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SHORT COMMUNICATION

Identification and characterization of 4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-*5H*-pyrimido[5,4-b]indole derivatives as *Salmonella* biofilm inhibitors

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Abstract

A screening of a small-molecule library was conducted, in search of *Salmonella* biofilm inhibitors active in a broad temperature range, both in prevention and in eradication of biofilms. Moreover, the inhibitors were selected not to influence the planktonic growth of *Salmonella* to diminish the selective pressure and to prevent or slow down resistance development. Out of the 20 014 compounds screened at 16 and 37 °C, 140 hits were identified. After characterization of the most promising hits at a broader set of temperatures (16, 25, 30 and 37 °C), we identified 7-methoxy-4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-*5H*-pyrimido[5,4-b]indole as an interesting preventive anti-biofilm compound. A first structure–activity relationship of this compound was delineated, revealing 8-fluoro-4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-*5H*-pyrimido[5,4-b]indole as a promising analogue in the prevention of *Salmonella* biofilms.

Biofilms are surface-associated structures in which microorganisms are enclosed in a gel-like matrix and are strongly protected against *i.a.* disinfectants and antibiotics (Costerton *et al.*, 1999; Hoiby *et al.*, 2011). An important biofilm forming pathogen is *Salmonella*. This enteric pathogen is one of the leading causes of food borne infections worldwide, yearly responsible for more than 1.3 billion infections and 3 million deaths (Pang *et al.*, 1995; Coburn *et al.*, 2007). *Salmonella* infections are treated with antibiotics only in severe cases. However, in people with a compromised immunosystem, like patients with HIV, children or the elderly, *Salmonella* is capable of causing life-threatening bacteraemia for which an antibiotic treatment is essential (Moir & Fauci, 2010). However, many *Salmonella* isolates have developed multi-drug resistance to several of the currently used antibiotics, including fluoroquinolones and third-generation cephalosporins (Parry & Threlfall, 2008).

The most common *Salmonella* infection sources are (raw) meat and eggs, but outbreaks linked to fresh produce are increasing (Heaton & Jones, 2008). Besides the broad host spectrum of many *Salmonella* serovars, this illustrates the wide distribution of *Salmonella* in the environment. The fact that *Salmonella* is able to form biofilms on various biotic surfaces, including seeds, vegetables, epithelial cells, gall stones (Prouty & Gunn, 2003; Lapidot *et al.*, 2006; Barak *et al.*, 2007) and abiotic surfaces, such as glass, plastics or stainless steel (Joseph *et al.*, 2001; Chmielewski & Frank, 2003; Wong *et al.*, 2010), is one of the major contributing factors to this wide distribution. *Salmonella* biofilms on surfaces, for example, in food industry, are very persistent reservoirs for cross- and re-contamination, even after extended cleaning of the surfaces (Joseph *et al.*, 2001; Chmielewski & Frank, 2003). The role of biofilm formation in the infection process of *Salmonella* is less

clear, but is likely inversely correlated with the invasiveness of *Salmonella* (Ahmad *et al.*, 2011). Therefore, biofilm formation inside a host is expected mostly to be important for the long-time survival and persistence of *Salmonella* (e.g. on gall stones), with chronic infections as a result (Prouty & Gunn, 2003). Despite the clear need for new anti-biofilm therapies, only a limited number of anti-biofilm compounds are being studied and even less compounds have made it to clinical trials or to the market (Landini *et al.*, 2010; Lynch & Abbanat, 2010; Steenackers *et al.*, 2012). This urges the need for new (*Salmonella*) biofilm inhibitors.

Therefore we conducted a high-throughput screening of 20 014 small-molecules [< 500 g mol^{-1}, provided by the Centre of Drug Design and Discovery of the KULeuven (Segers *et al.*, 2011)] for the prevention of *Salmonella* Typhimurium biofilms.

As the biofilm formation is a very complex process, which is regulated by an interplay between many cellular systems (see e.g. Steenackers *et al.*, 2012), we used a 'top-down' approach, that is, screening for prevention of the biofilm as a whole, as compared to a target-based screening ('bottom-up'), which depends on knowledge of biofilm targets already identified. In addition, by subsequently studying the mode of action of inhibitors identified in a 'top-down' screening, possible new important biofilm targets can be identified.

The screening was based on methods described (and validated with *Salmonella* mutants defective in biofilm formation) by De Keersmaecker *et al.* (2005) and Janssens *et al.* (2008). The device used is a platform carrying 96 polystyrene pegs (Nunc no.445497) that fits as a microtiter plate lid with a peg hanging into each microtiter plate well (Nunc no.269789), filled with a cell suspension of 1/100 diluted *Salmonella enterica* serovar Typhimurium ATCC14028 overnight culture in 1/20 diluted Tryptic Soy Broth (Becton Dickson and company, MacFaddin (1985)).

