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RESEARCH ARTICLE

Antiadhesion as a functional concept for prevention of pathogens: *N*-Phenylpropenoyl-L-amino acid amides as inhibitors of the *Helicobacter pylori* BabA outer membrane protein

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Scope: Besides flavan-3-ols, a family of *N*-phenylpropenoyl-L-amino acids (NPAs) has been recently identified as polyphenol/amino acid conjugates in the seeds of *Theobroma cacao* as well as in a variety of herbal drugs. NPAs were shown to exhibit antiadhesive activities against *Helicobacter pylori*.

Methods and results: For structure/activity relationship 24 homologous NPAs (2 mM) were investigated in a flow cytometric assay on potential antiadhesive effects against *H. pylori* adhesion to human gastric AGS cells. Dihydroxylation of the aromatic molecule part was shown to be necessary for activity; methoxylation decreases activity. High polarity of the amino acid is a prerequisite for activity. The model compound *N*-(*E*)-caffeoyl-L-glutamic acid 11 exerted a concentration-dependent inhibition of bacterial adhesion with saturation at 30% inhibition level. The antiadhesive effect was additionally confirmed by in situ adhesion assay on intact human gastric tissue. NPAs exhibited no cytotoxicity. Using immobilized ligands interaction 11 with bacterial adhesin BabA was demonstrated. RT-PCR indicated that the inhibition of BabA is not correlated with subsequent feed back regulations to express more adhesins or virulence factors (*vacA, cagA, cagL, cagα, fucT, ureI, ureA*, OMPs). The interaction of bacterial adhesins with the respective ligands does not automatically lead to a subsequent signal transduction towards induction of virulence processes.

Conclusion: The nutritional use of NPA-containing food may justify a positive antiadhesive effect against the recurrence of *H. pylori* infections.

Keywords:
Adhesion / BabA / Cytoprotection / *Helicobacter pylori* / *N*-phenylpropenoyl-L-amino acids

1 Introduction

N-Phenylpropenoyl-L-amino acids (NPAs), a homologous series of secondary products identified first in cocoa seeds from *Theobroma cacaco* and meanwhile in many other medicinal plants [1, 2] have been shown to exhibit beneficial biological activities. NPAs are untoxic against human epithelial cells under in vitro conditions [1], increase in vitro mitochondrial activity and proliferation rates in human liver cells and epidermal keratinocytes [1], exhibit antioxidant activity [3] and get absorbed in vivo after oral ingestion with unmetabolized excretion via urine [4]. Additionally,

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Abbrevation: Alp, adherence-associated lipoprotein; **AGS cells,** gastric adenocarcinoma epithelial cells; **BabA,** blood group binding adhesion; **cagA,** cytotoxin-associated gene A protein; **NPA,** *N*-phenylpropenoyl-L-amino acid amide; **Oip,** outer inflammatory protein; **OMP,** outer membrane protein; **SabA,** sialic acid-binding adhesion; **VacA,** vacuolating cytotoxin A

inhibitory activity towards the adhesion of *Helicobacter pylori* to human stomach tissue was described recently [1], getting NPA-containing cacao extract for prophylaxis against *H. pylori* infection into the focus of research.

H. pylori, a Gram-negative pathogen, colonizes highly specifically the human gastric mucosa. The organism is perfectly adapted to this environment. Concerning pathology, *H. pylori* causes gastritis and is classified as a major primary risk factor for the development of gastric or peptic ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma [5–7].

Adherence of *H. pylori* to the gastric epithelium has been accepted to be an important virulence factor for the initial and persistent colonization, as well as for the bacterium-to-cell signaling. Intimate contact of *H. pylori* to the gastric epithelium can lead to the exertion of virulence factors as the cytotoxin-associated gene A protein (cagA) and the type IV secretion system (TFSS) encoded in the cag pathogenicity island (cagPAI) [8–12] and the vacuolating cytotoxin A (VacA) [13, 14]. In addition, *H. pylori* flagella and cytoplasmic urease determine the bacterial survival in acidic conditions and therewith the enabling of bacterial adherence to the gastric epithelium [15]. The persistence of *H. pylori* in the stomach is mainly mediated by several outer membrane proteins (OMPs) of the *hop* protein group (*Helicobacter* outer membrane porins) and also by bacterial lipopolysaccharides (LPS) mimicking Lewis blood group antigens, especially Lewis^x [16]. The blood group antigen-binding adhesin (BabA) binds to fucosylated oligosaccharide structures present in H-1 and Lewis^b (Le^b) blood group antigens [17, 18]. BabA also plays as a key role for *H. pylori* binding to MUC5AC and MUC5B, even in non-secretors or those without Le^b and thereby acts as an important factor for the initial colonization [19].

Further, antigens such as sialyl-Lewis^a and sialyl-Lewis^x, which are reported to be predominantly expressed in inflamed gastric tissue, interact and bind to *H. pylori* sialic acid-binding adhesin (SabA) [20, 21]. Such fucosylated and sialylated antigens favour the colonization of *H. pylori* to the gastric mucosa and might even promote the chronicity of infection once gastritis is established [21]. Further, SabA is also reported for polymorphic binding to sialylated glycans, e.g. presented on the erythrocyte membrane, suggesting that SabA is the responsible haemagglutinin for the sialic acid-dependent haemagglutination [22].

A further bacterial adhesin HpaA, a subunit of the *N*-acetylneuraminyllactose-binding fibrillar haemagglutinin can be blocked by the glycoprotein fetuin and 3′-sialyllactose. It seems interesting that exogenous 3′-sialyllactose can even reverse haemagglutination and can detach adhering *H. pylori* from gastric cells [23–25].

A pair of highly homologous OMPs, the adherence-associated lipoproteins A and B (AlpA and AlpB), and others such as HopZ or the outer inflammatory protein (OipA), are also known to be associated with bacterial adherence [26–28]. However, a corresponding receptor for these proteins is known. In addition, interactions of *H. pylori* with extracellular matrix proteins such as laminin, fibronectin and type IV collagen have been described to function as receptors in the gastric region [29–31].

With regard to these interactions and the importance of *H. pylori* adherence in the development of its pathogenicity, a precise comprehension of an inhibition mechanism for new antiadhesive compounds is essential. The life-long eradication of *H. pylori* by antibiotics or the development of prophylaxis by vaccination has shown to be problematic [32–34]. Antiadhesive compounds could alternatively help for a preventive, cytoprotective strategy to control *H. pylori* colonization, especially for prevention of recurrence after antibiotic eradication therapy. Therefore, several NPA homologous (see Fig. 1) have been investigated in this work for their antiadhesive activity and specificity in inhibition of bacterial OMPs.

Despite all knowledge on *H. pylori* attachment to the gastric epithelium, the association and interaction between the individual virulence factors and the adhesion remain controversial [35]. Therefore, the specific interaction of a series of several potentially antiadhesive NPAs **1–26** and their influence on the mRNA expression of several OMPs as well as virulence factor encoding genes *ureA*, *ureI*, *fucT*, *cagA*, *cagα*, *cagL* and *vacA* have been studied within this work.

2 Materials and methods

2.1 Materials

3′-Sialyllactose (NeuAcα_{2-3}Galβ_{1-4}Glc) and fluoresceinisothiocyanate isomer I (FITC) were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals and reagents, if not specified otherwise, were purchased from Merck (Darmstadt, Germany) of analytical quality. Fresh Okra fruit extract, serving within the antiadhesion assays as the positive control [36, 37] was prepared according to Lengsfeld et al. [36]. NPAs **1–3**, **6–8**, **11**, **13**, **20** and **25** were synthesized and checked for purity (>98%) following the procedure reported recently [2]. Following the same synthetical strategy, the NPAs **4**, **5**, **9**, **10**, **12**, **14–19**, **21–24** and **26** were synthesized and their structure and purity (>98%) were confirmed by means of LC-MS and NMR spectroscopy. All NPAs are easily soluble in water and methanol. Physical data are described in [2]; stability of NPAs and the time of storage were ensured by HPLC analysis.

2.2 Spectroscopic data

N-(*E*)-*m*-Coumaroyl-L-aspartic acid **4** (Fig. 1): UV/VIS (MeOH/0.1% HCOOH, 1/1, v/v) λ_{max} = 216, 231, 278, 319 nm; exact mass: *m/z* 278.0674$^-$ (calculated for [$C_{13}H_{13}NO_6$-H]$^-$: 278.0670$^-$); LC/MS (ESI$^+$): *m/z* 279.9 (100, [M+H]$^+$), 302.0 (98, [M+Na]$^+$), 581.4 (95, [2M+H]$^+$); MS/MS 280 (ESI$^+$, CE 30 V): *m/z* 147 (100%), 280 (70%), 119 (25%); ^{1}H NMR (500 MHz, d_3-MeOD, COSY) δ 2.87

