**In vitro** anti-*Helicobacter pylori* activity of *Extractum liquiritiae*, glycyrrhizin and its metabolites

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**Objectives**: To investigate the *in vitro* activity of *Extractum liquiritiae* (EL), glycyrrhizic acid (GL), glycyrrhetinic acid (GA) and a novel lipophilic derivative of glycyrrhetinic acid monoglucuronide (GAMG), acetylated GAMG (aGAMG), against 29 *Helicobacter pylori* strains.

**Methods**: The MIC of each compound was determined by the agar dilution method, and the killing kinetics were monitored in brain heart infusion broth (10^6–10^7 cfu/mL) at 0, 4, 24, 48, 72 and 96 h.

**Results**: GA was the most potent compound (MIC 50/90, 50/100 mg/L), inhibiting 79.3% of the strains at MIC <_50 mg/L. Clarithromycin-resistant strains were susceptible at 12.5 and 25 mg/L, and metronidazole-resistant strains at 25–50 and at 200 mg/L. The MIC distribution (mg/L) of aGAMG was <_6.25 (29.2%), 50 (4.2%), 100–200 (12.5%) and >_400 (54.1%). EL and GL were less active (MICs >_400 mg/L).

**GA** exhibited rapid, concentration and strain-dependent bactericidal activity.

**Conclusions**: The potent *in vitro* activity of GA against *H. pylori* provides a further explanation for its beneficial effect on peptic ulcers. Its effectiveness against clarithromycin-resistant strains provides hope that it can form the basis for an alternative therapeutic agent against *H. pylori*.

Keywords: *Helicobacter*, liquorice, glycyrrhetinic acid, novel derivatives

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**Introduction**

Liquorice root (*Glycyrrhiza glabra*) has been used since antiquity for its medicinal effects on, among other ailments, peptic ulcers. In the first century A.D., Dioskorides described the use of liquorice syrup for heartburn. In 1946, F. E. Revers and in 1953, J. G. G. Borst first reported its anti-ulcerogenic effect. Glycyrrhizin, the main triterpene saponin of liquorice and a commonly used herbal medicine, and its biologically active metabolite glycyrrhetinic acid (GA), are both known for their anti-inflammatory properties. The structure of glycyrrhizin was identified 1989 as 3-β-D-glucurono-pyranosyl-(1–2)-β-D-glucurono-pyranosyl-glycyrrhetinic acid. Glycyrrhizin is formed from the Ca^2+ and K^+ salts of glycyrrhizic acid (GL). GL is the diglucuronide of 18β-glycyrrhetinic acid. Carbenoxolone (Biogastrone, Sterling Winthrop, London, UK; Uclos Tabilnen Samovania, Berlin, Germany), a hemisuccinate of GA, was developed as an ulcer-liquid and is used to treat gastroduodenal ulcers and gastritis. GL, GA and a number of liquorice flavonoids are reported to exhibit antiviral and antibacterial activity, and appear to inhibit *Helicobacter pylori* growth.

*H. pylori* is known to contribute to a wide spectrum of gastroduodenal diseases (gastric and duodenal ulcers and cancers of the stomach). GL has been used for many years in Japan, where it is reported to reduce the risk of hepatocellular carcinoma and is of clinical interest in the treatment of chronic hepatitis C. The beneficial effects of liquorice and GL on peptic ulcers and other clinical conditions might also be due, in part, to their bactericidal effect on *H. pylori*. Unfortunately, very few data are available to support this thesis. Some recent studies in Japan have attributed anti-*H. pylori* activity to GL. Orally administered GL is mainly absorbed after pre-systemic hydrolysis as GA. There are almost no data on GL metabolites.

