz, 1H). Anal. ($C_{10}H_{19}NO_4$) calculated: C, 55.28; H, 8.81; N, 6.45. Found: C, 55.12; H, 8.72; N, 6.50.

3-(4-Acetoxy-3,5-dimethoxy-phenyl)-acrylic acid (22). In a 50 mL round-bottom flask fitted with a stirring bar, 1.0 g Sinapic acid **21** (4.46 mmol), and 0.02g DMAP were combined in 15 mL anhydrous pyridine, then Ac_2O (0.56 mL, 0.57 g, 5.57 mmol) was added under ice bath. The reaction mixture was stirred for 2 hr, then poured onto crushed ice. The aqueous phase was acidified with 2 M aqueous HCl (pH =2) and extracted with EtOAc (3 x 80mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. Titration of the residue with hexane containing small amount of EtOAc afforded acetyl sinapic acid **22** 1.2 g (98% yield), white powder. MS: $[M-H]^-$ =265.

3-(4-Acetoxy-3,5-dimethoxy-phenyl)-acrylic acid 6-hydroxy-2,2-dimethyl-6-propylcarbamoyl-hexahydro-benzo[1,3]dioxol-4-yl ester (23). In a 100 mL round-bottom flask fitted with a stirring bar, lactone (**1**, 0.7g, 2.56 mMol), acetyl sinapic acid **22, (**0.72 g, 2.70 mmol) were combined in 50 mL anhydrous CH_2Cl_2 under argon, 0.05 g DMAP was added, then 0.6 mL DIC was added at rt, stirred for 2 days. Solid urine was filtered out. Reaction mixture was washed with 0.5 N HCl, extracted with CH_2Cl_2 (3 x 80mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. Residue was purified by flash column with solvent mixture of Hexane : Acetone (10/3, v/v). Collect white solid product **23**, 825 mg (62% yield). Rf 0.15 (Hexane:Acetone =10:3); MS: $[M+Na]^+$=544. ^1H NMR (300 MHz, DMSO) 0.81 (t, J=4.5 Hz, 3H), 0.85 (t, J=2.1 Hz, 1H), 1.27 (s, 3H), 1.40 -1.41 (m, 2H), 1.43 (s, 3H), 1.79 (d, J=4.8 Hz, 2H), 1.98 (d, J=9.0 Hz, 1H), 2.25(t, J=4.8 Hz, 3H), 3.02 (m, 2H), 3.80 (s, 6H), 4.14 (t, J=3.9 Hz, 1H), 4.44 (d, J=3.0 Hz, 1H), 5.38 (q, J=4.9 Hz, 1H), 5. 49 (s, 1H), 6.75 (d, J=9.9 Hz, 1H), 7.16 (s, 2H), 7.70 (d, J=9.9 Hz, 1H), 7.76 (t, J=3.6 Hz, 1H).

3-(4-Acetoxy-3,5-dimethoxy-phenyl)-acrylic acid 2,3,5-trihydroxy-5 propylcarbamoyl-cyclohexyl ester (24). In a 50 mL round-bottom flask fitted with a stirring bar, ester **23,** (0.23 g, 0.44 mmol) were dissoved in 5 mL THF, then 15 mL aqueous 1 N HCl was added at rt, stirred for 20 min. Reaction mixture was saturated with Na HCO_3 solution, extracted with EtOAc (3 x 80mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. Collect white solid product **24**, 200 mg (95% yield). MS: $[M+Na]^+$=504. ^1H NMR (300 MHz, DMSO) 0.81 (t, J=4.5 Hz, 3H), 1.25 (m, 2H), 1.41 (m, 2H), 1.78 (d, J=15 Hz, 1H), 1.93 (m, 2H), 2.25(s, 3H), 3.02 (m, 2H), 3.58 (m, 1H), 3.81 (s, 6H), 4.10 (t, J=3.9 Hz,

1H), 5.05 (d, J=6.0 Hz, 1H), 5.28 (q, J=6.3 Hz, 1H), 5. 59 (s, 1H), 5.70 (d, J=3.9 Hz, 1H), 6.75 (d, J=16 Hz, 1H), 7.16 (s, 2H), 7.63 (d, J=16 Hz, 1H), 7.76 (t, J=3.6 Hz, 1H).

3-(4-Hydroxy-3,5-dimethoxy-phenyl)-acrylic acid 2,3,5-trihydroxy-5-propylcarbamoyl-cyclohexyl ester (25). In a 100 mL round-bottom flask fitted with a stirring bar, ester **23, (**0.50 g, 0.96 mmol) were dissoved in 10 mL THF, then 30 mL aqueous 1 N HCl was added at rt. The reaction mixture was stirred at rt, and progress was monitored by ESI-MS. After 7 days, reaction was completed. Reaction mixture was killed with Na HCO_3 solution, and saturated with NaCl, extracted with EtOAc (3 x 80mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. The residue was purified by flash column ($CHCl_3$:MeOH:NH_3OH =100:10:1), Collect white solid product **25**, 210 mg (50% yield). R_f=0.16 ($CHCl_3$:MeOH:NH_3OH =100:10:1), MS: $[M+Na]^+$=462. ^1H NMR (300 MHz, DMSO) 0.80 (t, J=4.5 Hz, 3H), 1.41 (m, 2H), 1.75 (d, J=9.0 Hz, 1H), 1.89 (m, 1H),1.98 (m, 2H), 3.02 (m, 2H), 3.56 (m, 1H), 3.81 (s, 6H), 4.10 (s, 1H), 5.01 (d, J=3.3 Hz, 1H), 5.28 (q, J=3.0 Hz, 1H), 5. 58 (s, 1H), 5.67 (d, J=3.0 Hz, 1H), 6.52 (d, J=9.6 Hz, 1H), 7.02 (s, 2H), 7.56 (d, J=9.6 Hz, 1H), 7.75 (t, J=3.6 Hz, 1H), 8.92 (s, 1H). Anal. ($C_{21}H_{29}NO_9$. 0.33 H_2O) calculated: C, 56.62; H, 6.71; N, 3.14. Found: C, 56.53; H, 6.75; N, 3.00.

Acetic acid 4-(2-chlorocarbonyl-vinyl)-2,6-dimethoxy-phenyl ester (26). In a 50 mL round-bottom flask fitted with a stirring bar, 1.0 g Sinapic acid **21** (4.46 mmol), and 0.02g DMAP were combined in 15 mL anhydrous pyridine, then Ac_2O (0.56 mL, 0.57 g, 5.57 mmol) was added under ice bath. The reaction mixture was stirred for 2 hr, then poured onto crushed ice. The aqueous phase was acidified with 2 M aqueous HCl (pH =2) and extracted with EtOAc (3 x 80mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. Titration of the residue with hexane containing small amount of EtOAc afforded acetyl sinapic acid **22** 1.2 g (98% yield), white powder. MS: $[M-H]^-$ =265.

In a 50 mL round-bottom flask fitted with a stirring bar, 0.6g acetyl sinapic acid **22** (2.26 mmol), was dissolved in 20 mL CH_2Cl_2, then 0.8 mL $SOCl_2$ was added. The reaction mixture was refluxed 3 hr under argon. The solvent was removed under reduced pressure, residue was white solid **26**.

3-(4-Acetoxy-3,5-dimethoxy-phenyl)-acrylic acid 4,4-dimethyl-9-oxo-3,5,10-trioxa-tricyclo[6.2.1]undec-8-yl ester (27). In a 100 mL round-bottom flask fitted with a stirring bar, lactone (**14**, 0.6g, 2.56 mMol), compound (**26,** 2.26 mmol) were combined in 50 mL anhydrous CH_2Cl_2 under argon, 0.5 g DMAP was added, then 10 mL anhydrous pyridine was added at rt, stirred overnight under argon. The solvent was removed under reduced pressure. Reaction mixture was washed with 50 mL 0.5 N HCl, extracted with EtOAc (3 x 50mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. Residue was purified by flash column with solvent mixture of Hexane : EtOAc (7/3, v/v). Collect white solid product **27**, 605 mg (58% yield). Rf 0.14 (Hexane:EtOAc =7:3); MS: $[M+Na]^+$=485. ^1H NMR (300 MHz, DMSO) 1.27 (s, 3H), 1.47 (s, 3H), 2.26(t, J=4.8 Hz, 3H), 2.31 (d, J=3.3 Hz, 1H), 2.43 - 2.46 (m, 2H), 3.02 (m, 1H), 3.81 (s, 6H), 4.58 (d, J=4.9 Hz, 1H), 4.60 (td, J=8.8, 3.0 Hz,

1H), 4.96 (dd, J=6.0, 2.4 Hz, 1H), 6.81 (d, J=16 Hz, 1H), 7.19 (s, 2H), 7.70 (d, J=16 Hz, 1H).

1,3,4,5-tetrahydroxy-cyclohexane-carboxylic acid propylamide (28). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.40 g Lactone **27** (0.87 mmol), and propylamine (0.83 mL, 0.6g, 10.13 mmol) were combined, then glacial acetic acid (0.64 mL, 0.46 g, 7.80 mmol) was added. The solution was warmed to 85°C in oil bath for 45 min, at which time TLC ($CHCl_3$:MeOH:NH_3OH =100:10:1) indicated complete consumption of the starting lactone. The solvent was removed under reduced pressure. Residue was washed with Na HCO_3 solution, and saturated with NaCl, extracted with EtOAc (3 x 80mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. Collect product white solid **28,** one spot, R_f 0.5 ($CHCl_3$:MeOH:NH_3OH =100:10:1); MS: $[M+NH_4]^+$ =539; used for next reaction without further purification.

