Isoflavones at Concentrations Present in Soy Infant Formula Inhibit Rotavirus Infection in Vitro

Aline Andres, Sharon M. Donovan, Theresa B. Kuhlenschmidt, and Mark S. Kuhlenschmidt

Abstract
Rotavirus (RV) infections are a major cause of acute gastroenteritis in children and domestic animals, infecting virtually all children within their first 5 y of life. Infants consuming soy-based infant formula (SBIF) are exposed to high levels of isoflavones that exhibit antiviral activity on numerous viruses in vitro and in vivo. Thus, the hypothesis that isoflavones would inhibit RV infection was tested. All isoflavones at SBIF concentrations were tested individually and as a mixture (MIX). Virus infectivity was assessed in MA-104 cells using a focus forming unit assay. Genistin and MIX significantly reduced RV infectivity by 33–62% and 66–74%, respectively, compared with the control and across a wide range of RV concentrations. When tested without genistin, the MIX lost its anti-RV activity, suggesting that genistin is the biologically active isoflavone in our model. In a dose response assay, genistin significantly reduced RV infectivity at a concentration as low as 30 μmol/L. We investigated several possible mechanisms of action. Isoflavones decreased RV infectivity by modulating virion attachment to the host cells and by modulating a postbinding step. Isoflavones did not alter RV triple-layered structure and genistin did not act through inhibition of protein tyrosine kinases and topoisomerase II or by mimicking the effect of estrogens. To our knowledge, this is the first study showing the inhibition of RV infectivity by isoflavones present in SBIF. The modulation of SBIF isoflavone composition and concentration represents novel nutritional approaches to potentially reduce the severity of RV infection in human and production animals.

Introduction
Rotavirus (RV)

Isoflavones are biologically active compounds that modulate cellular functions (4,5). They are agonists of estrogen receptors and inhibit the activities of protein tyrosine kinases (PTK) and DNA topoisomerase II (6–8). Isoflavones also possess antiviral and antiinflammatory properties and are capable of modulating the immune response of the host. Importantly, the isoflavone genistein has been shown to possess antiviral properties in vitro against herpes simplex virus, bovine herpes virus type 1, bovine viral diarrhea virus, simian virus 40, and Epstein-Barr virus via an inhibition of tyrosine specific phosphorylation (9–13). These in vitro observations were supported by an in vivo study using a porcine model of porcine reproductive and respiratory syndrome viral infection (14). In this study, dietary genistein (13–30.57 mg·kg⁻¹·d⁻¹) reduced viral (P < 0.07) and IFNγ (P < 0.01) serum concentrations and increased α1-acetylglycoprotein serum concentration and spleen weight (P < 0.01), both indicating greater B-cell production. In these virally challenged pigs, genistein acted as an orally active immune modulator. Accordingly, isoflavones may have potential clinical applications in reducing the incidence and severity of RV infection.

Therefore, we investigated the potential effectiveness and underlying cellular and molecular mechanisms whereby dietary
isoflavones would inhibit RV infectivity in vitro. We focused our investigation on physiological isoflavone concentrations present in soy-based infant formulas (SBIF). We hypothesized that isoflavones would possess antiviral properties against RV infection via 2 potential mechanisms: inhibition of RV binding to the MA-104 cells and/or inhibition of intracellular phosphorylation reactions.

**Materials and Methods**

**Cells and virus.** Confluent monolayers of MA-104 cells were grown and maintained as described by Rolsma et al. (15). The MA-104 cell line is derived from embryonic African green monkey kidney cells (BioWhittaker) and is a well-established model to study RV infectivity (16–18). MA-104 cells are routinely used in RV infectivity experiments in vitro, because they support high levels of virus replication and were shown to discriminate between sialic acid- and nonsialic acid-dependent strains of RV. Group A porcine RV strain OSU [P9(7), G5] obtained from the American Type Culture Collection and propagated in MA-104 cells, was purified and cultured as previously described (15). The pH of the medium was monitored with phenol red.

