Treatment with Z-Ligustilide, a Component of Angelica sinensis, Reduces Brain Injury after a Subarachnoid Hemorrhage in Rats

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ABSTRACT
Subarachnoid hemorrhage (SAH) is a devastating stroke subtype accounting for approximately 3 to 7% of cases each year. Despite its rarity among the various stroke types, SAH is still responsible for approximately 25% of all stroke fatalities. Although various preventative and therapeutic interventions have been explored for potential neuroprotection after SAH, a considerable percentage of patients still experience serious neurologic and/or cognitive impairments as a result of the primary hemorrhage and/or secondary brain damage that occurs. Z-ligustilide (LIG), the primary lipophilic component of the Chinese traditional medicine Radix Angelica sinensis, has been shown to reduce ischemic brain injury via antiapoptotic pathways. Accordingly, in our study, we investigated the neuroprotective potential of LIG after experimental SAH in rats. Rats with SAH that was induced using the established double hemorrhage model were studied with and without LIG treatment. Mortality, neurobehavioral evaluation, brain water content, blood-brain barrier (BBB) permeability, and vasospasm assessment of the basilar artery were measured on days 3 and 7 after injury. Additional testing was done to evaluate for apoptosis using TdT-mediated dUTP-biotin nick end labeling staining as well as immunohistochemistry and Western blotting to identify key proapoptotic/survival proteins, i.e., p53, Bax, Bcl-2, and cleaved caspase-3. The results showed that LIG treatment reduced mortality, neurobehavioral deficits, brain edema, BBB permeability, and cerebral vasospasm. In addition, treatment reduced the number of apoptotic cells in the surrounding brain injury site, which accompanied a marked down-regulation of proapoptotic proteins, p53, and cleaved caspase-3. Our data suggest that LIG may be an effective therapeutic modality for SAH victims by altering apoptotic mechanisms.

Introduction
Subarachnoid hemorrhage (SAH) is a devastating stroke subtype with significantly high morbidity and mortality rates (Janjua and Mayer, 2003; Tseng et al., 2005). Despite promising therapeutic approaches, surgical treatment, triple-H therapy (hypertension, hypervolemia, and hemodilution) (Rosenwasser et al., 1999), calcium channel blockers, endothelin-receptor antagonists (Vajkoczy et al., 2005), and sodium nitroprusside therapy (Raabe et al., 2002), successful treatment after SAH remains inadequate. This is partly attributed to the lack of effective therapeutic approaches in dealing with cerebral ischemia as a result of cerebral vasospasm (CVS), one of the major consequences seen after SAH. As a result, a new therapeutic approach is warranted to combat the effects of cerebral vasospasm and hopefully improve prognosis.

Angelica sinensis, commonly known as the female ginseng, is a well known traditional Chinese medicine used for thousands of years to treat various gynecological diseases (Tsuchida et al., 1987; Liu et al., 2001) and immune system disorders (Wilsarumsee et al., 2002) and to prevent platelet aggregation (Shimizu et al., 1991) in patients. One of the major components of A. sinensis, Z-ligustilide (3-butylidene-4,5-dihydropthalide; LIG) (Fig. 1) (Kuang et al., 2006), has...
been considered to be the active ingredient responsible for its beneficial effects. Previously published works have alluded to the antioxidant effects induced by a decrease in malondialdehyde and increased activity of glutathione peroxidase and superoxide dismutase as the mechanism behind its protection. Yet other published data have directed attention more to the antiapoptotic mechanisms of LIG, specifically via up-regulation of Bel-2 and down-regulation of Bax and caspase-3 (Kuang et al., 2006). Nonetheless, LIG has been shown to significantly reduce infarction size and brain edema formation and improve neurobehavioral deficits caused by focal cerebral ischemia (Peng et al., 2007) as well as in transient forebrain cerebral ischemic models (Kuang et al., 2006).

Accordingly, in the present study we evaluated the potential therapeutic effects of LIG treatment after SAH brain injury, specifically hypothesizing that treatment with LIG may reduce brain injury by altering key players in the apoptotic pathway. To carry out our experiments, various parameters were studied, including neurobehavioral function, brain water content, blood-brain barrier (BBB) permeability, histopathological changes, and evaluation of proapoptotic and/or antiapoptotic protein expression.

