Inhibitors of *Helicobacter pylori* ATPase Cag α block CagA transport and cag virulence

Markus Hilleringmann,¹ Werner Pansegrau,¹ Michael Doyle,²
Susan Kaufman,² Mary Lee MacKichan,² Claudia Gianfaldoni,³
Paolo Ruggiero³ and Antonello Covacci¹

Correspondence

Antonello Covacci
Antonello_Covacci@Chiron.com

^{1,3}Cellular Microbiology and Bioinformatics Unit¹, and Serology and Animal Model Unit³,
Immunological Research Institute Siena (IRIS), Novartis Vaccines S.r.l., Via Fiorentina 1,
I-53100 Siena, Italy

²Research and Development, Biopharmaceuticals, Novartis, 4560 Horton St M/S 4.4,
Emeryville, CA 94608-2916, USA

With the steadily increasing occurrence of antibiotic resistance in bacteria, there is a great need for new antibacterial compounds. The approach described here involves targeting virulence-related bacterial type IV secretion systems (TFSSs) with small-molecule inhibitors. The cag TFSS of *Helicobacter pylori* was chosen as a model, and novel inhibitors directed against the cag VirB11-type ATPase Cag α were identified. The cag genes encode proteins that are components of a contact-dependent secretion system used by the bacterium to translocate the effector molecule CagA into host cells. Translocated CagA is associated with severe gastritis, and carcinoma. Furthermore, functional TFSSs and immunodominant CagA play a role in interleukin (IL)-8 induction, which is an important factor for chronic inflammation. Inhibitors of Cag α were identified by high-throughput screening of chemical libraries that comprised 524 400 small molecules. The ATPase activity of Cag α was inhibited by the selected compounds in an *in vitro* enzymic assay using the purified enzyme. The most active compound, CHIR-1, reduced TFSS function to an extent that cellular effects on AGS cells mediated by CagA were virtually undetectable, while reduced levels of IL-8 induction were observed. Gastric colonization by CHIR-1-pre-treated bacteria was found to be impaired in a dose-dependent manner using a mouse model of infection. Small-molecule Cag α inhibitors, the first described inhibitors of a TFSS, are potential candidates for the development of new antibacterial compounds that may lead to alternative medical treatments. The compounds are expected to impose weak selective pressure, since they target virulence functions. Moreover, the targeted virulence protein is conserved in a variety of bacterial pathogens. Additionally, TFSS inhibitors are potent tools to study the biology of TFSSs.

Received 10 March 2006

Revised 16 June 2006

Accepted 22 June 2006

INTRODUCTION

Widespread emergence of bacterial pathogens that are resistant to common antibiotics calls for innovative antibacterial drugs. Targeting virulence functions, instead of enzymic activities that are essential *in vitro*, is a new approach and may represent a way to reduce the rate of resistance formation. Virulence genes often encode functions involved in host–pathogen relationships, such as persistence and invasion, and therefore they are non-essential under laboratory conditions, but they are required for successful propagation of a pathogen within the host. Hence, the

resulting selective pressure that could give rise to resistance is expected to be weaker than for housekeeping genes.

Type IV secretion systems (TFSSs) have been recognized as major virulence determinants in bacteria that are pathogenic to animals and plants (*Agrobacterium*, *Brucella*, *Bartonella*, *Bordetella*, *Helicobacter*, *Legionella*, *Rickettsia* and *Wolbachia*). TFSSs are required for delivery of effector molecules, either directly into host cells or to the bacterial environment (Ding *et al.*, 2003; Christie *et al.*, 2005; Bourzac & Guillemin, 2005).

We have chosen the well-studied human gastroduodenal pathogen *Helicobacter pylori* as a model system to demonstrate that virulence functions encoded by TFSSs can be targeted for use in antibacterial therapy. While it is estimated that about half of the world population is infected

Abbreviations: ATPase, adenosine triphosphate hydrolase; HTS, high-throughput screening; IL, interleukin; NTPase, nucleoside triphosphate hydrolase; PAI, pathogenicity island; TFSS, type IV secretion system.

with *H. pylori* (Rothenbacher & Brenner, 2003), it has been recognized that severe outcomes of the infection, e.g. acute gastritis, peptic ulceration, formation of MALT lymphoma and adenocarcinoma, are associated with the presence of the *cag* pathogenicity island (PAI) (Bourzac & Guillemin, 2005; Censini *et al.*, 1996) in the *H. pylori* chromosome. The presence of the *cag* PAI has been linked to virulent type I strains, whereas strains lacking the PAI are less virulent, and classified as type II strains (Censini *et al.*, 1996). The *cag* PAI encodes protein components of a TFSS that has been shown to play a major role in *H. pylori* virulence (Covacci *et al.*, 1997; Censini *et al.*, 1996). To date, the only known effector molecule that has been identified to be secreted by *cag* is the CagA protein (Stein *et al.*, 2002, 2000; Backert *et al.*, 2000; Odenbreit *et al.*, 2000).

CagA is targeted to epithelial cells, where it is tyrosine phosphorylated at EPIYA sites by c-Src and Lyn host kinases (Stein *et al.*, 2002). Phosphorylated CagA triggers a signal cascade that promotes cell motility and proliferation, and leads to the characteristic 'hummingbird' phenotype (Segal *et al.*, 1999), involving formation of lamellipodia and filopodia. Another CagA-mediated effect results from the fact that translocated CagA localizes to the cell membrane, and promotes redistribution of the proteins ZO-1 and JAM, which are normally found at tight cell junctions (Amieva *et al.*, 2003). This redistribution occurs independently of the phosphorylation state of CagA phosphorylation state and leads to leakiness of the junctions (Amieva *et al.*, 2003).

The presence of a functional *cag* TFSS results in NF- κ B activation in infected gastric epithelial cells, and leads to pro-inflammatory responses, such as IL-8 release (Crabtree *et al.*, 1995). Although the mechanism by which a functional *cag* TFSS induces pro-inflammatory responses is still not fully understood, there are two candidate mechanisms, both of which may contribute to the observed effects: (i) the presence of *cag* facilitates entry of peptidoglycan fragments into host cells, and these are sensed by the intracellular receptor Nod1, resulting in NF- κ B activation (Viala *et al.*, 2004); and (ii) CagA itself seems to be capable of potentiating pro-inflammatory responses, as shown by gene replacement and mutagenesis studies (Brandt *et al.*, 2005).

The *cag* TFSS of *H. pylori* encodes several proteins that show significant similarity with gene products of the paradigmatic *vir* TFSS of the *Agrobacterium tumefaciens* T-DNA transfer system (Akopyants *et al.*, 1998; Christie & Vogel, 2000; Christie, 2001; Christie *et al.*, 2005). The *vir* TFSS is formed by 12 proteins (VirB1–11, plus the coupling protein VirD4) (Christie, 2001).

The assembly process is not yet defined at the molecular level, but mutations within the *virB* operon have resulted in lack of polymerization, and absence of functions. Mutants of VirB4, VirB7, VirB9, VirB10 and VirB11 all show functional impairment, and lack polymerization of the conduit used for delivery of the effectors (Fernandez *et al.*, 1996; Berger & Christie, 1994).

Three proteins that contain NTP-binding motifs have been identified: the prototypes are VirB4, VirB11 and VirD4. To date, *in vitro* NTPase activity has been shown for the VirB11-type proteins (Krause *et al.*, 2000b; Machon *et al.*, 2002) and for one member of the VirD4 family, TrwB (Tato *et al.*, 2005). For several VirB11-type proteins, a ring-like hexameric structure has been demonstrated (Krause *et al.*, 2000a), and this has been confirmed by X-ray structural analysis of the *H. pylori* Cag α NTPase (Yeo & Waksman, 2004; Yeo *et al.*, 2000).