3-(4-Hydroxy-3,5-dimethoxy-phenyl)-acrylic acid 2,3,5-trihydroxy-5-propylcarbamoyl-cyclohexyl ester (29). In a 100 mL round-bottom flask fitted with a stirring bar, ester **28,** (0.70 mmol) were dissoved in 5 mL THF, then 15 mL aqueous 1 N HCl was added at rt. The reaction mixture was stirred at rt, and progress was monitored by ESI-MS. After 5 days, reaction was completed. Reaction mixture was killed with $NaHCO_3$ solution, and saturated with NaCl, extracted with EtOAc (3 x 80mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. The residue was purified by flash column with solvent ($CHCl_3$:MeOH:NH_3OH =100:10:1), Collect white solid product **29**, 105 mg (34% yield). R_f=0.06 ($CHCl_3$:MeOH:NH_3OH =100:10:1), MS: $[M+Na]^+$=462. ^1H NMR (300 MHz, DMSO) 0.80 (t, J=7.2 Hz, 3H), 1.32 - 1.44 (m, 2H), 1.95 -2.05 (m, 4H),2.40(m, 1H), 3.02 (m, 2H), 3.57 (m, 2H), 3.81 (s, 6H), 4.35(d, J=4.5 Hz, 1H), 4.65 (d, J=4.5 Hz,1H), 5.34 (d, J=5.4 Hz, 1H), 6.45 (d, J=16 Hz, 1H), 7.00 (s, 2H), 7.50 (d, J=16 Hz, 1H), 8.03 (t, J=5.7 Hz, 1H), 8.94 (s, 1H). Anal. ($C_{21}H_{29}NO_9$. $1.33H_2O$) calculated: C, 54.42; H, 6.89; N, 3.02. Found: C, 54.56; H, 6.79; N, 2.98.

Acetic acid 2-acetoxy-4-(2-chlorocarbonyl-vinyl)-phenyl ester (31). In a 50 mL round-bottom flask fitted with a stirring bar, 1.0 g caffeic acid **30** (5.61 mmol), and 0.02g DMAP were combined in 15 mL anhydrous pyridine, then Ac_2O (1.4 mL, 1.42 g, 14.01 mmol) was added under ice bath. The reaction mixture was stirred for 2 hr, then poured onto crushed ice. The aqueous phase was acidified with 2 M aqueous HCl (pH =2) and extracted with EtOAc (3 x 80mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. Titration of the residue with hexane containing small amount of EtOAc afforded acetyl caffeic acid 1.2 g (98% yield), white powder. MS: $[M-H]^-$ =263.

In a 50 mL round-bottom flask fitted with a stirring bar, 0.7g acetyl caffeic acid (2.65 mmol), was dissolved in 20 mL CH_2Cl_2, then 0.8 mL $SOCl_2$ was added. The reaction mixture was refluxed 3 hr under argon. The solvent was removed under reduced pressure, residue was white solid **31**.

3-(3,4-Diacetoxy-phenyl)-acrylic acid 6-hydroxy-2,2-dimethyl-6-propylcarbamoyl-hexahydro-benzo[1,3]dioxol-4-yl ester (32). In a 100 mL round-bottom flask fitted with a stirring bar, compound **15** (0.7g, 2.56 mMol), compound (**31,** 2.26 mmol) were combined in 50 mL anhydrous CH_2Cl_2 under argon, 0.05 g DMAP was added, then 10 mL anhydrous pyridine was added at rt, stirred overnight under argon. The solvent was removed under reduced pressure. Reaction mixture was washed with 50 mL 0.5 N HCl, extracted with EtOAc (3 x 50mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. Residue was purified by flash column with solvent mixture of CH_2Cl_2:MeOH(98/2, v/v). Collect white solid product **32**, 750 mg (56% yield). Rf 0.44 (CH_2Cl_2:MeOH =98:2); MS: $[M+Na]^+$=442. ^{1}H NMR (300 MHz, DMSO) 0.81 (t, J=7.5 Hz, 3H),1.26 (s, 3H), 1.33 - 1.40 (m, 2H), 1.41(s, 3H),1.78 (d, J=9.0 Hz, 2H),1.99 (d, J=5.0 Hz, 1H), 2.24 – 2.30 (m, 2H), 2.31 (s, 6H), 2.98 – 3.06 (m, 2H), 4.13 (m, 1H), 4.42 (m, 1H), 5.31 – 5.41 (m, 1H), 5.46 (s, 1H), 6.64 (d, J=16 Hz, 1H), 7.32 (d, J=8.4, 1H), 7.65 (d, J=16 Hz, 1H), 7.64 - 7.68 (m, 2H).

3-(4-Hydroxy-3,5-dimethoxy-phenyl)-acrylic acid 2,3,5-trihydroxy-5-propylcarbamoyl-cyclohexyl ester (33). In a 100 mL round-bottom flask fitted with a stirring bar, ester **32** (0.84g, 1.62 mmol) were dissoved in 10 mL THF, then 40 mL aqueous 1 N HCl was added at rt. The reaction mixture was stirred at rt, and progress was monitored by ESI-MS. After 4 days, reaction was completed. Reaction mixture was killed with $NaHCO_3$ solution, and saturated with NaCl, extracted with EtOAc (3 x 80mL). The combined organic extracts were dried over Na_2SO_4, filtered, the solvent was removed under reduced pressure. The residue was purified by flash column with solvent (CH_2Cl_2 : MeOH =10:1), Collect white solid product **33**, 260 mg (41% yield). R_f=0.26 (CH_2Cl_2 : MeOH =10:1), MS: $[M+Na]^+$=418. ^{1}H NMR (300 MHz, DMSO) 0.80 (t, J=7.5 Hz, 3H), 1.37 - 1.44 (m, 2H), 1.71 (d, J=13.80 Hz, 1H), 1.87 -1.995 (m, 3H), 3.02 (m, 2H), 3.57 (m, 1H), 4.06 (s, 1H), 5.03 (s, 1H), 5.24(dt, J=4.0, 12.3 Hz, 1H), 5.57 (s, 1H), 5.65 (s, 1H), 6.23 (d, J=16 Hz, 1H), 6.76 (d, J=8.1 Hz, 1H), 7.01 (d, J=8.1 Hz, 1H), 7.03 (s, 1H), 7.50 (d, J=16 Hz, 1H), 7.73 (t, J=6.0 Hz, 1H), 9.14 (s, 1H), 9.56 (s, 1H). Anal. ($C_{19}H_{25}NO_8$. $0.5H_2O$) calculated: C, 56.79; H, 6.84; N, 3.40. Found: C, 56.63; H, 6.63; N, 3.21.

8-Hydroxy-4-phenyl-3,5,10-trioxa-tricyclo[6.2.1.0^{2,6}]undecan-9-one (34). In a 200 mL round-bottom flask fitted with a stirring bar, reflux condenser, Dean-Stark trap, and argon inlet, A mixture of quinic acid **1** (5.0g, 26.1 mmol), p-totuenesulfonic acid monohydrate (0.2g), benzaldehyde (4 mL, 39 mMol), and benzene (100 mL) was heated to reflux for overnight. The reaction mixture was cooled down, and solvent was removed under reduced pressure. Residue was purified by flash column with solvent mixture of Hex : EtOAc(1/1, v/v). Collected product **34** as oil that solidified on standing, 6.2g (91% yield). R_f=0.2 (EtoAC: Hex=1:1). MS: $[M+Na]^+$=285. ^{1}H NMR ($CDCl_3$) δ 2.42 – 2.53 (m, 4H), 2.82 (d, J=12.0 Hz, 1H), 4.40-4.43 (m, 1H), 4.55-4.60 (m, 1H), 4.84-4.87 (m, 1H), 5.79 (s, 1H), 7.40-7.53 (m, 5H).

Benzoic acid 3-bromo-1-hydroxy-7-oxo-6-oxa-bicyclo[3.2.1]oct-4-yl ester (35). In a 200 mL round-bottom flask fitted with a stirring bar, reflux condenser, and argon

inlet, A mixture of compound **34** (2.0g, 7.63 mmol), NBS (1.42g, 7.98 mmol), AIBN (0.01g), and benzene (120 mL) was heated to reflux for 1 hr under argon. The reaction mixture was cooled down, and solvent was removed under reduced pressure. Residue was dissolved in EtOAc, washed with saturated $NaHCO_3$ aqueous solution, collected organic phase. Rough compound was purified by flash column with solvent mixture of CH_2Cl_2: EtOAc (98/2, v/v). Collected product **35** as white solid, 2.2g (85% yield). R_f=0.4 (EtOAc: CH_2Cl_2=2:98). MS: $[M+Na]^+$=363. ^1H NMR ($CDCl_3$) δ 2.22 – 2.63 (m, 3H), 2.77-2.85(m, 2H), 4.50 (d, J=6.6, 1H), 5.03-5.07 (m, 1H), 5.70 (s, 1H), 7.48-7.54 (m, 2H), 7.63-7.68 (m, 1H), 8.03 (d, J=9.0 Hz, 2H).

Benzoic acid 1-(tert-butyl-dimethyl-silanyloxy)-7-oxo-6-oxa-bicyclo[3.2.1]oct-2-en-4-yl ester (36). In a 100 mL round-bottom flask fitted with a stirring bar, reflux condenser, and argon inlet, A mixture of compound **35** (1.85g, 5.44 mmol), DBU (2.4 mL, 16.1 mmol), *tert*-butyldimethylsilyl chloride (1.12 g, 7.43 mmol), and acetonitrile (50 mL) was heated to reflux for overnight under argon. The reaction mixture was cooled down, and solvent was removed under reduced pressure. Residue was purified by flash column. Collected product **36** as white solid, 1.42g (70% yield). MS: $[M+Na]^+$=397. ^1H NMR ($CDCl_3$) δ 0.19 (s, 3H), 0.23 (s, 3H), 0.95(s, 9H), 2.42 (d, J=11.0, 1H), 2.50-2.54 (m, 1H), 4.84 (t, J=2.5 Hz, 1H), 5.52 (t, J=1.8 Hz, 1H), 5.86 (dt, J=10.0, 1.8 Hz, 1H), 6.29 (d, J=10.0 Hz, 1H), 7.48 (t, J=7.5 Hz, 2H), 7.62 (t, J=7.5 Hz, 1H), 8.05 (d, J=7.5 Hz, 2H).