The biofilms were incubated during 48 h without shaking. After 24 h, the broth and compounds were refreshed and the optical density (600 nm) of the planktonic cells in the microtiter plate was measured. After 48 h, the biofilms were quantified using crystal violet staining as described by De Keersmaecker *et al.* (2005). As the compounds were present from the start of the incubation, the initial screening was directed towards biofilm prevention.

The screening was conducted at 16 and 37 °C because we observed that *Salmonella* forms distinct biofilm phenotypes at these temperatures, with intermediate phenotypes at 25 and 30 °C (Supporting information, Fig. S1). This points to a different regulation and different type of biofilm formed at different temperatures and as a consequence to a possible different response to anti-biofilm agents. By screening at both 16 and 37 °C, we were aiming to identify compounds that target general biofilm features instead of specific temperature-regulated processes. This ensures that the inhibitors have potential to be used both in- and outside a host environment.

Out of the 20 014 compounds screened, 140 hits were identified (0.7%).

Based on preliminary dose–response and planktonic growth analysis, the 31 most promising hits were selected and further characterized by determining their full dose–response relationship at different temperatures (16, 25, 30 and 37 °C). The tested 2/3 serial concentration gradient (ranging from 200 till 2.3 μM) of each compound was used to calculate the IC_{50}-value (concentration with 50% planktonic growth inhibition) and BIC_{50}-value (Biofilm Inhibitory Concentration with 50% biofilm inhibition) compared to the DMSO solvent control.

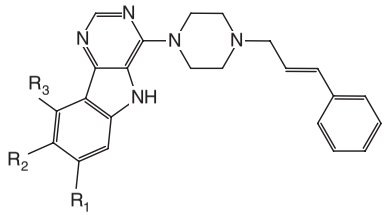
Additionally, the ability to eradicate or prevent further growth of existing biofilms was determined. Hereto biofilms were pre-grown during 24 or 48 h without compounds present, after which the biofilms were transferred to a challenge plate with the compounds and incubated for an additional 24 h.

Finally, using bioscreen analysis (Oy Growth Curves Ab Ltd), which measures the planktonic growth ($OD_{600\ nm}$) in time, the effects on the bacterial growth curves were determined.

Eleven compounds with a low BIC_{50} value (both on prevention and on eradication), high IC_{50} value and minimal influences on the planktonic growth of *Salmonella* were selected for further studies. One of the selected compounds was 7-methoxy-4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-*5H*-pyrimido[5,4-b]indole (Table 1, analogue 3), which will be discussed further.

A first structure–activity relationship (SAR) of 7-methoxy-4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-*5H*-pyrimido[5,4-b]indole was delineated following acquisition of commercially available analogues. These results indicate that the phenylpropenyl residue is essential for the activity of the compound. Furthermore, we found that by shortening the linker between the phenyl and piperazinyl moiety, the activity was lost. The indole moiety, part of the pyrimidoindole scaffold, was determined as a second essential feature because removing or replacing the indole group abolishes the anti-biofilm activity. Also, methylation of the indole nitrogen renders the compound inactive. Nonetheless certain substitutions at the R1, R2 or R3 position of the pyrimidoindole scaffold improve the activity of the compounds (see Table 1) as compared to the unsubstituted base structure (analogue 1).

Table 1. Tested substitutions at the indole moiety of the 4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-*5H*-pyrimido[5,4-b]indole derivatives and the corresponding preventive activities against *Salmonella* biofilm formation. Analogue 1 is the basic scaffold; Analogue 3 is the initial compound identified in the screening



	Substitution at the indole moiety			Dose–response *Salmonella* 16 °C				Dose–response *Salmonella* 37 °C			
	R_1	R_2	R_3	BIC_{50}*	95% confidence interval for BIC_{50}	IC_{50}†	95% confidence interval for IC_{50}	BIC_{50}*	95% confidence interval for BIC_{50}	IC_{50}†	95% confidence interval for IC_{50}
(1)	H	H	H	24.45	20.78–28.76	> 200‡		~51.01§		130.52	101.88–167.22
(2)	CH3	H	H	17.48	15.00–20.37	> 200‡		37.91	26.08–55.09	> 200‡	
(3)	O-CH3	H	H	11.02	7.00–17.34	~119.68§		21.38	17.27–26.47	> 200‡	
(4)	H	F	H	13.69	8.69–21.58	> 200‡		26.55	22.37–31.51	> 200‡	
(5)	O-CH3	O-CH3	H	16.84	15.82–17.92	> 200‡		53.08	38.18–73.8	114.89	83.55–157.99
(6)	H	H	O-CH3	11.54	8.57–15.54	38.33	31.96–45.98	> 200‡		23.19	19.39–27.75

