Geniposide and its iridoid analogs exhibit antinociception by acting at the spinal GLP-1 receptors

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A B S T R A C T

We recently discovered that the activation of the spinal glucagon-like peptide-1 receptors (GLP-1Rs) by the peptidic agonist exenatide produced antinociception in chronic pain. We suggested that the spinal GLP-1Rs are a potential target molecule for the management of chronic pain. This study evaluated the antinociceptive activities of geniposide, a presumed small molecule GLP-1R agonist. Geniposide produced concentration-dependent, complete protection against hydrogen peroxide-induced oxidative damage in PC12 and HEK293 cells expressing rat and human GLP-1Rs, but not in HEK293T cells that do not express GLP-1Rs. The orthosteric GLP-1R antagonist exendin(9-39) right-shifted the concentration—response curve of geniposide without changing the maximal protection, with identical pA2 values in both cell lines. Subcutaneous and oral geniposide dose-dependently blocked the formalin-induced tonic response but not the acute flinching response. Subcutaneous and oral geniposide had maximum inhibition of 72% and 68%, and ED50 values of 13.1 and 52.7 mg/kg, respectively. Seven days of multidaily subcutaneous geniposide and exenatide injections did not induce antinociceptive tolerance. Intrathecal geniposide induced dose-dependent antinociception, which was completely prevented by spinal exendin(9-39), siRNA/GLP-1R and cyclic AMP/PKA pathway inhibitors. The geniposide iridoid analogs geniposidic acid, genipin methyl ether, 1,10-anhydrogenipin, loganin and catalpol effectively inhibited hydrogen peroxide-induced oxidative damage and formalin pain in an exendin(9-39)-reversible manner. Our results suggest that geniposide and its iridoid analogs produce antinociception during persistent pain by activating the spinal GLP-1Rs and that the iridoids represented by geniposide are orthosteric agonists of GLP-1Rs that function similarly in humans and rats and presumably act at the same binding site as exendin(9-39).

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1. Introduction

The endogenous incretin glucagon-like peptide-1 (GLP-1) and exogenous exenatide have biological actions in the pancreas, such as stimulating glucose-dependent insulin synthesis and secretion, decreasing glucagon levels and notably altering β-cell proliferation and apoptosis (Baggio and Drucker, 2007; Li et al., 2003). They also have therapeutic value in treating type 2 diabetes mellitus (DeFronzo et al., 2005; Triplitt and Defronzo, 2006). These actions are mediated by activating the GLP-1 receptors (GLP-1Rs), which are members of the class B1 family of seven-transmembrane-spanning, heterotrimeric G-protein coupled receptors (GPCRs) and whose signal transduction is mainly effected through the calcium and cyclic AMP/protein kinase A (PKA) signaling pathways (Holst, 2007; Ramos et al., 2008; Roger et al., 2011). In humans and rodents, single, structurally identical GLP-1Rs are expressed in the pancreatic islets (Tornehave et al., 2008), lungs, brain, cardiovascular system, dorsal root ganglia and spinal cord, among others (Bullock et al., 1996; Gong et al., 2014a; Hanson et al., 1998; Himeno et al., 2011; Kim et al., 2013; Teramoto et al., 2011).

We recently discovered that GLP-1Rs are specifically expressed in spinal dorsal horn microglial cells and are up-regulated during microglial proliferation and hypertrophy after peripheral nerve injury. The intrathecal administration of the GLP-1R peptidic agonists exenatide and GLP-1(7-36) produced specific, potent antinociception of 60–90% during formalin-induced tonic pain, painful peripheral neuropathy, bone cancer-induced mechanical allodynia and diabetic pain. Our results suggested that the activation of the

Abbreviations: ANOVA, analysis of variance; DAPI, 4’,6-diamidino-2-phenylindole; DDA, 2’,5’-dideoxyadenosine; DMSO, dimethyl sulfoxide; Emax, maximum effect; ED50, half-effective dose; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; GPCR, G-protein coupled receptor; PEI, polyetherimide; PKA, protein kinase A.

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spinal GLP-1Rs by peptidic agonists leads to specific antinociception in chronic pain hypersensitivity states, notably refractory neuropathic pain, cancer pain and painful diabetic neuropathy (Gong et al., 2014a). The GLP-1R antinociception hypothesis can be confirmed by the application of small molecule GLP-1R agonists. Small molecule agonists with different chemical structures to exenatide and GLP-1 may be helpful for excluding the possibility that the antinociception induced by exenatide and GLP-1 is not related to the activation of the GLP-1Rs. Favorable results will also be useful for the development of orally-available analgesics that can easily penetrate the central nervous system.

The GLP-1Rs belong to a class B1 family of GPCRs that interact with peptidic ligands by a hypothesized two-step mechanism. The extracellular N-terminal ectodomain interacts with the C-terminal residues of cognate ligands and positions the N-terminus of the ligand to interact with the critical determinants in the receptor’s transmembrane regions, leading to full activation and signal transduction (Castro et al., 2005; Hoare, 2005; Laburthe et al., 2007). Small molecule compounds may not have sufficient mass or diameter to react with both the N-terminal ectodomains and the receptor transmembrane regions, and therefore possibly interact with only one binding site. It would be interesting to know whether small molecules produce antinociception by activating the GLP-1Rs with a full intrinsic efficacy. As peptidic exenatide and GLP-1 are not orally-active, the development of orally-available, non-peptidic GLP-1R agonists has been an active, although as yet unfruitful, area of research for both anti-diabetic drugs and analgesics (Wang et al., 2010; Willard et al., 2012). As exenatide and GLP-1 cannot readily cross the blood brain barrier, orally-available GLP-1R agonists with the ability to penetrate the central nervous system, leading to more specific, efficacious analgesics, must be developed.