1-(tert-Butyl-dimethyl-silanyloxy)-4,5-dihydroxy-cyclohex-2-enecarboxylic acid propylamide (37). In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, and argon inlet, A mixture of compound **36** (1.30g, 3.48 mmol), and propylamine (2.60 mL, 1.85g, 31.28 mmol) were combined, then glacial acetic acid (0.60 mL, 0.63 g, 10.43 mmol) was added. The solution was warmed to 85°C in oil bath for 60 min under argon. The reaction mixture was cooled down, and solvent was removed under reduced pressure. Residue was purified by flash column with solvent EtOAc: Hex=4:1. Collected product **37** as white solid, 0.52 g (45% yield). MS: $[M+H]^+$=330. R_f=0.17 (EtOAc: Hex=4:1). ^1H NMR (DMSO) δ 0.05(s, 3H), 0.07 (s, 3H), 0.82 (t, J=7.5 Hz, 3H), 0.85(s, 9H), 1.37-1.47 (m, 2H), 1.69 (t, J=12.6, 1H), 1.85-1.91(m, 1H), 2.96-3.12(m, 2H), 3.57-3.65 (m, 1H), 3.75 – 3.79 (m, 1H), 4.92 (d, J=4.8 Hz, 1H), 5.07 (d, J=4.8 Hz, 1H), 5.56 (d, J=10.0 Hz, 1H), 5.69 (dd, J=10.0, 1.8 Hz, 1H), 7.21 (t, J=3.0 Hz, 1H).

1,4,5-Trihydroxy-cyclohex-2-enecarboxylic acid propylamide (38). In a 100 mL round-bottom flask fitted with a stirring bar, compound **37** (0.42g, 1.28 mmol) was dissolved in 50 mL THF under ice bath, then 1 M solution of TBAF (2.0 mL, 2.0 mmol) was added. The solution was stirred for 1 hr at rt. The solvent was removed under reduced pressure. Residue was dissolved in methanol, the solution went through DOWEX 50W *8-200 strongly acidic cation exchanger column to remove TBAF. Product was further purified by flash column with solvent CH_2Cl_2: MeOH=96: 4. Collected product **38**, 0.15 g (50% yield). MS: $[M+Na]^+$=238. R_f=0.15 (CH_2Cl_2: MeOH =96:4). ^1H NMR (DMSO) δ 0.81 (t, J=7.5 Hz, 3H), 1.37-1.45 (m, 2H), 1.69-1.72(m, 1H), 1.87(t, J=12.3, 1H), 2.96-3.05(m, 2H), 3.57-3.65 (m, 1H), 3.73 – 3.79 (m, 1H), 4.8

(d, J=6.1 Hz, 1H), 5.0 (d, J=4.8 Hz, 1H), 5.37 (dt, J=10.0, 1.8 Hz, 1H), 5.61 (d, J=1.8 Hz, 1H), 5.64 (s, 1H), 7.78 (t, J=6.0 Hz, 1H). Anal. ($C_{10}H_{17}NO_4.0.5H_2O$) calculated: C, 53.56; H, 8.09; N, 6.25. Found: C, 53.75; H, 8.17; N, 5.93.

3.4.2 Biology

All chemicals and solvents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) if not specified, and were used without any further purification. TNF-alpha was purchased from Invitrogen (Carlsbad, CA). Great EscAPe SEAP Chemilumi Kit was purchase from Clontech Lab, Inc. (Mountain View, CA).

3.4.2.1 A549SN cell line and culture

A549 cells (ATCC), which are a human lung adenocarcinoma cell line representative of alveolar type II epithelium, were maintained in a 5% CO_2 incubator at 37°C using BME (Cellgrow) supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine (Gibco), 100U/mL penicillin-100 μg/mL streptomycin (Gibco). The plasmid NF-κB-SEAP-ntp was cloned.[117] For NF-κB transcription activity testing, A549 cells were transfected with NF-κB-SEAP-ntp plasmid, this is the A549SN cell line.

3.4.2.2 NF-κB activity – A549SN cell SEAP method

Day1: Plate NF-κB cells: (1) Plate NF-κB_SEAP A549 cells, 6.0x104 cells/well in three 24 well plates, 1000 μL DMEM complete medium: 10% heat inactivated FBS, 2mM L-Glu, 100U/mL or 100 μg/mL Penn/Strep, 500 μg/mL G418. (2) Incubate at 37°C incubator with 5% CO_2. Day2 : Treat cells with drugs: Cells are all alive, not confluent (~25-40%). Treat cells with QA=1μM, or other compounds @ 1 μM (990 μL 1% FBS DMEM + 10 μl (10/500) PBS diluted compounds from 5mM in DMSO), + 10 ng/mL TNFα, for 24 hrs. Day 3: Measure SEAP activity: 24 h later, take out 50μL medium. Store at –20°C. Use Clontech "Great EscAPe SEAP Chemiluminescence Detection Kit" (Cat# K2041-1) to measure luminescence through microplate luninometer (Packard αHT microplate reader). Measure 1 second for once at RT. Cells left in plate were lysed in 250 μl lysis buffer (10mMK_2HPO_4; 1mMKH_2PO_4; 1%Triton X100; 1mMDTT) for protein quantification (Pierce BCA Protein Assay Kit, Microplate Procedure) and SEAP activity was normalized to the total protein content.

3.4.2.3 Anti-oxidant activity test method

The DPPH and all standard antioxidant compounds including trolox were dissolved in 50% acetone. The DPPH stock solution at a concentration of 0.625 mM was prepared monthly and kept at 4°C in dark. The 0.208 mM fresh DPPH working solution was made daily by further diluting the stock solution in 50% acetone for each test. Stock

solutions of CA, CGA, AA and compound **3** were prepared in 50% acetone at concentrations of 10 mM, respectively, and stored at 4°C. A series of working solutions were made by appropriate dilutions of the above standard phenolic acid stock solutions with 50% acetone.

3.4.2.4 Conventional colorimetric analysis

The conventional colorimetric DPPH scavenging capacity assay was performed according to a previously described laboratory protocol.[116] Briefly, an aliquot of 500 μL of different concentrations of sample extracts in 50% acetone was added to 500 μL of 0.208 mM DPPH solution. The initial concentration was 0.104 mM for DPPH in all reaction mixtures. Each mixture was vortexed for a few seconds and test immediately. The absorbance (*A*) of each reaction mixture at 515 nm was measured against a blank of 50% acetone using a UV-visible spectrometer. The level of DPPH remaining for each reaction time was calculated as: % DPPH remaining=($A_{\text{sample-t}}$ / A_{control}) x 100.

CHAPTER 4. DETERMINATION OF QUINIC ACID AMIDES IN RAT PLASMA BY LC/MS/MS WITH A HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY COLUMN

4.1 Introduction

The interest in development of natural sources of anti-inflammatory activity has exploded in recent years. Quinic acid (QA) is a natural compound found widely in plants. Recently, QA has been identified as an active inflammatory ingredient in hot water extracts of the herbal Cat's claw (e.g., C-MED-100®), and shown to enhance immune cell response and DNA repair in humans.[3-5] Unfortunately, QA is utilized by gastrointestinal bacteria as a carbon source for aromatic acid synthesis. Consequently, only a small fraction of QA is absorbed after oral administration of QA.[27] Our group has focused on the discovery and development of stable QA derivatives. We have identified water soluble QA amide KZ-41(Compound **3**) which possess potent anti-inflammatory activity.[118,119] To study the pre-clinical pharmacokinetic properties of KZ-41, an analytical method is required for determination of the concentration of KZ-41 in pre-clinical samples.

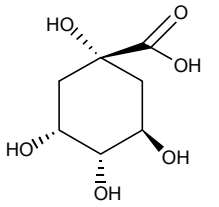
There is no determination method of quinic acid amides, such as KZ-41 in the literature. Several methods of determination of QA in plants and food have been described.[125-127] The reverse phase column (C_{18} or C_8) were used in the HPLC methods reported. Because QA is water soluble, the mobile phase used in RP column consisted of very high percentage of water, and re-equilibrated with 99% ACN in actually application.[125] Both QA and KZ-41 are water soluble and high polar. The determination of polar compounds in biological fluids is very efficient by using hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS) method.[128-130] The advantages of ESI-MS detection in conjunction with HILIC mode separations are obvious: better separation with high organic phase and high sensitivity.[131,132] UV detection could be much simpler, but the sensitivity for QA and KZ-41 were worse due to their lack of UV absorption function group.

This chapter has developed and validated a rapid, robust and sensitive HILIC-MSMS method which employs protein precipitation for the quantitative analysis of QA amide KZ-41 in rat plasma. It has lower limit of quantitation (LLOQ) of 0.5 ng /mL.

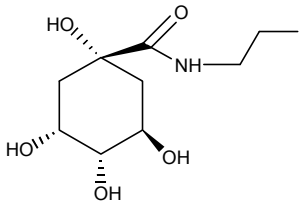
4.2 Experimental

4.2.1 Material and Reagents Synthesis of Quinic Acid Amides

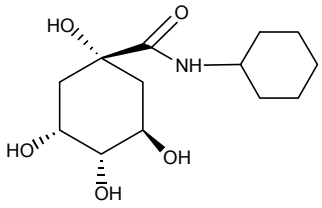
KZ-41 and internal standard (IS) (Figure 4-1) were synthesized in our medicinal chemistry Lab. Methanol, acetonitrile, acetone, and water (HPLC grade), were purchased from Sigma-Aldrich (St Louis, MO, USA) and the other chemicals were of HPLC grade



Quinic Acid, MW 192



KZ-41, MW 232



Internal Standard, MW 273

Figure 4-1. Chemical structure of quinic acid, KZ-41 and IS.

or the highest quality available. Drug-free rat plasma were obtained from Pelfreez Biologicals (Rogers, AR, USA).

4.2.2 Preparation of Calibration Standards and Quality Control Samples Biology Testing of Quinic Acid Amides

KZ-41 stock solution (1mg/mL) was prepared in acetonitrile. Serial working standard solutions of KZ-41 were prepared by further diluting each primary solution with acetonitrile to obtain desired concentrations. The drug-free rat plasma was spiked with KZ-41 and IS solution, and protein precipitated to prepare rat plasma solution.
The nominal rat plasma calibration standards of KZ-41 (0.5, 1.0, 5.0, 10.0, 50.0, 100.0, and 500.0 ng/mL) were prepared as above. The quality control samples were chosen at concentration levels (5.0, 25.0, and 125.0 ng/mL).

