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Modulating the Pharmacokinetics of Bioflavonoids

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Modulating the Pharmacokinetics of Bioflavonoids

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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ABSTRACT

One of the largest obstacles in drug development is to overcome solubility and bioavailability problems. Preformulation strategies such as nanoparticle formation are often employed but sometimes create new issues and are limited in their effectiveness and applications. Since the majority of drugs are marketed and sold as solid forms, drug delivery systems are not always desirable. This is where solid-state chemistry becomes important. Traditional solid-state chemistry approaches are often successful but are sometimes too restrictive and cannot be applied to certain compounds. Cocrystals have emerged as an alternative solid-state technique that can be applied to a broad range of compounds. However, the technology is still very new and its effectiveness in certain conditions had previously not been evaluated.

The studies detailed herein investigated the ability of two different technology platforms for overcoming drug design challenges for two promising bioflavonoids: EGCg and quercetin. Studies have shown that EGCg might be useful for the treatment of Alzheimer's disease and other neurodegenerative diseases. Quercetin is being investigated for numerous bioactivities and is currently being marketed as an energy dietary supplement. Both of these bioflavonoids exhibit poor bioavailability and water solubilities that are at opposite ends of the spectrum. In the chapters to follow, nanoparticle technology was
applied to EGCg and evaluated in cell models of Aβ production, a hallmark of Alzheimer’s disease. Bioavailability improvements were also evaluated in rats. Additionally, new forms of both flavonoids were created using cocrystallization. These new cocrystals were characterized using powder and single crystal x-ray diffraction, differential scanning calorimetry, and thermogravimetric analysis. Solubility and bioavailability changes were also evaluated. These data have strong implications in drug development since they elucidated the strengths and weaknesses of two major technologies in compounds with different design challenges.
CHAPTER 1

INTRODUCTION

1.1 Bioflavonoids

Bioflavonoids are a group of polyphenolic compounds that are common throughout the plant kingdom. They are widely studied and have been found to promote healthy living in epidemiologic studies (Hertog 1996; Arts 2008). Much of the attention that bioflavonoids have attracted is due mainly to the “French paradox”. This is the dietary anomaly in which people in the Mediterranean culture have a higher fat intake but a lower incidence of cardiovascular disease and increased longevity (Formica and Regelson 1995). This phenomenon has been largely attributed to increased dietary intake in bioflavonoids.

1.2 (-)-Epigallocatechin-3-Gallate

1.2.1 EGCg Therapeutic Properties

EGCc has been studied extensively during the last decade for its therapeutic potential in various cancers (Jung, Kim et al. 2001; Wang and Bachrach 2002; Hwang, Ha et al. 2007; Lim, Park et al. 2008; Wang, Hao et al. 2009), Alzheimer’s disease (Rezai-Zadeh, Shytle et al. 2005; Obregon, Rezai-Zadeh et al. 2006; Rezai-Zadeh, Arendash et al. 2008; Lin, Chen et al. 2009), obesity (Hwang, Park et al. 2005; Wolfram 2007), and diabetes (Lin and Lin 2008; Cai
and Lin 2009). In the chapters to come the focus will be on EGCg’s potential as an Alzheimer’s disease therapeutic. Therapies that oppose cleavage of amyloid precursor protein (APP) into Aβ peptides and resultant cerebral amyloidosis, a key pathological feature of Alzheimer’s disease (AD), have become a primary focus in the recent years. The main targets have been β- and γ-secretase, the two proteases that cleave APP at the amino and carboxyl-terminus of the Aβ peptide, respectively, and hence are directly responsible for Aβ peptide generation. However, numerous studies have shown that γ-secretases critically involve physiologically important signaling mechanisms required for cell-fate decisions during development and even during adulthood (De Strooper, Annaert et al. 1999; Walsh, Fadeeva et al. 2003). Most recently, it has been reported that β-secretase modulates myelination in the central and peripheral nervous system (Hu, Hicks et al. 2006). An alternative strategy, namely the activation of α-secretase, has scarcely been investigated for its therapeutic potential. α-secretase cleaves APP substrate within the Aβ peptide domain and precludes peptide generation, thereby promoting the non-amyloidogenic pathway of APP proteolysis. α-secretase activation may even have the added advantage of, not only preventing neurotoxic Aβ peptide formation, but also generating the putatively neuroprotective sAPP-α. Recently, we found that oral administration of green tea-EGCg reduces β-amyloid mediated cognitive impairment, reduces inflammation, and also modulates tau pathology in an Alzheimer’s disease transgenic mouse model (Rezai-Zadeh, Shytle et al. 2005; Arendash, Schleif et al. 2006; Obregón, Rezai-Zadeh et al. 2006; Rezai-Zadeh, Arendash et al. 2008).
For these reasons, EGCg was selected as one of the bioflavonoids for the studies to be discussed in the chapters to come.

1.2.2 EGCg Metabolism

The metabolism of EGCg has been identified as a major factor contributing to its low oral bioavailability. For this reason, EGCg metabolism has been studied in mice, rats, and humans. Because rats are most commonly used for preclinical bioavailability studies, there have been numerous studies on the absorption and metabolism of EGCg in rats (Chen, Lee et al. 1997; Nakagawa and Miyazawa 1997). Rats are typically the preferred species for bioavailability studies because they offer the logistical advantage of being more tolerant to the volumes of blood that must be withdrawn in order to generate a pharmacokinetic curve for each rat. In mice, multiple animals must be sacrificed to generate data for each time point. However, recent data suggests that mice may metabolize polyphenols in a manner that more closely resembles human metabolism from a mechanistic perspective (Lu, Meng et al. 2003). EGCg metabolism was studied in the presence of mice, rat, and human UDP-glucuronosyl-transferase. The investigators reported that mice and humans had a more similar EGCg metabolite profile than rats and humans (Lu, Meng et al. 2003). This is evidenced by the presence of similar levels of (-)-EGCg-4\(\text{"O}\)-glucuronide and (-)-EGCg-3\(\text{"O}\)-glucuronide in mice and in humans. In additional experiments, Lambert and colleagues reported that the rat liver cytosol had a substantially higher capacity for methylating and sulfating EGCg than mice or human liver cytosol (Lambert, Lee et al. 2003). This would account for the previously
reported lower bioavailabilities reported for rats, 1.6 ± 0.6% than for mice, 26.5 ± 
7.5% (Lambert, Lee et al. 2003). In mice and in humans, EGCg is reported to be 
extensively glucoronidated by UDP-glucuronosyl-transferase, which is present at 
high levels in the intestine and liver (Lambert, Lee et al. 2003; Lu, Meng et al. 
2003). This metabolic conjugation reduces the levels of free EGCg in systemic 
circulation for transport to the target organs. Because higher levels of free EGCg 
have been observed in mice than in humans, it would seem that UDP-
glucuronosyltransferases may not be as effective at metabolizing EGCg as 
sulfotransferases and methyltransferases in rats or is more easily saturated. 
Nonetheless, EGCg preformulation studies where metabolism is a targeted 
parameter for improving the pharmacokinetics should use a mouse model, as it is 
probably most relevant for translation in humans. Recently, a clinical trial was 
conducted to assess whether or not long term EGCg supplementation would 
modulate the cytochrome P450 (CYP) system in man, an essential system for 
drug metabolism. They reported that EGCg had no effects on CYP1A2, 
CYP2D6, and CYP2C9, but had very small effects on CYP3A4 activity (Chow, 
Hakim et al. 2006). Chow and colleagues concluded that it is unlikely that long 
term EGCg administration will have any clinically relevant metabolic drug 
interactions for drugs metabolized by the CYP isozymes. Because EGCg 
seemed to have no effect on CYP activity, one might also conclude that these 
enzymes are likely not to blame for the low bioavailability of EGCg. However, 
this presumption is yet to be determined directly.
1.2.3 EGCg Biodistribution

Many studies with animal models have shown that EGCg can prevent carcinogenesis of the skin, lung, oral cavity, esophagus, intestine, colon, prostate (Yang, Maliakal et al. 2002) and exert neuroprotective effects in the central nervous system (Xie, Jiang et al.; Lee, Bae et al. 2004; Giunta, Obregon et al. 2006; Avramovich-Tirosh, Reznichenko et al. 2007; Hou, Chen et al. 2008; Rezai-Zadeh, Arendash et al. 2008). Therefore, one can infer that EGCg must reach these target organs. Several groups have studied the distribution of tea catechins following oral consumption (Chen, Lee et al. 1997; Nakagawa and Miyazawa 1997; Lambert, Lee et al. 2003; Lin, Wang et al. 2007). Lambert et al reported that after intravenous administration of 21.8umol/kg of EGCg in mice, most was distributed to the lung, liver, kidney, small intestine and colon. However, when the EGCg was given per oral dose of 163.8umol/kg, very large quantities of EGCg could be measured in the small intestine and in the colon (Lambert, Lee et al. 2003). This confirms that EGCg is poorly absorbed in the small intestine and could also be due to substantial metabolic conjugation of the EGCg that is absorbed before it reaches systemic circulation, leading to it’s rapid elimination before being distributed to the organs. One of the newest proposed therapeutic uses of EGCg is that it might offer some neuroprotective properties. However, for a compound to have direct effects on the central nervous system, it should cross the blood brain barrier. Nakagawa measured tissue distribution of 500mg/kg EGCg 60 minutes after oral administration in rats. EGCg concentration was highest in the small intestine (565 nmol/g) and colon (68.6
nmol/g), but was also measured in the liver (48.3 nmol/g) and in the brain (0.5 nmol/g) (Nakagawa and Miyazawa 1997). These results indicated that although EGCg can penetrate the blood brain barrier, it does so poorly. Lin et al proposed that this might be due to EGCg’s bipolar functional group and the possibility that EGCg is largely protein-bound in the plasma and that the complex might be too large to traverse cells or capillary membranes in the brain (Lin, Wang et al. 2007). Thus, this is an important limitation that will need to be overcome or compensated for by substantial increases in the systemic absorption of EGCg in order for it to be therapeutically effective in the cerebrospinal compartment.

1.3 Quercetin

Quercetin, 3,3',4',5,7-Pentahydroxyflavone, (QUE) is one of the most common dietary flavonoids and is found throughout the plant kingdom. A typical Western diet was estimated to provide between 0 and 30 mg of QUE per day, most of which is consumed as tea, red wine, fruits and vegetables (Egert, Wolffram et al. 2008). This not only supports its safety for consumption by humans (albeit at a low dose), but also provides epidemiological evidence for its beneficial effects.

1.3.1 Quercetin Therapeutic Properties

Quercetin is best known for its antioxidant activity but has numerous other biological and pharmacological effects including metal chelation, anticarcinogenic, cardioprotective, bacteriostatic, and antiviral activity (Vargas and Burd; Bakay, Mucsi et al. 1968; Formica and Regelson 1995; Spencer, Kuhnle et al. 2003; Leopoldini, Russo et al. 2006; Murakami, Ashida et al. 2008).
1.4 Lipid Carriers

Self-assembled polymer micelles based on amphiphilic block copolymers have attracted substantial interest as delivery vehicles particularly for anti-cancer drugs (Yokoyama, Fukushima et al. 1998; Bontha, Kabanov et al. 2006; Liu, Lee et al. 2006; Tian, Bromberg et al. 2007; Huynh, Passirani et al. 2009; Kim, Kabanov et al. 2009; Siddiqui, Adhami et al. 2009). Additionally, lipid carriers with incorporated drugs have been demonstrated to increase the absorption and circulation time in the body versus stand-alone compounds secondary to minimized renal clearance (Maeda 2001; Huynh, Passirani et al. 2009). Liposomes have been shown to increase the permeability of tea catechins, including EGCg, in a mouse model of basal cell carcinoma (Fang, Lee et al. 2006). However, this study involved intratumor injection and, therefore, did not address the issue of oral bioavailability.

1.5 Cocrystals

Formulation of pharmaceutical ingredients is a fundamental process in drug development. Isolation of crystal forms of active pharmaceutical ingredients (API) is paramount in the industry in order to obtain pure forms. However, in many cases the crystalline API has less than favorable physicochemical characteristics. For this reason, a new branch of formulation is growing in popularity, crystal engineering. Crystal engineering can be used to create new multi-component crystalline solids coined cocrystals. Shan and Zaworotko have defined cocrystals as “multiple component crystals in which all components are solid under ambient conditions. These components co-exist as a stoichiometric
ratio of a target molecule or ion and a neutral molecular cocrystal former(s)” (Shan and Zaworotko 2008). Cocrystals are of growing interest because often various cocrystals can be formed that improve the physicochemical properties (e.g. solubility and stability), improve efficacy (e.g. bioavailability), and provide means for extending the life cycle of old APIs (Yadav, Shete et al. 2009). In a recent study, cocrystallization was used to improve the dissolution profile of a Class II BCS compound (low solubility, high permeability) such that it’s water solubility was 18 times greater, which translated into plasma concentration values that were three fold higher in dogs following oral administration (McNamara, Childs et al. 2006). These findings lend promise to the use of cocrystallization for improving APIs and could even prevent potentially useful compounds from being ignored due to poor clinical efficacy.
CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and Materials

Green tea-derived EGCg (>95% purity by HPLC) was purchased from www.herbs-tech.com. Quercetin dihydrate (98% purity), Isonicotinamide (INM) (99% purity), Caffeine (CAF), and Theobromine (TBR) (98% purity) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). The BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Anti-human amyloid-β antibodies 4G8 and 6E10 were obtained from Signet Laboratories (Dedham, MA, USA) and Biosource International (Camarillo, CA, USA), respectively.

2.2 Cell Models

2.2.1 SweAPP N2a cells; APP$_{695}$

Murine neuroblastoma cells (N2a) stably transfected with the “Swedish” mutant form of APP (SweAPP N2a Cells; APP$_{695}$), a well-established model for mimicking Aβ overproduction characteristic of Alzheimer’s disease were kind gifts from S. Gandy (Thomas Jefferson University, Philadelphia, PA, USA). These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal calf serum (FCS), and 200 µg/ml G418 (for selection). Prior to treatment
N2a cells were differentiated in serum-free Neurobasal medium supplemented with 300 µM dibutyryl-cAMP for 4 hours.

2.3 Sample Processing/ELISA

2.3.1 Lysate preparation

All cultured cells were lysed in ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 1 µg/mL leupeptin, 1 mM PMSF) for 5 minutes followed by scraping. Lysates were collected and centrifuged at 14,000 x g for 15 minutes at 4°C. Protein levels in cell lysate supernatants were determined and normalized by bicinchoninic acid assay (BCA; Pierce Biotechnology, Rockford, IL, USA) in accordance with the manufacturer's instruction.