Geniposide (Fig. 1) is one of the main iridoid glycosides of Gardenia jasminoides (Hu et al., 2005), which has been widely used in Chinese traditional medicine for its homeostatic, antiphlogistic, antinociceptive and antipyretic effects and indications. Geniposide can induce neuronal differentiation and protection against oxidative stress and can regulate insulin secretion (Guo et al., 2012; Liu et al., 2006, 2009). Its insulin regulation ability is blocked by the GLP-1R antagonist exendin(9-39) (Guo et al., 2012). The potency, efficacy and activation mode of geniposide’s activation of the GLP-1Rs has not yet been characterized. Many structurally related iridoid glycosides are found in plants (Ghule and Yeole, 2012; Jeong et al., 2012; Park et al., 2011a, 2011b; Sundaram et al., 2012; Xu et al., 2011). Understanding the structure–activity relationship of geniposide may suggest whether the iridoids represent a novel core structure for GLP-1R activation.

We studied the effects of geniposide and a series of structurally related iridoids in GLP-1R-sensitive in vitro and in vivo assays and compared them to exenatide. We tested the protective effect of geniposide against hydrogen peroxide-induced oxidative damage in PC12 cells that express rat GLP-1Rs, HEK293 cells that stably express human GLP-1Rs and HEK293T cells that do not express GLP-1Rs. This hydrogen peroxide assay is known to be GLP-1R-sensitive (Liu et al., 2009; Oeseburg et al., 2010).

We examined the antinociceptive effect of geniposide using a formalin test. Formalin induces an acute flinching response, representing an acute nociceptive reflex response without central sensitization, and a tonic flinching response, which represents
persistent pain involving central sensitization (Coderre et al., 1990; Dickinson and Sullivan, 1987). The primary active sites for geniposide antinociception and its ability to develop antinociceptive tolerance were also determined.

We explored the blocking effects of exendin(9-39), the GLP-1R gene silencer siRNA/GLP-1R and cyclic AMP/PKA signaling pathway inhibitors on spinal geniposide antinociception. We compared the activity of six iridoid analogs of geniposide, geniposidic acid, genipin, genipin methyl ether, 1,10-anhydrogenipin, loganin and catalpol (Fig. 1), in the hydrogen peroxide and formalin tests to geniposide. Our results indicate that orally-available geniposide and its iridoid analogs produce antinociception entirely via the activation of the spinal GLP-1Rs and suggest that the iridoids are novel orthosteric agonists of rat and human GLP-1Rs that possibly act at the same binding site as exendin(9-39).

2. Materials and methods

2.1. Drugs

Geniposide, geniposidic acid and genipin were purchased from the Linchuan Zhixin Biotechnology Co. (Jiangxi, China). Loganin was purchased from Fluka Chemicals (Shanghai, China) and catalpol from the Chengdu Purifa Technology Development Co. (Sichuan, China). 1,10-Anhydrogenipin was synthesized by dissolving genipin in methylene chloride with first triphenylphosphine and then diisopropyl azodicarboxylate, according to a previous report (Zhang et al., 2006). Genipin methyl ether was obtained by the methanolysis of geniposide in the

![Fig. 2.](image-url) Representative photomicrographs of GLP-1R expression and the concentration–response curves of exenatide, geniposide and the iridoid compounds geniposidic acid, genipin methyl ether, 1,10-anhydrogenipin, loganin and catalpol on hydrogen peroxide-induced oxidative damage in PC12 cells expressing rat GLP-1Rs (A, B, C), HEK293 cells stably expressing human GLP-1Rs (D, E, F) and HEK293T cells that do not express GLP-1Rs (G). Cells were incubated in 96-well plate for 24 h then treated with 500 or 800 μM hydrogen peroxide for 15 min (PC12 cells) or for 5 min (HEK293 and HEK293T cells). The cells were further treated with exenatide, geniposide or another iridoid and cultured for 12 h before the MTT assay. Scale bars: 100 μm in A, D and F. The data are mean ± SEM (n = 3 in each treatment) from two to three independent studies. * denotes a statistically significant difference from the control group (P < 0.05, calculated with a one-way ANOVA followed by a post-hoc Student–Newman–Keuls test).
presence of sulfuric acid (Yu et al., 2008). The structures of 1,10-anhydrogenipin and genipin methyl ether were confirmed by NMR spectroscopy with a purity of more than 95%. Morphine hydrochloride, exendin and exenin-(9-39) were obtained from Northp Pharmaceutical Co. (Shenyang, China), respectively. 2',5'-Dideoxyadenosine (DDA) and N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonylamide (H-89) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). All drugs and reagents were freshly dissolved in normal saline solution, except 1,10-anhydrogenipin and genipin methyl ether, which were dissolved in 25% dimethyl sulfoxide (DMSO) in saline.