**Chemicals.** Isoflavones (>98% chemical purity) were obtained from LC Laboratories and were diluted in dimethylsulfoxide (DMSO). The major isoflavones present in SBIF in their aglycone (genistein and daidzein), glycoside (genistin, daidzin, and glycitin), and acetylglycoside (acetylgenistin and acetyldaidzin) forms were tested individually and as a mixture (MIX). The concentrations studied were established by analyzing the isoflavone content by HPLC of 4 powdered SBIF (Table 1) in collaboration with Dr. Mark Berhow (USDA, Agriculture Research Service, Peoria, IL).

**Experimental design.** MA-104 cells and/or RV suspensions were treated with DMSO or isoflavones. Figures 1 and 2 and Tables 2 and 4 represent data that were obtained by treating the confluent MA-104 monolayers 24 h prior to and following RV infection with minimal essential medium (MEM) containing isoflavones or DMSO. Figure 3 and Table 3 present data that were obtained by treating the confluent MA-104 cells 24 h prior to and following RV infection as well as the RV suspensions with MEM containing isoflavones or DMSO. RV suspensions were treated for 30 min at room temperature prior to inoculating MA-104 cells.

**Focus forming unit assay.** The peroxidase immunocytochemical detection of virus progeny [focus forming unit (FFU) assay] was performed as previously described (15). Images were captured using a Nikon Eclipse TS100 inverted microscope (Nikon) connected to a SPOT RT camera (Diagnostic Instruments) and a motorized microscope stage (Optiscan, Prior Scientific). Integrated morphometric analysis was performed using Metamorph software (Molecular Devices). The number of foci, which were stained a brown color, was counted. A general fit model for nonlinear regression (GraphPad Prizm, GraphPad software) was used to generate a 50% inhibition (I50) concentration for genistin inhibition of RV infectivity.

**Virus binding assay.** 125I-labeled RV and the binding assay were prepared and performed as previously described (15). Confluent monolayers were treated with isoflavones or DMSO at concentrations described in Table 1 for 24 h prior to harvesting. Radioactivity was enumerated using a gamma counter. Sialyllactosyl-phosphatidylethanolamine (ganglioside-like RV receptor mimetic at 100 μmol/L) was used as a positive control for inhibition (M. S. Kuhlenschmidt, unpublished data).

**Transmission electron microscopy.** Transmission electron microscopy (TEM) analysis was performed by the Center for Microscopic Imaging (College of Veterinary Medicine). Briefly, RV suspensions in serum-free MEM were treated for 30 min at room temperature with either DMSO or MIX before being placed on ice and fixed with 2 drops of PBS Karnovsky’s fixative (2% glutaraldehyde, 2.5% paraformaldehyde) for 20 min. Samples were then stained with normal negative stain.

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**TABLE 1** Concentration of isoflavones in 4 powdered SBIF

<table>
<thead>
<tr>
<th></th>
<th>Carnation followup¹</th>
<th>Isomil²</th>
<th>ProSobee³</th>
<th>Alsoy¹</th>
<th>Mean μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistin</td>
<td>18.20</td>
<td>12.24</td>
<td>9.75</td>
<td>16.44</td>
<td>14.16</td>
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<td>Genistein</td>
<td>1.54</td>
<td>1.19</td>
<td>0.37</td>
<td>0.53</td>
<td>0.90</td>
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<tr>
<td>Acetylgensinin</td>
<td>9.56</td>
<td>5.78</td>
<td>6.78</td>
<td>8.30</td>
<td>7.61</td>
</tr>
<tr>
<td>Daidzin</td>
<td>10.77</td>
<td>7.92</td>
<td>5.66</td>
<td>8.07</td>
<td>8.10</td>
</tr>
<tr>
<td>Daidzin</td>
<td>3.75</td>
<td>2.20</td>
<td>1.60</td>
<td>3.07</td>
<td>2.65</td>
</tr>
<tr>
<td>Acetyldaidzin</td>
<td>11.67</td>
<td>5.85</td>
<td>5.82</td>
<td>7.94</td>
<td>7.82</td>
</tr>
<tr>
<td>Glycitin</td>
<td>2.22</td>
<td>1.98</td>
<td>1.27</td>
<td>1.03</td>
<td>1.59</td>
</tr>
<tr>
<td>Total isoflavones (MIX⁴)</td>
<td>57.70</td>
<td>37.03</td>
<td>31.24</td>
<td>45.38</td>
<td>42.84</td>
</tr>
</tbody>
</table>

¹ Nestlé.
² Ross Laboratories.
³ Mead Johnson.
⁴ Total isoflavones (MIX) is only expressed in milligrams per liter.

