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MONITORING OF PHARMACEUTICAL RESIDUES OF NON-STEROIDAL DRUGS WITH USE OF *Escherichia coli-GFP* BIOSENSORS

MONITOROWANIE POZOSTAŁOŚCI FARMACEUTYCZNYCH LEKÓW NIESTERYDOWYCH Z ZASTOSOWANIEM *Escherichia coli-GFP* BIOSENSORÓW

Abstract: *Escherichia coli* strains containing a three different plasmid-borne transcriptional fusion between genotoxin-inducible *recA, kat G* and *sodA* promoters involved in the SOS regulon and bacteria stress response and mutated form of *gfp* reporter gene, have been used. GFP-based bacterial biosensors allowed for detection of a cytotoxic and genotoxic activity of ibuprofenum, ketoprofenum and paracetamolum – conventional non-steroidal anti-inflammatory drugs in PBS buffer and surface water. For experimental tests drugs were used at concentration of 10^{-6}; 10^{-7}; 10^{-8}; 10^{-9} and 10^{-10} mg/dm^3, with bacteria strains time incubation of 3 and 24 hours. Experimental data indicated, that three promoters fusions with *gfp* gene as reporter were differently sensitive to applied drugs. Bacteria strains, *recA, kat G* and *sodA* promoters were a good bioindicator for cytotoxic and genotoxic effect monitoring of tested drugs in PBS buffer and surface water. The results showed, that applied in this experiment *E. coli gfp* biosensors strains could be potentially useful for environmental monitoring of cytotoxic and genotoxic effect of pharmacist residues of drugs in surface water.

Keywords: environmental monitoring, non-steroidal anti-inflammatory drugs, cytotoxicity, genotoxicity, *gfp* biosensors

Introduction

Environmental pollution by human pharmaceuticals (HPs) has become a major problem in many countries worldwide. Recently, numerous scientific research have detected the residual concentrations of human pharmaceuticals and their metabolites in wastewaters and surface waters. Pharmaceutically active chemicals (PhACs) are a class of emerging contaminants, which has led to increasing concern about potential

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environmental risks [1–4]. After excretion from human body, unchanged pharmaceuticals and their metabolites are discharged into hospital, ambulatory and domestic wastewaters. Nowadays, there are no effective sewage treatment system which can remove these chemicals and as a result untreated wastewater containing these drugs enters surface waters. Pharmaceuticals and metabolites were detected worldwide at ng/dm^3 to μg/dm^3 levels in surface water in different areas. Some of them, belong to the groups of very dangerous pollutants with strong cyto- and genotoxic activity, even at trace environmental levels. In addition, the metabolites of human drugs may be even more toxic than the parent compounds. Scientific research revealed, that exposure to pharmaceutical residues has been recognized as a potential health hazard [5–8]. Currently there is a lack of information regarding how these drugs influence on condition of aquatic organisms. These products have unpredictable impacts on the aquatic ecosystems in which they enter. Of a particular importance are pharmaceutical residues causing cytotoxic, genotoxic effect and DNA damage, carcinogenesis and a number of different diseases. So, there is a very important social need for development of effecitive biosystems for monitoring and toxicity assessment of PhACs residues in environmental samples, mainly in relation to antiinflammatory drugs, which are widely used and very often detected in aquatic environments [4–10].

Nonsalicylate, conventional nonsteroidal anti-inflammatory drugs (NSAIDs) have been widely used for human and animal therapy for decades. Among NSAIDs, ibuprofen (IB) (Fig. 1) is one of the most popular and has been shown to be more effective than acetaminophen for the treatment of pain associated with osteoarthritis (OA). IB is used in painful and inflammatory conditions. This drug was the first member of the group that came into general use [5, 8, 9, 11].