Materials and Methods

Animals. All animal experiments were performed in accordance with China Animal Welfare Legislation and approved by the Chongqing Medical University Committee on the Care and Use of Laboratory Animals. Adult male Sprague-Dawley (SD) rats (n = 215) weighing 230 to 310 g were purchased from the Animal Center of Chongqing Medical University. The rats were housed in light- and temperature-controlled environments with food and water available ad libitum.

Isolation of Z-Ligustilide. Radix A. sinensis was purchased from its cultivating base at the Good Agricultural Practice in Min Xian County, Gansu Province, China. Its identity was confirmed by comparison with descriptions of characteristics and the appropriate monograph in the Chinese Pharmacopoeia 2000. LIG was prepared as described previously (Kuang et al., 2006). In brief, A. sinensis essential oil was extracted using supercritical CO2 fluid. LIG was isolated from the oil by silica gel column chromatography and identified by electron impact ionization mass spectrometry, 1H NMR, and 13C NMR spectrometry. Purity was determined to be >98% based on the percentage of total peak area by gas chromatography. LIG was prepared with 3% Tween 80 before use.

SAH Model. Experimental SAH was induced using a modified rat double hemorrhage model as reported previously (Lee et al., 2008). In brief, male SD rats were anesthetized with chloral hydrate intraperitoneally at a dose of 350 mg/kg. Throughout the surgery, animals were allowed to breathe spontaneously while a rectal temperature was maintained at approximately 37 ± 0.5°C. A homeothermal operating table was used. After proper positioning in a stereotactic frame, a parieto-occipital incision was made. The nuchal muscle layers were then divided, exposing the atlanto-occipital membrane. The cisterna magna was punctured using a 27-gauge needle, and 0.1 ml of cerebral spinal fluid was gently aspirated. Nonheparinized autologous blood (0.1 ml/100 g) from the femoral artery was injected ascetically into the cisterna magna over 2 min to induce the first SAH. The needle was left in place for an additional 30 min after injection to prevent the possible leakage of blood. After removal of the needle, the skull hole was closed with glue. To permit blood distribution around the basal arteries, the rats were tilted 30° for 30 min with their heads down. After the animals recovered from the effects of anesthesia, they were returned to their cage in a room where the temperature was maintained at 24 to 26°C. The same procedure was repeated 48 h after the first induction of SAH, and 0.2 ml of autologous blood was injected to induce the second SAH. Sham-operated rats received two intracisternal injections of equal volume of physiological saline solution according to the same procedure.

Experimental Protocol. Male SD rats (n = 215) were randomly assigned to the following weight-matched groups: 1) sham group (surgery without SAH insult; n = 40); 2) SAH + vehicle group (SAH insult treated with vehicle; n = 61); 3) SAH + LIG5 group (SAH insult treated with 5 mg/kg LIG; n = 60); and 4) SAH + LIG20 group (SAH treated with 20 mg/kg LIG; n = 54). Thirty minutes after the first SAH, the SAH + LIG5 and SAH + LIG20 groups received intravenous injections of LIG administered at doses of 5 and 20 mg/kg via vena caudalis. No treatment was applied in the sham-operated group, whereas the SAH + vehicle groups were treated with a volume-matched vehicle (3% Tween 80). All treatments were given at the same time point for the next 7 days. Animals that died either on the table or within the first 3 h after the operation were not allocated to any group. The animals that died after surgery were replaced until the final group size reached an expected number in each group. The algorithm of the experimental protocol is summarized in Fig. 2.

Mortality. Mortality was calculated on day 7 after the first blood injection. The animals that were expected to be sacrificed on day 3 were not included in the mortality calculation. The numbers of animals in each group designated for the mortality study were: sham (n = 27), SAH + vehicle (n = 33), SAH + LIG5 (n = 32), and SAH + LIG20 (n = 29).

