INTRODUCTION

Diarrhoea is a major cause of morbidity and mortality in infants and children worldwide, and disease control is particularly problematic when drug-resistant bacterial pathogens are involved (Pickering, 2004). Many bacterial strains have already become resistant to current ‘first-line’ antibiotics through point mutations (Song et al., 2010), the spread of plasmids (Morita et al., 2010) or integrons (Yu et al., 2010), and it is only a matter of time before resistance occurs in the presence of any newly prescribed antibiotic. In this context, it has become a matter of urgency to seek novel strategies for effective treatment of bacterial infections.

Shigella and enteroinvasive Escherichia coli form a pathovar sharing similar virulence properties (Schroeder & Hilbi, 2008). These micro-organisms, which rely on the type 3 secretion system, are able to invade many types of cell, including epithelial cells and macrophages, by injecting effector proteins into the host cell. Shortly after invasion, the microbes are able to escape from the phagocytic vacuoles and live freely in the cell cytosol. In macrophages, the microbes induce apoptosis through the activation of caspase-1 via the invasion plasmid antigen B (Ipa-B), leading to the release of interleukin (IL)-1β and IL-18 from apoptotic cells (Thirumalai et al., 2007). Both cytokines are potent mediators of hyperinflammation in the gut, which is characteristic of shigellosis. In epithelial cells, the microbes can trigger the release of IL-8, which also contributes to inflammation. But, the microbes secrete effector proteins (e.g. IpgD) that inhibit apoptosis, not only preventing host cells from dying but also allowing long-term survival of the microbes in the cell (Pendaries et al., 2006).

Natural products modulate Shigella–host-cell interaction

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This study focused on identifying possible new options derived from natural sources for the treatment of bacterial infections. Several natural products were investigated for their potential in modulating Shigella–host-cell interactions. The proliferation of Shigella sonnei was effectively inhibited inside HEp-2 cells in the presence of 4-methoxycinnamic acid and propolin D. Propolin D also significantly reduced the apoptosis of infected macrophage-like U937 cells and moderately reduced the secretion of interleukin (IL)-1β and IL-18, which probably resulted from the inhibition of invasion plasmid antigen B secretion by this compound. Further characterization showed that propolin D did not prevent escape of Shigella from phagocytic vacuoles, as evidenced by actin-based motility and by the fact that addition of chloroquine did not further reduce the number of intracellular c.f.u. The role of propolin D in modulating autophagy could not be established under the experimental conditions used. As these compounds had no direct anti-Shigella activity in vitro, it was concluded that these compounds modulated Shigella–host-cell interactions by targeting yet-to-be defined mechanisms that provide benefits to host cells.

Abbreviations: DAPI, 4',6'-Diamidino-2-phenylindole; DMEM, Dulbecco’s minimal essential medium; IL, interleukin; LB, Luria–Bertani; MCA, 4-methoxycinnamic acid; MRSA, meticillin-resistant Staphylococcus aureus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; p.i., post-infection.

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resistance developed by micro-organisms (Clardy & Walsh, 2004; Gibbons, 2005; Payne et al., 2007). Only a small proportion of natural sources has been investigated thoroughly and therefore many natural products with potentially useful applications in therapeutics remain to be discovered (Cos et al., 2006). Most reports so far have investigated the direct in vitro antimicrobial activity of natural compounds. For example, one of us has previously demonstrated that propolis, a product obtained from beehives, has direct anti-staphylococcal activity in vitro (Raghukumar et al., 2010). Few studies have looked at the effect that natural compounds have on intracellular micro-organisms via host-cell modulation mechanisms. In this study, we started with a mini-screen on a small selection of natural compounds for their ability to fend off infection of epithelial and macrophage cells by a pathogen such as Shigella, which proliferates inside the cell cytoplasm (Sansonetti, 2002).

METHODS

Bacterial strains. We used a clinical Shigella sonnei isolate (20071599) from the Scottish Salmonella and Shigella Reference Laboratory (Glasgow, UK), a clinical MRSA isolate (LP97) from the New Royal Infirmary (Edinburgh, UK) and Pseudomonas aeruginosa strain ATCC 25668 from the American Type Culture Collection. Wild-type Salmonella enterica serovar Typhimurium SL1344 was a kind gift from Professor G. Dougan at the Sanger Institute, Cambridge, UK. All strains were maintained as a 15 % glycerol stock at −80 °C. Shigella sonnei was inoculated onto Luria–Bertani (LB) agar containing 0.1 % Congo red (Sigma-Aldrich) and incubated at 37 °C overnight to isolate red colonies for subsequent experiments. All other strains were inoculated on LB agar and incubated at 37 °C overnight before use.