2.3.2 Aβ ELISA

Conditioned media was collected and total Aβ1-40,42 species were quantified as previously described (Tan et al., 2002). Briefly, 96-well immunoassay plates were coated with monoclonal anti-Aβ1-17 antibody (6E10; 2 µg/mL in PBS; Signet Laboratories, Dedham, MA, USA) overnight at 4 °C. Plates were washed with 0.05% Tween 20 in PBS 5 times and incubated with blocking buffer (PBS with 1% bovine serum albumin (BSA), 5% horse serum) for 2 hours at room temperature. Conditioned media and standards were added to the plates, following appropriate dilutions, and incubated overnight at 4 °C. Following 3 washes, biotinylated anti-Aβ17–26 monoclonal antibody (4G8; 0.5 µg/mL in PBS with 1% BSA; Signet Laboratories) was added to the plates and incubated for 2
hours at room temperature. After 5 washes, streptavidin-horseradish peroxidase (HRP) (1:200 diluted in PBS with 1% BSA) was added to the 96-well plates for 30 minutes at room temperature. Tetramethylbenzidine (TMB) substrate was added to the plates and incubated for 15 minutes at room temperature. Stop solution (2N H2SO4) was added to stop the colorimetric reaction and optical density was determined immediately by a microplate reader at 450 nm. ELISA values from were reported as % change from control or pg of Aβ/mg of total protein, as determined by BCA assay.

2.3.3 sAPP-α ELISA

Conditioned media was collected and sAPP-α levels were quantified using an sAPP-α sandwich ELISA protocol as previously described (Bailey, Giunta et al. 2008). High binding 96-well plates (Nunc, Denmark) were coated with monoclonal antibody 22C11 diluted in 100 µL (1 µg/mL) of carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed five times with PBS-Tween buffer (0.05% Tween 20) and blocked with 300 µL of blocking buffer (1% BSA, 5% Horse Serum in PBS) for 2 hrs at 37°C. Synthetic sAPP-α protein (Abgent, San Diego, CA) was used as the positive control for this ELISA. All samples were analyzed in duplicate. 100 µL samples of conditioned media were added to each well of the plate. The plate was incubated for 2 hrs at 37°C. After washing 5 times, 100 µL of goat anti-human antibody 6E10 (Biosource; diluted 1:3,000 in reagent diluent) was added to each well of the plate. Following 2 hour-incubation at 37°C and 5-time washing, 100 µL of anti-goat IgG conjugated with HRP (1:1500) was added to each well of the plate. The plate was incubated
for 1 hour at 37°C. Following 5-time washing, 100 µL of substrate solution (TMB) was added to each well and plate was incubated at room temperature. Twenty minutes later, 50 µL of stop solution (2 N H$_2$SO$_4$) was added to each well of the plate. The optical density was determined using a microplate reader at 450 nm. Total intracellular protein was quantified using a BCA kit (Pierce Biotechnology, Rockford, IL) in accordance with the manufacturer's instructions. Data was reported as ng of sAPP-α/mg of total intracellular protein produced per well.

2.4 Syntheses

2.4.1 NanoEGCg synthesis

Nanolipidic particles (NanoEGCg) are prepared using a proprietary (US Provisional Patent Application #61/238,381) co-solubilization methodology involving use of monophasic liquid preparations developed by Nature’s Defense Systems, Tampa, Florida. These particles have a defined size range from 30 to 80nm. To form NanoEGCg co-solubilization methodology involving use of monophasic liquid preparations will be employed with proprietary starting materials. These materials are first solubilized into a water-in-ethanol solution (Step 1). Anhydrous EGCg was added to the materials in Step 1 and co-solubilized by mixing at room temperature (Step 2). NanoEGCg particles were formed by the addition of distilled water while mixing materials (Step 3). The final preparation of NanoEGCg particles was stirred for an additional 10 minutes prior to subjecting the preparation to sizing analysis with a Wyatt DynaPro Multiwell Reader (Wyatt Technology Corporation, Santa Barbara, California). The stock
formulations were stored at 20°C and protected from light until needed for no longer than 3 weeks to ensure integrity.

2.4.2 Bulk cocrystal synthesis
The cocrystals were made in bulk for dissolution study by slurry method. For each cocrystal stoichiometric amounts of the starting materials in 5-6 mL of ethanol and stirred overnight with the help of a magnetic stir bar on a stir plate, which produces the cocrystals with 100% yield. The purity of the bulk material is tested by powder X-ray diffraction (PXRD) and differential scanning calorimetry (DSC). For PXRD pattern of the bulk material is compared to the PXRD obtained from the single crystal X-ray diffraction and presence of any additional peaks other than the peaks in the calculated pattern indicates starting materials or impurities. Likewise, the DSC of the bulk cocrystal exhibits endotherm for the melting point of the cocrystals and the desolvation/dehydration.

2.5 Cocrystal characterization
2.5.1 Differential scanning calorimetry (DSC)
Thermal analysis was performed on a TA Instruments DSC 2920 Differential Scanning Calorimeter. Aluminum pans were used for all samples and the instrument was calibrated using an indium standard. For reference, an empty pan sealed in the same way as the sample was used. Using inert nitrogen conditions, the samples were heated in the DSC cell from 30 °C to the required temperature (melting point of the cocrystal) at a rate of 10 °C/min.
2.5.2 Powder x-ray diffraction (PXRD)

A Bruker AXS D8 powder diffractometer was used for all PXRD measurements with experimental parameters as follows: Cu Kα radiation (λ = 1.54056 Å); 40 kV and 30 mA. Scanning interval: 3–40° 2θ; time per step: 0.5 sec. The experimental PXRD patterns and calculated PXRD patterns from single crystal structures were compared to confirm the composition of bulk materials.

2.5.3 Single crystal x-ray data collection and structure determinations

Crystals suitable for X-ray crystallography were selected using an optical microscope. Single crystal X-ray diffraction data were collected and analyzed on a Bruker-AXS SMART-APEX CCD diffractometer with monochromatized Mo Kα radiation (λ = 0.71073 Å) connected to a KRYO-FLEX low temperature device.

2.5.4 Cocrystal water solubility

Each of the cocrystals was synthesized in bulk by taking stoichiometric ratios of the starting materials in methanol and slurrying them for 24 hours, which produced the cocrystals in 100% yield. The slurries were dried at room temperature and were sieved to attain a particle size between 53 and 75 µm. The solubility study was done by taking approximately 4 grams of the cocrystal in 30 mL of water and was stirred with magnetic stir bar at ca. 125 rpm for 24 hours. Aliquots were drawn from the slurry and filtered using a 0.45 µm nylon filters. The remaining solid was analyzed by PXRD and DSC and determined to be the cocrystal and not just a mixture of the starting materials. For every cocrystal, 1mL of the filtered solution from the slurry was transferred into a pre-weighed (w1) vial by using an analytical balance and was put on a hot plate (maintained at
50 °C) to evaporate the water. The vial was weighed again (w2) and the difference in the weights (w2-w1) gave the weight of the cocrystal in 1ml of water from which the weight of the API was calculated. The solubility studies and the gravimetric methods were done in replicates of three. Since the solubility of quercetin dihydrate is so low, it was impossible to obtain experimentally using our gravimetric method. Therefore, we used the reported solubility from the literature for comparison (Srinivas, King et al. 2010).

2.6 Intragastric gavage

All animals were food (not water) deprived for 18 hours prior to the start of the experiment. All formulations were delivered to the animals via oral gavage. The dosage used for all APIs was 100 mg/kg.

2.7 Chromatography

2.7.1 EGCg liquid chromatography with tandem mass spectroscopy

To accurately quantify the concentration of EGCg in the plasma, a previously described method was employed using liquid chromatography with tandem mass spectrometry (Wang, Hop et al. 2000; Sparidans, Lagas et al. 2007; Wang and Miksa 2007).

Stock preparation

A 2.00 mg/mL stock solution of EGCg in DMSO was prepared. The standard spiking solutions were prepared by diluting the stock solution to 1000 and 100 µg/mL using acetonitrile:water (1:1, v:v). Both solutions were protected from light using amber vials and all solutions were stored at −20 °C.

Standard curve preparation
For this analysis two standard curves were prepared one with a higher (10–0.100 µg/ml) dynamic range the other a lower (1000–10ng/ml). Both standard curves were prepared using the appropriate blank plasma containing the preservative. The results indicated that the standard curve performance was within acceptable range for bioanalytical method acceptance \((R^2>0.99)\) (Sparidans et al., 2007; Wang and Miksa, 2007; Wang et al., 2000).

### 2.7.2 Quercetin liquid chromatography with tandem mass spectroscopy

The plasma samples were analyzed for QUE content by the Burnham Institute for Medical Research Pharmacology Core (Orlando, FL). To accurately quantify the concentration of QUE in the plasma, a previously described method was employed using liquid chromatography with tandem mass spectrometry (Wang and Morris 2005). The standards were prepared as follows. A 2.00 mg/mL stock solution of QUE was accurately prepared in DMSO. The stock solution was protected from light using amber vials and stored at -20°C. Standards were prepared using the appropriate blank rat plasma with the ascorbic acid and EDTA preservative. The samples were aliquotted into pre-labeled 1.7 mL microcentrifuge tubes. 100 µL was used as the aliquot volume for all samples. Except for the double blanks, 400 µL of the internal standard spiking solution (Naproxen, 2.00 µg/mL in ACN) was added to all samples. Tubes were then capped, vortexed for 3 minutes and centrifuged for 10 minutes at 14,000 rpm. Approximately 300 µL was then transferred from each tube into a 96-well plate for analysis by LCMSMS. The concentration range of the standard curve was 10 µg/mL to 0.100 µg/mL of QUE. The results indicated that the standard curve
performance was within acceptable range for bioanalytical method acceptance ($R^2 > 0.99$) (Wang, Hop et al. 2000; Sparidans, Lagas et al. 2007; Wang and Miksa 2007).

2.8 Pharmacokinetic parameter calculations

Mean plasma EGCg concentrations ± the standard error in the mean (SEM) was calculated using GraphPad PRISM software (GraphPad Software, Inc.). Pharmacokinetic graphs and parameters were determined using GraphPad PRISM. Pharmacokinetic parameters included $C_{\text{max}}$, $T_{\text{max}}$, $T_{1/2}$, area under curve (AUC), and relative bioavailability ($F_{\text{rel}}$). Relative bioavailability was determined by dividing the AUC of each NanoEGCg formulation by the AUC of the control.
CHAPTER 3

NANOLIPIDIC PARTICLES IMPROVE THE BIOAVAILABILITY AND ALPHA-SECRETASE INDUCING ABILITY OF EPIGALLOCATECHIN-3-GALLATE (EGCg) FOR THE TREATMENT OF ALZHEIMER’S DISEASE

3.1 Introduction

The deterioration, malfunction, or death of neurons is a common etiological factor in several diseases including Alzheimer’s Disease (AD) and HIV-associated dementia (HAD) (Wojtera, Sikorska et al. 2005; Alisky 2007). As the number of elderly individuals continues to rapidly increase, neurodegenerative disease, marked by progressive loss of mnemonic and higher cortical functions, has led to a massive socioeconomic burden which is projected to worsen (Tarkowski, Liljeroth et al. 2003). Specifically, some 15% of the population greater than 65 years of age suffers from dementia (Meeuwsen, German et al. 2009). Its presentation is heterogeneous as it is caused by multiple disorders. Alzheimer’s disease (AD) and vascular dementia (VaD) are the two main causes of dementia affecting between 25–45% and 15–35%, respectively, of all patients suffering from dementia (Burns and Iliffe 2009). Among dementias where brain infectious viruses are etiologic, human
immunodeficiency virus type 1 (HIV-1) associated dementia (HAD) is the most common cause of dementia (Ghafouri, Amini et al. 2006). Prevention of amyloidogenic processing of amyloid precursor protein with the use of natural phytochemicals capable of enhancing α-secretase activity may be a therapeutic approach for treatment of neurodegenerative diseases including Alzheimer's disease (AD) and HIV-associated dementia (HAD). We have previously shown that modulation of apoptosis cascades (Giunta, Obregon et al. 2006; Giunta, Zhou et al. 2008), and APP (amyloid precursor protein) processing (Rezai-Zadeh, Shytle et al. 2005; Obregon, Rezai-Zadeh et al. 2006; Rezai-Zadeh, Shytle et al. 2008) with the green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCg) is a plausible therapy in mouse models of AD and HAD. In spite of these preclinical works, translating them to a human clinical trial has presented problems, primarily as a result of inefficient systemic delivery and bioavailability issues. To achieve maximum response of a neuroprotective agent, novel strategies are required to enhance the oral bioavailability of potentially useful agents. Self-assembled polymer micelles based on amphiphilic block copolymers have attracted substantial interest as delivery vehicles particularly for anti-cancer drugs (Yokoyama, Fukushima et al. 1998; Bontha, Kabanov et al. 2006; Liu, Lee et al. 2006; Tian, Bromberg et al. 2007; Huynh, Passirani et al. 2009; Kim, Kabanov et al. 2009; Siddiqui, Adhami et al. 2009). Additionally, lipid carriers with incorporated drugs have been demonstrated to increase the absorption and circulation time in the body versus stand-alone compounds secondary to minimized renal clearance (Maeda 2001; Huynh, Passirani et al.
This study investigated the ability of nanolipidic particle complexes for increasing the oral bioavailability of EGCg. These nanoparticles (NanoEGCg) differ from traditional liposomes because they do not require micelle formation. Rather, they are drug:lipid complexes. This enables the formation of smaller diameter particles that we hypothesized would be useful for increasing the oral bioavailability of EGCg.

3.2 Materials and Methods

3.2.1 Reagents

Green tea-derived EGCg (>95% purity by HPLC) was purchased from www.herbs-tech.com. The BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Anti-human amyloid-β antibodies 4G8 and 6E10 were obtained from Signet Laboratories (Dedham, MA, USA) and Biosource International (Camarillo, CA, USA), respectively.

3.2.2 Preparation of Nanolipidic EGCg Particles (NanoEGCg)

NanoEGCg was prepared as described in 2.4.1. Six nanoparticle formulations were prepared for the study with various ratios of lipid carrier to EGCg. Formulations prepared for this study were 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 (Nanocarrier material to EGCg on a mg/mg basis). Each formulation was subjected to sizing analysis using a Wyatt DynaPro Multiwell Reader (Wyatt Technology Corporation, Santa Barbara, California). This data is presented in figure 3.1. The particle size was found to range from 30 to 80 nm.
Dynamic light scattering data. A Wyatt DynaPro Multiwell Reader was used to characterize the diameter of the NanoEGCg particles. The data indicates a narrow size distribution, with a cumulants mean of 49.5nm and polydispersity of 0.052.