The objective of the present study was to investigate the *in vitro* activity and bactericidal effectiveness of liquorice extract, GL, GA and a novel lipophilic derivative of glycyrrhetinic acid monoglucuronide (GAMG), acetylated GAMG (aGAMG), against 29 different *H. pylori* strains.
Materials and methods

Bacterial strains

A total of 29 H. pylori strains [27 non-duplicate isolates from gastric biopsies of patients with gastritis and/or gastroduodenal ulcer and two reference (RF) strains (ATCC 43504 and 49503)] were tested. The isolates were identified by Gram staining and enzymatic activity (catalase, oxidase, urease). The cagA status as a virulence factor had been previously determined in 15 strains by immunofluorescence and/or PCR. Two clarithromycin-resistant (MICs ≥8 mg/L) strains were kindly provided by Prof. M. Kist, Freiburg, Germany. Five strains were resistant to metronidazole (MICs >_32 mg/L). All strains were susceptible (MICs 0.03–0.25 mg/L) to amoxicillin and clarithromycin (except for the two clarithromycin-resistant strains mentioned above). The strains were stored at -20°C until used.

Non-antibiotic compounds

The non-antibiotic compounds tested were liquorice extract of G. glabra [Extractum liquiritiae siccum norm. (EL) consisting of 6.4% GL (Caesar & Lorenz GmbH, Hilden, Germany), GL [3-O-(2-O-β-D-glucopyranosyl-α-D-glucopyranuronosyl)-18-β-glycyrrhetinic acid ammonium salt] (C$_{30}$H$_{46}$O$_{4}$), GA (Enoxolon, Merck, Darmstadt, Germany) and aGAMG. GAMG (50.4 mg) was acetylated yielding 73.3 mg of aGAMG. This acetylated form of GAMG, a more lipophilic derivative, was kindly produced for us by the Institute of Pharmacy of the University of Kiel. Each compound was newly prepared prior to use with a stock solution of 16 000 mg/L for EL, 4000 mg/L for GL and GA and 73 300 and 4000 mg/L (stock solutions A and B, respectively) for aGAMG. EL (range 0.78–400 mg/L), GL and GA (range 3.15–400 mg/L) and aGAMG (range 0.78–400 mg/L) were diluted at least 10-fold to minimize compound carryover to the BHI plates. Viable count determinations of culture controls with either DMSO or NaOH were indistinguishable from the ‘solvent-free control’ values.

MIC determination

The MICs were determined by the standard agar dilution method using BHI agar supplemented with 7% sheep blood containing two-fold serial dilutions of the non-antibiotic compounds [EL (range 3.15–1600 mg/L), seven of 29 strains were tested at a maximal concentration of 400 mg/L); GL and GA (range 3.15–1600 mg/L, seven of 29 strains were tested at a maximal concentration of 400 mg/L); GL and GA (range 3.15–1600 mg/L, seven of 29 strains were tested at a maximal concentration of 400 mg/L); GL and GA (range 3.15–1600 mg/L, seven of 29 strains were tested at a maximal concentration of 400 mg/L)]. The plates were inoculated with a bacterial suspension (~10$^6$–10$^7$ cfu/mL) in BHI broth with a multipoint inoculator (Titertek; Flow Laboratories, Meckenheim, Germany) and a volume of 10 μL per spot. Compound-free BHI agar media were used as controls. Inoculated plates were incubated at 37°C under microaerobic conditions and examined after 3 days. The MIC was defined as the lowest concentration that completely inhibited the development of visible growth on the agar plates and was determined in duplicate for each strain.

Time and dose–kill curves

The killing kinetics of the compounds were determined after exposing the H. pylori strains to concentrations of 400, 800 and 1600 mg/L for EL and GL, or of concentrations of 0.5×, 1×, 2× and 4× MIC for GA and aGAMG, by the microdilution test using BHI broth supplemented with 10% horse serum and 0.25% yeast extract and stored at -70°C until used.