Fig. 2. Schematic illustration of experimental design. The first and the second SAH were induced on days 0 and 2, respectively. The triangles indicate administration of LIG or vehicle. The neurobehavioral score was recorded daily from days 0 to 7. Twelve animals in each group were euthanized on day 3 for brain water content and blood-brain barrier permeability assessment. The rest were euthanized on day 7 for brain water content, blood-brain barrier permeability assessment, histology, immunohistochemistry, Western blot, and TUNEL staining.
Neurobehavioral Testing. Neurobehavioral testing was performed as described previously by Lee et al. (2008). In brief, animal subjects were observed for 5 min in a normal cage environment by two treatment-blinded investigators. Neurobehavioral condition was scored as follows: grade 1, no deficit, i.e., the rat moved about, explored the environment within a short period of time, and approached at least three walls of the cage without motor deficits; grade 2, slightly affected, i.e., the rat did not move in the cage with a delay but did not approach all sides and hesitated to move, although it eventually reached at least one upper rim of the cage; grade 3, moderately affected, i.e., the rat did not rise up at all and barely moved in the cage without abnormality; and grade 4, severely affected, i.e., the rat did not move at all and showed tetraplegia or paraplegia. The first evaluation (day 0) was performed 6 h after the treatment, i.e., the rat did not move at all and showed tetraplegia or paraplegia. The first evaluation (day 0) was performed 6 h after the treatment, and examined under a Toshiba (Tokyo, Japan) 7000S transmission electron microscope.

Immunohistochemistry. Tissue preparation was conducted as previously noted under Histological Examination. In brief, coronal sections of the brain were processed into paraffin blocks, and 10-µm slices were cut using a microtome. After dewaxing, the slices were heated and boiled for 30 min in citrate buffer solution (0.01 M, pH 6.0) for retrieval antigen. Each section was treated with 3% hydrogen peroxide for 20 min at room temperature to diminish nonspecific staining. After rinsing with PBS, the slices were blocked with 5% normal goat serum in PBS (0.01 M, pH 7.4) for 30 min at room temperature. The slides were then incubated with the primary antibody (rabbit anti-Bcl-2 (1:100), rabbit anti-Bax (1:200), rabbit anti-Bax (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and rabbit anticleaved-caspase-3 (1:200) (Cell Signaling Technology, Danvers, MA)) overnight at 4°C. After rinsing with PBS, the specimens were incubated with biotinylated secondary antibody at room temperature for 30 min and then reincubated with peroxidase-labeled streptavidin for 15 min. The immunoreactivity was visualized by 3,3-diaminobenzidine tetrahydrochloride solution. For the negative control, some slices from each group were incubated in a medium omitting the primary antibody. After being counterstained with hematoxylin, slices were dehydrated and cover-slipped.

TUNEL Staining. Coronal brain slices were stained using the in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining kit (Roche Diagnostics, Basel, Switzerland) to evaluate apoptotic neuronal cells. Cells showing nuclear condensation or fragmentation and apoptotic bodies in the absence of cytoplasmic TUNEL reactivity were considered apoptotic neuronal cells. The apoptosis index was calculated as described previously (Wang et al., 2005).

Western Blotting. Under deep anesthesia, rats were perfused intracardially with 200 ml of ice-cold physiological saline solution on day 7. The cerebral cortex was isolated and homogenized for 30 min in radioimmunoprecipitation assay lysis buffer with phenylmethylsulfonyl fluoride. The insoluble material was removed by centrifugation at 12,000g for 15 min. Fifteen micrograms of each lysate sample was denatured for 5 min in sample buffer and separated by 10% SDS-polyacrylamide gel electrophoresis. After electrophoretic transfer of the separated polypeptides to the polyvinylidene difluoride membranes at 100 V for 90 min, the membranes were probed with horseradish peroxidase-conjugated rabbit anticleaved caspase-3 (1:200) (Cell Signaling Technology, Danvers, MA) overnight at 4°C. After rinsing with PBS, the slices were blocked with 1% bovine serum albumin in Tween/Tris-buffered saline for 1 h at room temperature. The membranes were then incubated with the primary antibody at 4°C overnight. The following primary antibodies were used: anti-p53 antibody (1:500), anti-Bax (1:500), anti-Bcl-2 (1:500), anti-β-actin (1:500) (Santa Cruz Biotechnology, Inc.) and anticleaved-caspase-3 (1:1000) (Cell Signaling Technology). The membranes were probed with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000) (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The immunoreactive bands were visualized using an enhanced chemiluminescence method and quantified with Quantity One Software (Bio-Rad Laboratories, Hercules, CA). As a loading control, β-actin was blotted on the same membranes after stripping (Nakamura, 2004). Five animals were used in each group for Western blot analysis.

