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PCT

Abstract: The present invention relates to a pharmaceutical composition comprising at least one inhibitor of SHP-2, optionally together with pharmaceutically acceptable carriers, adjuvants, diluents and/or additives. The present invention also relates to screening methods for identification of a compound suitable as inhibitor of SHP-2.
Pharmaceutical composition comprising an inhibitor of SHP-2

*Helicobacter pylori* inhibits EGF receptor to shut down expression of antimicrobial defensin hBD3. Defensins are short peptides that are crucial for the first line of innate immune responses in mammals. Several defensins are known to be induced after infection with the human gastric pathogen *Helicobacter pylori*, including the human β-defensin hBD3. Here, we report on the molecular mechanism by which *H. pylori* escapes from the highly potent antimicrobial effect of hBD3. During prolonged infection, *H. pylori* disables hBD3 expression by directly inhibiting transactivation of the epidermal growth factor receptor (EGFR). EGFR blockage is mediated by the translocated bacterial effector protein CagA and the tyrosine phosphatase SHP-2. Our study provides a tantalizing mechanistic explanation of how the pathogenicity factor CagA enables *H. pylori* to persist in the human stomach over decades.

Introduction

The highly adapted human gastric pathogen *H. pylori* colonizes at least half of the world's population and elicits a tremendous socio-economic burden because of its etiological association with peptic ulcer disease and gastric cancer. Infections with strains, expressing a functional type IV secretion system (TFSS), result in the translocation of the CagA effector protein into host epithelial cells, thereby enhancing the pathology of infection (Hatakeyama, 2004). Once inside host cells, CagA targets the oncogenic tyrosine phosphatase SHP-2 to manipulate host cell signaling (Higashi et al., 2002). Previous studies have reported the strong induction of several potent antimicrobial peptides, referred to as defensins, during the initial stages of infection with different bacteria, including *H. pylori* (Lee and Josenhans,
Defensins are short peptides and comprise key elements of the initial innate immune response against infection, exerting their antimicrobial impact via charge-mediated binding to bacterial membranes. Following membrane integration, defensins form pores resulting in bacterial lysis (Selsted and Ouellette, 2005). Recent work has shown that Nodl-dependent NF-κB signaling mediates *H. pylori*-induced expression of human β-defensin 2 (hBD2) (Grubman et al., 2009; Wada et al., 2001), but knowledge regarding the functional role of other signal transduction pathways, including the epidermal growth factor receptor (EGFR), remains fragmentary (Boughan et al., 2006). Moreover, the strategies employed by *H. pylori* to circumvent the antimicrobial action of β-defensins and to maintain persistent gastric colonization for decades remain entirely unknown.

**Results**

*H. pylori* stimulates synthesis and secretion of antimicrobial hBD3 in gastric epithelial cells.

Since hBD3 has a known potent antimicrobial impact on a variety bacterial pathogens (Moranta et al., 2010), we wanted to assess its role during *H. pylori* infection in greater detail. To test the spectrum of the microbicidal effect on *H. pylori* we tested three different strains (P1, P12 and G27). Infection of human gastric epithelial cells (AGS) with all three strains increased expression levels of hBD3 after 4 h (Fig. 1A). Moreover, the viability of all tested *H. pylori* strains was drastically reduced in comparison to untreated controls, following incubation for 1 h with different concentrations of hBD3 peptides (Fig. 1B). Confocal images of PI infected AGS cells confirmed the increased expression of hBD3 and further revealed that hBD3 colocalized exclusively with coccoid bacteria (Fig. 1C), thought to be less viable than spiral shaped forms (Andersen and Rasmussen, 2009). Additionally, hBD3 colocalized with coccoid forms of *H. pylori* in infected human tissue culture (Fig. 1D). Taken together, our findings corroborate the microbicidal potency of hBD3.
Expression of hBD3 is EGFR and MAPK dependent

To delineate the host cell signaling pathways inducing expression of hBD3, we treated *H. pylori* infected cells with chemical inhibitors targeting EGFR and the MAP kinase MEK. Interestingly, hBD3 mRNA and protein expression levels were substantially reduced upon application of two different EGFR-specific inhibitors (AG1478, Gefitinib; Fig. 2A and fig. S1). Similarly, hBD3 protein expression was markedly diminished in infected cells treated with U0126, a common inhibitor of MEK (Fig. 2B). This data demonstrates a key role of the EGFR-MEK signaling cascade in the *H. pylori* dependent up-regulation of hBD3.

Initial hBD3 expression strongly decreases during prolonged infections in a CagA-dependent manner.

To elucidate the mechanism by which *H. pylori* naturally circumvents the antimicrobial activity of hBD3 we extended our analyses to later stages of an infection. Surprisingly, the increased hBD3 expression observed at early time points of infection appeared to vanish after prolonged infection. We found that the initial up-regulation of hBD3 already ceased after 24 h of infection on the protein level whereas at the same time point no mRNA could be detected, indicating a long protein half-life (Fig. 3A and B). Immunofluorescence microscopy corroborated these results demonstrating no colocalization of hBD3 with bacteria after two days of infection (Fig. 3C). To exclude the possibility that the observed decrease of hBD3 was not the result of reduced cell viability we used a WST-1 assay as an indicator of cellular fitness revealing no detrimental effects. Accordingly, abrogation of hBD3 expression after 24 h of infection cannot be attributed to reduced cellular fitness but instead indicates a direct bacterial effect on hBD3 expression (fig. S2). Moreover, prolonged infection (24 h) with the isogenic mutant PlAcagA did not result in reduced hBD3 expression but rather showed continued elevation of hBD3 mRNA levels (Fig. 3D), further demonstrating that the translocated bacterial effector CagA is directly involved in hBD3 inhibition.
Down-regulation of hBD3 is mediated by the CagA-dependent inhibition of EGFR.

