Biological activity of flufenamic acid and synthesized derivatives (PFCs) that may occur in the environment

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Abstract
Polyfluorinated compounds (PFCs) are used in the field of pharmaceuticals, pesticides, impregnating agents, etc. These are predominantly compounds that are difficult to degradate in the environment, and therefore are highly persistent. Many these compounds are known to have toxic effects and thus a negative effect on humans and the entire ecosystem. PFCs include, for example, flufenamic acid (antipyretic). The aim of the study is to verify the biological activity of flufenamic acid and derivatives prepared from this initial compound by organic synthesis. Flufenamic acid is a known substance, however the prepared derivatives are chemical compounds that have not been described and characterized in detail.
In the synthesis of prepared compounds, whose structure was confirmed by NMR spectroscopy, antimicrobial potential was tested against a wide range of microorganisms by the disc diffusion method and microdilution method in microtiter plates. Some antimicrobial properties of some of the prepared derivatives have been found to be close to the antimicrobial effect of the previously described and known antibiotic agents. These are significant and extremely stable pollutants in the environment, which, however, may have interesting antimicrobial properties according to the obtained results.
Keywords: flufenamic acid, biological activity, antimicrobial activity, inhibitory concentration

1. Introduction

There are many opportunistic pathogens (e.g. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, etc.) that can cause human and animal diseases. Furthermore, there has been increasing attention to drugs belonging to different pharmacological classes for possible antimicrobial activity, including a number of non-steroidal anti-inflammatory drugs (NSAIDs), which have been discovered to possess potent antibacterial activities [Allem and Douglas, 2004; Allem and Douglas, 2005]. The antimicrobial activity of NSAIDs is thought to be due to inhibition of prostaglandin biosynthesis; interestingly, prostaglandin biosynthesis contributes to fungal hyphal formation and biofilm development [Allem and Douglas, 2005].

Flufenamic acid (FFA; N-(α,α,α-trifluoro-m-tolyl) anthranilic acid) is a potent non-steroidal analgesic and anti-inflammatory agent used especially in the management of rheumatic disorders. FFA is a member of a group of compounds known as the fenamates. Fenamates have a wide variety of actions at both the clinical and cellular level. Clinically, fenamates are classified as non-steroidal anti-inflammatory drugs (NSAIDs) and are used for the relief of pain and pyrexia. It is likely that the clinical mechanism of action of fenamates is through inhibition of the cyclooxygenase pathway. The therapeutic daily dose of fenamates varies from 200 to 1200 mg and the most frequent side effects are referable to the gastrointestinal tract such as alteration in bowel habits, nausea, vomiting, and abdominal pain [Glazko, 1967; Ferrugia et al., 1998].
The aim of the present study was to evaluate antimicrobial activity of FFA and prepared derivatives (ion pairs) in case of several bacteria. The testing was performed using disk diffusion method and microdilution method for MIC determining.

2. Methods

2.1. Flufenamic acid and derivatives preparing

FFA was purchased from Sigma-Aldrich. Structure of six laboratory prepared derivatives (ion pares) was checked using NMR spectroscopy and used for other testing.

2.2. Antimicrobial assay

For antibacterial testing, the agar disc diffusion method was used. Plates containing Mueller–Hinton agar were spread with bacterial suspension adjusted to 1.5×10^8 CFU/mL. Filter paper discs (6 mm diameter, Oxoid Ltd.) were placed onto inoculated agar surfaces and impregnated with 10 µl of stock solution (100 mg/mL dissolved in DMSO). Simultaneously, pure DMSO were

used as a negative control. The plates were cultivated for 24 h at 37 °C under aerobic conditions.

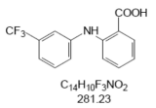
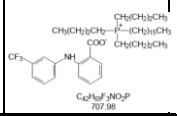
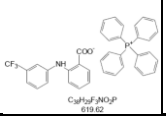
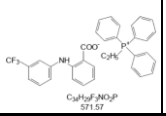
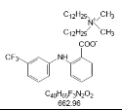
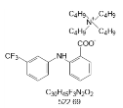
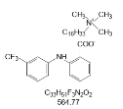
Inhibition zones were measured using a Bacmed 6iG2 automatized reader (Aspiag, Czech Republic). Experiments were performed in triplicates and the resulting activities (mm) were expressed as a mean of inhibition zones (mm) with standard deviation.