Statistical Analysis. SPSS software (version 10.0; SPSS Inc., Chicago, IL) was used for all statistical calculations. All data are expressed as mean ± S.D. The statistical significant of differences between means was evaluated by the one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test for multiple comparisons. A probability value of p < 0.05 was considered statistically significant.
Results

Mortality. Of 215 rats, 39 died either on the table or within the first 3 h after operation. Those animals were excluded from further analysis. The mortality on day 7 after the first SAH was 21.21% (7 of 33) in the SAH + LIG5 group, 18.75% (6 of 32) in the SAH + LIG20 group, and 3.70% (1 of 27) in the sham group.

Neurobehavioral Evaluation. Generally speaking, neurobehavioral deficits were encountered in all SAH-injured groups and characterized by lethargy, drowsiness, and minimal movements. The neurobehavioral scores revealed the worst scores for every group at 6 h after the first SAH (day 0) and the second SAH (day 2). Although neurobehavioral scores in all SAH groups were poor between days 3 and 5, the scores in the SAH + LIG20 group significantly improved compared with the SAH + vehicle and SAH + LIG5 groups on days 3 and 4 (Fig. 3). After day 5, all animals showed a rapid improvement in their neurobehavioral status.

Brain Water Content. Brain edema, as indicated by increased brain water content, was seen at 24 h after the second SAH (day 3). In the SAH + vehicle group, the brain water content increased significantly compared with the sham group (79.12 ± 0.23 versus 78.34 ± 0.40%, p < 0.01). This increase was significantly reduced on day 3 with LIG20 therapy (78.66 ± 0.35 versus 79.12 ± 0.23%, p < 0.05). The administration of LIG (5 mg/kg) did not reduce brain edema at this time point (78.94 ± 0.18 versus 79.12 ± 0.23%, p > 0.05). On day 7, there was no significant difference among the groups (Fig. 4).

BBB Permeability. The BBB analysis showed a significant protection afforded by LIG (20 mg/kg) on day 3 compared with the SAH + vehicle group. The value of EB dye extravasations in the SAH + vehicle group was significantly higher than that in the sham group (9.05 ± 2.48 versus 3.86 ± 1.14 μg/g, p < 0.01) and the SAH + LIG20 group (9.05 ± 2.48 versus 5.65 ± 1.04 μg/g, p < 0.05). Low-dose (5 mg/kg) treatment failed to produce a significant difference. On day 7, there was not a significant difference among the four groups (Fig. 5).

Cerebral Vasospasm. Representative pictographs of the BA can be seen in Fig. 6A. No vasospasm was noted in the animals in the sham group (Fig. 6Ad). However, severe morphological vasospasm was observed in the SAH + vehicle groups (Fig. 6Aa), characterized by corrugation of the internal elastic lamina and contraction of the smooth muscle cells. In addition, the vessel lumen was decreased in size and the vessel wall thickness was increased. A dose-dependent improvement was found in the BA treated with 5 or 20 mg/kg LIG (Fig. 6A, b and c).

The average cross-sectional area of the basilar artery in the SAH + vehicle group was 12,411.93 ± 2008.04 μm², which was increased to 13,417.14 ± 3690.16 μm² (p < 0.05) and 18,092.23 ± 4270.35 μm² (p < 0.05) with 5 and 20 mg/kg LIG treatment, respectively (Fig. 6B). The average wall thickness of the basilar artery in the SAH + vehicle, SAH + LIG5, SAH + LIG20, and sham groups was 25.91 ± 10.65, 27.51 ± 7.37, 15.69 ± 5.22, and 14.42 ± 4.09 μm, respectively (Fig. 6C). The wall thickness in the SAH + LIG20 (p < 0.05) and sham (p < 0.05) groups was significantly lower than in the SAH + vehicle group.