Non-antibiotic compounds

The non-antibiotic compounds tested were liquorice extract of G. glabra [Extractum liquiritiae siccum norm. (EL) consisting of 6.4% GL (Caesar & Lorenz GmbH, Hilden, Germany), GL [3-O-(2-O-β-D-glucopyranosyl-α-D-glucopyranuronosyl)-18-β-glycyrrhetinic acid ammonium salt] (C$_{30}$H$_{46}$O$_{4}$), GA (Enoxolon, Merck, Darmstadt, Germany) and aGAMG. GAMG (50.4 mg) was acetylated yielding 73.3 mg of aGAMG. This acetylated form of GAMG, a more lipophilic derivative, was kindly produced for us by the Institute of Pharmacy of the University of Kiel. Each compound was newly prepared prior to use with a stock solution of 16 000 mg/L for EL, 4000 mg/L for GL and GA and 73 300 and 4000 mg/L (stock solutions A and B, respectively) for aGAMG. EL (range 0.78–400 mg/L), GL and GA (range 3.15–400 mg/L) and aGAMG (range 0.78–400 mg/L) were diluted at least 10-fold to minimize compound carryover to the BHI plates. Viable count determinations of culture controls with either DMSO or NaOH were indistinguishable from the ‘solvent-free control’ values.

MIC determination

The MIC values of EL were >1600 and 800 mg/L for 21 and one of H. pylori strains, respectively. For EL, the maximum concentration was 1600 mg/L for 22 of the 29 tested strains, for all other compounds it was 400 mg/L for all strains.

BHI broth supplemented with 5% calf serum. Seven strains were tested: the RF strain ATCC 49503 and six isolates (two strains were clarithromycin-resistant). Rates of killing were determined in duplicate by measuring the reduction in viable bacteria (log$_{10}$ cfu/mL) by serial dilution and plating onto BHI agar at 0, 4, 24, 48, 72 and 96 h at defined concentrations. The starting inoculum was ~10$^8$–10$^9$ cfu/mL.

The minimum detection level was 10 cfu/mL. Culture samples containing compounds were diluted at least 10-fold to minimize compound carryover to the BHI plates. Viable count determinations of culture controls with either DMSO or NaOH were indistinguishable from the ‘solvent-free control’ values.

Results

MIC determination

GA was the most potent substance (MIC$_{50/90}$, 50/100 mg/L), followed by aGAMG (MIC$_{50/90}$ 400/>400 mg/L), GL and EL (MIC$_{50/90}$ 400/>400 mg/L). GA inhibited 79.3% (23/29) of the strains at ≤50 mg/L. The percentage of inhibition by aGAMG at this concentration was 33.3% [eight of 24 strains (24 of all 29 strains were available at the time of testing aGAMG)]. However, seven of these strains showed a very low MIC of ≤6.25 mg/L. EL and GL were less active, exhibiting high MICs of >400 mg/L against all the strains (Figure 1). The two clarithromycin-resistant strains were inhibited by GA at 12.5 and 25 mg/L; the MIC values of aGAMG against these strains were 400 mg/L. Metronidazole-resistant strains were susceptible to GA at concentrations of 25 and 50 mg/L (one and three strains) and 200 mg/L (one strain). The MICs of aGAMG were 200 and 400 mg/L. No significant difference in the susceptibility was detected between cagA$^+$ and cagA$^-$ strains.

Time and dose–kill curves

Figure 2(a–f) shows the time and dose–kill curves of EL, GL and GA against two selected H. pylori isolates. The three compounds exhibited concentration- and strain-dependent
bactericidal effects. GA (Figure 2c and f) showed the best activity followed by GL (Figure 2b and e) and EL (Figure 2a and d). EL and GL were most effective at the highest concentration (1600 mg/L). A concentration of 800 mg/L either slightly inhibited *H. pylori* growth or had a bactericidal effect within 48–96 h (Figure 2a–b and d–e). At a lower concentration of 400 mg/L, no influence or a slight decrease in cfu/mL was noted. The results were comparable for these compounds against the clarithromycin-resistant isolates and the RF strain (data not shown).

GA generally exhibited rapid killing against all the five strains tested, demonstrating concentration- and strain-dependent bactericidal activity. At 2 × and 4 × MIC, inhibition occurred immediately after the addition of *H. pylori* suspension to the concentrations or within 4 and 24 h. The time to 99.9% killing at 1 × MIC was 4 and 48 h (Figure 2c and f). The sub-MIC [0.5 × MIC, examined only in one strain (*Hp* 287)] achieved an immediate reduction of viable cells of >4 log_{10}, compared with control (Figure 2f). Clarithromycin-resistant strains showed comparable time–kill curves (data not shown).

