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Phenobarbital Treatment at a Neonatal Age Results in Decreased Efficacy of Omeprazole in Adult Mice

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Running Title: Long-term interaction between phenobarbital and omeprazole

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ABBREVIATIONS:

DDIs: drug-drug interactions; OME: omeprazole; P450s: cytochrome P450s; PB: phenobarbital.

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ABSTRACT

Drug-drug interactions (DDIs) occur when the action of one drug interferes with or alters the activity of another drug taken concomitantly. This can lead to decreased drug efficacy or increased toxicity. Because of DDIs, physicians in the clinical practice attempt to avoid potential interactions when multiple drugs are co-administrated, however there is still a large knowledge gap in understanding how drugs taken in the past can contribute to DDIs in the future. The goal of this study is to investigate the consequence of neonatal drug exposure on efficacy of other drugs administered to adult life. We selected a mouse model to test phenobarbital exposure at a neonatal age and its impact on efficacy of omeprazole in adult life. The results of our experiment show an observed decrease in omeprazole's ability to raise gastric pH in adult mice that receive single or multiple doses of phenobarbital at a neonatal age. This effect may be associated with the permanent induction of cytochrome P450 enzymes in adult liver after neonatal phenobarbital treatment. Our data indicates that DDIs may result from drugs administered in the past in an animal model and should prompt reevaluation of how DDIs are viewed and avoided long term DDIs in clinical practice.

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Introduction

Polypharmacy-induced drug-drug interactions (DDIs) significantly raise the risks for decreasing therapeutic efficacy and increasing adverse reactions with particular higher risks for the elder, children, and women populations (Sharifiet al. 2014). Approximately 50% of the population aged over 65 years now takes at least 5 different medications with 35-60% of these elderly patients exposed to a potential DDI and 5-15% suffering clinically significant adverse reactions (Magro et al. 2012). Additionally, it is estimated that 49% of hospitalized patients under the age of 21 are exposed to a potential DDI (Feinstein et al. 2015).

Based on their mechanisms of actions, DDIs are classified into two main categories: pharmacokinetics and pharmacodynamics. DDIs that are considered in the category of pharmacokinetics occur when one drug alters the absorption, distribution, metabolism, or excretion of another drug in the patient's body. Altering any of these pharmacokinetic factors can increase or decrease the concentrations of a drug or its metabolites in circulation (Seymour and Routledge 1998). Certain drugs possess the ability to increase or decrease the rate, at which another drug is metabolized, leading to significantly lower or higher serum concentrations of the drug or its metabolites. These types of interactions are common due to the induction of gene expression of certain number of cytochrome P450 enzymes (P450s) that are responsible for the biotransformation of 80% prescription drugs (Jana and Paliwal 2007). Induction of P450s by drugs occurs shortly after the inducing drug is taken, with a delay depending on the half-life of the drug. Induction is considered to be a temporary event in adults, in which P450 levels will return to normal once the inducing drug is ceased (Lynch and Price 2007). However, a large body of works from the Shapiro's laboratory have demonstrated that neonatal exposure to

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phenobarbital can cause a permanent elevation of basal levels of enzyme activities in a variety of P450-mediated drug metabolizing enzymes (Agrawal et al. 1995, Agrawal and Shapiro 2000, Agrawal and Shapiro 2005). Recently, a research in our lab has shown that the permanent elevation of gene expression and enzyme activities of several P450s in adult mouse liver by neonatal treatment with phenobarbital is dependent on two key factors: the age, at which the mouse is treated in early life, and the dose, at which phenobarbital is given (Tien et al. 2015). . Phenobarbital is still the first drug of choice for treating acute neonatal seizures, which occur in 2-3 out of 1,000 live births (Hellstrom-Westas et al. 2015) and is still widely administered to babies. It is also a known inducer of the CYP2B6, 2C9, 2C19, and 3A4 in human and CYP2B10, 2C29, and 3A11 in mice (Czekaj 2000). Treatment at earlier ages with high doses of phenobarbital produces a permanent induction of P450 enzymes at the adult age in mouse liver (Tien et al. 2015). However, whether the permanent induction of P450 enzymes has an effect on the efficacy of other drugs administered to adults was not investigated.