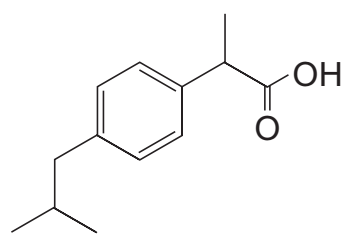

Fig. 1. Chemical structure of ibuprofenum

IB is used in rheumatoid arthritis, osteoarthritis, for relief of mild to moderate pain, primary dysmenorrhoea and reduction of fever. Ibuprofen is usually detected in influents in highest concentrations amongst the PhACs. In Europe, the fate of 13 PhACs has been traced in different municipal Wastewater Treatment Plants (WWTPs). Ibuprofen was found in effluent at maximum 3.9 mg/dm^3. In Poland the presence of IB in surface waters was detected at concentration of 0.05–0.1 μg/dm^3. Various experiments have determined that exposure to IB alone can affect aquatic organisms. IB exposure inhibits the growth of the mollusk *Planorbis carinatus*, reduces the reproductive capacity of the crustacean *Daphnia magna*, and causes abnormal behavior in *Gammarus pulex*; moreover, IB concentrations in water of greater than 100 mg/ dm^3 are

known to be fatal to the fish *Oryzias latipes*. Ragugnetti et al [11] observed ibuprofen genotoxic effects in experimental model using *Oreochromis niloticus*. IB has been reported to induced gastrointestinal bleeding, meningitis, lymphopenia and hepatotoxicity in human bodies. It inhibits the synthesis of prostacyclin and prostaglandin of type E. Acute ingestion of IB results in nephritis, proteinuria, renal failure, adult respiratory distress syndrome and metabolic acidosis [4, 7, 9, 11–13].

Ketoprofen (KP) similarly to other non-steroidal anti-inflammatory drugs has strong antiinflammatory, analgesically and antipyretic activity. In 2007 Struwea et al [14] assessed photochemical genotoxicity of ketoprofen *in vitro* with use of photo comet assay with L5178Y mouse lymphoma cells. In 2013 Da Silveira et al [15] showed anti-cancer ability of ketoprofen, where ketoprofen-loaded polymeric nanocapsules selectively inhibit cancer cell growth *in vitro* and in preclinical model of glioblastoma multiforme (GBM) [14–16].

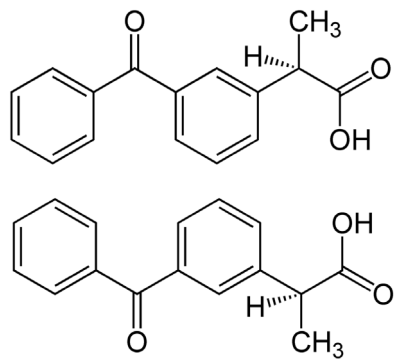

Fig. 2. Chemical structure of ketoprofenum

Paracetamol (PCM; N-(4-hydroxyphenyl) acetamide) as similar as IB and KP is a widely used analgesic and antipyretic agent that is utilized in human and animal medicine. PCM is one of the most common "first line use" drug which is present in global water bodies where it reaches concentrations up to μg /dm^3. Chemically, paracetamol is a phenol (Fig. 3) [17–22].

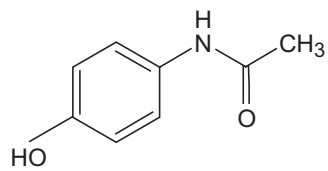

Fig. 3. Structure of paracetamol

Depending on the pharmacological mechanisms of action, NSAIDs have been divided into conventional and COX-2-selective categories. Conventional NSAIDs such as phenoprofen, ibuprofen, indomethacin, ketoprofen, keterolac, naproxen, paracetamol,

piroxicam and aspirin are nonselective COX inhibitors, whereas etodolac, meloxicam, diclofenac, celecoxib, nimesulide, valdecoxib, parecoxib, rofecoxib and etoricoxib are selective COX-2 inhibitors and are also known as coxibs [19–26].

Data revealed the capacity of PCM to induce moderate genotoxicity in bivalves exposed to environmental concentrations [24]. PCM is a hepatotoxic agent and is the leading cause of acute liver failure (ALF). Research studies showed that toxic activity of PCM in living cells caused the increase in oxidative stress and/or had a direct interaction with DNA.