The killing kinetics of aGAMG was examined on two isolates with significantly different MICs (0.78 and 200 mg/L). aGAMG did not exhibit a bactericidal effect on the strains within 72 h. Higher concentrations of 2 × and 4 × MIC did not lead to a decrease in cfu/mL.

**Discussion**

The medicinal use of liquorice and its main constituent, glycyrhrizin, for peptic ulcers has a long tradition. Orally administered
glycyrrhizin is converted into the active metabolite GA by human intestinal bacteria via β-glucuronidase, followed by its absorption into the blood.1,4,5

Among the compounds studied here, GA was the most active in inhibiting H. pylori, a finding in accordance with previous studies.4 The majority of the strains (~80%) were inhibited at a low concentration of ≤50 mg/L GA, i.e. at concentrations two- to four-fold lower than those reported to be toxic in humans.1 Comparable results were reported previously.5 In contrast, Kim et al.4 described higher MICs of 200 mg/L. It is of interest that GA was also effective against clarithromycin-resistant strains. H. pylori-resistance to clarithromycin is largely responsible for treatment failure.7 GA also exhibited rapid killing of clarithromycin-resistant and -sensitive isolates. Thus, GA may become clinically relevant, particularly for antibiotic-resistant strains. However, more resistant strains have to be studied to confirm these findings.

EL and GL were not effective, even at higher concentrations of 400 and 1600 mg/L, which agrees with previous results.4 These compounds did, however, exhibit a dose- and strain-dependent bactericidal effect in the killing studies, the highest concentration being the most effective. Chung5 reported a similar finding, although they unexpectedly found a very high concentration of 16 mmol GL to be the most effective, with an inhibition of 76–90%. At a four-fold lower concentration (2 mmol), the inhibition of 400 and 1600 mg/L, which is about equivalent to our highest concentration (1600 mg/L) the inhibition of H. pylori reached only about 30%. At this concentration, 99.9% killing was achieved within 4 h in our studies. These discrepancies might be due to differences in the methods used (incubation time and determination of H. pylori growth). GL impairs H. pylori growth by inhibiting arylamine N-acetyltransferase activity.5

aGAMG exhibited lower anti-H. pylori activity than did GA, but higher than either EL or GL. However, it is interesting that aGAMG elicited the most potent activity, with significantly lower MICs (≤6.25 mg/L) for about 30% of the strains. The reason for this is unclear since the mechanisms by which aGAMG inhibits H. pylori are as yet unknown. To the best of our knowledge, these are the first reported results on the anti-H. pylori activity of this novel derivative. It remains for further investigations to determine the significance of this finding. Future studies also have to clarify whether gastric pH values cause the hydrolytic breakdown of aGAMG and whether it is bioavailable within the pH milieu of the gastric mucosa. Hydrolysis of aGAMG in a base pH milieu could account for the absence of in vitro bactericidal activity.

Overconsumption of liquorice and/or its active metabolites GL or GA can produce pseudo-aldosteronism.1,2,8 A maximum dosage of up to 100 mg GL a day is considered to be acceptable and safe. After intravenous injection of an 80 mg dose of GL in healthy volunteers, the maximum GL plasma concentration was 29.3 mg/L. After an oral intake of 100 mg GL, no GL was found in the plasma; GA was found at <200 ng/mL.8 No data exist about the concentration of these compounds in gastric mucosa. Orally administered GL is almost completely hydrolysed into GA in the stomach and absorbed in the intestinal tract. Carbenoxolone (a derivative of GA) has been shown to successfully treat gastric ulcers at dosages of 150–300 mg/day (~123.7–247.4 mg GA/day). Further studies are needed to determine whether doses of this size deliver GA to the gastric mucosa in concentrations at or above the MIC for H. pylori, and to establish whether GA’s bactericidal properties explain its anti-ulcerogenic activity.

In conclusion, the potent in vitro activity of GA against various H. pylori isolates provides a further explanation for its positive effect on peptic ulcers. Its effectiveness against clarithromycin-resistant strains provides hope that it can serve as the basis for an alternative therapeutic agent against these pathogens, which it remains for future in vivo studies to determine.

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References