Original Research Article

Antimicrobial effect of salicylamide derivatives against intestinal sulfate-reducing bacteria

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A B S T R A C T

Sulfate-reducing bacteria (SRB) are most likely involved in both the initiation and maintenance of inflammatory bowel disease (IBD); unfortunately present antibacterial chemotherapeutics used in the treatment of IBD have been ineffective. Thus, the antimicrobial activity of salicylamide derivatives against two different genera of intestinal SRB, Desulfovibrio and Desulfomicrobium, was investigated. Six 2-(phenylcarbamoyl)phenyl N-(benzyloxycarbonyl)alkanoates and three 2-hydroxy-N-(2S)-1-oxo-1-(phenylamino)alkan-2-yl]benzamides showed MIC values in the range from 0.22 to 0.35 mM against Desulfovibrio Vib-7 and in the range from 0.27 to 8.52 mM against Desulfomicrobium sp. Rod-9, while MIC values of ciprofloxacin were 41.2 μM and 39.3 μM. The highest potency against the two strains was observed for 4-chloro-N-(2S)-1-[(3,4-dichlorophenyl)amino]-3-methyl-1-oxobutan-2-yl]2-hydroxybenzamide (MIC 0.22 μM and 0.27 μM). 4-Chloro-2-[(4-nitrophenyl)carbamoyl]phenyl (2S)-2-[(benzyloxy)carbonyl]amino]-3-methylbutanoate showed high activity against D. Vib-7 (MIC = 0.26 μM), while 4-chloro-2-[(4-methylphenyl)carbamoyl]phenyl (2S)-2-[(tert-butoxycarbonyl)amino]-3-(1H-indol-2-yl)propanoate expressed high activity against Desulfomicrobium sp. Rod-9 (MIC = 0.31 μM). Structure–activity relationships are discussed.

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Introduction

Ulcerative colitis (UC) is one of the two major forms of idiopathic inflammatory bowel disease (IBD) (Cummings et al., 2003). Both acute and chronic forms of the illness affect the colon and rectum and can be a highly disabling condition (Barton and Hamilton, 2010). This disease is more common in North America and Western Europe with the increasing incidence in Asia. The reported incidence is 1.2–20.3 cases per 100,000 persons per year, and the prevalence is 7.6–245 cases per 100,000 per year (Feuerstein and Cheifetz, 2014). Ulcerative colitis usually has a relapsing/remitting pattern and current medical approaches focus on treating active disease to address symptoms, to improve the quality of life and thereafter to maintain remission. Bloody diarrhoea, an urgent need to defecate and abdominal pain are the main symptoms of active disease or relapse. The treatment chosen for active disease depends not only on clinical severity, but also on the extent of disease and the person’s preference (Loubinoux et al., 2000, 2002a,b; Kornbluth and Sachar, 2010). Conventional drug therapy for UC involves the use of 5-aminosalicylates (the mainstay of treatment for mild to moderate disease), corticosteroids (for patients who failed 5-aminosalicylates therapy and for acute episodes), azathioprine/6-mercaptopurine, cyclosporine and anti-tumour necrosis factor therapy (Lissner and Siegmund, 2013).

Several reports suggested the possible involvement of sulfate-reducing bacteria (SRB), a group of phylogenetically diverse anaerobic microorganisms, in both the initiation and maintenance of the disease (Loubinoux et al., 2000, 2002a,b; Zinkevich and Beech, 2000; Cummings et al., 2003). SRB such as Desulfovibrio and Desulfomicrobium genera, are normal inhabitants of the human and animal large intestine, capable of dissimilatory sulfate reduction (Gibson et al., 1991, 1993; Kushkevych, 2012a,b; Kushkevych and Moroz, 2012). Most of the SRB utilize sulfate or other sulfur compounds such as thiosulfate, sulfate and sulfur as terminal electron acceptors. The main product of SRB metabolism, hydrogen sulfide, is a compound that may act through inhibition of butyrate oxidation, the main energy source for colonocytes. In addition it is cytotoxic, mutagenic and cancerogenic to epithelial oxidation, the main energy source for colonocytes. In addition, a compound that may act through inhibition of butyrate metabolism of digestive materials (Cummings et al., 2003). The compounds were fully characterized by melting point, CHN analyses, IR and NMR spectroscopy (Pauk et al., 2013). The investigated salicylamide derivatives showed high potency against different bacterial strains as was published recently (Pauk et al., 2013; Zadrazilova et al., 2015a). Both SRB are Gram-negative strictly anaerobe genera. Desulfovibrio piger is usually considered as a commensal bacterium in humans. More recently, D. piger has attracted more interest as it was found to be the most prevalent species of SRB in faeces of patients with inflammatory bowel disease (Holt et al., 1994; Barton and Hamilton, 2010).