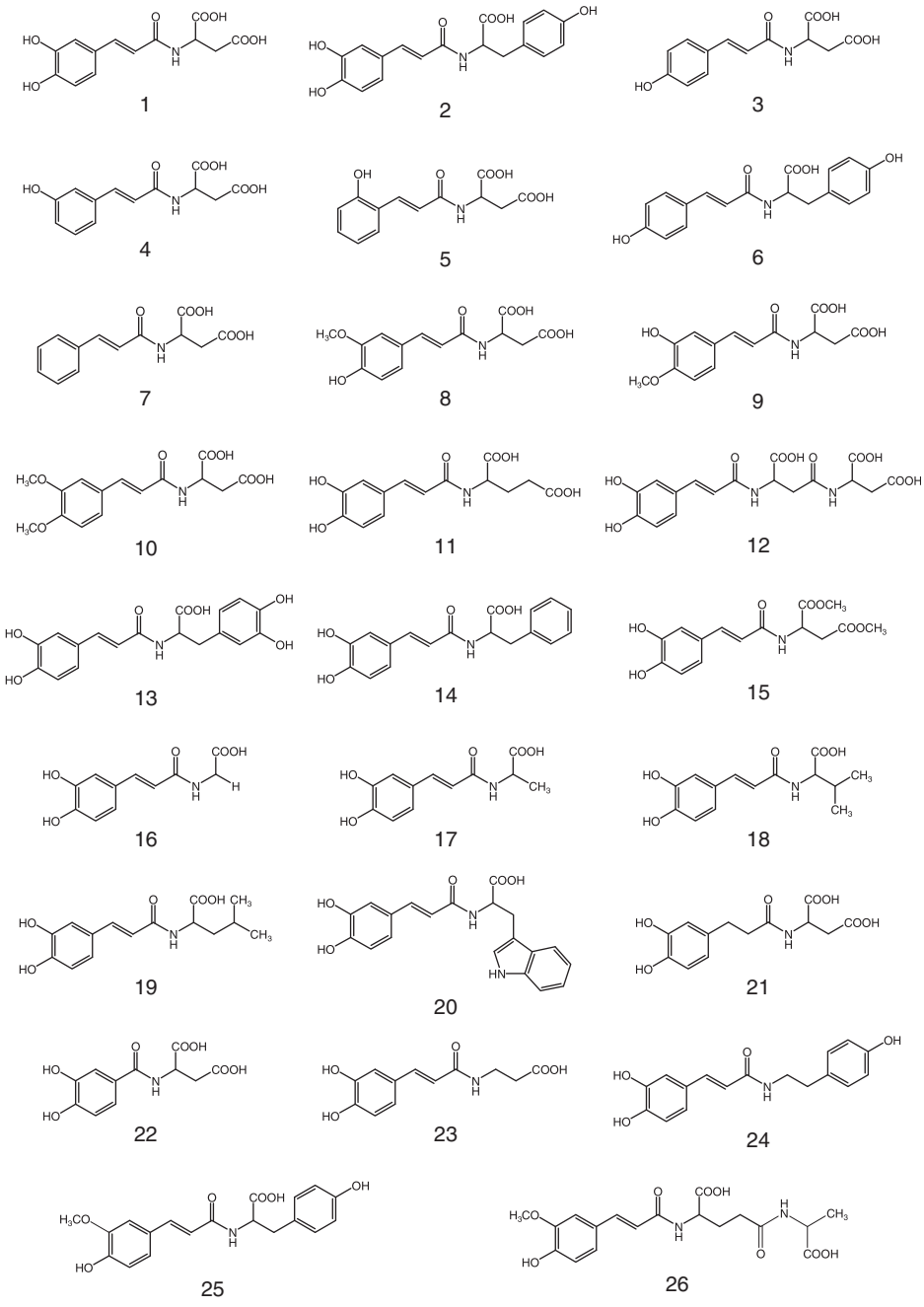


Figure 1. Chemical structures of NPA amides (**1–26**).

[dd, 1H, $J = 6.8$, 16.8 Hz, H-C(3α)], 2.92 [dd, 1H, $J = 5.3$, 16.8 Hz, H-C(3β)], 4.88 [dd, 1H, $J = 5.4$, 6.8 Hz H-C(2)], 6.64 [d, 1H, $J = 16.8$ Hz, H-C(8′)], 6.80 [ddd, 1H, $J = 2.3$, 2.4, 8.0 Hz, H-C(4′)], 6.98 [pt, 1H, $J = 1.8$, 2.0 Hz, H-C(2′)], 7.03 [d, 1H, $J = 7.7$ Hz, H-C(6′)], 7.20 [pt, 1H, $J = 7.8$, 7.9 Hz, H-C(5′)], 7.48 [d, 1H, $J = 16.8$ Hz, H-C(7′)]; ^{13}C NMR (125 MHz, d_3-MeOD, HMQC, HMBC): δ 37.2 [C-3], 50.6 [C-2], 115.3 [C-2′], 118.2 [C-4′], 120.6 [C-6′], 121.3 [C-8′], 131.1 [C-5′], 137.7 [C-1′], 142.8 [C-7′], 159.1 [C-3′], 168.6 [C-9′], 174.1 [C-4/1], 174.2 [C-1/4].

N-(*E*)-O-Coumaroyl-L-aspartic acid **5** (Fig. 1): UV/VIS (MeOH) $\lambda_{max} = 220$, 272, 324 nm; exact mass: *m/z* 302.0634$^+$ (calculated for $[C_{13}H_{13}O_6N+Na]^+ = 290.0635^+$); LC/MS (ESI$^+$): *m/z* 147 (100%), 280 (74% $[M+H]^+$), 302 (29% $[M+Na]^+$), 581 (12% $[2M+Na]^+$); MS/MS 280 (ESI$^+$, CE 30 V) 147 (100%), 280 (34% $[M+H]^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.62 [dd, 1H, $J = 7.2$, 16.6 Hz, H-C(3α)], 2.73 [dd, 1H, $J = 5.8$, 16.6 Hz, H-C(3β)], 4.63 [dd, 1H, $J = 6.8$, 7.0 Hz, H-C(2)], 6.74 [d, 1H, $J = 16.0$ Hz, H-C(8′)], 6.83 [pt, 1H, $J = 7.5$, H-C(5′)], 6.89 [d, 1H, $J = 8.2$ Hz, H-C(3′)], 7.19 [dt, 1H, $J = 1.6$, 7.8, 8.0 Hz, H-C(4′)], 7.43 [dd, 1H, $J = 1.0$, 7.6 Hz, H-C(6′)], 7.64 [d, 1H, $J = 16.0$ Hz, H-C(7′)], 8.34 [d, 1H, $J = 8.0$ Hz, H-N]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 36.1 [C(3)],

48.7 [C(2)], 115.9 [C(3′)], 119.3 [C(5′)], 121.0 [C(1′), 121.5 [C(8′)], 128.1 [C(6′)], 130.5 [C(4′)], 135.0 [C(7′)], 156.2 [C(2′)], 165.3 [C(9′)], 171.5 [C(4)], 172.3 [C(1)].

N-(*E*)-Isoferuoyl-L-aspartic acid **9** (Fig. 1): UV/VIS (MeOH/0.1% HCOOH, 1/1, v/v) λ_{max} = 219, 303 nm; LC/MS (ESI^+): *m/z* 177 (100, $[M-132]^+$), 332 (58, $[M+Na]^+$), 310 (8, $[M+H]^+$); MS/MS 310 (ESI^+, CE 20 V): 177 (100%), 117.0 (31%), 145 (24%), 149 (18%), 134 (15%) 310 (2%); exact mass: *m/z* 332.0735^+ (calculated for $C_{14}H_{15}NO_7Na^+$ 332.0741^+); ^{1}H NMR (400 MHz, d_3-MeOD, COSY): δ 2.90 [2xdd, 2H, *J* = 5.2, 6.4, 16.4 Hz, H-C(3α, β)], 3.88 [s, 3H, H-C(10′)], 4.87 [dd, 1H, H-C(2)], 6.51 [d, 1H, *J* = 15.6 Hz, H-C(8′)], 6.93 [d, 1H, *J* = 8.4 Hz, H-C(5′)], 7.04 [dd, 1H, *J* = 2.0, 8.0 Hz, H-C(6′)], 7.08 [d, 1H, *J* = 2.0 Hz, H-C(2′)], 7.44 [d, 1H, *J* = 15.6 Hz, H-C(7′)]; ^{13}C NMR (100 MHz, MeOD): δ 37.3 [CH_2, C(3)], 50.6 [CH, C(2)], 56.5 [CH_3, C(10′)], 112.7 [CH, C(5′)], 114.6 [CH, C(2′)], 118.7 [CH, C(8′)], 122.6 [CH, C(6′)], 129.3 [C, C(1′)], 142.6 [CH, C-7′], 147.6 [HO-C, C(3′)], 151.0 [HO-C, C(4′)], 168.9 [CO, C(9′)], 171.8 [COO, C(4)], 174.7 [COO, C(1)].

N-(*E*)-3,4-Dimethoxy-cinnamoyl-L-aspartic acid **10** (Fig. 1): UV/VIS (MeOH) λ_{max} = 296, 324 nm; exact mass: *m/z* 346.0900^+ (calculated for $[C_{15}H_{17}O_7N+Na]^+$ = 346.0897^+); LC/MS (ESI^+): *m/z* 191 (100% $[M-132]^+$), 163 (48% $[M-160]^+$), 324 (29% $[M+H]^+$); MS/MS 324 (ESI^+, CE 25 V) 191 (100%), 324 (31%), 163 (15%); ^{1}H-NMR (400 MHz, DMSO-d_6, COSY) δ 2.66 [dd, 1H, *J* = 7.0, 16.6 Hz, H-C(3α)], 2.74 [dd, 1H, *J* = 5.5, 16.6 Hz, H-C(3β)], 3.77 [s, 3H, H-C(3′a/4′a)], 3.79 [s, 3H, H-C(4′a/3′a)], 4.65 [m, 1H, *J* = 5.7, 7.1, 7.8 Hz, H-C(2)], 6.65 [d, 1H, *J* = 15.6 Hz, H-C(8′)], 6.98 [d, 1H, *J* = 8.4 Hz, H-C(5′)], 7.11 [dd,1H, *J* = 1.8, 8.4 Hz, H-C(6′)], 7.17 [d, 1H, *J* = 1.8 Hz, H-C(2′)], 7.36 [d, 1H, *J* = 15.6 Hz, H-C(7′)], 8.28 [d, 1H, *J* = 8.0 Hz, H-N]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 36.1 [C(3)], 48.7 [C(2)], 55.3 [C(3′a/4′a)], 55.5 [C(4′a/3′a)], 109.9 [C(2′)] 111.7 [C(5′)], 119.4 [C(8′), 121.5 [C(6′)], 127.6 [C(1′)], 139.3 [C(7′)], 148.8 [C(4′)], 150.1 [C(3′)], 165.0 [C(9′)], 171.6 [C(4)], 172.4 [C(1)].