The EGFR is known to be activated by *H. pylori* during the early stages of infection (Keates et al., 2005; Wallasch et al., 2002), however, its activation status after prolonged infection periods in human cells remains unknown. Since initial hBD3 up-regulation is EGFR dependent (Fig. 2A and fig. S1), we examined EGFR transactivation during the course of an infection. Upon binding to its receptor, the EGFR-specific ligand EGF induces receptor dimerization and subsequent receptor autophosphorylation, referred to as transactivation, which can be monitored by immunoblotting. Therefore, we analyzed EGFR transactivation in non-infected and PI infected AGS cells after treatment with EGF for five minutes (Fig. 4A). Both non-infected and short-term infected cells (0.5-8 h) displayed strong receptor phosphorylation levels, whereas no EGFR phosphorylation could be detected in cells infected for prolonged periods (24 h). The degree of EGFR phosphorylation blockage was multiplicity of infection (MOI)-dependent, indicating a direct bacterial effect on EGFR inhibition (fig. S3). During transactivation the EGFR is phosphorylated at multiple tyrosine moieties (Hubbard and Miller, 2007). To check whether inhibition is specific for individual tyrosine residues, we analyzed the phosphorylation level of EGFR Y845, Y1045, Y992 and Y1068 during prolonged infection (Fig. 4B and fig. S4). All examined tyrosine residues showed strong reduction of phosphorylation after 24 h infection with *H. pylori*. Moreover, as indicated by the loading control, decreased EGFR phosphorylation was not due to reduced EGFR protein levels (Fig. 4B; lower panel). Remarkably, treatment of infected AGS cells with an alternative ligand (HB-EGF) gave rise to the same phenotype (fig. S5), indicating a ligand independent EGFR-specific effect. To ensure the observed inhibition of signal transduction was not a general feature of prolonged infections, we analyzed the transactivation of c-Met, an EGFR related receptor tyrosine kinase (fig. S6); however, no reduced phosphorylation levels were observed, providing strong evidence for selective EGFR inhibition during prolonged infection.

Since the translocated bacterial effector protein CagA is known to interfere with numerous signal transduction pathways during the course of infection, we investigated its putative role in
EGFR inhibition, infection of AGS cells with a *H. pylori* Cag A mutant strain (*PlAcagA*) did not result in a reduction of EGFR phosphorylation, clearly showing that CagA is involved in the inhibition of both hBD3 (Fig. 3D) and EGFR (Fig. 4C). To ultimately demonstrate that CagA-mediated EGFR inhibition represents the underlying mechanism of reduced hBD3 expression, we tested whether constitutively active EGFR could restore hBD3 expression during prolonged infection. Plasmids encoding for either constitutive active EGFR (EGFR L858R) or EGFR wt (Uchida et al., 2007) were transiently expressed in non-infected and infected cells. Notably, non-infected cells expressing EGFR L858R also produced hBD3, thus confirming the EGFR-dependent induction of hBD3 (Fig. 4D). Moreover, hBD3 mRNA levels were substantially higher in infected cells expressing EGFR L858R in comparison to non-infected cells. This finding is consistent with our previous observation that prolonged infection with *H. pylori* leads to enhanced surface exposition of EGFR (Bauer et al., 2009). Interestingly, an increased level of wt EGFR is sufficient to overcome the *H. py/or/-induced repression of hBD3. Taken together, these data clearly demonstrate that hBD3 expression is blocked by selective EGFR inhibition during prolonged infection.

**CagA dependent EGFR blockage is induced by the tyrosine phosphatase SHP-2.**

CagA is known to activate the tyrosine phosphatase SHP-2 (Higashi et al., 2002). Further, SHP-2 is known to regulate EGFR signaling by mediating either direct EGFR dephosphorylation or dephosphorylation of downstream signaling molecules like GTPase activating proteins (Neel et al., 2003). We speculated blockage of EGFR could be the result of constant dephosphorylation; we therefore investigated the role of SHP-2 in the EGFR-mediated reduction of hBD3. Both chemical inhibition (ortho-vanadate) and siRNA-mediated depletion of SHP-2 resulted in the rescue of EGFR transactivation in infected cells (Fig. 5A and fig. S7). Notably, in infected cells, increased concentrations of orthovanadate were needed to elevate tyrosine phosphorylation levels, consistent with the strong CagA-mediated activation of SHP-2 (Higashi et al., 2002).
Accordingly, elevated SHP-2 activity was observed in infected cells, as indicated by increased SHP-2 phosphorylation levels (fig. S8).

Next, we investigated the influence of SHP-2 knockdown on the hBD3 expression level. Since EGFR transactivation is recovered when SHP-2 is depleted, we anticipated that the expression of hBD3 would not be blocked. Consistent with this hypothesis, hBD3 protein and mRNA production was increased in infected cells transiently depleted of SHP-2 (Fig. 5B and C). In a SHP-2 stable knockdown cell line (SHP2_4_KD) hBD3 expression was even more pronounced (Fig. 5D; fig. S9). Thus, our data provide evidence for a SHP-2 and CagA-dependent blockage of EGFR transactivation, which ultimately leads to inhibition of hBD3 expression during prolonged infections.

**SHP-2 mitigates the antimicrobial impact of hBD3 on bacterial viability.**

Confocal imaging revealed that high hBD3 expression in stable SHP-2 knockdown cells is reflected in reduced bacterial survival (Fig. 6A). While the colocalization of hBD3 and bacteria vanished during prolonged infection of AGS cells, it was still detectable in SHP-2 knockdown cells two days after infection. In addition, bacterial viability was considerably reduced in SHP-2 knockdown cells, as quantified by CFU assays (Fig. 6B). The antimicrobial effect of hBD3 was equally potent for attached (Pellet; PE) and free swimming (Supernatant; SN) bacteria.

