Inhibition of Genistein Glucuronidation by Bisphenol A in Human and Rat Liver Microsomes^[S]

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Received August 15, 2011; accepted December 6, 2011

ABSTRACT:

Genistein is a natural phytoestrogen of the soybean, and bisphenol A (BPA) is a synthetic chemical used in the production of polycarbonate plastics. Both genistein and BPA disrupt the endocrine system in vivo and in vitro. Growing concerns of altered xenobiotic metabolism due to concomitant exposures from soy milk in BPA-laden baby bottles has warranted the investigation of the glucuronidation rate of genistein in the absence and presence (25 μM) of BPA by human liver microsomes (HLM) and rat liver microsomes (RLM). HLM yield V_{max} values of $0.93 \pm 0.10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $0.62 \pm 0.05 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in the absence and presence of BPA, respectively. K_m values for genistein glucuronidation by HLM in the absence and presence of BPA are $15.1 \pm 7.9 \mu\text{M}$ and $21.5 \pm$

$7.7 \mu\text{M}$, respectively, resulting in a K_i value of $58.7 \mu\text{M}$ for BPA. Significantly reduced V_{max} and unchanged K_m in the presence of BPA in HLM are suggestive of noncompetitive inhibition. In RLM, the presence of BPA resulted in a K_i of $35.7 \mu\text{M}$, an insignificant change in V_{max} ($2.91 \pm 0.26 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $3.05 \pm 0.41 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in the absence and presence of BPA, respectively), and an increase in apparent K_m ($49.4 \pm 14 \mu\text{M}$ with no BPA and $84.0 \pm 28 \mu\text{M}$ with BPA), indicative of competitive inhibition. These findings are significant because they suggest that BPA is capable of inhibiting the glucuronidation of genistein in vitro, and that the type of inhibition is different between HLM and RLM.

Introduction

Glucuronidation is a major form of phase II xenobiotic metabolism, which is catalyzed by isoforms of the UDP-glucuronosyltransferase (UGT) family. UGTs are promiscuous enzymes capable of conjugating structurally diverse substrates. UGTs are constitutively expressed in a tissue-specific manner, with their expression and activity altered by genetic and environmental factors (Tukey and Strassburg, 2000). Whereas some UGTs exist only in extrahepatic tissues, most UGTs are expressed abundantly in the liver (Tukey and Strassburg, 2000; Miners et al., 2006). Although UGT isoforms vary between species, several interspecies homologs have been identified. Humans and rats have several orthologous UGTs, including UGT1A1, UGT1A3, UGT1A6, and UGT1A10. In general, rats have an overall greater

capacity for glucuronidation than humans (Elsby et al., 2001; Völkel et al., 2002). Along with the required cofactor UDP-glucuronic acid (UDPGA), UGTs catalyze the synthesis of a β -D-glucuronide conjugate. Glucuronide conjugates are generally less biologically active and are more readily excreted than their parent substrates, making UGTs key players in the regulation of xenobiotic metabolism and toxicity.

Genistein [4',5,7-trihydroxyisoflavone] is a natural phytoestrogen abundantly present in soybeans. Because nearly 60% of processed foods contain soy, humans consume a nearly continuous supply of genistein in their everyday diet (Patisaul and Jefferson, 2010). The pleiotropic effects of genistein remain controversial. Whereas some laboratories report therapeutic effects of genistein such as chemoprevention, improved bone health, and amelioration of menopausal symptoms (Goldwyn et al., 2000; Evans et al., 2011), other groups emphasize the phytoestrogen's adverse health effects, namely endocrine disruption (Casanova et al., 1999; Jefferson et al., 2005; Wisniewski et al., 2005). In both humans and rats, genistein causes precocious puberty and altered menstrual cycles (Casanova et al., 1999; Strom et al., 2001). Additional adverse effects of genistein in rats include inhibited embryonic development and impaired spatial learning (Ball et al., 2010; Xing et al., 2010).