N-(*E*)-Caffeoyl-L-asparagyl-L-aspartic acid **12** (Fig. 1): exact mass: *m/z* 433.0844^+ (calculated for $[C_{17}H_{18}O_{10}N_2+Na]^+$ = 433.0854^+); MS (ESI^+): *m/z* 433 (100%M+Na^+), 163 (98%), 249 (50%), 411 (35%); MS/MS 411 (ESI^+, CE 20 V) 163 (100%), 135 (48%), 117 (46%), 145 (36%), 198 (23%), 278 (15%); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ2.76dd, 1H, *J* = 8.0, 16.8 Hz, H-C(7α), 2.86dd, 2H, *J* = 5.6 Hz, H-C(3), 2.91dd, 1H, *J* = 5.6, 16.8 Hz, H-C(7β), 4.74t, 2H, *J* = 5.6 Hz, H-C(2,6), 6.43d, 1H, *J* = 15.6 Hz, H-C(8′), 6.76d, 1H, *J* = 8.4 Hz, H-C(5′), 6.92dd, 1H, *J* = 2.0, 8.4 Hz, H-C(6′), 7.02d, 1H, *J* = 2.0 Hz, H-C(2′), 7.43d, 1H, *J* = 15.6 Hz, H-C(7′); ^{13}C-NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ36.7C(3), 36.9C(7), 50.2C(2), 51.4C(6), 115.2C(2′), 116.5C(5′), 117.7C(8′), 122.4C(6′), 128.3C(1′), 143.3C(7′), 146.8 [C(3′)], 149.0C(4′), 169.2C(9′), 173.0C(5), 173.7 [C(1), 174.0C(4,8)

N-(*E*)-Caffeoyl-L-phenylalanine **14** (Fig. 1): UV/VIS (MeOH) λ_{max} = 322 nm; exact mass: *m/z* 350.1002^+ (calculated for $[C_{18}H_{17}O_5N_1+Na]^+$ = 350.0998^+); MS (ESI^+): *m/z* 350 (100% $[M+Na]^+$), 328 (32% $[M+H]^+$), 163 (12%); MS/MS 328 (ESI^+, CE 20 V) 163 (100%), 328 (23% $[M+H]^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.91 [dd, 1H, *J* = 9.4, 13.8 Hz, H-C(7α)], 3.11 [dd, 1H, *J* = 4.6, 13.8 Hz, H-C(7β)], 4.53 [m, 1H, *J* = 4.8, 8.6, 8.8 Hz, H-C(8)], 6.40 [d, 1H, *J* = 15.8 Hz, H-C(8′)], 6.73 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.82 [dd, 1H, *J* = 1.6, 8.0 Hz, H-C(6′)], 6.93 [d, 1H, *J* = 1.6 Hz, H-C(2′), 7.17–7.29 [m, 6H, H-C(2,3,4,5,6,7′)], 8.25 [d, 1H, *J* = 8.0 Hz, H-N]; ^{13}C-NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 37.4 [C(7)], 54.2 [C(8)], 114.3 [C(2′)], 116.2 [C(5′)], 118.4 [C(8′)], 121.0 [C(6′)] 126.7 [C(1′)], 126.8 [C(4)], 128.6 [C(3,5)], 129.6 [C(2,6)], 138.3 [C(1)], 140.1 [C(7′)], 146.0 [C(4′)], 147.9 [C(3′)] 165.8 [C(9′)], 173.7 [C(9)].

N-(*E*)-Caffeoyl-L-aspartic acid dimethylester **15** (Fig. 1): exact mass: *m/z* 346.0897^+ (calculated for $[C_{15}H_{17}O_7N+Na]^+$ = 346.0897^+); LC/MS (ESI^+): *m/z* 324 (100% $[M+H]^+$), 364 (45% $[M+Na]^+$), 362 (32% $[M+K]^+$); MS/MS 324 (ESI^+, CE 20 V) 163 (100%), 310 (60% $[M+H]^+$), 135 (22%), 117 (20%), 145 (15%); ^{1}H-NMR (400 MHz, DMSO-d_6, COSY) δ 2.76 [d, 1H, *J* = 7.2, 16.8 Hz, H-C(3α)], 2.86 [dd, 1H, *J* = 5.8, 16.8 Hz, H-C(3β)], 3.62 [s, 3H, H-C(5)], 3.64 [s, 3H, H-C(6)], 4.75 [ddd, 1H, *J* = 5.8, 7.2, 8.0, H-C(2)], 6.39 [d, 1H, *J* = 16.0 Hz, H-C(8′)], 6.76 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.86 [dd, 1H, *J* = 2.0, 8.0 Hz, H-C(6′)], 6.96 [d, 1H, *J* = 2.0 Hz, H-C(2′)], 7.27 [d, 1H, *J* = 16.0 Hz, H-C(7′)], 8.50 [d, 1H, *J* = 8.0 Hz, H-N]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 36.2 [C(3)], 49.1 [C(2)], 52.2 [C(5/6)], 52.7 [C(6/5)], 114.4 [C(2′)] 116.2 [C(5′)], 117.9 [C(8′), 121.1 [C(6′)], 126.6 [C(1′)], 140.8 [C(7′)], 146.0 [C(3′)], 148.1 [C(4′)], 165.8 [C(9′)], 171.1 [C(4)], 171.8 [C(1)].

N-(*E*)-Caffeoyl-glycine **16** (Fig. 1): UV/VIS (MeOH/0.1% HCOOH, 77/23, v/v) λ_{max} = 204, 219, 235, 290, 298, 321 nm; exact mass: *m/z* 260.0530^+ (calculated for $C_{11}H_{11}NO_{10}Na^+$ 260.0529^+); MS (ESI^+): *m/z* 238 (100%M+H^+), 260 (30%M+Na^+); MS/MS 238 (ESI^+, CE 20 V): *m/z* 163 (100%), 238 (5%); ^{1}H NMR (400 MHz, d_3-MeOD, COSY): δ4.02s, 2H, H-C(2), 6.43d, 1H, *J* = 15.68 Hz, H-C(8′), 6.77d, 1H, *J* = 8.13 Hz, H-C(6′), 6.92dd, 1H, *J* = 1.82, 8.24 Hz, H-C(6′), 7.02d, 1H, *J* = 1.82 Hz, H-C(2′), 7.42d, 1H, *J* = 15.86 Hz, H-C(7′); ^{13}C NMR (100 MHz, d_3-MeOD, HMQC, HMBC): δ42.1C(2), 115.3C(2′), 116.6C(5′), 118.0C(8′), 122.4C(6′), 128.4C(1′), 143.0C(7′), 146.9C(3′), 149.0C(4′), 169.7C(9′), 173.3C(1).

N-(*E*)-Caffeoyl-L-alanine **17** (Fig. 1): UV/VIS (MeOH) λ_{max} = 322 nm; exact mass *m/z* 274.0680^+ (calculated for $[C_{12}H_{13}O_5N_1+Na]^+$ = 274.0685^+); MS (ESI^+): *m/z* 274 (100% $[M+Na]^+$), 163 (50%), 290 (23%), 252 (12% $[M+H]^+$); MS/MS 252 (ESI^+, CE 13 V) 163 (100%), 252 (22% $[M+H]^+$), 89 (5%); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 1.30 [d, 3H, *J* = 7.3 Hz, H-C(3)], 4.30 [dq, 1H, *J* = 7.3 Hz, H-C(2)], 6.40 [d, 1H, *J* = 15.6 Hz, H-C(8′)], 6.74 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 6.84 [dd,1H, *J* = 2.0, 8.0 Hz, H-C(6′)], 7.00 [d, 1H, *J* = 2.0 Hz, H-C(2′)], 7.24 [d, 1H, *J* = 15.6 Hz, H-C(7′)], 8.26 [d, 1H, *J* = 7.3 Hz, H-N]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 17.0 [C(3)],

46.9 [C(2)], 113.1 [C(2′)] 115.1 [C(5′)], 117.3 [C(8′), 119.8 [C(6′)], 125.6 [C(1′)], 138.9 [C(7′)], 144.8 [C(4′)], 146.7 [C(3′)], 164.4 [C(9′)], 174.1 [C(1)].

N-(*E*)-Caffeoyl-L-valine **18** (Fig. 1): UV/VIS (MeOH/0.1% HCOOH, 40/60, v/v) λ_{max} = 204, 221, 239, 297, 323 nm; exact mass: *m/z* 302.1009$^+$ (calculated for $C_{14}H_{17}NO_5Na^+$ 302.0999 $^+$); MS (ESI$^+$): *m/z* 280 (100%M+H$^+$), 302 (75%M+Na$^+$); MS/MS 280 (ESI$^+$, CE 20 V): *m/z* 163 (100%), 145 (10%), 280 (5%); ^{1}H NMR (400 MHz, DMSO-d_6, COSY): δ0.90d, 3H, *J* = 6.8 Hz, H-C(4), 0.92d, 3H, *J* = 6.8 Hz, H-C(5), 2.09m, 1H, *J* = 6.8, 6.8 Hz, H-C(3), 4.27dd, 1H, *J* = 5.7, 8.6 Hz, H-C(2), 6.57d, 1H, *J* = 15.7 Hz, H-C(8′), 6.75d, 1H, *J* = 8.1 Hz, H-C(5′), 6.85dd, 1H, *J* = 1.9, 8.2 Hz, H-C(6′), 6.96d, 1H, *J* = 1.9 Hz, H-C(2′), 7.24d, 1H, *J* = 15.7 Hz, H-C(7′), 8.09d, 1H, *J* = 8.6 Hz, H-C(NH), ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC): 18.1C(5), 19.2C(4), 29.9C(3), 57.3C(2), 113.9C(2′), 115.8C(5′), 118.2C(8′), 120.5C(6′), 126.4C(1′), 139.7C(7′), 145.5C(3′), 147.4C(4′), 165.6C(9′), 173.2C(1).