An IS stock solution (1mg/mL) was prepared in methanol. The IS working solution was diluted with methanol to a final concentration of 10 ng/mL. All solutions were stored at −20°C.

4.2.3 Sample Processing

Rat plasma samples were processed by protein precipitation. A 50 µl volume of plasma sample was diluted with 150 µl of methanol with IS (10 ng/mL). The samples were vortexed vigorously for 5 min, refrigerated sample for 2 hrs, and the mixture was centrifuged for 15 min at 10 000 *g*. Supernatant aliquots of 100 µl were transferred to vials and evaporated to dry by TurboVap LV concentration workstation (Caliper Life sciences, Hopkinton, MA) at 30°C under nitrogen. The residues were reconstituted to 200 µl with pure acetonitrile and vortex-mixed for 2 min. Aliquot of 20 µl was injected into LC-MS/MS for analysis.

4.2.4 Instrumentation

4.2.4.1 Chromatographic conditions

Chromatographic separation of analyte was carried out using Shimadzu (Columbia, MD) LC-10ADvp pumps with a Leap (Carrboro, NC) CTC PAL autosampler. The separation was performed on a PolyHYDROXYETHYL A^{TM} HILIC column (5 µm, 100 x 2.1 mm, 100 Å, The Nest Group, Inc., South borough, MA, USA). The mobile phase consisted of eluent A (water) and eluent B (acetonitrile), and separation achieved using a gradient program of 0 min: 15% A; 4 min: 40% A; 4.5 min: 15% A, and the total analytical run time was 5.5 min. The flow rate was 0.2 mL/min with the column at ambient temperature.

4.2.4.2 Mass spectrometric conditions

An API 4000 Q TRAP mass spectrometer equipped with an electrospray ion source (Applied Biosystems Sciex, Foster city, CA) operated in negative-ion mode was used for MS detection. Quantitation was performed using MRM mode to study parent→ product ion transitions for KZ-41 (232 → 178) and IS (272 → 218) with unit resolution. Source dependent parameters optimized were gas 1 (nebulizer gas): 30 psi, gas 2 (heater gas): 40 psi, ion spray voltage (ISV): -4500 V, temperature [(transmission electron microscope (TEM)]: 700°C. Compound dependent parameters were declustering potential (DP), entrance potential (EP), collision energy (CE), cell exit potential (CXP). DP: -50 V, CE: -28 V, EP: -10 V, CXP: -15 V were set for KZ-41, and DP: -60 V, CE: -32 V, EP: -10 V,CXP: -15 V were set for IS. Focusing potential (FP) was 400 V for both analyte and IS. Nitrogen was used as collision-activated dissociation (CAD) gas and was set at 6 psi. Quadrupole 1 and quadrupole 3 were maintained at unit resolution and dwell time was set at 250 ms.

4.2.4.3 Data processing and quantification

Mass spectrometric data acquisition and data analysis were done with the Analyst Version 1.5. Software (Applied Biosystems). A weighted 1/*x* linear regression was used to generate calibration curve from standards and calculate the concentrations of quality control and unknown samples. Equation of the standard curve: $y=mx + b$, where '*y*' is the peak area ratio of the analyte to IS, '*x*' is the theoretical concentration of the analyte divided by the theoretical concentration of IS, '*m*' is the slope and '*b*' is the intercept of the regression line.

4.2.5 Method Validation

4.2.5.1 Linearity, accuracy and precision

Rat plasma calibration standards were analyzed in three separate analytical runs. The peak areas generated by KZ-41 and IS were used to calculate their area ratios. The calibration curves were plotted by weighted 1/*x* linear regression analysis of concentrations of KZ-41 versus the peak ratios of KZ-41 and IS.

The intra-batch precision and accuracy were assessed by determinations of KZ-41 in rat plasma at nominal concentrations of 5.0, 25.0, and 125.0 ng/mL, on the same day. The inter-batch precision and accuracy were carried out by analyzing the same samples on three different days. Five replicates of each sample were analyzed together with a set of calibration standards, independently prepared from the control samples, in five analytical runs. The CV (%) values of measured concentrations were used to show the precision of the method. The deviation of the mean measured concentrations away from the corresponding nominal concentration served as a measure of accuracy.

4.2.5.2 Matrix effect and recovery

Matrix effect and recovery were evaluated according to a published literature.[133] Briefly, matrix effect was determined by comparing the analytical response of the standards spiked into plasma extracts (Solution B) with that of the net standard solutions (Solution A). The loss of signal represents the ion suppression. The extraction recovery of KZ-41 from rat plasma was determined by comparing the analytical response of processed quality control samples (Solution C) with that of blank net plasma extracts spiked with standard working solutions (Solution B). They are expressed as: matrix effect (ME%)=(B/A)*100; recovery rate (RR%)=(C/B)*100. These experiments were performed in triplicate at concentration level 5.0, 25.0, and 125.0 ng/mL.

4.2.5.3 Stability

Storage stability tests were performed with quality control (QC) samples subjected to different storage conditions (4°C or at 25°C for 48 h). The stability was calculated by comparing injected freshly prepared QC samples with re-injected samples 48 h later. Five replicates were analyzed with each sample at each concentration. Three concentration levels (5.0, 25.0 and 125.0 ng/mL) were studied. The storage stability of both KZ-41 and IS in the working solution and in the processed extracts was investigated.

4.3 Results and Discussion

4.3.1 Optimization of Sample Preparation Procedure

Protein precipitation is a valid and fast rat plasma sample preparation procedure. The commonly used solvent is acetonitrile. We started with acetonitrile as plasma splash solvent (150 μl organic solvent into 50 μl plasma), the recovery rate was poor. Then other organic solvents (methanol, acetone, and chloroform) were tested. We found methanol provided a good protein precipitation and drug extraction recovery. Methanol was selected as plasma splash organic solvent.

4.3.2 Optimization of Chromatography Condition

Good chromatography separation with sharp peak shape will ensure high selectivity, specificity and sensitivity of the analytical method. This optimization will be achieved by good matching within analyte, column, and mobile phases. KZ-41 is an amide, neutral in acidity, and water soluble (LogP=-2.03). We tested it on a C_8 reverse-phase symmetry (3.5 μm, 2.1 × 50 mm, 100 Å) column, 95% aqueous phase in mobile phase was needed to obtain 2 min retention time, and the peak shape was distorted. Furthermore, too much aqueous reduce the sensitivity of MS detection, and the stability of the C_8 column. When KZ-41 was separated on a PolyHYDROXYETHYL A™ HILIC

column (5 μM, 100 x 2.1 mm, 100 Å, The Nest Group, Inc., Southborough, MA, USA), with 85% acetonitrile in mobile phase (acetonitrile and water), this amide had about 3 min retention time with good peak shape (Figure 4-2). The higher percentage of organic phase improved the sensitivity of MS detection. Several compounds from KZ-41 chemical library were investigated to find a suitable IS, a structurally similar analog was found to be the most appropriate for this study. The structures of quinic acid, KZ-41, and IS were shown in Figure 4-1.

4.3.3 Optimization of MS Instrumentation

MS instrument parameters for determination each compound were optimized by directly infusion each compound (1 μg/mL acetonitrile solution) into the mass spectrometer at a flow rate of 600 μl/h. On the negative full scan mass spectra, the de-protonated molecular ions $[M - H]^-$ of KZ-41 and IS were observed at a mass to charge ratio (*m*/*z*) of 231.7 and 271.9, respectively. The product ion scan resulted in a major fragment at *m*/*z* 177.8 for KZ-41 and *m*/*z* 217.9 for IS (Figure 4-3). The fragments were the products of parent molecules by lost three water molecules (mass=18*3). These MRM ion pair selections contributed to the compound specificity of the MS method. The CE and collision CXP were optimized in order to obtain the best product ion/precursor ion intensity ratio. The mass spectrometry detection MRM ion pairs for KZ-41 and IS were selected as 232/178 and 272/218 at unit resolution, respectively. Parameters including ionization voltage, focusing and DP, flow of curtain and nebulizer gas were further investigated in order to obtain the maximum intensity as a HILIC/MS/MS analysis method.

4.3.4 Linearity, Accuracy and Precision

A linear dynamic range was obtained over the concentration range from 0.5 to 500 ng /mL (Figure 4-4). The standard curve equation is y=0.0059x + 0.0154. The correlation coefficient (r^2) is greater than 0.999.

The intra- and inter-day accuracy and precision are presented in Table 4-1. The intra-day accuracy was 112.67%, 98.89%, 98.06% at concentrations of 5.0, 25.0, and 125.0 ng/mL respectively. They are all within the accepted deviation range 85% to 115%. The CV (%) value is also within accepted range.