Because of the activity of pharmaceutical residues of conventional NSAIDs and their impact on the environment and the public health, it is mandatory to provide highly sensitive and robust analytical methodologies to control them and their active metabolites, at trace levels [25–30].

Although classical analytical methods can detect most chemicals in environmental sample with great precision, they are elaborate and expensive and also do not differentiate between the unavailable and bio-available fractions. Biological assays with use of living cells are able to showing the bioavailability and ecotoxicological effects of compounds [31–38]. Nowadays, various *in vitro* biotest systems have been developed for genotoxic risk assessment of pharmaceuticals in environmental samples. Several genotoxin-specific recombinant bacterial biosensors have been constructed to determine genotoxic potency of analytes. These tests are based on the ability of compounds to induce DNA damage, oxidative stress formation, enhance the formation of mutations or chromosomal aberrations or initiate a cellular stress response. To establish genotoxicity, various genetically modified bacteria-based assays have been developed. The DNA – damage and oxidative stress formation assays are based on activation of bacterial SOS and oxidative stress response upon exposure to genotoxic compounds. The SOS, SoxRS and OxyR regulons with *recA*, *sodA* and *katG* promoters are one of the most thoroughly studied genotoxic stress regulons for bacteria. These specific genes are induced in response to reactive oxygene species (superoxide anion, hydrogen peroxide and hydroxyl radicals) and other DNA-damaging (*eg* alkylating) agents, such as anticancer drugs and different chemicals [38–46]. The genotoxin-sensitive *recA*, *katG* and *sodA* promoters transcription is induced upon DNA damage (genotoxic and mutagenic effect). The application of these promoters in order to create some effective genotoxicity bacteria biosensors is connected with broad involvement of RecA protein and *katG* and *sodA* expression products in several DNA repair pathways, including the repair of daughter-strand gaps and double-strand breaks, as well as in an error prone damage tolerance mechanisms called SOS mutagenesis and stress oxidation response [37, 39, 41, 45].

The green fluorescent protein (*gfp*) reporter gene is one of the common used in microbial biosensors creation, because it is stable, direct and convenient tracing tool, without external substrate. *Gfg* expression can be observed in living cells [39].

Among the many classes of pharmaceuticals, the conventional non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most important groups with an annual production of several kilotons. NSAIDs are the sixth most sold drugs worldwide. Many authors have reported levels of these drugs exceeding 1 $\mu g/dm^3$ in wastewaters and in

the effluents of sewage treatment plants (STP), while lower concentrations have been found in surface waters. Due to the biological activity of these drugs, there are considered to be hazardous to living organisms and human health. There is a need to monitor of their presence in environmental samples and assess their cytotoxic and genotoxic risk to living organisms [1–10]. Thus, the present study showed the possibility of application of *E. coli* K-12 *gfp* microbial biosensor strains for cytotoxic and genotoxic effects monitoring of ibuprofen, ketoprofen and paracetamol – one of the most popular drugs use in human and veterinary medicine.

Methodology

Chemicals

Ibuprofen, ketoprofen and paracetamol were commercially obtained from Bialystok pharmacy. These drugs had been dissolved in PBS buffer (1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 0.2 g KCl, 8 g NaCl per dm^3 of destilled water, pH = 7) at determined experimental concentrations before they were used.

Bacteria strain and plasmid

In the experiment *Escherichia coli* K-12 MG1655 stationary phase cells: *Escherichia coli* K-12 *recA*::*gfpmut2*, *Escherichia coli* K-12 *katG*::*gfpmut2*, *Escherichia coli* K-12 *sodA*::*gfpmut2* and *Escherichia coli* K-12 *promoterless*::*gfpmut2*, genetically modified were used. They contained a pUA66 plasmid-borne transcriptional fusion between DNA-damage inducible, oxidative stress *recA*, *katG* and *sodA* promoters involved in the SOS regulon and oxidative stress response and fast folding GFP variant reporter gene-*gfpmut2*. The genetic structure of pUA66 plasmid is described in the work of Zaslaver et al [44]. In the present work a more stable and fast folding mutant of *gfp* gene – *gfpmut2* with excitation and emission wavelengths of 485 and 507 nm was used [44].