This study aims to determine whether treatment with the P450-inducing drug phenobarbital early in life can affect the efficacy of a drug taken at a separate time later in adult life. Omeprazole is a proton pump inhibitor commonly used to treat stomach ulcers, gastroesophageal reflux disease, and heartburn (Marostica et al. 2007). Its action involves blocking the release of acid by proton pumps in the stomach so as to raise the pH of the gastric lumen. We chose omeprazole as a model drug to test for changes in efficacy in adult mice after neonatal treatment with phenobarbital. Its efficacy can be tested by measuring pH of gastric juices after daily dosing. Omeprazole is known to be primarily metabolized by CYP2C19 and CYP3A4 in humans (Andersson et al. 1994, Chang et al. 1995, Andersson 1996). Its metabolism can be altered by co-administrated with phenobarbital (Park et al. 2005). Although the primary P450 enzymes

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93 metabolizing omeprazole in mice are not defined yet, we select CYP2C29 and CYP3A11 as a
94 representative member of CYP2C and CYP3A subfamily, respectively, in this study.
95 Investigating whether neonatal phenobarbital exposure affects the ability of omeprazole to
96 increase stomach pH in adults can give insight into how drug treatment in early life can impact
97 drug interactions in later life. This knowledge may prompt a reevaluation of how DDIs in the
98 clinic are viewed and predicted. In order for a drug-drug interaction to occur, it may not be
99 necessary for two drugs to be taken concomitantly.

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Materials and Methods

Chemicals. Phenobarbital and omeprazole were purchased from Sigma-Aldrich (St. Louis, MO).

Animal treatment with drugs. The use of animals in the current study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. C57BL/6 mice were bred and housed under the standard conditions in the Animal Care Service Facility at the University of Connecticut according to the animal care guidelines provided by the American Association for Laboratory Animal Science. Treatment schedules of phenobarbital or omeprazole in each experiment are outlined in figures. Phenobarbital at a dose of 200 mg/kg or control saline (PBS) was given to the mice via intraperitoneal injection. The selected dose of phenobarbital was proved to be able to permanently induce P450 expression in adult liver when treatment occurs at a neonatal age (Tien et al. 2015). Three consecutive doses of omeprazole at 150 mg/kg/day or control saline (PBS) were given to the mice via intraperitoneal injection. The dose of omeprazole at 150 mg/kg/day was selected because this dose has previously been demonstrated to efficiently inhibit the gastric H^+/K^+ -ATPase pump in mice (Aristoteli et al. 2006). Blocking the activity of the gastric proton-pump prevents the release of hydrochloric acid from parietal cells and increases the pH of the stomach as the cavity becomes less acidic (Marostica et al. 2007). Because no difference between male and female was found in a previous study (Tien et al., 2015) on the permanent induction of gene expression of CYP3A11 and CYP2C29 in adult by phenobarbital treatment at early life, only male mice were used in each control and treatment group.

pH measurement in gastric stomach. One hour after the final omeprazole or control PBS treatment, all adult mice were anesthetized with isoflurane and an incision was made in the

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stomach. An Orion 9863BN micro pH electrode was placed in the stomach and the pH of gastric liquids was measured by a pH probe using a previous described procedure (Brenneman et al. 2014). Mice were then sacrificed and livers were harvested.

Quantitative real-time PCR and Western blot analysis. Total RNAs and proteins were isolated from the harvested livers and expression of CYP2C29 and CYP3A11 in liver at mRNA level was determined by RT-PCR and protein level by Western blot with procedures described in a previous study (Tien et al. 2015).

Statistics. Data are presented as mean \pm SD with $n = 3$ mice per group in the control or treatment group. Statistical analyses were performed using GraphPad Prism 6. Comparisons between control and treatment groups were performed using unpaired t -test and a p -value < 0.05 was considered statistically significant. The sample size ($n=3$) had a power over 80% to find $p < 0.05$ if the means of the treatment groups compared to the control group are greater than 2 fold and standard deviations are less than 30%.