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**FIGURE 1** Effect of isoflavones at concentrations present in SBIF on RV infectivity in MA-104 cells. Data are expressed in percent of control as means ± SEM, n = 6–10. Means without a common letter differ, P ≤ 0.05.
protocol using 2% ammonium molybdate at pH 6. Samples were counted on the TEM scope (Hitachi H600, Hitachi) at 25× magnification. Results were expressed as a percentage of fully shelled particles per total particles.

Statistical analysis. Data are reported as means ± SEM and unless stated otherwise are expressed as a percentage of the control group. Statistical analyses were performed using 1-way ANOVA under the MIXED procedure (SAS, SAS Institute) with a post hoc least significant difference test to evaluate the main effect of each isoflavone and the MIX (25). A 2-way ANOVA under the MIXED procedure was performed to determine the effect of isoflavones and time or concentration and their interactions. A random term was introduced in the model to account for interexperimental variations. Probabilities for all pairwise differences were computed with the PDIF option. Statistical significance was set at $P \leq 0.05$.

Results

Isoflavones were initially tested at the mean concentrations present in SBIF (Table 1) for their effects on RV infectivity using the FFU assay. Only genistin and the MIX significantly reduced RV infectivity by 34.4 ± 7.1% and 41.3 ± 3.5%, respectively, compared with the DMSO control. No other isoflavone inhibited RV infectivity in our model. Daidzein significantly increased RV infectivity by 62.0 ± 24.7% compared with the control (Fig. 1). To determine whether genistin was the active anti-RV isoflavone, the MIX was tested without genistin. The MIX (28.71 mg/L) devoid of genistin did not significantly reduce RV infectivity, whereas genistin and the MIX significantly reduced RV infectivity compared with the control and were significantly different from each other (Table 2).

To investigate whether the timing of exposure of MA-104 cells to isoflavones was important, genistin and MIX treatments

### Table 2 Genistin is necessary for the MIX of isoflavones to inhibit RV infectivity in MA-104 cells

<table>
<thead>
<tr>
<th>Isoflavones concentrations</th>
<th>% Control</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 4.3*</td>
</tr>
<tr>
<td>MIX (42.87 mg/L)</td>
<td>39.8 ± 1.3*</td>
</tr>
<tr>
<td>Genistin (14.16 mg/L)</td>
<td>56.4 ± 3.9*</td>
</tr>
<tr>
<td>MIX without genistin (28.71 mg/L)</td>
<td>88.6 ± 4.7*</td>
</tr>
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</table>

*Values are means ± SEM, $n = 5$ or 6.

*Means without a common letter differ, $P \leq 0.05$.

The MIX, the MA-104 cells that were exposed to the MIX were exposed to higher genistein concentrations than when genistin or genistein were tested individually. Indeed, if all genistein forms were converted to genistin, the MA-104 cells were exposed to 52 μmol/L genistein equivalents, which correspond to genistin (32.7 μmol/L genistein equivalent), genistein (3.4 μmol/L genistein equivalent), and acetylgensistein (16 μmol/L genistin equivalent) added together. Thus, all genistein forms were evaluated at the same molar concentration as the total genistein equivalents in SBIF (52 μmol/L). The MIX, genistin, genistein, and acetylgensistein at 52 μmol/L significantly decreased RV infectivity to a similar degree and did not differ from each other (39.4 ± 4.1, 29.5 ± 5.8, 40.1 ± 6.4, 48.5 ± 7.5% inhibition compared with the control, respectively).

To delineate the potential underlying mechanisms of action whereby isoflavones reduced RV infectivity, 3 approaches were
The combination of genistin, genistein, and acetylgenistin also increased RV infectivity by 49.0%. No other isoflavone inhibited RV binding activity (Fig. 4). As evidenced by TEM, isoflavones present in MIX did not affect the triple-layered structure of RV particles, suggesting that isoflavones reduce RV infectivity via mechanisms other than virion outer capsid dissociation.