Taken together, our data demonstrate that the bacterial effector CagA enables survival of *H. pylori* in the stomach over prolonged periods by selectively blocking EGFR transactivation (Fig. 6C). During prolonged infection the tyrosine phosphatase SHP-2 is activated, inhibiting the EGFR by dephosphorylation. This mechanism accounts for the drastic reduction of hBD3 expression, which substantially boosts bacterial survival.

Our study provides the first mechanistic explanation of how the persistent pathogen *H. pylori* can neutralize a human β-defensin, which functions both in microbial defense and wound
healing. Furthermore, our delineation of the CagA-dependent activation of SHP-2 during *H. pylori* infection promotes SHP-2 as a promising target for antimicrobial and ulcer therapies.
Summary

*H. pylori* selectively disrupts SHP-2 dependent EGFR signaling to evade the antimicrobial impact of the human beta defensin hBD3

Endogenous antimicrobial peptides have been identified as key elements of innate host defence against infection. Within this peptide family, defensins exhibit a variety of microbicidal activities against Gram-positive and -negative bacteria, mycobacteria, fungi and certain viruses. Recently, the human beta defensin 3 (hBD3) was shown to have antimicrobial potential: it efficiently killed the human gastric pathogen *Helicobacter pylori* after short contact *in vitro*. *H. pylori* type I strains persistently colonize the human stomach, thereby causing severe diseases like ulcer and gastric cancer. Whereas most bacteria are free swimming organisms in the gastric mucosa, a small percentage adheres to epithelial cells. Here, pathogenic type I strains translocate the effector protein CagA into the host cell, which dramatically interferes with cellular signaling pathways like apoptosis and cytoskeletal rearrangements. The mechanisms how *H. pylori* establishes a protective biological niche remain poorly understood. Since *H. pylori*-mediated pathogenesis is a long-term process, this particular question is an important scientific issue. Therefore, we wanted to investigate if and how *H. pylori* escapes from the antimicrobial impact of hBD3 to enable persistent colonization. Intriguingly, we found that initially induced hBD3 expression vanishes over time in order to provide extracellular survival of *H. pylori* during longer infection. Moreover, we could reveal the underlying molecular mechanism that directly leads to *H. pylori* mediated blockage of hBD3. Within this scenario both, the bacterial effector protein CagA as well as the cellular tyrosine phosphatase SHP-2 play essential roles in complex manipulations of EGFR signaling, thereby allowing long term survival of *H. pylori*. Our data provide new mechanistic insights of how *H. pylori* controls complex host cell pathways to conduct them into beneficial directions. Furthermore, they lead to the hypothesis that attachment of free swimming bacteria cause a positive impact on the bacterial population by setting up a protective biological environment.
References


Materials & Methods

Bacterial cultivation

Type I *H. pylori* wild type strain PI, P12 and G27 are clinical isolates, that has been described previously (Censini et al., 1996; Corthesy-Theulaz et al., 1996; Schmitt and Haas, 1994). Construction of the isogenic knock out mutants *PlAcagA* and *PlAvirBII* have been described elsewhere (Backert et al., 2001). Wildtype strains were cultivated on horse serum agar plates supplemented with vancomycin (10 ug/ml). Mutants were cultivated on selective chloramphenicol (4 ug/ml) agar plates. Bacteria were incubated 1 day before infection at 37°C in a humidified anaerobic atmosphere containing 5% CO₂, 5% O₂ and gasmixture of 9:1 N₂/H₂ (Thermo Fischer).

CFU assay

1x10⁶ cells were infected with *H. pylori* for 24 h by using a MOI of 50. Supernatant was diluted in BHI medium in a final volume of 1 ml (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) and 20 µl of each dilution was plated on agar plates and incubated by 37°C (see bacterial cultivation). Cells with attached bacteria were washed 3 times with RPMI medium. 700 µl 0.5% Saponin/BHI was added and incubated for 10 min by 37°C. Lysed cells were homogenized thoroughly by pipetting and bacterial dilutions were prepared in a final volume of 1 ml (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴). 20 µl of each dilution was plated on agar plates (see bacterial cultivation) and incubated by 37°C. CFU (colony forming units) of attached (PE) and free swimming bacteria in the supernatant (SN) were calculated 3 days later.

Antimicrobial activity assay

1x10⁵ bacteria were incubated for 1 h by 37°C with or without indicated concentrations of hBD3. Bacteria were diluted subsequently in BHI medium in a final volume of 1 ml (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴). 20 µl of each bacterial suspension was plated on agar plates and incubated for 3
days (see bacterial cultivation). CFU (colony forming units) were counted and normalized to untreated bacterial samples.

**Cell Culture, EGF and HB-EGF stimulation and inhibitor treatment**

The human gastric adenocarcinoma cell line AGS (ATCC) was grown in RPMI 1640 medium containing 4 mM glutamine (Invitrogen) and 10% fetal calf serum (Gibco). Cells were incubated in a humidified atmosphere with 5% CO₂. 2 days before infection cells were seeded in tissue culture plates. EGF and HB-EGF stimulations were performed after serum starvation for 20 h by adding 100 ng/ml EGF (Sigma) or 100 ng/ml HB-EGF (Sigma) for the indicated length of time. For inhibitor experiments cells were incubated 1 h before infection with indicated concentrations of AG1478 (Calbiochem), U0126 (Cell Singaling) or Gefitinib (Selleckchem). Inhibitors were not removed during infection. For general inhibition of tyrosine phosphatases sodium orthovanadate was added 1 h before EGF stimulation. Orthovanadate was activated before usage. Here, 37.5 µl from a 0.2 M stock solution were incubated with 70 µl H₂O₂ (3%) in 500 µl RPMI medium (Gibco). After incubation for 10 min at RT, suspension was dilution with RPM to reach a final stock concentration of 100 µM.

**WST-1 Viability Assay**

In order to monitor cellular viability reduction of WST-1, a marker for proliferative and viably cells was measured via a spectometrical assay by following manufactory instructions (Roche, Mannheim, Germany).