Ultrastructural Appearances. Representative ultrastructural micrographs of the basilar arteries obtained from the SAH + vehicle, SAH + LIG20, and sham groups are shown in Fig. 7. The endothelial cells in the sham group were flat and spindle in shape, tightly attached to the internal elastic lamina, and characterized by a single continuous layer of contacts of varying length with tight junctions and occasional indentations. The smooth muscle cells were glabrous without corrugation. There was no vacuolization in either endothelial or smooth muscle cells (Fig. 7c). Pathomorphological changes were observed in the SAH + vehicle group on day 7. These changes include swelling, vacuolization, disorientation, and...
A. sinensis has been used as a medicinal plant and included in numerous traditional Chinese herbal prescriptions for thousands of years. Besides being used to modulate the immune system, treat obstetrical and gynecological disorders, and prevent platelet aggregation, it has been clinically administered to treat cerebrovascular and cardiovascular diseases. Previous studies show that the effective constituents of A. sinensis extract are classified into water-soluble parts and essential oil. The former, including polysaccharides and ferulic acid, is widely used in the clinic. The effective substances of the essential oil are still largely unknown. It has been reported that LIG is one of the major essential oil components and characteristic phthalide component of A. sinensis. It has numerous pharmacological actions, including antiasthmatic and analgesic effects, antiproliferative effects on smooth muscle cells, and improved microcirculation and smooth muscle relaxation. Multiple studies have reported that LIG provider significant neuroprotective effects on transient forebrain ischemia in mice (Kuang et al., 2006), permanent forebrain ischemia in rats (Kuang et al., 2008), and focal cerebral ischemia in rats (Peng et al., 2007). However, much less is known about the potential effects of LIG on brain injury after SAH.

The aim of this study was to evaluate the potential therapeutic use of LIG as a treatment option after a SAH brain injury. We were able to show for the first time that LIG treatment could provide effective neuroprotection against SAH by reducing apoptotic damage through decreased expression of p53 and cleaved caspase-3, and in doing so, subsequently limit the formation of secondary brain injuries. Specifically, we found that it reduced mortality among our rat population, increased neurobehavioral scores, and decreased expression of p53 and cleaved caspase-3.

**Discussion**

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Secondary brain injury as a result of SAH has been recognized as a leading cause of death and disability in patients (Bederson et al., 1995). These injuries include brain edema formation, cerebral arterial vasospasm, BBB disruption, and neurobehavioral deficits. Mounting evidence has suggested that apoptosis may be the key orchestrator of secondary brain injury after SAH (Cahill et al., 2006). Therefore, therapeutic agents that can block and/or slow down the activation...
of apoptotic signals can translate into reduced short- and long-term clinical outcomes. In the present study, we found that TUNEL-positive cell counts, which are a strong marker for neuronal cell death, were significantly increased after SAH injury and could be effectively reduced after LIG treatment. Specifically, we were able to demonstrate a markedly elevated expression of p53 after SAH injury, which was reduced after treatment. This is important because p53, a well known tumor suppression protein, has been implicated in a host of intracranial pathologies, including cerebral ischemia and Alzheimer's disease (Daily et al., 1999; Mattson, 2000; Leker et al., 2004; Cahill et al., 2007) and has been shown to orchestrate the development of vasospasm and neuronal cell death after SAH injury (Zhou et al., 2004, 2005; Cahill et al., 2007).

Other key players in the apoptotic cascade are members of the Bcl family, which are important endogenous regulators in the mitochondrial apoptotic pathway (Oltvai et al., 1993). Cell survival during the apoptotic cascade depends on the balance between the proapoptotic and antiapoptotic proteins of this family. Bax, a proapoptotic protein, and Bcl-2, an antiapoptotic protein, are the two primary members of the Bcl-2 family (Gross et al., 1999; Antonsson, 2001; Perfettini et al., 2002; Tsujimoto, 2003). This is important because one of the largest target groups of p53 is the Bcl-2 family. A growing body of evidence suggests that p53 can function as a direct transcriptional activator of Bax and may either directly or indirectly down-regulate expression of the Bcl-2 gene via a p53-dependent negative response element. The inhibition of p53 allows for the balance of proapoptotic to antiapoptotic members of the Bcl-2 family to tip in favor of survival, thereby preventing mitochondrial pore formation and, ultimately, the release of apoptosis-promoting factors (Miyashita et al., 1994a,b; Miyashita and Reed, 1995). In our study, we found that the immunoreactivity of Bcl-2 was decreased in brain regions after SAH injury, whereas Bax expression was increased. Although treatment with LIG in the SAH group did not influence Bax expression after injury, it resulted in a dose-dependent increase in Bcl-2 levels, which led to a large shift of Bcl-2/Bax ratio in favor of the antiapoptotic Bcl-2. In fact, some studies have shown that the Bcl-2/Bax ratio may be a stronger predictor of apoptotic activity than the concentrations of either Bax or Bcl-2 alone (Oltvai et al., 1993).

