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Title Page

Flavonoids modulate Monocarboxylate transporter-1 mediated transport of Gamma-hydroxybutyrate *in vitro* and *in vivo*

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Running Title Page

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c) Abbreviations: GHB, γ-hydroxybutyrate; MCT, monocarboxylate transporter; AUC, area under curve

Abstract

The objective of this study was to determine the effects of flavonoids on the in vitro Monocarboxylate Transporter 1 (MCT1)-mediated transport and in vivo disposition of the drug of abuse, γ-hydroxybutyrate (GHB). The uptake of GHB in rat MCT1 gene-transfected MDA-MB231 cells was significantly decreased in the presence of the flavonoids apigenin, biochanin A, chrysin, diosemin, fisetin, genicitin, hesperitin, kaempferol, luteolin, morin, narigenin, phloretin and quercetin, but was not affected by the flavonoid glycosides phloridzin and rutin. The IC_{50} values for luteolin, morin and phloretin were 0.41 ± 0.14, 6.41 ± 2.01 and 2.57 ± 0.48 μM, with the inhibition mechanism for luteolin being competitive. ^{3}H-kaempferol and ^{3}H-biochanin A did not exhibit MCT1-mediated uptake, suggesting that these flavonoids are not substrates for MCT1. The combination of luteolin and phloretin inhibited the uptake of GHB in a synergistic manner; however, the combination of luteolin and morin was antagonistic. GHB 1000 mg/kg was administered to rats by *iv* bolus, with or without the concomitant administration of luteolin 10 mg/kg *iv*. Following luteolin treatment, the renal and total clearances of GHB were significantly increased, likely due to inhibition of the MCT1-mediated renal reabsorption of GHB, and the sleep time significantly decreased (121 ± 5 min versus 165 ± 10 min) compared with control rats. Overall the results of this study indicate that flavonoids from food or herbal products may significantly alter the pharmacokinetics and pharmacodynamics of MCT substrates.

Introduction

Nonlinear pharmacokinetics of the drug of abuse, γ-hydroxybutyric acid (GHB), occurs in animals (Lettieri and Fung, 1979; Shumate and Snead, 1979) and humans (Ferrara et al., 1992; Palatini et al., 1993), as a result of its capacity-limited metabolism (Lettieri and Fung, 1979; Ferrara et al., 1992; Palatini et al., 1993) and/or absorption (Arena and Fung, 1980). Recently, clinical reports have suggested the importance of renal clearance of GHB following overdoses, since high concentrations of GHB are present in urine samples of intoxicated patients who ingested GHB or the precursors of GHB, γ-butyrolactone and 1,4-butanediol (Zvosec et al., 2001; Sporer et al., 2003). Using the rat as an animal model, we demonstrated that the renal clearance of GHB also contributes to the nonlinear pharmacokinetics of GHB (Morris et al., 2005). The renal clearance of GHB is significantly increased at higher GHB plasma concentrations, and plays a more important role in the overall elimination of GHB following overdoses.

The mechanism of this capacity-limited renal clearance is due to the capacity-mediated renal reabsorption by monocarboxylic acid transporter(s) (MCT) and sodium-dependent monocarboxylic transporter(s) (SMCT) (Wang et al., 2006). Among the 14 members of the MCT family that have been described, only MCT 1-4 have been shown to be proton-coupled monocarboxylate transporters (Halestrap and Meredith, 2004). Among these four MCTs, MCT1, the first member identified, is ubiquitously distributed throughout the body, such as in red blood cells, intestine, colon, muscle, heart, brain, and kidney (Halestrap and Meredith, 2004). We have recently reported that MCT1 represents an important transporter for GHB in kidney cortex (Wang et al., 2006). The substrates of MCT1 include L-lactate, pyruvate, butyrate, GHB and ketone bodies (Garcia et al., 1994;

Halestrap and Price, 1999; Wang et al., 2006). The inhibitors for MCT1 include α–cyano-4-hydroxycinnamic acid (CHC), phenyl-pyruvate, niflumic acid, 4, 4′-diisothiocyanostilbene-2, 2′-disulphonate (DIDS), p-chloromercuribenzene sulphonate, phloretin and quercetin (Halestrap and Meredith, 2004). Quercetin and phloretin belong to a family of food-derived products called flavonoids, which have been widely studied for their antioxidative, anti-inflammatory, antiallergic, antiviral and anticarcinogenic properties (Nijveldt et al., 2001). Flavonoids have been shown to inhibit for various efflux transporters, including ABCB1 (Pgp), ABCC1 (MRP1), ABCC2 (MRP2), and ABCG2 (BCRP) (Di Pietro et al., 2002; Zhang and Morris, 2003; Ahmed-Belkacem et al., 2005; Morris and Zhang, 2006), and OATP influx transporters (Wang et al., 2005b; Fuchikami et al., 2006). Membrane transport of lactate in rat tumor cells has also been shown to be inhibited by a number of flavones from a subclass of flavonoids (Belt et al., 1979). In that study the authors demonstrated that the flavones apigenin, quercetin, and morin, are inhibitors of L-lactate transport, while the flavonoid glycosides were not (Belt et al., 1979).

The objectives of this study were 1) to determine the effects of several classes of flavonoids, i.e., chalcone, flavone, flavonol, and isoflavonone on the MCT1-mediated transport of GHB in MCT1 gene transfected MDA-MB231 cells; 2) to characterize the inhibition kinetics of selected flavonoids on GHB uptake; and 3) to evaluate the effect of the flavonoid luteolin on GHB pharmacokinetics (PK) and pharmacodynamics (PD) in rats.

Methods

GHB, α-cyano-4-hydroxycinnamate (CHC), and all the flavonoids were purchased from Sigma-Aldrich (St Louis, MO). [2, 3-^{3}H]- γ-Hydroxybutyric acid (50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). DMEM medium, fetal bovine serum and genecitin (G418) were purchased from Invitrogen (Invitrogen Corp. Carlsbad, CA).

Cell Culture

Rat MCT1 gene-transfected MDA-MB231 cells and mock cells were kindly provided by Professors I. Tamai and A. Tsuji (Kanazawa University, Japan), and cultured as previously described (Wang et al., 2006). Briefly, cells were cultured at 37 ºC in 5% CO_2 / 95% air environment in T-75cm^2 flasks in DMEM Medium with 10% fetal bovine serum, 1 mg/ml genecitin, 100 units penicillin, and 100 μg/ml of streptomycin added, with the fresh media added every 2 to 3 days. Two or three days before the uptake studies, cells were seeded in 35mm diameter plastic culture dishes with a cell density of 1x10^5 cells per ml.

Cellular uptake studies

For the uptake study, the medium was removed from cell monolayers and cells were washed three times with wash buffer (150mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, pH7.4). One ml of uptake buffer (150mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 10mM MES, pH6.0) containing [^{3}H]-GHB (5 μM) was added to the dishes. The uptake was stopped by aspirating the buffer, and washing three times with ice-cold wash buffer. The cells were lysed and collected in 1ml of lysis buffer containing 0.3 N NaOH and 1% SDS. Radioactivity was determined by liquid scintillation spectroscopy. Protein concentrations were determined by the Bicinchoninic Acid protein

assay with bovine serum albumin as the protein standard. The results were normalized for the protein content of the cell lysate and accumulation was expressed as pmol or nmol /mg protein/minute. The uptake of GHB (5 μM) was examined in the absence or presence of various flavonoids over 1 min at pH 6.0 at room temperature. We used 5 μM GHB to allow the inhibitor concentration to be at least 10 fold higher than the substrate concentration. A higher GHB concentration of 1 mM has been used in the presence of flavonoids quercetin and phloretin (100 μM) , and similar results were observed (data not shown).

To investigate the effect of flavonoid concentration on GHB uptake, three flavonoids, luteolin, morin, and phloretin were used to inhibit GHB uptake at different concentrations, ranging from 0.01 to 50 μM. To investigate the mechanism of inhibition by luteolin, the effect of 50 μM of luteolin on GHB uptake, at different concentrations of GHB, was studied. To investigate the effects of flavonoid combinations on GHB uptake, uptake of GHB was determined in presence of two flavonoids, phloretin and luteolin, or luteolin and morin. To test if flavonoids are substrates for the MCT1 transporter, the uptake of radiolabeled (^{3}H)-kaempferol and (^{3}H)-biochanin A were examined in the presence or absence of the MCT inhibitor alpha-cyano-4-hydroxycinnamic acid (CHC) at pH 6.0 at room temperature. (^{3}H)-kaempferol and (^{3}H)-biochanin A and belongs to different subclass of flavonoids, flavone and isoflavonone, respectively; so the uptake of two compounds may represent the general mechanism of transport for flavonoids.