**Infection Assays**

Bacteria were collected from plates and resuspended in PBS. To avoid BHI components derived from the agar plates during infection, bacteria were washed once with PBS. After centrifugation for 3 min by 4000 g, bacteria were resuspended again in PBS. The amount of bacterial
suspension required for indicated MOI (Multiplicity of infection) was calculated by measuring optical densities (OD\textsubscript{550}) and by calculating respective volumes using a standard curve. Eucaryotic cells were infected under serum starved conditions and incubated at 37°C in a 5% CO\textsubscript{2} incubator for indicated time points. For long term infections (1 to 3 days) cells were grown in FCS containing medium to a confluency of 20% and infected with a MOI of 20 or 100. Infection of fallopian tubes was performed by adding $1 \times 10^8$ bacteria directly on the epithelial surface from tubular tissue.

**siRNA and DNA Transfection**

AGS cells were transiently transfected with DNA or siRNA respectively. For DNA transfections Lipofectamine 2000 was used following the manufacturer's instructions (Invitrogen). RNA transfections were performed by using RNAfect (Invitrogen). DNA transfected cells were incubated at 37°C and 5% CO\textsubscript{2} 24 h prior infection. siRNA transfected cells were incubated under the same conditions for 24 h, subsequently splitted and further incubated for additional 48 h. The plasmid encoding the constitutive active EGFR (EGFR L858R) and EGFR wildtype (EGFR WT) were a generous gift from Prof. Katsuyuki Kiura and Dr. Akiko Uchida (Uchida et al., 2007). The plasmid encoding for SHP-2 was kindly provided by Dr. Paul Crocker (Avril et al., 2004). For knock down experiments we used a siRNA pool containing 20 µM of the following siRNA molecules to obtain optimal knock down with minor off target effects: SHP-2 (2r) AAGGUGGUUUCAUGGACAUCU; SHP-2 (1) GAAUAUGGCUGUCAUGCGUGTT; SHP-2 (2) CCUUAACACGACUGUAUAAA; SHP-2 (3) GCGUGCAUGCGUUGGAAGCG. Controls were performed under the application of siRNA oligos targeting Luciferase (AACUUACGCUGAGUACUG), which have no specific target in the genome.

**Construction of stable SHP-2 knock down cell lines**

shRNAs for SHP-2 constructs were stably transduced into AGS cells using a lentiviral based expression system (Wiznerowicz and Trono, 2003). Design of shRNAs was performed with the
BLOCK-iTTM RNAi Designer from Invitrogen and cloned into the vector pLVTHM. 293T cells were transfection using calcium phosphate transfection together with the packaging vectors psPAX2 and pMD2G. Viruses were harvested from the supernatant and used for infection of HeLa cells in the presence of polybrene (5 μg/ml). GFP-positive cells were selected 7 days p.i. and sorted into 96-well plates using flow cytometry. Target sequences for SHP-2 constructs: SHP2_1: GCCTCATATGTTGAATCATCC; SHP2_4: GGGATCACTTTACACATAG;

**Immunofluorescence in cell culture**

For immunofluorescence staining cells were fixed with icecold methanol, incubated with 0.1% Triton X-100 (Sigma) for 20 min at RT and blocked with 1% bovine serum albumin. Staining were performed with anti a polyclonal hBD3 antibody (Santa Cruz), a phosphospecific polyclonal antibody against EGFR pY 1068 (Cell Signaling) and a monoclonal anti CagA antibody (Santa Cruz). All secondary antibodies were purchased from Jackson ImmunoResearch. Nuclei were stained by using DraQ5 (Alexis) following manufactures instructions. Samples were mounted in moviol and analyzed by epifluorescence microscopy with a Leica DM/R microscope or by confocal laser scanning microscopy using a Leica TCS SP microscope.

**Immunofluorescence staining of fallopian tubes**

Fallopian tubes were obtained from women undergoing operative procedures for tubal ligation or hysterectomy. Material was transported on ice within 2 hours after operation. Extra connective tissue was removed by dissection under continuous washing with PBS. Tubular lumen was identified and rinsed with PBS by using a syringe. Transversal sections (approx. 1-2 mm) were generated by using a scalpel. Tissue slices were transferred to small petri dishes in MEM Earl medium+10 % FCS and incubated at 37 °C prior to infection. 1 h post infection tissue was fixed by incubating samples 24 h in 4% PFA. Samples were stored in PBS at 4°C prior to paraffin embedding. Paraffin embedding and immunofluorescence labelling was performed as described by (Robertson et al., 2008). Polyclonal anti Urease-B antibody was used to stain H.
*pylori*, DraQ 5 (Alexis) was applied to stain nuclei and monoclonal anti-hBD3 antibody (Abnova) was used to visualize hBD3. All secondary antibodies were purchased from Jackson ImmunoResearch. Samples were analyzed by confocal laser scanning microscopy using a Leica TCS SP microscope.

**Immunoprecipitation**

For immunoprecipitation of c-Met and SHP-2 1 x 10^7 AGS cells were lysed on ice in RIPA-Buffer (1 % Triton X-100, 50 mM Tris HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02 % sodium azide, 1 mM Na_2VO_4, Roche complete protease inhibitors) by 15 passages through a 20-gauge syringe. Cell debris was removed by centrifugation at 12,000 g for 5 min. Lysates were pre-cleared with G-sepharose (Pharmacia) for 30 min at 4 °C. Lysates were incubated with 1 μg monoclonal anti c-Met antibody (Upstate 05-237) or anti SHP-2 (Santa Cruz) antibody by 4 °C overnight under subsequent addition of protein G-sepharose beads for 2 h by 4 °C. Precipitates were analyzed by SDS-PAGE and immunoblotting.