Although VirB11-type NTPases have been very well characterized structurally (Krause *et al.*, 2000a; Savvides *et al.*, 2003; Yeo *et al.*, 2000) and biochemically (Krause *et al.*, 2000b; Machon *et al.*, 2002; Rivas *et al.*, 1997), their function in TFSSs remains unclear. Members of this class of enzymes are also present in the type II secretion systems, and type 4 pilus systems (Planet *et al.*, 2001; Krause *et al.*, 2000a; Rivas *et al.*, 1997), and a role in the formation of the pilus-like cell appendages, which are commonly associated with TFSS, has been suggested (Sagulenko *et al.*, 2001). VirB11-type proteins are essential for TFSS function, as shown by mutagenesis studies (Berger & Christie, 1994), and their NTPase activity is detectable *in vitro* using purified protein, without the requirement of complex cofactors (Krause *et al.*, 2000b; Rivas *et al.*, 1997); these two properties make VirB11-type proteins, such as *H. pylori* Cag α , ideal targets for use in high-throughput screening (HTS) to search for compounds that specifically inhibit TFSS function.

Here, we describe the identification and validation of three specific inhibitors of Cag α , which is the VirB11 homologue of *cag*. The compounds efficiently inhibit Cag α ATPase activity *in vitro*. We demonstrate that one of the compounds reduces *cag* activity to the limits of detection, as measured by transfer of CagA protein from *H. pylori* to eukaryotic host cells. In a mouse model of infection, bacteria that had been pre-treated with the inhibitor showed impaired ability to colonize the gastric mucosa. Implications for application of TFSS inhibitors as a new type of anti-infective, representing the first described inhibitor of a TFSS, as well as a new tool for studying the mechanism of action of TFSSs, are discussed.

METHODS

Bacterial strains, cell lines and culture conditions. *H. pylori* strains G27 and SPM 326, and mutants G27 Δ HSP527 (*cagY*) and G27 Δ cagA, have been described (Marchetti *et al.*, 1995). Strains were cultivated on Columbia agar base (Oxoid) containing 5% (v/v) defibrinated horse blood or 5% (v/v) fetal bovine serum (FBS), and Dent's antibiotic supplement (Oxoid). BHI and Brucella broth (BB) media were supplemented with 5% FBS and appropriate antibiotics. Bacitracin (Sigma-Aldrich) was used at a concentration of 200 μ g ml⁻¹. Bacteria were incubated at 37 °C, under microaerophilic conditions. AGS cells (European Collection of Cell Cultures no. 89090402) were grown at 37 °C in 5% CO₂/95% air in Dulbecco's modified Eagle's medium (DMEM), with 10% (v/v) FBS and 1.5 mM L-glutamine.

Cag α in vitro assay. Native Cag α protein (HP0525) was obtained from induced *Escherichia coli* SCS1 (pWP4525) cells, as described previously (Krause *et al.*, 2000b). The ATPase activity of Cag α was assessed using a chromogenic reagent based on the classical malachite green reagent for detection of P_i (BIOMOL Green reagent). Briefly, Cag α (approx. 0.4 μ g in a total reaction volume of 40 μ l) was incubated for 45 min at 37 °C in reaction buffer (20 mM Tris/HCl, pH 8.0, 50 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, and 0.05 % Brij-35) in the presence of 1 mM ATP. Following the incubation period, 60 μ l reaction buffer was added, followed by 1 ml BIOMOL Green reagent. The concentration of P_i was determined by measuring the absorbance at 620 nm, after an incubation period of 30 min at 25 °C.

Chemical library. The compound collection belonged to the Chiron Corporation (Emeryville) substance libraries. For HTS, 524 400 diverse chemicals were selected, all of which were small molecules (M_r < 600), and of diverse structure. Five hundred thousand of the compounds were derived from Chiron's combined chemical libraries (CombiChem). We did not use a focused library, such as one containing analogues of ATP or ADP.

Assay methodology for Cag α inhibitors (HTS). Test compounds were added to DMSO at 40 $\times$ final concentration, and dispensed into 384-well polypropylene plates (1 μ l per well). Using a Multidrop dispenser (Thermo), 20 μ l buffer/salt solution (20 mM Tris, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, and 200 μ M ATP; Roche Applied Sciences) was added to each well, and the plates were shaken briefly to mix in the compounds. The Cag α enzyme (HP0525) was diluted to 2.25 μ g ml⁻¹ in a buffer/salt solution consisting of 20 mM Tris, pH 8.0, 50 mM NaCl, and 0.1 % Brij-35. Again using the Multidrop dispenser, 20 μ l of the enzyme solution was added to the plates to start the reaction. The plates were incubated at 37 °C for 45 min, at which time the reaction was terminated, and detection of free phosphate was initiated by the addition of 60 μ l Green reagent. After 30 min at room temperature, the plates were stacked, ten at a time, onto two Victor 2 plate readers (Perkin Elmer), and absorbance at 625 nm was measured. Two readers were used for each set of 20 plates, so that all were read within 1 h of the addition of Green reagent. Data were analysed using ActivityBase software (IDBS).

Hit evaluation (re-screening of hit candidates). Evaluation of hits was performed by re-screening the compounds in a dose-response assay. The most potent compounds were analysed by LC/MS to determine purity, and to confirm the intended structure. For further study, selected compounds were repurified by HPLC and reassayed to calculate their IC₅₀ values. Some compounds were contaminated with heavy metals; therefore, 0.1 mM EDTA was included in follow-up assays to avoid inhibition of Cag α activity by heavy metal ions. Inhibitory activity was crosschecked against non-VirB11-type ATPases, e.g. *E. coli* RecA protein (Sigma-Aldrich) in the presence of ssDNA, and canine Na⁺/K⁺-ATPase (EC 3.6.3.9).

Inhibitor studies. *H. pylori* Cag α inhibitor CHIR-1 was used at concentrations of 0–25 μ M (100 mM stock solution in DMSO) for the *in vitro* enzymic assay. For cell culture experiments with AGS cells, 25, 50 and 75 μ M solutions of CHIR-1 were applied. All controls were performed with the respective amount of DMSO. Preceding the infection protocol, overnight cultures of *H. pylori* or AGS cells were pre-incubated with CHIR-1 at 37 °C for up to 30 min; otherwise, the bacteria were added to the monolayer simultaneously with the supplemented medium. Cell viability was tested using the cell proliferation reagent WST-1 (Roche), according to the manufacturer's instructions.

Infection and fractionation of AGS cells. AGS cells (seeded in 10 cm tissue-culture dishes) were infected with an overnight culture

of *H. pylori* (in BB supplemented with antibiotics, or DMEM) for 3–5 h at an m.o.i. of about 100:1. Fractionation of AGS cells into an RIPA-buffer-soluble fraction (0.15 M NaCl, 0.05 M Tris/HCl, pH 7.2, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, containing 200 mM vanadate and 5 mg protease inhibitors ml⁻¹), and a membrane fraction, was performed as described by Stein *et al.* (2000).

Antibodies and immunoprecipitation. Immunoprecipitation with anti-phosphotyrosine (PY99; Santa Cruz Biotechnology) was carried out according to the protocols provided by the supplier. Anti-tubulin antibody (T-9026) was obtained from Sigma. Antibodies against G27 CagA (rabbit) and G27 urease (rabbit) were produced in our laboratory (F. Bagnoli, L. Buti, S. Censini, W. Pansegrau, I. Russo & M. Stein, unpublished results; Stein *et al.*, 2000). Secondary antibodies were obtained from Bio-Rad.