*BIC_{50}: concentration (in μM) of inhibitor needed to inhibit biofilm formation ($OD_{570\ nm}$) by 50% as compared to a DMSO solvent control.
†IC_{50}: concentration (in μM) of inhibitor needed to inhibit planktonic growth ($OD_{600\ nm}$) by 50% as compared to a DMSO solvent control.
‡The (B)IC_{50} value is higher than the highest tested concentration (200 μM).
§~: The (B)IC_{50} value could not be determined accurately because of the steepness of the dose–response curve.
The 95% confidence interval is based on tree repeats per test.

Table 2. Activity of 8-fluoro-4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-*5H*-pyrimido[5,4-b]indole on *Salmonella* Typhimurium ATCC14028

Biofilm prevention				Existing biofilms		
	BIC_{50}*	95% Confidence interval	IC_{50}†		BIC_{50}‡	95% Confidence interval
16 °C	13.69	8.69–21.58	> 200§	16 °C – 24 + 24 h	> 200§	
25 °C	22.14	19.95–24.56	~153.28¶	16 °C – 48 + 24 h	> 200§	
30 °C	36.08	25.34–51.37	~191.87¶	37 °C – 24 + 24 h	45.78	36.58–57.30
37 °C	26.55	22.37–31.51	> 200§	37 °C – 48 + 24 h	45.46	40.96–50.45

*BIC_{50}: concentration (in μM) of inhibitor needed to inhibit biofilm formation ($OD_{570\,nm}$) by 50% compared to a DMSO solvent control.
†IC_{50}: concentration (in μM) of inhibitor needed to inhibit planktonic growth ($OD_{600\,nm}$) by 50% compared to a DMSO solvent control.
‡In this context, the calculated BIC_{50} value is the concentration with 50% biofilm inhibition compared to the DMSO solvent control after 24 h additional incubation in the challenge plate (48 or 72 h old biofilms).
§The (B)IC_{50} value is higher than the highest tested concentration (200 μM).
¶~: The (B)IC_{50} value could not be determined accurately because of the steepness of the dose–response curve.
The 95% confidence interval is based on tree repeats per test.

Based on the results above, we selected 8-fluoro-4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-*5H*-pyrimido[5,4-b]indole (Table 1, analogue 4) for further testing (Table 2), as this compound shows the lowest BIC_{50} values at both 16 and 37 °C, without growth effects (high IC_{50} values). The strong preventive activity of this compound against *Salmonella* biofilms was found to be consistent over the tested temperatures. At 37 °C, but not at 16 °C, also inhibitory effects on existing biofilms of *Salmonella* were observed (Table 2). This last finding points again at differently regulated and different types of biofilms depending on the temperature. The cause of this difference is not fully unravelled, but temperature sensitive genes are likely to be involved (Gerstel & Romling, 2003; Steenackers *et al.*, 2012).

The bioscreen analysis (data not shown) shows a (limited) 7.2% reduction in surface area below the *Salmonella* growth curve at 37 °C at 20 μM. Below 20 μM < 3% inhibition was observed, which was not considered as a significant effect. Intriguingly at higher concentrations (starting with 40 μM), growth inducing effects were observed in a concentration-dependent manner, reaching an increase of up to a 100% at 80 μM. At 16 °C, no inhibitory effects were shown, although similar inducing effects were observed starting with 1 μM and reaching the same maximum at 20 μM. An explanation for this increase in planktonic growth is not obvious at this point and will have to be elucidated. Nevertheless, as the bacteria remain planktonic, they will stay more susceptible to antibacterials and host immune defences, as compared to the biofilm state.

In conclusion, the identified 4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-*5H*-pyrimido[5,4-b]indole class shows a biofilm specific, preventive effect on *Salmonella* biofilms in a broad temperature range. Further optimization and more extended structure–activity relationship studies are being conducted to further improve the activity range. In addition, we are conducting extensive 'mode of action' studies, which can help to improve the activity of this compound further and, from a more fundamental point of view, possibly yield new knowledge about the *Salmonella* biofilm formation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *Salmonella* biofilm formation on the pegs of the static biofilm assay.

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