3.2.3 Rats

Male Sprague Dawley rats weighing 200–250 g were purchased from Harlan Laboratories (Indianapolis, IN). The rats were pre-cannulated by Harlan. The rounded tip catheters were surgically implanted into the jugular vein of the rats making multiple, precise blood draws painless to the animal. The rats were food (not water) deprived for 18 h prior to the start of the experiment. The EGCg formulations were delivered via oral gavage at a dosage of 100 mg EGCg/kg body weight (n=3 per group; 9 total). Blood was collected at the following time points: 0, 5, 10, 30, 60, 120, 240, and 480 min. Because heparin was kept in the catheter lines to prevent clotting, a small amount of blood was drawn and discarded before collecting each sample. Approximately 300 μL of blood was collected in EDTA tubes for each time point. The samples were kept on ice to
preserve their integrity, then centrifuged at 4000 rpm for 10 min, after which the plasma was transferred to sterile centrifuge tubes. A preservative solution was added to each plasma sample at 10% (v/v) concentration to ensure the integrity of the EGCg during storage (Lambert, Lee et al. 2006). This preservative was comprised of 20% ascorbic acid (to prevent oxidation of EGCg) and 0.1% EDTA (to scavenge any metal contaminants). The samples were stored at −80 °C until they were analyzed for EGCg content via LC-MSMS. The LC-MSMS method is described in 2.7.1.

3.2.4 Bioactivity assessment of NanoEGCg formulations

Murine neuroblastoma cells that were stably transfected with the human APP gene (APP; SweAPP N2a cells) were cultured in 24-well tissue-culture plates at 1 x 10^5 cells/well (n = 2 for each condition) with 0.5 mL of complete medium (MEM medium with 10% fetal calf serum). Prior to treatment, the MEM was aspirated and replaced with 0.5 mL of neurobasal media and differentiated with cAMP for 4 hours. Following differentiation, the cells were treated with various nanoparticle formulations and controls (25μM - 3μM) for 18 hours. Controls included two formulations of EGCg without a lipid carrier: one dissolved in water and another in ethanol and water at the same ratio that the nanoparticles were formed (2.4.1). The conditioned media was collected and sAPP-α levels were quantified using a sAPP-α sandwich ELISA protocol as described in 2.3.3.

3.2.5 Pharmacokinetic screening of EGCg formulations in rats

Male Sprague Dawley rats weighing 200-250 g were purchased from Harlan Laboratories (Indianapolis, IN). The rats were pre-cannulated by Harlan. The
rounded tip catheters were surgically implanted into the jugular vein of the rats making multiple, precise blood draws painless to the animal. The rats were food (not water) deprived for 18 hours prior to the start of the experiment. The EGCg formulations were delivered via oral gavage at a dosage of 100 mg EGCg/kg body weight. Blood was collected at the following time points: 0, 5, 10, 30, 60, 120, 240, and 480 minutes. Because heparin was kept in the catheter lines to prevent clotting, a small amount of blood was drawn and discarded before collecting each sample. Approximately 300 µL of blood was collected in EDTA tubes for each time point. The samples were kept on ice to preserve their integrity, then centrifuged at 4000 rpm for 10 minutes, after which the plasma was transferred to sterile centrifuge tubes. A preservative solution was added to each plasma sample at 10% (v/v) concentration to ensure the integrity of the EGCg during storage (Lambert, Lee et al. 2006). This preservative was comprised of 20% ascorbic acid (to prevent oxidation of EGCg) and 0.1% EDTA (to scavenge any metal contaminants). The samples were stored at -80°C until they were analyzed for EGCg content.

3.2.6 Quantification of EGCg in Rat Plasma

The plasma samples were blinded and sent to be analyzed for EGCg content by the Burnham Institute for Medical Research Pharmacology Core (Orlando, FL). The LC-MSMS method described in 2.7.1 was utilized.

3.2.7 Pharmacokinetic Calculations

Mean plasma EGCg concentrations ± the standard error in the mean (SEM) were calculated using GraphPad PRISM software (GraphPad Software, Inc.).
Pharmacokinetic graphs and parameters were determined using GraphPad PRISM. Pharmacokinetic parameters included $C_{\text{max}}$, $T_{\text{max}}$, area under curve (AUC), and relative bioavailability. Relative bioavailability was determined by dividing the AUC of each NanoEGCg formulation by the AUC of the control.

### 3.2.8 Statistical Analysis

**sAPP-α ELISA**

A Two-way ANOVA was performed using GraphPad PRISM software (GraphPad Software, Inc.). This was followed by Bonferroni post-tests to assess the significance of each NanoEGCg formulation versus the EGCg/10%EtOH/H2O Control at each concentration.

**Pharmacokinetics**

A Two-way ANOVA was performed using GraphPad PRISM software (GraphPad Software, Inc.). This was followed by Bonferroni post-tests to assess the significance of the 1:16 NanoEGCg formulation versus the EGCg/10%EtOH/H2O Control at each time point.

### 3.3 Results

#### 3.3.1 Encapsulating EGCg increases sAPP-α generation in cultured SweAPP N2a cells

We utilized an *in vitro* model for Alzheimer’s disease to test the hypothesis that formation of nanoparticle complexes would increase the bioactivity of EGCg by promoting α-secretase activity in cultured SweAPP N2a cells. These cells overproduce human APP, making them ideal for screening compounds that modulate APP processing (Obregon, Rezai-Zadeh et al. 2006). Additionally, we
used this assay as a criterion to select the most effective nanoparticle formulations to carry through to the pharmacokinetic pilot study. Figure 3.2 shows the mean ng of sAPP-α per mg of total protein produced ± standard deviation for all EGCg formulations. Because ethanol was used to solubilize the lipid carrier and EGCg during the NanoEGCg productions process, it was appropriate to include a similarly formulated EGCg solution (10% EtOH solution v/v) to rule out any potential gains in α-secretase activity being due to the alcohol content of the nanoparticle formulations.

From figure 3.2, not all NanoEGCg formulations were effective. In fact, the 1:1 and 1:2 formulations were outperformed by the EGCg and 10% EtOH/H2O control at all concentrations tested. The 1:8 and 1:16 NanoEGCg formulations were selected to be advanced to the pharmacokinetics phase of the study because they outperformed the control at all concentrations tested. The 1:8 formulation was statistically significant at all concentrations, whereas the 1:16 was only statistically significant at the lower two concentrations. Not only did these formulations show marked increases in sAPP-α generation but, perhaps more importantly, they continued to promote enhanced levels of α-secretase activity even at the lowest EGCg concentration tested.
Estimated sAPP-α generation for each treatment group. The sAPP-α concentration (ng/ml) was normalized to the total protein content (mg/ml). Data is presented as mean ng of sAPP-α per mg of total protein produced ± standard deviation. The 1:8 and 1:16 formulations were superior to the other formulations, with the 1:8 showing 92% improvement in α secretase activity over the EtOH control at the 3µM concentration. The 1:8 NanoEGCg formulation was statistically higher than the control at all concentrations tested. The 1:16 NanoEGCg formulation was statistically higher at the lower two concentrations (**P<.01, *P<.05).

3.3.2 Encapsulation improves the bioavailability of EGCg in rats

There have been numerous groups to report the poor oral bioavailability of EGCg (Cai, Anavy et al. 2002; Lambert, Hong et al. 2004; Zhang, Zheng et al. 2004; Feng 2006; Lambert, Sang et al. 2006; Chan, Zhang et al. 2007; Lin, Wang et al. 2007; Henning, Choo et al. 2008). Recent reports suggest that this poor oral bioavailability is mostly due to factors such as poor absorption and intestinal metabolism, rather than elimination via first pass metabolism (Cai, Anavy et al. 2002). Larger lipid-based bilayer delivery systems have been shown to increase
the absorption of poorly permeable compounds (Allen 1998). This preliminary study evaluated the ability of proprietary lipid nanoparticle complexes to increase the oral bioavailability of EGCg in rats. Our results indicate that nanoparticles are highly effective at increasing the absorption of EGCg into systemic circulation. Figure 3.3 shows a compilation of the mean pharmacokinetic curves for the nanoparticle formulation tested and the control. Because EGCg is poorly water soluble, 10% EtOH was added to fully solubilize the EGCg at a concentration equivalent to the NanoEGCg stock (50mg/ml) and ensure accurate dosing in the control. Our data suggests that the nanoparticle formulations result in substantial increases in the absorption of EGCg. Although Figure 3.3 indicates only one NanoEGCg curve, both 1:8 and 1:16 formulations were tested. However, both nanoparticle formulations were similarly absorbed and not statistically different, so the 1:16 preparation was selected to represent the NanoEGCg pharmacokinetic curve. The control was very poorly absorbed in comparison to the NanoEGCg. Statistical significance (\( **P<.01 \)) was observed at the 5 and 10-minute time points. Table 3.1 shows some important pharmacokinetic parameters: \( C_{\text{max}} \), \( T_{\text{max}} \), AUC, and relative bioavailability. The relative bioavailability (defined by the AUC) of the NanoEGCg was 2.31 and 2.50 for the 1:16 and 1:8 formulations, respectively, in comparison to the free EGCg in 10%EtOH solution (10%EtOH Control).
Figure 3.3

EGCg pharmacokinetic curve (mean plasma concentration ± SEM vs. time) for the 1:16 NanoEGCg formulation (n=3) and free EGCg in 10%EtOH solution (n=3). The nanoparticle formulation resulted in substantial increase in systemic EGCg absorption. Statistical significance (**P<.01) was observed at the 5 and 10-minute time points. The 10% EtOH control had very poor absorption, with plasma concentration peaking at 116.57 ng/ml. In comparison, the 1:16 NanoEGCg reached a maximum plasma concentration of 599.33 ng/ml.

Table 3.1 Pharmacokinetic Parameters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cmax</th>
<th>Tmax</th>
<th>AUC (0-240min)</th>
<th>Relative Bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCg+10% EtOH (Control)</td>
<td>116.57</td>
<td>5</td>
<td>14621</td>
<td>1</td>
</tr>
<tr>
<td>NanoEGCg (1:16)</td>
<td>599.33</td>
<td>10</td>
<td>33722</td>
<td>2.31</td>
</tr>
<tr>
<td>NanoEGCg (1:8)</td>
<td>704.67</td>
<td>5</td>
<td>36524</td>
<td>2.50</td>
</tr>
</tbody>
</table>

3.4 Discussion and Conclusion

Nanoparticles and larger liposomes have been investigated extensively for increasing the oral bioavailability of poorly absorbed compounds (Pandey, Ahmad et al. 2005; He, Horn et al. 2007; Kumar, Chandrasekar et al. 2007; Frezard, Martins et al. 2008; Rao, Yajurvedi et al. 2008). It has been recently reported that encapsulating EGCg into liposomes can improve its anti-cancer efficacy (Siddiqui, Adhami et al. 2009) and antioxidant capacity (Italia, Datta et al. 2008), probably by increasing its bioavailability. However, these studies utilized
larger diameter particles (>100nm) and focused primarily on improved efficacy of EGCg for specific disease modifying parameters. Here, we have tested the ability of small diameter nanolipidic particle formation as a method for increasing not only the α-secretase inducing ability of EGCg, but also its oral bioavailability.

It has been shown that an oral dose of 800mg/70kg/day provides approximately 400ng/ml EGCg in human plasma (Chow, Cai et al. 2001). Given that we have recently shown that 1000 - 2000ng/mL of free EGCg is necessary for promoting APP α-secretase cleavage in SweAPP N2a cells (Rezai-Zadeh, Shytle et al. 2005), using linear approximation, an oral dose of EGCg of 1800mg/70kg/day would be required to reach therapeutically effective plasma concentrations of EGCg. From a safety and practicality point of view, this dose might be unacceptable for clinical trials (Ullmann, Haller et al. 2003; Ullmann, Haller et al. 2004). Since the oral EGCg dosage in most clinical trials for cancer therapy is typically not more than 800 mg/day (Chow, Cai et al. 2001) regimens which enhance EGCg bioavailability, effecting reductions in neuropathology and cognitive decline at minimum doses, are very desirable. Thus, the bioavailability of EGCg is an important issue for oral administration of EGCg to clinical trials.

It has been previously reported that decreased bioavailability of EGCg is greatly associated with the glucuronidated form, which is largely present in the plasma of treated mice (Lambert, Lee et al. 2003). Additionally, it has been shown that piperine, an alkaloid derived from black pepper, enhances the bioavailability of EGCg by inhibiting glucuronidation (Lambert, Hong et al. 2004). Unfortunately the consumption of piperine also influences the metabolism of all
other ingested food and drugs (Khajuria, Thusu et al. 2002). For example it increases the plasma concentration of phenytoin (Pattanaik, Hota et al. 2006), propanalol, and theophylline in healthy volunteers (Bano, Raina et al. 1991) and plasma concentrations of rifamipicin (rifampin™) in patients with pulmonary tuberculosis (Zutshi, Singh et al. 1985). By forming EGCg nanolipidic complexes as we have, it is possible to increase the oral bioavailability of EGCg as well as its AD and HAD preventative and therapeutic actions, without affecting the absorption of other ingested compounds. This may be an important factor to consider when bringing an EGCg therapeutic into the clinical setting.

In this study, we have modified EGCg such that it requires no co-administration of other drugs. Rather, it is co-solubilized with a lipid carrier using proprietary methodology to form 30-80nm diameter nanoparticle complexes. The importance of particle diameter for drug delivery is particularly important for delivery of drugs to the brain (Wissing, Kayser et al. 2004). Previously, even smaller diameter liposomes (100nm) have had trouble penetrating the tight junctions between the endothelial cells of the blood brain barrier without osmotic disruption (Sakamoto and Ido 1993). This highlights an important distinction between this nanoparticle technology and previous liposomal technologies, which require micelle formation. NanoEGCg does not involve encapsulating the EGCg into a micelle. Instead, lipid:EGCg complexes are formed. Because the EGCg is not fully encased in a micelle structure, it is possible to achieve smaller diameter particles without compromising the stability of the carrier. Although this preliminary study has demonstrated the ability of nanoparticles to increase the
systemic absorption of EGCg taken orally, it is likely that the small diameter of these particles will also lead to improved blood brain barrier penetration. Further studies will be performed to investigate the possibility that nanolipidic particles can be used to enhance the delivery of poorly absorbed drugs to the brain.

