Baicalin Inhibits the Lethality of Shiga-Like Toxin 2 in Mice

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Shiga-like toxins (Stxs), produced by pathogenic Escherichia coli, are a major virulence factor involved in severe diseases in human and animals. These toxins are ribosome-inactivating proteins, and treatment for diseases caused by them is not available. Therefore, there is an urgent need for agents capable of effectively targeting this lethal toxin. In this study, we identified baicalin, a flavonoid compound used in Chinese traditional medicine, as a compound against Shiga-like toxin 2 (Stx2). We found that baicalin significantly improves renal function and reduces Stx2-induced lethality in mice. Further experiments revealed that baicalin induces the formation of oligomers by the toxin by direct binding. We also identified the residues important for such interactions and analyzed their roles in binding baicalin by biophysical and biochemical analyses. Our results establish baicalin as a candidate compound for the development of therapeutics against diseases caused by Stxs.

Materials and Methods

Chemicals and reagents. Baicalin (BAI; purity, 98.5%) (Fig. 1A) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and the stock solution at a concentration of 40.96 mg/ml was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). For in vitro studies, BAI was dissolved in sterile phosphate-buffered saline (PBS) at a concentration of 100 mg/ml.

Expression and purification of Stx2. (i) Construction of plasmid encoding Stx2. The recombinant plasmid was constructed as described previously (21). Briefly, the E. coli stx2 gene, coding for the mature Stx2 protein, was amplified from the genome of E. coli O157:H7 strain ATCC 43895 with the primer pair 5′-CCGGAATTCACTAGTTGATATTATTATAATG-3′ (forward) and 5′-ACCGCTCGACCTAGTGGTGCCTGTTGGGTGCATATTAAACTGCACTTC-3′ (reverse). An EcoRI site and a SalI site (underlined) were added to the 5′ and 3′ ends of the primers and used for subcloning of the gene into the expression vector pET32a.

Received 15 June 2015 Returned for modification 10 July 2015 Accepted 2 September 2015


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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.01416-15.
Cleared by centrifugation at 16,000 g. bovine serum, at 37°C in 5% CO2 in a humidified incubator. Cells for penicillin and 100 U/ml streptomycin), and 10% heat-inactivated fetal rStx2, and the expression of luciferase was measured. All data are means and standard errors from three independent experiments. *, P < 0.05; **, P < 0.01.

(ii) Procedure of protein expression and purification. Cells of strain BL21(DE3)(pET32a-stx2) grown in LB to saturation were diluted 1:100 into 1,000 ml of LB broth with 0.08% glucose. Protein expression was induced with 1 mM IPTG when the optical density at 600 nm (OD600) of the culture reached 0.6 to 0.8 for 30 min at 4°C. Cells harvested by centrifugation at 3,800 × g for 30 min at 4°C were suspended in lysis buffer (1X PBS, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and broken by sonication. The lysates were cleared by centrifugation at 16,000 × g for 40 min at 4°C. The supernatant was loaded onto a self-packed nickel-nitrilotriacetic acid (Ni-NTA) affinity column (2 ml Ni-NTA Hi-Bind resin; Qiagen). Unbound proteins were removed by washing with 10 column volumes of washing buffer (lysis buffer containing 60 mM imidazole). The protein was eluted with 200 mM imidazole in lysis buffer. The protein was further purified with a Superdex 75 16/60 column (GE Healthcare) on an AKTA system. The purity of all proteins was greater than 95%, as assessed by SDS-PAGE and Coomassie bright blue staining.

Cell-free translation assay. The TNT coupled reticulocyte lysate systems and luciferase assay system (Promega, Madison, WI) were employed to investigate the ability of BAI to block protein synthesis inhibition imposed by rStx2 (22). One nanogram of rStx2 was used in all reactions, and the concentration of BAI ranged from 9 μM to 72 μM. The reaction was allowed to proceed for 90 min at 30°C in a water bath and was terminated by placing the plates on ice. Luciferase activity was measured per the manufacturer’s instructions.