Bisphenol A [(BPA); 4,4'-isopropylidenediphenol] is a synthetic monomer used in the synthesis of polycarbonate plastics and epoxy resins. BPA is a fairly ubiquitous compound, found as a common

This work was supported by the National Institutes of Health National Institute of Environmental Health Sciences [Grants T32-ES007148, ES05022]; and the Environmental and Occupational Health Sciences Institute.

Coughlin JL (2011) Characterization of the Inhibition of Genistein Glucuronidation by Bisphenol A in Human and Rat Liver Microsomes. Master's thesis, Rutgers University, Piscataway, New Jersey.

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

<http://dx.doi.org/10.1124/dmd.111.042366>.

[S] The online version of this article (available at <http://dmd.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; BPA, bisphenol A; HLM, human liver microsomes; RLM, rat liver microsomes; BPA gluc, bisphenol A β -D-glucuronide; genistein gluc, genistein 4'- β -D-glucuronide; HPLC, high-performance liquid chromatography; SPE, solid-phase extraction; MS/MS, tandem mass spectrometry.

component in plastic baby bottles, as well as food and beverage containers. BPA can leach from plastic containers into contents, especially when containers are exposed to heat and/or excessive wear, placing humans at a direct risk of exposure (Brede et al., 2003). BPA has estrogenic activity both in vitro and in vivo across numerous species (Kuiper et al., 1998; Perez et al., 1998; vom Saal et al., 1998). Endocrine disrupting properties of BPA are routinely reported and include inhibited embryonic development, altered postnatal growth rate, and induction of precocious puberty (Howdeshell et al., 1999; Xing et al., 2010).

The UGT isoforms that are primarily responsible for the glucuronidation of genistein and BPA have been identified. The primary human UGTs implicated in the glucuronidation of genistein are hepatic UGT1A1, UGT1A6, and UGT1A9, as well as extrahepatic UGT1A10 and UGT2B17 (Pritchett et al., 2008; Doerge et al., 2000; Liu et al., 2007; Tang et al., 2009). Although the predominant UGTs responsible for the glucuronidation of genistein in rats have not been definitively identified, they are suspected to be UGT1A1, UGT1A6, and/or UGT1A10, because these isoforms are orthologous to the human UGTs (Tukey and Strassburg, 2000). The predominant UGT responsible for the glucuronidation of BPA in rats is UGT2B1, which is homologous to human UGT2B7 and UGT2B17, neither of which is the primary UGT involved in human BPA glucuronidation (Elsby et al., 2001). Instead, human UGT2B15 is the primary UGT responsible for glucuronidation of BPA in humans (Hanioka et al., 2008a).

Genistein and BPA share common metabolic pathways, with glucuronides being the predominant conjugate of each substrate formed in both human liver microsomes (HLM) and rat liver microsomes (RLM). In addition to similar metabolism, genistein and BPA also exhibit comparable adverse effects of endocrine disruption when individuals are exposed during gestation and/or neonatal development. Humans and rats are routinely exposed to both endocrine disrupting compounds simultaneously in their everyday lives, and interactions between BPA and genistein have been observed both in vitro and in vivo, warranting the analysis of genistein and BPA in a coexposure scenario (Dolinoy et al., 2007; Xing et al., 2010). The goal of the present study was to investigate the inhibitory effect of BPA on the glucuronidation of genistein in vitro during simultaneous exposure to both endocrine disrupting compounds. Hepatic UGT inhibition was investigated in HLM and RLM using binary incubations of BPA and genistein.

Materials and Methods

Chemicals and Reagents. Chemicals were purchased from the sources indicated: BPA, genistein, ammonium acetate, UDPGA, alamesticin, and magnesium chloride (Sigma-Aldrich, St. Louis, MO); BPA β -D-glucuronide (BPA gluc) and genistein 4'- β -D-glucuronide (genistein gluc; TRC Canada, North York, Ontario); ethyl acetate (pesticide grade), methanol (liquid chromatography/mass spectrometry grade), and formic acid (Thermo Fisher Scientific, Waltham, MA); and high-purity solvents acetonitrile and water, both of high-performance liquid chromatography (HPLC) grade (Honeywell Burdick & Jackson, Muskegon, MI). Bond Elut Plexa cartridges (60 mg, 1 ml) were purchased from Varian Inc. (Palo Alto, CA). HLM (pooled from 50 donors, mixed gender) and RLM (pooled from 100 female and 100 male Wistar rats) were purchased from XenoTech, LLC (Lenexa, KS).