This study provides important preliminary evidence that nanolipidic particles might be useful for safely translating EGCg into human clinical trials. Not only did NanoEGCg more than double the oral bioavailability of EGCg in rats (Figure 3.3) but also was more effective at promoting α-secretase activity in vitro, even at reduced concentrations (Figure 3.2). Taken together, it is possible that NanoEGCg will be therapeutically effective at doses that would be considered acceptable in the clinical setting.
CHAPTER 4

COCRYSTALS OF QUERCETIN WITH IMPROVED SOLUBILITY AND ORAL BIOAVAILABILITY

4.1 Introduction

Flavonoids in general are widely studied and have been found to promote healthy living in epidemiologic studies (Hertog 1996; Arts 2008). Quercetin, 3,3’,4’,5,7-Pentahydroxyflavone, (QUE) is one of the most common dietary flavonoids and is found throughout the plant kingdom (Figure 1). A typical Western diet was estimated to provide between 0 and 30 mg of QUE per day, most of which is consumed as tea, red wine, fruits and vegetables (Egert, Wolffram et al. 2008). This not only supports its safety for consumption by humans (albeit at a low dose), but also provides epidemiological evidence for its beneficial effects. QUE is perhaps best known for its antioxidant activity but has numerous other biological and pharmacological effects including metal chelation, anti-carcinogenic, cardioprotective, bacteriostatic, and antiviral activity (Vargas and Burd; Bakay, Mucsi et al. 1968; Formica and Regelson 1995; Spencer, Kuhnle et al. 2003; Leopoldini, Russo et al. 2006; Murakami, Ashida et al. 2008).
Even though QUE has been found to have many potential beneficial effects, its usefulness \textit{in vivo} is questionable due to unfavorable pharmacokinetics in its pure form. The bioavailability of QUE has been studied extensively (Hollman, van Trijp et al. 1997; Manach, Morand et al. 1997; Graefe, Derendorf et al. 1999; Manach, Texier et al. 1999; Ader, Wessmann et al. 2000; Graefe, Wittig et al. 2001; Cermak, Landgraf et al. 2003; Lesser, Cermak et al. 2004; Erlund, Freese et al. 2006). Unfortunately, it appears that QUE is absorbed poorly, is highly susceptible to metabolic conjugation, and exists mostly as a conjugated form in systemic circulation, resulting in low bioavailability (Manach, Texier et al. 1999; Ader, Wessmann et al. 2000). Furthermore, QUE bioavailability has been evaluated in humans following a single 4 g oral dose. The investigators reported that following oral administration, no measurable plasma or urine QUE concentrations could be detected (Gugler, Leschk et al. 1975). They concluded that less than 1\% of the QUE was absorbed unchanged in the gastrointestinal tract (Gugler, Leschk et al. 1975). This is not surprising given that QUE has extremely low water solubility, a major factor in drug absorption. Not only is QUE absorbed poorly, but also it is conjugated extensively in the liver following oral administration (Reinboth, Wolffram et al.; Manach, Morand et al. 1997; Ader, Wessmann et al. 2000). In dogs, more than 80\% of the circulating flavonols existed as conjugated metabolites (Reinboth, Wolffram et al.). Since many of the proposed health effects of QUE were observed \textit{in vitro} with the unconjugated aglycone, it is questionable whether the QUE metabolites will remain bioactive. Few investigators have studied the
bioactivity of QUE metabolites and those that do typically only look at one potential clinical indication for QUE. Spencer et al studied the bioactivity of in vivo QUE metabolites. They found that 3′-O-methyl quercetin and 4′-O-methyl quercetin had a reduced capacity to protect fibroblasts from peroxide-induced cell damage, whereas quercetin 7-O-β-D-glucuronide was completely inactive (Spencer, Kuhnle et al. 2003). Even though this study only investigated one activity of QUE, it is probable that the in vivo effectiveness of QUE will be limited by its absorption and metabolism.

Thus, novel strategies that can increase the absorption of QUE or affect its metabolism are desirable. The pharmaceutical industry typically employs several methods for correcting compounds that exhibit undesirable physicochemical characteristics including screening for salts, polymorphs, and hydrates/solvates of the active pharmaceutical ingredient (API) (Shan and Zaworotko 2008). However, one long known methodology has remained relatively unexplored by many. Cocrystallization is beginning to attract the attention of the pharmaceutical industry because it can be used to both modify the physicochemical properties of a compound without affecting the intrinsic bioactivity and extend the product life of the API (Sarma, Chen et al. 2011). Cocrystals have been defined as “a multiple component crystal in which all components are solid under ambient conditions when in their pure form” (Shan and Zaworotko 2008). The API and cocrystal former(s) exist at a stoichiometric ratio and form complexes that are generally stabilized by hydrogen bonds. This technique has been used to improve the physicochemical properties of numerous
different APIs (Cheney, Weyna et al.; Yadav, Dabke et al.; Brader, Sukumar et al. 2002; Remenar, Morissette et al. 2003; Childs, Chyall et al. 2004; McNamara, Childs et al. 2006; Hickey, Peterson et al. 2007; Bak, Gore et al. 2008; Basavoju, Bostrom et al. 2008). Most notably, cocrystallization is often used to modulate the solubility of an API. Whenever solubility is a limiting factor in the bioavailability of a compound (like with QUE), modulation of solubility can produce drastic effects. Thus, we determined that QUE would be a good candidate for cocrystallization.

In this report, we evaluate the solubility and pharmacokinetic profile of four novel QUE cocrystals. Each of the cocrystals described were selected due to improved physicochemical properties that we hypothesized would lead to improved bioavailability.

4.2 Materials and Methods

4.2.1 Reagents
Quercetin dihydrate (98% purity), Isonicotinamide (INM) (99% purity), Caffeine (CAF), and Theobromine (TBR) (98% purity) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).
4.2.2 Synthesis of the cocrystals

**QUE**

**QUECAF**

The cocrystal was prepared by desolvating QUECAF•MeOH. It was prepared by overnight slurrying of QUE dihydrate (101.46 mg, 0.300 mmol) and caffeine (58.23 mg, 0.300 mmol) in 2 mL of methanol. The solid was filtered and dried in the vacuum oven for 3 hours at 150 °C, thereby affording QUECAF. (melting point of dried solid = 245.95°C).

**QUECAF•MeOH**: QUE dihydrate (34.0 mg, 0.101 mmol) and CAF (19.0 mg, 0.100 mmol) were dissolved in 6 mL of methanol by heating. The resulting solution was placed in a refrigerator overnight. Golden yellow crystals of the cocrystal were harvested (melting point = 246°C).

---

**Figure 4.1**

QUE cocrystal reaction sheme.
QUEINM: QUE dihydrate (34.0 mg, 0.101 mmol) and INM (12.3 mg, 0.100 mmol) were dissolved in 6 mL of methanol by heating on a hotplate. The resulting solution was placed in a refrigerator. After 2 days, yellow plate crystals of QUEINM were obtained (melting point = 262°C).

QUETBR•2H₂O: QUE dihydrate (33.8 mg, 0.099 mmol) and TBR (18.0 mg, 0.099 mmol) were dissolved in 5 mL of ethanol and 10 mL of 1:1 mixture of water and ethanol respectively by heating. Resulting solutions were filtered together and placed in the refrigerator for slow evaporation. The crystals were harvested after 4 days (melting point = 293.8°C).

Synthesis of cocrystals in bulk for dissolution:

The cocrystals were made in bulk for dissolution study by slurry method. For each cocrystal stoichiometric amounts of the starting materials in 5-6 mL of methanol and stirred overnight with the help of a magnetic stir bar on a stir plate, which produces the cocrystals with 100% yield. The purity of the bulk material is tested by powder X-ray diffraction (PXRD) and differential scanning calorimetry (DSC). For PXRD pattern of the bulk material is compared to the PXRD obtained from the single crystal X-ray diffraction and presence of any additional peaks other than the peaks in the calculated pattern indicates starting materials or impurities. Likewise, the DSC of the bulk cocrystal exhibit endotherm for the melting point of the cocrystals and the desolvation/dehydration.

4.2.3 Characterization of Cocrystals

Differential scanning calorimetry (DSC): Thermal analysis was performed on a TA Instruments DSC 2920 Differential Scanning Calorimeter. Aluminum pans
were used for all samples and the instrument was calibrated using an indium standard. For reference, an empty pan sealed in the same way as the sample was used. Using inert nitrogen conditions, the samples were heated in the DSC cell from 30 °C to the required temperature (melting point of the cocrystal) at a rate of 10°C/min.

**Infrared spectroscopy (FT-IR):** To characterize the cocrystals by infrared spectroscopy a Nicolet Avatar 320 FT-IR instrument was used. Sample amounts of 1-2 mg were used and spectra were measured over the range of 4000–400 cm⁻¹ and analyzed using EZ Omnic software.

**Ultraviolet/Visible Spectroscopy (UV/vis):** UV/vis analysis was performed on a Perkin-Elmer Lambda 900 UV/VIS/NIR spectrometer.

**Powder X-ray diffraction (PXRD):** A Bruker AXS D8 powder diffractometer was used for all PXRD measurements with experimental parameters as follows: Cu Kα radiation (\(\lambda = 1.54056 \text{ Å}\)); 40 kV and 30 mA. Scanning interval: 3–40° 2θ; time per step: 0.5 sec. The experimental PXRD patterns and calculated PXRD patterns from single crystal structures were compared to confirm the composition of bulk materials.

**Single-Crystal X-ray Data Collection and Structure Determinations:** Suitable crystals for X-ray crystallography were selected using an optical microscope. The X-ray diffraction data were collected on Bruker-AXS SMART-APEXII CCD diffractometer using Cu Kα (\(\lambda = 1.54178 \text{ Å}\)) for QUECAF•MeOH and Mo Kα radiation (\(\lambda = 0.71073 \text{ Å}\)) for QUEINM. Indexing was performed using *APEX2* (2010) (Difference Vectors method). Data integration and reduction were
performed using SaintPlus 6.01 (2009). Absorption correction was performed by multi-scan method implemented in SADABS (Sheldrick 2008). Space groups were determined using XPREP implemented in APEX2 (2010). The structure was solved using SHELXS-97 (direct methods) and refined using SHELXL-97 (full-matrix least-squares on $F^2$) contained in APEX2 (2010) and WinGX v1.70.01 (Sheldrick 1990; Sheldrick 1997; Farrugia 1999) program packages. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed in geometrically calculated positions and included in the refinement process using riding model with isotropic thermal parameters: $U_{iso}(H) = 1.5U_{eq}(-CH_3,-OH)$, $U_{iso}(H) = 1.2U_{eq}(-CH)$. Hydrogen atoms of the NH$_2$ group were found on a difference Fourier map and were freely refined.

**4.2.4 Solubility evaluation**

Solubility studies were performed on QUECAF•MeOH, QUECAF, QUEINM, and QUETBR•2H$_2$O using 1:1 mixture of water and ethanol as QUE could not be quantified by using UV/VIS/NIR spectrophotometry in water alone. However the solubility of QUE and its dihydrate in water has been reported by Srinivas et al as 0.00215 mg/mL and 0.00263 mg/mL respectively (Srinivas, King et al. 2010). Each of the cocrystal was synthesized in bulk by taking stoichiometric ratios of the starting materials in methanol and slurring them for 24 hours, which produced the cocrystals in 100% yield. The slurries were dried at room temperature and were sieved to attain a particle size between 53 and 75 µm. The solubility study was done by taking approximately 4 grams of the cocrystal in 30 mL of water and was stirred with magnetic stir bar at ca. 125 rpm for 24 hours.
The solubility studies were conducted by taking approximately 4 grams of the cocrystal in 70 mL of 50% ethanol and were stirred with magnetic stir bar at ca. 125 rpm for 24 hours at room temperature. Aliquots were drawn from the slurry at regular time intervals (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 75, 90, 105, 120, 150, 180, 240 and 1440 minutes) and filtered using a 0.45 µm nylon filters. The filtrates were immediately diluted appropriately and were analyzed to measure the concentration of QUE by using UV/VIS/NIR spectrometer at 360 nm where the interference of the cocrystals formers was not observed. The remaining solids were analyzed by PXRD and DSC. The solubility measurements were done in replicates of three.

4.2.5 Pharmacokinetic screening of quercetin formulations in rats

Male Sprague Dawley rats (n=3 per group) weighing 200-250 g were purchased from Harlan Laboratories (Indianapolis, IN). The rats were purchased precannulated by Harlan. The rounded tip catheters were surgically implanted into the jugular vein of the rats making multiple, precise blood draws painless to the animal. The rats were food (not water) deprived for 18 hours prior to the start of the experiment. Vegetable oil was selected as the gavage vehicle because all crystal forms were observed to be insoluble in it. The QUE formulations were delivered via oral gavage at a dosage of 100 mg quercetin/kg body weight. Blood was collected at the following time points: 0, 5, 10, 30, 60, 120, 240, and 480 minutes. Because heparin was kept in the catheter lines to prevent clotting, a small amount of blood was drawn and discarded before collecting each sample. Approximately 300 µL of blood was collected in EDTA tubes for each
time point. The samples were kept on ice to preserve their integrity, then centrifuged at 4000 rpm for 10 minutes, after which the plasma was transferred to sterile centrifuge tubes. A preservative solution was added to each plasma sample at 10% (v/v) concentration to ensure the integrity of the QUE during storage (Lambert, Lee et al. 2006). This preservative was comprised of 20% ascorbic acid (to prevent oxidation) and 0.1% EDTA (to scavenge any metal contaminants). The samples were stored at -80°C until they were analyzed for QUE content.

4.2.6 Quantification of QUE in Rat Plasma

The plasma samples were analyzed for QUE content by the Burnham Institute for Medical Research Pharmacology Core (Orlando, FL). To accurately quantify the concentration of QUE in the plasma, a previously described method was employed using liquid chromatography with tandem mass spectrometry (Wang and Morris 2005). The standards were prepared as follows. A 2.00 mg/mL stock solution of QUE was accurately prepared in DMSO. The stock solution was protected from light using amber vials and stored at -20°C. Standards were prepared using the appropriate blank rat plasma with the ascorbic acid and EDTA preservative. The samples were aliquotted into pre-labeled 1.7 mL microcentrifuge tubes. 100 µL was used as the aliquot volume for all samples. Except for the double blanks, 400 µL of the internal standard spiking solution (Naproxen, 2.00 µg/mL in ACN) was added to all samples. Tubes were then capped, vortexed for 3 minutes and centrifuged for 10 minutes at 14,000 rpm. Approximately 300 µL was then transferred from each tube into a 96-well plate.
for analysis by LCMSMS. The concentration range of the standard curve was 10 µg/mL to 0.100 µg/mL of QUE. The results indicated that the standard curve performance was within acceptable range for bioanalytical method acceptance ($R^2> 0.99$) (Wang, Hop et al. 2000; Sparidans, Lagas et al. 2007; Wang and Miksa 2007).