N-(*E*)-Caffeoyl-L-leucine **19** (Fig. 1): UV/VIS (MeOH/0.1% HCOOH, 42/58, v/v) λ_{max} = 204, 221, 240, 297, 323 nm; exact mass: *m/z* 316.1159$^+$ (calculated for $C_{15}H_{19}NO_5Na^+$ 316.1155$^+$); MS (ESI$^+$): *m/z* 294 (100%M+H$^+$), 316 (80%M+Na$^+$); MS/MS 294 (ESI$^+$, CE 20 V): *m/z* 163 (100%), 294 (5%); ^{1}H NMR (400 MHz, DMSO-d_6, COSY): 0.86d, 3H, *J* = 6.45 Hz, H-C(5/6), 0.91d, 3H, *J* = 6.45 Hz, H-C(6/5), 1.55m, 1H, *J* = 5.1, 5.8 Hz, H-C(3), 1.65m, 1H, *J* = 6.0, 6.1, 6.4, 6.7, 7.5 Hz, H-C(4), 4.34ddd, 1H, *J* = 5.8, 6.4 6.5, 8.0 Hz, H-C(2), 6.41d, 1H, *J* = 15.7 Hz, H-C(8′), 6.75d, 1H, *J* = 8.0 Hz, H-C(5′), 6.84d, 1H, *J* = 8.0 Hz, H-C(6′), 6.94brs, 1H, H-C(2′), 7.24d, 1H, *J* = 15.7 Hz, H-C(7′), 8.20d, 1H, *J* = 8.0 Hz, H-C(NH); ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC): δ21.3C(5/6), 22.9C(6/5), 24.4C(4), 39.5C(3), 50.3C(2), 113.8C(2′), 115.8C(5′), 118.0C(8′), 120.5C(6′), 126.3C(1′), 139.6C(7′), 145.5C(3′), 147.4C(4′), 165.4C(9′), 174.3C(1).

N-Dihydro-caffeoyl-L-aspartic acid **21** (Fig. 1): UV/VIS (MeOH) λ_{max} = 236, 284 nm; exact mass: *m/z* 320.0740$^+$ (calculated for $[C_{13}H_{15}O_7N_1+Na]^+$ = 320.0740$^+$); MS (ESI$^+$): *m/z* 298 (100% [M+H]$^+$), 320 (62% [M+Na]$^+$), 134 (24%), 336 (16% [M+K]$^+$), 165 (10%); MS/MS 298 (ESI$^+$, CE 20 V) 134 (100%), 165 (40%), 298 (10% [M+H]$^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.31 [m, 2H, *J* = 6.2, 7.3 Hz, H-C(8)], 2.53 [dd, 1H, *J* = 7.0, 16.6 Hz, H-C(3α)], 2.60 [m, 2H, *J* = 7.3 Hz, H-C(7)], 2.66 [dd, 1H, *J* = 6.0, 16.6 Hz, H-C(3β)], 4.53 [m, 1H, *J* = 6.8, 7.4 Hz, H-C(2)], 6.43 [dd, 1H, *J* = 1.8, 8.0 Hz, H-C(6′)], 6.56 [d, 1H, *J* = 1.8 Hz, H-C(2′)], 6.60 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 8.28 [d, 1H, *J* = 8.0 Hz, H-N]; ^{13}C-NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 36.1 [C(3)], 48.7 [C(2)], 109.9 [C(2′)] 111.7 [C(5′)], 119.4 [C(8′), 121.5 [C(6′)], 127.6 [C(1′)], 139.3 [C(7′)], 148.8 [C(4′)], 150.1 [C(3′)], 165.0 [C(9′)], 171.6 [C(4)], 172.4 [C(1)].

N-3,4-Dihydroxybenzoyl-L-aspartic acid **22** (Fig. 1): exact mass: *m/z* 292.0403$^+$ (calculated for $[C_{11}H_{11}O_7N+Na]^+$ = 292.0427$^+$); LC/MS (ESI$^-$): *m/z* 268 (100% [M-H]$^-$), 174 (75%), 132 (50%), 153 (33%); MS/MS 268 (ESI$^-$, CE −20 V) 132 (100%), 153 (20%), 268 (10%); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.43 [dd, 1H, *J* = 3.4, 15.8 Hz, H-C(3α)], 2.66 [dd, 1H, *J* = 9.8, 15.8 Hz, H-C(3β)], 4.36 [dd, 1H, *J* = 3.4, 15.8 Hz, H-C(2)], 6.77 [d, 1H, *J* = 8.2 Hz, H-C(5′)], 7.16 [dd,1H, *J* = 2.2, 8.2 Hz, H-C(6′)], 7.24 [d, 1H, *J* = 2.2 Hz, H-C(2′)], 8.34 [s, 1H, H-N]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC): 39.6 [C(3)], 49.4 [C(2)], 115.0 [C(2′)] 115.4 [C(5′)], 119.4 [C(6′), 125.8 [C(1′)], 146.1 [C(4′)], 148.1 [C(3′)], 165.8 [C(7′)], 174.2 [C(4)], 174.4 [C(1)].

N-(*E*)-Caffeoyl-β-alanine **23** (Fig. 1): UV/VIS (MeOH/0.1% HCOOH, 83/17, v/v) λ_{max} = 204, 218, 239, 300, 325 nm; exact mass: *m/z* 274.0688$^+$ (calculated for $[C_{12}H_{13}NO_5+Na]^+$: 274.0686$^+$); LC/MS (ESI$^+$): *m/z* 252 (100% [M+H]$^+$), 274 (22% [M+Na]$^+$); MS/MS 252 (ESI$^+$, CE 20 V): *m/z* 163 (100%), 252 (13%); ^{1}H NMR (400 MHz, d_3-MeOD, COSY) δ 2.58 [t, 2H, *J* = 6.7 Hz, H-C(2)], 3.54 [t, 2H, *J* = 6.7 Hz, H-C(3)], 6.36 [d, 1H, *J* = 15.7 Hz, H-C(8′)], 6.77 [d, 1H, *J* = 8.2 Hz, H-C(5′)], 6.90 [dd, 1H, *J* = 2.0, 8.2 Hz, H-C(6′)], 7.01 [d, 1H, *J* = 2.0 Hz, H-C(2′)], 7.40 [d, 1H, *J* = 15.7 Hz, H-C(7′)]; ^{13}C NMR (100 MHz, d_3-MeOD, HMQC, HMBC): δ 34.9 [C-2], 36.7 [C-3], 115.2 [C-2′], 116.6 [C-5′], 118.3 [C-8′], 122.3 [C-6′], 128.4 [C-1′], 142.5 [C-7′], 146.8 [C-3′], 148.9 [C-4′], 169.5 [C-9′], 175.5 [C-1].

N-(*E*)-Caffeoyl-tyramine **24** (Fig. 1): UV/VIS (MeOH) λ_{max} = 228, 296, 322 nm; exact mass: *m/z* 322.1055$^+$ (calculated for $[C_{17}H_{17}O_4N_1+Na]^+$ = 322.1049$^+$); MS (ESI$^+$): *m/z* 322 (100% [M+Na]$^+$), 300 (74% [M+H]$^+$), 121 (33% [M-178]$^+$), 163 (22% [M-136]$^+$), 338 (10% [M+K]$^+$); MS/MS 300 (ESI$^+$, CE 25 V) 163 (100%), 121 (42%), 300 (7%); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.68 [t, 2H, *J* = 7.5 Hz, H-C(7)], 3.34 [m, 2H, *J* = 6.8, 13.8 Hz, H-C(8)], 6.67 [d, 2H, *J* = 8.4 Hz, H-C(3,5)], 6.74 [d, 1H, *J* = 8.2 Hz, H-C(5′)], 7.00 [d, 2H, *J* = 8.4 Hz, H-C(2,6)], 7.15 [dd, 1H, *J* = 2.0, 8.2 Hz, H-C(6′)], 7.25 [d, 1H, *J* = 2.0 Hz, H-C(2′)], 8.16 [t, 1H, *J* = 5.8 Hz, H-N], 9.10, 9.16, 9.33 [3xbrs, 3H, HO-C(3′,4′,4);^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 34.4 [C(7)], 41.1 [C(8)], 114.7 [C(2′)], 114.9 [C(5′)], 115.0 [C(3,5)], 118.7 [C(6′)], 125.9 [C(1′)], 129.4 [C(2,6)], 129.6 [C(1)], 144.7 [C(4′)], 148.1 [C(3′)], 155.5 [C(4)] 165.2 [C(9′)].

N-(*E*)-Feruoyl-α-L-glutamyl-L-alanine **26** (Fig. 1): exact mass: 417.1259$^+$ (calculated for $[C_{18}H_{22}O_8N_2+Na]^+$ = 417.1268$^+$); LC/MS (ESI$^+$): *m/z* 417 (100% [M+H]$^+$); 177 (90%); MS/MS 417 (ESI$^+$, CE 30 V) 177 (100%), 145 (62%), 117 (41%), 417 (9%); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 1.43 [d, 3H, *J* = 7.2 Hz, H-C(3)], 1.99 [m, 1H, *J* = 4.4, 6.4, 8.4 Hz, H-C(6α)], 2.17 [m, 1H, *J* = 6.0, 8.0, 14.0 Hz, H-C(6β)], 2.48 [m, 2H, *J* = 6.4, 8.0, H-C(7α,β)], 3.89 [s, 3H, *J* = 7.3 Hz, H-C(10′)], 4.40 [q, 1H, *J* = 7.2, H-C(2)], 6.52 [d, 1H, *J* = 16.0 Hz, H-C(8′)], 6.80 [d, 1H, *J* = 8.0 Hz, H-C(5′)], 7.04 [dd, 1H, *J* = 2.0, 8.0 Hz, H-C(6′)], 7.14 [d, 1H, *J* = 2.0 Hz, H-C(2′)], 7.47 [d, 1H, *J* = 15.6 Hz, H-C(7′)]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC): δ 17.6 [C(3)], 28.8 [C(6)], 31.2 [C(7)], 49.5 [C(2)], 54.0 [C(5)], 56.4 [C(10)], 111.7 [C(2′)] 116.5 [C(5′)], 118.3 [C(8′), 123.5 [C(6′)], 128.3 [C(1′)], 142.9 [C(7′)], 149.3 [C(3′)], 150.1 [C(4′)], 169.1 [C(9′)], 173.6 [C(4)], 175.9 [C(1)], 176.7 [C(8)].