Formation of Glucuronide Metabolites. Reagent pool was prepared by mixing HLM (0.0625 mg/ml) or RLM (0.025 mg/ml) with alamesticin (25 μ g/mg protein in incubation), magnesium chloride (4 mM), and Tris-HCl buffer (pH 7.4 at 25°C, 50 mM). Microsomal protein concentrations were optimized through range-finding experiments with varying concentrations of HLM and RLM ranging between 0.5 and 0.025 mg/ml (data not shown). For enzyme kinetics studies, varying concentrations of either BPA or genistein were added (10, 25, 50, 100, 175, and 250 μ M). Equal aliquots of reagent pool were placed in incubation vials (1.5-ml center drain glass vials; Sigma-

Aldrich) and were preincubated in a shaking water bath (80 strokes/min) at 37°C for 15 min. Reactions were initiated by the addition of UDPGA (5 mM), bringing each incubation volume to a total of 200 μ l. After a 30-min incubation period, reactions were terminated via addition of 400 μ l of ice-cold acetonitrile. Duration of incubation was optimized by testing and analyzing total metabolite formation after 0, 15, 30, 45, and 60 min incubations (data not shown). Samples were then centrifuged at 2800 rpm for 20 min, and supernatant was collected for eventual solid-phase extraction (SPE) cleanup and HPLC-tandem mass spectrometry (HPLC-MS/MS) analysis.

Inhibition Studies. Reagent pools were prepared as described previously. To determine K_i , samples contained varying concentrations of genistein (10, 25, 50, 100, 175, and 250 μ M) in the absence (0 μ M) and presence (25 μ M) of BPA. The concentration range 10 to 250 μ M for genistein was chosen because it represented the high end of the environmentally relevant dose while allowing for good quantitative measurement with the available instrumentation. For IC₅₀ determination in HLM, samples contained varying concentrations of BPA (0, 5, 10, 25, 50, 100, and 250 μ M) in the presence of 100 μ M genistein.

Sample Preparation. Analytes were isolated from their matrix using SPE with Bond Elut Plexa cartridges and sequential additions of elution solvents ethyl acetate, methanol, and acetonitrile, as described previously by Coughlin et al. (2011). SPE extracts were evaporated to dryness under vacuum and a stream of nitrogen before being reconstituted in 100 μ l of 50% acetonitrile in water.

Standard Solutions. Initial genistein stock solutions were prepared by dissolving genistein in methanol; BPA, BPA gluc, and genistein gluc were initially dissolved in acetonitrile. All subsequent standards, ranging from 1 to 1000 μ g/ml, were prepared via serial dilution in acetonitrile and were stored at -20°C.

Blank Controls. Two sets of blank controls were used: incubation blanks and SPE blanks. Incubation blank controls contained all of the same reagents and substrates as the samples; however, the blanks were terminated via addition of ice-cold acetonitrile before initiation of the reaction with UDPGA. SPE blank controls contained high purity water in place of standard solutions and were processed alongside samples during SPE using all of the same supplies and reagents. The use of blank controls is especially important when analyzing ubiquitous compounds such as BPA, which is known to be prevalent in numerous laboratory supplies because of its use as a plasticizer (Stiles et al., 2007). The use of plastics was minimized wherever possible, such as using glass incubation vials instead of plastic, in an effort to reduce contamination with BPA.

Analytical Conditions. Analytical conditions were similar to those optimized by Coughlin et al. (2011), with minor modifications. Chromatographic separation was performed with a Discovery C8 column (Supelco, Bellefonte, PA; 50 $\times$ 4.6 mm i.d., 5 μ m) and a Krud Katcher Ultra In-Line Filter guard column (0.5 μ m) (Phenomenex, Torrance, CA). A gradient of solution A (10% acetonitrile in 2 mM ammonium acetate, pH 9) and solution B (acetonitrile) was used as follows: 0 to 3 min 45% B, 3 to 14 min 45 to 89% B, 14 to 18 min 89% B, 18 to 18.2 min 89 to 45% B, and 18.2 to 20 min 45% B.