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Results

Omeprazole inhibits the gastric proton-pump to increase pH in the gastric stomach in adult mice. A control experiment illustrated in Fig. 1A was performed to examine efficacy of omeprazole in inhibition of proton pumps in adult mouse stomach. The adult mice that received PBS as a control had a gastric stomach pH of 2.4 ± 0.2 at a non-fasting condition (Fig. 1B). The adult mice that received omeprazole for 3 days had a gastric stomach pH of 4.5 ± 0.2 under the non-fasting condition after the final treatment. This indicates that omeprazole efficiently blocked the activity of gastric proton pump to reduce the amount of acids released and significantly raised the pH in the stomach (** $p < 0.01$). Expression of CYP2C9 and CYP3A11 at mRNA level and CYP3A11 at protein level in mouse livers was further examined by RT-PCR and Western blot, respectively. Because of no available antibody specific against CYP2C9, CYP2C9 protein level wasn't determined by Western blot in this study. No differences in gene expression of CYP3A11 and CYP2C9 at mRNA level were noted after treatment with omeprazole compared to the control (Fig. 1C and 1D). CYP3A11 expression at the protein level is also consistent between the control and omeprazole treatment (Fig. 1E). These results show that treatment with omeprazole has no effect on the induction of expression of CYP2C9 and CYP3A11.

Concurrent administration of phenobarbital and omeprazole temporarily reduces efficacy of omeprazole in proton-pump inhibition in adult mice. Figure 2A illustrates an experimental design to examine phenobarbital-omeprazole (PB-OME) interaction on efficacy of omeprazole in proton pump inhibition in adult mice. Mice in the OME control group had a gastric pH of 4.0 ± 0.1 , comparable to the result shown in Fig. 1B. Mice in the co-treatment

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group had a gastric pH of 3.5 ± 0.1 that was significantly lower ($***p < 0.001$) than the control group. This indicates that administering phenobarbital at the same time with omeprazole in adult mice causes a drug-drug interaction, diminishing the efficacy of omeprazole in increase of gastric stomach pH. Mice in the post-treatment group had a gastric pH of 4.2 ± 0.1 that was similar to the OME control mice and showed no statistically significant difference. This indicates that in adult mice, the efficacy of proton-pump inhibition by omeprazole is not affected by a previous administration of phenobarbital. To exclude an effect on the gastric pH by phenobarbital, a group of mice ($n=3$) was treated with phenobarbital (200 mg/kg) at day 57 after birth and followed by PBS treatment at day 58 and 59, the mice had a gastric pH of 2.7 ± 0.3 at a non-fasting condition, which was not different compared to the control group received PBS treatment at day 57, 58, and 59 ($pH = 2.4 \pm 0.2$).

Gene expression of CYP2C29 and CYP3A11 at mRNA level was also determined by RT-PCR in the livers collected after the completion of omeprazole treatment (Fig. 2C and 2D). Compared to the OME control group, expression of both CYP2C29 and CYP3A11 were significantly induced to 7.3 ± 0.9 ($***p < 0.001$) and 12.6 ± 0.8 ($***p < 0.001$) fold higher, respectively, in the PB/OME co-treatment mice. Mice that received phenobarbital three days prior to beginning omeprazole treatment had no significant changes in either CYP3A11 or 2C29 expression after the last dose of omeprazole. Similar changes at protein level for CYP3A11 were observed in Fig. 2E. These results indicate that phenobarbital-mediated P450 induction in adult mice is not long-term and induced levels of enzymes will return to normal at 6 days after phenobarbital treatment is ceased.

Here we demonstrated that induction of the expression of CYP2C29 and CYP3A11 by phenobarbital was associated with the decreased efficacy of omeprazole in proton-pump

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inhibition shown as a decreased raise of pH in mouse gastric stomach when phenobarbital and omeprazole were co-administrated, but such effect was temporary and wasn't observed in a treatment, in which omeprazole administration was three days later than the phenobarbital treatment.

Neonatal administration of phenobarbital reduces the efficacy of omeprazole in proton-pump inhibition in adult mice. An experimental design is illustrated in Fig. 3A. In the Neo vehicle/adult OME control group, omeprazole treatment at the adult age repeatedly showed efficient proton-pump inhibition to raise gastric pH to 4.5 ± 0.2 . In the Neo single-PB/adult vehicle (PBS) group, no omeprazole was treated at the adult age, therefore, no gastric pH was increased (pH = 2.3 ± 0.8). Mice in the Neo single-PB/adult OME group had a significantly lower pH level of 3.6 ± 0.1 than the control mice that were administered with PBS at day 5 (** $p < 0.01$). Mice in the Neo multi-PB/adult OME group experienced an even lower gastric pH of 3.1 ± 0.2 (** $p < 0.01$). These results suggest that phenobarbital exposure at a neonatal age could result in a long-term interaction with omeprazole to lower efficacy in proton pump inhibition in adult stomach. To ensure that phenobarbital treatment has no effect on gastric stomach pH, we also included a group of mice (n=3) receiving a single dose of 200 mg/kg/day phenobarbital at day 5 and 3 consecutive doses of PBS vehicle at 57, 58, and 59 days after birth. Their gastric pH values were comparable to a normal physiological level at 2.4 ± 0.4 , indicating phenobarbital treatment at day 5 had no effect on gastric stomach pH.