Caspase-3 is a downstream member of the caspase-dependent apoptotic pathway. The decrease of Bcl-2/Bax ratio may initiate apoptosis by causing the loss of outer mitochondrial membrane integrity. This results in the release of apoptogenic proteins such as cytochrome c from the intermembrane space of mitochondria. The released cytochrome c leads to the formation of the apoptosome complex, which, in turn, activates downstream caspases such as caspase-3. Furthermore, activated caspase-3 cleaves numerous nuclear enzymes associated with apoptosis such as poly(ADP-ribose) polymerase. So caspase-3 is considered one of the central effectors of apoptosis. Previous studies have alluded to the role of caspase-3 in cortical neuron apoptosis post-SAH (Park et al., 2004). In the current study, we found that the cleaved caspase-3 level was increased in cortex after SAH and that this was reversed with LIG treatment. Thus, the present data suggest that the neuroprotection of LIG treatment after SAH probably is associated with the shift of Bcl-2/Bax ratio.
in favor of Bcl-2, resulting in decreased activation of caspase-3 in cortical neuron post-SAH.

Although upstream regulators of these apoptotic proteins were not detected in the present study, previous studies have investigated the actions of LIG on some regulators in other models. A study of LIG in vascular smooth muscle cells demonstrated that LIG had the potential to suppress extracellular signal-regulated kinase 1/2, p38, and c-Jun NH2-terminal kinase, the members of mitogen-activated protein kinases associated with decreased intracellular reactive oxygen species (ROS) production, which resulted in the inhibition of vascular smooth muscle cell proliferation and cell cycle progression (Lu et al., 2006). Moreover, a study showed that pretreatment of the PC12 cells with LIG significantly attenuated H2O2-induced cell death via reducing increased intracellular ROS levels, down-regulation of Bax, cleaved-caspase 3, and cytochrome c expression, and up-regulation of Bcl-2 expression (Yu et al., 2008). Based on these observations, we could reasonably speculate that LIG treatment might act as a ROS or mitogen-activated protein kinase inhibitor to alter the levels of apoptotic proteins, and then promote survival of cortical neurons after SAH. To elucidate the mechanisms of LIG-altering apoptotic proteins, further studies on the effects of LIG treatment on alterations of the above-mentioned regulators after SAH are needed.

In addition to apoptosis, CVS after SAH is another secondary brain injury complication. Although the pathogenesis of CVS remains inconclusive, considerable studies have shown that calcium ions play a critical role in almost all stages of CVS formation. Previous research has suggested that LIG treatment can induce vasodilation in the rat mesenteric artery by inhibiting voltage-dependent calcium channels, thus preventing receptor-mediated Ca2+ influx and release (Cao et al., 2006). Similar to that work, our study found that the SAH-injured rats had shown traits after hematoxylin and eosin staining that were characteristic of SAH-induced vasospasm, including an increase in wall thickness, luminal narrowing, shrunken endothelial cells, and corrugation of the internal elastic lamina of the basilar arteries. Moreover, after LIG treatment these changes were notably reduced, and additional histogram analysis revealed a significant improvement in cross-sectional area and basilar wall thickness. This suggests that LIG may in fact alter the mechanism responsible for inducing CVS after injury.

In summary, the results of the present study strongly suggest that LIG treatment may in fact ameliorate brain injury after experimental SAH through mechanisms that reduce neuronal apoptosis and subsequently ameliorate secondary brain injury. Further studies will be needed to determine more mechanistic detail with regard to the role of LIG and apoptosis.
Authorship Contributions

Participated in research design: Chen, Tang, and S. Wang.
Conducted experiments: Chen, Zhu, Li, Jiang, and Tu.
Contributed new reagents or analytic tools: C. Wang.
Wrote or contributed to the writing of the manuscript: Chen, Tang, Khatibi, and S. Wang.

References


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