GHB pharmacokinetics

A study was conducted using male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighting 280-340 g to investigate the *in vivo* interaction between luteolin and GHB. The

animal housing room had controlled environmental conditions with temperature and relative humidity of approximately 20 ± 2°C and 40-70%, respectively, and artificial lighting, alternating on a 12-h light-dark cycle. All care and experiments were approved by the Institutional Animal Care and Use Committee at the University at Buffalo. Two or three days before the PK study, cannulas were inserted into rat right jugular veins and bladders under anesthesia (ketamine 90 mg/kg and xylazine 9 mg/kg, im injection, Henry Schein, Melville, NY) as previously described (Morris et al., 2005). During the study, the rats were placed in metabolism cages for collection of urine samples. GHB was dissolved in water and administered via an *iv* bolus injection; luteolin was dissolved in a vehicle composed of DMSO (5%) and hydroxypropyl-beta-cyclodextrin (20%) and injected right after the GHB injection. The control group was given GHB (1g/kg) and the luteolin vehicle; the luteolin treatment group was given GHB (1 g/kg) and luteolin (10 mg/kg). Blood samples (120 μl each) were withdrawn from the jugular vein at different time points (2, 5, 10, 30, 60, 120, 180, 240, and 360 min) and placed in heparinized 0.6-ml microcentrifuge tubes. The plasma was separated from whole blood by centrifugation at 2,000 × *g* for 5 min at 4°C. The urine was collected from the bladder cannulas over 6 hour and urine pH and volume were measured. All plasma and urine samples were stored at -80°C until analysis by liquid chromatography-tandem mass spectrometry (LC/MS/MS) to determine GHB concentrations. GHB in rat plasma and urine samples were measured by a validated LC/MS/MS assay as previously described with some modifications (Fung et al., 2004). Briefly, after protein precipitation of plasma and urine samples with 50% methanol, the mixture was centrifuged at 20,000 g for 20 min, and the supernatant was used for GHB analysis. Compounds were separated on an Aqua C-18 5

μm 125A column (150 × 4.6 mm i.d., Phenomenex, Torrance, CA) with a mobile phase consisting of 5 mM formic acid/methanol (33:67, v/v) at a flow rate of 0.25 ml/min. The injection volume was 10 μl. Multiple reaction monitoring was used to detect GHB and GHB-D6 using a PE SCIEX API 3000 triple-quadruple tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a turbo ion spray (PE SCIEX) measuring the ion pair transitions of *m/z* 105 (parent ion) to *m/*z 87 (product ion) and of *m/z* 111 (parent ion) to *m/z* 93 (product ion), respectively. The Analyst software version 1.4.1 (PE SCIEX) was used for instrument control and data analysis.

Data Analysis

The uptake data are presented as mean ± SD. One-way analysis of variance (ANOVA) followed by a Dunnett's test was used to detect statistical significance among the means of more than two groups. Differences with $p < 0.05$ were considered as statistically significant. Data analysis was performed using GraphPad Prism (GraphPad Inc., San Diego CA). The inhibition of GHB uptake by flavonoids (IC_{50}) was calculated by fitting with equation below using weighted nonlinear regression analysis (Adapt II, BMSR University of South California, Los Angels, CA).

$$F = 100 * (1 - \frac{I_{\max} * C^r}{IC_{50}{}^r + C^r}) \qquad \text{Equation 1}$$

F is the percentage of uptake rate of GHB in presence of flavonoids compared with the control and C is the concentration flavonoids. The goodness of fit was determined by the sum of the squared derivatives, the residual plot and the Akaike Information Criterion (AIC).

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The percentage of inhibition was calculated using the following equation.

$$\%inhibition = 100 * (1 - \frac{V_{mct\_flav} - V_{con\_flav}}{V_{mct} - V_{con}}) \qquad \text{Equation 2}$$

V_{mct_flav} is the uptake rate of GHB by MCT1 cells in the presence of flavonoids; $V_{con\text{-}flav}$ is the uptake rate of GHB by control cells in the presence of flavonoids; V_{mct} is the uptake rate of GHB by MCT cells in the absence of flavonoids; and V_{con} is the uptake rate of GHB by control cells in the absence of flavonoids.

Non-compartmental analysis was used to analyze the animal PK data using WINNONLIN 2.1 software (Pharsight Corp. Cary, NC) and area under the plasma concentration versus time curve (AUC), apparent volume of distribution at steady state (Vss), apparent clearance (Cl), and terminal slope (λ_z), apparent renal clearance (Cl_R) were calculated. The apparent metabolic clearance (Cl_m) was calculated by Cl minus Cl_R. Unpaired t-tests were used to detect the statistical difference among two groups.

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Results

Uptake studies

The protein expression of rat MCT1 in MDA-MB231 cells has been examined previously in our laboratory, and we have also examined the endogenous human MCT forms using RT-PCR. These results have been published previously (Wang et al., 2006) and we have demonstrated that rat MCT1 gene is expressed only in rat MCT1 gene-transfected MDA-MB231 cells, but not in control MDA-MB231 cells and that no human MCT1 isoform was present.

Effects of Flavonoids on GHB uptake. To investigate the effects of flavonoids on GHB uptake, 1 min uptake rates of ^{3}H-GHB (5 μM) at pH 6.0 were determined in the absence and presence of flavonoids (50 μM) (Fig 1). GHB uptake was significantly inhibited by all the flavonoids tested in the study, namely, apigenin, biochanin A, chrysin, diosmetin, fisetin, genistein, hesperitin, kaempferol, luteolin, morin, narigenin, phloretin, and quercetin (Fig 1). The flavonoid glycosides demonstrated different effects on GHB uptake (Fig 2): narigin moderately inhibited GHB uptake while phlorizin and rutin had no effects.

Inhibition mechanism of luteolin. . To investigate the mechanism of luteolin inhibition of GHB uptake by MCT1, the concentration-dependent uptake of GHB by MCT1 cells was examined in the absence or presence of luteolin (50 μM). The Lineweaver-Burk plot suggested that the inhibition was competitive in nature (Fig 3). Preincubation of luteolin with MCT1 gene transfected cells did inhibit GHB uptake; however, this inhibition of

GHB uptake can be reversed by washing the cells with uptake buffer before the uptake. This suggested that this inhibition was reversible (data not shown).

Concentration-dependent inhibition of GHB uptake by flavonoids. Concentration-dependent inhibition GHB (5 μM) uptake by flavonoids was demonstrated in this study for three flavonoids tested, luteolin, morin and phloretin (Fig 4). Using Equation 1, the inhibition IC_{50} values were calculated for luteolin, morin and phloretin and were 0.41, 6.21 and 2.57 μM, respectively (Table 1).

Effects of flavonoid combinations on GHB uptake. To investigate the effects of flavonoid combinations, luteolin and phloretin at a ratio of 1:1 or 1:2, and luteolin and morin at a ratio of 1:1 were used to inhibit the uptake of GHB (Fig 5). The IC_{50} values were determined as described above; the total concentration of the combined flavonoids was used as the sole inhibitor concentration (Fig 5 and Table 2). The combination index (CI) using the Loewe additivity equation (Loewe and Muischnek, 1926) was calculated as $CI = C_1/IC_{50\_1} + C_2/IC_{50\_2}$; where C_1 represents the concentration of flavonoid 1; C_2 represents the concentration of flavonoid 2. The CI values were listed with the 90% confidence intervals (Table 2). Using isobologram analysis (Gessner, 1974), the two combinations of luteolin and phloretin were both below the additive line (Fig 6), while, the combination of luteolin and morin was above the additive line (Fig 6).

Flavonoid uptake in MCT1 gene transfected cells. To investigate the transport of flavonoids by MCT1, ^{3}H-kaempferol uptake studies were conducted at pH 6.0 in the

absence or presence of the MCT1 inhibitor CHC. The uptake of ^{3}H-kaempferol in MCT1 gene transfected cells was similar to that in control cells, and this uptake was not affected by CHC (Fig 7). Since the uptake of ^{3}H-kaempferol was not stimulated either by the MCT1 gene, or by low pH, it was unlikely that ^{3}H-kaempferol was transported by rat MCT1 protein. Additionally, a MCT1 inhibitor, CHC, failed to inhibit the uptake of ^{3}H-kaempferol also suggested that MCT1 may not contribute to the cellular uptake of kaempferol. Similar results were observed with ^{3}H-biochanin A (data not shown).

Luteolin-GHB interaction in vivo. The effects of a 10 mg/kg iv dose of luteolin on the PK and PD of GHB, after administration of a 1g/kg iv dose of GHB, were examined in this study. The plasma concentration versus time profiles of control and luteolin-treated groups are presented in Fig 8. The average AUC of the luteolin-treated group (103 mg/ml*min) was significantly lower that of the control group (187 mg/ml*min) (Table 3). The apparent clearance of the luteolin-treated group was significantly higher than that of the control group (Table 3). No significant change was observed for V_{ss} or the terminal slope λ_z. The Cl_R of the luteolin-treated group was significantly greater than that of the control group, and the Cl_R of the luteolin-treated was about 3 times of that of control (Table 3), while the Cl_m was not changed by the treatment of luteolin. The hypnotic effect of GHB, which was characterized using the sleep time of the animals, was attenuated by the treatment of luteolin. The mean sleep time of luteolin-treated group (121 ± 5 min) was significantly less than that of control group (165 ± 10 min) (Table 3). The sleep time of control group obtained here was very similar to previous determined sleep time in rats with the same dose (Macmillan, 1980).