After the measurement of gastric pH, livers of the all mice were collected for further analysis of gene expression of CYP2C29 and CYP3A11 at mRNA level (Fig. 3C and 3D) and CYP3A11 at protein level (Fig. 3E). Compared to the Neo vehicle/adult OME control group, phenobarbital treatment at the neonatal age with either single or multiple doses resulted in a long-term

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208 elevation of mRNA expression of CYP2C29 and CYP3A11 in adult life. Phenobarbital treatment
209 at the neonatal ages also resulted in increases of CYP3A11 at protein level (Fig. 3E).

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Discussion

Phenobarbital, along with several other first-generation antiepileptic and sedative drugs, is known to cause induction of several different P450 enzymes (Perucca 2006). Because of this, physicians are currently aware of the risk of DDIs with many common medications in patients prescribed phenobarbital (Lebowitz et al. 2016). However, the current clinical practice only takes the DDIs into consideration when multiple drugs are co-administrated at same times. A historical usage of drugs in previous weeks, months, or years has not been a consideration factor for DDIs as in most cases induction of P450 expression by a drug is a temporary event and will disappear in a short period after administration of the inducer drug is stopped. This is true for adults, however, our study may have a significant impact to change the concept of DDIs for people who received drug treatment at neonatal and infant ages.

Studies from Shaprio's laboratory have demonstrated that permanent induction of P450 expression in adult liver can be achieved when phenobarbital is exposed at neonatal and infant ages (Agrawal et al. 1995, Agrawal and Shapiro 2000, Agrawal and Shapiro 2005). Our previous study further illustrated that the permanent induction is dose- and age-dependent (Tien et al. 2015). In the current study, we further demonstrated that the permanent induction of P450 expression in adult mice by neonatal phenobarbital exposure may be associated with a significant decrease of efficacy in inhibition of proton pumps by omeprazole in adult life. Although previous studies have shown that neonatal treatment with phenobarbital can decrease sleep time *in vivo* in adult rats treated with hexobarbital (Agrawal and Shapiro 2000, Agrawal and Shapiro 2005) and neonatal treatment with TCPOBOP can decrease paralysis time in adult mice treated zoxazolamine (Chen et al. 2012), neither hexobarbital nor zoxazolamine are current commonly

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used drugs. In the current study by using the commonly used clinical drugs of omeprazole and phenobarbital, we have shown a long-term impact on therapeutic efficacy of a drug, which is metabolized by P450s, by neonatal exposure to another drug, which can induce P450 expression, in an animal model. However, this concept on long-term drug-drug interactions needs to be further confirmed by more drugs, such as midazolam, which are primarily metabolized by the inducible P450 enzymes in adults, and by more drugs, such as phenytoin and dexamethasone, which are capable to induce P450 expression in adult when they are exposed at neonatal ages in animal models. Dose ranges and exposure sensitive windows need to be established for each inducible drugs. Furthermore, the underline molecular mechanisms also need to be explored for explanation of the permanent induction of P450 expression by neonatal drug exposure. Epigenetic mechanisms, including DNA methylation, histone modifications, and microRNAs have all been implicated in regulating the expression of drug metabolizing enzymes in both neonatal and adult livers (Kacevska et al. 2012, Ingelman-Sundberg et al. 2013, Bonder et al. 2014). Nuclear receptors are also known to control the induction and expression of many hepatic P450 enzymes and transporter genes (Chai et al. 2013, Kandel et al. 2016) and likely play a role in causing their permanent induction with activation during the neonatal developmental period (Chen et al. 2012). Further mechanistic studies for this phenomenon can give greater insight to factors responsible for permanent induction of drug metabolizing enzymes by other drugs, environmental toxins, and nutritive components.