The siRNA targeting the GLP-1Rs and its non specific oligonucleotide were synthesized by GenePharma Co. (Shanghai, China), following Yin et al. (2010). The 19 nucleotide duplexes and two unpaired nucleotides overhanging 3’ methylsulfonyl synthesized by GenePharma Co. (Shanghai, China), following Yin et al. (2010). The 19 nucleotide duplexes and two unpaired nucleotides overhanging 3’ ends were as follows:

**Table 1**

<table>
<thead>
<tr>
<th>Iridoids or exendin</th>
<th>Molecular weight</th>
<th>EC_{50} (10^{-8} M)</th>
<th>ED_{50} (μg/rat)</th>
<th>ED_{50} (mmol/rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exenadine</td>
<td>4186</td>
<td>3.8 × 10^{-8} M</td>
<td>2.5 ng (0.9–6.8 ng)</td>
<td>0.6 pmol</td>
</tr>
<tr>
<td>Geniposide</td>
<td>388</td>
<td>16.5</td>
<td>17.3 (12.54–23.83)</td>
<td>44.5</td>
</tr>
<tr>
<td>Geniposidic acid</td>
<td>374</td>
<td>23.7</td>
<td>15.3 (7.35–31.85)</td>
<td>40.9</td>
</tr>
<tr>
<td>Genipin</td>
<td>226</td>
<td>NA</td>
<td>20.4 (8.12–51.12)</td>
<td>90.2</td>
</tr>
<tr>
<td>Genipin methyl ether</td>
<td>240</td>
<td>7.7</td>
<td>11.5 (0.15–857.31)</td>
<td>59.9</td>
</tr>
<tr>
<td>1,10-Anhydrogenipin</td>
<td>208</td>
<td>6.0</td>
<td>11.3 (6.062–20.98)</td>
<td>54.3</td>
</tr>
<tr>
<td>Loganim</td>
<td>390</td>
<td>16.3</td>
<td>23.8 (11.20–50.54)</td>
<td>61.0</td>
</tr>
<tr>
<td>Catalpol</td>
<td>362</td>
<td>20.1</td>
<td>12.2 (5.656–26.42)</td>
<td>33.7</td>
</tr>
<tr>
<td>Shanzhise methylester</td>
<td>406</td>
<td>9.3</td>
<td>23.3 (5.7–95.6)</td>
<td>57.4</td>
</tr>
<tr>
<td>8-O-Acetyl shanzhise methylester</td>
<td>448</td>
<td>12.0</td>
<td>20.5 (15.5–27.1)</td>
<td>45.8</td>
</tr>
</tbody>
</table>

a Data were derived from Gong et al. (2011a).

b Data were derived from Zhu et al. (2014).
Fig. 3. The specific GLP-1R antagonist exendin(9-39) acts antagonistically against the effect of exenatide, geniposide and the iridoid compounds geniposide acid, genipin methyl ether, 1,10-anhydrogenipin, loganin and catalpol on hydrogen peroxide-induced oxidative damage in PC12 cells expressing rat GLP-1Rs (A, B, C) or in HEK293 cells that stably express human GLP-1Rs (D, E). Cells were cultured in a 96-well plate for 24 h, then were treated with 500 μM hydrogen peroxide for 15 min (PC12 cells) or for 5 min (HEK293 cells). The cells were treated with a range of concentrations of exenatide, geniposide or an iridoid compound, in the absence or presence of exendin(9-39), and cultured for another 12 h before the MTT assay. The Schild plots of exendin(9-39) competing with exenatide and geniposide are inserted in A and B, and D and E. The data are mean ± SEM (n = 3 in each treatment) from two to three independent studies.
2.8. Data analysis

For a dose–response curve analysis, the minimum effect, maximum effect (\(E_{max}\)), half-effective dose (\(ED_{50}\)) and Hill coefficient (n) were calculated for each dose–response curve. The values of a response (Y) were fitted by nonlinear least-squares curves to the relationship Y = a + b ln x, where x = \([D]/[D_0] + [D]^n\), to give the value of \(D_0\) and b (\(E_{max}\)), yielding a minimum residual sum of squared deviations from the theoretical curve (Wang and Pang, 1993).

For a Schild plot and \(pA_2\) analysis, the ratio of the dose of an agonist (A) required to produce the \(ED_{50}\) in the presence of an antagonist (B) to the dose required to produce the same response in the absence of the antagonist (A) was calculated using \([A]/[A] = [A][B]/[A][B] + [B]/[K_B]_0\), where \(K_B\) and \(K_B_0\) are the equilibrium dissociation constants for A and B, respectively (Wang et al., 1993). The \(pA_2\) was determined by applying at least three doses of antagonist and plotting log \((A/A_1 - 1)\) against the negative log B. If the regression of log \((A/A_1 - 1)\) on -log B was linear with a slope of \(-1\), the antagonism was competitive and, by definition, the agonist and antagonist acted at the same recognition sites. The x-intercept of the fitted regression line was an estimate of the \(pA_2\), which was the dose of the antagonist required for a twofold increase in the agonist concentration.

The data are expressed as mean ± standard error of the mean (SEM) and there were no missing data. The statistical significance was evaluated with an unpaired Student’s t-test or a one-way analysis of variance (ANOVA) in Prism (version 5.01, GraphPad Software Inc., San Diego, CA). A post-hoc Student–Newman–Keuls test was applied when a statistically significant drug (dose) effect was observed. The probability values were two-tailed and the statistical significance criterion P value was 0.05.