Assay for IL-8 induction by epithelial cells. AGS cells were cultivated in 75 ml tissue-culture flasks in a medium containing DMEM supplemented with L-glutamine and 5 % FBS. Cells were harvested by trypsinization, and resuspended in supplemented DMEM to a concentration of 2 $\times$ 10⁵ cells ml⁻¹. A 2 ml volume of the cell suspension was added to each well of a six-well plastic tissue-culture plate, and incubated until the monolayer was confluent to about 80 %. A *H. pylori* G27 culture grown overnight was harvested in PBS, and resuspended to a density of approximately 10⁸ c.f.u. ml⁻¹. Dilutions of 1:3 and 1:10 were prepared. Where indicated, bacteria were pre-incubated with DMEM/CHIR-1 at 37 °C, before adding 1.5 ml of the suspension to the monolayer. Otherwise, bacteria were added simultaneously with the respective medium to the AGS cells. After 4 and 24 h microaerophilic incubation, samples (500 μ l) were taken and centrifuged (15 000 g), before freezing at -80 °C, until assayed. The volume removed was replaced by fresh DMEM/CHIR-1, and phenotypic changes of AGS cells were estimated by light microscopy. Additionally, 20 μ l samples were removed and incubated under microaerophilic conditions in 1 ml BB medium for up to 5 days to control the viability of *H. pylori* G27.

Cytokine assay. The level of IL-8 in cell culture supernatants was determined by use of a commercially available human IL-8 ELISA kit (OptEIA; BD Biosciences), and expressed in pg ml⁻¹.

Confocal immunofluorescence. Infected AGS monolayers on glass coverslips were fixed with 3 % formaldehyde in PBS, with 1 mM CaCl₂ and 1 mM MgCl₂. Following permeabilization with 0.2 % Triton X-100, anti-*H. pylori* antibodies (α -CagA, rabbit) were applied to the coverslips. Green Alexa Fluor 488 goat anti-rabbit IgG (A-11034; Molecular Probes) was used as a secondary antibody.

Mouse colonization assays. Specific-pathogen-free CD1 mice (Charles River Laboratories) were housed and treated in compliance with current Italian law. After a 16-h starvation period, 6-week-old CD1 mice (10 animals per group) were infected, as previously reported (Marchetti *et al.*, 1995), with minor modifications. Immediately before challenge, bacteria were harvested from plates by a sterile cotton swab, resuspended in supplemented BHI medium, and their concentration was determined spectrophotometrically. An OD₅₃₅ of 1 in a cuvette with a 1 cm path length corresponds to 10⁹ c.f.u. ml⁻¹. *H. pylori* exists as an actively dividing, highly motile, spiral form, and a non-culturable, but viable, metabolizing coccoid form. Others (Cole *et al.*, 1997) have shown that the coccoid form of *H. pylori*, in contrast to the spiral form, binds poorly to gastric epithelial cells. Therefore, the phenotype of bacterial cultures, i.e. motility and spiral shape, was monitored microscopically so that we were able to initiate infection experiments with motile spiral cells. Mice were given 0.25 ml of 0.2 M NaHCO₃, intragastrically, to neutralize acidity, about 15 min before challenge. Through the same

route, either 10^9 (high dose of *H. pylori*) or 10^8 (standard dose of *H. pylori*) c.f.u. in 100 μ l saline was delivered directly into the stomach of each mouse. Where indicated, bacteria were pre-treated with 25 or 50 μ M CHIR-1 in DMSO (3.5 and 7 mM, respectively) for approximately 30 min before challenge (controls received 7 mM DMSO). The number of c.f.u. in inocula was determined by subsequent plate culture. Groups that received the standard infecting dose of *H. pylori* were further infected with the same bacterial dose 2 days later; infection was not repeated in mice that received the high dose. Forty-eight hours after each infection, a second dose of inhibitor (in 150 mM NaCl) was administered. Four and eleven days after infection with high and low dose, respectively, the animals were euthanized by carbon dioxide asphyxiation and their stomachs were removed immediately. Gastric mucosa was scraped from the stomach of each mouse, and, after serial dilution, this was spread on selective plates, which had been prepared as described above, with addition of 0.2 mg bacitracin ml^{-1} . *H. pylori* c.f.u. were counted after 6 days.

Statistical analysis. The statistical significance of the data was evaluated by a one-tailed Mann–Whitney *U* test or Fisher's exact test. For all comparisons, probability values less than 5% ($P < 0.05$) were considered statistically significant.

RESULTS

HTS of Cag α identifies three potent small-molecule inhibitors

The ATPase activity of Cag α was measured by assessing the P_i liberated during the Cag α *in vitro* assay. Quantification of free phosphate was performed by measuring absorbance at 620 nm using BIOMOL Green reagent. The laboratory-scale enzymic assay was adapted to HTS in 384-well plates (see Methods). A chemical library of 524 400 compounds was screened to identify inhibitors of Cag α ATPase activity. CombiChem compounds were tested in pools, at a final concentration of 1 μ M for each substance; other compounds were added at a final concentration of 5 μ M to the assay mixture, either as single compounds or as mixtures of five compounds. In an initial screen, 675 compounds were found to inhibit Cag α ATPase activity by at least 50%. Re-screening and verification of primary hits was performed in dose–response assays, resulting in 200 confirmed hits that had preliminary IC_{50} values of less than 10 μ M. After deconvolution of any pooled compound hits, 33 single compounds, thereof, showed IC_{50} values of 5 μ M or less, and 20 showed values of 1 μ M or less. Structural considerations to exclude undesirable structures with reactive groups, or structures difficult to modify, led to a selection of seven compounds. These compounds were analysed by LC/MS for purity, and to confirm their intended structure, followed by further studies after repurification by HPLC. The seven compounds considered for more comprehensive evaluation had not been identified as significant kinase inhibitors in other screens, nor had they shown inhibitory activity in other HTS assays.

Three of the seven compounds (CHIR-1, -2 and -3) retained IC_{50} values of < 1 μ M (Table 1) in further screens. Remarkably, two compounds, CHIR-1 and CHIR-2, were

Table 1. Small-molecule inhibitors of *H. pylori* Cag α

Inhibitor	Structure	IC_{50} (μ M)	M_r
CHIR-1		0.45	292.2
CHIR-2		0.78	316.4
CHIR-3		0.55	280.7

structurally closely related, falling into the class of thiazolidine-3,5-diones. The compound with the lowest IC_{50} value, CHIR-1, was acquired in larger amounts to confirm the inhibition properties of the substance, and to perform the experiments described below. Crosschecking its *in vitro* activity against non-VirB11-type ATPases [a mammalian ATPase (canine Na^+/K^+ -ATPase), and bacterial RecA protein] revealed 10- and 5-fold higher IC_{50} values, respectively (data not shown).

CHIR-1 suppresses the hummingbird phenotype in infected AGS cells

Infection of gastric cell lines by type I strains of *H. pylori* induces the so-called hummingbird phenotype, characterized by lamellipodia and filopodia, with spreading and elongated growth of the cells (Segal *et al.*, 1999). We investigated the effect of CHIR-1 on *H. pylori* in an infection experiment in which the translocation of CagA, and the resulting hummingbird phenotype, were indicators of the functionality of the *H. pylori* TFSS.

AGS cells (Fig. 1a) were infected with the wild-type strain G27, or with G27 ΔcagA as a negative control. To study the influence of CHIR-1, AGS cells were co-cultivated with the wild-type strain pre-incubated in the presence of CHIR-1. Wild-type *H. pylori* G27 induced cytoskeletal rearrangement, resulting in the hummingbird phenotype (Fig. 1b), whereas the ΔcagA mutant of G27 did not (Fig. 1c). Following pre-incubation of *H. pylori* for 30 min in the presence of 50 μ M CHIR-1, and co-cultivation with AGS cells in the presence of 50 μ M CHIR-1 for 4 h, a reduction in the number of cells showing the hummingbird phenotype, which was comparable with that for the ΔcagA mutant, was observed (Fig. 1e). A similar reduction of the hummingbird phenotype was not observed with bacterial cells that had not been pre-incubated with CHIR-1 (Fig. 1d).

The MIC value of CHIR-1 with *H. pylori* G27 was calculated to be 90 μ M, as determined by serial dilution of the inhibitor in a bacterial suspension. Human AGS cell toxicity of CHIR-1 was tested using a colorimetric assay, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases. AGS cell viability was not affected by CHIR-1 concentrations up to 100 μ M (data not shown).