Materials and methods

Tested compounds

The discussed salicylamide derivatives (see Table 1) were synthesized previously (Pauk et al., 2013) by means of microwave-assisted synthesis and rearrangement described in literature (Imramovsky et al., 2006, 2009a, 2010, 2011; Pauk et al., 2013). The compounds were fully characterized by melting point, CHN analyses, IR and NMR spectroscopy (Pauk et al., 2013).

In vitro antibacterial susceptibility testing

The synthesized compounds were evaluated for in vitro antibacterial activity against the intestinal sulfate-reducing bacteria D. piger Vib-7 and Desulfomicrobium sp. Rod-9 that were isolated from the healthy human large intestine as described previously (Kushkevych, 2013; Kushkevych et al., 2014). The strains have been kept in the collection of microorganisms at the Department of Molecular Biology and Pharmaceutical Biotechnology of the Faculty of Pharmacy at the University of Veterinary and Pharmaceutical Sciences Brno (Czech Republic). Ciprofloxacin (Sigma-Aldrich) was used as the standard. Prior to testing, each strain was passaged onto nutrition modified Kravtsov-Sorokin’s (KS) agar medium (Kushkevych and Moroz, 2012). Bacterial inocula were prepared by suspending a small portion of bacterial colony in sterile KS liquid medium (pH 7.5). The cell density was adjusted to 0.5 McFarland units using a densitometer (Densi-La-Meter, LIAP, Latvia). The final inoculum was made to a 1:20 dilution of the suspension with KS liquid medium. Before bacterial passage in the medium, 10 mL/L of sterile Mohr’s salt solution [(NH₄)₂SO₄Fe(SO₄)₂·6H₂O] (10%) for detecting colonies of the
sulfate-reducing bacteria was added. As a result, FeS was formed by the bacterial cells that caused black coloured colonies. The compounds were dissolved in DMSO (Sigma), and the final concentration of DMSO in the KS liquid medium did not exceed 0.1% of the total solution composition. The final concentrations of the evaluated compounds ranged from 100 to 0.05 μM. The medium dilution micro-method modified according to NCCLS guidelines (CLSI, 2012, 2014) in KS medium was used to determine the minimum inhibitory concentration (MIC). Drug-free controls, sterility controls and controls consisted of KS medium and DMSO alone were included. Petri plates were introduced into an anaerobic box with oxygen uptake generators (GENbox anaer, France) for anaerobiosis. The determination of results was performed visually after 72 h of static incubation in the darkness at 37 °C under anaerobic conditions. The MICs were defined as the lowest concentration of the compound at which no visible bacterial growth was observed. The results are summarized in Table 1.

**Results**

The studied compounds can be divided into two groups based on their chemical structure: Group I includes N-protected amino esters of N-phenylsalicylamides 1a-f, and Group II includes compounds 2a-c that can be named as diamides, since they contain two amidic moieties. The activity of both groups of salicylamide derivatives against sulfate-reducing bacteria D. piger Vib-7 and Desulfovibrio sp. Rod-9 were compared with the effect of ciprofloxacin as a clinically used drug. The in vitro antibacterial activity of compounds was expressed as the minimum inhibitory concentration (MIC) that is defined for bacteria as a 90% (IC90) or greater reduction of growth in comparison with the control. The MIC/IC90 value is routinely and widely used in bacterial assays, being a standard detection limit according to the Clinical and Laboratory Standards Institute (CLSI, 2012, 2014). In the case of potency against D. piger all the compounds showed a narrow range of the MICs from 0.22 to 0.35 μM, while the activity against Desulfovibrio sp. ranged from 0.27 to 8.52 μM. Nevertheless, the potency of all discussed compounds was much higher against both genera than that of ciprofloxacin (MIC 41.2 μM or 39.3 μM). All the results are listed in Table 1.