3. Results

3.1. Activation of rat and human GLP-1Rs via the exendin(9-39)-sensitive site

The activation of the GLP-1Rs is known to protect against hydrogen peroxide-induced oxidative damage (Liu et al., 2007; Oeseburg et al., 2010). We evaluated the protective effects of geniposide and exenatide in PC12 cells that express GLP-1Rs to identify whether geniposide can directly activate the GLP-1Rs (Perry et al., 2002). We confirmed the presence of GLP-1Rs in the PC12 cells by immunostaining (Fig. 2A). Treatment with exenatide (up to \(3 \times 10^{-8}\) M) or geniposide (up to \(3 \times 10^{-3}\) M) in PC12 cells was not associated with any cellular dysfunction. Exenatide completely protected the PC12 cells from hydrogen peroxide-induced oxidative damage in a concentration-dependent manner (EC50 = \(3.8 \times 10^{-9}\) M). Geniposide produced the same concentration-dependent protection (EC50 = \(1.7 \times 10^{-4}\) M), with an EC50 approximately 45,000-fold less potent than exenatide (Table 1 and Fig. 2B). Treatment with geniposide, genipin methyl ether, 1,10-anhydrogenipin, loganin or catalpol at up to \(3 \times 10^{-3}\) M in PC12 cells was not associated with any cellular dysfunction. However, \(10^{-4}\) M genepin caused significant cell death, probably due to its protein cross-linking activity, whereas its dehydrated derivative 1,10-anhydrogenipin lacked this activity (Zhang et al., 2006). All of the compounds except genipin were therefore tested further. Geniposidic acid, genipin methyl ether, 1,10-anhydrogenipin, loganin and catalpol exhibited similar concentration-dependent protection against hydrogen peroxide-induced viability loss (Fig. 2C), with EC50 values ranging from \(6.0 \times 10^{-5}\) to \(2.4 \times 10^{-4}\) M (Table 1). In a recent study, we investigated the protective effects of two other iridoid compounds, shanzhiside methylester and 8-O-acetyl shanzhiside methylester (Fig. 1) (Zhu et al., 2014). Their \(ED_{50}\) values of \(9.3 \times 10^{-3}\) M and \(12 \times 10^{-3}\) M are also listed in Table 1.

To test whether geniposide can activate human GLP-1Rs, the protective effects of exenatide and geniposide were examined in HEK293 cells that stably expressed human GLP-1Rs. The expression of GLP-1Rs in the HEK293 cells was again confirmed with specific immunostaining (Fig. 2D). Treatment with exenatide (up to \(3 \times 10^{-8}\) M) or geniposide (up to \(3 \times 10^{-3}\) M) in HEK293 cells was not associated with any cellular dysfunction. Both exenatide (EC50 = \(2.8 \times 10^{-9}\) M) and geniposide (EC50 = \(1.3 \times 10^{-4}\) M) produced concentration-dependent complete protection against hydrogen peroxide-induced oxidative damage, at a ratio of approximately 1:46,000 (Table 1 and Fig. 2E). These results suggest that there is no difference in geniposide and exenatide’s ability to activate GLP-1Rs in rats or in humans. We tested geniposide and its iridoid analogs in HEK293 cells that do not express GLP-1Rs (http://webserver.mbi.ufl.edu/~shaw/293.html) to confirm that their protective effects against hydrogen peroxide-induced oxidative damage are via the activation of the GLP-1Rs. There was no specific GLP-1R immunostaining in the HEK293T cells (Fig. 2F). As shown in Fig. 2G, treatment with exenatide (30 nM) did not have any protective effect against hydrogen peroxide oxidative damage in HEK293T cells. Conversely, treatment with nicotinamide (3 mM) exhibited approximately 60% protection, in agreement with previous findings (Wilkinson et al., 2008). Geniposide and its iridoid analogs at 1 mM had none or only residual protective effects, which were probably nonspecific.

The specific orthosteric GLP-1R antagonist exendin(9-39) was used to test whether geniposide’s protective effect could be competitively antagonized. As shown in Fig. 3A, B, exenatide (EC50 = \(4.0 \times 10^{-9}\) M) and geniposide (EC50 = \(1.3 \times 10^{-4}\) M) inhibited the hydrogen peroxide-induced viability loss in PC12 cells in a concentration-dependent manner to a maximal protection of 100%. Co-treatment with exendin(9-39) at \(1 \times 10^{-7}\), \(3 \times 10^{-7}\), \(1 \times 10^{-6}\) and \(3 \times 10^{-6}\) M concentration-dependently shifted the concentration response curves of both exenatide and geniposide to the right without affecting the maximal protection. The Schild plots of exendin(9-39) inserted in Fig. 3A, B show the slope and \(pA_2\) values against exenatide and geniposide. The slope was \(-1.19\) against exenatide and \(-1.27\) against geniposide. The \(pA_2\) value was 7.32 against exenatide and 6.94 against geniposide.