Fig. 1. Interference contrast microscopy of infected AGS cells. AGS cells were incubated for 4 h in the presence of *H. pylori* strains, as described in Methods. Where indicated, co-cultivation was performed in the presence of CHIR-1; for one experiment, bacteria were pre-incubated for 30 min with CHIR-1. (a) AGS cells; (b) AGS cells incubated with *H. pylori* G27; (c) AGS cells incubated with G27 Δ cagA; (d) AGS cells incubated with G27 in the presence of 50 μ M CHIR-1; (e) G27 pre-incubated for 30 min in the presence of 50 μ M CHIR-1, and AGS cells incubated with the pre-treated G27 cells (*) in the presence of 50 μ M CHIR-1. Unstained cells were examined by using interference contrast microscopy.

CHIR-1 causes accumulation of CagA protein in *H. pylori* during AGS cell infection

The effect of CHIR-1 on CagA translocation was studied by confocal fluorescence microscopy (Fig. 2). AGS cells were infected with wild-type *H. pylori*, and CagA protein was visualized in permeabilized cells using a polyclonal anti-CagA antibody. A secondary antibody conjugated with Green Alexa Fluor 488 was used for CagA detection. AGS cells (60 % confluent) were infected with 10^7 c.f.u wild-type *H. pylori* in 12-well culture plates. In the untreated controls, the signal intensity of CagA within the bacterial cells was relatively low, while in the AGS cells, a faint and diffuse fluorescence signal, due to CagA translocation, was observed (Fig. 2a–c). However, upon co-cultivation of AGS cells with *H. pylori* cells that had been pre-cultivated in the presence of 50 μ M CHIR-1, CagA was visible virtually exclusively within the bacterial bodies, in which the protein accumulated in significant amounts (Fig. 2d–f).

As demonstrated by fluorescence intensity, at least a threefold higher concentration of CagA was demonstrated in *H. pylori* cells in AGS–*H. pylori* co-cultivates that were treated with CHIR-1, compared with untreated co-cultivates (Fig. 2g). Correspondingly, as compared with the untreated control, a similar (three- to fourfold) decrease in mean fluorescence intensity was observed in AGS cells in

AGS–*H. pylori* co-cultivates treated with CHIR-1 (Fig. 2g). These findings demonstrate that translocation of CagA is much reduced by the action of CHIR-1. The effect was comparable with that seen with *H. pylori* cag mutants defective in Cag α (VirB11) (Backert *et al.*, 2000).

CagA translocation into AGS cells is blocked by CHIR-1

To quantify the inhibitory effect of CHIR-1 on CagA translocation more precisely, we took advantage of the fact that CagA becomes tyrosine phosphorylated in the host cell. Therefore, the amount of tyrosine-phosphorylated CagA (pCagA) can be used as a readout for CagA translocation. Following co-cultivation of AGS cells with *H. pylori* G27, infected cells were harvested together with the attached bacteria, and a total extract was prepared. Phosphorylated proteins were enriched by immunoprecipitation from the total cell extract using anti-phosphotyrosine antibodies. Phosphorylated CagA (pCagA) protein was detected in the precipitate of untreated cells by Western blot analysis (Fig. 3, lanes 2 and 3). Again, 50 μ M CHIR-1 was sufficient to inhibit CagA translocation into AGS cells to the limits of detection for our study (Fig. 3, lanes 6 and 7). Once more, a short period of pre-incubation of *H. pylori* cells in the presence of the inhibitor was necessary to achieve complete

Fig. 2. Inhibition of CagA activity by CHIR-1 results in CagA accumulation in *H. pylori*. AGS cells were incubated in the presence of *H. pylori* G27, as described in Methods. CagA protein was labelled by immunofluorescence using polyclonal anti-CagA antibodies and Green Alexa-Fluor-488-labelled IgG, as primary and secondary antibodies, respectively. (c, f) Phase-contrast images (a, d) were overlaid with the corresponding confocal fluorescent images (b, e) showing the distribution of CagA. Bar, 50 μ m. (a–c) No inhibitor was added; (d–f) G27 was pre-incubated for 30 min in the presence of 50 μ M CHIR-1, followed by co-cultivation of AGS cells with G27 in the presence of 50 μ M CHIR-1. (g) Quantification of CagA-protein-related fluorescence within *H. pylori* and AGS cells, respectively. Mean fluorescence intensities were measured in selected regions of digital image sets corresponding to either AGS or *H. pylori* cells. Intensities are given in arbitrary units. Error bars indicate SD.

inhibition of CagA transfer. Without pre-incubation, a detectable amount of CagA reached the recipient cells (Fig. 3, lanes 4 and 5). Importantly, the total amount of CagA remained constant, indicating that the different quantities of pCagA measured were not due to variable overall amounts of CagA resulting from CHIR-1 treatment (Fig. 3, lower panel). Intracellular concentrations of *H. pylori* α -urease and AGS α -tubulin were not altered during a 4 h incubation period in the presence of 50 μ M CHIR-1, when compared with a control without CHIR-1 (data not shown).

To determine the minimum period of time required for pre-incubation, and to compare it with the time required for CagA transfer, *H. pylori* G27 cells were exposed to CHIR-1.

Aliquots of the treated *H. pylori* cells were applied in co-cultivation experiments with AGS cells, in the presence of CHIR-1, and the amount of phosphorylated CagA (pCagA) was determined as described above (Fig. 3). CagA transfer activity was reduced significantly between 1 and 3 min of pre-incubation (Fig. 4, bars 6 and 7). The incubation period required for this effect to occur was short (<3 min) compared with the time required for CagA transfer, which was in the range of 30 min. The finding that a significant quantity of CagA reaches the AGS cells in the presence of CHIR-1, without the pre-incubation step, suggests that the inhibitor is effective only for a short time period at the beginning of the secretion process, and that transfer of CagA then becomes resistant to the action of CHIR-1. This might be related to the observation that

Fig. 3. Complete inhibition of CagA translocation requires pre-incubation of *H. pylori* G27 with CHIR-1. AGS cells were co-cultivated with *H. pylori* G27. Upper panel, transfer of CagA protein to AGS cells was analysed by immunoprecipitation of phosphotyrosine-containing proteins from the cell extracts with anti-phosphotyrosine IgGs, separation on a SDS-polyacrylamide gel, and blotting to a nitrocellulose membrane. Hence, only CagA protein that was phosphorylated after transfer to AGS cells (pCagA) is seen. Immunoprecipitated pCagA protein was detected using polyclonal anti-CagA serum and the ECL system. Lower panel, CagA protein was detected with polyclonal anti-CagA serum, following electrophoresis of total cell extracts and blotting. Lanes: 1, AGS cells were co-cultivated with G27 Δ cagY (*virB10*); 2 and 3, AGS cells were co-cultivated with G27 in the absence of the inhibitor; 4 and 5, AGS cells were co-cultivated with G27 in the presence of 50 μ M CHIR-1; 6 and 7, G27 cells were pre-incubated for 30 min in the presence of 50 μ M CHIR-1. AGS cells were co-cultivated with the pre-treated G27 in the presence of 50 μ M CHIR-1.

contact establishment between AGS cells and *H. pylori* is a fast process that requires less than 5 min (F. Bagnoli, personal communication). Pre-incubation of the AGS cells with the inhibitor had no effect on CagA transfer (Fig. 4, bar 2).