Natural compounds. Nine natural compounds were investigated in this study: oleanolic acid (1), β-sitosterol (2), usnic acid (3), coumaric acid (4), 4-methoxycinnamic acid (MCA) (5), propolin D (6), totarol (7), longifolene (8) and betulinic acid (9). Compounds 1–5 were from Sigma-Aldrich; compound 6 was isolated from propolis (Raghukumar et al., 2010) and 7–9 were purified from plants (Gordien et al., 2009; Shilpi et al., 2010). These compounds were selected because they had previously demonstrated some antimicrobial activity (Seidel et al., 2008; Shilpi et al., 2010) and/or because they were part of our laboratory database of authentic standards available in sufficient amounts for biological screening. All compounds were dissolved in DMSO (2 mg ml⁻¹) to prepare stock solutions and diluted with cell-culture medium to 18 μg ml⁻¹ (i.e. 9 μl ml⁻¹) for all cell assays. A solution of culture medium containing 9 μl DMSO ml⁻¹ was also prepared and used as a control.

Gentamicin-killing assay. HEp-2 (ATCC CCL-23) and U937 (ATCC CRL-1593) cell lines were cultured in Dulbecco’s minimal essential medium (DMEM) and RPMI 1640 (both from Sigma-Aldrich), respectively, containing 10 % fetal bovine serum with 5 % CO₂ at 37 °C, and assays were carried out as described previously (Lucchini et al., 2005). Briefly, cells were seeded in 24-well plates and cultured to ~80 % confluency. U937 cells were differentiated for 7 days with phorbol myristate acetate at 200 ng ml⁻¹ before the assay. Bacteria from the mid-exponential phase were added to the cells at an m.o.i. of 10 or 100 and Shigella bacteria (not Salmonella) were centrifuged at 2000 r.p.m. in a microcentrifuge for 10 min at room temperature to enhance invasion. Extracellular bacteria (for both Shigella and Salmonella) were removed after 40 min incubation and the cells washed with PBS. Medium containing gentamicin (50 μg ml⁻¹) and the natural compounds (18 μg ml⁻¹, i.e. 9 μl stock solution in 1 ml medium) were added for further incubation. At appropriate time intervals, the cells were washed with PBS and lysed with 0.1 % Triton X-100 in water. The cell lysates were plated onto LB agar to determine the number of c.f.u.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. An MTT assay was carried out as described previously (Li et al., 2006). HEp-2 and differentiating U937 cells were seeded into 96-well plates and left to grow to ~60 % confluency. The cells were then treated with DMSO (9 μl ml⁻¹) or the compounds (18 μg ml⁻¹) overnight. MTT was added to a final concentration of 0.02 μg ml⁻¹. The cell-culture medium was carefully removed and 150 μl DMSO was added to each well to solubilize the purple precipitate. The absorbance at 540 nm (A540) was measured with a plate reader (LabSystems Genesis).

Detection of activated caspases. U937 cells were differentiated for 7 days on coverslips and infection was carried out as described previously (Lucchini et al., 2005). At 6 h post-infection (p.i.), cells were washed with PBS, fixed with 3.7 % paraformaldehyde and stained with sulforhodamine-1-valylalanasparglutaric acid fluoromethyl ketone according to the manufacturer’s instructions (FLICA kit; Immunochimistry Technologies). Coverslips were mounted onto glass slides with Pro-Long Gold antifade reagent (Invitrogen) containing 4',6'-diamidino-2-phenylindole (DAPI) for nuclear staining. Images were captured using a confocal microscope (LSM 510; Carl Zeiss).

Detection of cytokines secreted from infected U937 cells. Cell infection was carried out as described previously (Lucchini et al., 2005). At various time intervals, the medium was collected and cytokines were detected using ELISA kits (R&D Systems). Standard curves were constructed using purified IL-1β and IL-18 supplied in the kits, and the concentrations in the samples were calculated by regression analysis.

Determination of MICs. A broth microdilution method was used to determine MICs, as described previously (Seidel et al., 2008).