**Immunoblotting**

SDS-PAGE, protein transfer to PVDF membranes and probing with primary and secondary antibodies were performed by standard procedures. We used antibodies against EGFR (Cell Signaling), β-Actin (Sigma), EGFR pY 845, 1045, 992, 1068 (Cell Signaling), hBD3 (Santa Cruz), SHP-2 (Santa Cruz), c-Met (Santa Cruz), pY99 (anti phosphotyrosine antibody; Santa Cruz), CagA (Santa Cruz). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham) were used and detected with the Renaissance western blot kit system for ECL immunostaining (ICN).

**Quantification of Immunoblots**
Immunoblot quantification of band intensities were performed from digital pictures which were processed using the software Photoshop CS3 (Adobe). Pictures were inverted and region of interest was defined. Histogram of selected region displayed median of intensity and region size. All bands were normalized to background regions and respective loading control band intensities.

RT-PCR

Quantification of mRNA levels was conducted by quantitative real-time (qRT) PCR. RNA was isolated using RNeasy Mini Kit (Qiagen) and qRT-PCR was performed using QuantiFast Sybr Green PCR Kit (Qiagen) and Applied Biosystems 7500 fast real-time PCR system. House-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for relative quantification. Data was analyzed using software SDS 2.2.2 (Applied Biosystems) and Excel (Microsoft). Data were normalized to GAPDH expression. Following primer pairs were used for quantification of hBD3 and GAPDH expression: hBD3 FW: 5’ TCTG CCTTACCATTGGGTTC 3’; hBD3 RW: 5’ CAC GCTGAGACTGGATGAAA 3’; GAPDH FW: 5’ GGTATCGTGGAGACTCATGAC 3’; GAPDH RW: 5’ ATGccAGTGCTCCCGTTCAG 3’

Statistical analysis:

Student’s r-fest was performed to determine statistical significance shown in bar graphs (p-value < 0.05; asterisks). Error bars indicate mean ± SD of three independent experiments.
Figure Legends

Figure 1: Infection with *H. pylori* induce expression of antimicrobial hBD3 in gastric epithelial cells. (A) AGS cells were infected with PI, P12 and G27 for indicated time points using an MOI (multiplicity of infection) of 100. Infected and non-infected (NI) cells were lysed and analyzed for hBD3 expression by immunoblot. Actin was used as a loading control. (B) Antimicrobial activity assay of hBD3 with three different *H. pylori* wildtype strains (PI, P12, G27). Bacteria (lxIO ⁵) were incubated for 1 h with indicated peptide concentrations. Colony forming units (CFU) were quantified three days later. Data are mean ± SD of three independent experiments. (C) Immunofluorescence analysis of hBD3 expression (red) in PI infected (lower panel) and non-infected (NI) AGS cells (upper panel). Bacteria were visualized using an anti CagA antibody (green). Nuclei were stained with DraQ5 (blue). Scale bar represents 10 μm. (D) Immunofluorescence staining of PI infected tissue culture (fallopian tubes). hBD3 expression was analyzed by using an anti hBD3 antibody (green). *H. pylori* was visualized with an anti CagA antibody (red). Nuclei were stained with DraQ5 (blue). Scale bar represents 50 μm. Immunofluorescence staining was analyzed by confocal microscopy.

Figure 2: hBD3 expression is mediated by EGFR and MAP kinases. AGS cells were infected with PI using an MOI of 100. Before and during infection cells were treated with inhibitors against EGFR (AG1478, Gefitinib) or against B) MAP kinases (U0126). (A) RT-PCR analysis of hBD3 mRNA expression in PI infected and non-infected (NI) cells after 8 h of infection. Inhibitor added at 10 μM). DMSO was used as control in infected cells. Error bars indicate mean ± SD of three independent experiments. (B) hBD3 expression of infected and non-infected cells treated with (left panel) or without (right panel) U0126 was analyzed by immunoblot. Inhibitor used at 100 nM. Actin was used as a loading control.

Figure 3: Initially induced hBD3 expression decreases during prolonged infections. (A) AGS cells were infected with PI using the indicated MOI. To compare hBD3 expression between prolonged and short infections, cells were infected for 2 h on different days (1, 2 and 3 days). Final hBD3 expression was analyzed by immunoblotting. Actin was used as a loading control. (B, D) RT-PCR analysis of hBD3 mRNA expression in infected and non-infected (NI) AGS cells (MOI 100). Error bars indicate mean ± SD of three independent experiments. (B) Cells were infected with PI for indicated time points. D) Cells were infected with PI or the isogenic deletion mutant *PIAcagA* and analyzed 24 h after infection. (C)
Immunofluorescence staining of PI infected AGS cells 1 day (Id) and 2 days (2d) after infection. hBD3 was stained by using an anti hBD3 antibody (red), bacteria are visualized with an anti CagA antibody (green). Nuclei are stained with DraQ5 (blue). Scale bar represents 10 μm. Analysis was performed by using confocal microscopy.

Figure 4: Reduction of hBD3 is caused by selective inhibition of EGFR in a CagA-dependent manner. AGS cells were infected with PI using an MOI of 100. After infection cells were treated with 100 ng/ml EGF for 5 min. (A) EGFR phosphorylation was measured by immunoblotting using an anti-phosphotyrosine antibody. Phosphorylation intensities were quantified and normalized to non-infected unstimulated cells. Error bars indicate mean ± SD of three independent experiments. (B) Immunoblot analysis of EGFR phosphorylation at specific tyrosines in infected (PI) and non-infected (NI) cells. Antibodies against pY845, pY1045, pY992 and pY1068 were used. EGFR was used as a loading control. (C) Cells were infected with either PI or the isogenic deletion mutant PiAcagA. EGFR and CagA phosphorylation were analyzed by immunoblot using an anti-phosphotyrosine antibody. Actin was used as a loading control. (D) AGS cells were transfected with EGFR constructs encoding either constitutive active EGFR (EGFR L858R) or wildtype EGFR (EGFR WT). At 24 h post transfection cells were infected with PI. Cellular RNA was extracted and hBD3 mRNA measured by RT-PCR, 24 h after infection.