Second, the binding capacity of RV particles to MA-104 cells was tested using 125I-radiolabeled RV. Genistin and MIX reduced RV binding to MA-104 cells by 28.9% ± 5.5% and 54.3% ± 8.5%, respectively, compared with the DMSO control (P < 0.05). No other isoflavone inhibited RV binding activity (Fig. 5). The combination of genistin, genistein, and acetylgenistin also significantly reduced RV binding by 58.9% ± 5.9%, which was not significantly different from the MIX (data not shown). Sialyllactosyl-phosphatidyethanolamine, a synthetic RV virus receptor-mimetic known as a virus binding competitor, decreased RV infectivity by 49% ± 0.6% compared with the control.

Third, we investigated whether a PTK inhibitor (herbimycin A), DNA topoisomerase II inhibitor (etoposide), or estrogen receptor agonist (17β-estradiol) modulated RV infectivity using the FFU assay. None of the compounds tested reduced RV infectivity, except genistin (32.7 μmol/L), which significantly decreased RV infectivity compared with the control (Table 4).

### Discussion

RV remains the most common cause of diarrheal disease worldwide, affecting virtually all children by the age of 5 (1,2). Nutritional intervention is 1 approach to reduce the burden of this disease. In this study, we focused on isoflavones, because previous studies have demonstrated that they exert antiviral activity both in vitro (9–13) and in vivo (14). In addition, soy isoflavones are present at high concentrations in SBIF consumed by ~25% of formula-fed infants in the US (27,28), representing potential natural dietary anti-RV agents. Herein, we evaluated the ability of purified isoflavones at concentrations present in SBIF to inhibit RV infectivity using MA-104 cells, a well-established in vitro assay. We hypothesized that isoflavones will act via 2 potential mechanisms: inhibition of RV binding to the cells and/or inhibition of PTK activity.

Only genistin and the MIX significantly reduced RV infectivity over a 16-fold range of RV concentrations compared with the control. The specificity of this effect was demonstrated when MIX without genistin did not inhibit RV infectivity. These data reveal for the first time, to our knowledge, that genistin possesses anti-RV properties and that both genistin and MIX are effective along a continuum of RV infection severity. These results are in agreement with Shaneyfelt et al. (29), who recently identified the potential for genistein to inhibit RV infectivity in a cell-based moderate-throughput screening assay using the bovine RV Nebraska calf diarrhea virus strain.

In a dose response assay, genistin reduced RV infectivity in the concentration range of 30–100 μmol/L. This effective range includes the typical genistin concentrations (~22–42 μmol/L) that infants consuming SBIF are exposed to on a daily basis (27; Table 1). To determine whether this anti-RV effect was influenced by the chemical form of genistein, all 3 forms present [

### Table 3

<table>
<thead>
<tr>
<th>FFU across RV concentrations, ng/100 μL</th>
<th>% Control</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 13.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Genistin (14.16 μg/mL)</td>
<td>55.9 ± 5.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MIX (42.87 mg/mL)</td>
<td>33.9 ± 4.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± SEM, n = 4.

<sup>b</sup> Means without a common letter differ, P < 0.05.

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**FIGURE 4** Integrity of RV particles. TEM pictures of RV particles treated with either DMSO (control) or the MIX (42.87 mg/L).

**FIGURE 5** Isoflavones, at concentrations present in SBIF (Table 1), partially inhibit RV binding to MA-104 cells. Sialyllactosyl-phosphatidylethanolamine (100 μmol/L) was used as a positive control. Data are expressed in percent of control as means ± SEM, n = 6. Means without a common letter differ, P < 0.05.
in SBIF were tested at 52 μmol/L, which corresponds to the total genistein equivalent present in SBIF. At this concentration, genistein, genistin, and acetylgenistin decreased RV infectivity to a similar degree, which was not significantly different from each other or the MIX. These results suggest that genistin in any form (aglycone, glycoside, or acetylglycoside) possesses anti-RV properties, which further implies that MA-104 cells possess the capacity to convert the glycoside and acetylglycoside forms of genistin into the aglycone, biologically active form, or that the region of the genistein molecule that is bioactive against RV is not impacted by the presence of the side chain. Moreover, these data explain the observed differences in anti-RV efficacy of genistin alone vs. the MIX. These results are in accordance with Greiner et al. (14,30), who found that dietary genistein but not daidzein reduced the severity of porcine reproductive and respiratory syndrome virus infection in swine.