Bacteria growth condition

Escherichia coli K-12 MG1655 strains: *Escherichia coli* K-12 *recA*::*gfpmut2*, *Escherichia coli* K-12 *katG*::*gfpmut2*, *Escherichia coli* K-12 *sodA*::*gfpmut2* and *Escherichia coli* K-12 *promoterless*::*gfpmut2* were cultured overnight in LB agar medium (Merck, Germany) at 30°C supplemented with 10^4 μg/dm^3 of kanamycin (Sigma-Aldrich, Germany). Colonies were carried to LB broth medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per 1 dm^3 of destilled water) with 100 μg/cm^3 of kanamycin and incubated overnight at 30°C. Afterwards the cells were washed with PBS buffer.

Monitoring of bacteria growth and concentration

At the beginning of the experiment the initial bacteria cells density was standardized to OD (Optical Density) value by using spectrophotometer (Multi Detection System,

Promega) at the wavelength of 600 nm. The concentration of bacteria cells per cm^3 of PBS was assessed by series dilutions system and expressed as CFU/dm^3 values.

Dynamic growth of bacteria strains treated with drugs was monitored by the use of standard spectrophotometer analysis of Optical Density values at the wavelength of 600 nm.

Bacteria cells treatment with ibuprofen, ketoprofen and paracetamol in PBS buffer

10^{-4} dm^3 of stationary phase bacteria cells ($2 \cdot 10^{11}$ CFU/dm^3) were suspended in 40^{-4} dm^3 of PBS buffer and the following drugs were used for genotoxicity testing: ibuprofen (PBS buffer solution of 200 mg of ibuprofenum) (IB), ketoprofen (PBS buffer solution of 50 mg of ketoprofenum) (KP) and paracetamol (PBS buffer solution of 500 mg of paracetamolum) (PCM). Drugs were applied at five different concentrations: 10^{-6}; 10^{-7}; 10^{-8}; 10^{-9} and 10^{-10} mg/dm^3. The concentration range of the drugs analysed in research was selected experimentally from the minimum level of *recA::gfp*, *katG::gfp* and *sodA::gfp* constructs sensitivity and according to the reviewed references recommendation, which indicated the concentrations observed in the environment [26]. The time of bacteria incubation with drugs (3 h and 24 h) was estimated for monitoring of sensitivity of genetic constructs for quick (3 h) and later (24 h) response. The control samples – *Escherichia coli* K-12 *recA*::*gfpmut2*, *Escherichia coli* K-12 *katG*::*gfpmut2* and *Escherichia coli* K-12 *sodA*:*gfpmut2* strains in PBS buffer were not treated with drugs. For verification the correct activity of *recA*, *katG* and *sodA* promoters, *Escherichia coli* K-12 strain containing pUA66 plasmid without the promoter – *Escherichia coli* K-12 *promoterless*::*gfpmut2* – was used as the control one. Additionally, for assessment of genotoxic sentivity of *recA::gfp* construct, 4% acetone was used as the negative control and 50 μM methylnitronitrosoguanidine (MNNG, known genotoxin) as the positive control.

Bacteria cells treatment with drugs in surface water

Surface water samples were collected in sterile flasks from the Bialka river. The samples were sterilized by filtration. 10^{-4} dm^3 of stationary phase bacteria cells ($2 \cdot 10^{11}$ CFU/dm^3) were suspended in 40^{-4} dm^3 of surface water and the following drugs were used for genotoxicity testing: ibuprofen and ketoprofen at concentration of 10^{-8} mg/dm^3 for IB and 10^{-7} mg/dm^3 for KP with use of *recA::gfp* and *katG::gfp* genetic constructs. Drugs concentrations were selected for the highest stimulation of *gfp* gene expression in PBS buffer (for IF = 8.01 and IF = 8.37, respectively).

The conditions of bacteria incubations and the control protocols were the same as above.