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Discussion

In this study, we investigated the effects of four classes of flavonoids and their glycosides on the transport of GHB by rat MCT1 protein. The flavonoids included in this study encompassed several sub-classes of flavonoids, e.g., chalcones, flavones, flavonols, and isoflavonones. GHB shares similar transporters as lactate, and we have recently reported that MCT1 was an important transporter for the renal reabsorption of GHB in rat kidney (Wang et al., 2006). One way to increase the elimination of GHB is to increase its renal clearance using transport inhibitors, as we have demonstrated in a previous study (Morris et al., 2005). However, the doses of inhibitors used in that study were very high, and unlikely to be clinically applicable. The flavonoids examined in this study have demonstrated a very high potency with IC_{50} values as low as 0.41μM and flavonoids have reported to have very low toxicity (Nijveldt et al., 2001); therefore, flavonoids represent a class of potent and nontoxic MCT1 inhibitors.

There are some structural elements that appear to be important for the MCT1-inhibitory activity of flavonoids, although more detailed studies are needed for confirmation. The presence of a 4′-hydroxy group appears to moderately increase the inhibitory effect (for example, the inhibitory effect of apigenin is greater than that of chrysin), while methylation of this hydroxyl group decreases the inhibitory activity (for example, diosmetin is much less potent than luteolin). The addition of an additional hydroxyl group at position 3′ or 2′ increases the inhibitory effect (for example, luteolin is more potent than apigenin and morin is more potent than kaempferol) while the addition of a 5-hydroxyl group on ring A has minimal effect on the inhibitory effect (for example, fisetin has similar inhibitory effect as quercetin).

The inhibition of MCT1-mediated transport by luteolin was competitive and reversible, which is consistent with previous reports for phloretin and quercetin as reversible non-covalent inhibitors of L-lactate transport by tumor cells or red blood cells (Belt et al., 1979; Deuticke, 1989). The inhibition of MCT1 by other flavonoids may follow the same mechanism. Using radiolabeled kaempferol and biochanin A, we demonstrated that both flavonoids were not transported by MCT1; similar results have been reported for other flavonoids. The flavonoid aglycone epicatechin was not transported by MCT but epicatechin-gallate was transported by MCT (Vaidyanathan and Walle, 2003); additionally, quercetin is not transported by MCT, while its metabolites are (Konishi, 2005).

The combination of luteolin and phloretin produced a synergistic effect on the inhibition of GHB transport, while the combination of luteolin and morin produced an antagonistic effect. The mechanism underlying these differences has not been elucidated. No reports have suggested multiple substrate binding sites for lactate or other monocarboxylates, and no reports have suggested the binding of phloretin or quercetin to the hydrogen binding site on the MCT transporter. Interestingly, the proper membrane expression and function of MCT1 is associated with CD147, an ancillary protein (Kirk et al., 2000; Wilson et al., 2005). The flavonoids may also interact with this protein on the cell membrane, since it was suggested that p-chloromercuribenzoic acid, a potent MCT inhibitor, targeted CD147 (Wilson et al., 2005). The flavonoid flavone can stimulate the uptake of fluorescein and L-lactate into mitochondria of HT-29 cancer cells by an allosteric effect (Wenzel et al., 2005); the transporter responsible for this uptake is likely to be MCT1 (Wenzel et al., 2005). This study suggested that different flavonoids may

bind to potentially different sites on the MCT1 protein or may bind to its ancillary protein CD147.

The renal clearance of GHB increases with increasing dose, and could be increased by the MCT substrates L-lactate and pyruvate (Morris et al., 2005), resulting in an increase in the total clearance of GHB (Morris et al., 2005). Similar results were observed in this study in that the apparent total clearance of the luteolin-treated group was significantly higher that of control group. The plasma protein binding of GHB is minimal, as determined previously (Morris et al., 2005), so the increase of clearance in the luteolin-treated group was not due to changes in protein binding. The metabolism of GHB at this dose level is saturated, and exhibits Michaelis-Menten kinetics. To our knowledge, there is no report of luteolin affecting the metabolism of GHB, and in this study, the apparent metabolic clearance in luteolin treated group was not significantly different from that of the control group. On the other hand, the apparent Cl_R for the luteolin-treated groups was increased to 3 times of that for the control group, which accounted for the increase of apparent total clearance of GHB. Whether MCT1 is the sole transporter of GHB is speculative, although we found that it represents a major MCT for GHB transport in human kidney HK-2 cells (Wang et al., 2005a). MCT2 also represents a possible GHB transporter (Lin et al., 1998) and it is expressed, albeit at lower amounts than MCT1, in the rat kidney (Wang et al., 2006).

Previous studies reported that MCT1 represents a major MCT for butyrate transport in the gastrointestinal tract (Ritzhaupt et al., 1998a; Ritzhaupt et al., 1998b), and GHB is a substrate of MCT1 (Wang et al., 2006). When ingested as food or herbal medicines, the concentration of flavonoids in the gastrointestinal tract would be expected to be high

(~50 μM or higher), so that flavonoid-GHB interactions are likely in the intestine. MCT1 is also the single most important transporter for monocarboxylates at the blood-brain barrier (Gerhart et al., 1997; Pellerin et al., 2005), and it was suggested as a GHB transporter (Bhattacharya and Boje, 2004); therefore, the interaction of flavonoids with GHB at this barrier is also possible. The plasma concentration of luteolin can reach as high as 15.5 μM in rats after an oral dose of 50 μmol/kg, and remain over 1 μM for 9 hours (Shimoi et al., 1998). The results of the in vivo study suggested that a low dose of luteolin could inhibit MCT1-mediated transport; however, interaction studies need to be conducted to investigate whether luteolin might alter intestinal MCT-mediated transport.

A significant reduction in the GHB-induced hypnotic effect was observed after luteolin treatment. The loss of righting reflex occured in 2-4 minutes and was not affected by luteolin treatment; however, the regain of righting reflex was significantly shortened by luteolin treatment compared with control values. This decrease in sleep time was consistent with a decrease of GHB exposure as measured by the AUC of GHB, although there was no direct correlation with the plasma concentration of GHB.

In summary, the results of this study have demonstrated that flavonoids aglycones from several classes of flavonoids (chalcone, flavone, flavonol, and isoflavonone) are effective inhibitors of MCT1-mediated transport, while the flavonoids glycosides have much less inhibitory effect compared to the aglycones. Secondly, we demonstrated that combining flavonoids may result in effects on MCT1-mediated transport ranging from synergistic to antagonistic. Lastly, this study provides the first in vivo evidence that the flavonoid luteolin is effective in increasing the renal and total elimination of GHB, resulting in a decrease in its pharmacological effect of hypnosis.

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References

Ahmed-Belkacem A, Pozza A, Munoz-Martinez F, Bates SE, Castanys S, Gamarro F, Di Pietro A and Perez-Victoria JM (2005) Flavonoid structure-activity studies identify 6-prenylchrysin and tectochrysin as potent and specific inhibitors of breast cancer resistance protein ABCG2. *Cancer Res* **65**:4852-4860.

Arena C and Fung HL (1980) Absorption of sodium gamma-hydroxybutyrate and its prodrug gamma-butyrolactone: relationship between in vitro transport and in vivo absorption. *J Pharm Sci* **69**:356-358.

Belt JA, Thomas JA, Buchsbaum RN and Racker E (1979) Inhibition of Lactate Transport and Glycolysis in Ehrlich Ascites Tumor Cells by Bioflavonoids. *Biochemistry* **18**:3506-3511.

Bhattacharya I and Boje KM (2004) GHB (gamma-hydroxybutyrate) carrier-mediated transport across the blood-brain barrier. *J Pharmacol Exp Ther* **311**:92-98.

Deuticke B (1989) Monocarboxylate Transporter in Red Blood Cells: Kinetics and Chemical Modification. *Methods in Enzymology* **173**:300-329.

Di Pietro A, Conseil G, Perez-Victoria JM, Dayan G, Baubichon-Cortay H, Trompier D, Steinfels E, Jault JM, de Wet H, Maitrejean M, Comte G, Boumendjel A, Mariotte AM, Dumontet C, McIntosh DB, Goffeau A, Castanys S, Gamarro F and Barron D (2002) Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein and related ABC transporters. *Cell Mol Life Sci* **59**:307-322.

Ferrara SD, Zotti S, Tedeschi L, Frison G, Castagna F, Gallimberti L, Gessa GL and Palatini P (1992) Pharmacokinetics of gamma-hydroxybutyric acid in alcohol dependent patients after single and repeated oral doses. *Br J Clin Pharmacol* **34**:231-235.

Fuchikami H, Satoh H, Tsujimoto M, Ohdo S, Ohtani H and Sawada Y (2006) Effects of herbal extracts on the function of human organic anion-transporting polypeptide OATP-B. *Drug Metab Dispos* **34**:577-582.

Fung HL, Haas E, Raybon J, Xu J and Fung SM (2004) Liquid chromatographic-mass spectrometric determination of endogenous gamma-hydroxybutyrate concentrations in rat brain regions and plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **807**:287-291.