The protective effects of five iridoid compounds were also antagonized with exendin(9-39) in PC12 cells. Treatment with geniposide, 1,10-anhydrogenipin, genipin methyl ether, loganin and catalpol at 1 mM produced approximately 65–85% protection against hydrogen peroxide-induced oxidative damage. Co-treatment with \(3 \times 10^{-6}\) M exendin(9-39) nearly completely prevented the protective effects of these iridoid compounds (Fig. 3C). The protective effects of shanzhiside methylester and 8-O-acetyl shanzhiside methylester were also nearly completely prevented by exendin(9-39) in our recent study (Zhu et al., 2014). Exendin(9-39)’s competitive antagonism of exenatide and geniposide was confirmed in HEK293 cells expressing human GLP-1Rs. Co-treatment with exendin(9-39) at \(1 \times 10^{-7}\), \(3 \times 10^{-7}\) and \(1 \times 10^{-6}\) M concentration-dependently shifted the concentration response curves of both exenatide and geniposide to the right without affecting the maximum protection (Fig. 3D, E). Schild plots of exendin(9-39) showed that the slope against exenatide was...
Fig. 5. The antinociceptive effects of an intrathecal injection of geniposidic acid, genipin, genipin methyl ether, 1,10-anhydrogenipin, loganin or catalpol on the formalin-induced acute nociceptive response and tonic pain in rats (A–F). Rats received an intrathecal injection of saline, 25% DMSO in saline or a variety of doses of iridoid compounds 30 min before a formalin challenge. The nociceptive behavior was quantified by counting the number of times that the formalin-injected paw flinched in 1 min epochs. G. Dose response analysis of the iridoid compounds’ effect on formalin-induced tonic pain, measured with the AUC_{10–90 min}, best-fitted by the nonlinear least-squares method. H. The effects of intrathecal exenatide and geniposide on random blood sugar (representing postprandial sugar) in rats. The data are presented as mean ± SEM (n = 6 in each group, except for the blood sugar study, in which n = 4 in each group).
dependently prevented the formalin-induced tonic response before a formalin challenge. The subcutaneous geniposide dose-saline (10 ml/kg) or geniposide (3, 10, 30, 100 or 300 mg/kg) 30 min (Fig. 4C). A dose–response analysis of exenatide by best fit revealed that the $E_{\text{max}}$ was 82.0% and the $E_{\text{50}}$ was 22.7 mg/kg (5.4 mmol/kg, 95% confidence limits: 21.4–24.0 mg/kg) (Fig. 4B).

Six groups of mice received a subcutaneous administration of saline (10 ml/kg) or geniposide (3, 10, 30, 100 or 300 mg/kg) 30 min before a formalin challenge. The subcutaneous geniposide dose-dependently prevented the formalin-induced tonic flinching response, with maximum inhibition at 100 mg/kg or less, but did not block the formalin-induced acute nociceptive response (Fig. 4A). A dose–response analysis of exenatide by best fit revealed that the $E_{\text{max}}$ was 71.5% and the $E_{\text{50}}$ was 13.1 mg/kg (33.8 μmol/kg, 95% confidence limits: 9.0–19.1 mg/kg) (Fig. 4B). The $E_{\text{50}}$ dose ratio for subcutaneous geniposide vs. exenatide in mole base was roughly 6200:1.

Six groups of fasting mice (12 h) received an oral gavage of saline (10 ml/kg) or geniposide (10, 30, 100, 300 or 1000 mg/kg) 1 h before a formalin challenge. The oral gavage of geniposide dose-dependently prevented the formalin-induced tonic response but did not block the formalin-induced acute nociception (Fig. 4G). A dose–response analysis showed that the $E_{\text{max}}$ was 58% and an $E_{\text{50}}$ of 52.7 mg/kg (95% confidence limits: 50.1–55.4 mg/kg) (Fig. 4B). The apparent oral bioavailability was 25% that of the subcutaneous route. No apparent sedative or motor side effects were observed after the oral gavage or subcutaneous administration of geniposide or exenatide.

The antinociceptive effects of geniposide via direct injection to the spinal cord, brain and peripheral afferent nerve terminals were tested. Six groups of rats chronically implanted with intrathecal cannulas received an intrathecal single injection of saline (10 μl) or geniposide (3, 10, 30, 100 or 300 μg) 30 min before a formalin injection. The paw subcutaneous injection of formalin in control rats produced a characteristic biphasic flinching response consisting of an initial, rapidly decaying acute phase (within 10 min after the formalin injection) followed by a slowly rising, long-lived (10–90 min) tonic phase. Although up to 300 μg geniposide did not block the flinching response in the acute phase, it did prevent the formalin-induced tonic flinching response in a dose-dependent manner (Fig. 4E).

The ineffectiveness of exenatide and geniposide induction of antinociceptive tolerance to the formalin-induced acute phase nociceptive response (A) and tonic phase pain (B) after chronic treatment. Mice received multiple bidaily subcutaneous injections of saline, morphine, exenatide or geniposide for seven continuous days. A single injection of saline, morphine, exenatide or geniposide was given on the eighth day 30 min before a formalin challenge. The cumulated licking duration from zero to five minutes after the formalin injection represented the acute phase nociceptive response and 20–40 min after the injection represented the tonic phase pain. The data are presented as mean ± S.E.M. (n = 6 in each group). a, b denote a statistically significant difference from the saline + saline control group or the saline + morphine group, respectively (P < 0.05, tested with a one-way ANOVA, followed by a post-hoc Student–Newman–Keuls test).
Fig. 7. The blockade effects of an intrathecal injection of the specific GLP-1R antagonist exendin(9–39) (A, B) and the GLP-1R gene silencer siRNA/GLP-1R (C, D) on the antinociceptive effect of geniposide in the rat formalin test. For the antagonist study, rats received an intrathecal single injection of saline, geniposide or exendin(9–39) + geniposide 30 min before a formalin challenge. In the gene silencing study, rats received multidaily intrathecal injections of the vehicle, nonspecific oligonucleotide or siRNA/GLP-1R for seven continuous days. On the eighth day, an intrathecal single injection of saline or geniposide was given 30 min before a formalin injection. The nociceptive behavior was quantified by counting the number of times that the formalin-injected paw flinched in 1 min epochs. B, D. The formalin-induced tonic pain, measured with the AUC10−90 min. The data are presented as mean ± SEM (n = 6 in each group). a, b denote a statistically significant difference from the saline control or the geniposide group, respectively (P < 0.05, tested with a one-way ANOVA, followed by a post-hoc Student–Newman–Keuls test).
100 or 300 μg) 30 min before a formalin injection. Up to 300 μg of 
any of the iridoid compounds did not inhibit the acute nociceptive 
response but did prevent the formalin-induced tonic pain in a dose-
dependent manner (Fig. 5A–F). No apparent sedative or motor side 
effects were observed for these iridoids, except for 300 μg genipin, 
which paralyzed rats 24 h after injection, probably due to its pro-	ein cross-linking effect (Zhang et al., 2006). A dose–response 
analysis using the AUC10–90 min showed that the Emax values for the 
iridoids ranged from 62.8% to 88.6% and that the ED50 values ranged 
from 11.3 μg to 23.8 μg (33.7–90.2 nmol) (Fig. 5G, Table 1). Shanzhiside 
methylester (ED50 = 23.3 μg) and 8-O-acetyl shanzhiside (ED50 = 20.5 μg) were also shown to alleviate the formalin-
induced tonic flinching response in our recent study (Zhu et al., 
2014) and are included in Table 1.