CHIR-1 reduces IL-8 secretion in infected AGS cells

IL-8 induction by *H. pylori* in epithelial cells is dependent on the presence of a functional TFSS (Fischer *et al.*, 2001; Selbach *et al.*, 2002), and is influenced by mediators such as CagA (Brandt *et al.*, 2005) and peptidoglycan (Viala *et al.*, 2004). The function of Cag α (VirB11) has been shown to be necessary for IL-8 induction (Fischer *et al.*, 2001). Furthermore, systematic mutagenesis of the *cag* PAI revealed that the mechanism is independent of Cag β (VirD4), which is another TFSS protein that contains an NTP-binding motif. We tested Cag α -ATPase inhibitor CHIR-1 for its effect on IL-8 secretion. It has been shown that IL-8 secretion of infected AGS cells is strongly enhanced after co-cultivation for 24–36 h (Brandt *et al.*, 2005). In particular, the

Fig. 4. Kinetics of CagA translocation inhibition by CHIR-1. *H. pylori* cells were incubated for various periods of time in the presence of 50 μ M CHIR-1. Following pre-incubation, bacteria were co-cultivated with AGS cells and 50 μ M CHIR-1. Phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies, separated on a polyacrylamide gel, and blotted to a nitrocellulose membrane. pCagA protein (phosphorylated following transfer to AGS cells) was detected using a polyclonal anti-CagA antiserum, and quantified densitometrically. Relative amounts of pCagA are given in arbitrary units. Internal controls for quantification: total CagA protein and α -urease for *H. pylori*; α -tubulin for the AGS cells (not shown). Bars: 1, AGS cells were co-cultivated with *H. pylori* G27 Δ cagA; 2, AGS cells were pre-incubated with CHIR-1 for 15 min (*) before co cultivation with G27; 3, AGS cells were co-cultivated with *H. pylori* G27; 4–9, *H. pylori* G27 was pre-incubated with CHIR-1 for the indicated periods of time, before co-cultivation with AGS cells (*). Grey bars, data points for the kinetic assays; white bars, control experiments. Quantifications were performed in triplicate. The results are means $\pm$ SD.

potentiating effect of CagA on IL-8 production can be observed only after prolonged periods of co-cultivation (Brandt *et al.*, 2005). In our experiment, we quantified the amount of IL-8 release by AGS cells infected with CHIR-1-treated *H. pylori* G27 by ELISA after 24 h (Fig. 5).

IL-8 production by infected AGS cells was found to be inhibited by CHIR-1 in a concentration-dependent manner (Fig. 5). The effect varied with the amount of viable bacteria added (not shown), being most evident for a 1 : 10 dilution of the stock suspension. Interestingly, inhibition of IL-8 production was more pronounced after pre-incubation of infecting bacteria with CHIR-1. In the presence of 50 μ M CHIR-1, the amount of secreted IL-8 dropped by 19.8%. Using *H. pylori* cells that had been pre-incubated for 15 min with 50 μ M CHIR-1, IL-8 secretion dropped by 36.6%. In both cases, IL-8 concentrations were measured after 24 h co-cultivation, and the viability of the treated bacteria was tested by incubating an aliquot in BB medium at 37 $^{\circ}$ C for several days, under microaerophilic conditions. It was found that the CHIR-1 treatment did not impair viability of *H. pylori* under the conditions described (not shown).

Fig. 5. CHIR-1 inhibits IL-8 secretion by infected AGS cells. IL-8 secretion was induced in AGS cells, as described in Methods, and the amount of secreted IL-8 was detected by ELISA. Bars: 1, AGS cells were co-cultivated with *H. pylori* G27; 2–4, co-cultivation was performed in the presence of 25, 50 and 75 μM CHIR-1, respectively; 5, G27* was pre-incubated for 15 min with 50 μM CHIR-1, and co-cultivation with AGS cells was in the presence of the same concentration of CHIR-1; 6, G27 in the absence of AGS cells or CHIR-1; 7, AGS cells in the absence of G27 or CHIR-1; 8, AGS cells in the presence of 50 μM CHIR-1. A square bracket joins data points obtained with and without pre-incubation of bacteria (PI) in the presence of 50 μM CHIR-1. IL-8 concentration in the culture supernatant was determined after 24 h co-cultivation. Quantifications were performed in triplicate. The results are means ± SD.

CHIR-1 impairs gastric colonization of mice by *H. pylori*

To study the effect of the Cagx inhibitor on early phase of colonization, we inoculated CD1 mice intragastrically with *H. pylori* strain SPM 326 that had been pre-treated with CHIR-1 (Marchetti *et al.*, 1995). SPM 326 is a mouse-adapted type I strain, which responds to CHIR-1 in a way that is similar to G27 in various assays (data not shown). Mice were given roughly equal numbers of c.f.u. of non-treated and CHIR-1-treated bacteria. All animals tested survived treatment with bacteria plus Cagx inhibitor at concentrations as high as 50 μM.

Infection/colonization was quantified by determining the number of bacteria in the stomach of each mouse after scraping gastric mucosa, and plating out serial dilutions. Increasing concentrations of CHIR-1 resulted in decreased colonization by *H. pylori* SPM 326, as measured by the number of c.f.u. recovered, in comparison with the c.f.u. recovered in the corresponding infected controls. When the stomachs of mice that received standard bacterial dose were analysed after sacrifice, 8 out of 10 mice were found to be *H. pylori*-positive in the infected control group, while a significantly lower number of *H. pylori*-positive mice (2 out of 10, $P=0.011$) were found infected in the group that concomitantly received 50 μM CHIR-1; the group that received 25 μM CHIR-1 showed a non-significant reduction in the number of infected mice (6 out of 10, $P=0.31$). In terms of bacterial colonization level, the group that received the standard bacterial dose showed a highly significant decrease in bacterial colonization (the geometric mean of c.f.u. per stomach was more than 2 log units lower than control group, $P=0.0014$) when concomitantly treated with 50 μM CHIR-1, and only a marginal reduction ($P=0.139$) when treated with 25 μM CHIR-1. The group that received the high bacterial dose showed a similar trend after 4 days of infection, although the differences with the control group were not significant: 9 out of 10 mice were found to be infected in the control group, 5 out of 10 ($P=0.07$) treated with 50 μM CHIR-1 were infected, and 8 out of 10 ($P=0.5$) treated with 25 μM CHIR-1 were infected; in quantitative terms, the reduction of infection almost reached a significant level (a geometric mean of more than 1 log unit lower than the control group, $P=0.052$) with 50 μM CHIR-1, and it was not significant ($P=0.3$) with 25 μM CHIR-1 (Fig. 6).

DISCUSSION

Using the well-studied organism *H. pylori* as a model, we sought to find small molecules that inhibited the activity of the cag-encoded TFSS, which is an important factor in the pathogenicity of the bacterium (Bourzac & Guillemin, 2005; Censini *et al.*, 1996). A HTS involving 524 400 molecules identified 53 compounds that inhibited the TFSS-encoded ATPase Cagx *in vitro*, with IC₅₀ values below 5 μM. Three selected compounds (CHIR-1, -2 and -3) had IC₅₀ values below 1 μM, and we considered these to be a reasonable starting point for identifying candidates as lead structures (Table 1). The most potent substance, CHIR-1 (IC₅₀ 0.45 μM), was chosen for further characterization.

The potential of CHIR-1 to block cag activity was assessed in several cell-based assays. We used CagA transfer and phosphorylation as early indicators of TFSS functionality, since phosphorylated CagA can be detected in AGS cells as early as 30 min after the start of co-cultivation (Selbach *et al.*, 2003). Furthermore, it has been verified recently that all translocated CagA is phosphorylated at tyrosine residues, before being processed in the Golgi apparatus (A. Covacci, F. Bagnoli & L. Buti, unpublished observations).

Fig. 6. Dependence of c.f.u. recovered in a mouse model of infection on CHIR-1 pre-treatment of *H. pylori*. CD1 mice (10 animals per group) were infected twice with 1×10^8 (a) or once with 1×10^9 (b) *H. pylori* SPM 326 that had been pre-treated for 30 min with different concentrations of CHIR-1 (0, 25 and 50 μ M). A second dose of CHIR-1 was given 48 h p.i. Eleven and four days p.i., for low- and high-infection doses, respectively, groups were euthanized, and gastric mucosa was assessed for colonization by inoculating serial dilutions of mucosal cells on selective blood agar plates. Statistical analysis was performed using a Mann-Whitney *U* test. Symbols represent values for individual animals in a group, and horizontal bars represent geometric means. *, A significant reduction in c.f.u. compared with the non-treated control ($P=0.0014$).