Our study, although performed in a mouse model, can prompt a reevaluation of how DDIs are presently viewed and predicted. Further translational studies in human subjects will need to be completed in order to prove that this concept can make a clinical impact. Historical usage of

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256 drugs particularly during neonatal and infant ages may serve as a consideration factor for
257 predicting drug response.
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259 **Author contributions**

260 Participated in research design: Tien, Piekos, Pope, and Zhong.

261 Conducted experiments: Tien, Piekos, and Pope.

262 Performed data analysis: Tien and Zhong

263 Wrote or contributed to the writing of the manuscript: Tien, Piekos, Pope, and Zhong.

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334 **Footnotes**

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Figure legends

Fig. 1. Omeprazole treatment increases gastric pH without altering P450 expression in adult mice. (A) An illustration of animal treatment. The adult mice were treated with either vehicle control (PBS) (n=3) or omeprazole (OME) at 150 mg/kg/day (n=3) for 3 consecutive days at the age of 57, 58, and 59 days after birth. (B) Gastric pH with mean \pm SD in the mouse stomach at one hour after the last treatment of PBS or OME. (C) Relative fold changes of mRNA of CYP2C29, (D) mRNA of CYP3A11, and (E) protein of CYP3A11 in the mouse livers with the control and OME treatment. ** $p < 0.01$.

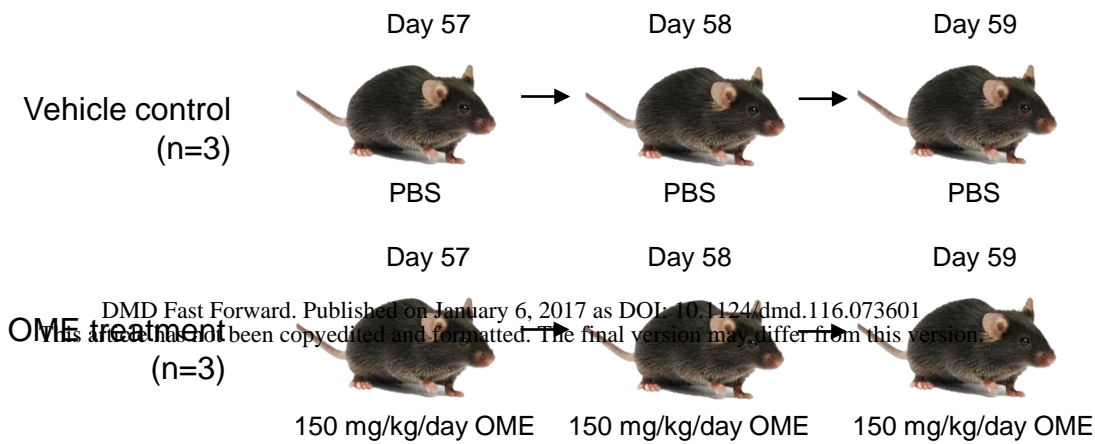
Fig. 2. Concurrent administration of phenobarbital and omeprazole results in a drug-drug interaction and reduces omeprazole efficacy. (A) An illustration of animal treatment. Adult mice in the OME-control group (n=3) were treated with 3 consecutive doses of 150 mg/kg/day of omeprazole at 57, 58, and 59 days after birth. Adult mice in the PB-OME co-treatment group (n=3) received a single dose of 200 mg/kg phenobarbital together with a dose of 150 mg/kg omeprazole at day 57, followed by two days of treatment with just omeprazole at day 58 and 59. Adult mice in the PB-OME post-treatment group (n=3) received a same single dose of 200 mg/kg phenobarbital at day 57, then the 3 consecutive doses of 150 mg/kg/day of omeprazole were started 3 days later at day 60, 61, and 62. (B) Gastric pH with mean \pm SD in the mouse stomach at one hour after the last treatment of OME. (C) Relative fold changes of mRNA of CYP2C29, (D) mRNA of CYP3A11, and (E) protein of CYP3A11 in the mouse livers with phenobarbital (control, co-, and post-) and omeprazole treatment. *** $p < 0.001$.