Cell-based assay for protection against rStx2. We examined the effects of BAI on cell toxicity of Stx2 with HeLa cells as described previously (23). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 3 mM glutamine, antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), and 10% heat-inactivated fetal bovine serum, at 37°C in 5% CO2 in a humidified incubator. Cells for experiments were plated on a 96-well plate at a density of 1.5 × 104 cells per well in DMEM and incubated for 16 h at 37°C in 5% CO2.

The cells were incubated with 50 pg/ml rStx2 (capable of killing about 80% cells) (see Table S1 in the supplemental material) in the presence of BAI at different concentrations for 72 h at 37°C. Samples without BAI or toxin were established as controls. Cytotoxicity was evaluated by measuring lactate dehydrogenase (LDH) release using the cytotoxicity detection kit (LDH) (Roche, Basel, Switzerland) according to the manufacturer’s directions. Plates were read on a microplate reader (Tecan, Austria) at 490 nm.

Ethics statement. All animal studies were performed according to the Regulations for the Administration of Affairs Concerning Experiments Animals (1988.11). The experimental protocols were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) of Jilin University.

Mice injection protocols. Assays for lethality, clinical chemistry, histopathology, and cytokine secretion were performed to detect the ability of BAI to protect mice from rStx2. Eight-week-old BALB/c mice weighing 16 to 18 g were obtained from the Experimental Animal Center of Jilin University (Changchun, China). Mice were injected intraperitoneally with 100 μl of either sterile PBS or various amounts of rStx2. For mortality studies, each mouse was injected with a single dose of rStx2 at 100 ng (about twice the dose required for killing 50% of the animals [LD50]) (see Table S2 in the supplemental material); for other studies, mice were administered a single dose of rStx2 (50 ng per mouse). For mortality studies, the infected mice were treated with 100 mg BAI/kg of body weight subcutaneously 6 h after injection and then at 6-h intervals for a total of 6 days. Mice were weighed at 24-h intervals for 8 days after receiving all the treatments.

Hematology and clinical chemistry analysis. For blood studies, mice were euthanized and blood was collected 72 h after toxin injection. For blood urea nitrogen (BUN) and serum creatinine (Cr) level analysis, blood samples were coagulated for 10 min at 37°C and then were centri-
FIG 2 Baicalin protects mice against lethality induced by rStx2. (A) Groups (n = 10) of mice injected with rStx2 were treated with PBS solution or with BAI 6 h after toxin injection, and survival of mice was monitored for 6 days. The death of mice was followed up to 6 days after rStx2 injection, with no additional mortalities shown in the figure. The curves for BAI-treated mice are statistically significantly different from those for rStx2-injected mice, as evaluated by the log rank test (P = 0.0002 for 100 mg/kg of BAI against rStx2). (B) Effects of BAI on body weight caused by sublethal doses of rStx2. Groups (n = 10) of mice receiving Stx2 or a control solution were administered BAI or PBS 6 h after toxin injection. The body weight was monitored at 24-h intervals for 8 days. Similar results were obtained from more than three independent experiments. *, P < 0.05 for rStx2 versus rStx2 plus BAI and PBS at day 6; **, P < 0.01 for rStx2 versus PBS at day 7 and for rStx2 versus rStx plus BAI and PBS at day 8.

fuged at 2,900 × g for 10 min at 4°C to obtain serum. BUN and Cr levels were determined with a Hitachi 7600 biochemical analyzer. For hemoglobin level determinations, 100 μl serum was used to obtain an absorbance from 300 nm to 600 nm with a spectrophotometer.

Histopathology analysis. Mice receiving the different treatments were anesthetized and sacrificed by cervical dislocation 72 h after rStx2 injection. Kidneys were removed and fixed in 1% formalin. Fixed kidneys were then stained with hematoxylin and eosin (H&E) and were visualized by light microscopy.

Cytokine detection in kidneys. Kidneys were dissected from mice treated with the different regimens 72 h after toxin injection. Kidney tissues were homogenized in PBS buffer. After centrifugation, the supernatants were collected and stored at −20°C until analysis. Cytokine levels were measured using an enzyme-linked immunosorbent assay (ELISA) by specific mouse ELISA kits (BioLegend, CA).