In this work, we did not use *cag α* (*virB11*) mutants as controls. Although this seems to be an obvious control to use, Cag α mutants do not reflect the action of the inhibitor. TFSSs are multi-protein complexes consisting of a core of ten or more essential components. If any of these components is missing, the entire complex is absent from the cells. Cag α is one of the essential components, and, consequently, using a Cag α mutant as a control would not only eliminate the target for the inhibitor, but destroy the entire TFSS. Hence, one would neither see any transfer of CagA, nor see the phenotypic effects caused by either phosphorylated CagA or the mere presence of the TFSS. These effects are well studied, and described extensively in the literature (Christie *et al.*, 2005; Fischer *et al.*, 2001). Exposing cells that bear a functional TFSS to an inhibitor such as CHIR-1 is a completely different situation. The effect is directed against the NTPase activity of VirB11, but the TFSS complex is present, and the effect is transitory. Nevertheless, in this situation, which is not comparable with that of a VirB11 mutant, we see that in the presence of CHIR-1, CagA transfer can be virtually abolished.

We demonstrated that CHIR-1 (i) inhibits CagA secretion into AGS cells, as shown by the marked reduction of intracellular CagA in AGS cells, and accumulation of CagA protein in bacterial cells when co-cultivated with AGS cells (Fig. 2); (ii) reduces the amount of phosphorylated CagA in infected AGS cells to the limits of detection (Fig. 3); and (iii) strongly reduces the number of cells showing the hummingbird phenotype, which is known to be a consequence of the presence of phosphorylated CagA in AGS cells (Fig. 1).

We did not observe a reduction of viability in bacteria after several hours of incubation in the presence of 50 μ M CHIR-1. In addition to general viability tests, the intracellular concentrations of two chosen proteins of *H. pylori*,

CagA and α -urease, were found to be unaltered during a 4 h incubation period in the presence of 50 μ M CHIR-1, when compared with an untreated control (Fig. 3, and data not shown). Furthermore, there was no influence on AGS cell viability at CHIR-1 concentrations of up to 100 μ M, when using the cell proliferation reagent WST-1.

A complete block of the TFSS-driven CagA translocation by inhibition of Cag α ATPase activity was dependent on pre-incubation of the bacteria with CHIR-1 for a short period of time before allowing the bacteria to come into contact with the recipient cells (Figs 3 and 4). Without a pre-incubation step, CagA translocation into AGS cells was not totally blocked, even in the presence of CHIR-1, and the hummingbird phenotype (Fig. 1) was observed. These findings indicate that the inhibitor interferes with a very early step in the TFSS mechanism, but not with the translocation step of the secretion process itself, or with other processes resulting in CagA-induced cellular changes.

In the common model of the TFSS machinery, it is generally assumed that VirB11-like proteins, such as Cag α , are required for pilus assembly, functioning either as chaperones for pilus subunits or as a pump that drives pilus subunits across the inner membrane and assembles them into filamentous structures (Savvides *et al.*, 2003; Sagulenko *et al.*, 2001). Interestingly, NTPases related to VirB11-type proteins are also found in type-4 pilus systems, in which they are supposed to be involved in active pilus extrusion and retraction (Planet *et al.*, 2001). Using electron microscopy to observe TFSSs during the initial phase of the transfer process, pilus structures have been seen to make the first contact between donor and recipient cells (Rohde *et al.*, 2003). Cag α has also been found to be structurally similar to the p97 AAA ATPase, a protein involved in membrane fusion and organelle biogenesis (Atmakuri *et al.*, 2004; Yeo & Waksman, 2004). Therefore, one aspect of Cag α activity

might involve promoting intimate bacterial–host interactions, as a result of membrane fusion events or pilus-mediated attachment.

Involvement of Cag α during initial phase of contact might explain the requirement for a short period of pre-incubation of the bacteria with the inhibitor to inhibit CagA transfer completely (Figs 3–5). Following formation of the initial contact, which is a very brief process of a few minutes only, there is evidence that a stable aggregate is formed involving tight contact between the host cells and the bacteria (Rohde *et al.*, 2003). At this point, it is conceivable that VirB11-related NTPase activity is no longer required, and that the subsequent secretion process is therefore resistant to any inhibitor of VirB11-type NTPases. In this context, it would be interesting to find out if the surface structures that have been identified recently in *H. pylori* (Tanaka *et al.*, 2003; Rohde *et al.*, 2003) are affected by CHIR-1.

IL-8 secretion, an important factor for chronic inflammation during *H. pylori* inflammation, has been found to be dependent on a functional cag PAI TFSS (Fischer *et al.*, 2001), and influenced by mediators, such as CagA (Brandt *et al.*, 2005) and peptidoglycan (Viala *et al.*, 2004). Cag α plays a crucial role in IL-8 induction, as shown in several mutagenesis studies (Fischer *et al.*, 2001; Selbach *et al.*, 2002). Interestingly, besides individual strain characteristics influencing cytokine induction, IL-8 release has been found to be time dependent, and strongly enhanced at 24–36 h after infection (Brandt *et al.*, 2005). We investigated the influence of the Cag α inhibitor on cytokine production in a cell culture model. After 24 h of co-cultivation at concentrations of CHIR-1 that reduced CagA translocation from pre-treated bacteria into AGS cells to detection limits (Fig. 3), the secretion of IL-8 was also found to be significantly reduced (Fig. 5). The effect was concentration dependent, and again it was enhanced by pre-incubation of infecting bacteria (Fig. 5). Reduced IL-8 induction caused by missing translocated CagA is in accordance with published results (Brandt *et al.*, 2005) that have identified CagA as a contributing factor for IL-8 induction.

Generally, in experiments in which CagA is missing, or does not contain EPIYA sites (Brandt *et al.*, 2005), or does not reach the recipient cells, higher IL-8 levels are observed than in respective studies involving deletion mutants of Cag α . In the absence of Cag α , IL-8 induction as low as 5–20 %, when compared with wild-type levels, has been observed (Selbach *et al.*, 2002). One reason for our observation of higher residual levels of IL-8 in the presence of CHIR-1 might be the substantial difference between the complete deletion of a gene and the inhibition of the enzymic activity of its product with a small molecule inhibitor. Regarding the multimeric composition of a TFSS, the deletion of a component results in more drastic changes, affecting also the overall structural integrity. In line with this, it has been found that other than TFSS-driven substrate transfer, overall structural intactness, and attachment of bacteria to epithelial cells, are crucial for cytokine production (Rieder *et al.*, 1997).

We studied the influence of CHIR-1 on bacterial colonization in a mouse model during the early stages of *H. pylori* infection. cag-PAI-positive type I strains are associated with increased virulence and severe clinical outcomes (Marchetti *et al.*, 1995). *H. pylori* type I strain SPM 326 was pre-incubated with different concentrations of CHIR-1 before infecting CD1 mice. After 11 days of infection with a standard bacterial dose, increasing concentrations of Cag α inhibitor resulted in (i) decreased numbers of infected animals per group, and (ii) reduced numbers of recovered c.f.u. per stomach in infected mice (Fig. 6). The reduced number of infected animals in the group pre-treated with 50 μ M CHIR-1 was found to be significant when compared with the non-treated control. Whereas a trend in c.f.u. reduction was measured for the control group not treated with the inhibitor, and the group treated with 25 μ M CHIR-1, almost no c.f.u. were recovered from mice infected with bacteria that had been pre-treated with 50 μ M CHIR-1 (significant decrease of about 2 log units). Although not significant, a downward trend of infected animals and recovered c.f.u. with increasing amounts of CHIR-1 used to pre-treat bacteria was also found after 4 days of infection with the high bacterial dose. This suggests that CHIR-1 interferes with early events in *H. pylori* SPM 326 colonization of mouse gastric mucosa. In the literature, contradictory results can be found regarding whether or not cag PAI is essential for gastric colonization, and how it facilitates persistent infection; the results, apart from the specific phase of infection, depend highly on the bacterial and host phenotypes observed during *in vivo* study (Eaton *et al.*, 2001; Marchetti & Rappuoli, 2002). Results reported by Eaton *et al.* (2001) indicate that deletion of cag PAI in *H. pylori* strain SS1 is not essential for colonization. However, during the very early phase of infection [2 days post-infection (p.i.)], a cag-negative strain of *H. pylori* 26695 tended to colonize piglets to a lesser extent than the respective wild-type strain (Eaton *et al.*, 2001). Studies using the well-established Mongolian gerbil model suggest that intact TFSS of *H. pylori* has a role in colonization of the gastric corpus (Rieder *et al.*, 2005). It has been suggested that cag PAI might be important for a specific step in colonization (Monack *et al.*, 2004). Our results are in line with the work performed by Marchetti and Rappuoli (2002), in which, during the early phase of infection, colonization efficiency in CD1 mice was reduced by 200-fold for a Cag α mutant of *H. pylori* strain SS1; inactivation of cag genes essential for TFSS function also led to a reduced number of infected animals (mean infection rate was 20–40 % when compared with animals infected with the wild-type). Inactivation of Cag α , and the resulting loss of TFSS functionality, suggest a reduced bacterial virulence for type I strains of *H. pylori* in animal models.