Lipophilicity is the most frequent physicochemical parameter employed in structure-activity relationship analysis. In a number of studies examining the biological activity of potential drugs, the relationship between lipophilicity and/or other descriptors (e.g., electronic parameters or molar volume of substituents) and their potency have been investigated. In the present study the calculated lipophilicity (Clog P values), see Table 1, was found as the main parameter influencing general antibacterial potency. Clog P value is the logarithm of n-octanol/water partition coefficient based on established chemical interactions.

Within Group I, 4-chloro-2-[[4-nitrophenyl]carbamoyl]phenyl (2S)-2-[[benzylxycarbonyl]amino]-3-methylbutanoate (1d) showed the highest activity (MIC = 0.26 μM) against D. piger, while compound 1c (R1 = 4-Cl, R2 = 4-Br, R3 = -CH2-chx) demonstrated the lowest activity (MIC = 0.35 μM). Within Group II 4-chloro-N-[2S]-1-[[3,4-dichlorophenyl]amino]-3-methyl-1-oxobutan-2-yl]-2-hydroxybenzamide (2b) was found to be the most effective compound with MIC = 0.22 μM. Fig. 1 illustrates the dependence of the antibacterial activity against D. piger expressed as log(1/MIC) of all the tested compounds on the lipophilicity expressed as Clog P. Activity within Group I decreases with the lipophilicity increase almost linearly.
Fig. 1 – Dependence of antibacterial effect of tested compounds 1a–f (Group I) and 2a–c (Group II) against Desulfovibrio piper Vib-7 expressed as log(1/MIC [M]) on lipophilicity.

Fig. 2 – Dependence of antibacterial effect of tested compounds 1a–f (Group I) and 2a–c (Group II) against Desulfomicrobium sp. Rod-9 expressed as log(1/MIC [M]) on lipophilicity.

Thus, based on the MIC values, it can be concluded that diamide 2b is the most potent compound against both SRB genera. From Group I, ester of N-phenylsalicylamides 1d is the most potent against D. piper Vib-7, while compounds 1b, 1e and 1a showed an acceptable activity against Desulfomicrobium sp. Rod-9. Based on these results and the limited number of the compounds it is not possible to assess if R1 substitution in C(4) or C(5) is more advantageous. Rather electron-withdrawing R2 substituent and less lipophilic (isopropyl, benzyl) R3 substituent seem to be also more favourable for higher activity. Compound 2b is much more potent than any compound from Group I. These results are opposite to the results of the recently published study (Pauk et al., 2013), where just N-protected amino esters of N-phenylsalicylamides showed high activity against Staphylococcus aureus, methicillin-resistant Staphylococcus aureus, Clostridium perfringens, Pasteurella multocida and, overall, were more effective than diamides. However, these microorganisms were more resistant to the effect of the compounds compared with D. piper Vib-7 and Desulfomicrobium sp. Rod-9. On the other hand, it is important to note that diamides were found to have bactericidal effect against three clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA) and S. aureus ATCC 29213 as the reference and quality control strain (Zadrazilova et al., 2015a). None of above discussed compounds did not show any effect against typical Gram-negative bacterial strains, such as Escherichia coli, Pseudomonas aeruginosa, Salmonella, Proteus or Helicobacter (Zadrazilova, 2013; Zadrazilova et al., 2015a,b).