Garcia CK, Goldstein JL, Pathak RK, Anderson RG and Brown MS (1994) Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. *Cell* **76**:865-873.

Gerhart DZ, Enerson BE, Zhdankina OY, Leino RL and Drewes LR (1997) Expression of monocarboxylate transporter MCT1 by brain endothelium and glia in adult and suckling rats. *Am J Physiol* **273**:E207-213.

Gessner P (1974) in *Drug Interactions* (Moselli P, Garattini S and Cohen S eds) p 349, Raven Press, New York.

Halestrap AP and Meredith D (2004) The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch* **447**:619-628.

Halestrap AP and Price NT (1999) The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J* **343 Pt 2**:281-299.

Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN and Halestrap AP (2000) CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *Embo J* **19**:3896-3904.

Konishi Y (2005) Transepithelial transport of microbial metabolites of quercetin in intestinal Caco-2 cell monolayers. *J Agric Food Chem* **53**:601-607.

Lettieri JT and Fung HL (1979) Dose-dependent pharmacokinetics and hypnotic effects of sodium gamma-hydroxybutyrate in the rat. *J Pharmacol Exp Ther* **208**:7-11.

Lin RY, Vera JC, Chaganti RS and Golde DW (1998) Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter. *J Biol Chem* **273**:28959-28965.

Loewe S and Muischnek H (1926) *Arch. Exp. Pathol. Pharmakol* **114**:313-326.

Macmillan V (1980) Sequential alterations of cerebral carbohydrate metabolism associated with gamma-hydroxybutyrate. *Brain Res* **183**:123-134.

Morris ME, Hu K and Wang Q (2005) Renal clearance of gamma-hydroxybutyric acid in rats: increasing renal elimination as a detoxification strategy. *J Pharmacol Exp Ther* **313**:1194-1202.

Morris ME and Zhang S (2006) Flavonoid-drug interactions: effects of flavonoids on ABC transporters. *Life Sci* **78**:2116-2130.

Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K and van Leeuwen PA (2001) Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr* **74**:418-425.

Palatini P, Tedeschi L, Frison G, Padrini R, Zordan R, Orlando R, Gallimberti L, Gessa GL and Ferrara SD (1993) Dose-dependent absorption and elimination of gamma-hydroxybutyric acid in healthy volunteers. *Eur J Clin Pharmacol* **45**:353-356.

Pellerin L, Bergersen LH, Halestrap AP and Pierre K (2005) Cellular and subcellular distribution of monocarboxylate transporters in cultured brain cells and in the adult brain. *J Neurosci Res* **79**:55-64.

Ritzhaupt A, Ellis A, Hosie KB and Shirazi-Beechey SP (1998a) The characterization of butyrate transport across pig and human colonic luminal membrane. *J Physiol* **507 (Pt 3)**:819-830.

Ritzhaupt A, Wood IS, Ellis A, Hosie KB and Shirazi-Beechey SP (1998b) Identification and characterization of a monocarboxylate transporter (MCT1) in pig and human colon: its potential to transport L-lactate as well as butyrate. *J Physiol* **513 (Pt 3)**:719-732.

Shimoi K, Okada H, Furugori M, Goda T, Takase S, Suzuki M, Hara Y, Yamamoto H and Kinae N (1998) Intestinal absorption of luteolin and luteolin 7-O-beta-glucoside in rats and humans. *FEBS Lett* **438**:220-224.

Shumate JS and Snead OC, 3rd (1979) Plasma and central nervous system kinetics of gamma-hydroxybutyrate. *Res Commun Chem Pathol Pharmacol* **25**:241-256.

Sporer KA, Chin RL, Dyer JE and Lamb R (2003) Gamma-hydroxybutyrate serum levels and clinical syndrome after severe overdose. *Ann Emerg Med* **42**:3-8.

Vaidyanathan JB and Walle T (2003) Cellular uptake and efflux of the tea flavonoid (-)epicatechin-3-gallate in the human intestinal cell line Caco-2. *J Pharmacol Exp Ther* **307**:745-752.

Wang Q, Darling IM and Morris ME (2006) Transport of Gamma-hydroxybutyrate in Rat Kidney Membrane Vesicles --- Role of Monocarboxylate Transporters. *J Pharmacol Exp Ther* **318**:751-761.

Wang Q, Lu Y, Yuan M, Repasky E and Morris M (2005a) Monocarboxylate Transporter-1 (MCT1) Mediates γ-Hydroxybutyrate Transport In Human Kidney HK2 Cells. *The AAPS Journal* **7**:R6290.

Wang X, Wolkoff AW and Morris ME (2005b) Flavonoids as a novel class of human organic anion-transporting polypeptide OATP1B1 (OATP-C) modulators. *Drug Metab Dispos* **33**:1666-1672.

Wenzel U, Schoberl K, Lohner K and Daniel H (2005) Activation of Mitochondrial Lactate Uptake by Flavone Induces Apoptosis in Human Colon Cancer Cells. *J Cellular Physiology* **202**:379-390.

Wilson M, Meredith D, Manning FJ, Manoharan C, Davies A and Halestrap A (2005) Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate isoforms 1 and 4: The ancillary protein for the insensitive MCT2 is embigin (gp70). *J Biol Chem* **280**:29213-29221.

Zhang S and Morris ME (2003) Effects of the flavonoids biochanin A, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. *J Pharmacol Exp Ther* **304**:1258-1267.

Zvosec DL, Smith SW, McCutcheon JR, Spillane J, Hall BJ and Peacock EA (2001) Adverse events, including death, associated with the use of 1,4-butanediol. *N Engl J Med* **344**:87-94.

Footnotes

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Legends for Figures

Figure 1. Effects of flavonoid aglycones on the uptake of GHB by MCT1 gene transfected MDA-MB231 and control cells. The closed bars represent the uptake of GHB by MCT1 cells; the open bars represent the uptake of GHB by control cells. The uptake studies were conducted at pH 6.0, room temperature, and the uptake time was 1 minute. The concentration of GHB was 5 μM and the concentrations of flavonoids were 50 μM. All the uptake values were normalized to that of control cells in the absence of any flavonoids. The data are presented as mean ± SD. The experiments have bee repeated three times, with at least triplicate determinations for each experiment. One-way ANOVA followed by Dunnett's test was used for statistical analysis, * $p<0.05$, ** $p<0.01$.

Figure 2. Effects of flavonoids and their glycosides on the uptake of GHB by MCT1 gene transfected and control cells. The closed bars represent the uptake of GHB by MCT1 cells; the open bars represent the uptake of GHB by control cells. The uptake studies were conducted at pH 6.0, room temperature, and the uptake time was 1 minute. The concentration of GHB was 5 μM and the concentrations of flavonoids were 50 μM. The uptake values were normalized to that of control cells in the absence of any flavonoids or their glycosides. The data are presented as mean ± SD. The experiments have bee repeated two times, with at least triplicate determinations for each experiment. One-way ANOVA followed by a Dunnett's test was used for statistical analysis, * $p<0.05$, ** $p<0.01$.

Figure 3. Line-weaver Burk plot of the uptake of GHB in the presence of 50 μM of luteolin. The lines represent the fitted values, as described under Methods. The open circles represent the uptake in the presence of luteolin (50 μM); the closed circles represent the uptake in the absence of luteolin. The uptake studies were conducted at pH 6.0, room temperature, and the uptake time was 1 minute. The concentration of GHB used was 5 μM. Uptake by control cells has been subtracted from the values. The data are presented as mean ± SD. The experiments have bee repeated three times, with at least triplicate determinations for each experiment.

Figure 4. Concentration-dependent inhibition of MCT1-mediated GHB transport by luteolin (A), morin (B) and phloretin (C). The lines represent the fitted IC_{50} values. The uptake results were normalized by the control values (in the absence of flavonoids). Uptake by control cells has been subtracted from the values. The uptake studies were conducted at pH 6.0, room temperature, and the uptake time was 1 minute. The concentration of GHB used was 5 μM. The experiments have bee repeated three to four times, with at least triplicate determinations for each experiment. The data are presented as mean ± SD for one representative experiment.

Figure 5. Combination-dependent effects of selected flavonoids on the uptake of GHB. The total concentration of combined flavonoids is given on the x-axis. The % of GHB uptake is the uptake in the presence of flavonoids to that in the absence of flavonoids. The line represents the fitted line using the equation for the IC_{50} determination. Effects of the combination of (A) luteolin with phloretin (1:1), (B) luteolin with phloretin (1:2), and

(C) luteolin with morin (1:1). Uptake by control cells has been subtracted from the values. The uptake studies were conducted at pH 6.0, room temperature, and the uptake time was 1 minute. The concentration of GHB used was 5 μM. The experiments have bee repeated three to four times, with at least triplicate determinations for each experiment. The data are presented as mean ± SD for a representative experiment.

Figure 6. Isobolograms of the effects of combinations of two flavonoids. The line represent the line of additivity. The open triangles represent the combination of phloretin and luteolin and the closed square represents the combination of luteolin and morin.