Figure 5: EGFR and hBD3 expression are inhibited via SHP-2. AGS cells were infected with PI (MOI 100). Cells were analyzed by immunoblotting 24 h after infection. For EGFR phosphorylation analysis infected and non-infected (NI) cells were stimulated with EGF (100 ng/ml) for 5 min. EGFR phosphorylation was analyzed by immunoblotting using an anti-phosphotyrosine antibody. Actin was used as a loading control. Error bars indicate mean ± SD of three independent experiments. (A, B, C) AGS cells were transfected with siRNAs targeting SHP-2 or luciferase as control. Three days post transfection cells were infected and analyzed by immunoblot (A, B) or RT-PCR (C). (A) Before lysis cells were treated with EGF. Phosphorylation intensities were quantified and normalized to non-infected unstimulated cells. (B) Immunoblotting analysis of hBD3 protein expression. Knockdown efficiency of SHP-2 was monitored using an anti SHP-2 antibody. (C) hBD3 mRNA quantification by RT-PCR of siRNA transfected cells. (D) RT-PCR of hBD3 mRNA expression in infected and non-infected AGS and SHP-2 knockdown cells (SHP2_4_KD).
Figure 6: SHP-2 enables bacterial survival during prolonged infections. (A) Immunofluorescence analysis of PI infected AGS and SHP-2 knock down cells (SHP2_4_KD). Cells were infected (MOI 100) and stained with an anti-hBD3 antibody (red) or anti-CagA antibody (blue) to visualize H. pylori. Knockdown cells express GFP (green) as a reporter construct for efficient lentiviral integration. Scale bar represents 10 µm. Analysis was performed using confocal microscopy. (B) CFU assay of free swimming (supernatant; SN) and attached (pellet; Pe) PI after infection of AGS and SHP-2 knockdown cells. Bacteria were plated after 24 h of infection. (C) Systematic model of CagA and SHP-2-mediated blockage of hBD3 expression during later infection time points.

Figure S1: hBD3 expression is mediated by EGFR. AGS cells were infected with PI (MOI 100). Before and during infection cells were treated with the indicated inhibitor concentrations targeting EGFR (AG1478). hBD3 expression of infected (PI) and non-infected (NI) cells was analyzed by immunoblotting. Actin was used as a loading control.

Figure S2: Cellular viability of infected AGS cells. AGS cells (1x10^5) were infected with PI (MOI 100). Cellular viability was measured 24 h after infection using a WST assay kit (company) and normalized to non-infected (NI) cells. Error bars indicate mean ± SD of three independent experiments. Cells treated for 5 min with TritonX-100 (TX) as a control.

Figure S3: EGFR inhibition is MOI dependent. AGS cells were infected with PI at the indicated MOI. Non-infected and infected cells were treated with EGF (100 ng/ml) 24 h after infection. Immunoblot analysis of EGFR phosphorylation was performed by using an anti phosphotyrosine antibody. Actin was used as a loading control.

Figure S4: EGFR Y1068 phosphorylation is inhibited during prolonged infections. AGS cells were infected with PI (MOI 100). Non-infected (NI) and infected cells were stained with an antibody directed to EGFR pY1068 (red). Scale bar represents 50 µM. Immunofluorescence was performed by epifluorescence microscopy.

Figure S5: Blockage of EGFR transactivation is ligand-independent. AGS cells were infected with PI (MOI 100). Non-infected (NI) and infected cells were stimulated at 24 h after infection with EGF or HB-
EGF respectively for the indicated time points. Immunoblotting analysis of EGFR phosphorylation was performed using an anti-phosphotyrosine antibody. Actin was used as a loading control.

Figure S6: *H. pylori* induced inhibition is selectively directed against EGFR. c-Met was precipitated from infected and non-infected (NI) cells 24 h p.i. Before lysis cells were treated with 100 ng/ml HGF. Phosphorylation of precipitated c-Met was analyzed by immunoblotting using an anti-phosphotyrosine antibody. Equal loading was confirmed with an anti c-Met antibody.

Figure S7: EGFR inhibition is dependent on tyrosine phosphatases. AGS cells were infected with PI (MOI 100). Non-infected (NI) and infected cells were treated at 24 h after infection with different concentrations of activated orthovanadate. Cells were subsequently stimulated with EGF (100 ng/ml) for 5 min and cellular lysates were analyzed by immunoblot. Long and short exposure times are shown to visualize EGFR phosphorylation under different inhibitor conditions. Immunoblot analysis of EGFR phosphorylation was performed by using an anti-phosphotyrosine antibody. Actin was used as a loading control.

Figure S8: SHP-2 is phosphorylated during *H. pylori* infection. AGS cells were transfected with a plasmid encoding for SHP-2. SHP-2 was precipitated using an anti SHP-2 antibody. Precipitates of non-infected (NI) and infected cells were analyzed by immunoblotting (lower panel). SHP-2 phosphorylation was monitored using an anti-phosphotyrosine antibody. Equal loading was confirmed with an antibody directed to actin. Phosphorylation intensity was normalized to non-infected cells (upper panel). Error bars indicate mean ± SD of three independent experiments.