Analytical method for the intensity of *gfp* gene fluorescence (IF) analysis

After exposition of bacteria cultures to tested drugs, the strains were washed with PBS buffer and the intensity of fluorescence of *gfp* gene in the volume of 10^{-4} dm^3 of

bacteria cells suspension ($1 \cdot 10^7$ CFU/dm^3) in PBS buffer was measured with the spectrofluorometer (Multi Detection System, Promega). The measurements were done at excitation and emission wavelengths of 485 and 507 nm.

Assessment of SFI values

The specific fluorescence intensity (*SFI*) value which is defined as the raw fluorescence intensity (*IF*) divided by the optical density (*OD*) measured at each time point at 600 nm was calculated according to the below formula for monitoring the dynamic of *gfp* expression after bacteria treatment with drugs:

$$SFI = \frac{IF}{OD} \tag{1}$$

where: *SFI* – Specific Fluorescence Intensity,
IF – The raw fluorescence intensity of the strains at excitation and emission wavelengths of 485 and 507 nm,
OD – Optical Density at 600 nm of the strains.

Detection of $S_{gfpexp.}$ value

For the increased *SFI* values with the level of *gfp* expression in comparison with the control sample the percentage stimulation of *gfp* ($S_{gfpexp.}$) was calculated according to the formulas:

$$S_{gfpexp.}(\%) = I_{TS}\,(\%) - SFI_{CS}\,(\%) \tag{2}$$

where: $I_{TS}\,(\%)$ – the increase for SFI values for tested drugs sample in comparison with the control sample,
$SFI_{CS}\,(\%)$ – *SFI* for the control sample = 100%.

Assessment of F_I values

For each concentration of tested drugs the induction factors (F_I) were calculated.

$$F_I = (Fl_I / OD_0) / (Fl_0 / OD_I) \tag{3}$$

where: Fl_I – the raw fluorescence of the culture treated with DNA – damaging compound,
Fl_0 – the raw fluorescence of the control sample without genotoxin,
OD_I – the optical density at 600 nm of treated culture,
OD_0 – the optical density of the control sample.

The *SFI*, $S_{gfpexp.}$ and F_I, values express the potency of influence of tested drugs on the sensitivity of genotoxicity and oxidative stress *recA::gfp*, *katG::gfp* and *sodA::gfp* constructs.

Classification of tested drugs as genotoxins

The F_I values were calculated for classification of tested drugs as genotoxins. According to Ptitsyn et al [45] and Kostrzynska et al [39] a chemical was identified as a genotoxin if its induction factor was 2 or more ($F_I \geq 2$).

Statistical analysis

Statistical data obtained in this study are expressed as mean ± standard deviation (SD) for $n = 8$. The data were analyzed by the use of standard statistical analyses, including one-way Student's test for multiple comparisons to determine the significance between different groups. The values for $P < 0.05$ were considered significant.

Results

In the experiment the positive fluorescence reactivity of *Escherichia coli* K-12 was obtained for each tested genetic constructs with three different promoters *recA*, *katG* and *sodA* and for all tested drugs, especially at concentration of 10^{-6}; 10^{-7}; 10^{-8} mg/dm^3

Escherichia coli K-12 MG1655 *recA*::*gfpmut2* strain treatment with ibuprofen, ketoprofen and paracetamol showed that administration of three drugs caused a significant increase ($p \leq 0.05$) in *SFI*, F_I and $S_{gfpexp.}$ values compared to non-treated cells (Table 1). Bacteria cells reacted with different efficiency in *gfp* expression after incubation with drugs which possessed different chemical structure.

Longer treatment of *recA::gfp* bacteria strain with KP and PCM (up to 24 h) intensified *SFI*, F_I and S_{gfpexp} values at concentration of 10^{-8} mg/dm^3. Bacteria cells incubation with IB caused the strongest stimulation of *gfp* expression for 3 h incubation at concentrations of 10^{-7}; 10^{-8} mg/dm^3, compared to the control sample. A maximum point of *recA::gfp* stimulation (about 701% higher *gfp* stimulation comparable to control sample) was observed for KP at concentration of 10^{-8} mg/dm^3 and 24 h incubation with drug (Table 1).