RESULTS

Intracellular fate of Shigella sonnei in HEp-2 cells in the presence of natural compounds

The natural compounds being tested underwent a preliminary screen using a gentamicin-killing assay to determine their effects on HEp-2 cells infected with Salmonella strain SL1344 or Shigella strain 20071599. Each compound was added to the DMEM after the bacterial invasion phase (i.e. 40 min after the addition of bacteria) at a final concentration of 18 μg ml⁻¹ in the presence of gentamicin (50 μg ml⁻¹). The number of intracellular c.f.u. was determined 4 h after infection. Treatment of cells with each of the nine compounds made no difference to Salmonella c.f.u. counts, whilst an approximate tenfold reduction in intracellular c.f.u. was repeatedly observed for Shigella infection in the presence of MCA, propolin D and totarol compared with the DMSO control (data not shown). This prompted us to focus our investigation on the effect of these three compounds (Fig. 1a) on Shigella infection. We
performed a time-course analysis to evaluate bacterial intracellular proliferation inside HEp-2 cells in the presence of these three compounds. Three independent experiments produced similar results, and one set of data is shown in Fig. 1(b). The number of intracellular c.f.u. increased very little from 2 to 6 h in the presence of each of the compounds. In contrast, the intracellular c.f.u. counts increased steadily (doubling time of approximately 30–40 min) during the 2–6 h interval in the presence of DMSO, which was consistent with previous studies (Yu, 1998). It was noted that the addition of DMSO reduced the Shigella intracellular c.f.u. count compared with the non-treatment control (see Supplementary Fig. S1, available in JMM Online; \( P < 0.05 \); Student's \( t \)-test). Because all compounds tested were dissolved in DMSO, only the DMSO control is shown in the figures used in the main text.

An MTT cell viability assay was performed to eliminate the possibility that the observed reduction in intracellular c.f.u. counts was due to cytotoxicity (i.e. the natural products could have damaged the host-cell membrane, allowing gentamicin penetration and killing of intracellular bacteria). The results showed that MCA and propolin D had no cytotoxic effect, whereas totarol significantly reduced cell viability overnight (Fig. 1c). It is therefore unlikely that MCA and propolin D facilitated the gentamicin-mediated killing of intracellular bacteria at the concentrations used.

**Intracellular fate of Shigella sonnei in U937 cells in the presence of natural compounds**

We evaluated the three compounds for their activity on U937 cells during Shigella infection using the gentamicin-killing assay.
assay. Three independent experiments were performed with similar results, and one set of results (carried out in triplicate for all groups) is presented in Fig. 2(a). Regardless of whether the compounds were present or not, the intracellular c.f.u. counts increased steadily with a doubling time of approximately 30–40 min in the 2–4 h period p.i. Between 4 and 6 h, the intracellular c.f.u. dropped significantly from the peak c.f.u. at 4 h in all groups. However, in the presence of the compounds, the intracellular c.f.u. counts dropped to a lesser degree compared with the DMSO control. Propolin D was more potent than either totarol or MCA in maintaining the intracellular c.f.u. load. The intracellular c.f.u. counts were statistically higher ($P=0.02$; Student’s $t$-test) in the presence of propolin D compared with the DMSO control (Fig. 2a).

The MTT assay revealed that none of the three compounds had any significant cytotoxicity towards U937 cells at a concentration of 18 $\mu$g ml$^{-1}$ overnight (Fig. 2b). This eliminated the possibility that the drop in intracellular c.f.u. by 6 h in the experimental groups was due to a cytotoxic effect of the compounds on U937 cells. The observed reduction in intracellular c.f.u. by 6 h could be attributed to $Shigella$-induced apoptosis of U937 cells. Such a mechanism would damage the cell membrane, thus allowing gentamicin to penetrate the cells and kill intracellular bacteria 4 h after infection.

**Fate of U937 cells in the presence of propolin D during Shigella infection**

As propolin D treatment resulted in a significantly higher intracellular bacterial load in U937 cells at 6 h p.i. (Fig. 2a), we reasoned that this compound might slow down apoptosis. To substantiate this hypothesis, we performed confocal microscopy in conjunction with a FLICA kit, which detects all activated caspases. At 6 h p.i., every cell in the DMSO control stained strongly for activated caspases, whereas only ~30 % of cells treated with propolin D stained positively. Many cells in the DMSO control group had nuclear fragmentation, characteristic of the late stage of apoptosis (Fig. 3a). Most of the cells treated with totarol or MCA were positively stained for activated caspases (data not shown), consistent with the results of the gentamicin-killing assay (Fig. 2a).

It is known that $Shigella$ induces apoptosis by secreting IpaB protein (Thirumalai et al., 1997). Therefore, IpaB secretion was assessed in the presence of propolin D. It was clear that 0.1 % Congo red was sufficient to trigger IpaB secretion; however, this was inhibited by propolin D in a dose-dependent manner (see Supplementary Fig. S2, available in JMM Online).