Figure 7. Transport of kaempferol in MCT1 gene transfected and control cells. The black bars represent the uptake of ^{3}H-kaemperferol by MCT1 cells; the grey bars represent the uptake of ^{3}H-kaemperferol by control cells. The pH 6.0 group represents the uptake of GHB at pH6.0 in the absence of alpha-cyano-4-hydroxycinnamate (CHC). The CHC group represents the uptake of GHB in the presence of CHC. The uptake time was 1 minute at room temperature. The data are presented as mean ± SD for a representative experiment. The experiments have repeated twice.

Figure 8. The pharmacokinetic profile of GHB when administered alone or with concomitant luteolin administration. The rats were dosed with GHB 1 g/kg with concomitant administration of luteolin 10 mg/kg or the vehicle. Plasma concentration of GHB was measured using the LC/MS/MS method as described. The data are presented as mean ± SD, n = 3 rats for each group.

Tables

Table 1. IC_{50} values of the flavonoids luteolin, morin and phloretin for the inhibition of GHB (0.1mM) uptake at pH 6.0 by rat MCT1 gene transfected MDA-MB231 cells. The values were presented as mean ± SD. Three to four experiments were carried out with each experiment consisted of triplicate measurements.

	Luteolin	Morin	Phloretin
IC_{50} (μM)	0.41±0.14	6.21±2.01	2.57±0. 48

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Table 2 Effects of combinations of flavonoids on the uptake of GHB in MCT1 gene transfected MDA-MB231 cells. Data are presented as mean ± SD, n= 3-4. CI index was calculated as CI = $C_1/IC_{50\_1} + C_2/IC_{50\_2}$; C_1 represents the concentration of flavonoid 1; C_2 represents the concentration of flavonoid 2.

	Luteolin + Phloretin (1:1)	Luteolin + Phloretin (1:2)	Luteolin+ Morin (1:1)
IC_{50} (μM)	0.53±0.13	0.60±0.03	1.07 ±0.16
CI index (90% confidence interval)	0.75±0.19 (0.93,0.57)	0.75±0.18 (0.92, 0.58)	1.39±0.21 (1.11, 1.51)

Table 3

Effect of luteolin administration on the pharmacokinetics and pharmacodynamics of GHB. Data presented as mean ± SD, n= 3 rats for each group. An unpaired t-test was used to test for statistically significant differences between the two groups; ** $P<0.01$

Parameters	Control	Luteolin (10mg/kg)
AUC (mg/ml•min)	187 ± 30	103 ± 4 **
λ_z	0.056 ± 0.007	0.086 ± 0.019
Cl (ml/min/kg)	5.45 ± 0.91	9.76 ± 0.39 **
V_{ss} (ml/kg)	493 ± 52	519 ± 33
Urinary recovery (%)	25.9 ± 10.4	44.1 ± 11.7
Cl_R (ml/min/kg)	1.36 ± 0.36	4.28 ± 1.02**
Cl_m (ml/min/kg)	4.08± 1.15	5.48 ± 1.31
Sleep time (min)	165 ± 10	121 ± 5**

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Table 4. Structural elements affecting the inhibitory activity of flavonoids.

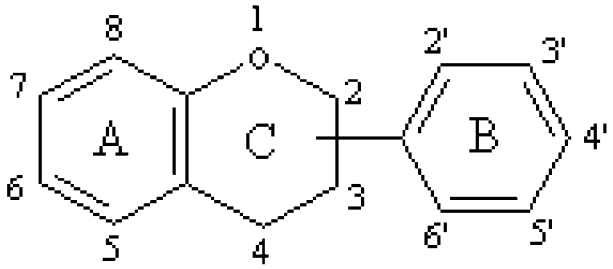
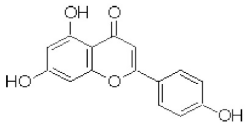
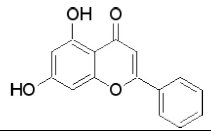
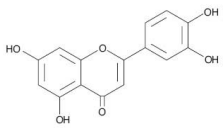
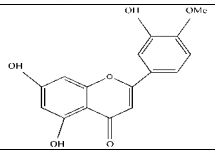
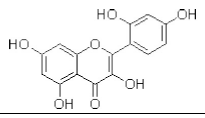
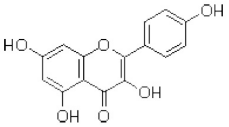
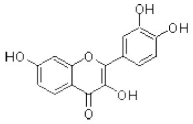
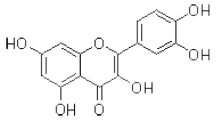
Structure of flavonoids			
Functional group	Compound Name	Structure	% of inhibition
4′-hydroxyl group	Apigenin		52.5%
	Chrysin		48.6%
Methylation of 4′-hydroxyl group	Luteolin		100%
	Diosmetin		49.1%
2′-hydroxyl group	Morin		100%
	Kaempferol		71.3%
3′-hydroxyl group	Luteolin	See above	100%
	Apigenin	See above	52.5
5-hydroxyl group	Fisetin		93.9%
	Quercetin		84.7%