As the activation of the peripheral GLP-1Rs produces hypogly-
cemia and the spinal cord is the primary site for GLP-1R anti-
nociception, we examined whether the spinal exenatide and 
geniposide antinociception observed was due to hypoglycemia. 
Three groups of intrathecally cannulated rats received an intra-
thechal single injection of saline (10 μL), exenatide (300 ng) or 
geniposide (300 μg). Random blood sugar (representing postprandial 
sugar) levels were measured prior to and 1 h after the intrathecal 
injection using the glucose oxidase method and the OneToucha 
Ultraa 2 Blood Glucose Meter (Johnson & Johnson Company, 
California, USA) (Gong et al., 2011b). Compared to the saline control 
group, neither exenatide nor geniposide induced hypoglycemia in 
rats (Fig. 5H). Our results rule out the possibility that spinal GLP-1R 
antinociception is due to hypoglycemia.

Morphine tolerance to analgesia is a common phenomenon and 
hampers morphine’s clinical usefulness for the treatment of cancer 
and non-cancer pain. We tested whether a chronic treatment of 
geniposide induced antinociceptive tolerance. Seven groups of 
mice received multiple bidaily subcutaneous injections of saline 
(10 ml/kg, bid), exenatide (100 μg/kg, bid), geniposide (100 mg/kg, 
bid) and morphine (10 mg/kg, bid) for seven continuous days, fol-
lowed by a single subcutaneous injection of saline (10 ml/kg), 
exenatide (100 μg/kg), geniposide (100 mg/kg) or morphine (5 mg/
kg) on the eighth day, 30 min before a formalin challenge. A single 
injection of morphine, but not exenatide or geniposide, blocked the 
formalin-induced acute nociceptive response, whereas morphine, 
geniposide and exenatide all inhibited the tonic flinching response in 
mice treated multidaily with saline (Fig. 6A, B). In the tonic phase, multiple bidaily injections of geniposide and exenatide over 
seven days maintained their antinociceptive efficacies, whereas the 
same long-term regimen of morphine completely abolished its 
antinociception (Fig. 6B). These results indicate that geniposide and 
exenatide, unlike morphine, do not induce antinociceptive toler-
ance after chronic treatment.

**Fig. 8.** The blockade effect of an intrathecal injection of the specific GLP-1R antagonist exendin(9-39) on the antinociceptive effects of geniposidic acid, genipin, genipin methyl ether, 1,10-anhydrogenipin, loganin and catalpol in rats (A, B). Rats received an intrathecal single injection of saline, an iridoid or exendin(9-39) + an iridoid 30 min before a formalin challenge. The nociceptive behavior was quantified by counting the number of times that the formalin-injected paw flinched in 1 min epochs. C. The formalin-induced tonic pain, measured with the AUC10–90 min. The data are presented as mean ± SEM (n = 6 in each group). ^a^b denote a statistically significant difference from the saline control or the corresponding iridoid group, respectively (P < 0.05, measured with a one-way ANOVA, followed by a post-hoc Student–Newman–Keuls test).
3.3. Antinociception by the activation of the spinal GLP-1Rs and subsequent cyclic AMP/PKA signaling pathway

To test whether geniposide produced antinociception via the activation of the spinal GLP-1Rs, a GLP-1R gene silencer (siRNA) (Yin et al., 2010) was used in the rat formalin test. Four groups of intrathecally cannulated rats received multidaily intrathecal injections of 7.5 μg PEI (control vehicle), 5 μg nonspecific oligonucleotide or 5 μg siRNA/GLP-1R for seven continuous days before an intrathecal single injection of saline or geniposide followed by a formalin challenge. The single intrathecal injection of geniposide effectively blocked the formalin-induced tonic pain by 76.7% in PEI-treated rats or 88.0% in nonspecific oligonucleotide-treated rats. The intrathecal injections of siRNA/GLP-1R completely blocked geniposide’s antinociceptive effect (Fig. 7A, B). The rats were sacrificed immediately after the behavior test and spinal homogenates were obtained from lumbar enlargements of the L3–L5 cord for a Western blot. GLP-1R protein expression was significantly reduced by siRNA/GLP-1R (Fig. 7C insert). The GLP-1R/β-actin band intensity ratio was calculated after scanning. Spinal GLP-1R protein expression was reduced 54.0% from the vehicle control and 53.5% from the nonspecific oligonucleotide control by siRNA/GLP-1R (Fig. 7C).