As propolin D significantly protected U937 cells from apoptosis and inhibited IpaB secretion, its effect on the release of IL-1$\beta$ and IL-18 from $Shigella$-infected U937 cells was analysed using ELISA kits (R&D Systems). Indeed, treatment with propolin D moderately reduced cytokine secretion at all time intervals (Fig. 3b). The release of IL-18 at 6 h p.i., however, was significantly lower compared with the DMSO control ($P<0.05$, Student’s $t$-test).

**Further characterization of the role of propolin D in modulating Shigella−HEp2 cell interaction**

Propolin D appeared to be the most effective of the products tested. Therefore, we undertook further characterization of the potential mechanism(s) in the control of $Shigella$ proliferation in HEp-2 cells. It is known that $Shigella$ escapes from phagocytic vacuoles shortly after...
invasion, which is a prerequisite for actin-based motility and intracellular growth (Schroeder & Hilbi, 2008). If propolin D could prevent vacuole escape, *Shigella* would be impaired in actin-based motility. Hence, we performed actin staining to visualize intracellular motility. At 2 h p.i. in the presence of propolin D, typical actin tails and protrusions were observed (see Supplementary Fig. S3, available in JMM Online); typical protrusions and actin tails were also observed in cells treated with DMSO (data not shown). To further substantiate the fact that propolin D did not prevent *Shigella* vacuole escape, we included chloroquine in the gentamicin-killing assay. Chloroquine selectively kills bacteria in vacuoles (Fernandez-Prada et al., 2000) and would further reduce the intracellular c.f.u. counts in conjunction with propolin D if the latter restricted *Shigella* in vacuoles. As shown in Supplementary Fig. S1, cells treated with chloroquine and propolin D together produced an intracellular c.f.u. count similar to cells treated with chloroquine alone; propolin D alone was more effective in controlling intracellular c.f.u. (*P*<0.001;

![Fig. 3.](a) Confocal microscopy on U937 cells at 6 h p.i. Activated caspases were stained with FLICA (red; panels i and iv), and cell nuclei were stained with DAPI (blue; panels ii and v). Panels (iii) and (vi) show merged images. All cells in the controls (i–iii) stained strongly with FLICA (i.e. underwent apoptosis) and some nuclei were fragmented (arrows). In the presence of propolin D (iv–vi), only ~30% of cells stained weakly with FLICA and no nuclear fragmentation was observed. Bars, 50 μm. (b) Detection of secreted IL-1β and IL-18 by ELISA. Infection of U937 cells was carried out as described previously (Lucchini et al., 2005). At the indicated time intervals, the culture medium was collected and the levels of cytokines were determined as described in Methods. Results are shown as means±sd. A statistically significant difference in IL-18 release (*, *P*<0.05, Student’s *t*-test) was observed between the propolin D-treated and control (DMSO) groups at 6 h.
Student’s t-test). Thus, inhibition of Shigella intracellular growth by propolin D was unlikely to be due to prevention of Shigella vacuole escape.

Shigella is also known to escape from autophagy, which is mediated by the invasion protein IcsB encoded by the virulence plasmid (Ogawa et al., 2005). Therefore, we investigated whether propolin D could facilitate autophagy in HEp-2 cells. We exploited tamoxifen and wortmanin, a well-established autophagy inducer and inhibitor, respectively. HEp-2 cells were treated for 1 h with either tamoxifen (10 mM) or wortmanin (1 mM), both of which resulted in a significantly reduced Shigella intracellular c.f.u. count compared with the DMSO control (P<0.01; Student’s t-test) at 4 h p.i. (Supplementary Fig. S1). The addition of propolin D (18 µg ml⁻¹) together with either tamoxifen or wortmanin led to further reduction in intracellular c.f.u. However, these results could not establish whether inducing or inhibiting autophagy helps the control of Shigella growth in HEp-2 cells. Furthermore, supplementing tamoxifen or wortmanin in L-broth did not affect Shigella sonnei growth compared with non-supplemented controls at 37°C with shaking (200 r.p.m.). Thus, it did not appear that either wortmanin or tamoxifen killed intracellular Shigella directly.

**In vitro susceptibility of Shigella sonnei to MCA and propolin D**

As MCA and propolin D showed no cytotoxicity to HEp-2 and U937 cells, they were selected for *in vitro* susceptibility tests. The results showed that neither MCA nor propolin D had any direct antimicrobial activity on the Shigella strain used (MIC >50 µg ml⁻¹ in both cases) compared with a ciprofloxacin control (MIC=0.19 µg ml⁻¹). The two compounds were inactive (MIC >50 µg ml⁻¹) against the *Pseudomonas aeruginosa* reference strain. Propolin D showed moderate activity (MIC=12.5 µg ml⁻¹) against an MRSA clinical isolate, consistent with previous studies (Raghukumar et al., 2010).