Our initial studies on CHIR-1 activity *in vivo* encourage further investigations using different systems, such as the Mongolian gerbil model, to gain a deeper understanding of the properties of CHIR-1 *in vivo*. At this point, we cannot exclude the fact that additional effects of CHIR-1 may

contribute to the measured reduction of colonization during early stages of infection. These effects include interaction with other VirB11-like proteins in *H. pylori*, such as the VirB11-homologue located within the flagellar export locus (Porwollik *et al.*, 1999), which has been found to be essential for gastric colonization in a Mongolian gerbil model of infection (Kavermann *et al.*, 2003).

For TFSS Cag β , which is another protein containing NTP-binding motifs, the effect of CHIR-1 could not be tested, since we did not succeed in demonstrating the NTPase activity *in vitro*. However, the primary sequences, including the NTP-binding motifs of Cag α and β , differ markedly from each other; therefore, it is very unlikely that a molecule selected for its activity on Cag α would also interact with Cag β .

Our study formally demonstrates that it is possible to identify inhibitors of virulence-related TFSSs, as shown in our model of study *H. pylori*. Nevertheless, many additional questions need to be addressed before an enzymic inhibitor can be transformed into an efficient antibiotic. Studies on the suitability of a compound for use as a drug (e.g. solubility and uptake), and its toxicity and pharmacokinetics, together with further optimization of lead compounds, must be performed in order to obtain the desired properties.

Presently, we are studying the interaction between Cag α and CHIR-1 using co-crystallization and molecular modelling techniques. Detailed structural information might allow rational modifications of the lead structure of the inhibitor to further improve its efficacy. The crystal structure of Cag α has been solved by Yeo *et al.* (2000). In addition, the synthesis of radioactively labelled CHIR-1 might be helpful for a detailed investigation of the uptake, and further interaction of the inhibitor with Cag α , in the cellular system.

To our knowledge, this is the first report that describes a specific inhibitor of a TFSS that is capable of interfering with an essential enzymic function of the injectosome. The compound efficiently inhibited the function of a single component of the *H. pylori* *cag* TFSS, Cag α , and thereby blocked the biological activity of the system as a whole. Initial *in vivo* evaluation studies indicated that CHIR-1 mediated the reduction of pathogenic effects of *H. pylori* in mice. Recently, for several conjugative systems, inhibition by unsaturated fatty acids was demonstrated, although the mechanism of action of these compounds remains to be elucidated (Fernandez-Lopez *et al.*, 2005). Others have shown that type III secretion systems are also potential targets for new antimicrobials (Nordfelth *et al.*, 2005; Kauppi *et al.*, 2003). Our ongoing work will extend these studies to other pathogens in which TFSSs are required virulence factors. Future experiments could give more information on the specificity of the inhibitors identified using *H. pylori* Cag α that, in principle, might also be active on similar NTPases from other pathogens that carry a TFSS.

ACKNOWLEDGEMENTS

We gratefully acknowledge A. Harris and S. Harrison for providing inhibitors CHIR-1, -2 and -3, J. Bach for carrying out many of the assays, and K. Dungca for technical assistance. We are indebted to L. Pancotto and the animal care team for excellent help during *in vivo* experiments, and to M. de Bernard (University of Padua) for valuable help with confocal microscopy. We would like to thank S. Barone and F. Bagnoli for helpful discussions, and providing protocols, and the other members of our laboratory for the supportive comments, and supplying various reagents and antibodies. Finally, we are grateful to G. Corsi for his support doing the artwork. M.H. is supported by a Marie Curie Fellowship of the European Commission (contract number QLK2-CT-2001-51028). This work was supported by grant QLRT-2001-01200 (TFSS) of the European Union framework 5 program.

REFERENCES

- Akopyants, N. S., Clifton, S. W., Kersulyte, D. & 7 other authors (1998). Analyses of the *cag* pathogenicity island of *Helicobacter pylori*. *Mol Microbiol* **28**, 37–53.
- Amieva, M. R., Vogelman, R., Covacci, A., Tompkins, L. S., Nelson, W. J. & Falkow, S. (2003). Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science* **300**, 1430–1434.
- Atmakuri, K., Cascales, E. & Christie, P. J. (2004). Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial type IV secretion. *Mol Microbiol* **54**, 1199–1211.
- Backert, S., Ziska, E., Brinkmann, V., Zimny-Arndt, U., Fauconier, A., Jungblut, P. R., Naumann, M. & Meyer, T. F. (2000). Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cell Microbiol* **2**, 155–164.
- Berger, B. R. & Christie, P. J. (1994). Genetic complementation analysis of the *Agrobacterium tumefaciens* *virB* operon: *virB2* through *virB11* are essential virulence genes. *J Bacteriol* **176**, 3646–3660.
- Bourzac, K. M. & Guillemin, K. (2005). *Helicobacter pylori*-host cell interactions mediated by type IV secretion. *Cell Microbiol* **7**, 911–919.
- Brandt, S., Kwok, T., Hartig, R., Konig, W. & Backert, S. (2005). NF- κ B activation and potentiation of proinflammatory responses by the *Helicobacter pylori* CagA protein. *Proc Natl Acad Sci U S A* **102**, 9300–9305.
- Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R. & Covacci, A. (1996). *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci U S A* **93**, 14648–14653.
- Christie, P. J. (2001). Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol Microbiol* **40**, 294–305.
- Christie, P. J. & Vogel, J. P. (2000). Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol* **8**, 354–360.
- Christie, P. J., Atmakuri, K., Krishnamoorthy, V., Jakubowski, S. & Cascales, E. (2005). Biogenesis, architecture, and function of bacterial Type IV secretion systems. *Annu Rev Microbiol*
- Cole, S. P., Cirillo, D., Kagnoff, M. F., Guiney, D. G. & Eckmann, L. (1997). Coccoid and spiral *Helicobacter pylori* differ in their abilities to adhere to gastric epithelial cells and induce interleukin-8 secretion. *Infect Immun* **65**, 843–846.
- Covacci, A., Falkow, S., Berg, D. E. & Rappuoli, R. (1997). Did the inheritance of a pathogenicity island modify the virulence of *Helicobacter pylori*? *Trends Microbiol* **5**, 205–208.