Figure S9: SHP-2 knockdown in stable SHP2_4_KD cells. AGS derived stable SHP-2 knockdown cells were lysed and analyzed by immunoblot. AGS wildtype cells were used as control. SHP-2 protein levels were monitored by using an anti SHP-2 antibody. Actin was used as a loading control.
Table 1:

SHP-2 Inhibitors

Inhibitors targeting specifically SHP-2

2 main common inhibitors:

1) 8-hydroxy-7-(6-sulfonaphthalen-2-yl)diazenyl-quinoline-5-sulfonic acid (NSC-87877)
   Found in a screen of National Cancer Institute (NCI) Diversity Set chemical library; binds to catalytic cleft; cross-inhibition of SHP-1 in vitro;
   Chen et al.: 2006; Molecular Pharmacology
   Available: Acros Organics; L.8-hydroxy-7-(6-sulfo-2-naphthylazo)-5-quinolinesulfonic acid disodium salt, 412210100

2) Calpeptin (Calpain inhibitor)
   Here we identify the SH2 (Src homology region 2)-containing PTPase Shp-2 as a calpeptin-sensitive PTPase, and show that calpeptin interferes with the catalytic activity of Shp-2 in vitro and with Shp-2 signaling in vivo.
   Schoenwaelder et al.: 2000; Curr Biol
Table 1 (continued)

Others:

3) Nine compounds

Wen-Mei Yu et al., 2008; J Med Chem

Inhibitory effects of the nine active compounds on the phosphatase activity of SHP-2. (A) Candidate compounds identified by CADD were screened using the in vitro tyrosine phosphatase assay as described in the Experimental Section. Compounds were dissolved in DMSO at 100 µM. Shown are results of the nine active compounds. (B) Chemical structures of the nine active compounds. (C) The inhibitory effect of compound no. 162 on SHP-2 activity was tested at the indicated concentrations using the in vitro phosphatase assay.

For (A) and (C) see Figure S10.

4) p-halosulfonamides \((S0_2NHCH_2(4-ClC_6H_4); S0_2NHCH_2(4-FC_6H_4))\); Isatins (C0_2H)

Screening of the NCI diversity set of compounds has led to the identification of 5 (NSC-117199) which inhibits the protein tyrosine phosphatase (PTP) Shp2 with an IC₅₀ of 47 µM.

Lawrence et al.; 2008; J Med Chem

Table 1

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SUBSTITUTE SHEET (RULE 26)
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*Values are means and standard deviations of at least 4 experiments, each performed in duplicate.*
Table 1 (continued)

1. IC_{50} (Shp2) 10 µM

2. IC_{50} (Shp2) 1.8 µM

3. IC_{50} (Shp2) 2.5 µM

4. IC_{50} (Shp2) 0.3 µM

Known non-selective inhibitors for SHP-2

5) NAT6-297775 (Tetrazole)
Discovered from a screen of a natural product-like library
Noren-Muller et al., 2006; Proc Natl Acad Sci

Table 1. Selected phosphatase inhibitors identified in the screens

2a

2.47 ± 0.93 Shp-2

2b

3.95 ± 1.49 Shp-2'

6) PHPSI (selective Bidentate Inhibitor; Polypeptide; based on Sun et al., 2003 JBC; [Protein Data Bank (PDB) ID code 1N6W1]; http://www.rcsb.org/pdb/explore/explore.do?structureId=1PXH

PHPSI is the first compound that specifically inhibits Shp2 over the closely related phosphatases Shpi and PTP1B. PHPSI is an active site-directed small molecule inhibitor of Shp2. K_i value of PHPSI for inhibition of Shp2 was 0.73 (±0.34) µM, which is in agreement with the value, 0.7 µM

Hellmuth et al., 2008; Proc Natl Acad Sci

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7) Sodium Stibogluconate
Using in vitro protein tyrosine phosphatase (PTPase) assays, we found that sodium stibogluconate, a drug used in treatment of leishmaniasis, is a potent inhibitor of PTPases Src homology PTPase (SHP-1), SHP-2, and PTP1B but not the dual-specificity phosphatase mitogen-activated protein kinase phosphatase 1.
Pathak et al., 2001; The J of Immunology

Unspecific for SHP-2

8) bis(trifluorosulfonamide)
Inhibitor of PTPIB
Chen et al., 2001; J Biol Chem

9) Bisindolylmaleimide (PKC inhibitor)
Blocks PKC mediated Ser phosphorylation of SHP-2
Strack et al., 2002; Biochemistry

Common tyrosine phosphatase inhibitors

10) Orthovanadate (Na$_2$V$_0$_4)
Potent Tyrosine Phosphatase inhibitor.

(Company) Santa Cruz:

11) Phosphatase inhibitor Cocktail B: sc45045
Effectively inhibits tyrosine protein phosphatases, acid and alkaline phosphatases
Each vial contains 200 mM Imidazole, 100 mM Sodium Fluoride, 115 mM Sodium Molybdate, 100 mM Sodium Orthovanadate and 400 mM Sodium Tartrate Dihydrate.

12) bpV(biPY) (K[VO(O2)2C10H8N2]); sc-221376/bpV(HOpic) (K2[VO(02)2C6H4N03]); sc-221377/bpV(phen) (K[VO(02)2C12H8N2] . 3H20); sc-221378
These are protein phosphotyrosine phosphatase inhibitors.

13) BVT 948 (4-Hydroxy-3,3-dimethyl-2H-benz[g]indole-2,5(3H)-dione); sc-203536
Non-competitive protein tyrosine phosphatase inhibitor; enhances insulin signalling and insulin tolerance in ob/ob mice. Displays irreversible inhibition via catalysis of hydrogen peroxide-dependent oxidation of PTP.

14) Dephostatin (C$_7$H$_8$N$_2$O$_3$); sc-202131
Protein tyrosine phosphatase inhibitor. Competitively inhibits protein tyrosine phosphatase activity in membrane preparations from human neoplastic T cell line (IC$_{50}$ = 7.7 µM).
Table 1 (continued)

15) L-p-Bromotetramisole Oxalate (C11H11BrN2S-C2H2O4); sc-201431
p-Bromotetramisole is a well known inhibitor of alkaline phosphatases which was recently found to inhibit tyrosine phosphatase. This activity was identified by its ability to mimic the action of orthovanadate (a well known tyrosine phosphatase inhibitor) (1) in the potentiation of fluorouracil antiproliferative activity (2).