2.3 HPLC-MS/MS (for characterization of the NPAs)

Agilent 1200 HPLC-system connected to the API 4000QTrap LC-MS/MS (Applied Biosystems, Darmstadt, Germany) running in the negative electrospray ionization (ESI^-) mode. Zero-grade air served as the nebulizer gas (45 psi) and as turbo gas (400°C) for solvent drying (55 psi). Nitrogen served as the curtain (20 psi) and collision gas (4.5×10^{-5} Torr). Both quadrupoles were set at unit resolution. ESI^+ mass and product ion spectra were acquired with direct flow infusion. For ESI^+, the ion spray voltage was set at +5500 V in the positive mode and −4500 in the negative mode. The MS/MS parameters were tuned for each individual compound, detecting the fragmentation of the $[M+H]^+$ or $[M\text{-}H]^-$ molecular ions into specific product ions after collision with nitrogen (4.0×10^{-5} Torr).

2.4 LC-time-of-flight mass spectrometry (LC/TOF-MS) (for characterization of the NPAs)

Mass spectra of the compounds were measured on a Bruker Micro-TOF mass spectrometer (Bruker Daltronics, Bremen, Germany) with flow injection and on a Quattro LCZ mass spectrometer (Waters-Micromass, Manchester, UK) with NanoSpray interface and referenced on sodium formate and polyethylene glycol (PEG) 600, respectively. The compounds were dissolved in methanol and mixed with a solution of sodium formate (5 mM) in aqueous methanol to measure the exact mass of the sodium adducts.

2.5 NMR

^1H, *gs*COSY, *gs*HMQC, *gs*HSQC, *gs*HMBC and ^{13}C NMR measurements were performed on a DMX 400 spectrometer (Bruker, Rheinstetten, Germany) as well as on an Avance 3 DRX 500 MHz spectrometer. Chemical shifts were referenced to the solvent signal. Data processing was performed by using the Topspin Version 1.3 and MestReNova version 5.2.3 software (Mestrelab Research, Santiago de Compostela, Spain).

2.6 Bacteria and growth conditions

H. pylori ATCC 700824 (strain J99, identification for quality control by PCR for vacA, cacA genes) was cultivated for two or three passages to minimize the risk of phase-variable switching of OMP genes. Cultivation was performed according to Niehues et al. [37].

2.7 Cell culture

Human adherent gastric adenocarcinoma epithelial cells (AGS, ATCC CRL-1730) were kindly provided by Prof. W. Beil (Medizinische Hochschule Hannover, Germany). Cells were grown as described by Niehues et al. [37].

2.8 Labeling of bacteria, *H. pylori* adhesion assays, dot blot overlay assay

The respective assays have been described in detail in [37, 47].

2.9 Haemagglutination assay

Erythrocyte suspension (2%) from fresh human EDTA-blood (blood group O^+) in PBS was prepared. Fresh agar-grown *H. pylori* (48 h) were harvested and resuspended in PBS. Before incubating with erythrocytes, bacteria were pretreated with test compounds and PBS as untreated control (optical density OD 2.0). Suspensions were incubated for 2 h in dark, followed by three times washing with PBS-Tween® 20 and resuspension in 250 μL PBS. Nearly, 50 μL of these solutions were transferred into 96-well plates, followed by serial dilution in PBS. Nearly, 50 μL of the erythrocyte suspension was added to each well. The plate was gently shaken for 5 min and incubated for 1 h at room temperature. Haemagglutination was measured by MicroWin® 2000/4.38 (Microtek Labsystems, Overath, Germany). As positive control the total fraction of acidic human milk oligosaccharides at 1.0 mg/mL was used (Danone Research – Centre for Specialised Nutrition, Friedrichsdorf, Germany) [47]. The fraction of acidic human milk oligosaccharides includes a variety of structures bearing α-2,3-linked sialic acids capable of inhibiting the adhesins SabA and HpaA [35, 38].

2.10 *H. pylori* pretreatment and total RNA preparation

Total RNA was prepared by RNA-extraction kit (innuSPEED Bacteria/Fungi RNA Kit, Analytik Jena AG, Jena, Germany). The relative amount and purity of RNA was estimated from ODs of the preparations. One OD_{260} unit was assumed to be equivalent to 40 μg RNA/mL.

For the pretreatment with test compounds *H. pylori* (OD_{550} 0.1) were incubated for 4 h with the respective NPAs in liquid bacterial medium. Incubation was stopped by centrifugation ($3150 \times g$, 5 min) and by washing with PBS buffer (2 ×). Pelleted bacteria were treated for RNA isolation as described in the manufacture's protocol (Analytik Jena AG). An untreated bacterial control in liquid culture medium was used with an identical procedure.

2.11 Reverse transcription and preparation of single-stranded cDNA (ss-cDNA)

Total RNA was treated with rDNase (Macherey-Nagel, Düren, Germany) in accordance with the recommendations

of the supplier, 300 ng total RNA was converted into ss-cDNA using a commercially available QuantiTect RT-PCR Kit (Qiagen, Kebo, Stockholm, Sweden). Prior to ss-cDNA synthesis, all rDNase-treated RNA preparations were tested for the absence of contaminating DNA (data not shown).

2.12 Real-time PCR analysis

The Primers for: OMP genes *alpA*, *alpB*, *babA*, *hopZ*, *oipA*, *hpaA*, α-1,3-fucosyltransferase gene (*fucT*), cytotoxin-associated gene (*cagA*), cagPAI genes (*cagα*, *cagL*), vacuolating enzyme gene (*vacA*), urease A gene (*ureA*), urease I gene (*ureI*) and espective sequences are shown in Table 1. The reaction volumes were 20 μL, and RT PCR was performed in optical 96-well plates (Amersham – Pharmacia Biotech) in a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) as follows: initial enzyme activation and denaturation step at 95°C/15 min, followed by extension at 76°C/30 s, denaturation at 94°C/15 s and annealing at 61°C/30 s (40 cycles). Gene expression was calculated by the comparative C_T method with the following formula:

$$RQ = 2^{-(\Delta C_{T\ \text{sample}} - \Delta C_{T\ \text{reference}})} \quad (1)$$

As the reference the 23S rRNA housekeeping gene was used.

2.13 Statistical analysis

Statistical tests were performed by using SPSS®. The experimental results were expressed as the mean ± SD. Data were assessed by analysis of variance. In case the analysis indicated significant differences between group means, each group was compared by Dunnett *T* test (two-sided), and $p < 0.05$ was considered to be statistically significant.

3 Results

3.1 Antiadhesive activity, concentration dependency, cytotoxicity and structure–activity relationship

To determine the influence of potential antiadhesive NPAs **1–26** (Fig. 1) against the tissue-specific adherence of *H. pylori* two different assays were performed. For quantification of antiadhesive effects and for mechanistic studies an in vitro assay with AGS cells and FITC-labeled bacteria was used to quantify *H. pylori* adhesion by flow cytometry [37, 40]. Additionally, a semi-quantitative in situ assay, based on human tissue sections, was performed to confirm results of the in vitro assay [36].

For investigation of concentration/activity relationship *N*-(*E*)-caffeoyl-L-glutamic acid **11** was used as the model compound due to the fact that in prestudies this compound exhibited good antiadhesive activity. Maximal activity with a mean bacterial adhesion of about 73% was found at 1 mM, with obvious saturation effects at higher concentrations (Fig. 2). The 20–30% inhibition of bacterial adhesion has to be assessed as an typical indicator for blocking of single adhesins; unspecific blockers with interaction against many or most of the OMPs will typically result in inhibition rates >70% [37]. Also, the concentrations during measurement are in the millimolar range and assessed to be quite high for

Table 1. Primer sequences for *H. pylori* (Qiagen)

Primer	Gene/locus tag (Ref.)		Sequence, 5′→3′ orientation
23S rRNA	jhpr2 [39]	F	CCTTAGATTTACGGCGGATACA
		R	CAGTGGTAGAAGAGCGTTCATA
		P	TCACTTCATACCGCTCC
AlpA	jhp0848 [39]	F	GCTCACTAAAAACACCATT
		R	CATGCTTCTCACCTGATTGTTG
		P	TCCAACCAAGCTAACGC
AlpB	jhp0849 [39]	F	GAGTCAAAACATCAGCAAGA
		R	ACTGAGCTGGTTGGAGAGATT
		P	GACAACAACACCACGA
BabA	jhp0833 [39]	F	GATTTGTTATCGTTTGTCCTAC
		R	AGGCTTAGCGGGACTTTT
		P	GTTGATGG*GTTGTTGC
HopZ	jhp0007 [39]	F	CCAAGAAATCGTAACGCAAG
		R	TGTTTTGAGCGAAAGCCTATC
		P	TCCTTACACCTCTGCT
OipA	jhp0581 [39]	F	ATAAGCGAGCGTGTCAAGAA
		R	ATGCCAATCACAAGCCCTGAA
		P	GAAAGAAGG*GTAAAAGG
HpaA	jhp0733 [39]	F	CAAACCAGTGGAGAATAATAAC
		R	GGATAGCAGCGATAAAGACGAT
		P	CCATTCATAGCGACAG
FucT	jhp0596 [39]	F	TGCAAGTATCTCACGTAATCAA
		R	CTCAAGGCTATGGCTATGTAAC
		P	TGG*GAATGGTGTGGCT
CagA	jhp0495 [39]	F	TGGCAGTGGGTTAGTCATA
		R	CCTGTGAGTTGGTCTTCTTTGT
		P	AGGTGGTGAGAAAGG*GA
CagL	jhp0487 [39]	F	CTCGATTTTCAGCTTCCC
		R	TCAATCCCTTAGACCAAAAGACT
		P	ATTCCGCATTGTTGCT
Cagα	jhp1344 [39]	F	GAGACAAGCTCCATGAGA
		R	ACCCCCGGTTCATAAAGACT
		P	ACTTATTCTCCCACTTGC
VacA	jhp0819 [39]	F	AAACGACAAGAAAGAGATCAGT
		R	CCAGCAAAAGGCCCATCAA
		P	CAATAGCAACACAGAGG
Ure I	jhp0066 [39]	F	AGTGTTGATCGCTACGAATAAG
		R	AGCGACTGGGTTATTGTTTGG
		P	AGTGTGGTTGATAGCGG
UreA	jhp0068 [39]	F	TTGCCTTCGTTGATAGTGATG
		R	CTGATGGGACCAAACTCGTAA
		P	AACAACTCACCAGGAA

F, Forward Primer; R, Reverse Primer; P, QuantiProbe (label FAM); *, modified nucleotide.