- Crabtree, J. E., Covacci, A., Farmery, S. M., Xiang, Z., Tompkins, D. S., Perry, S., Lindley, I. J. & Rappuoli, R. (1995). *Helicobacter pylori* induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. *J Clin Pathol* **48**, 41–45.
- Ding, Z., Atmakuri, K. & Christie, P. J. (2003). The outs and ins of bacterial type IV secretion substrates. *Trends Microbiol* **11**, 527–535.
- Eaton, K. A., Kersulyte, D., Mefford, M., Danon, S. J., Krakowka, S. & Berg, D. E. (2001). Role of *Helicobacter pylori* *cag* region genes in colonization and gastritis in two animal models. *Infect Immun* **69**, 2902–2908.
- Fernandez, D., Spudich, G. M., Zhou, X. R. & Christie, P. J. (1996). The *Agrobacterium tumefaciens* VirB7 lipoprotein is required for stabilization of VirB proteins during assembly of the T-complex transport apparatus. *J Bacteriol* **178**, 3168–3176.
- Fernandez-Lopez, R., Machón, C., Longshaw, C. M., Martin, S., Molin, S., Zechner, E. L., Espinosa, M., Lanka, E. & de la Cruz, F. (2005). Unsaturated fatty acids are inhibitors of bacterial conjugation. *Microbiology* **151**, 3517–3526.
- Fischer, W., Puls, J., Buhrdorf, R., Gebert, B., Odenbreit, S. & Haas, R. (2001). Systematic mutagenesis of the *Helicobacter pylori* *cag* pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. *Mol Microbiol* **42**, 1337–1348.
- Kauppi, A. M., Nordfelth, R., Uvell, H., Wolf-Watz, H. & Elofsson, M. (2003). Targeting bacterial virulence: inhibitors of type III secretion in *Yersinia*. *Chem Biol* **10**, 241–249.
- Kavermann, H., Burns, B. P., Angermüller, K., Odenbreit, S., Fischer, W., Melchers, K. & Haas, R. (2003). Identification and characterization of *Helicobacter pylori* genes essential for gastric colonization. *J Exp Med* **197**, 813–822.
- Krause, S., Barcena, M., Pansegrau, W., Lurz, R., Carazo, J. M. & Lanka, E. (2000a). Sequence-related protein export NTPases encoded by the conjugative transfer region of RP4 and by the *cag* pathogenicity island of *Helicobacter pylori* share similar hexameric ring structures. *Proc Natl Acad Sci U S A* **97**, 3067–3072.
- Krause, S., Pansegrau, W., Lurz, R., de la Cruz, F. & Lanka, E. (2000b). Enzymology of type IV macromolecule secretion systems: the conjugative transfer regions of plasmids RP4 and R388 and the *cag* pathogenicity island of *Helicobacter pylori* encode structurally and functionally related nucleoside triphosphate hydrolases. *J Bacteriol* **182**, 2761–2770.
- Machon, C., Rivas, S., Albert, A., Goni, F. M. & de la Cruz, F. (2002). TrwD, the hexameric traffic ATPase encoded by plasmid R388, induces membrane destabilization and hemifusion of lipid vesicles. *J Bacteriol* **184**, 1661–1668.
- Marchetti, M. & Rappuoli, R. (2002). Isogenic mutants of the *cag* pathogenicity island of *Helicobacter pylori* in the mouse model of infection: effects on colonization efficiency. *Microbiology* **148**, 1447–1456.
- Marchetti, M., Arico, B., Burrone, D., Figura, N., Rappuoli, R. & Ghiara, P. (1995). Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science* **267**, 1655–1658.
- Monack, D. M., Mueller, A. & Falkow, S. (2004). Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nat Rev Microbiol* **2**, 747–765.
- Nordfelth, R., Kauppi, A. M., Norberg, H. A., Wolf-Watz, H. & Elofsson, M. (2005). Small-molecule inhibitors specifically targeting type III secretion. *Infect Immun* **73**, 3104–3114.
- Odenbreit, S., Puls, J., Sedlmaier, B., Gerland, E., Fischer, W. & Haas, R. (2000). Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* **287**, 1497–1500.
- Planet, P. J., Kachlany, S. C., Desalle, R. & Figurski, D. H. (2001). Phylogeny of genes for secretion NTPases: identification of the widespread *tadA* subfamily and development of a diagnostic key for gene classification. *Proc Natl Acad Sci U S A* **98**, 2503–2508.
- Porwollik, S., Noonan, B. & O'Toole, P. W. (1999). Molecular characterization of a flagellar export locus of *Helicobacter pylori*. *Infect Immun* **67**, 2060–2070.
- Rieder, G., Hatz, R. A., Moran, A. P., Walz, A., Stolte, M. & Enders, G. (1997). Role of adherence in interleukin-8 induction in *Helicobacter pylori*-associated gastritis. *Infect Immun* **65**, 3622–3630.
- Rieder, G., Merchant, J. L. & Haas, R. (2005). *Helicobacter pylori* *cag*-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. *Gastroenterology* **128**, 1229–1242.
- Rivas, S., Bolland, S., Cabezon, E., Goni, F. M. & de la Cruz, C. F. (1997). TrwD, a protein encoded by the IncW plasmid R388, displays an ATP hydrolase activity essential for bacterial conjugation. *J Biol Chem* **272**, 25583–25590.
- Rohde, M., Puls, J., Buhrdorf, R., Fischer, W. & Haas, R. (2003). A novel sheathed surface organelle of the *Helicobacter pylori* *cag* type IV secretion system. *Mol Microbiol* **49**, 219–234.
- Rothenbacher, D. & Brenner, H. (2003). Burden of *Helicobacter pylori* and *H. pylori*-related diseases in developed countries: recent developments and future implications. *Microbes Infect* **5**, 693–703.
- Sagulenko, E., Sagulenko, V., Chen, J. & Christie, P. J. (2001). Role of *Agrobacterium* VirB11 ATPase in T-pilus assembly and substrate selection. *J Bacteriol* **183**, 5813–5825.
- Savvides, S. N., Yeo, H. J., Beck, M. R. & 7 other authors (2003). VirB11 ATPases are dynamic hexameric assemblies: new insights into bacterial type IV secretion. *EMBO J* **22**, 1969–1980.
- Segal, E. D., Cha, J., Lo, J., Falkow, S. & Tompkins, L. S. (1999). Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc Natl Acad Sci U S A* **96**, 14559–14564.
- Selbach, M., Moese, S., Meyer, T. F. & Backert, S. (2002). Functional analysis of the *Helicobacter pylori* *cag* pathogenicity island reveals both VirD4-CagA-dependent and VirD4-CagA-independent mechanisms. *Infect Immun* **70**, 665–671.
- Selbach, M., Moese, S., Hurwitz, R., Hauck, C. R., Meyer, T. F. & Backert, S. (2003). The *Helicobacter pylori* CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation. *EMBO J* **22**, 515–528.
- Stein, M., Rappuoli, R. & Covacci, A. (2000). Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after *cag*-driven host cell translocation. *Proc Natl Acad Sci U S A* **97**, 1263–1268.
- Stein, M., Bagnoli, F., Halenbeck, R., Rappuoli, R., Fantl, W. J. & Covacci, A. (2002). c-Src/Lyn kinases activate *Helicobacter pylori* CagA through tyrosine phosphorylation of the EPIYA motifs. *Mol Microbiol* **43**, 971–980.
- Tanaka, J., Suzuki, T., Mimuro, H. & Sasakawa, C. (2003). Structural definition on the surface of *Helicobacter pylori* type IV secretion apparatus. *Cell Microbiol* **5**, 395–404.
- Tato, I., Zunzunegui, S., de la Cruz, F. & Cabezon, E. (2005). TrwB, the coupling protein involved in DNA transport during bacterial conjugation, is a DNA-dependent ATPase. *Proc Natl Acad Sci U S A* **102**, 8156–8161.
- Viala, J., Chaput, C., Boneca, I. G. & 13 other authors (2004). Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* *cag* pathogenicity island. *Nat Immunol* **5**, 1166–1174.
- Yeo, H. J. & Waksman, G. (2004). Unveiling molecular scaffolds of the type IV secretion system. *J Bacteriol* **186**, 1919–1926.
- Yeo, H. J., Savvides, S. N., Herr, A. B., Lanka, E. & Waksman, G. (2000). Crystal structure of the hexameric traffic ATPase of the *Helicobacter pylori* type IV secretion system. *Mol Cell* **6**, 1461–1472.