4.2.7 Pharmacokinetic Calculations
Mean plasma QUE concentrations and the standard error in the mean (SEM) were graphed using GraphPad PRISM software (GraphPad Software, Inc.). Pharmacokinetic parameters were determined using a commercially available computer modeling program, PK Solutions v2.0.7 (Summit Research Services, Ashland, OH). The reported pharmacokinetic parameters included $C_{\text{MAX}}$, $T_{\text{MAX}}$, area under curve (AUC), relative bioavailability ($F_{\text{REL}}$), absorption half life ($A \ T_{1/2}$), distribution half life ($D \ T_{1/2}$), and elimination half life ($E \ T_{1/2}$). Relative bioavailability was determined by dividing the AUC of each QUE formulation by the control.

Statistical Analysis
A post hoc t-test with Bonferroni correction was used to assess the statistical significance at each time point for the pharmacokinetic study. Each QUE cocrystal was compared to the QUE dihydrate control. The criterion for rejection of the null hypothesis was $P<0.05$.

4.3 Results
4.3.1 Dissolution study of the QUE cocrystals
QUE has very poor water solubility. In fact, when we tried to determine the water solubility of QUE dihydrate it was below our lower limit of detection. Therefore, 1:1 ethanol/water solvent mixture was used for the dissolution studies. The solubility of QUE dihydrate in 1:1 ethanol/water solvent mixture is found to be 0.267 mg/mL. We found that the QUECAF and QUECAF•MeOH cocrystals exhibit the highest solubilities. The maximum solubilities exhibited by QUECAF, QUECAF•MeOH, QUEINM, QUETBR•2H₂O cocrystals were 3.627, 2.018, 1.22 and 0.326 mg/mL respectively. From the dissolution profiles it is evident that QUECAF (desolvated form of QUECAF•MeOH) displayed the highest concentration of QUE. This cocrystal increased the solubility of QUE by 14 fold, and the lowest was by QUETBR•2H₂O, which was only slightly improved over QUE alone. Figure 4.2 represents the dissolution profiles of QUE dihydrate and its cocrystals in the first 4 hours and Figure 4.3 in 24 hours. From the DSC and PXRD obtained from the leftover powders after 24-hour dissolution reveal that QUECAF, QUECAF•MeOH, QUETBR•2H₂O and QUEINM convert back to QUE dihydrate.
Figure 4.2
Dissolution profiles of QUE dihydrate and its cocrystals in 1:1 ethanol/water mixture (a) first 4 hours.

Figure 4.3
Dissolution profiles of QUE dihydrate and its cocrystals in 1:1 ethanol/water mixture (a) first 4 hours.
4.3.2 Pharmacokinetics of the QUE cocrystals

We hypothesized that the improvements in solubility would lead to improved bioavailability for the QUE cocrystals. This hypothesis was supported in our preliminary pharmacokinetic evaluation. The pharmacokinetic curves are shown in Figure 4.4.

![Pharmacokinetic profiles](image)

**Figure 4.4**
Pharmacokinetic profiles (mean plasma concentration + SEM versus time). There were n=3 rats per group. Statistical significance was achieved for QUEINM at t=10 (p<0.01) and for QUECAF•MeOH at t=5 (p<0.001).

As expected, the QUE dihydrate was absorbed very poorly. In fact, only one of the three rats exhibited levels of QUE that were above our lower limit of quantification (0.100 µg/mL) and only at one time point, the T\text{MAX}, t=30 minutes. This is easily explained by the extremely poor solubility of QUE dihydrate.
Because the QUE dihydrate was so poorly absorbed, we were unable to calculate half-life values for the three pharmacokinetic phases (absorption, distribution, and elimination). All four of the cocrystals exhibited favorable solubility and improved pharmacokinetic parameters. These pharmacokinetic parameters including the T_{MAX}, C_{MAX}, AUC, F_{REL}, A T_{1/2}, D T_{1/2}, and E T_{1/2} can be found in Table 4.1. Interestingly, the QUETBR•2H$_2$O cocrystal was only slightly more soluble than QUE dihydrate but exhibited vastly superior pharmacokinetic properties. The QUETBR•2H$_2$O cocrystal had the highest F_{REL} at 9.93. This means that the AUC was nearly 10-fold higher than with QUE dihydrate. Additionally, the elimination of QUETBR•2H$_2$O appears to be slowed. The E T_{1/2} was the highest for this cocrystal at 145 minutes (Table 4.1). All of the cocrystals reached systemic circulation more quickly than the QUE control, which is consistent with the improved solubility. Perhaps most importantly, the relative bioavailability (F_{REL}) was much higher with the cocrystals. QUEINM had the second greatest F_{REL} at 5.46, followed by the QUECAF•MeOH and QUECAF at 4.01 and 2.57, respectively. The E T_{1/2} followed the same trend as the F_{REL}. The QUEINM exhibited an E T_{1/2} of 77 minutes and the QUECAF•MeOH and QUECAF cocrystals were 52 and 26 minutes, respectively.
### Table 4.1. Pharmacokinetic Parameters

<table>
<thead>
<tr>
<th></th>
<th>QUE</th>
<th>QUEINM</th>
<th>QUECAF•MeOH</th>
<th>QUECAF</th>
<th>QUETBR•2H₂O</th>
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</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;MAX&lt;/sub&gt;(min)</td>
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<td>5</td>
<td>10</td>
<td>5</td>
</tr>
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<td>C&lt;sub&gt;MAX&lt;/sub&gt;(ng/mL)</td>
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<td>1401</td>
<td>2612</td>
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<tr>
<td>AUC (ng•min/mL)</td>
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<td>9.93</td>
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<td>A T&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
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<td>D T&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
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### 4.3.3 Crystal structures of the QUE cocrystals

**Table 4.2.** Crystallographic data and structure refinement parameters for the cocrystals reported herein.

<table>
<thead>
<tr>
<th></th>
<th>QUEINM</th>
<th>QUECAF•MeOH</th>
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<tr>
<td>Formula</td>
<td>C&lt;sub&gt;21&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;8&lt;/sub&gt;</td>
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<td>Crystal system</td>
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</tr>
<tr>
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</tr>
<tr>
<td>&lt;i&gt;a&lt;/i&gt; (Å)</td>
<td>4.978 (1)</td>
<td>10.309 (3)</td>
</tr>
<tr>
<td>&lt;i&gt;b&lt;/i&gt; (Å)</td>
<td>12.636 (3)</td>
<td>14.853(4)</td>
</tr>
<tr>
<td>&lt;i&gt;c&lt;/i&gt; (Å)</td>
<td>15.571 (3)</td>
<td>15.199 (5)</td>
</tr>
<tr>
<td>α (deg)</td>
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<td>90</td>
</tr>
<tr>
<td>β (deg)</td>
<td>97.63 (3)</td>
<td>100.612 (2)</td>
</tr>
<tr>
<td>γ (deg)</td>
<td>99.39 (3)</td>
<td>90</td>
</tr>
<tr>
<td>V /Å&lt;sup&gt;3&lt;/sup&gt;</td>
<td>885.7 (3)</td>
<td>2287.51 (12)</td>
</tr>
<tr>
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<td>1.535</td>
</tr>
<tr>
<td>Z</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>θ range</td>
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<td>4.20 to 67.88</td>
</tr>
<tr>
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<td>4031/353</td>
</tr>
<tr>
<td>T /K</td>
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<td>100 (2)</td>
</tr>
<tr>
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<td>0.0434</td>
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<td>0.1090</td>
</tr>
<tr>
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<td>1.035</td>
</tr>
<tr>
<td>Abs coef.</td>
<td>0.124</td>
<td>0.1033</td>
</tr>
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</table>

**QUEINM:** The 1:1 cocrystal of QUEINM contains two molecules of QUE and INM in the unit cell and crystallizes in <i>P</i>-<i>L</i> space group. The catechol moiety of QUE molecules form the O···O-H supramolecular homosynthet with hydrogen
bond distance of 2.765 (3) Å. The carbonyl and the syn-hydrogens of the amide functionality of INM molecules hydrogen bond to the catechol dimer on either side and results in the formation of a ring described by \( R_2^2 \) (8) graph set (Etter, MacDonald et al. 1990) (N-H···O: 2.610 (3) Å and O···O-H: 3.028 (4) Å). Thus, a four-component assembly is generated by two molecules of INM and QUE molecules which can be described by \( R_4^4 \) (18) graph set. The \( \text{N}_{\text{arom}} \) and the anti-hydrogen of the amide moiety of INM molecules interacts with one of the O-H moieties (O-H···\( \text{N}_{\text{arom}} \): 2.688 (3) Å) and the carbonyl moieties (O···N-H: 3.019 (3) Å of QUE molecule respectively. The supramolecular interactions between QUE and INM molecules in QUEINM cocrystal overall lead to the generation of a 2-D sheet as shown in Figure 4.5.
Figure 4.5
Representation of supramolecular sheet in QUEINM. In the cocrystal the QUE and INM molecules interact via $\text{O-H\cdots N}_{\text{arom}}$ supramolecular H-bonds. Two QUE molecules form the catechol dimer through $\text{O-H\cdots O}$ H-bonds and the amide functionalities of the INM molecules H-bond on either side of the catechol dimer. Overall H-bonding results in the generation of supramolecular sheet.
**Table 4.2.** Crystallographic data and structure refinement parameters for the cocrystals reported herein.

<table>
<thead>
<tr>
<th></th>
<th>QUEINM</th>
<th>QUECAF•MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formula</strong></td>
<td>C_{21}H_{16}N_{2}O_{8}</td>
<td>C_{24}H_{24}N_{4}O_{10}</td>
</tr>
<tr>
<td><strong>MW</strong></td>
<td>424.36</td>
<td>528.47</td>
</tr>
<tr>
<td><strong>Crystal system</strong></td>
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<td>Monoclinic</td>
</tr>
<tr>
<td><strong>Space group</strong></td>
<td>P-1</td>
<td>P2(1)/c</td>
</tr>
<tr>
<td><strong>a (Å)</strong></td>
<td>4.978 (1)</td>
<td>10.309 (3)</td>
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</tr>
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<td><strong>R_1 [I&gt;2sigma(I)]</strong></td>
<td>0.0592</td>
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<td><strong>wR_2</strong></td>
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<td>1.091</td>
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<tr>
<td><strong>Abs coef.</strong></td>
<td>0.124</td>
<td>0.1033</td>
</tr>
</tbody>
</table>

**QUECAF•MeOH:** This cocrystal crystallizes in a P2_1/c space group. The N_{arom} of the imidazole ring and the CO of the imide ring of CAF interacts with O-H moieties of QUE through O-H···N_{arom} (2.821 (3) Å) and CO···O-H (2.716 (3) Å) hydrogen bonds. Both the O-H in the catechol moiety of QUE molecule hydrogen bond to the O-H of neighboring QUE molecule through O-H···O and O···O-H (2.774 (3) Å and 2.847 (3) Å respectively). Supramolecular heterosynths: One of the O-H adjacent to the CO functionality engages in a strong intramolecular H-bonding (2.602 (3) Å). The methanol molecule interacts with the remaining CO group on the imide ring of CAF molecule with a hydrogen bond distance of 2.712 (3) Å. The overall interaction between QUE, CAF and...
MeOH results in the formation of a supramolecular sheet as shown in Figure 4.6.

**Figure 4.6**
Intermolecular hydrogen bonding in QUECAF•MeOH. QUE molecules interact with CAF molecules through O-H⋯N_{arom} and O-H⋯C=O supramolecular H-bonds and the MeOH molecules just interact with the CAF molecules. A supramolecular sheet is produced by hydrogen bonding between QUE, CAF and MeOH molecules.

The MeOH molecules in one layer interact with one of the O-H of the catechol moieties of QUE in another supramolecular sheet through O⋯O-H hydrogen bonds. This results in the formation of bilayers as shown in Figure 4.7.

**Figure 4.7**
Illustration of bilayers in QUECAF•MeOH. Each supramolecular sheet in QUECAF•MeOH interacts with the other other sheets through MeOH molecules and results in the formation of bilayers which can be described as ABAB and so on.
The crystal structure of QUETBR•2H$_2$O has been published elsewhere (Clarke, Arora et al. 2010). The crystal structure of QUECAF has not been determined yet but the chemical identity of the cocrystal is confirmed from the PXRD, IR, DSC and TGA. The PXRD reveals that QUECAF is isostructural to QUECAF•MeOH (Figure 4.8a) and the DSC confirms that after the loss of MeOH molecule from QUECAF•MeOH the cocrystal is still stable and has the same melting point as that of the solvated one (Figure 4.8b).

![Figure 4.8a](image)

Comparison PXRDs of QUECAF•MeOH and QUECAF; (b) Comparison of DSCs of QUECAF•MeOH and QUECAF. The PXRD patterns of QUECAF•MeOH calculated and experimental (bulk powder) matches exactly indicating the total conversion of the starting materials into cocrystal and no presence of starting materials. The similarity in the PXRD patterns of QUECAF•MeOH and QUECAF indicates isostructural nature of both cocrystals.
Figure 4.8b
Comparison of DSCs of QUECAF•MeOH and QUECAF. The DSC of QUECAF reveals that the crystal packing is intact even after the removal of MeOH molecule as its melting point matches with that of QUECAF•MeOH.

4.4 Discussion and Conclusion

Given that QUE exhibits numerous useful bioactivities it is not surprising that it remains to be studied even after the discovery of its poor bioavailability in 1975 (Gugler, Leschik et al. 1975). Since then, there have been several follow-up reports of QUE deposition and bioavailability in various animal models (Manach, Morand et al. 1997; Manach, Texier et al. 1999; Ader, Wessmann et al. 2000; Morand, Manach et al. 2000; Cermak, Landgraf et al. 2003; Khaled, El-Sayed et al. 2003; Lesser, Cermak et al. 2004; Mullen, Rouanet et al. 2008). The reoccurring theme in the existing literature is that QUE is present in systemic circulation mainly as conjugated metabolites. Piskula et al (1998) studied the
bioavailability of QUE in various vehicles in a rat model and reported circulating levels of QUE and its metabolites (Piskula and Terao 1998). Not surprisingly, the free, unconjugated QUE was detected only when QUE was administered in a propylene glycol vehicle, in which it was most soluble compared to the other vehicles. They later hypothesized that this phenomenon was likely due to animals' overdosing. It is not clear whether they meant experimental error (animals given more than 50 mg/kg) or a saturation of liver first pass enzymes due to more rapid absorption in the vehicle that exhibited the highest solubility. Either of these are feasible explanations. Nonetheless, it was clear that QUE was present in vivo mainly as a conjugated metabolite and that its solubility was an important factor in overall absorption. The premise that modulating the solubility of QUE could affect its absorption is what fueled our study and others before it. Kim et al (2009) reported several new quercetin-amino acid conjugates that exhibited improved water solubility and in vitro stability and permeability (Kim, Park et al. 2009). Even though this will presumably lead to improved bioavailability, this remains to be seen due to the lack of an in vivo evaluation.