Cell-free translation assays for mutant proteins. Cell-free translation assays were performed as described above. Briefly, 1 ng rStx2 was mixed with BAI at a concentration of 36 μM, and the mixture was then added to the reaction system according to the manufacturer’s directions. After incubation for 90 min at 30°C in a water bath, the reaction was terminated by placing the plates on ice and analyzed by the luciferase assay system.

Statistical analysis. The experimental data were assessed using independent Student’s t test with SPSS 14.0 statistical software (SPSS Inc., Chicago, IL), and a P value less than 0.05 was considered statistically significant.

RESULTS

BAI protects against rStx2-induced cytotoxicity. BAI is able to neutralize the toxicity of ricin (20). Because Stxs are also ribosome-inactivating enzymes, we first evaluated whether BAI is able to protect cultured cells caused by Stx2. To this end, we incubated various amounts of BAI with HeLa cells treated with 50 pg/ml rStx2 and evaluated the integrity of cell membranes by measuring LDH release. In samples from mice that did not receive BAI, 82.96% LDH release was detected (Fig. 1B). Significant protection was detected when BAI was used at 4.5 μM, and maximal protection was achieved with 36 μM under our experimental conditions (Fig. 1B).

Similar protection was achieved when BAI was mixed with rStx2 30 min prior to addition to the cells under the same experimental conditions. Again, 4.5 μM BAI was able to significantly protect HeLa cells, and maximal protection was achieved when the concentration used was 36 μM (Fig. 1C). These results suggest that BAI is able to block the activity of the toxin, and this inhibition likely occurs by direct interactions.

Stxs induce cell death by inhibiting protein synthesis. We thus employed the cell-free translation assay to evaluate the effects of BAI on the inhibition of ribosomal activity. Under our experimental conditions, 36 μM BAI significant rescued protein synthesis in reactions received 1 ng rStx2 (Fig. 1D). Taken together, these results establish that BAI is able to neutralize the cytotoxicity of rStx2 in vitro.

BAI significantly reduces rStx2-induced lethality in mice. The strong protective effects of BAI against rStx2-induced cytotoxicity in in vitro systems prompted us to explore its potential usefulness in therapeutics. To this end, we employed the mouse model for hemolytic-uremic syndrome (HUS) (9). Under our experimental conditions, a single dose (~2 LD50, 100 ng per mouse) of rStx2 caused 100% animal deaths within 6 days (Fig. 2A). Mice receiving rStx2 alone showed tremors and ataxia 2 days postinjection, and death began to occur on the third day (Fig. 2A). In mice receiving BAI subcutaneously at 6 h postexposure and subsequently at 6-hour intervals for a total of 23 doses, all at 100 mg/kg, the death rate at day 6 was about 30% (Fig. 2A), indicating that this compound can provide approximately 70% protection.

We further evaluated the ability of BAI to protect against the weight loss caused by sublethal doses of rStx2 (9). BAI alone, even at high doses, does not detectably affect mouse body weight over 8 days (24). In our experiments, mice injected with PBS or a single dose of rStx2 (50 ng per mouse) were monitored for body weight at 24-h intervals. In the treated group, 100 mg/kg BAI was administered 6 h after toxin injection and subsequently at 6-h intervals for 8 days. Untreated mice in the group injected with the toxin exhibited decreased food and water intake, body weight loss became apparent on day 3, and weights reached the lowest point at day 6 (Fig. 2B). In agreement with the lethality protection results, BAI treatment delayed the occurrence of body weight loss for 24 h; further, the severity of the loss was significantly lower throughout the experiment (Fig. 2B). As expected, consistent with their normal health status, the body weights of control mice receiving PBS displayed detectable increases throughout the experiment (Fig. 2B).