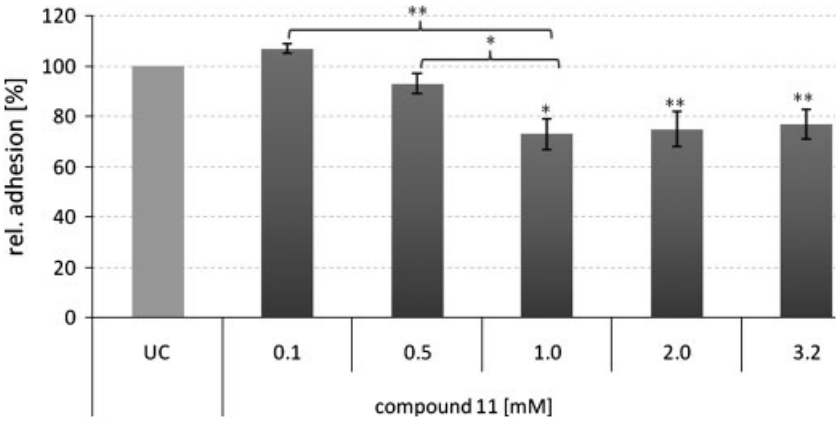


Figure 2. Mean adhesion in (%) of FITC-labeled *H. pylori* to AGS cells, after pretreatment with different concentrations of *N*-(*E*)-caffeoyl-L-glutamic acid amide **11**. Values are mean ± SD; $n = 3$; * $p \leq 0.05$, ** $p \leq 0.01$.

Table 2. Mean adhesion (%), related to the untreated control) of FITC-labeled *H. pylori* to AGS cells after 2 h pretreatment with different NPAs (2 mM)

Nr.	NPA Compound (all as amides)	Adhesion (%)	Nr.	Compound	Adhesion (%)
	Variable: aromatic substation of phenylpropenoic acid constant: amino acid		16	*N*-(*E*)-Caffeoyl-glycine	82 ± 9
1	*N*-(*E*)-Caffeoyl-L-aspartic acid	**83 ± 2****	17	*N*-(*E*)-Caffeoyl-L-alanine	85 ± 7
2	*N*-(*E*)-Caffeoyl-L-tyrosine	**84 ± 3****	18	*N*-(*E*)-Caffeoyl-L-valine	93 ± 10
3	*N*-(*E*)-*p*-Coumaroyl-L-aspartic acid	91 ± 4	19	*N*-(*E*)-Caffeoyl-L-leucine	112 ± 14
4	*N*-(*E*)-*m*-Coumaroyl-L-aspartic acid	104 ± 7	20	*N*-(*E*)-Caffeoyl-L-tryptophan	116 ± 23
5	*N*-(*E*)-*o*-Coumaroyl-L-aspartic acid	101 ± 8		*Variable: propenoic acid spacer constant: amino acid*	
6	*N*-(*E*)-*p*-Coumaroyl-L-tyrosine	104 ± 5	1	*N*-(*E*)-Caffeoyl-L-aspartic acid	**83 ± 2****
7	*N*-(*E*)-Cinnamoyl-L-aspartic acid	100 ± 8	21	*N*-Dihydrocaffeoyl-L-aspartic acid	**85 ± 2****
8	*N*-(*E*)-Feruoyl-L-aspartic acid	101 ± 5	22	3,4-Dihydroxybenzoyl-L-aspartic acid	88 ± 4
9	*N*-(*E*)-Isoferuoyl-L-aspartic acid	100 ± 9		*Relevance of carboxylic group of the amino acid*	
10	*N*-(*E*)-3,4-Dimethoxycinnamoyl-L-aspartic acid	101 ± 1	1	*N*-(*E*)-Caffeoyl-L-aspartic acid	**83 ± 2****
	Variable: polarity of the amino acid Constant: phenylpropenoic acid		2	*N*-(*E*)-Caffeoyl-L-tyrosine	**84 ± 3****
11	*N*-(*E*)-Caffeoyl-L-glutamic acid	**75 ± 7****	23	*N*-(*E*)-Caffeoyl-ß-alanine	103 ± 12
1	*N*-(*E*)-Caffeoyl-L-aspartic acid	**83 ± 2****	24	*N*-(*E*)-Caffeoyl-L-tyramine	119 ± 21
12	*N*-(*E*)-Caffeoyl-L-aspartyl-L-aspartic acid	**82 ± 7***		*Controls*	
13	*N*-(*E*)-Caffeoyl-L-dihydroxyphenylalanine	**78 ± 13****	–	Positive control	70 ± 5
2	*N*-(*E*)-Caffeoyl-L-tyrosine	**84 ± 3****	–	(*E*)-Caffeic acid	104 ± 6
14	*N*-(*E*)-Caffeoyl-L-phenylalanine	98 ± 2	–	L-Glutamic acid	101 ± 4
15	*N*-(*E*)-Caffeoyl-L-aspartic acid-dimethylester	104 ± 12	–	–	–

NPAs were classified into four different groups, dependent on their structural properties. Data are related to the untreated control (= 100%); positive control 3′-sialyllactose (15 mM); $n = 3$; * $p \leq 0.05$, ** $p \leq 0.01$.

antibacterial treatment. It has to be kept in mind that anti-adhesive treatment against *H. pylori* is a local treatment and higher concentrations of NPAs easily to be reached after oral application. For further mechanistic investigation NPA concentrations ≥ 1 mM were used.

The absence of cytotoxic effects of different NPAs against *H. pylori* was verified by the agar diffusion test. Compounds were tested in concentrations up to 4 mM over 48 h. With none of the NPAs tested no toxic effects were detected (data not shown).

For structure/activity relationship investigations, 24 homologous NPAs (Fig. 1, Table 2) were synthesized and clustered into four different groups according to the respective structural features. The amplitude of the effects between the NPAs was small (about 20%) due to the fact that the NPAs act only against a single adhesion (see later points of our investigation) data could be reproduced very reliable and also systematic evaluation indicated in cases of active compound statistical significance.

NPAs with identical amino acid (L-aspartic acid) but differences in the phenylpropenoyl part of the molecule

indicated dihydroxylation of the aromatic system as the favoured substitution pattern (**1** and **2** with 83 rep. 84% adhesion). With decreasing number of hydroxyl groups or increase in methoxyl groups the antiadhesive effect is significantly reduced (**6–10**). Hydroxylation in para-position is preferential to meta- and ortho-substitution (**3–5**). In total, this could be indicative for hydrogen bond interactions as one active principle within the NPA–*H. pylori* adhesin interaction. Concerning the nature of the amino acid polarity best activities were found for polar amino acid (e.g. aspartic acid **1**, glutamic acid **11** or 3,4-dihydroxyphenylalanine **13**). Acidic dipeptides with two aspartic acid residues **12** did not further increase the activity. Deesterification of the carboxyl groups **15** of aspartic acid completely abolished the activity, indicating the necessity of a highly polar functionality.

Again, the data indicate the requirement of a polar amino acid to establish possible hydrogen bond interactions with an *H. pylori* adhesin. Further, strong unpolar amino acids binding partners such as valin, leucin and tryptophan inhibited the antiadhesive activity (**18–20**).

For investigation of the spacer functionality between the aromatic and the amino acid parts a dihydrocaffeic acid derivative **21** and dihydroxybenzoyl-L-aspartic acid **22** were studied, indicating that a certain spacer between the two molecule parts is not necessary for the activity.

The necessity of an amino acid coupled to the aromatic acid was shown by amidation of β-alanin **23** or tyramin to (*E*)-caffeic acid **24**: both derivatives were completely inactive.

(*E*)-Caffeic acid and L-glutamic acid were tested as controls. No antiadhesive activity was observed here, which proved the necessity of the amide linkage in NPAs for the inhibition of *H. pylori* adherence.

Therefore, an antiadhesive NPA should link a 3,4-dihydroxylated aromatic acid with or without spacer via amide linkage to an acidic, polar amino acid in order to obtain optimized activity. To confirm the antiadhesive activity of the selected NPAs detected within the in vitro assays, representative compounds were tested on *H. pylori* within an in situ assay on human gastric tissue sections. Okra fresh extract, prepared from the immature fruits from *Abelmoschus esculentus* served as the positive control [36], a known blocker of *H. pylori* adhesion to human stomach tissue [37, 41]. Therefore, three NPAs were selected: one antiadhesive active **11**, a moderate active one **3** and an inactive one **26** (Fig. 1). Tests of these compounds on human gastric tissues and evaluation of the binding of FITC-labeled *H. pylori* by fluorescence imaging confirmed the results from the flowcytometric assay in vitro assay on AGS cells (Fig. 3): Again, **11** exhibited the highest antiadhesive activity with a mean reduction of *H. pylori* adhesion by approximately 50% (Fig. 3C). Compound **3** exerted a lower effect reducing bacterial adherence to the gastric sections by approximately 20% (Fig. 3D), while **26** was inactive (Fig. 3E). From these data, the potential antiadhesive effects have been reproduced within an independent further assay system.