4.3.5 Specificity and Selectivity

MRM chromatograms of blank, KZ-41, IS and QC samples were showed in Figure 4-2. LC/MS/MS system with MRM mode provides high selectivity and specificity. The LC separation selectivity and specificity was good, there was no peaks that co-eluted with either KZ-41 or the IS, and each analyte had specific retention time

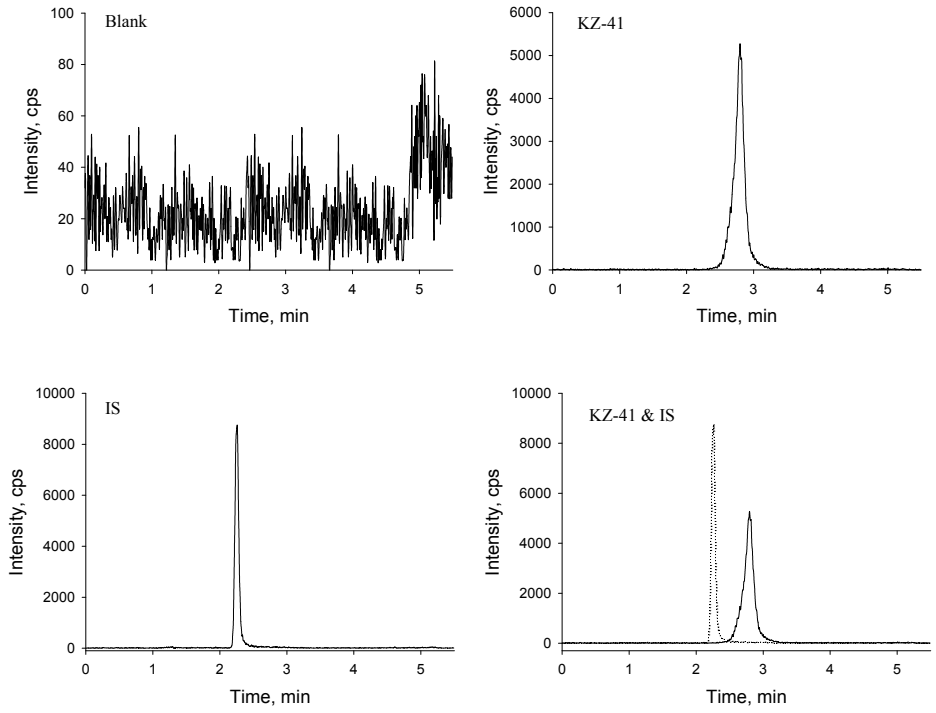


Figure 4-2**.** Representative HILIC-MS MRM chromatograms of KZ-41 and IS in extraction from rat plasma. The separation was performed on a PolyHYDROXYETHYL HILIC column with 85% acetonitrile in water as the mobile phase. The flow rate was 0.2 mL/min. Detection was performed using triple quad mass spectrometer in negative MRM mode.

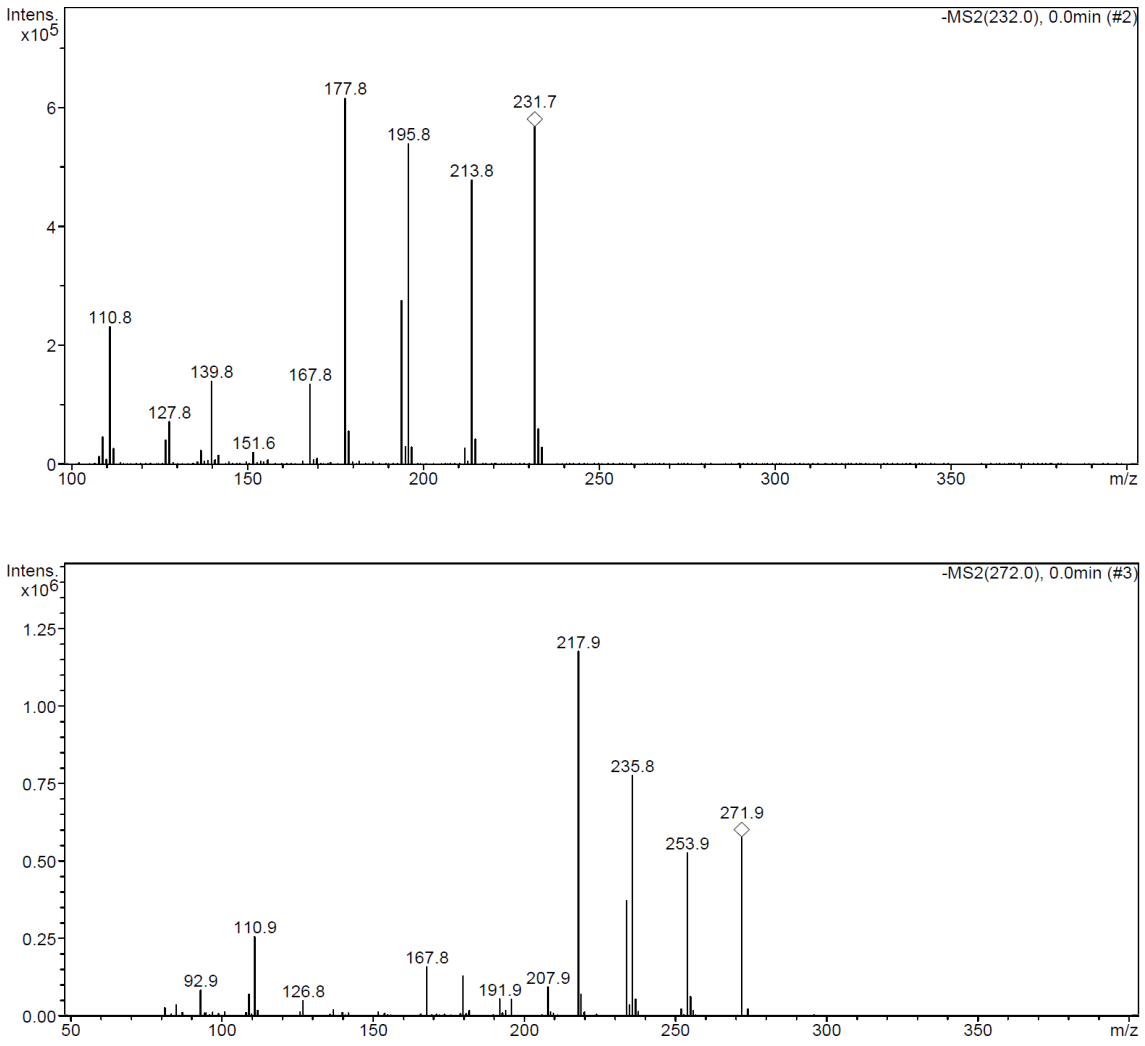


Figure 4-3. Product mass spectra of KZ-41 and IS. Upper spectrum. KZ-41, parent ion [M-H]=232, dominated daughter ions: 214, 196, 178. Lower spectrum. IS, parent ion [M-H]=272, dominated daughter ions: 254, 236, 218.

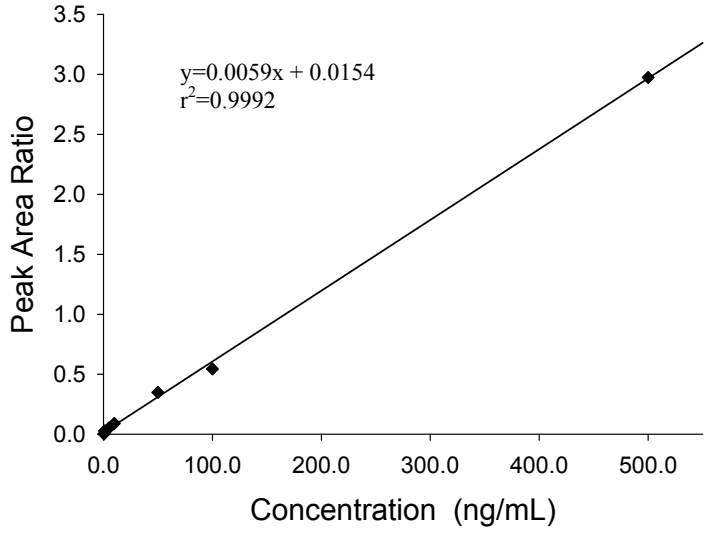


Figure 4-4. Representative standard curve of KZ-41 in rat plasma. The standard curve equation is y=0.0059x + 0.0154. The correlation coefficient (r^2) is greater than 0.999 over the concentration range of 0.5 to 500 ng /mL.

Table 4-1. Intra- and inter-day accuracy and precision of KZ-41 in rat plasma.

Time	Nominal concentration (ng/mL)	Found concentration (ng/mL)	Accuracy (%)[a]	CV (%)[b]
Intra-day (n=5)	5.0	5.63	112.67	14.08
	25.0	24.72	98.89	5.38
	125.0	122.57	98.06	3.75
Inter-day (n=3)	5.0	5.36	107.20	11.20
	25.0	24.12	96.48	5.80
	125.0	123.15	98.52	3.30

[a] Accuracy (%) is calculated as mean found concentrations over nominal concentration x 100%.

[b] CV (%) is calculated as standard deviation of found concentrations over nominal concentration x 100%.

(Figure 4-2). MRM ion pairs were parent ions with moiety-specific fragment ions, there was no mass transition interference from rat plasma with either of the compounds.

4.3.6 Matrix Effect and Recovery

In this study, the values of matrix effect were 79.4% and 81.3% for KZ-41 and IS, respectively, which meant the biological matrix had similar effect on the ionization of KZ-41and IS. Extraction recoveries for KZ-41 and the IS were 100.9% and 99.2%, respectively. Only a negligible carryover effect (about 0.1%) was observed after injection of a blank plasma extract following injection of the upper calibration standard.

4.3.7 Stability

The result showed both KZ-41 and IS in the working solution and in the processed extracts were stable over 48 h storage (4°C or at 25°C for 48 h). KZ-41 and IS did not show any significant degradation after protein plasma precipitation even after 48 hr. This suggests that plasma protein precipitation can be conveniently performed within 2 days of the LC-MS/MS measurement analysis. In the freeze/thaw investigations and long-term stability experiments, the observed maximum degradation was within 20% and fulfilled the acceptance criteria.

4.4 Conclusions

A robust and sensitive HILIC-MS/MS method for the analysis of KZ-41 in rat plasma has been developed and validated. This assay used protein precipitation method for sample preparation, has a lower limit of detection of 0.5 ng /mL. The standard curve equation is $y=0.0059x + 0.0154$. The correlation coefficient (r^2) is greater than 0.999 over the concentration range of 0.5 to 500 ng /mL. It is novel and useful for the pharmacokinetic studies of KZ-41.