Data Analyses. All incubations for enzyme activity and inhibition were performed in triplicate for each substrate concentration, and each reconstituted extract was injected into the HPLC-MS/MS in triplicate. Replicate injections were averaged together for each sample. All incubations were performed three times, and reported values represent the overall average of these nine determinations $\pm$ relative S.D. Reported values were blank-subtracted from raw data before calculation of analyte quantity. Michaelis-Menten values of apparent K_m and V_{max} were obtained by analyzing Lineweaver-Burk plots using GraphPad Prism 5.04 (GraphPad Software Inc., San Diego, CA) and were confirmed via manual calculation using the Michaelis-Menten equation:

$$V_0 = \frac{(V_{max}) \times [S]}{(K_m + [S])}$$

where V_{max} represents the maximal reaction rate, $[S]$ represents the substrate concentration, and K_m is the Michaelis constant, which is defined as the substrate concentration at half of the maximal reaction rate. K_i values were found graphically using Lineweaver-Burk plots and were verified via manual calculation using the following equation:

$$K_m \text{ apparent} = \left(1 + \frac{[I]}{K_i}\right) \times K_m$$

where $K_m \text{ apparent}$ is the Michaelis constant in the presence of inhibitor, K_m is the Michaelis constant in the absence of inhibitor, $[I]$ represents the inhibitor concentration, and K_i represents the dissociation constant. IC_{50} values were found graphically on the basis of plots created using GraphPad Prism 5.04. Unpaired Student's t tests were performed using GraphPad Prism 5.04 to determine statistical significance between curves of 0 and 25 μM BPA. Data acquisition was performed using Xcalibur 2.0.7 and were analyzed using Qual Browser 2.0.7 (Thermo Fisher Scientific). Quantitation was calculated in Excel 2003 (Microsoft, Redmond, WA), using manually integrated peak areas using genesis peak integration and 15-point smoothing with Xcalibur 2.0.7.

Results

BPA Glucuronidation Kinetics. BPA glucuronidation kinetics were best modeled using the Michaelis-Menten equation (Fig. 1). When incubated with pooled male and female HLM, the $V_{\max}$ for BPA glucuronidation was determined to be $4.71 \pm 0.30 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, and the K_m was $45.8 \pm 8.9 \mu\text{M}$.

Genistein Glucuronidation Kinetics. The enzyme kinetics of genistein glucuronidation were best fit using the Michaelis-Menten model. With pooled male and female HLM, the mean $V_{\max}$ for genistein glucuronidation was $0.93 \pm 0.10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. When coincubated with 25 μM BPA, the mean $V_{\max}$ for genistein glucuronidation significantly decreased to $0.62 \pm 0.05 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. K_m values remained relatively unchanged between samples incubated with HLM in the absence ($15.1 \pm 7.9 \mu\text{M}$) and presence ($21.5 \pm 7.7 \mu\text{M}$) of 25 μM BPA (Fig. 2). When genistein glucuronidation was performed with pooled male and female RLM, mean $V_{\max}$ values of 2.91 ± 0.26 and $3.05 \pm 0.41 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ were achieved in the absence and presence of 25 μM BPA, respectively. K_m values for genistein glucuronidation by RLM were $49.4 \pm 14 \mu\text{M}$ in the absence of BPA and $84.0 \pm 28 \mu\text{M}$ in the presence of 25 μM BPA (Fig. 3).