In this study, we evaluated the bioavailability of four cocrystals of QUE with varying degrees of improved water solubility in comparison to QUE alone. Our in vivo pharmacokinetic evaluation is in agreement with existing literature in that modulating solubility did impact absorption patterns of the QUE cocrystals. Since solubility often limits the rate of absorption of a compound, one would expect that improving the solubility of a poorly soluble compound would shift to the left the time at which maximal plasma levels were achieved. This was the
case for all of the cocrystals presented in this study. In table 4.1, the QUE dihydrate control had a $T_{MAX}$ of 30 minutes, whereas the cocrystals peaked at 5 and 10 minutes. Not only did the QUE cocrystals reach systemic circulation more quickly than QUE dihydrate but they also reached significantly higher concentrations. The QUECAF•MeOH cocrystal peaked rapidly at 5 minutes with 7.7 µM free QUE. For comparison, the QUE dihydrate control peaked at 30 minutes with 0.84 µM QUE aglycone.

Even though solubility is a limiting factor of the oral bioavailability of QUE, it is apparent that it is not the only factor. Modulation of metabolism can also have drastic effects on oral bioavailability. Recently, one study reported that several ester-based precursors to QUE might be useful for increasing systemic aglycone concentrations (Biasutto, Marotta et al. 2007). The investigators demonstrated that some ester precursors were resistant to phase II conjugation by tight monolayers of MDCK-1, MDCK-2, and Caco-2 cells. They hypothesize that in vivo the residual acyl groups will be eliminated leaving the QUE aglycone that is known to be bioactive. Although this data is promising, the study is limited due to the lack of in vivo evaluation. Additionally, one of the major limitations to QUE is its very low water solubility. Each of these precursors was reported to exhibit solubility similar to QUE. This might pose a formulation dilemma due to insolubility and poor dissolution at physiologically relevant doses in vivo.

Interestingly, post-hoc analysis of the pharmacokinetic curve (Fig 4.4) revealed that modulation of solubility is probably not the only contributing factor to the improved bioavailability of some of the QUE cocrystals. This is evident
when the relative solubilities of each cocrystal (Fig 4.3) are compared to the pharmacokinetic profile (Fig 4.4). Based on solubility differences alone, the QUECAF cocrystal would be expected to have the highest $C_{\text{MAX}}$ since it would be solubilized, and thus absorbed, more completely than the others. However, the QUECAF cocrystal had the lowest $C_{\text{MAX}}$ of all of the cocrystals, peaking at just 656 ng/mL at 10 minutes. Furthermore, the QUEINM cocrystal had the second lowest solubility and caused systemic QUE aglycone levels to reach 1401 ng/mL, second only to the QUECAF•MeOH cocrystal. Therefore, there must be factors other than solubility at play. It is possible that the INM might reduce the first pass effect of the liver enzymes on QUE. Perhaps most interesting is the pharmacokinetic profile of the QUETBR•2H$_2$O cocrystal (Fig 4.4). Despite having a solubility that is only slightly higher than QUE dihydrate, this cocrystal achieved sustained levels of quercetin aglycone, indicative of modulated elimination. The resultant $F_{\text{REL}}$ of the QUETBR•2H$_2$O cocrystal was 9.93, the largest increase observed in this study (Table 4.1). This cocrystal also exhibited the longest $E_{1/2}$, 145 minutes (Table 4.1). Still, we cannot reach any definitive conclusion about the metabolism of the cocrystals since we did not measure circulating QUE metabolites.

These results add to existing evidence that cocrystallization is a useful methodology for improving the physicochemical properties of a compound and that these improvements can lead to drastically enhanced bioavailability when applied to compounds that are limited by their solubility. This same ideology was previously shown to be successful in several preclinical studies. Carbamazepine
(CBZ) is used for the treatment of epilepsy and bipolar disorder but also suffers from low solubility and oral bioavailability. Cocrystals of CBZ and saccharin were administered orally to dogs and were found to achieve greater plasma concentrations than the API alone (Hickey, Peterson et al. 2007). This improvement in bioavailability was attributed to the improved solubility of the cocrystal. AMG 517, VR1 (vanilloid receptor 1) antagonist is a practically insoluble drug, which limits its bioavailability. Bak et al reported that the cocrystallization of AMG 517 with sorbic acid dramatically increases its bioavailability in rats (Bak, Gore et al. 2008). Similarly, Jung et al used cocrystallization to improve the solubility, and consequent bioavailability of indomethacin (Jung, Kim et al. 2010). Thus, modulating the solubility of a poorly soluble API using cocrystallization appears to be a proven, highly effective, and broadly applicable method for improving oral bioavailability.

Taken with the existing evidence from the literature, the results of this study further implicate the potential for cocrystallization in drug development. One limitation is that the plasma samples were analyzed for free, unconjugated QUE content alone. This is because modulated metabolism was not an expected outcome of cocrystallization. However, given the unexpected discrepancies between the solubility and pharmacokinetic profiles of the cocrystals, modulated metabolism is probable. Future studies should investigate whether cocrystallization with certain cocrystal formers can be used to modulate the metabolism of the API. Nevertheless, this study implicates cocrystallization as a potential solution to the solubility and bioavailability problems that thwart the
success of QUE as an effective treatment option for its numerous clinical indications.
CHAPTER 5

MODULATING THE SOLUBILITY OF EGCg USING COCRYSTALLIZATION
AND ITS EFFECTS ON PHARMACOKINETICS

5.1 Introduction

EGCg and its therapeutic uses, metabolism, and problematic clinical translation are discussed in detail in Chapter 1. Due to extremely poor bioavailability, novel methods for improving its absorption and/or therapeutic efficacy are desirable. Nanolipidic particles were used to improve the absorption of EGCg in Chapter 3. This chapter aims to modulate the dissolution, and consequent pharmacokinetics of EGCg using cocrystallization.

In the previous chapter, modulating the solubility of quercetin using cocrystallization drastically improved its bioavailability. However, unlike quercetin, EGCg is highly soluble in water. Therefore, it was selected to determine the effects that reducing the solubility through cocrystallization might have on the pharmacokinetic profile. Compounds that are highly water-soluble are typically absorbed well in the gastrointestinal tract but are consequently easily excreted and, thus, exhibit low half lives ($T_{1/2}$). We hypothesized that we could improve the circulation time of EGCg by reducing its water solubility through cocrystallization. This chapter will describe the synthesis, solubility, and
bioavailability of four new EGCg cocrystals: EGCg/Nicotinamide hydrate (EGCgNIC•9H2O), EGCg/Isonicotinamide hydrate (EGCgINM•5H2O), EGCg/Isonicotinic Acid (EGCgINA), EGCg/Isonicotinic Acid hydrate (EGCgINA•3H2O).

5.2 Materials and Methods

5.2.1 Reagents

EGCg (>95% purity by HPLC) was purchased from www.herbs-tech.com. Isonicotinamide (INM) (99% purity), Nicotinamide (99% purity), and Isonicotinic Acid (99% Purity) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

5.2.2 Synthesis of the cocrystals

![Figure 5.1]

EGCg cocystal reaction scheme.
EGCgNIC•9H$_2$O: EGCg (90% pure, 45.83 mg, 0.1000 mmol) and nicotinamide (99% pure, 12.21 mg, 0.1000 mmol) were dissolved in 2 mL of water. Colorless crystals were harvested after 3 days. The cocrystal can also be obtained by solvent drop grinding and slurrying with water. (melting point = 90.0 °C).

EGCgINM•5H$_2$O: EGCg (90% pure, 45.83 mg, 0.1000 mmol) and iso-nicotinamide (99% pure, 12.21 mg, 0.1000 mmol) were dissolved in 6 mL of water. Colorless crystals were harvested after ca. 5 minutes. (melting point = 155.0 °C).

EGCgINA•3H$_2$O: EGCg (90% pure, 45.83 mg, 0.1000 mmol) and isonicotinic acid (99% pure, 12.31 mg, 0.1000 mmol) were dissolved in 10 mL of water. Colorless crystals were harvested after 1 day. The cocrystal can also be obtained by solvent drop grinding and slurrying with water. (melting point = 190 °C).

EGCgINA: EGCg (90% pure, 45.83 mg, 0.1000 mmol) and isonicotinic acid (99% pure, 12.31 mg, 0.1000 mmol) were dissolved in 10 mL of water. Colorless crystals were harvested after 1 day. The cocrystal can also be obtained by solvent drop grinding and slurrying with water. The cocrystal loses water after heating, converting to an anhydrous cocrystal. (melting point = 190 °C).

5.2.3 Solubility evaluation

Solubility studies were performed on EGCgNIC•9H$_2$O, EGCgINM•5H$_2$O, EGCgINA•3H$_2$O, and EGCgINA. Each of the cocrystals was synthesized in bulk by taking stoichiometric ratios of the starting materials in methanol and slurrying them for 24 hours, which produced the cocrystals in 100% yield. The slurries were dried at room temperature and were sieved to attain a particle size between
53 and 75 µm. The solubility study was done by taking approximately 4 grams of the cocrystal in 30 mL of water and was stirred with magnetic stir bar at ca. 125 rpm for 24 hours. Aliquots were drawn from the slurry and filtered using a 0.45 µm nylon filters. The remaining solid was analyzed by PXRD and DSC. For every cocrystal, 1mL of the filtered solution from the slurry was transferred into a pre-weighed (w1) vial by using an analytical balance and was put on a hot plate (maintained at 50 °C) to evaporate the water. The vial was weighed again (w2) and the difference in the weights (w2-w1) gave the weight of the cocrystal in 1ml of water from which the weight of EGCg was calculated. The solubility studies and the gravimetric methods were done in replicates of three.

5.2.4 Pharmacokinetic screening of EGCg formulations in rats

Male Sprague Dawley rats (n=3 per group) weighing 200-250 g were purchased from Harlan Laboratories (Indianapolis, IN). The rats were purchased pre-cannulated by Harlan. The rounded tip catheters were surgically implanted into the jugular vein of the rats making multiple, precise blood draws painless to the animal. The rats were food (not water) deprived for 18 hours prior to the start of the experiment. Vegetable oil was selected as the gavage vehicle because all crystal forms were observed to be insoluble in it. Crystallographic data was used to calculate the equivalent weight of each multiple component cocrystal to provide a 100 mg/kg dose of EGCg (Mukherjee, Clarke et al. 2011). The EGCg formulations were delivered via oral gavage at a dosage of 100 mg EGCg/kg body weight. Blood was collected at the following time points: 0, 5, 10, 30, 60, 120, 240, and 480 minutes. Because heparin was kept in the catheter lines to
prevent clotting, a small amount of blood was drawn and discarded before collecting each sample. Approximately 300 µL of blood was collected in EDTA tubes for each time point. The samples were kept on ice to preserve their integrity, then centrifuged at 4000 rpm for 10 minutes, after which the plasma was transferred to sterile centrifuge tubes. A preservative solution was added to each plasma sample at 10% (v/v) concentration to ensure the integrity of the EGCg during storage (Lambert, Lee et al. 2006). This preservative was comprised of 20% ascorbic acid (to prevent oxidation) and 0.1% EDTA (to scavenge any metal contaminants). The samples were stored at -80°C until they were analyzed for EGCg content.

5.2.5 Quantification of EGCg in rat plasma

Refer to 2.7.1.

5.2.6 Statistical analysis

A post hoc t-test with Bonferroni correction was used to assess the statistical significance at each time point for the pharmacokinetic study. Each EGCg cocrystal was compared to the EGCg control. The criterion for rejection of the null hypothesis was $P<0.05$.

5.3 Results

5.3.1 Crystallography

$EGCgNIC\cdot 9H_2O$: The single crystal x-ray diffraction data revealed that EGCgNIC$\cdot 9H_2O$ is a 2:2 cocrystal of EGCg and nicotinamide with 9 water molecules. The intermolecular hydrogen bonding of the cocrystal is shown in Figure 5.2.
EGCgNIC•9H₂O intermolecular hydrogen bonds. The intermolecular hydrogen bonds are indicated by blue dotted lines on the left. As a hydrate, water molecules exist in the crystal structure and are indicated by red balls on the right.

**EGCgINM•5H₂O:** The single crystal x-ray structure analysis revealed that EGCgINM•5H₂O is a hydrated, 1:1 cocrystal of EGCg and isonicotinamide. EGCg molecules and isonicotinamide molecules interact through single-point hydrogen bonds (O-H···N, O···N: 2.756Å) between the hydroxyl group of EGCg molecule and the aromatic nitrogen of isonicotinamide molecules. These dimeric units are connected further by O-H···O (O···O: 2.662Å) hydrogen bonds formed between the hydroxyl moieties of EGCg and carbonyl moieties of isonicotinamide molecules. This results in the zig-zag chain formation shown in Figure 5.3.

**Figure 5.2**
EGCgNIC•9H₂O intermolecular hydrogen bonds. The intermolecular hydrogen bonds are indicated by blue dotted lines on the left. As a hydrate, water molecules exist in the crystal structure and are indicated by red balls on the right.

**Figure 5.3**
EGCgINM•5H₂O intermolecular hydrogen bonds. The intermolecular hydrogen bonds are indicated by blue dotted lines.
EGCgINA•3H₂O: The single crystal x-ray structure analysis revealed that EGCgINA•3H₂O is a trihydrate co-crystal of EGCG and nicotinamide at a 1:1 ratio. The intermolecular hydrogen bonds are illustrated in Figure 5.4.

![Figure 5.4](image)

**Figure 5.4** EGCgINA•3H₂O intermolecular hydrogen bonds. The intermolecular hydrogen bonds are indicated by blue dotted lines.

EGCgINA: It is possible to generate a stable anhydrous form of the EGCgINA•3H₂O cocrystal by heating to 85°C. The water loss can be confirmed by comparing the differential scanning calorimetry data before and after heating (Figure 5.5).
Figure 5.5
Differential scanning calorimetry data of EGCgINA and EGCgINA•3H₂O. The first dip in the green line is indicative of water loss for the hydrate. The first dip is nonexistent in the red line because water is not present in this cocrystal.

5.3.2 Solubility of the EGCg cocrystals
EGCg is considered to have good aqueous solubility (approximately 20 mg/ml). We determined the solubility profiles of four new EGCg cocrystals. All of the cocrystals exhibited reduced water solubility. Figure 5.6 shows the dissolution profiles of EGCg and the EGCg cocrystals. The maximum solubility of EGCg determined experimentally was in agreement with published values, approximately 22 mg/ml. The EGCG cocrystals exhibited far lower aqueous solubility. This is apparent in Figures 5.6 and 5.7. The most soluble cocrystal was EGCgNIC•9H₂O at 2.95±0.11 mg/ml. This was followed by EGCgINA, EGCgINM•5H₂O, and EGCgINA•3H₂O at 1.227±0.036, 1.401±0.122, 0.97±0.07, respectively.
Figure 5.6
Dissolution profiles of EGCg and EGCg cocrystals in water.