We used the specific orthosteric antagonist exendin(9-39) to verify the action site of geniposide and the other iridoids. Three groups of intrathecally cannulated rats received an intrathecal injection of 10 μl saline, 100 μg geniposide or 2 μg exendin(9-39) + 100 μg geniposide 30 min before a formalin challenge. A previous study showed that 2 μg exendin(9-39) did not alter the formalin-induced flinch response, but completely prevented the antinociceptive effect of exenatide and GLP-1(7-36) in the tonic phase (Gong et al., 2014a). As exhibited in Fig. 7D and E, the intrathecal geniposide injection significantly inhibited the formalin-induced tonic pain by 57%, but did not alter the acute nociceptive response. The intrathecal co-injection of 2 μg exendin(9-39) completely blocked geniposide’s antinociceptive effect. Our results further support the spinal cord as the primary site and suggest that the activation of the spinal GLP-1Rs is responsible for geniposide’s antinociceptive effect.

Eleven groups of intrathecally cannulated rats received an intrathecal injection of 10 μl saline or 100 μg geniposidic acid, genipin, genipin ether, 1,10-anhydrogenipin, loganin or catalpol in the absence or presence of 2 μg exendin(9-39) 30 min before a formalin injection. The intrathecal injection of 100 μg of any of the iridoids markedly inhibited formalin-induced tonic pain by approximately 60%, but did not alter the acute nociceptive response. The intrathecal injection of 2 μg exendin(9-39) completely blocked the iridoids’ antinociceptive effects (Fig. 8A–C). The antinociceptive effects of shanzhishide methylester and 8-O-acetyl shanzhishide methylester were also demonstrated to be completely blocked by exendin(9-39) (Zhu et al., 2014).

The recognition of GLP-1 by its cognate receptors in the pancreatic islets activates a classical signaling cascade through stimulatory G-protein and adenylyl cyclases (Ramos et al., 2008; Fig. 9. The blockade effects of an intrathecal injection of the adenylyl cyclase inhibitor DDA (2’5’-dideoxyadenosine) (A, B) and the protein kinase A inhibitor H89 [N-(2-(p-bromocinnamylamino)ethyl)-5-isouquinolinesulfonamide)] (C, D) on geniposide’s antinociceptive effect in the rat formalin test. Rats received an intrathecal injection of saline, DDA or H89, followed by an intrathecal injection of saline or geniposide 30 min before a subcutaneous injection of formalin. The nociceptive behavior was quantified by counting the number of times that the formalin-injected paw flinched in 1 min epochs. B and D. The formalin-induced tonic pain measured with the AUC10–90 min. The data are presented as mean ± SEM (n = 6 in each group). * and ** denote a statistically significant difference from the saline control or geniposide group, respectively (P < 0.05, measured with a one-way ANOVA, followed by a post-hoc Student-Newman-Keuls test).
Roger et al., 2011). Cellular levels of cyclic AMP increase and PKA activates its downstream effectors, resulting in insulin secretion (Holst, 2007). We tested whether the cyclic AMP/PKA signaling pathway is also involved in geniposide-induced antinoception, by using the adenylyl cyclase inhibitor DDA (Ramos et al., 2008) and the PKA inhibitor H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) (Li et al., 2010). Four groups of rats received an intrathecal injection of 10\(\mu\)l saline or 1\(\mu\)g DDA. Ten minutes later, they received a second intrathecal injection of 10\(\mu\)l saline or 100 \(\mu\)g geniposide, followed 30 min later by a formalin challenge. The intrathecal DDA did not alter either the formalin-induced acute nociceptive response or the tonic pain but completely prevented geniposide’s antinoceptive effect in the tonic phase (Fig. 9A, B).

Four groups of rats received an intrathecal injection of 10\(\mu\)l saline or 10 \(\mu\)g H89. After 10 min, they received a second intrathecal injection of 10\(\mu\)l saline or 100 \(\mu\)g geniposide, followed 30 min later by a formalin challenge. The intrathecal injection of H89 did not alter the formalin-induced acute nociception or tonic pain, but did completely prevent geniposide’s antinoceptive effect in the tonic phase (Fig. 9C, D). These results suggest that spinal geniposide antinoceception is mediated by the activation of the cyclic AMP/PKA signaling pathway secondary to GLP-1R activation, which is consistent with GLP-1 action in insulin release and glucose regulation (Holst, 2007).

4. Discussion

The therapeutic success of peptidic GLP-1R agonists for the treatment of type 2 diabetes mellitus has inspired discovery efforts aimed at developing orally-available small molecule GLP-1R agonists. Although the GLP-1R is a member of the structurally complex class B1 family of GPCRs, there have been increasing reports of structurally diverse small molecule orthosteric and allosteric agonists with intrinsic efficacy for the GLP-1Rs (Wang et al., 2010; Willard et al., 2012). It has been reported that substituted cyclobutanes such as Boc5 and BW4-24 (Chen et al., 2007; Liu et al., 2012) and pyrimidines such as Compound B (Sloop et al., 2010) are orthosteric agonists, whereas 6,7-dichloroquinolines (Irwin et al., 2010; Teng et al., 2007) and Compound 2 (Knudsen et al., 2007) are allosteric agonists or regulators. This study adds our new attempts to this list. We demonstrate that geniposide is similar but less potent than exenatide. It reverses hydrogen peroxide-induced oxidative damage in both rat and human GLP-1R-expressing PC12 and HEK293 cells, but not in HEK293T cells that do not express GLP-1Rs. Exendin(9-39) competitively blocks geniposide’s protective effect with similar pA2 values to those when competing with exenatide. Geniposide 100\% reverses hydrogen peroxide oxidative damage and its maximal spinal antinocepcion of formalin pain is between 60 and 80\%, which identical to the action of exenatide and GLP-1(7-36) (Gong et al., 2014a). Our results confirm and extend the findings of Guo et al. (2012) by demonstrating that geniposide appears to be a reversible orthosteric agonist of GLP-1Rs and possibly acts at the same binding site as exendin(9-39) and exenatide. Our results also suggest that full intrinsic efficacy may not be required for GLP-1R activation that simultaneously binds both the presumed extracellular N-terminal ectodomain and the determinant in the receptor transmembrane region.