Salicylanilides show broad spectrum of antimicrobial activities, because due to their structure they can affect a wide range of targets, although exact mechanisms have not been elucidated. For example, they inhibit the two-component regulatory systems of bacteria, bind to protein kinase epidermal growth factor receptor, inhibit interleukin-12p40 production, inhibit bacterial enzymes, such as transglycosylases, n-alanine-n-alanine ligase, isocitrate lyase and methionine aminopeptidase or destroy the cellular proton gradient due to
their function as proton shuttles (Zadrazilova, 2013; Zadrazilova et al., 2015a,b). Nevertheless, such sensitivity of studied intestinal SRB to salicylamides derivatives can be caused by the peculiarities of their metabolism including their physiological, biochemical, cytological and ecological properties (Barton and Hamilton, 2010; Kushkevych, 2012a,b).

Despite the fact that both evaluated bacteria belong to the same group and even physiological subgroup, it is known that Desulfovibrio species is different from Desulfomicrobium in biochemical and physiological properties. Genera Desulfovibrio and Desulfomicrobium have the same basic structure of bacterial cell wall (peptidoglycan), but Desulfovibrio have such phenotypic features as the presence of desulfoviridin, cytochrome C₃ and menaquinone MK-6. Desulfomicrobium contains desulfurubidin, not desulfovirdin, and also the structure of cytochrome is different. The activity, structure and properties of vital enzymes of Desulfovibrio and Desulfomicrobium genera differ (Holt et al., 1994; Brenner et al., 2005; Barton and Hamilton, 2010).

Based on above-mentioned facts, the inhibitory effect of salicylamides 1a-f and 2a-c can be obviously caused by their action on the process of dissimilatory sulfate reduction in both Desulfovibrio and Desulfomicrobium genera and their growth and production of hydrogen sulfide. However, the difference in these parameters may be due to different mechanisms of sulfate transport, the presence in their cells of different transport systems or, even, enzymes providing this process. Relative survival in D. piger Vib-7 or Desulfomicrobium sp. Rod-9 cells and antimicrobial effect under the influence of salicylamides derivatives 1a-f and 2a-c is different. It is due to the chemical structure of these compounds (diamides were described as bactericidal agents) and the genus characteristics of Desulfovibrio and Desulfomicrobium.

Conclusions

Compounds of Group I 1a-f (2-(phenylcarbamoyl) phenyl N-(benzoyloxy)carbonyl)alkanoates and Group II 2a-c (2-hydroxy-N-[25]-1-oxy-1-phenylamino)alkan-2-yl)benzamides, were found to inhibit the intestinal bacterial growth of D. piger Vib-7 and Desulfomicrobium sp. Rod-9. 4-Chloro-N-{(25)-1-{(3,4-dichlorophenyl)amino}-3-methyl-1-oxobutan-2-yl}-2-hydroxybenzamide (2b) demonstrated the highest potency against both strains: MIC = 0.22 μM against D. piger and MIC = 0.27 μM against Desulfomicrobium sp. 4-Chloro-2-[(4-nitrophenyl)carbamoyl] phenyl (2S)-2-[[benzoyloxy]carbonyl]amino)-3-methylbutanoate (1d) showed high activity against D. piger Vib-7 (MIC = 0.26 μM) and 4-chloro-2-[(4-methylphenyl)carbamoyl] phenyl (2S)-2-[[tert-butoxycarbonyl]amino]-3-(1H-indol-2-yl)propanoate (1b) showed high activity against Desulfomicrobium sp. Rod-9 (MIC = 0.31 μM). Lipophilicity was recognized as a significant parameter affecting biological activities. It is not possible to assess if R² substitution in C₉ or C₁₀ is more advantageous, but in general for both SRB strains rather electron-withdrawing R² substituent and less lipophilic (isopropyl or benzyl) R² substituent are also more favourable for higher activity. Based on the results it can be hypothesized that salicylamide derivatives interact with enzymatic systems of the bacteria affecting vital cell functions, and diamide 2b could probably serve as bactericidal active agent. Therefore, salicylamide derivatives seem to be promising candidates of potential agents with activity against sulfate-reducing bacteria.

Conflict of interest

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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