Figure 5.7
Dissolution profiles of EGCg cocrystals alone in water.

5.3.3 Pharmacokinetics of EGCg cocrystals

The pharmacokinetic curves for EGCg and the EGCg cocrystals are shown in figure 5.8. EGCg exhibited the prototypical pharmacokinetic curve for a highly water-soluble small molecule: rapid peak and elimination. The EGCg cocrystals
exhibited very different pharmacokinetic profiles. The pharmacokinetic parameters are shown in Table 5.1. EGCg peaked rapidly at 5 minutes ($T_{\text{max}}$) due to its high solubility and, consequent, high rate of dissolution. The cocrystals peaked in the plasma in the following order: EGCgNIC•9H2O, EGCgINM•5H2O, EGCgINA•3H2O, EGCgINA. Surprisingly, only two of the cocrystal forms resulted in improved bioavailability: EGCgINA•3H2O and EGCgINA. These cocrystals had modest improvements in relative bioavailability at $F_{\text{REL}}$ of 1.53 and 1.18, respectively. Free EGCg peaked at higher concentrations and more quickly than the cocrystal forms. However, cocrystallization did change the overall shape of the pharmacokinetic curve. In some cases, these pharmacokinetic profiles might be advantageous to the typical peak and elimination profile of free EGCg.

![Pharmacokinetic profiles of EGCg and EGCg cocrystals.](image)

**Figure 5.8**
Pharmacokinetic profiles of EGCg and EGCg cocrystals.
Table 5.1. Pharmacokinetic parameters

<table>
<thead>
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<th>EGCg</th>
<th>EGCgNIC•9H2O</th>
<th>EGCgINM•5H2O</th>
<th>EGCgINA•3H2O</th>
<th>EGCgINA</th>
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<tbody>
<tr>
<td>T(_{\text{MAX}}) (min)</td>
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<td>30.0</td>
<td>60.0</td>
<td>120.0</td>
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<tr>
<td>C(_{\text{MAX}}) (ng/ml)</td>
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<td>126.0</td>
<td>297.9</td>
<td>149.6</td>
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<td>AUC</td>
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<td>25237.0</td>
<td>22707.0</td>
<td>54560.0</td>
<td>41937.0</td>
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<tr>
<td>F(_{\text{REL}})</td>
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<td>0.64</td>
<td>1.53</td>
<td>1.18</td>
</tr>
<tr>
<td>A T(_{1/2}) (min)</td>
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<td>3.75</td>
<td>10.44</td>
<td>30.53</td>
<td>62.71</td>
</tr>
</tbody>
</table>

5.4 Discussion and conclusions

Tea is the most widely consumed beverage in the world. One of the most popular types of tea is green tea. Green tea contains numerous bioactive ingredients but the most beneficial class of compounds is thought to be the polyphenols. Of the polyphenols found in green tea, EGCg is the most abundant. EGCg research increased drastically when researchers reported that epidemiological evidence indicated lower rates of certain cancers in populations that consumed the most green tea. Since then, numerous molecular mechanisms for these beneficial properties have been discovered. Not surprisingly, EGCg has become one of the most popular nutraceutical ingredients in the world. However, reports of its poor bioavailability are troublesome to product development (Landis-Piwowar, Huo et al. 2007; Dube, Nicolazzo et al. 2010; Kale, Gawande et al. 2010; Smith, Giunta et al. 2010). EGCg is highly susceptible to oxidation, first pass metabolism and rapid efflux (Kale, Gawande et al. 2010). Numerous groups have evaluated delivery systems to overcome these limitations but none have tried cocrystallization.

In this study, we report four new cocrystals of EGCg all with reduced water solubility. We hypothesized that these changes in solubility would change the pharmacokinetics of EGCg, perhaps improving its bioavailability. At first glance,
it seems that cocrystallization might not be the most useful method for improving the bioavailability of EGCg, since only two cocrystals exhibited improved bioavailability and the increases were very modest (Table 5.1). However, that does not mean that cocrystallization cannot benefit the commercialization of EGCg. The ability to have multiple solid forms of EGCg with different physicochemical properties and pharmacokinetic profiles could be very useful. Further examination of Figure 5.8 indicates that some of the EGCg cocrystals exhibited very unique pharmacokinetic profiles. The EGCgINA•3H2O and EGCgINA cocrystals not only had the highest relative bioavailabilities, but also resulted in higher concentrations of free EGCg after 60 and 120 minutes. This plateau effect could be useful for achieving more sustained levels of EGCg in the blood. Additionally, because nutraceutical products often contain combinations of ingredients with synergistic effects, cocrystallization could be used to select forms with similar pharmacokinetic curves as the other ingredients, thus, optimizing the synergisms.

Our results generally had the hypothesized effect: reducing the solubility reduced the rate of dissolution and delayed the time at which maximal plasma levels were reached ($T_{\text{max}}$). EGCgNIC•9H2O was the most water-soluble cocrystal form and had the most rapid $T_{\text{max}}$ after free EGCg. EGCgINM•5H2O and EGCgINA•3H2O had expected $T_{\text{max}}$ values as well. However, EGCgINA peaked last at 120 minutes (Table 5.1) and was the second most soluble cocrystal (Figure 5.7). This phenomenon cannot be explained by our solubility hypothesis. Normally, it might be reasonable to assume that perhaps other
pharmacokinetic parameters are being changed, like elimination rate or metabolism. However, this would normally be attributed to some effect of the cocrystal former. This theory is debunked in our experiment because we evaluated EGCgINA and EGCgINA•3H2O. As expected, the hydrate exhibited lower water solubility and peaked later in comparison to the other more soluble EGCg forms. Because anhydrous and hydrous forms were evaluated, we are able to conclude that the presence of isonicotinic acid does not explain the discrepancy observed with the EGCgINA cocrystal. This phenomenon could be due to experimental error since this was a relatively low n study.

In conclusion, cocrystallization might not be the best option for improving the bioavailability of highly water-soluble cocrystals. However, it could be very useful when absorption kinetics is critical such as it is in synergistic combination products. Additionally, cocrystallization is capable of broadening intellectual property since the only requirement for cocrystal formation is complementary functional groups. This is unlike salts, which require the presence of ionizable species severely limiting the number of possibilities and, in many cases, rendering the potential for salt formation impossible for certain compounds. This study is good proof of principle for the use of cocrystallization to modulate the pharmacokinetics of highly water-soluble compounds. In this study, we described the creation of four new solid forms of EGCg, each with unique physicochemical properties and pharmacokinetic characteristics.
CHAPTER 6

DISCUSSION

6.1 Nanolipidic flavonoid complexes

Nanoparticles have been long studied as a means of preformulation for improving the oral bioavailability of various compounds. Most often, the compounds selected for nanoparticle preformulations exhibit low water solubility and, thus, dissolve slowly or incompletely thwarting their absorption and bioavailability. These compounds generally fall under the Biopharmaceutics Classification System (BCS) as Class II active pharmaceutical ingredients because they present low solubility and high permeability. BCS Class II compounds are generally limited by their rate of dissolution, which slows their absorption. Recently, one group has utilized solid lipid nanoparticles to drastically improve the bioavailability of another natural compound, curcumin (Kakkar, Singh et al. 2011). Curcumin has very poor bioavailability due to inadequate water solubility. Kakkar et al reported increases in oral bioavailability of up to 155 times, dependent on the dose administered.

Nanolipidic particles have been reported to increase the absorption of compounds via the lymphatic system (Porter, Trevaskis et al. 2007). Lymphatic drug absorption requires a compound to be highly lipophilic or to be complexed
to a lipidic material as is the case with nanolipidic particles. Lymphatic absorption offers the advantage of bypassing first pass metabolism, resulting in elevated systemic circulation levels of the free xenobiotic. These were key factors that contributed to our development of EGCg nanolipidic particles and provided rationale for our pharmacokinetic studies. We evaluated the pharmacokinetics of nanolipidic EGCg particles using Sprague dawley rats. We administered the “free” EGCg in the same vehicle that was used in the NanoEGCg formulation minus the lipid carrier. What we found was that EGCg was absorbed very poorly, peaking at 116 ng/ml. In comparison, the NanoEGCg formulations exhibited more than double the relative bioavailability. Given that lipidic formulations have been shown to increase lymphatic absorption (Peng, Zhang et al. 2010), it is possible that this is one mechanism for the improved bioavailability of NanoEGCg. Additionally, EGCg has been shown to be highly susceptible to metabolism in the gastrointestinal tract pre-absorption (Takagaki and Nanjo 2010), thus, it is possible that the NanoEGCg is less susceptible metabolism by intestinal flora. Unfortunately, one major limitation to this technology is that it requires ethanol for nanoparticle formation. This is not ideal for the commercialization of EGCg as a dietary supplement or as a drug. Not only this, but the ethanol content alone probably effected the absorption of EGCg. This is evidenced by the fact that the plasma levels reached by our EGCg control were lower than previously reported (Lin, Wang et al. 2007). This limitation must be considered when interpreting the results but does not change the fact that the systemic absorption of free EGCg in the nanoEGCg formulation
was vastly superior to the control. Alternative methods for nanoEGCg production without alcohol should be explored in future studies.

6.2 Solid-state chemistry

The fact that the majority of drugs are marketed and sold as solid dosage forms is a testament to the importance of solid-state chemistry in the pharmaceutical industry. They have important implications in clinical, legal, and even regulatory perspectives. Perhaps the most pertinent factor contributing to the emphasis being placed on solid-state chemistry is the fact that novel solid forms offer a way to change the physicochemical characteristics of compounds with less than desirable properties in their pure form. One of the most rapidly emerging new solid forms are cocrystals. Shan and Zaworotko have defined cocrystals as “multiple component crystals in which all components are solid under ambient conditions. These components co-exist as a stoichiometric ratio of a target molecule or ion and a neutral molecular cocrystal former(s)” (Shan and Zaworotko 2008). Cocrystals are particularly useful because they involve noncovalent hydrogen bonds, which pose fewer limitations than covalent modifications and ionic complexes (salts). Because cocrystals can be formed from compounds without ionizable species, the number of possible combinations are staggering in comparison to other solid forms.

Recent advances in crystal engineering have propelled cocrystallization to the forefront of solid-state chemistry. Now, supramolecular heterosynthons that seem to favor the formation of cocrystals have been discovered. Those relevant to the studies discussed in chapters 4 and 5 are exemplified by carboxylic acid-
aromatic nitrogen, carboxylic acid-amide, and alcohol-pyridine (Shan and Zaworotko 2008). This supramolecular synthon approach was employed in the development of all of the cocrystals evaluated in this report. This took much of the trial and error out of cocrystal development and allowed for effective selection of cocrystal formers that were most likely to hydrogen bond with the bioflavonoids: EGCg and quercetin. Furthermore, these synthons can be applied to other structurally similar bioflavonoids. The studies described in previous chapters of this report attest to the effectiveness of this crystal engineering approach.

**6.2.1 Pharmacokinetic implications of cocrystals of quercetin**

Quercetin was chosen as one of the bioflavonoids for these cocrystallization studies because it exhibits extremely low water solubility, a very common problem in drug development. Quercetin’s water solubility of 0.00215 mg/mL (Srinivas, King et al. 2010) is the major contributor to its poor bioavailability. For a compound to be absorbed by the body it must dissolve, which is dependent on its solubility in aqueous mediums. Our hypothesis was that we could improve the solubility of quercetin by forming new cocrystal forms. In turn, this would produce predictable changes in the pharmacokinetic profiles leading to improvements in the bioavailability.

Our results supported this hypothesis in a broad sense. Each of the four cocrystals exhibited marked improvements in solubility. However, our ability to evaluate the changes in pharmacokinetics that could be produced by small differences in aqueous solubility was limited because we were forced to use a
50% ethanol medium to produce quantifiable levels of quercetin for the quercetin dihydrate control. Although this makes it difficult to make accurate conclusions that are physiologically relevant, it is very obvious that the cocrystals exhibited increased solubility and improved bioavailability. Additionally, we would expect that increasing the solubility would increase the rate of dissolution and cause the plasma levels to peak earlier. This was the case for all of the quercetin cocrystal formulations. The quercetin dihydrate control was absorbed slowly ($T_{\text{max}}$ at 30 minutes) and poorly ($C_{\text{max}}$ at 285 ng/mL). In comparison, the quercetin cocrystals reached peak levels at 5 and 10 minutes. The QUECAF•MeOH peaked the highest at 2612 ng/mL followed by QUEINM at 1401 ng/mL, QUETBR•2H$_2$O at 840 ng/mL, and QUECAF at 656 ng/mL. This increases in absorption resulted in increases in bioavailability of up to nearly 10 fold. The lowest performing cocrystal in terms of bioavailability was QUECAF, which still produced increases in relative bioavailability of 2.57 fold. The cocrystals were absorbed similarly and there was little meaningful difference in the absorption half lives. However, the elimination half lives were indicative of major changes in the pharmacokinetics of the cocrystals. This was unexpected. We anticipated that we would change the rate of dissolution, improving the absorptive phase of the cocrystals. However, it appears that cocrystallization also produces profound effects on the elimination phase. This was most evident for QUETBR•2H$_2$O that exhibited an elimination half life of 145 minutes. This parameter can be visualized by looking the shape of the pharmacokinetic curve (Figure 4.4). The
shape is not a peak with rapid elimination, but a peak with a plateau effect. This is what produced huge gains in bioavailability ($F_{REL}=9.93$).

The impact of these studies is two fold. It provides more evidence that poorly soluble compounds can be improved drastically using cocrystallization (Hickey, Peterson et al. 2007; Bak, Gore et al. 2008; Jung, Kim et al. 2010). Also, it could help commercialize quercetin as a dietary supplement and eventually as a therapeutic agent. QUE is best known for its antioxidant activity but has numerous other biological and pharmacological effects including metal chelation, anti-carcinogenic, cardioprotective, bacteriostatic, and antiviral activity (Vargas and Burd; Bakay, Mucsi et al. 1968; Formica and Regelson 1995; Spencer, Kuhnle et al. 2003; Leopoldini, Russo et al. 2006; Murakami, Ashida et al. 2008). However, these bioactivities are meaningless unless its poor absorption and bioavailability can be overcome. Our studies offer four quercetin cocrystals that accomplish this. Future studies will need to assess the translation of these results into humans.