Treatment with BAI improves renal function and blood pathology induced by rStx2. Severe renal damage is another symp-
tom associated with Stxs toxicity (5); we thus performed a pathological study to evaluate whether BAI treatment can alleviate the manifestation of renal injury. Mice injected with 50 ng rStx2 were administered BAI at 100 mg/kg or with a PBS buffer 6 h after receiving the toxin and subsequently at 6-h intervals as described above. Kidneys from mice in the different treatment groups were sectioned for pathological examination 72 h after toxin injection. In mice not receiving BAI treatment, significant damage was observed in tubules, characterized by swelling and casts in the lumen; the glomerulus also appeared to be hyperemic and smaller (Fig. 3A). Such renal injury was not observed in kidneys from mice treated with BAI after toxin challenge, which was similar to those from control animals not receiving the toxin (Fig. 3A).

To further determine kidney function after BAI treatment, we examined levels of blood urea nitrogen (BUN) and creatinine (Cr), the two important indicators of kidney function, 72 h after injection (9). The BUN level in mice receiving rStx2 was 6.56 ± 0.32 mmol/liter. BAI treatment significantly reduced the BUN level (4.56 ± 0.28 mmol/liter). In fact, this level is comparable to the 4.53 ± 0.43-mmol/liter concentration observed in control mice that were not injected with the toxin (Fig. 3B). Similar results were obtained when Cr was measured (Fig. 3C). Significantly higher levels of hemoglobin were detected at 72 h in the serum of untreated mice that had been injected with the toxin; similarly, treatment with BAI led to reduction of hemolysis (Fig. 3D).

We also examined the levels of several relevant cytokines to determine whether BAI reduces inflammation accompanied by kidney damage. As expected, rStx2 caused elevated levels of interleukin 1β (IL-1β), IL-4, IL-6, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) (Fig. 3E). Consistent with its effect in protecting kidney damage caused by Stx2, the levels of these cytokines significantly decreased in mice treated with BAI at 72 h after injection (Fig. 3E). Taken together, these results indicate that BAI is able to effectively reduce the damage to the kidney caused by rStx2.
BAI inhibits rStx2 activity by directly interacting with the toxin to induce protein oligomerization. Our results from both in vitro and in vivo experiments suggest that BAI exerts its protective activity by targeting rStx2 directly. Our previous study revealed that BAI inhibits the activity of ricin, another RIP, by inducing the A subunit of ricin (RTA) to form enzymatically less active oligomers (20). Given the fact that the activities of RTA and Stx2a are highly similar and that these two toxins share high-level resemblance in their structures (Fig. 4), we attempted to identify residues on Stx2 potentially involved in binding baicalin. Comparative structural analysis allowed us to identify R179, Q180, S183, E184, and V218 as candidate residues involved in the binding of Stx2 to BAI (Fig. 5). We thus replaced each of these sites with an alanine residue and studied the relevant phenotypes associated with the mutant proteins. In HeLa cells, only the Q180A mutant lost considerable toxicity (Fig. 6A), and it was still sensitive to BAI. The S183A mutant maintained toxicity but was sensitive to BAI (Fig. 6B). In contrast, the R179A, E184A, and V218A mutants retained toxicity similar to that of wild-type rStx2 (Fig. 6B). Importantly, all these mutations abolished or significantly reduced the sensitivity of rStx2 to BAI (Fig. 6B), suggesting the involvement of these residues in engaging BAI. Taken together, these results suggest that BAI interacts with Stx2a directly, probably by

**FIG 4** Overlay of structures of ricin subunit A (cyan) and Shiga-like toxin 2 subunit A1 (purple). The crystal structures of RTA and Stx2A1 are displayed in ribbon representation, with coils for α-helices and arrows for β-strands. BAI is superimposed in stick mode, with carbon and oxygen atoms in yellow and red, respectively.

**FIG 5** Structure-based sequence alignment of the A subunit of ricin and the A1 subunit of Shiga-like toxin 2. Identical residues are highlighted in red, and similar residues are shown in red font. Secondary structures are shown for both proteins above and below their respective sequences, with coils representing α/β-helices and arrows representing β-strands.
The toxin 2 (Stx2) is the primary virulence factor for HUS (30). Stx2 is linked to severe diseases caused by STEC infections, and Shiga-like toxins (Stxs) are the key virulence factor associated with hemolytic uremic syndrome. Epidemiol Rev 13:60–98.