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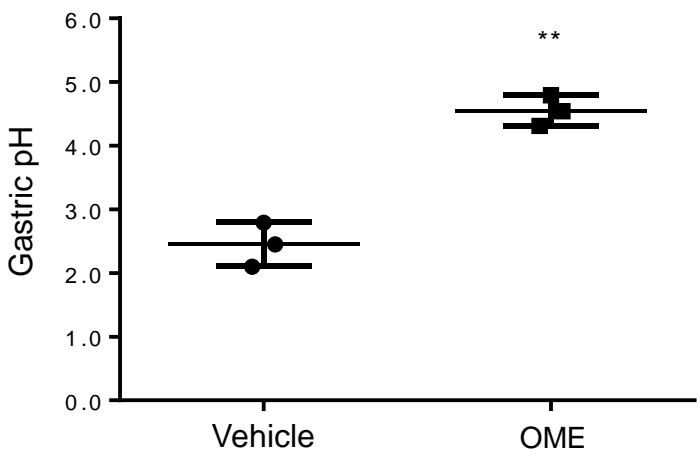
Fig. 3. Neonatal administration of phenobarbital causes a drug-drug interaction and reduces efficacy of omeprazole in adult mice. (A) An illustration of animal treatment. The mice in the Neo vehicle/adult OME control group (n=3) received vehicle (PBS) at a neonatal age of day 5 and 3 consecutive doses of 150 mg/kg/day of omeprazole at adult ages of 57, 58, and 59 days after birth. The mice in the Neo single-PB/adult vehicle group (n=3) received a single dose of 200 mg/kg/day phenobarbital at day 5 and three consecutive treatment of vehicle (PBS) at 57, 58, and 59 days after birth. The mice in the Neo single-PB/adult OME group (n=3) received a single dose of 200 mg/kg/day phenobarbital at day 5 and 3 consecutive doses of 150 mg/kg/day of omeprazole at 57, 58, and 59 days after birth. The mice in the Neo multi-PB/adult OME group (n=3) received 3 consecutive doses of 200 mg/kg/day phenobarbital at day 5, 6, and 7 and 3 consecutive doses of 150 mg/kg/day of omeprazole at 57, 58, and 59 days after birth. (B) Gastric pH with mean \pm SD in the mouse stomach at one hour after the last treatment of OME. (C) Relative fold changes of mRNA of CYP2C29, (D) mRNA of CYP3A11, and (E) protein of CYP3A11 in mouse livers with the neonatal phenobarbital (control, single-, and multiple-) and adult omeprazole treatment. * p <0.05 and ** p <0.01.

Figure 1

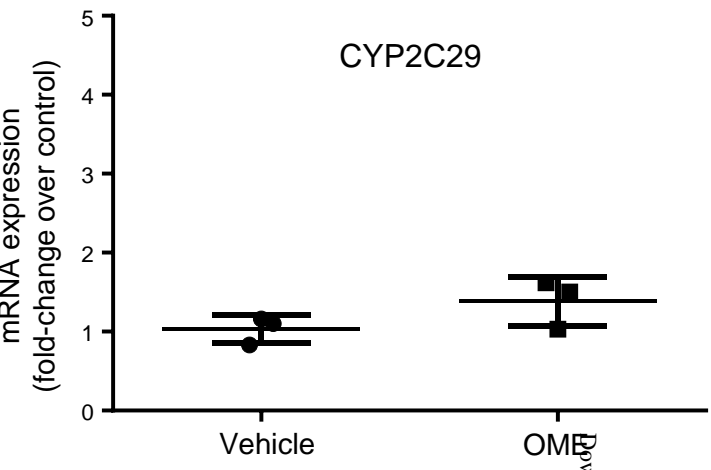
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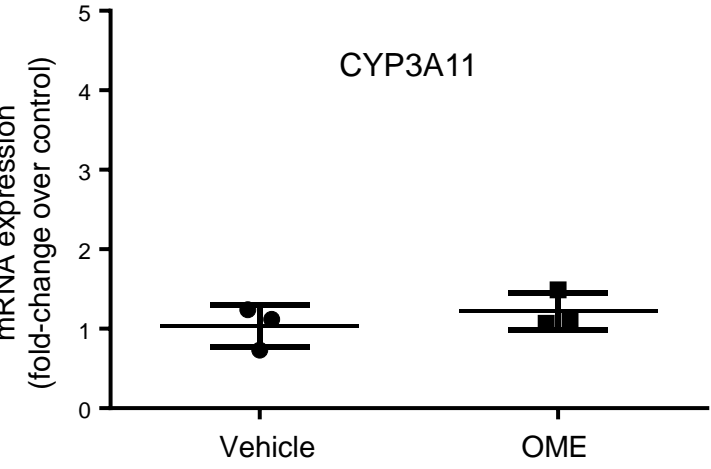
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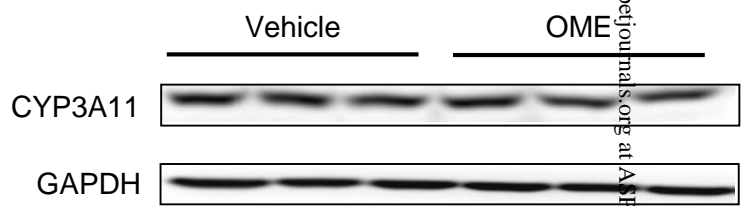