6.2.2 Pharmacokinetic implications of cocrystals of EGCg

EGCg was selected for this study to represent the opposite end of the solubility spectrum. EGCg is a highly water-soluble compound. We used the supramolecular heterosynthon approach to identify cocrystal formers with complementary functional groups to form hydrogen bonds with the hydroxyl groups abundant on EGCg. This crystal engineering approach to cocrystal design was very effective and we generated several new EGCg cocrystals. The
four presented in this study were selected to advance to the in vivo pharmacokinetic studies.

EGCg usually exhibits a prototypical pharmacokinetic profile for highly water soluble compounds: it peaks rapidly and is eliminated rapidly from systemic circulation via tissue distribution and metabolism at the liver. We hypothesized that cocrystals with reduced solubility would change the dissolution and consequent absorption of the compound in the gastrointestinal tract. This hypothesis was mostly supported by our results.

We evaluated four new cocrystal forms of EGCg in a rat model of pharmacokinetics and bioavailability. The EGCg formulations were administered in vegetable oil to prevent presolubilization of the powder forms. Sieving the powders prior to slurring in vegetable oil and dosing the animals via oral gavage regulated the particle size. The EGCg control was absorbed rapidly absorbed ($T_{\text{MAX}}=5$ minutes) and eliminated quickly. The EGCg cocrystals exhibited a delayed absorption pattern that coincided with the decreases in solubility in all cases except one: EGCgINA. This cocrystal was the second most soluble but appeared in the plasma last. We can only attribute this to experimental error since there is no other explanation for it. That aside, our hypothesis was supported by our results. Interestingly, oral bioavailability was not improved from EGCg alone except very modestly with the EGCgINA•3H2O and EGCgINA cocrystals. These cocrystals had the latest $T_{\text{MAX}}$ values.

Although, at first glance, it might be easy to conclude that cocrystallization is not useful for improving the bioavailability of highly water-soluble compounds
this might not be true. In this study, we generated four new cocrystal forms of EGCg with different physicochemical and pharmacokinetic properties. During commercialization, it is possible to formulate the EGCg cocrystals into a combination product. This, in theory, has the potential to create sustained plasma levels of free EGCg. This possibility was simulated in Figure 6.1.

Figure 6.1
Pharmacokinetic curve of theoretical combination delivery of all cocrystal forms simultaneously (25 mg/kg each).

Figure 6.1 was generated by combining the experimental pharmacokinetic curves of all of the cocrystals divided by 4 (to adjust the dose to 100 mg/kg total of EGCg). In theory, this is the pharmacokinetic curve that would occur if all of the cocrystal forms were dosed into an animal at once. As shown in Figure 6.1, this would cause the pharmacokinetic curve to plateau similar to other sustained release preformulation techniques like transdermal delivery. Sustained plasma levels are preferred so that the therapeutic bioactivity of the active ingredient is
maintained for a longer duration. Even though transdermal delivery can also accomplish sustained plasma levels, it has numerous disadvantages to oral formulation such as complicated production, increased cost, and reduced patient compliance due to common side effects like skin irritation at the adhesion site.

A single cocrystal with reduced solubility from the pure form of a highly water-soluble compound is unlikely to produce improved bioavailability. However, the ability to modulate the pharmacokinetic curve could be very useful in the clinical translation of active ingredients. The opportunities are there to create combination products that produce sustained plasma levels of a single active ingredient.
REFERENCES


Bak, A., A. Gore, et al. (2008). "The co-crystal approach to improve the exposure of a water-insoluble compound: AMG 517 sorbic acid co-crystal


Wang, M. and I. R. Miksa (2007). "Multi-component plasma quantitation of antihyperglycemic pharmaceutical compounds using liquid chromatography-


APPENDIX 1:

ADDITIONAL QUERCETIN COCRYSTAL FIGURES

IR
DSC

PXRD (Experimental and Calculated)

Figure A1.1
QUECAF•MeOH (Methanol solvate of 1:1 cocrystal Quercetin and Caffeine)
Figure A1.2
QUECAF (1:1 cocrystal Quercetin and Caffeine)
Figure A1.3
QUEINM (1:1 cocrystal of Quercetin and Isonicotinamide)
Figure A1.4
QUETBR.2H₂O (Dihydrate of 1:1 cocrystal of Quercetin and Theobromine)
APPENDIX 2:

DATA SHEET FOR EGCGNIC•9H2O: HYDRATE OF 1:1 COCRYSTAL OF EPIGALLOCATECHIN GALLATE (EGCG) AND NICOTINAMIDE

Description:
The single crystal x-ray structure analysis reveals that EGCGNIC•9H2O is a decahydrate of 2:2 co-crystal of EGCG and nicotinamide.

Figure A2.1: FT-IR of EGCGNIC•9H2O. (Nicolet Avatar 320 FTIR, solid state)
Figure A2.2: DSC of EGCGNIC•9H2O (TA instrument 2920)
Figure A2.3: Powder x-ray diffraction patterns of EGCGNIC•9H2O
Calculated (top) and Experimental (bottom)
(Bruker AXS D8, Cu radiation)

Major peaks were observed in the experimental PXRD pattern at the following positions: 8.8, 11.9, 13.3, 15.8, 17.2, 18.4, 19.3, 20.3, 21.2, 23.5, 24.2, 25.1, 26.1, 27.7, 28.8, 29.7, 33.0 and 34.4 degrees.
Figure A2.4: Comparison of powder x-ray diffraction patterns of EGCGNIC•9H2O.

Major peaks were observed in the experimental PXRD pattern at the following positions: 8.8, 11.9, 13.3, 15.8, 17.2, 18.4, 19.3, 20.3, 21.2, 23.5, 24.2, 25.1, 26.1, 27.7, 28.8, 29.7, 33.0 and 34.4 degrees.

Figure A2.5: TGA analysis of EGCGNIC•9H2O
**Table A2.1: Single crystal x-ray diffraction data for EGCGNIC•9H2O**  
(Bruker-AXS SMART APEX CCD diffractometer)

<table>
<thead>
<tr>
<th>Crystallographic data</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification code</td>
<td>EGCgNIC•9H2O</td>
</tr>
<tr>
<td>Empirical formula</td>
<td>2(C_{22} H_{18} O_{11}) • 2(C_{6} H_{6} N_{2} O) • 10(O)</td>
</tr>
<tr>
<td>Formula weight</td>
<td>660.49</td>
</tr>
<tr>
<td>Temperature</td>
<td>100(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>1.54178 Å</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Triclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P1</td>
</tr>
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<td>Unit cell dimensions</td>
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<tr>
<td></td>
<td>b = 11.769(2) Å \ b= 85.80(3)^°.</td>
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<tr>
<td></td>
<td>c = 12.203(2) Å \ g = 83.00(3)^°.</td>
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<td>Density (calculated)</td>
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<td>Absorption coefficient</td>
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<td>Independent reflections</td>
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<td>Final R indices [I&gt;2sigma(I)]</td>
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<td>R indices (all data)</td>
<td>R1 = 0.0832, wR2 = 0.2045</td>
</tr>
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</table>
Figure A2.6: Digital microscope image of EGCGNIC•9H2O crystals

Figure A2.7: Intermolecular hydrogen bonding in EGCGNIC•9H2O
APPENDIX 3:

DATA SHEET FOR EGCGINM•5H2O: HYDRATE OF 1:1 COCRYSTAL OF EPIGALLOCATECHIN GALLATE (EGCG) AND ISO-NICOTINAMIDE

Description:
The single crystal x-ray structure analysis reveals that EGCGINM•5H2O is a hydrate of 1:1 cocrystal of EGCG and iso-nicotinamide. EGCG molecules and iso-nicotinamide molecules interact through one point hydrogen bonds (O-H···N, O···N: 2.756Å) between the hydroxyl group of EgCG molecule and the aromatic nitrogen of iso-nicotinamide molecules. These dimeric units are further connected by O-H···O (O···O: 2.662Å) hydrogen bonds formed between hydroxyl moieties of EgCG and carbonyl moieties of iso-nicotinamide molecules and thereby form zig-zag chains.

Figure A3.1: FT-IR of EGCGINM•5H2O (Nicolet Avatar 320 FTIR, solid state)
Figure A3.2: DSC of EGCGINM•5H2O (TA instrument 2920)

Sample: ka10153_1
Size: 6.0000 mg
Method: Ramp

File: C:\kapil\ka10153_1.001
Operator: kapil
Run Date: 3-Mar-08 15:50

Exo Up

Heat Flow (W/g)

Temperature (°C)
Major peaks were observed in the experimental powder x-ray diffraction pattern at about the following positions: 7.6, 8.5, 9.2, 11.5, 12.6, 15.4, 18.7, 19.1, 20.0, 24.7, 26.4, 27.7 and 35.2 degrees.
Figure A3.4: Comparison of experimental and calculated powder x-ray diffraction patterns of EGCGINM•5H2O
## Table A3.1: Single crystal x-ray diffraction data for EGCGINM•5H2O
(Bruker-AXS SMART APEX CCD diffractometer)

<table>
<thead>
<tr>
<th>Crystallographic data</th>
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<tbody>
<tr>
<td><strong>Empirical formula</strong></td>
<td>( \text{C}<em>{28}\text{H}</em>{24}\text{N}<em>{2}\text{O}</em>{17} )</td>
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<tr>
<td><strong>Formula weight</strong></td>
<td>660.49</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
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<tr>
<td><strong>Wavelength</strong></td>
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<td><strong>Crystal system</strong></td>
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<td><strong>Space group</strong></td>
<td>( \text{C}\text{2} )</td>
</tr>
<tr>
<td><strong>Unit cell dimensions</strong></td>
<td>( a = 19.603(8) \text{ Å} ) ( a = 90° ) &lt;br&gt;( b = 14.491(6) \text{ Å} ) ( b = 92.089(7)° ) &lt;br&gt;( c = 10.585(4) \text{ Å} ) ( g = 90° )</td>
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<tr>
<td><strong>Volume</strong></td>
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<td><strong>Z</strong></td>
<td>4</td>
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<td><strong>Density (calculated)</strong></td>
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<td><strong>Absorption coefficient</strong></td>
<td>0.124 mm(^{-1})</td>
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<td><strong>Independent reflections</strong></td>
<td>4761 [R(int) = 0.0238]</td>
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<td><strong>Final R indices [I&gt;2sigma(I)]</strong></td>
<td>( R1 = 0.0671, , wR2 = 0.1782 )</td>
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<td><strong>R indices (all data)</strong></td>
<td>( R1 = 0.0964, , wR2 = 0.2235 )</td>
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</table>
Figure A3.5: Digital microscope image of EGCGINM•5H2O crystals

Figure A3.6: Intermolecular hydrogen bonding in EGCGINM•5H2O

Figure A3.7: An EGCG molecule as it exists in EGCGINM•5H2O
APPENDIX 4:

DATA SHEET FOR EGCGINA•3H2O: HYDRATE OF 1:1 COCRYSTAL OF EPIGALLOCATECHIN GALLATE (EGCG) AND ISO NICOTINIC ACID AND EGCGINA: ANHYDRATE OF 1:1 COCRYSTAL OF EPIGALLOCATECHIN GALLATE (EGCG) AND ISO NICOTINIC ACID

Description:
The single crystal x-ray structure analysis reveals that EGCGINA•3H2O is a trihydrate of 1:1 co-crystal of EGCG and nicotinamide.

Figure A4.1: FT-IR of EGCGINA. (Nicolet Avatar 320 FTIR, solid state)
Figure A4.2: DSC of EGCGINA•3H2O and EGCgINA (TA instrument 2920)

Green = EGCG:Isonicotinic acid trihydrate = EGCGINA•3H2O
Red = EGCG:Isonicotinic acid anhydrate = EGCgINA
Figure A4.3: Powder x-ray diffraction patterns of EGCGINA•3H2O
Calculated (top) and Experimental (bottom)
(Bruker AXS D8, Cu radiation)

Major peaks were observed in the experimental PXRD pattern at the following positions: 12.3, 13.8, 15.2, 18.3, 18.8, 20.6, 21.5, 24.2, 24.8, 25.5, 26.5, 27.6, 28.1, 29.0, 30.6, 31.5, 32.7, 35.6 and 38.4 degrees.
Figure A4.4: Comparison of powder x-ray diffraction patterns of EGCGINA•3H2O.

Major peaks were observed in the experimental PXRD pattern at the following positions: 12.3, 13.8, 15.2, 18.3, 18.8, 20.6, 21.5, 24.2, 24.8, 25.5, 26.5, 27.6, 28.1, 29.0, 30.6, 31.5, 32.7, 35.6 and 38.4 degrees.

Figure A4.5: Experimental powder x-ray diffraction patterns of EGCgINA (Bruker AXS D8, Cu radiation)

Major peaks were observed in the experimental PXRD pattern at the following positions: 6.9, 13.7, 14.5, 17.3, 18.4, 19.3, 20.5, 23.8, 24.7, 27.3, 28.1, 29.9, 30.6, 34.3, 36.5 and 38.3 degrees.
Figure A4.6: Comparison of powder x-ray diffraction patterns of EGCGINA•3H2O and EGCgINA.

Major peaks were observed in the experimental PXRD pattern of EGCgINA at the following positions: 6.9, 13.7, 14.5, 17.3, 18.4, 19.3, 20.5, 23.8, 24.7, 27.3, 28.1, 29.9, 30.6, 34.3, 36.5 and 38.3 degrees.
Figure A4.7: Comparison of powder x-ray diffraction patterns of EGCgINA, EGCG form-1 and isonicotinic acid

Major peaks were observed in the experimental PXRD pattern of EGCgINA at the following positions: 6.9, 13.7, 14.5, 17.3, 18.4, 19.3, 20.5, 23.8, 24.7, 27.3, 28.1, 29.9, 30.6, 34.3, 36.5 and 38.3 degrees.
Figure A4.8: TGA analysis of EGCGINA•3H2O

Calculated = 8.5% weight loss for 3 water molecules

Experimental = 6.9% weight loss
Table A4.1: Single crystal x-ray diffraction data for EGCGINA•3H2O
(Bruker-AXS SMART APEX CCD diffractometer)

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<tr>
<td>Wavelength</td>
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<td>Space group</td>
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<td>a = 7.5176(7) Å, a = 90°.</td>
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<td>b = 14.0259(11) Å, b = 90°.</td>
<td></td>
</tr>
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<td>c = 26.174(2) Å, g = 90°.</td>
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<td>Crystallographic data</td>
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<td>Crystal system</td>
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<td>Space group</td>
<td>P2_{1}2_{1}2_{1}</td>
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| Unit cell dimensions  | a = 7.3367(22) Å  
|                       | b = 13.1192(39) Å  
|                       | c = 25.9406(77) Å  |
| Volume                | 2496.83 Å³ |
Figure A4.9: Intermolecular hydrogen bonding in EGCGINA•3H2O