**DISCUSSION**

The purpose of this work was to explore an alternative approach to combat intracellular bacterial pathogens such as *Salmonella* and Shigella that does not rely on the identification of compounds with direct antimicrobial activity but rather is aimed at revealing existing mechanism(s) of the host cells involved in the control of invading microbes. Such an approach offers a lower risk of selecting drug-resistant micro-organisms compared with the direct use of antibiotics.

We demonstrated that three natural compounds had the potential to slow down Shigella-induced apoptosis in macrophage-like U937 cells, with propolin D exhibiting the strongest activity (Fig. 2). Furthermore, propolin D was able to moderately reduce the secretion of functional IL-1β and IL-18 (Fig. 3b), which might show significance in appropriate animal models where reduced levels of these cytokines would reduce inflammation through poorer neutrophil infiltration and natural killer cell activation (Schoeder & Hilbi, 2008). The reduced apoptosis and cytokine secretion probably resulted from inhibition of IpaB secretion by propolin D (Supplementary Fig. S2).

The most striking phenomenon observed in this study was that the use of MCA and propolin D prevented *Shigella sonnei* growth inside epithelial HEp-2 cells (Fig. 1b). Because the compounds and gentamicin were added to the cells 40 min after bacterial invasion, they played no part in preventing bacterial entry into the cells. Furthermore, propolin D did not affect actin-based motility (Supplementary Fig. S3), and addition of chloroquine to propolin D did not further reduce the number of intracellular c.f.u. (Supplementary Fig. S1). These data eliminated the possibility that propolin D prevents Shigella vacuole escape. One other possibility was that propolin D could promote autophagy by suppressing the anti-autophagy activity mediated by the Shigella IcsB protein (Ogawa et al., 2005). We tested the autophagy inducer tamoxifen and inhibitor wortmanin at a concentration tenfold less than that recommended by the manufacture (Invitrogen), and both significantly reduced the Shigella intracellular c.f.u. count (Supplementary Fig. S1). As neither wortmanin nor tamoxifen had direct anti-Shigella activity *in vitro*, it was plausible that both agents were highly toxic to HEp-2 cells so that the cells were no longer able to support bacterial growth. In fact, 2 h after exposure to wortmanin (1 mM) or tamoxifen (10 mM), the HEp-2 cells were all detached (data not shown), which was the reason that cells were treated for only 1 h in the experiments conducted (Supplementary Fig. S1). Use of an icsB mutant will be helpful in investigating the potential of propolin D in modulating autophagy.

Although MCA and propolin D had no direct anti-*Shigella* activity *in vitro*, they might have direct activity against Shigella in the cell cytosol, which has a different profile of gene expression compared with Shigella grown in broth (Lucchini et al., 2005). The altered gene expression in the cell might increase the sensitivity of Shigella to natural products such as propolin D and MCA. Given the fact that these products had no effect on *Salmonella* infection of HEp-2 cells, such a hypothesis deserves further investigation. *Salmonella* and Shigella share similar profiles of gene expression inside epithelial cells (Lucchini et al., 2005). However, *Salmonella* exists in specialized vacuoles known as Salmonella-containing vacuoles (SCVs) – in contrast to Shigella, which exists in the cytosol. SCVs might protect Salmonella from being killed by propolin D or MCA in the cell, which might explain the Shigella-specific activity. Furthermore, SCVs are also targeted by autophagy (Birmingham et al., 2006). A reduction in Salmonella intracellular c.f.u. could have been observed if any of the compounds enhanced SCV autophagy. However, this scenario does not exclude the possibility that the natural
products promoted *Shigella* autophagy, which occurs through completely different mechanisms from SCV autophagy (Birmingham et al., 2006; Ogawa et al., 2005).

Finally, it would be interesting to expand on this observation by carrying out further experiments on other cytosolic bacteria such as *Listeria monocytogenes*. This would allow us to differentiate the specific cytosolic activity (i.e. treatment of HEp-2 cells with the compounds prevents the proliferation of all cytosolic microbes) from species specificity (i.e. treatment of HEp-2 cells with the compounds only works for *Shigella*). In addition, as the three compounds are not structurally related, it remains to be determined whether they target the same cellular mechanism(s).

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**REFERENCES**