Figure 2

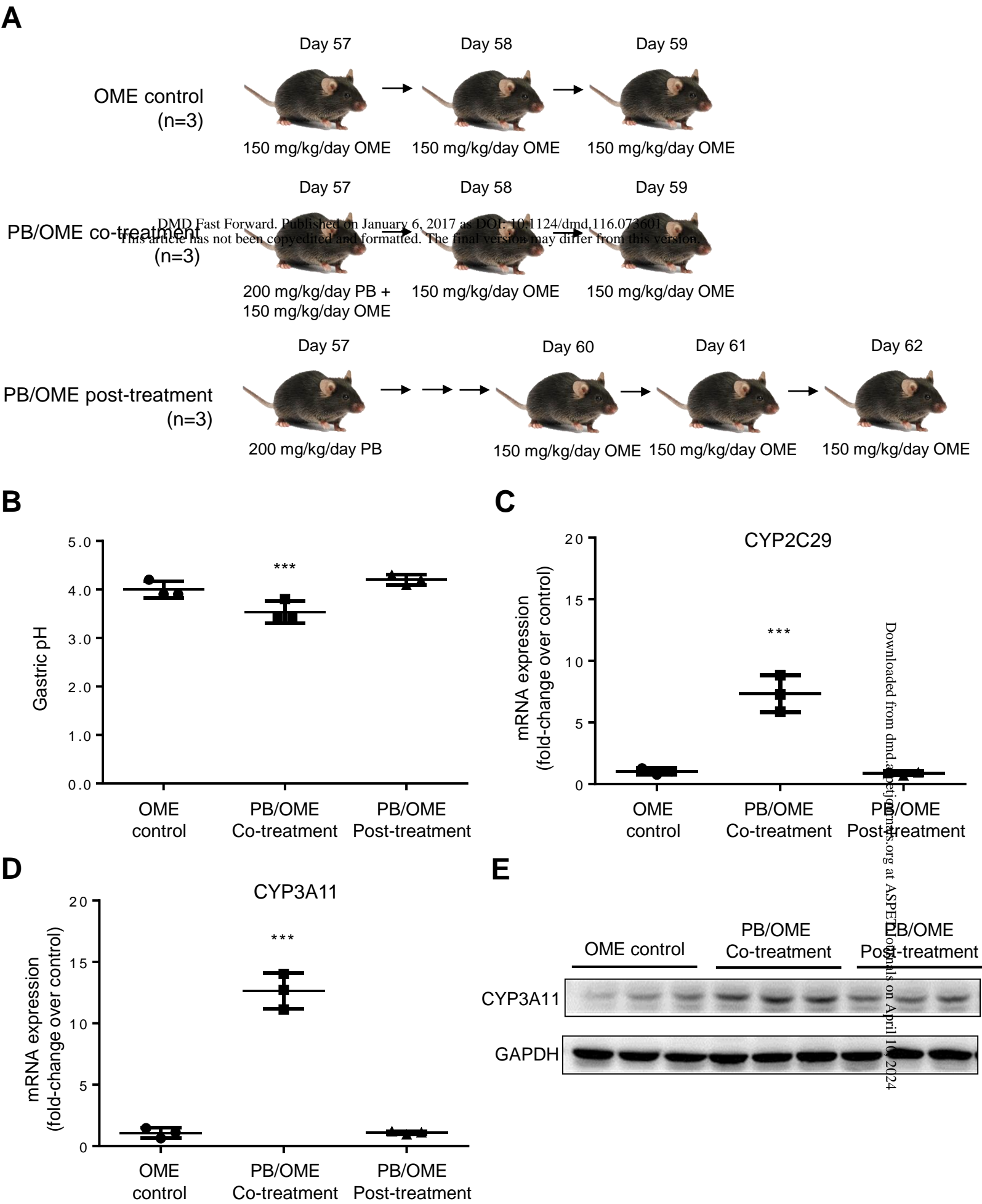


Figure 3