3.2 NPAs interact with BabA

For pinpointing the respective bacterial adhesins blocked by the active NPAs, a semi-quantitative dot blot overlay assay was implemented. Therefore putative ligands, known to interact specifically with *H. pylori* adhesins, were immobilized by spotting on PVDF membranes [48]. A representative selection of ligands identified for *H. pylori* adhesins used for these experiments was: Le^b- and H-type I-conjugates, interacting with BabA; 3′-sialyllactose and fetuin interacting specifically with HpaA, sialyl-Lewis a, sialyl-Lewis x and laminin known for interacting with SabA and fibronectin with a not yet determined bacterial adhesin affinity. Further, human serum albumin (HSA) and bovine serum albumin (BSA) were used as controls to exclude non-specific binding of *H. pylori* to spotted compounds on the membrane. In addition 6′-sialyllactose was used to demonstrate the binding specificity of HpaA to 3′-sialylactose.

For the respective binding assays, bacteria were preincubated with test compounds, followed by FITC-labeling. The membranes, containing the immobilized glycoproteins and neoglycoproteins, were incubated with the pretreated bacteria. Non-adhering bacteria were washed off and adhering fluorescence labeled *H. pylori* were visualized by imaging. As expected, significant bacterial adhesion of untreated *H. pylori* to the immobilized ligands was obvious (Fig. 4I). As the positive control, bacteria were preincubated with 3′-sialyllactose, interacting specifically with the hpaA adhesin. As expected no adhesion to spotted 3′-sialyllactose-HSA conjugate was observed (data not shown).

In cases of pretreatment of *H. pylori* with compound **11** a reduction of bacterial interaction with Le^b- (slight reduction) and H-type I-HSA conjugates (strong reduction) was observed (Fig. 4II). Thereby, a strong interaction of **11** with BabA is obvious while other adhesins investigated were not influenced. For a further proof that NPAs do not interact with SabA, haemagglutination assay was performed. Haemagglutination of erythrocytes induced by *H. pylori* is sialic acid dependent and is mediated by bacterial SabA haemagglutinin [23]. Therefore, *H. pylori,* pretreated with **3** and **11**, were incubated after a serial dilution together with human erythrocytes. The positive control consisted of complex human milk oligosaccharides, containing α-2-3-linked sialic acids and fucosylated oligosaccharides, known to interact with SabA and BabA adhesins [35, 38], leading to a reduction of agglutination by 2.5 ± 0.4 logarithmic titres. In comparison, bacteria pretreated with compounds **3** and **11** showed only weak, non-significant inhibition of haemagglutination (Table 3).

In summary, antiadhesive NPAs interact with the *H. pylori* BabA surface protein leading to a distinct reduction of bacterial adhesion with gastric epithelial cells.

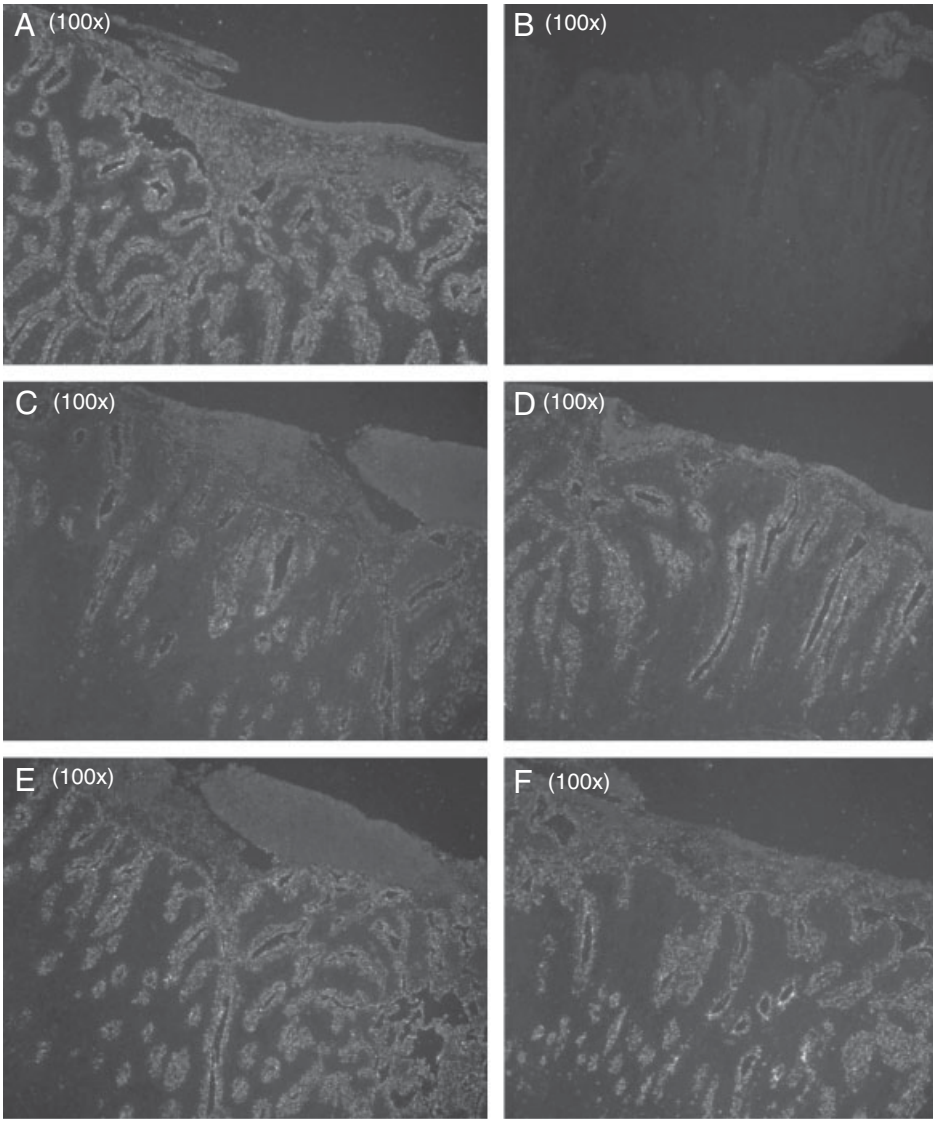
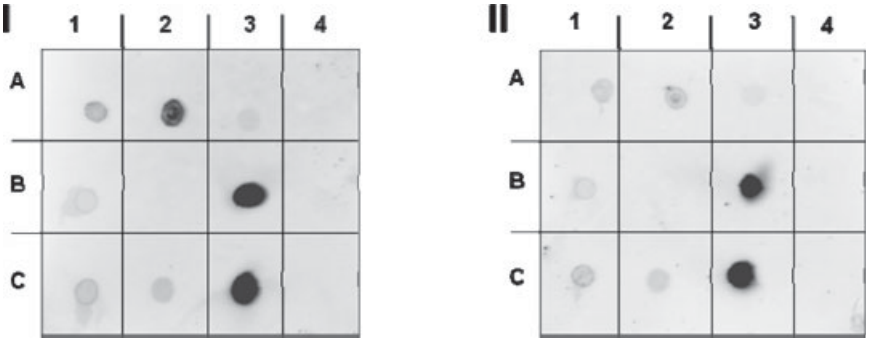


Figure 3. Representative fluorescence microscopy images of FITC-labeled *H. pylori* adhering to human gastric mucosa sections: untreated *H. pylori* negative control with full bacterial adhesion (**A**, **F**), positive control: *H. pylori* pretreated with Okra FE, nearly complete inhibition of bacterial adhesion (**B**), *N*-(*E*)-caffeoyl-L-glutamic acid **11** (**C**) *N*-(*E*)-*p*-coumaroyl-L-aspartic acid **3** (**D**) and *N*-(*E*)-feruoyl-L-glutamyl-L-alanine **26** (**F**). Images are equalized in brightness and fluorescence intensity assessed by double-blinded microscopic evaluation as well as by fluorescent imaging by the ImageJ® software; ∗++++, strong adhesion; +, weak adhesion.

	Compound	Concentration	Fluoresc. Intensity*	rel. Fluoresc. area [%]
(**A, F**)	untreated negative control	-	+ + + + +	100
(**B**)	positive control	1 mg/mL	(+)	3
(**C**)	N-(E)-Caffeoyl-L-glutamic acid (**11**)	2 mM	+ + (+)	48
(**D**)	N-(E)-p-Coumaroyl-L-aspartic acid (**3**)	2 mM	+ + + +	76
(**E**)	N-(E)-Feruloyl-L-glutamic acid-L-alanine (**26**)	2 mM	+ + + + +	101

3.3 Differential gene expression

To determine the influence of the antiadhesive BabA blocker **11** on the gene expression of bacterial adhesins and virulence factors and especially on a potential correlation of adhesion with expression of virulence factors, *H. pylori* was incubated with *N*-(*E*)-caffeoyl-L-glutamic acid amide **11** and with inactive *N*-(*E*)-feruoyl-L-tyrosine amide **25**. Using 23S rRNA as the endogenous control, the influence on several OMPs (*babA*, *alpA*, *alpB*, *hopZ*, *oipA* and *hpaA*) was studied. Moreover, the gene encoding the α-1,3-fucosyltransferase (*fucT*) which is involved in catalysis of the Lewis^x trisaccharide, a major component of *H. pylori* LPS, was included in the study [42]. Additionally, genes encoding for *vacA* and



A1: Lewis b-HSA
A2: H type I-HSA
A3: Fetuin
A4: HSA
B1: 3`-Sialyllactose-HSA
B2: 6'-Sialyllactose-HSA
B3: Fibronectin
B4: BSA
C1: Sialyl-Lewis a-HSA
C2: Sialyl-Lewis x-HSA
C3: Laminin
C4: unspotted control

Figure 4. Representative images of pretreated FITC-labeled *H. pylori* wild-type (wt) strain J99 adhering to immobilized ligands: (**I**) untreated control and pretreated bacteria with (**II**) *N*-(*E*)-caffeoyl-L-glutamic acid **11**. (Neo)glycoproteins spotted on PVDF membranes (1 μg per spot) were overlaid with FITC-labeled *H. pylori* and adherent bacteria detected by a fluorescence scanner. The locations of spotted (neo)glycoproteins are indicated below.

cagA, as well as *cagL* and *cagá* encoded in the cagPAI for the TFSS, were studied, beside the metalloenzyme urease (*ureA*) and a regulator for the transport of urea by an acid-gated urea-channel (*ureI*) [43].