E. coli K-12 *katG::gfp* treatment with IB, KP and PCM resulted in a progressive significant stimulation of *SFI* values for IB and PCM at concentration of 10^{-7}; 10^{-8} mg/dm^3 and for KP at concentration of 10^{-6}; 10^{-7}; 10^{-8} mg/dm^3 for 3 and 24 h incubation with drugs compared to the control sample (Table 2). The maximum point for *SFI* value ($S_{gfpexp.}$= 737%) was for KP at the concentration of 10^{-7} mg/dm^3 and 24 h of incubation time.

E. coli K-12 *sodA::gfp* cells administrated with IB, KP and PCM exerted some influence on *SFI* and the parameters with the maximum point for *SFI* ($S_{gfpexp.}$ = 599%) were for 10^{-7} mg/dm^3 of KP after 24 h incubation with drug. KP almost at each concentration significantly modulated (in 80% of cases) *gfp* expression. Only in the case of 10^{-9} mg/dm^3 KP concentration no significant differences in *SFI* between KP and control sample were observed (Table 3).

The monitoring of bacteria cultures density as optical density value (*OD*) of *E. coli recA::gfp* and *E. coli katG::gfp* and *E. coli sodA::gfp* at the start of bacteria treatment (time 0) and after 3 and 24 h of incubation with drugs indicated a significant decrease in

Table 1

SFI values for *E. coli* K-12 *recA::gfp mut2* treated with ibuprofen, ketoprofen and paracetamol in PBS buffer in comparison with the control sample (bacteria strain in PBS buffer), T – time of bacteria strain incubation with drugs, F_I – induction factor values, $S_{gfpexp.}$ [%] – the percent of stimulation of *gfp* expression after treatment of bacteria cells with drugs in comparison with the control sample [100%]

C [mg/dm^3]	T [h]	Control sample $SFI \pm SD$	Ibuprofen $SFI \pm SD$	F_I	S_{gfpexp} [%]	Ketoprofen $SFI \pm SD$	F_I	$S_{gfpexp.}$ [%]	Paracetamol $SFI \pm SD$	F_I	$S_{gfpexp.}$ [%]
10^{-6}	3	26.5 ± 3.4	24.3 ± 3.6	—	—	27.6 ± 4.3	—	—	32.6 ±3 .4	—	—
	24	41.0 ± 5.4	36.4 ±2 .8	—	—	49.8 ± 4.1	—	—	65.4 ± 3.8	—	—
10^{-7}	3	26.5 ± 3.4	26.3 ± 3.1	—	—	30.0 ± 3.4	—	—	27.3 ± 3.6	—	—
	24	41.0 ± 5.3	39.2 ± 3.0	—	—	56.4 ± 5.3^{ab}	—	—	140.3 ± 5.2^{abc}	2.42	142
10^{-8}	3	26.5 ± 3.4	45.7 ± 4.6^{a}	—	72	33.3 ± 4.6	—	—	90.4 ± 6.4^{abc}	2.41	141
	24	41.0 ± 5.4	283.0 ± 7.2^{a}	6.89	589	356.0 ± 8.3^{ab}	7.70	670	114.0 ± 7.1^{abc}	2.78	178
10^{-9}	3	26.5 ± 3.4	59.4 ± 3.6^{a}	2.24	124	64.4 ± 4.2^{ab}	2.43	143	105.5 ± 6.3^{abc}	2.98	198
	24	41.0 ± 5.3	298.0 ± 8.3^{a}	7.27	627	370.0 ± 6.4^{ab}	8.01	701	176.0 ± 6.8^{abc}	3.30	230
10^{-10}	3	26.5 ± 3.4	29.4 ± 3.5	—	—	29.4 ± 3.4	—	—	32.4 ± 4.1	—	—
	24	41.0 ± 5.3	43.5 ± 4.0	—	—	53.5 ± 4.9	—	—	72.4 ± 5.4^{abc}	—	—

Mean values ± SD; $n = 8$; a – significantly different from control ($p < 0.05$); b – significantly different from ibuprofen (IB) group ($p < 0.05$); c – significantly different from ketoprofen (KP) group ($p < 0.05$).