Figure 1

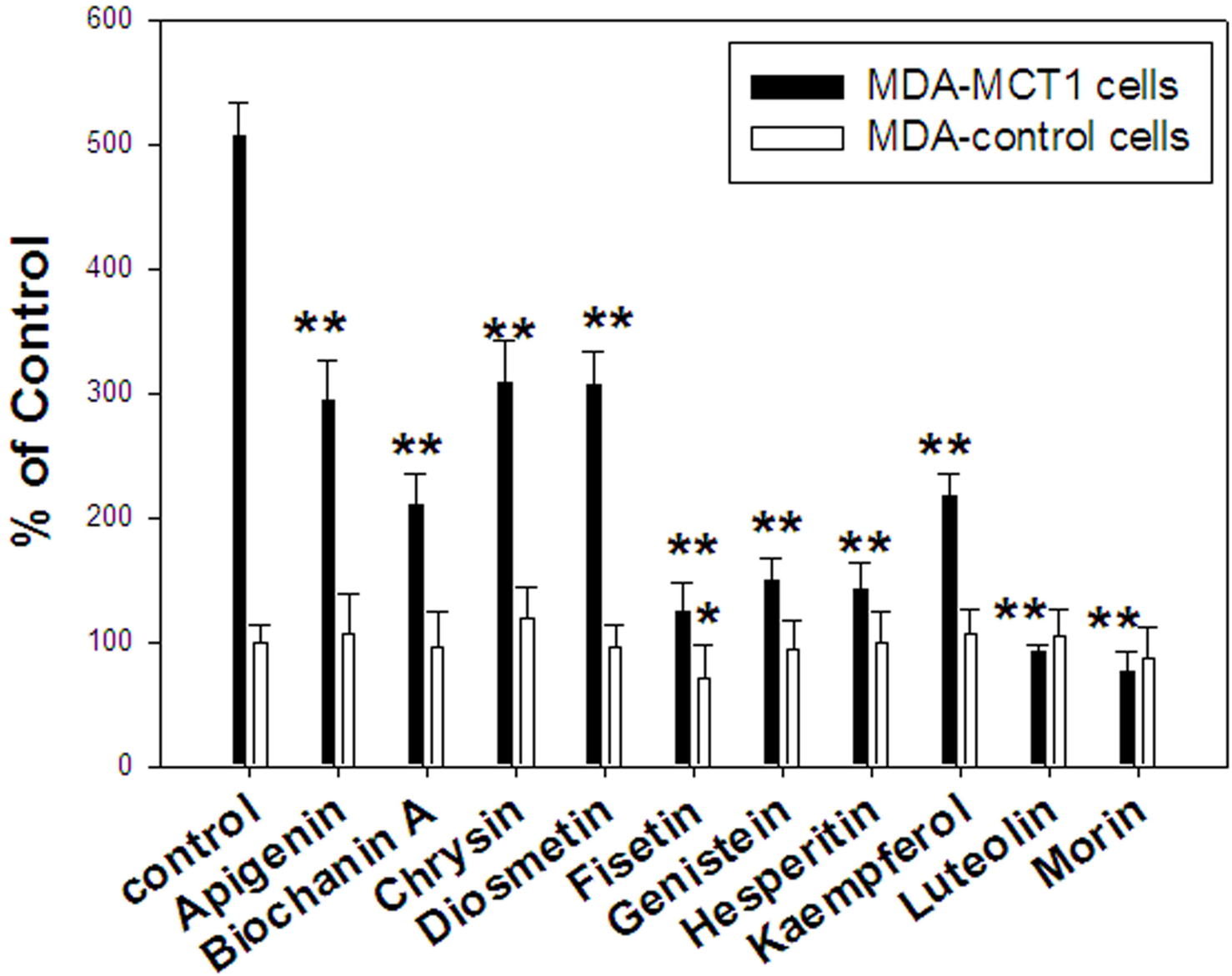


Figure 2

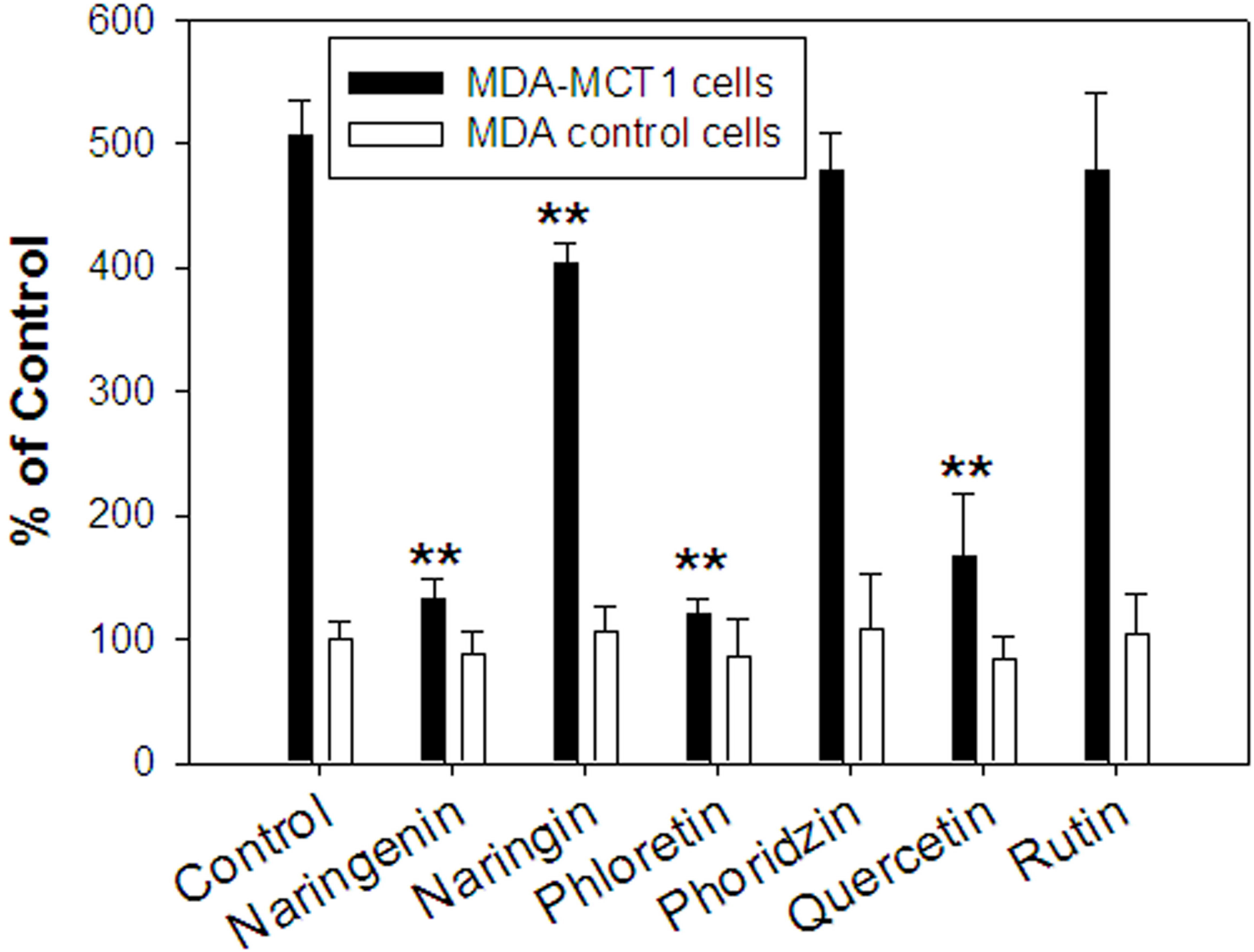


Figure 3

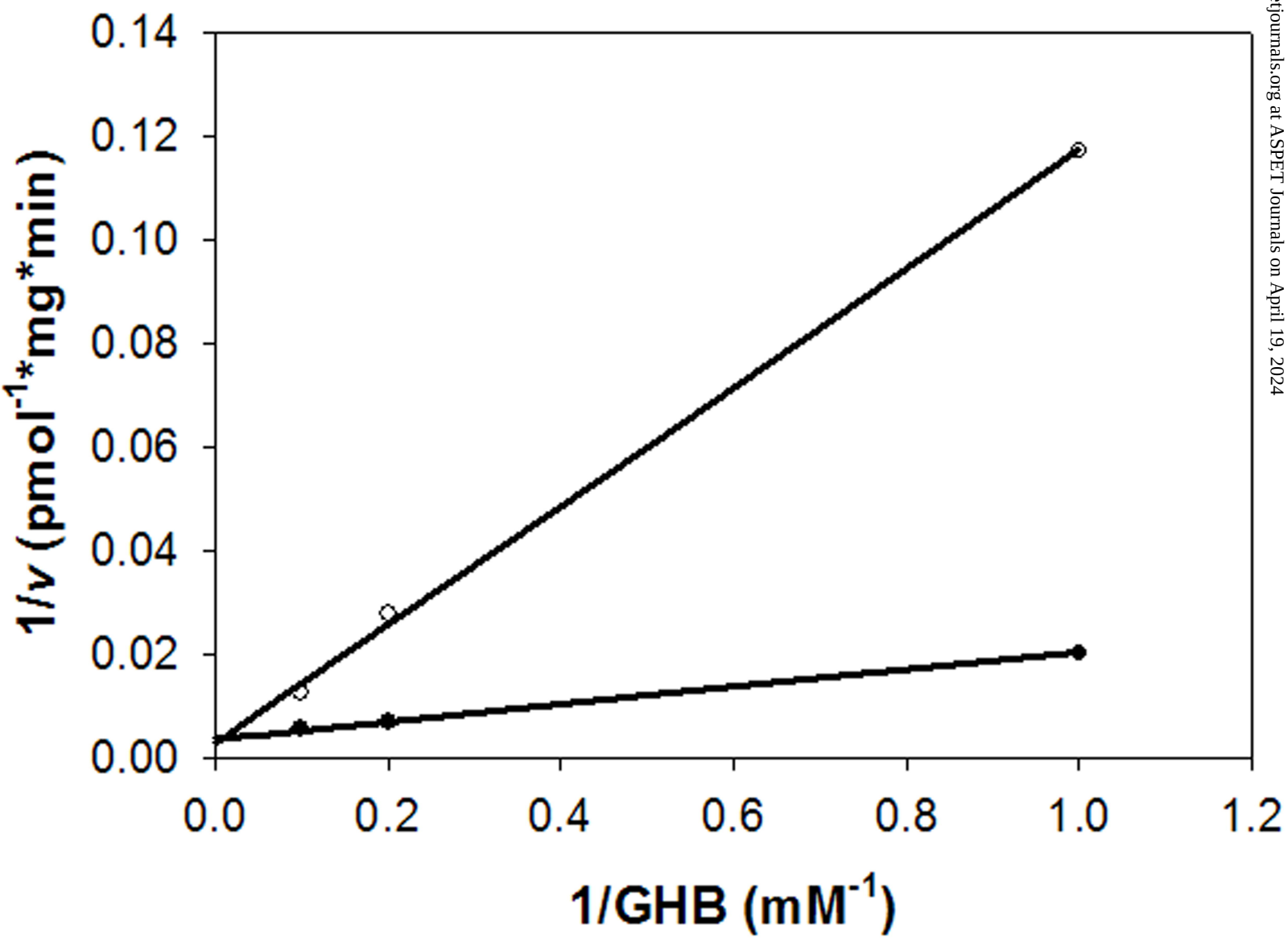


Figure 4

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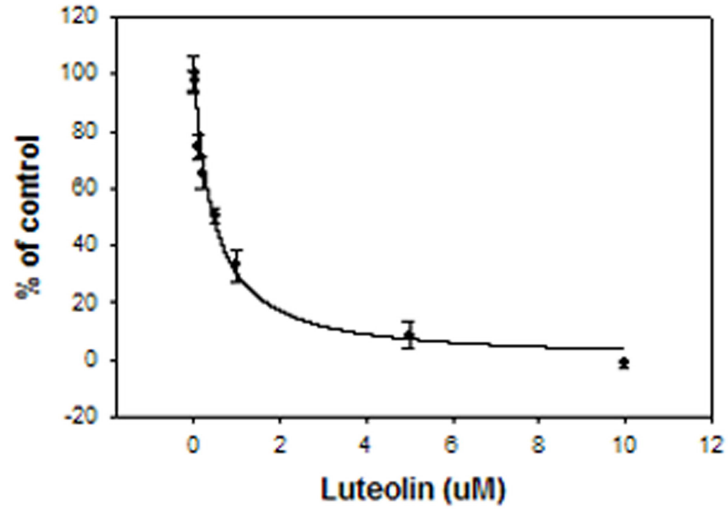