CHAPTER 5. PHARMACOKENETICS AND BIOAVAILABILITY OF NOVEL ANTI-INFLAMMATORY AGENT KZ-41

5.1 Introduction

Many interests have been raised in development of natural products or modified products having anti-inflammatory activity in recent years. Quinic acid (QA) is a natural compound found widely in plants.[8-10] Recently, QA has been identified as an active ingredient in hot water extracts of the herbal Cat's claw (e.g., C-MED-100®), and shown to enhance immune cell response and DNA repair in humans.[3-5] In particular, such extracts have been found to have various anti-inflammatory effects such as inhibition of the production of the inflammatory cytokine TNFα, scavenges free radicals[45] and the activation of the central transcription factor nuclear factor κB (NF-κB).[46-49] Unfortunately, QA is utilized by microorganisms and plants through the shikimate biosynthetic pathway leading to aromatic amino acids phenylalanine, tyrosine, and tryptophan.[16,19,20,134,135] In 1967, a QA oxidizing enzyme produced by acetic acid bacteria was discovered demonstrating that bacteria could catabolize QA.[25] Subsequently, the QA catabolic pathway was described as a result of characterization of fungal mutants with lesions in genes encoding biosynthetic shikimate pathway enzymes.[26] Bacteria and fungi utilize QA as a growth substrate and thus express an abundance of the aforementioned enzymes involved in QA transformation. High levels of QA catabolizing enzymes in gut bacteria is consistent with the finding that less than 10% of an orally administered dose QA is recovered in rats.[27]

In short, rapid degradation by gut flora potentially limits the oral effectiveness of current quinic acid preparations. Our study group has focused to discover new anti-inflammatory agents that will be orally active based on the quinic acid structure. We have identified water soluble QA amide KZ-41 which possess potent anti-inflammatory activity.[118,119] There are no biopharmaceutical and pharmaceutics data available for this compound to establish the dosing for in vivo animal study. This study was conducted to investigate the bacterial stability, and absorption, distribution, metabolism and elimination of KZ-41 in rat after intravenous and oral administration.

5.2 Experimental

5.2.1 Material and Reagents

KZ-41 and internal standard (IS) were synthesized in our medicinal chemistry Lab. Methanol, acetonitrile, acetone, and water (HPLC grade), were purchased from Sigma-Aldrich (St Louis, MO, USA) and the other chemicals were of HPLC grade or the highest quality available. Drug-free rat plasma was obtained from Pelfreez Biologicals (Rogers, AR, USA).

5.2.2 Microsomal Stability

Microsomal metabolic stability of KZ-41 was assessed in Pooled IGS Sprague-Dawley Rat Liver Microsomes (Xenotech, LLC, Lenexa, Kansas) preparations by monitoring disappearance of the parent compound over an incubation period of 90 minutes. The concentration change of intact parent compound in the samples was estimated by comparing analyte concentrations before and after incubation using LC-MS/MS assay. The detailed procedure is described in the following. The stock solution (10 mM stock in methanol) was diluted in PBS buffer (50 mM, pH 7.4) to make 500 µM working solution. Test compounds were incubated at a final concentration of 5.0 µM. A concentration of 50.0 µM was utilized to allow for detection of possible minor metabolites. Rat liver microsomes were utilized at a final concentration of 1.0 mg/mL. Reactions mixture were prepared by adding 25 µl of microsomal sprotein solution (20mg/mL) to 50 µl test compound (500 µM) and 425 µl of NADPH regenerating system (NRS) "master mix" solution. The combined components, with the exception of the microsomes, were preincubated at 37°C for 10 min, after which the microsomes were added. The mixture was incubated in a water bath at 37ºC. Deactivated microsome solution was used as negative control. The NRS "master mix" is a solution of glucose 6-phosphate dehydrogenase, NADP+, MgCl2, and glucose 6-phosphate, prepared per manufacturer's instructions (BD Biosciences, Waltham, MA). Each 5.0 mL stock of NRS "master mix" solution contains 3.8 mL H20, 1.0 mL solution "A" (Cat. #451220), and 0.2 mL solution "B" (Cat. #451200).

At each time point (0, 45 and 90 minutes), 100 µl of reaction was removed and the reaction was terminated by addition of 100 µL of acetonitrile containing internal standard IS (150 ng/mL). Samples were then centrifuged at 10,000 rpm for 10 minutes at 4°C to remove debris and precipitated protein. Approximately 150 µl of supernatant was subsequently transferred to a new sample vial for analysis.

5.2.3 Plasma Protein Binding

Plasma protein binding of KZ-41 was determined by using rapid equilibrium dialysis. The RED device for rapid equilibrium dialysis was obtained from Pierce Biotechnology Inc (Rockford, IL). The experimental procedure was followed to the manual protocol with optimization. Briefly, biologically relevant concentrations of test compound were prepared (low, middle and high) in rat plasma. The RED inserts were placed in the base plate. 300 µL of the plasma sample was placed in the sample chamber and 500 µL of dialysis buffer, pH 7.4 in the buffer chamber. The chambers were covered with a seal and incubated at 37ºC for 4-6 hours at approximately 100 rpm on an orbital shaker. At the end of incubation, remove seal and confirm volume of the sample chamber. Minimal to no volume change should have occurred. Aliquots of plasma and buffer were used to determine the drug concentration using an LC-MS/MS assay. Pipette 50 µL each of post-dialysis samples from the buffer and the plasma chambers into separate microcentrifuge tubes. Add 50 µL of plasma to the buffer samples, and an equal volume of PBS to the collected plasma samples. Add 100 µL of precipitation Methanol

to precipitate protein and release compound. Vortex and incubate 30 minutes on ice. Centrifuge for 10 minutes at 13,000-15,000 × *g*. Transfer supernatant to a vial, dry the supernatant under nitrogen, and reconstitute to 200 µL with acetonitrile before LC/MS/MS. Determine the concentration of test compound in the buffer and plasma chambers from peak areas relative to the internal standard. Calculate the percentage of the test compound bound as follows:

% Free=(Concentration buffer chamber/Concentration plasma chamber) × 100%
% Bound=100% - % Free

5.2.4 Bacterial Stability

Bacteria *Gluconobacter oxydans* was selected to test the bacterial stability of QA analogs.[30,31] The log growth phase in which this bacterial strain has the most active growth, was obtained by plotting the growth curve of this bacterial. The growth curve of this bacteria strain was obtained by taking OD readings generally at 0, 0.5, 1, 2, 4, 8, 12, and 24 hours when bacterial is cultured. Then plot the log OD versus time and find the linear portion of the graph which is the log growth phase of this bacterial. *Gluconobacter oxydans* was incubated in mannitol media at 26°C and 150 rpm, and used to test the stability of QA analogs when it was in the log growth phase. Test was divided into three groups: negative control (QA in autoclaved bacterial media), positive control (QA in active bacterial media), and treatment (KZ-41 in active bacterial media). Compounds were dissolved in fresh culture media. 200 µl test solution (2mg/mL) was added into 3 mL bacterial media in a 6 well plate. Samples were taken at different time points after treatment. Sampling procedure: removed 200uL sample from each well and put into labeled tubes, quenched sample with 300uL of methanol. Between samplings, plate was placed on C24 Incubator Shaker (New Brunswick Scientific, Edison, NJ) at 26°C and 150 rpm. Samples were store at -80°C for future use.

5.2.5 Pharmacokinetic Studies in Rats

All animal procedures used in this research were approved by the University of Tennessee Animal Care and Use Committee. Pre-cannulated Sprague-Dawley male rats (200 -300 g, jugular vein alone for oral study and jugular vein and femoral vein for intravenous study) were obtained from Harlan Bioscience (Indianapolis, IN). Animals were fed a normal laboratory diet, allowed ad libitum access to water and maintained on a 12-hr light-dark cycle (25ºC). The catheter was exteriorized between the shoulder blades to ensure the catheter remains in place over the sampling interval. Catheter patency was maintained with heparin glycerol solution (100 IU heparin/mL in 75% glycerol saline solution). Groups of animals (n=6) received a dose (10 mg/kg) of the compound of KZ-41 via the oral or intravenous route. For oral administrations, the animals were fasted overnight and until 4 hr after administration of test compound. Blood samples (200 to 300 uL) were withdrawn from the jugular vein catheter pre-dose and at regular intervals after dosing (5, 15, 30, 45 minutes and 1, 2, 4, 8, 12, and 24 hrs). An equal volume of

sterile saline solution will be administered via the jugular vein catheter after each blood collection. All experiments will be performed on un-restrained animals. A maximum of twelve blood samples, representing no more than 2% of the body weight in blood volume, were taken from individual animals. Blood was transferred into Microtainer® brand tubes with lithium heparin (BD, Franklin Lakes, NJ). Plasma was separated immediately by centrifugation (3000g for 2 min at 4ºC). Urine and fecal samples were pooled and collected up to 24 hr respectively. All samples were stored at -80°C until future analysis.

5.2.6 Analytical Methods

A LC-MS/MS method was developed to quantitate KZ-41 concentration in rat plasma. This method was validated and published separately. Chromatographic separation of analyte was carried out using Shimadzu (Columbia, MD) LC-10ADvp pumps with a Leap (Carrboro, NC) CTC PAL autosampler. The separation was performed on a PolyHYDROXYETHYL A^{TM} HILIC column (5 µM, 100 x 2.1 mm, 100 Å, The Nest Group, Inc., South borough, MA, USA). The mobile phase consisted of eluent A (water) and eluent B (acetonitrile), and separation achieved using a gradient program of 0 min: 15% A; 4 min: 40% A; 4.5 min: 15% A, and the total analytical run time was 5.5 min. The flow rate was 0.2 mL/min with the column at ambient temperature. An API 4000 Q TRAP mass spectrometer equipped with an electrospray ion source (Applied Biosystems Sciex, Foster city, CA) operated in negative-ion mode was used for MS detection. Quantitation was performed using MRM mode to study parent→ product ion transitions for KZ-41 (232 → 178) and IS (272 → 218) with unit resolution. Source dependent parameters optimized were gas 1 (nebuliser gas): 30 psi, gas 2 (heater gas): 40 psi, ion spray voltage (ISV): -4500 V, temperature [(transmission electron microscope (TEM)]: 700°C. Compound dependent parameters were declustering potential (DP), entrance potential (EP), collision energy (CE), cell exit potential (CXP). DP: -50 V, CE: -28 V, EP: -10 V, CXP: -15 V were set for KZ-41, and DP: -60 V, CE: -32 V, EP: -10 V,CXP: -15 V were set for IS. Focusing potential (FP) was 400 V for both analyte and IS. Nitrogen was used as collision-activated dissociation (CAD) gas and was set at 6 psi. Quadrupole 1 and quadrupole 3 were maintained at unit resolution and dwell time was set at 250 ms.