Table 2

SFI values for *E. coli* K-12 *katG::gfp mut2* treated with ibuprofen, ketoprofen and paracetamol in PBS buffer in comparison with the control sample (bacteria strain in PBS buffer), T – time of bacteria strain incubation with drugs, F_I – induction factor values, $S_{gfpexp.}$ [%] – the percent of stimulation of *gfp* expression after treatment of bacteria cells with drugs in comparison with the control sample [100%]

C [mg/dm^3]	T [h]	Control sample *SFI* ± SD	Ibuprofen *SFI* ± SD	F_I	$S_{gfpexp.}$ [%]	Ketoprofen *SFI* ± SD	F_I	$S_{gfpexp.}$ [%]	Paracetamol *SFI* ± SD	F_I	$S_{gfpexp.}$ [%]
10^{-6}	3	20.4 ± 2.8	31.3 ± 3.9	—	—	24.3 ± 3.9	—	—	24.8 ± 3.7	—	—
	24	35.6 ± 3.5	39.6 ± 3.4	—	—	45.0 ± 4.6^{a}	—	—	42.6 ± 4.2	—	—
10^{-7}	3	20.4 ± 2.8	32.6 ± 3.8	—	—	120.6 ± 7.6^{ab}	5.9	491	29.8 ± 3.2	—	—
	24	35.6 ± 3.5	49.8 ± 4.3	—	—	280.0 ± 8.4^{ab}	7.8	686	88.0 ± 5.3^{abc}	2.5	147
10^{-8}	3	20.4 ± 2.8	85.4 ± 5.5^{a}	4.2	318	124.0 ± 6.3^{ab}	6.1	507	70.8 ± 4.6^{ac}	3.4	247
	24	35.6 ± 3.5	210.0 ± 7.50	5.9	490	298.0 ± 8.5	8.4	737	110.0 ± 7.4	3.1	209
10^{-9}	3	20.4 ± 2.8	14.0 ± 8.0^{a}	6.8	586	99.0 ± 5.3^{a}	4.8	385	102.0 ± 7.2^{ab}	5.0	400
	24	35.6 ± 3.5	240.0 ± 7.6^{a}	6.7	574	167.0 ± 7.3^{ab}	4.7	370	123.0 ± 8.1^{abc}	3.4	245
10^{-10}	3	20.4 ± 2.8	31.4 ± 4.0	—	—	26.0 ± 3.4	—	—	31.2 ± 5.1	—	—
	24	35.6 ± 3.5	46.8 ± 4.3	—	—	49.0 ± 3.8	—	—	43.3 ± 5.6	—	—

Mean values ± SD; $n = 8$; a – significantly different from control ($p < 0.05$); b – significantly different from ibuprofen (IB) group ($p < 0.05$); c – significantly different from ketoprofen (KP) group ($p < 0.05$).

Table 3

SFI values for *E. coli* K-12 *sodA::gfp mut2* treated with ibuprofen, ketoprofen and paracetamol in PBS buffer in comparison with the control sample (bacteria strain in PBS buffer), T – time of bacteria strain incubation with drugs, F_I – induction factor values, $S_{gfpexp.}$ [%] – the percent of stimulation of *gfp* expression after treatment of bacteria cells with drugs in comparison with the control sample [100%]