The mechanisms of action whereby isoflavones inhibited RV infectivity were then investigated. First, the effect of timing of exposure to isoflavones relative to the RV infection was examined. MA-104 cells were exposed to isoflavones before and/or after infection with RV suspensions that were also exposed to isoflavones or DMSO prior to infection. In all scenarios, the MIX significantly decreased RV infectivity. Treatment of cells with genistin only before infection reduced RV infectivity to a level that was intermediate between control and treatment with isoflavones only after infection. Thus, isoflavones could modulate RV infectivity by acting on a binding or a postbinding event, which could include entry into the cell or viral replication.

To rule out a direct effect of isoflavones on RV structure, RV particle integrity was assessed by TEM following exposure to isoflavones. The physical structure of RV particles was unaffected by genistin or MIX. Although we cannot rule out an effect of isoflavones on the outer layer of RV particles at the molecular level, these data revealed that isoflavones were not directly disrupting the three-layered structure of RV that is essential for its infectivity properties.

Next, the impact of isoflavones on binding of RV to host cells was investigated. The pattern of inhibition of RV binding by isoflavones was similar to the inhibition of RV infectivity, supporting modulation of RV binding by genistin and the MIX. Kvistgaard et al. (31) previously demonstrated the modulation of RV binding by human milk lactadherin resulting in a reduction of RV infectivity. The inhibition of RV binding to host cells by isoflavones is most likely mediated by an inhibition of PTK-induced activation of a2p1 binding to RV, as suggested by Graham et al. (32). In fact, inhibitors of protein kinase A and p38 mitogen-activated protein kinase have already been shown to decrease RV infection by 50 and 40%, respectively, in vitro (33). To test that hypothesis, we utilized herbimycin A, which is a well-described PTK inhibitor, over a range of concentrations (22,23). Herbimycin A, at the concentrations tested herein, did not inhibit RV infectivity. Although herbimycin A has been previously used to mimic genistein effects (22,23), work by Gozlan et al. (34) on HIV-1 virus found that herbimycin A did not replicate the effect of genistein on virus replication. Ongoing investigation in our laboratory will determine whether isoflavones inhibit RV infectivity via inhibition of PTK phosphorylation or via another mechanism of action. In addition to the inhibitor of PTK, we also investigated the effect of etoposide and 17β-estradiol on RV infectivity. Neither compound affected RV infectivity, suggesting that isoflavones do not act via an inhibition of DNA topoisomerase II or by mimicking estrogen. In summary, genistin or a MIX of isoflavones inhibited RV infectivity. More importantly, this inhibition occurred at physiological concentrations and across a continuum of RV concentrations in vitro, which suggest that their anti-RV properties could protect against a range of RV infection severities. The mechanisms of action appear to occur by reducing RV binding to the cells and by acting at a postbinding step, which remains to be determined.

There are currently no epidemiological data on the incidence of RV infection between infants fed cow’s milk-based or SBIF. In light of these results, epidemiological studies may be warranted to evaluate the effect of SBIF on the incidence of RV infection in formula-fed infants. A lactose-free, soy-based formula reduced the duration and severity of diarrhea in infants with prolonged and acute gastroenteritis compared with infants fed a cow’s milk-based infant formula containing lactose (36–38). However, in these studies, the potential effects of soy protein or soy isoflavones could not be separated from the impact of removing lactose from the diet. In addition, feeding a soy formula supplemented with soy polysaccharides reduced diarrhea duration by 13 h in outpatients and by 120 h in hospitalized patients compared with feeding a fiber-free soy formula (39,40). However, no cow’s milk formula-fed control group was included in either of these studies. Thus, future studies are warranted to evaluate the in vivo efficacy of isoflavones alone and within the complex matrix of a SBIF on RV infectivity in neonates. We have previously shown that dietary genistein at a concentration present in SBIF was bioactive within the piglet intestine (35); thus, our laboratory is currently investigating this question using the neonatal piglet model.

Acknowledgments

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Literature Cited