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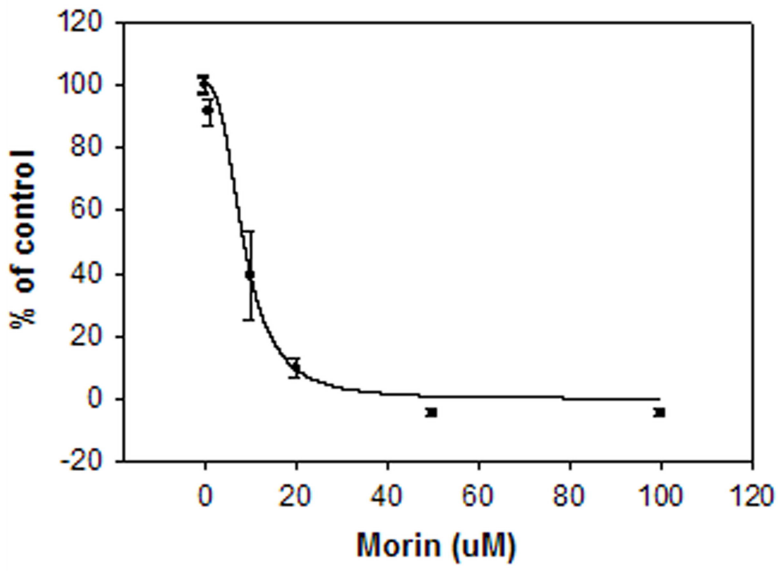

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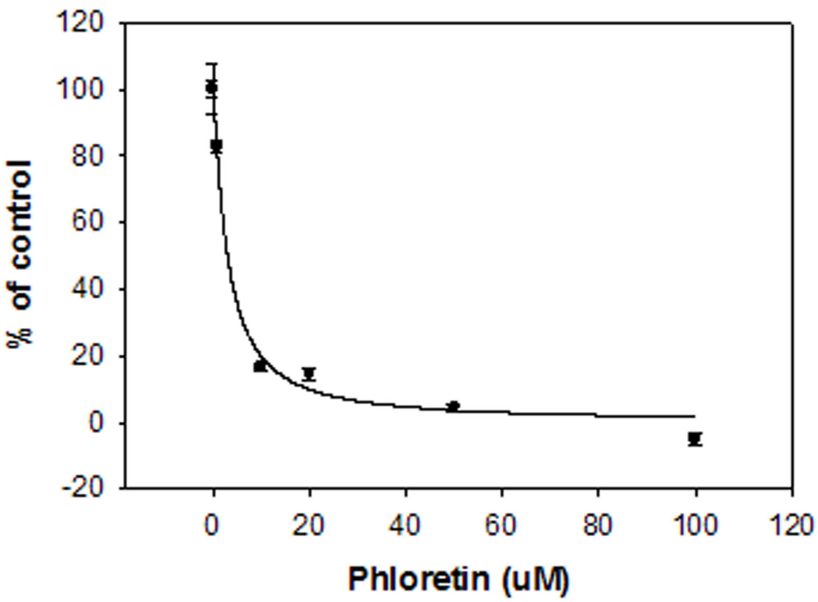

Figure 5

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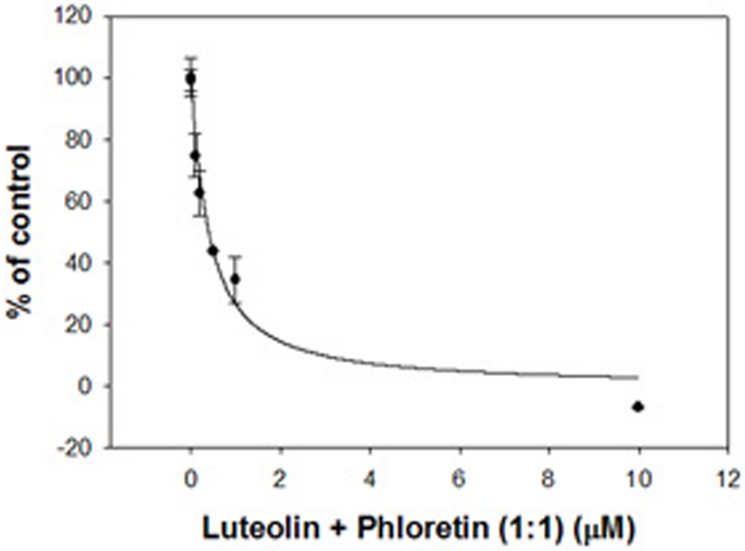

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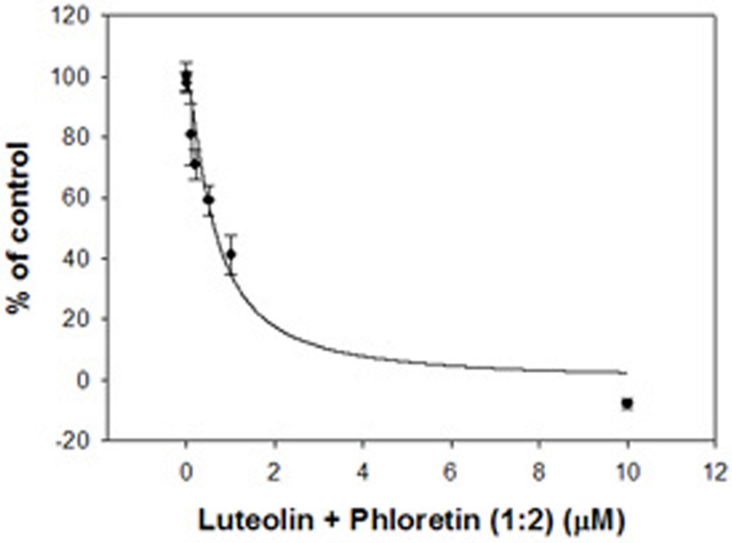

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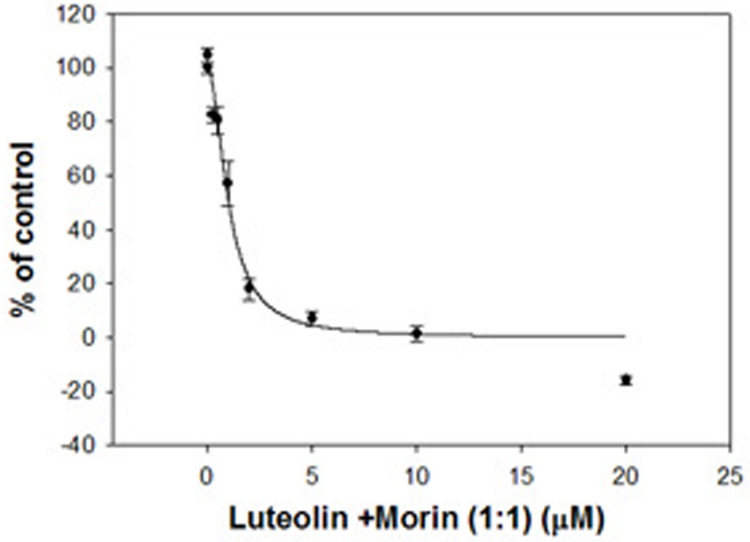