This and our previous study (Zhu et al., 2014) tested the iridoid analogs of geniposide, geniposidic acid, genipin methyl ether, 1,10-anhydrogenipin, loganin, catalpol shanzhiside methylester and 8-O-acetyl shanzhiside methylester, using GLP-1R sensitivity assays. The in vitro results and the in vivo antinoceptive effects reveal that all of these compounds similarly activate the GLP-1Rs. If geniposide’s C-1 glycoside moiety is removed, resulting in genipin (Fig. 1), the antinoceptive activity remains the same. Genipin-induced GLP-1R activation is also not significantly altered by replacing the free C1-hydroxyl with a methyl group, forming genipin methyl ether, or by dehydration, forming 1,10-anhydrogenipin. Geniposide-induced GLP-1R activity is not significantly altered by the removal of the free C4-carboxyl group, forming the C4-decarboxylated iridoid catalpol. Although a detailed structure-activity relationship cannot be extrapolated from our results due to the small number of compounds and the low activities, the results do suggest that the iridoid structure represented by geniposide and genipin analogs is a novel core structure for activating GLP-Rs. They can be chemically modified further to obtain iriods with increased activities.

We previously demonstrated that the activation of the spinal GLP-1Rs by peptidic agonists leads to specific antinoceception in chronic pain hypersensitivity states. Spinal GLP-1Rs may represent a promising potential therapeutic target molecule for the management of chronic pain (Gong et al., 2014a). Geniposide and its iridoid analogs produce antinoceception via the same mechanism. Geniposide and its iridoid analogs dose-dependently block formalin-induced tonic pain but not acute nociceptive responses. Our previous results also showed that other two iridoid analogs, shanzhiside and 8-O-acetyl shanzhiside methylester, exhibited specific antinoceception in peripheral nerve injury- and bone cancer-induced mechanical allodynia and in formalin-induced tonic pain (Zhu et al., 2014). The antinoceptive effects of geniposide and its iridoid analogs are produced via the spinal cord, rather than the brain ventral area or peripheral nociceptors. The antinoceptive effects of geniposide and its iridoid analogs are completely prevented by exendin(9-39), the GLP-1R silent siRNA/ GLP-1R and the cyclic AMP/PKA signaling pathway inhibitors (DDA and H89). Geniposide’s in vivo spinal antinoceception appears to be correlated to its GLP-1R-mediated in vitro activity, as supported by the similarity of the potency ratios between geniposide and exenatide’s activation of the GLP-1Rs in the cell protection assay (6500:1) and during direct spinal antinoceception (7400:1). The assessment of geniposide and the other iriods in PC12 and HEK293 cells suggests a direct interaction between these compounds and the spinal GLP-1Rs, rather than via an interaction with a signal transduction pathway after the activation of the GLP-1Rs. Our study using geniposide and its iridoid analogs with chemical structures distinct from exenatide and GLP-1(7-36) further validates the hypothesis that the activation of the spinal GLP-1Rs produces specific antinoceception.

Exenatide and GLP-1(7-36) are oligopeptides and must be administered parenterally. The ED90 value for the subcutaneous injection of exenatide to produce antinoceception is 22.7 \(\mu\)g/kg, roughly 38-folds higher than the 0.6 \(\mu\)g/kg required for its peripheral blood sugar lowering effect (Gong et al., 2011b). The geniposide vs. exenatide potency ratio for spinal antinocepcion is 74,000:1, whereas the ratio for subcutaneous injection is 6200:1. The more than 10-fold-reduction in the potency of exenatide antinocepcion indicates that it does not easily reach its spinal target molecule and that intrathecal or epidural administration may be more desirable. We therefore evaluated the feasibility of geniposide and its iridoid analogs producing antinocepcion via peripheral routes. Although geniposide and its iridoid analogs are less potent GLP-1R agonists in vitro, their subcutaneous injection (ED90: 13.1 mg/kg) and oral gavage (ED90: 52.7 mg/kg with an apparent oral bioavailability of 25% of the subcutaneous route) produce a fairly potent antinocepcion. This is clinically significant, given that gabapentin, a widely-prescribed pain medicine for chronic neuropathic pain and painful diabetic neuropathy, is dosed in a range of 100–200 mg/kg for rats and mice and 900–1200 mg for human patients. Geniposide appears to activate rat and human
GLP-1Rs similarly and long-term exposure to geniposide after chronic treatment does not induce antinociceptive tolerance. Our data therefore suggests that orally-available geniposide and its iridoid analogs are good analgesics lead for further development and for chemical modifications to increase their potency.

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