Rat plasma samples were processed by protein precipitation. A 50 µl volume of plasma sample was diluted with 150 µl of methanol with IS (10 ng/mL). The samples were vortexed vigorously for 5 min, refrigerated sample for 2 hrs, and the mixture was centrifuged for 15 min at 10 000 g. Supernatant aliquots of 100 µl were transferred to vials and evaporated to dry by TurboVap LV concentration workstation (Caliper Life sciences, Hopkinton, MA) under nitrogen at 30°C. The residues were reconstituted to 200 µl with pure acetonitrile and vortex-mixed for 2 min. Aliquot of 20 µl was injected into LC-MS/MS for analysis.

An aliquot, 600 µl, of the pooled urine was evaporated to dryness under nitrogen, 200 µl of acetonitrile with IS (10 ng/mL) was added. Approximately 1.8 g of pooled fecal sample was extracted with 10 mL of methanol for 1 hr in a polypropylene centrifuge tube

using a rotating mixer. The extract was centrifuged at 4,000g for 10 min. The supernatant was transferred to a new tube. The pellet was re-extracted as described above and the supernatants were combined. The extract was evaporated to dryness under a stream of nitrogen, residue was dissolved in 0.5mL methanol water mixture (50/50 v/v), and transferred into a 1.8 mL vial. This mixture was evaporated to dryness under nitrogen, and 200 µl of acetonitrile with IS (10 ng/mL) was added. The samples were vortexed vigorously for 5 min, and the mixture was centrifuged for 15 min at 10 000 g. Supernatant aliquots of 20 µl was injected into LC-MS/MS for analysis.

5.2.7 Pharmacokinetic Data Analysis

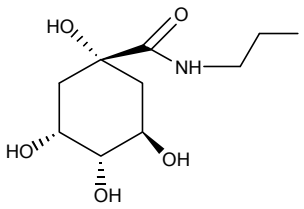
Plasma concentration-time profiles were analyzed by non-compartmental analysis using WinNonlin 5.0.1 (Pharsight Corporation, Mountain View, CA). The maximum plasma concentration (C_{max}) observed after oral dosing and the time at which it was observed (T_{max}) were determined by direct inspection of the individual plasma concentration-time profiles. The terminal half-life ($t_{1/2}$) was calculated as 0.693 divided by the terminal phase rate constant λ_z. The area under the plasma concentration-time curve from time 0 to infinity (AUC_{inf}) was calculated by the trapezoidal rule with extrapolation to time infinity. The total plasma clearance (CL) of i.v. KZ-41 was calculated as the i.v. dose divided by the AUC_{inf} of i.v ($CL=Dose_{iv}/AUC_{inf,iv}$). Volume of distribution based on terminal phase (Vz) was calculated as the i.v. dose divided by the product of AUC_{inf} of i.v and λ_z ($Vz=Dose_{iv}/(\lambda_z * AUC_{inf,iv})$). The parameters of oral dose were calculated same way as iv dose. The relative oral bioavailability (F) was calculated as $F=(AUC_{inf,oral} * Dose_{iv})/(AUC_{inf,iv} * Dose_{oral})$.

Statistical analyses were performed using a two-tailed t test at a 5% level of significance.

5.3 Results

5.3.1 Pharmacokinetics of KZ-41

The structure of KZ-41 is shown in Figure 5-1. Figure 5-2 shows the plasma mean concentration – time profile of KZ-41 (n =6) after administration of i.v. and oral doses at dose of 10 mg / kg. The derived pharmacokinetic parameters is listed in Table 5-1. The total plasma clearance of KZ-41 in rats was 13.8 mL/min/kg (i.v.), or 12.1 mL/min/kg (oral). This moderate clearance indicated the compound would have reasonable duration in the body. The terminal plasma elimination half-lives was 183 min, or 3.1 hr. The compound was moderately distributed in the body with volume of distribution of 3.65 L/kg. The amount of compound excreted unchanged into urine and feces was approximately 60% of the dose by both routes, this suggested low metabolism of KZ-41 in the rat body. The oral bioavailability of this compound in rat was about 111%. Theoretically, Bioavailability should not exceed 100%. The result which is over



KZ-41, MW 232

Figure 5-1. Chemical structure of quinic acid amide KZ-41.

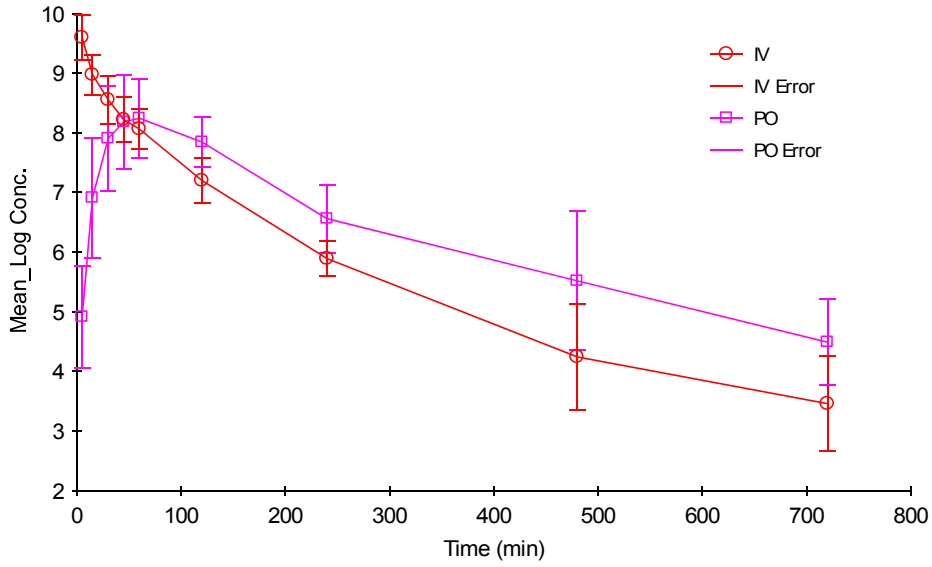


Figure 5-2. Rat plasma meanlog concentration – time plot of KZ-41 after a single dose (10 mg/kg, n=6). The curve without absorption peak is IV dose curve, the curve with a peak is oral dose curve. Each points represents the mean ±S.D.

Table 5-1. Key pharmacokinetic parameters of KZ-41 in rats (n=6) calculated by mean concentrations versus time profile (Dose: 10mg/kg).

Route	C_{max} ng/mL	T_{max} min	AUC min*ug/mL	$T_{1/2}$ min	CL mL/min/kg	V_z mL/kg	F
IV	15688.7	5.0	722.2	183.1	13.8	3654.3	
PO	4591.3	45.0	803.1	147.7	12.1	2579.8	1.11*

*p=0.67.

100% may due to experimental error. T-test has been done to compare the AUC of i.v. to that of oral. The p-value was 0.67. Statistically, bioavailability of KZ-41 in rat was 100%. Key pharmacokinetic parameters of KZ-41 in individual rat by i.v. and oral administration were listed in Table 5-2 and Table 5-3 respectively.

5.3.2 Microsomal Metabolic Stability

KZ-41 was metabolically stable when incubated with pooled IGS Sprague-Dawley rat liver microsomes (Table 5-4.). The parent compound was about 100% remaining after 90 min incubation, while the positive control (propanolol) was 32% remaining.

5.3.3 Plasma Protein Binding

The plasma protein binding data of KZ-41 is listed in Table 5-5. The plasma protein binding was moderate and not concentration dependent over the concentration range of 50 ng/mL to 5000 ng/mL, with an average of 27.46% bound to plasma proteins. At concentrations of 50 ng/mL, 500 ng/mL, and 5000ng/mL, the percentage of KZ-41 bound was 21.27%, 28.83%, and 32.29% respectively.

5.3.4 Bacterial Stability

Bacteria *Gluconobacter oxydans* was unable to utilize KZ-41 as food by transforming it to another form (Figure 5-3). The parent compound KZ-41 remained 100% unchanged with cultured bacterial over 24 hr. As expected, QA was not stable in bacterial cultured media, less than 10% unchanged QA remained after one day.

5.4 Discussion

The present study demonstrates that KZ-41 had high oral bioavailability (about 100%). This could be the result of numerous factors, including being stable with gut microflora, good absorption, slight first-pass metabolism in the intestinal wall and/or in the liver. First, KZ-41, not like QA, was not growth substrate target by bacteria *Gluconobacter oxydans* as we shown in bacterial stability study. It was stable in bacterial cultured medium. The enzymes responsible for QA transformation are quinate dehydrogenase (QDH), 3-dehydroquinate dehydratase (DQD), and 3-dehydroshikimate dehydratase.[16, 30, 31] KZ-41 is not the substrate of QDH or DQD, may be the inhibitor of these enzymes of the bacterial. Further mechanism study is on going. Second, KZ-41 was well absorbed after oral administration, with a mean C_{max} value of 4.59 μg/mL achieved at 45 min. KZ-41 is a highly water soluble small molecular QA amide, with molecular weight 233 and negative CLogP 2.03. Therefore, it appears to be permeable through the gastrointestinal tract. This suggestion is also evidenced by the fact that about 60% of the

Table 5-2. Key pharmacokinetic parameters of KZ-41 in rats by intravenous administration (Dose: 10mg/kg).

Animal	C_{max} ng/mL	T_{max} min	AUC min*ug/mL	$T_{1/2}$ min	CL mL/min/kg	V_z mL/kg
1	12760.0	5.0	637.4	202.2	15.3	4447
2	24400.0	5.0	1024.3	136.6	9.7	1920
3	24560.0	5.0	1070.2	147.4	9.2	1960
4	12320.0	5.0	585.7	148.8	16.8	3600
5	11920.0	5.0	530.1	149.8	18.6	404
6	8948.0	5.0	473.4	131.5	21.0	3984
Mean	15818.0	5.0	720.2	152.7	15.1	3325.5
S.D.	6841.8	0.0	259.6	25.3	4.8	1106.4

Table 5-3. Key pharmacokinetic parameters of KZ-41 in rats by oral administration (Dose: 10mg/kg).