16) Mpv(pic) (V0(02)(C6H4N02)(Water)2); sc-221958
A protein phosphotyrosine phosphatase inhibitor and insulin receptor kinase (IRK) activator

(Company) Calbiochem:

17) Ptp inhibitor IV (j/s(4-Trifluoromethylsulfonamidophenyl)-1,4-diisopropylbenzene); 540211
A potent, reversible, competitive, and active-site directed inhibitor of protein tyrosine phosphatases (PTP; IC50 = 1.8, 2.5, 8.4, 13, 20, 6.4, and 6.7 µM for SHP-2, PTP1B, PTP-ε, PTP-Meg-2, PTP-α, PTP-β, and PTP-μ, respectively).

(Company) SIGMA:

18) Phenylarsine oxide (C6H5As0); P3075
Inhibits internalization of cell surface receptors; inhibits tyrosine phosphatases, with no effect on tyrosine kinase. Metabolic poison.

19) Alendronate sodium trihydrate (4-amino-l-hydroxy-l-phosphonobutyl phosphonic acid); A4978
Alendronate sodium trihydrate is a bone resorption inhibitor; farnesyl diphosphate synthase inhibitor (IC50 = 460 nM); CD45 protein tyrosine phosphatase inhibitor.

Inhibitors mentioned in a book (no access yet):

Design and synthesis of inhibitors of the protein tyrosine phosphatase, SHP-2
Table 1 (continued)

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Claims

1. A pharmaceutical composition comprising at least one inhibitor of SHP-2, optionally together with pharmaceutically acceptable carriers, adjuvants, diluents or/and additives.

2. The pharmaceutical composition as claimed in claim 1 for the treatment or/and prevention of a Helicobacter infection, in particular a Helicobacter pylori infection.

3. The pharmaceutical composition as claimed in claim 1 or 2, wherein the at least one inhibitor specifically inhibits the interaction of CagA with SHP-2, in particular the binding of CagA to SHP-2.

4. The pharmaceutical composition as claimed in any of the preceding claims, wherein the at least one inhibitor inhibits /-e/ac-acier-induced inactivation of the EGFR receptor.

5. The pharmaceutical composition as claimed in any of the preceding claims, wherein the at least one inhibitor prevents or/and reduces Helicobacter-induced down-regulation of hBD3 expression or/and exportation from the eukaryotic host cell.

6. The pharmaceutical composition as claimed in claim 5, wherein hBD3 expression is mediated by EGFR signalling.

7. The pharmaceutical composition as claimed in any of the preceding claims, wherein the at least one inhibitor prevents or/and reduces colonization of the stomach by Helicobacter, in particular Helicobacter pylori.
8. The pharmaceutical composition as claimed in any of the claims 1 to 7, wherein the at least one inhibitor is selected from the inhibitors of Table 1.

9. The pharmaceutical composition as claimed in any of the claims 1 to 7, wherein the at least one inhibitor is selected from the group of nucleic acids, nucleic acid analogues such as ribozymes, peptides, polypeptides, and antibodies, wherein the nucleic acid encodes SHP-2, or/and a fragment thereof, and wherein the antibody is directed against SHP-2 or/and a fragment thereof.

10. The pharmaceutical composition as claimed in claim 9, wherein the RNA molecule is a double-stranded RNA molecule, preferably a double-stranded siRNA molecule with or without a single-stranded overhang alone at one end or at both ends.

11. The pharmaceutical composition as claimed in claim 9 or 10, wherein the nucleic acid has a length of at least 15, preferably at least 17, more preferably at least 19, most preferably at least 21 nucleotides, or/and has a length of at the maximum 29, preferably at the maximum 27, more preferably at the maximum 25, especially more preferably at the maximum 23, most preferably at the maximum 21 nucleotides.

12. A screening method for identification of a compound suitable as inhibitor in a pharmaceutical composition defined in any of the claims 1 to 11, comprising the steps
   (a) providing a eukaryotic host cell or/and a transgenic non-human animal capable of being infected with *Helicobacter*, in particular *Helicobacter pylori*,
   (b) contacting a compound with the cell or/and the transgenic non-human animal of (a), and
   (c) selecting a compound which
      (i) inhibits the interaction of CagA with SHP-2, in particular the
binding of CagA to SHP-2,
(ii) inhibits Helicobacter-induced inactivation of the EGFR receptor,
or/and
(iii) prevents or reduces Helicobacter-induced down-regulation of hBD3 expression or/and exportation from the eukaryotic host cell.

13. The screening method of claim 12, wherein in step (a), the cell is contacted with Helicobacter, in particular H. pylori.

14. The screening method of claim 12 or 13, wherein the cell or/and animal expresses, in particular overexpresses, SHP-2, EGFR receptor, MAPK, or/and hBD3.

15. A screening method for identification of a compound suitable as inhibitor in a pharmaceutical composition defined in any of the claims 1 to 11, comprising the steps
(a) contacting a compound with isolated SHP-2,
(b) determining the activity of SHP-2 to interact with the EGFR receptor, and
(c) selecting a compound which reduces the interaction of SHP-2 with the EGFR receptor.

16. A method for treating or/and prevention of a Helicobacter infection, in particular a Helicobacter pylori infection comprising the administration of a pharmaceutical composition of any of the claims 1 to 11 to a subject in need thereof.

17. Use of an inhibitor of SHP-2 for the manufacture of a medicament for the treatment or/and prevention of a Helicobacter infection, in particular a Helicobacter pylori infection.
Figure 1 Bauer et al.

A

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B

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C

CagA

hBD3

Overlay

Magnification

NI

24 h p.i.

D

hBD3

H. pylori

Overlay

24 h p.i.
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Figure S1 Bauer et al.

WO 2011/110546

PCT/EP2011/053446

7/16
Figure S3 Bauer et al.

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EGFR pY
CagA pY
Actin
Figure S7 Bauer et al.

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long exposure
short exposure

EGFR pY
EGFR pY
Actin

NI
P1
Figure S8 Bauer et al.

SHP-2 phosphorylation in %

NI  P1
SHP-2 pY
SHP-2