The MIC determination (0.02–20 mg/mL) of the tested compounds was performed in 96-well polystyrene flat–bottomed microtiter plates (SPL Live Sciences Co., Ltd., Korea) as previously described (Christensen et al., 1985) with modifications. Briefly, the twofold dilutions of samples were prepared in MH broth to obtain a final concentration ranging from 100 to 0.05 mg/mL in the wells after the addition of 10 µl of the freshly diluted tested cell culture containing 10^6 CFU/mL. After incubation at 37 °C for 24 h under aerobic conditions, the MIC was evaluated as the last position with yellow color after adding thiazolyl blue tetrazolium bromide (Sigma-Aldrich).

3. Results and Discussion

The inhibition effect of FFA as well as prepared derivatives was observed. The results are presented in Table 1. The obtained results confirmed the expected antimicrobial effects of FFA, as previously confirmed in the literature [Chavez-Dozal et al., 2014]. Different ion pairs were synthetized from this compound, which were found to have even higher antimicrobial activity compared to the base FFA. Generally, a very sensitive bacterium was *Bacillus subtilis* (MIC 0.76-24.41 µg/mL). On the contrary, *Pseudomonas aeruginosa* was very resistant against these samples (MIC <3,125 µg/mL). In general, the prepared derivatives were more effective against Gram-positive bacteria. The results indicate low inhibitory concentration especially in case of sample 2 and 6. The inhibitory effect of some samples is comparable or even higher compared to some antibiotics (results not shown here).

Table 1. Antimicrobial effect of flufenamic acid and prepared derivatives – disk diffusion method and MIC determination.

	Flufenamic acid		**Sample 1**		**Sample 2**		**Sample 3**		**Sample 4**		**Sample 5**		**Sample 6**	
														
	IZ [mm]	MIC [µg/mL]	IZ [mm]	MIC [µg/mL]	IZ [mm]	MIC [µg/mL]	IZ [mm]	MIC [µg/mL]	IZ [mm]	MIC [µg/mL]	IZ [mm]	MIC [µg/mL]	IZ [mm]	MIC [µg/mL]
St. aureus	19±1.0	195.3	11±0.5	1.53	34±1.5	0.76	20±1.0	48.82	10±0.5	24.41	14±1.0	195.31	20±1.0	0.19
St. epidermidis	23±1.0	24.41	10±1.0	50,000	37±2.5	0.19	23±0.5	12.21	7±0.0	24.41	20±1.0	48.83	9±0.0	0.19
M. luteus	29±1.5	6.10	17±1.0	195.31	54±3.5	0.02	42±2.0	0.38	9±0.5	3.05	25±1.0	6.10	20±1.0	<0.01
B. subtillis	23±1.5	24.41	8±0.0	1.53	29±1.0	24.41	19±0.5	24.41	8±0.0	0.76	19±1.0	24.41	13±0.0	0.76
E. coli	7±0.0	3,125	7±0.0	195.3	7±0.0	97.7	7±0.0	97.7	7±0.0	12,500	7±0.0	781.3	7±0.0	6,250
P. aeruginosa	7±0.0	6,250	7±0.0	3,125	7±0.0	3,125	7±0.0	3,125	7±0.0	6,250	6±0.0	3,125	6±0.0	6,250
Kl. pneumoniae	7±0.0	6,250	7±0.0	6,250	7±0.0	781.25	7±0.0	390.63	7±0.0	12,500	7±0.0	3,125	7±0.0	25,000

Strain specifications: *Staphylococcus aureus* CCM 4223, *Staphylococcus epidermidis* CCM 4418, *Micrococcus luteus* CCM 732, *Bacillus subtilis* CCM 2215, *Escherichia coli* CCM 3954, *Pseudomonas aeruginosa* CCM 3955, *Klebsiella pneumonia* NPK1
Notes: IZ – *inhibition zone*, MIC – *minimal inhibitory concentration*

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