Animal	C_{max} ng/mL	T_{max} min	AUC min*ug/mL	$T_{1/2}$ min	CL mL/min/kg	V_z mL/kg	F
1	4220.0	45.0	855.6	130.8	11.5	2164	1.19
2	4284.0	45.0	858.2	147.6	11.3	2412	1.19
3	1540.0	120.0	303.1	301.0	31.5	13677	0.42
4	6192.0	60.0	1021.8	178.4	9.3	2390	1.42
5	11680.0	45.0	1337.0	213.8	7.2	2230	1.86
6	2668.0	45.0	447.0	130.5	22.0	4136	0.62
Mean	5097.3	60.0	803.8	183.7	15.5	4501.5	
S.D.	3592.1	30.0	378.3	65.8	9.4	4555.7	

Table 5-4. KZ-41 rat liver microsomal stability study.

	KZ-41		Positive control		Negative control	
Time (Min)	% Remaining	SD	% Remaining	SD	% Remaining	SD
0	100.0	13.3	100.0	9.0	100.0	9.3
45	109.9		32.1		104.2	
90	106.5		42.3		87.9	

Positive control (propranolol), Negative control (propranolol with deactivated microsomes).

Table 5-5. KZ-41 protein binding in rat plasma.

Concentration, ng/mL	% Bound	% Free drug	Standard deviation (%)
50	21.27	78.73	10.01
500	28.83	71.17	9.61
5000	32.29	67.71	7.89
Mean	27.46	72.54	9.17

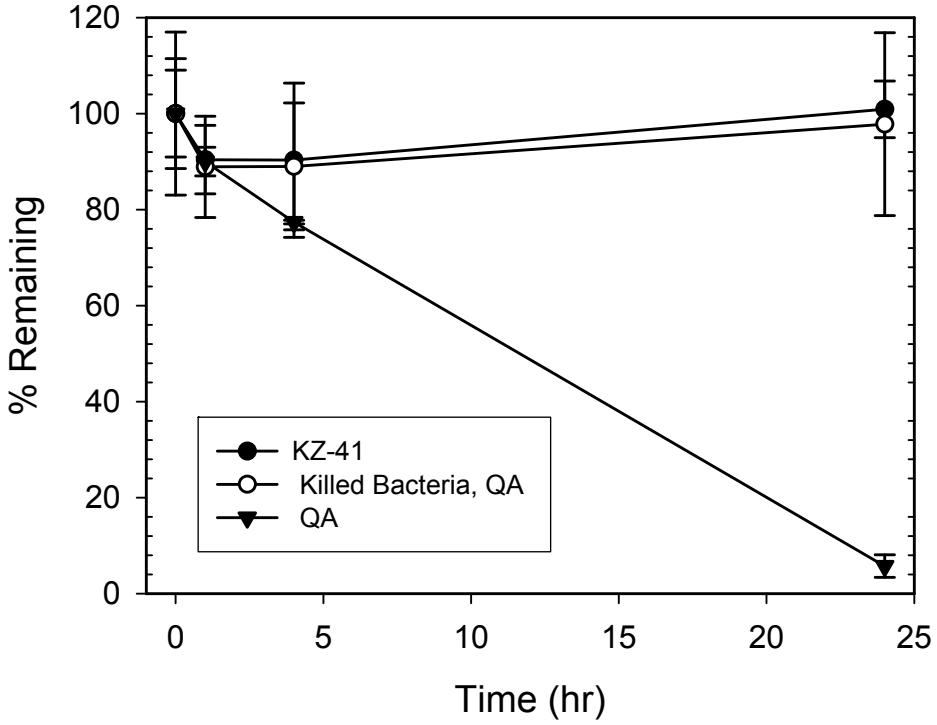


Figure 5-3. Bacterial stability of KZ-41. The parent compound KZ-41 remained 100% unchanged with cultured bacteria over 24 hr. As expected, QA was not stable in bacterial cultured media, less than 10% unchanged QA remained after one day.

administered dose was excreted in urine and feces. Third, the mean plasma clearance after i.v. administration was 13.8 mL/min/kg, comparing to a typical hepatic blood flow of 55.2 mL/min/kg,136 the theoretical hepatic extraction ratio (ER) for this compound could be estimated as 25% (13.8/55.2). Considering first-pass inactivation alone, this would suggest that maximum oral bioavailability for KZ-41 could theoretically reach 75% (100%-ER). The actually observed bioavailability of about 100% indicated there was almost no first-pass inactivation in the intestinal wall and/or the liver. It was supported by liver microsomal stability study data. Those data suggested that plasma clearance mainly come from renal and/or other routes.

The plasma protein binding of KZ-41 was moderate and not concentration dependent over the concentration range of 50 ng/mL to 5000 ng/mL. This finding will be very useful when choosing dose in animal study, the higher dose will not cause dose dependent pharmacokinetics problems due to plasma protein binding.

5.4 Conclusion

In summary, KZ-41 was well absorbed with approximately 100% bioavailability after oral dose in rat. It was not the substrate of bacterial *Gluconobacter oxydans*. It has high aqueous solubility and metabolic stability. Its plasma protein binding was moderate. KZ-41 has good biopharmaceutical and pharmaceutics properties, is ideal for further development of an orally active anti-inflammatory drug.

CHAPTER 6. SUMMARY

Quinic acid (QA) esters found in hot water extracts of *Uncaria tomentosa* (a.k.a. Cat's claw) exert anti-inflammatory activity through mechanisms involving inhibition of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB). Herein, we describe the synthesis and biological testing of novel QA derivatives. Inhibition of NF-κB was assessed using A549 (Type II alveolar epithelial-like) cells that stably express a secreted alkaline phosphatase (SEAP) reporter driven by an NF-κB response element. A549- NF-κB cells were stimulated with TNF-α (10 ng/mL) in the presence or absence of QA derivative for 18 hours followed by measurement of SEAP activity. Amide substitution at the carboxylic acid position yielded potent inhibitors of NF-κB. A variety of modifications to the amide substitution were tolerated with the N-propyl amide derivative **3** being the most potent. Compound **3** was named as KZ-41. The NF-κB inhibitory potency (IC_{50}) of our most active analog KZ-41 was determined to be 2.83±1.76 μM. Further examination of the SAR demonstrated that acetylation of the hydroxyl groups reduced NF-κB inhibitory activity. QA amide derivatives lacked anti-oxidant activity and were found to be neither anti-proliferative nor cytotoxic at concentrations up to 100 μM.

We investigated the importance of the hydroxyl groups on the cyclohexane ring by preparing the dehydroquinate amide analogs. We hypothesized these modifications will retain anti-inflammatory activity with enhanced resistance to microbial degradation. The dehydroxyl QA amides **18** and **20** were prepared. They showed NF-κB inhibition at concentration 1 μM. The extent of NF-κB inhibition was close to positive control drug dexamethasone. The dehydroxyl QA amide with a double bond **38** was also synthesized. The QA amide esters **24**, **25**, **29** and **33** were designed and synthesized to retain anti-inflammatory activity with additional antioxidant properties. As expected, compound **25** showed strong anti-oxidant activity. Furthermore, they were hydrolyzed by the microflora, for an example, **25** was hydrolyzed into SA and **3**. Both SA and **3** will not be consumed by gut bacteria, and are easily absorbed in animal digestive tract. This was done by other researchers, and not reported here.

To facilitate pre-clinical biopharmaceutic and pharmacokinetic (B/PK) studies of our lead QA amide analog KZ-41, we developed and validated a novel hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC-MS/MS) analytical assay. An analog of KZ-41 was used as internal standard (IS). KZ-41 and the IS were obtained by protein precipitation and separated by HILIC chromatography using acetonitrile and water. A triple quadrupole mass spectrometer operating in the negative electrospray ionization mode with multiple reaction monitoring was used to detect KZ-41 and IS transitions of m/z 232 → 178 and 272 → 218 respectively. The lower limit of quantification (LLOQ) was 0.5 ng/mL in plasma. The method was validated for selectivity, linearity, accuracy and precision in rat plasma. The ion suppression, recovery and stability of the analyte in the biological matrix were also tested. The assay developed is rapid, sensitive and robust enough to support preclinical B/PK studies of KZ-41.

The study was also conducted to characterize the biopharmaceutics and pre-clinical pharmacokinetics of the lead QA amide analog, KZ-41. Rats (n=6/group) received a dose of either an i.v. or p.o. dose (10 mg/kg) of KZ-41. Pharmacokinetic parameters were determined from concentration - time profiles by non-compartmental analysis. Bacterial stability study was conducted in cultured bacterial *gluconobacter oxydans*. Plasma protein binding and metabolic stability were determined using equilibrium dialysis and rat liver microsomes respectively. Following i.v. administration, KZ-41 demonstrated a medium clearance (15.1±4.8 mL/min/kg), medium volume of distribution (3.3±1.1 L/kg), and a terminal half-life of 2.6±0.4 hrs. KZ-41 was rapidly absorbed with complete oral bioavailability (F≈1), which was consistent with the fact that KZ-41 was not susceptible to degradation in bacterial and liver microsomal studies. KZ-41 binding to plasma proteins was about 30%. These studies demonstrate that new QA analog KZ-41 is an orally active anti-inflammatory agent.

In summary, we have discovered a novel series of non-toxic QA amides that potently inhibit NF-κB activity in TNF-α-stimulated human alveolar Type II-like epithelial cells (A549). The NF-κB inhibitory potency (IC_{50}) of our most active analog KZ-41 was determined to be 2.83±1.76 μM. We have demonstrated that the QA analogs presented in this work do not exert their activity via anti-oxidant, cytotoxic, or carrageenan induced paw edema mechanisms. KZ-41 was rapidly absorbed with complete oral bioavailability (F≈1). It was not the substrate of bacterial *Gluconobacter oxydans*. It has high aqueous solubility and metabolic stability. Its plasma protein binding was moderate. KZ-41 has good biopharmaceutical and pharmaceutics properties, is idea for further development of an orally active anti-inflammatory drug. Mechanistic studies and pre-clinical efficacy studies of these newly designed compounds in various in vitro and in vivo models are on-going by other researchers, and are not reported in this dissertation.

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