Lineweaver-Burk plots were constructed to determine K_i values as well as the type of enzyme inhibition on genistein glucuronidation by BPA. According to the Lineweaver-Burk plots, BPA exerted noncompetitive inhibition for genistein glucuronidation in HLM, with a K_i value of $58.7 \mu\text{M}$, and BPA exerted competitive inhibition for genistein glucuronidation in RLM, with a K_i value of $35.7 \mu\text{M}$ (Figs. 2B and 3B, respectively). Variation in $V_{\max}$, K_m , and K_i values among

FIG. 1. Enzyme kinetic plots of BPA glucuronidation using pooled human liver microsomes. A, Michaelis-Menten plot. B, Lineweaver-Burk plot. $V_{\max} = 4.71 \pm 0.30 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_m = 45.8 \pm 8.9 \mu\text{M}$.

FIG. 2. Enzyme kinetic plots of genistein glucuronidation (pooled data) using pooled HLM in the absence (0 μM ; open circles) and presence (25 μM ; closed circles) of BPA. A, Michaelis-Menten plot. B, Lineweaver-Burk plot. In the absence of BPA, $V_{\max} = 0.93 \pm 0.10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_m = 15.1 \pm 7.9 \mu\text{M}$. In the presence of BPA, $V_{\max} = 0.62 \pm 0.05 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_m = 21.5 \pm 7.7 \mu\text{M}$.

replicates was minimal for both HLM and RLM (Supplemental Figs. 1 through 6).

The effects of varying concentrations of BPA on glucuronidation activity in HLM are shown in Fig. 4. The IC_{50} value of BPA for genistein glucuronidation in HLM was $37.0 \mu\text{M}$.

Discussion

The work presented here shows that BPA inhibits the glucuronidation activity of genistein in both HLM and RLM. The glucuronide is the predominant metabolite of genistein formed in both humans and rats. Inhibition of genistein's glucuronidation, therefore, increases the bioavailability of genistein in the presence of BPA, thus allowing genistein to have a prolonged pharmacological effect. Extended biological action of genistein could exacerbate the compound's adverse effects, especially among populations that are particularly vulnerable to the endocrine disrupting effects of the natural phytoestrogen, such as neonates. Alternatively, metabolism of genistein and BPA could be

FIG. 3. Enzyme kinetic plots of genistein glucuronidation (pooled data) using pooled RLM in the absence (0 μM ; open circles) and presence (25 μM ; closed circles) of BPA. A, Michaelis-Menten plot. B, Lineweaver-Burk plot. In the absence of BPA, $V_{\max} = 2.91 \pm 0.26 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_m = 49.4 \pm 14 \mu\text{M}$. In the presence of BPA, $V_{\max} = 3.05 \pm 0.41 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_m = 84.0 \pm 28 \mu\text{M}$.

FIG. 4. The effects of BPA at concentrations ranging from 0 to 250 μM on the glucuronidation of genistein in pooled male and female HLM. The IC_{50} value of BPA for genistein glucuronidation in HLM is 37.0 μM .

transferred to susceptible extrahepatic organs, further contributing to endocrine disruption. Such an alteration in metabolic capacity is noteworthy, considering that both humans and laboratory rodents are routinely exposed to BPA and genistein simultaneously in their everyday lives (Thigpen et al., 2004; Patisaul and Jefferson, 2010).

It is interesting that the type of UGT enzyme inhibition elicited by BPA is different in HLM and RLM. In HLM, the presence of BPA induced a significant decrease in V_{max} , whereas K_m remained unaffected. These characteristics, along with the Lineweaver-Burk plot for coexposures to genistein and BPA in HLM, are suggestive of non-competitive enzyme inhibition (Fig. 2B). On the other hand, increased K_m values and unchanged V_{max} values in the presence of inhibitor are hallmarks of competitive inhibition. These trends, coupled with the Lineweaver-Burk plots obtained from incubations of genistein and BPA with RLM, suggest competitive inhibition (Fig. 3B).

The disparity in UGT inhibition type caused by BPA between species may be due to a variety of reasons. One possible explanation for the difference in inhibition type of genistein glucuronidation observed between HLM and RLM is that different UGT isoforms may be responsible for genistein's metabolism in the different species. It is reasonable to consider that UGT isoforms are present at different levels in HLM, as they are in RLM, thus contributing to different glucuronidation capacities between the two species. In addition, it has been reported previously that BPA may inhibit the activity of human UGT1A6, which is one of the key isoforms responsible for the metabolism of genistein (Hanioka et al., 2008b). This interaction could be implicated in the altered metabolism of genistein observed during binary exposures in HLM and may help to explain why the glucuronidation rate of BPA appears to be independent of the presence of genistein (previous experiments, data not shown).