Figure 6

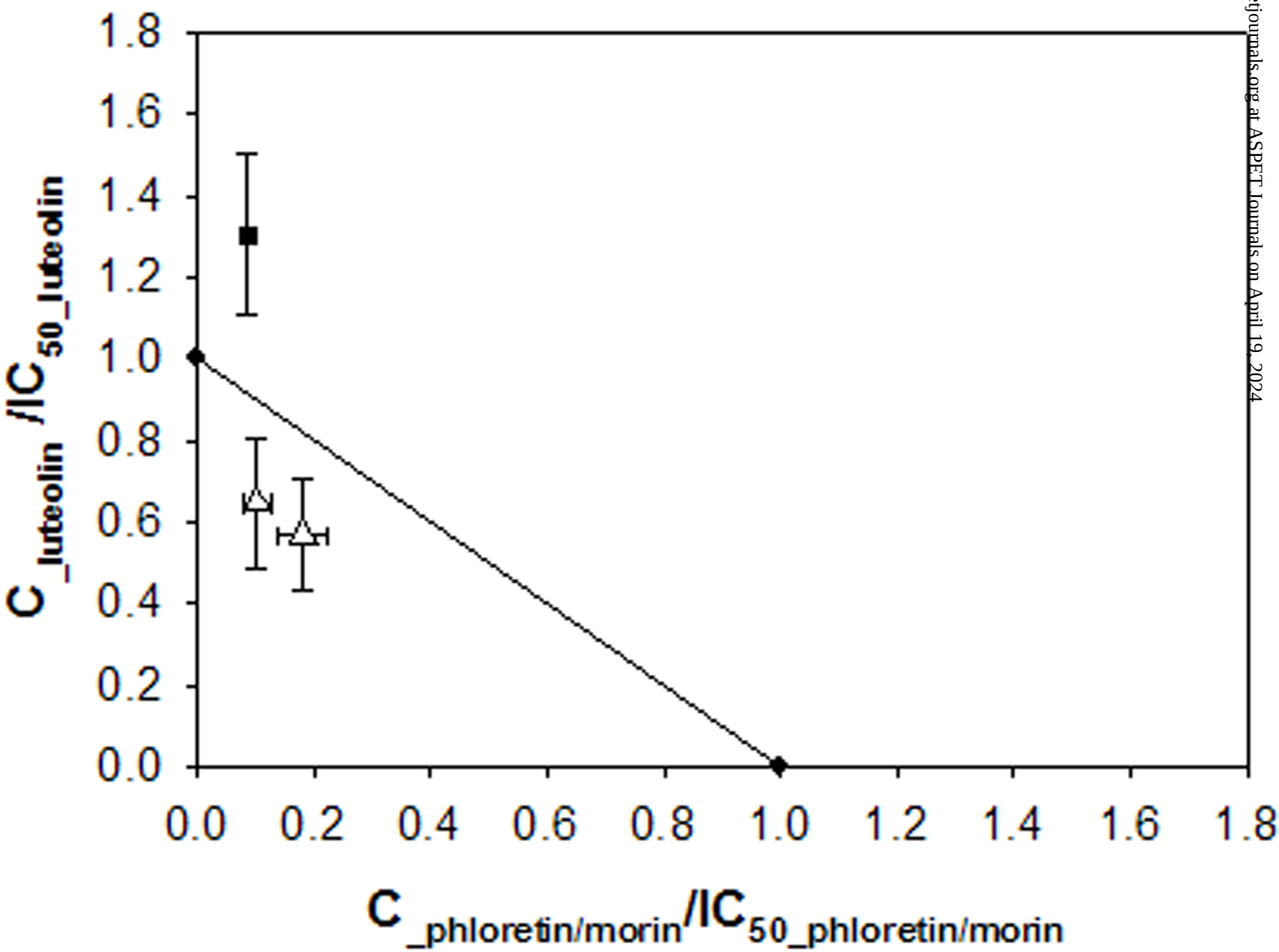


Figure 7

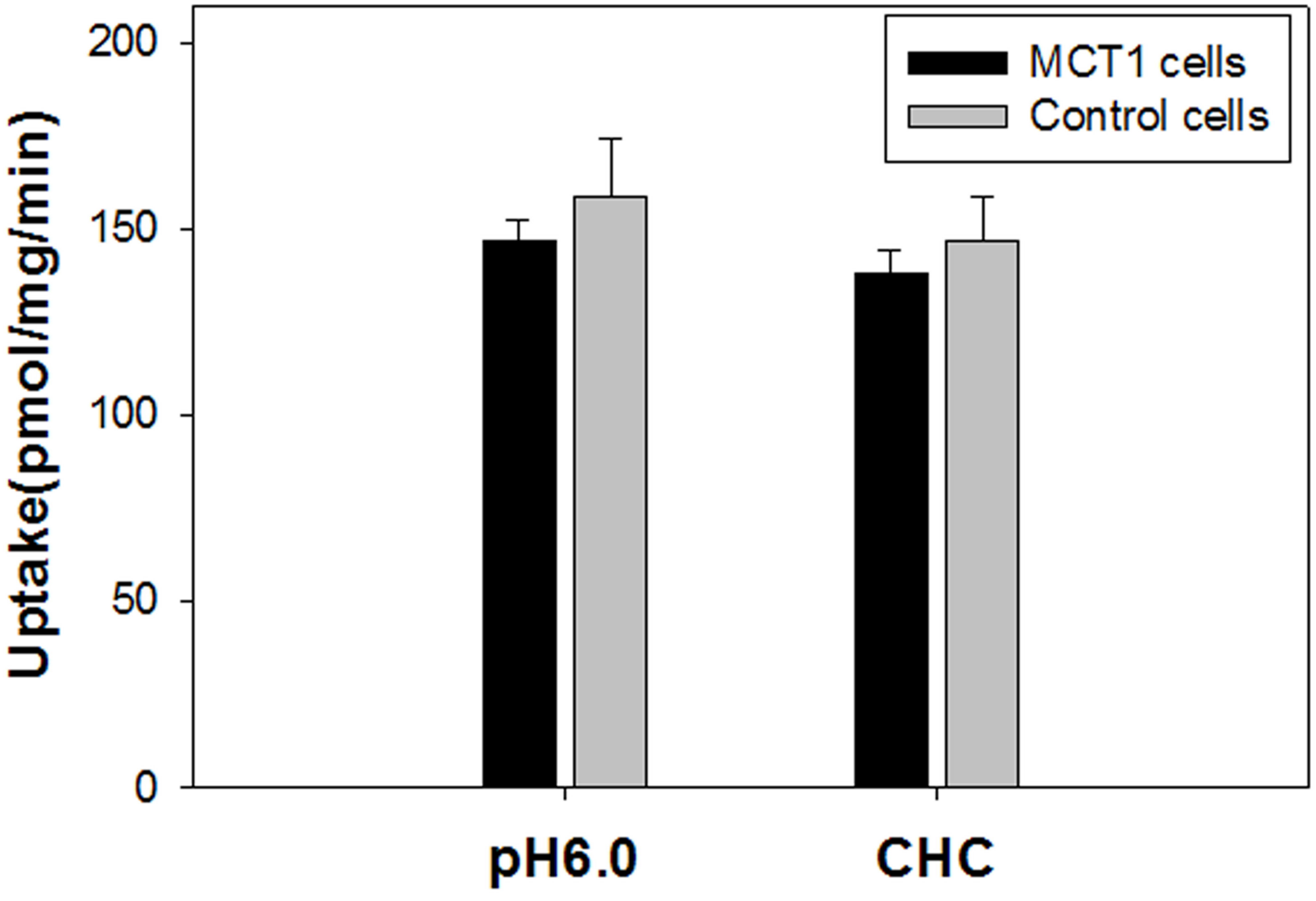
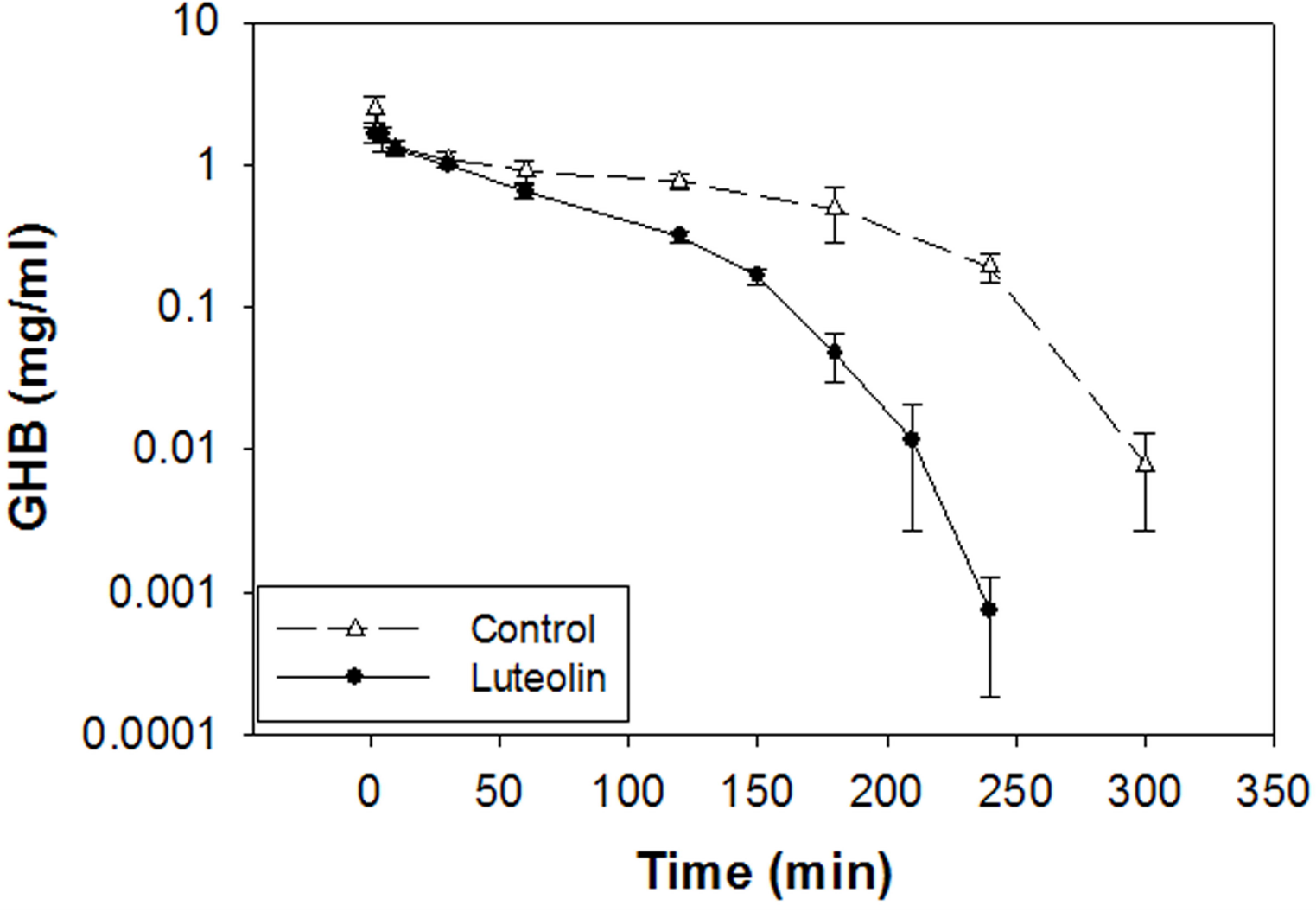


Figure 8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