**DISCUSSION**

Pathogenic *E. coli* is one of the most common pathogens that often is contracted from contaminated food and water as well as animal and human effluents (25). Shiga-like toxin (Stx)-producing *E. coli* (STEC), particularly enterohemorrhagic *E. coli* (EHEC) strains such as O157:H7, are a leading cause of outbreaks and sporadic cases of bloody diarrhea and hemolytic-uremic syndrome (HUS) (26). These infections pose grave challenges to public health. In the United States, 265,000 STEC infections occur each year, of which about 36% are caused by EHEC O157:H7. Although antibiotics can be used when oligoanuric HUS is established, the wide spread of resistance has threatened the effectiveness of such treatments (6, 27). Furthermore, in some cases, the problem is further compounded by the fact that conventional treatments such as antibiotics aggravate symptoms (5, 28). Thus, there is an urgent need for agents complementary to antibiotics that are clinically effective in treating diseases caused by these pathogens. With better understanding of how bacteria cause diseases, some new approaches based on our understanding of virulence mechanisms have been established in recent years (29). Among these, agents that target virulence factors hold great promise.

Shiga-like toxins (Stxs) are the key virulence factor associated with severe diseases caused by STEC infections, and Shiga-like toxin 2 (Stx2) is the primary virulence factor for HUS (30). Stx2 thus has been recommended as a potent target for drug development (15). Recently, several excellent reports described the identification of novel small-molecule inhibitors and the development of specific antibodies against Stx2. Significant achievements have been made in therapies against STEC infections using specific neutralizing monoclonal antibodies, and several antibodies have been tested in phase I clinical trials (14, 25, 31). Small-molecule inhibitors presumably have some advantages over neutralizing antibodies, such as having a lower cost, being relatively easier to store and transport among different locations, and being less likely to be contaminated. To date, only two small-molecule inhibitors for Stx2 toxicity have been described. These compounds, called Retro-1 and Retro-2, have been shown to protect mice from ricin- and Stx1-induced lethality (18). However, to achieve the protection, the animals need to receive these compounds at least 1 h prior to toxin exposure (18). Clearly, these inhibitors are not appropriate for therapeutic purposes, where the treatment of infection or toxin exposure is the focus. Furthermore, these two compounds function by targeting the retrograde trafficking of the host cells (18), the potential side effects of which are unknown.

Our identification of baicalin (BAI) as an inhibitor for the activity of Stx2 and the observation that this compound at a low concentration is able to significantly protect mice from toxin-induced mortality after toxin exposure represent a significant step forward in this research direction. The successful use of the structure information of the ricin–BAI complex to identify the key residues on Stx2 important for its interactions with BAI suggests that BAI targets Stx2 with a similar mechanism, namely, induction of protein oligomerization to occlude its active site (20). These results also suggest that chemical modification of BAI to obtain molecules with higher affinity for the toxins is feasible and clearly is a direction worth pursuing in future research. Moreover, BAI is reported as a safe agent in mice; the dosage can reach 15 g/kg of body weight when administered intragastrically and 2.74 g/kg of body weight when injected intraperitoneally (24, 32).

Given the similarity in the mechanism of action between ricin and Stxs, the activity of BAI against Stx2 is not completely unexpected. Because toxin released from dead bacteria killed by antibiotics can potentially compound the disease symptoms, agents that target the toxin directly will be very beneficial. It will be interesting to determine how a combination of antibiotics and BAI functions in the treatment of HUS and other infectious diseases caused by Stx toxin-producing pathogens. Because BAI directly targets the toxin released by the bacteria killed by the antibiotics, such a treatment regimen may be more efficacious than BAI administered alone.

**ACKNOWLEDGMENTS**

This work was supported by the National Basic Research Program of China (grant 2013CB127205 to X.D.), the National Nature Science Foundation of China (grant 31130053 to X.D.), and the National 863 program (grant 2012AA020303).

We declare that no competing interests exist.

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