Interactions between BPA and genistein and their subsequent biological effects have been reported both in vitro and in vivo. Xing et al. (2010) demonstrated a significant synergistic interaction between BPA and genistein in embryonic development of cultured postimplantation rat embryos when these two compounds are present simultaneously during organogenesis, supporting the hypothesis that BPA and genistein act in a synergistic manner when present simultaneously. Conversely, Dolinoy et al. (2007) report a nonsynergistic effect between the two by demonstrating that although BPA exposure during early stem cell development alters epigenetic patterning in mice, concomitant exposure to genistein, a dietary component that provides methyl donors, negates the DNA hypomethylation effect. The study

described here showed a competitive inhibition in rat and a noncompetitive inhibition of glucuronidation in human microsomal culture. Such contributions have added to our understanding of the effects of simultaneous exposures to BPA and genistein, but more work needs to be done to fully elucidate the true nature and extent of the interactions. Although effects of chemical mixtures are often assumed to be additive, there are likely multiple levels of regulatory interactions between genistein and BPA that may be synergistic or antagonistic depending on the level and species selection. The study described in this article contributes to this growing body of research and highlights the complexity of outcomes that occur with coexposures of these endocrine disrupting compounds.

In vitro analyses offer distinct advantages, but they are also limited in their utility. Xenobiotic incubations with liver microsomes provide a great model for quick screenings of drug-to-drug interactions. Because the majority of predominant UGTs involved in the metabolism of BPA (rat UGT2B1, human UGT2B15) and genistein (UGT1A1, UGT1A6, and UGT1A9) are hepatically expressed, the glucuronidation of genistein in the absence and presence of BPA can be easily assessed using liver microsomes. Overall conjugation pathways, however, cannot be investigated using liver microsomes, because of their inherent limited capacity for sulfation. Finally, studies using liver microsomes exclude the analysis of enzymes that are expressed extrahepatically, whereas in vivo studies allow for the analysis of the contribution of extrahepatic as well as hepatic enzymes in the metabolism of xenobiotics.

Potential human risk from exposures to xenobiotics is often assessed by data generated from animal models. Such a method is often sufficient, because the animal data frequently mirrors human data. In some circumstances, however, data generated from laboratory animals does not directly parallel the effects observed in humans, and alternative models are then required to predict risk to human health. The data presented here offer such an example of an incidence where animal data and human data do not match at the levels of genistein and BPA tested; therefore, human risk assessment for coexposures to genistein and BPA should not necessarily be based solely on data generated from rat models.

Countless xenobiotics share the metabolic pathway of glucuronidation; however, not all xenobiotics exhibit similar biological endpoints and increased susceptibility to adverse effects when exposed during particular windows of exposure. In addition, practical binary exposure scenarios do not abound for all compounds. Genistein and BPA make for a unique mixture of xenobiotics to be investigated simultaneously because of their common metabolic pathways, similar biological effects, and prevalent everyday coexposures.

In conclusion, BPA induced noncompetitive inhibition for genistein glucuronidation in HLM, as well as competitive enzyme inhibition for genistein glucuronidation in RLM. Altered metabolism of genistein in the presence of BPA may affect the phytoestrogen's toxicity and demonstrated the need for analysis of the effect on glucuronidation of the two endocrine disrupting compounds in a binary exposure.

Acknowledgments

We thank Ill Yang for technical assistance and maintenance of the HPLC-MS/MS and Elizabeth McCandlish for helpful discussions.

Authorship Contributions

Participated in research design: Coughlin, Thomas, and Buckley.

Conducted experiments: Coughlin.

Contributed new reagents or analytic tools: Thomas.

Performed data analysis: Coughlin.

Wrote or contributed to the writing of the manuscript: Coughlin and Buckley.

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