For this study, the gene expression of untreated *H. pylori* was set as the reference (RQ = 1) in relation to the pretreated bacteria. *H. pylori* preincubated with compound **11** did not influence significantly the expression of OMPs associated with adhesion or either expression of the *fucT* gene (Fig. 5). Similarly, the specific interaction of compound **11** to BabA did not lead to a significant difference in expression of the virulence factors *cagA*, *cagL*, *cagá*, *vacA*, *ureA* or *ureI*. Also, pretreatment of *H. pylori* with the non-antiadhesive compound **25** did not induce substantial difference in expression of the studied virulence factors. These data clearly indicate that the interaction of **11** with BabA does not induce an automatic feedback mechanism in the bacterial cell, associated with increased or decreased functionality. This again proves that the bacterial adhesins act probably not in a cooperative manner with signal transduction systems, but have to be seen as more or less isolated systems.

Table 3. Haemagglutination of human erythrocytes by *H. pylori*, pretreated with *N*-(*E*)-caffeoyl-L-glutamic acid **11** and *N*-(*E*)-*p*-coumaroyl-L-aspartic acid **3**

Compound	Concentration	Reduction of log. titre
Positive control	1.0 mg/mL	2.5 ± 0.4
Positive control	0.5 mg/mL	1.9 ± 0.3
N-(*E*)-Caffeoyl-L-glutamic acid (11)	2.0 mM	0.5 ± 0.3
N-(*E*)-Caffeoyl-L-glutamic acid (11)	1.0 mM	0.2 ± 0.3
N-(*E*)-*p*-Coumaroyl-L-aspartic acid (3)	2.0 mM	0.6 ± 0.3
N-(*E*)-*p*-Coumaroyl-L-aspartic acid (3)	1.0 mM	0.1 ± 0.3

Values from three independent experiments indicate the reduction of the logarithmic titres as mean MW ± SD related to untreated control. Positive control: human milk oligosaccharides, acidic fraction [35, 38].

4 Discussion

Among the *H. pylori* adhesins the interaction of BabA with its complementary receptors on host cells, namely the Lewis^b antigen and related fucosylated ABO-blood group antigens, is one of the best characterized proteins of the bacterium. Several studies investigated these interactions and postulated the central domain in BabA to be the determinant region for the specificity of receptor binding [17, 18, 44, 45]. Still, the motifs of the BabA gene, involved in the target binding are still to be determined.

Independent of the BabA binding motif, the interaction with specific ligands as the Le^b or H-1 antigens is associated with the respective carbohydrate structure. H-1 antigen has a Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4(Glc) terminal region and Le^b additionally a second branched fucose (Fuc), connected to *N*-acetylgalactosamine (GlcNAc). These carbohydrate regions demonstrate that the nature of the receptor binding specificity with BabA is based most probably on hydrogen bond interactions with hydroxyl groups of the terminal regions.

The structure–activity relationship study of several NPAs reflects this mode of interaction. Not only a high hydroxylation degree within the phenylpropenoyl part of the amide, but also a polar/acidic amino acid were shown to be essential for optimized antiadhesive activity. Further, the strong activity of compound **11** shown within in vitro and in situ assays was also demonstrated to be specific against the *H. pylori* BabA adhesin.

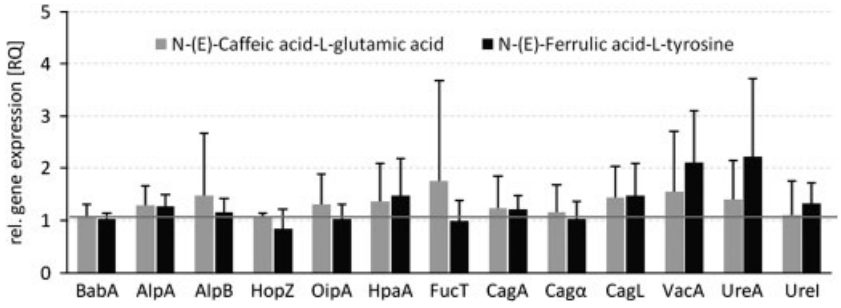


Figure 5. Differential gene expression of *H. pylori* pretreated with *N*-(*E*)-caffeoyl-L-glutamic acid **11** (2 mM) and *N*-(*E*)-feruoyl-L-tyrosine **25** (2 mM). Endogenous control: 23S rRNA. Data related to untreated control (UC): *H. pylori* in liquid growth medium (RQ = 1). *babA*, *alpA*, *alpB*, *hopZ*, *hpaA*: OMPs associated with adhesion; *fucT*: fucosyltransferase for LPS. *cagA*; *cagα* and *cagL*: T4SS; *vacA*; *ureA* and *urel*: Urease protein and urea transporter. $n = 3$ (mean ± SD).

Noteworthy is the major role of BabA for the initial gastric colonization by *H. pylori*, enhancing this OMP as a good potential target for prophylactic applications with antiadhesive compounds. Since the dot blot overlay assay was representative only for interactions with BabA, SabA, HpaA and an unknown fibronectin-binding adhesin, it cannot be excluded that NPAs also interact with other *H. pylori* adhesins.

A structural comparison, for instance between the highly active compound **11** and the BabA-binding terminal region of the H-1 antigen is challenging without proper molecular modeling data. Yet, active NPAs show to H-1 some similarities within structural properties (see Fig. 1, Table 2). There is on one hand the amide bond and the need for a high hydroxylation on the phenylpropenoyl part. On the other hand, the amino acid substituent should exhibit a polar or acidic character for a good antiadhesive activity. In summary, these properties indicate the possibility of hydrogen bond interactions with the BabA binding motif, similar to the Fucα1-2Galβ1-3GlcNAc-terminal region of H-1 and Le^b antigens. However, a detailed definition of the necessary motifs for the NPA-BabA interaction demands further investigations as the identification of the BabA-binding region.

The expression of single *H. pylori* OMPs and other virulence factors has been studied extensively (1). Thus, investigations correlating the expression of these with inhibitors of bacterial adhesins (e.g. **11** interacting with BabA) are novel. The presence of mRNA from studied targets does not necessarily correlate with the presence of translated proteins, since for instance OMPs are coded by contingency genes and post-transcriptional regulation in bacteria is also a frequent event [46]. With regard to the OMPs, *H. pylori* uses several strategies to generate diversity. As shown in a Rhesus Macaque model, in which animals were infected with a BabA-producing *H. pylori* strain, reisolated strains exhibited a loss of BabA expression by slipped-strand mispairing (SSM) or recombination of the BabA gene locus [45]. As a result this led to a loss of binding to Le^b.

The incubation period of 4 h for *H. pylori* with two representative NPAs chosen in this work is probably not sufficient so that regulatory mechanisms as the SSM take place.

Still, the regulation of virulence gene expression, especially within a specific inhibition of an adhesin, is of major interest. The specific inhibition of BabA with compound **11** simulated on one hand the situation of adhesion in initial gastric colonization. On the other hand, the incubation of *H. pylori* with NPAs should demonstrate the influence of these compounds on the bacterial virulence. The results reflect that the expression of the targeted *H. pylori* OMPs was not significantly stimulated by the BabA inhibition with compound **11**. Within this short incubation period a change in the expression pattern of OMPs was therefore not observed. Similarly, other virulence factors (see Table 1) for instance as the *vacA* or *cagA* encoding genes were also not stimulated in expression.

Nevertheless, these results demonstrate that antiadhesive compounds, as the NPAs, do not enhance at mRNA level the *H. pylori* virulence. Together with the specific inhibition of BabA, which carries an important role in the initial gastric colonization (19), one further step for the development of prophylactic or post-therapeutic applications with such antiadhesive compounds is established by this study. NPAs can be assessed as follows: the compounds are easily manufactured by simple synthetic routes, galenical formulation of the hydrophilic compounds should not result in big problems, stability as drug compounds and in the formulation must be ensured by additional antioxidative arrangements, the compounds have to be given in high doses in the millimolar range to interact with the bacterial adhesion, the compounds are bioavailable in the systemic compartment [3] and toxicity against human cells is not to be expected [1, 2]. Infections studies have to evaluate whether the inhibition of one adhesin can prevent effectively bacterial adhesion and lead to diminished infection rates.

These data enhance again the potential of antiadhesive compounds for future applications for prophylactic control of *H. pylori* infections within a cytoprotective strategy. However, due to the genetic heterogeneity of *H. pylori* strains and the resulting complexity of the adhesion mechanism, further investigations with additional clinical isolates are suggested In principle development of such antiadhesive compounds towards products for use in food or health products can be seen for prevention of very early infection in children, due to the fact that during the first 2–5 years of life most infections with *H. pylori* take place. On the

other side, also food supplements are thinkable to be used during and after an antibiotic eradication therapy, due to the fact that most patients after antibiotic treatment will suffer from recurrence of the infection after several months. From these aspects, a translational development of NPAs for interesting products for the future can be discussed.

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