C [mg/dm^3]	T [h]	Control sample $SFI \pm SD$	Ibuprofen $SFI \pm SD$	F_I	$S_{gfpexp.}$ [%]	Ketoprofen $SFI \pm SD$	F_I	$S_{gfpexp.}$ [%]	Paracetamol $SFI \pm SD$	F_I	$S_{gfpexp.}$ [%]
10^{-6}	3	22.7 ± 3.1	29.5 ± 3.4	—	—	70.3 ± 7.6^{ab}	2.1	110	29.6 ± 3.8	—	—
	24	38.3 ± 3.9	41.0 ± 3.6	—	—	93.2 ± 8.2^{ab}	—	43	45.7 ± 4.6	—	—
10^{-7}	3	22.7 ± 3.1	34.6 ± 3.6	—	—	126.0 ± 8.1^{ab}	4.5	355	32.0 ± 3.9^{c}	—	—
	24	38.3 ± 3.9	52.5 ± 5.2^{a}	—	—	301.0 ± 9.0^{ab}	6.8	586	85.0 ± 6.2^{abc}	2.22	122
10^{-8}	3	22.7 ± 3.1	74.3 ± 5.3^{a}	2.3	127	156.0 ± 7.4^{ab}	5.8	487	82.0 ± 5.2^{ac}	2.6	161
	24	38.3 ± 3.9	175.0 ± 6.8^{a}	3.6	257	306.0 ± 8.9^{ab}	6.9	599	134.0 ± 8.2^{ac}	2.5	150
10^{-9}	3	22.7 ± 3.1	98.0 ± 7.4^{a}	3.3	232	85.0 ± 5.7^{a}	2.7	174	111.0 ± 8.3^{a}	3.9	290
	24	38.3 ± 3.9	190.0 ± 7.4^{a}	3.9	296	133.0 ± 7.6^{ab}	2.5	147	133.0 ± 8.9^{ab}	2.4	147
10^{-10}	3	22.7 ± 3.1	34.6 ± 4.5	—	—	29.0 ± 3.7	—	—	33.4 ± 6.2	—	—
	24	38.3 ± 3.9	64.5 ± 5.3^{a}	—	—	52.0 ± 4.3	—	—	42.7 ± 5.6	—	—

Mean values ± SD; $n = 8$; a – significantly different from control ($p < 0.05$); b – significantly different from ibuprofen (IB) group ($p < 0.05$); c – significantly different from ketoprofen (KP) group ($p < 0.05$).

OD (growth inhibition) values for 10^{-6}, 10^{-7}, 10^{-8} mg/dm^3 tested concentrations of ketoprofen for 24 h treatment and for IB at concentrations of 10^{-6}, 10^{-7} mg/dm^3 for 24 h incubation. There were no statistical differences in the case of paracetamol and a shorter time (3 h) of IB and KP influence on bacteria cells. Prolonged treatment (up to 24 h) of bacteria cells with IB and KP significantly influenced the *OD* value of bacteria strains, especially at higher concentration. The *OD* inhibition values after treatment of bacteria strains with IB and KP were very similar for the three strains with *recA*, *katG* and *sodA* promoters.

Bacteria incubation with PBS buffer (the control sample) without any drugs addition resulted in no statistically differences in *OD* value from 0 to 24 hours continuous cultivation.

Treatment of *E. coli recA::gfp* and *E. coli katG::gfp* biosensor bacteria strains in surface water enhanced the sensitivity of *recA::gfpmut2* and *katG::gfp* genotoxic system and increased the stimulation of *gfp* expression and *SFI* value in comparison to incubation in PBS buffer (Table 4). Prolonged treatment (up to 24 h) of bacteria cells with the ketoprofen significantly influenced *gfp* expression with the maximum values for F_I = 9.06 for *recA::gfp* genetic construct and F_I = 11.41 for *katG::gfp* genetic construct comparable to the control sample.

In this experiment, for assessment of genotoxic sensitivity of a *recA::gfp* genetic biosensing system, 4% acetone was tested as the negative control. In the case of this chemical there was no increased in F_I values for 3 h and 24 h of incubation. Methylnitronitrosoguanidine (MNNG) – known genotoxin at the concentration of 50 μM – was used as the positive control. For this analyte F_I = 8.4 for 24 h incubation time and F_I = 2.8 for 3h (data not shown). These results proved stronger sensitivity of a *recA::gfp* biosensing system for MNNG than for an acetone stressor.

Table 4

SFI values for *E. coli* K-12 *recA::gfp mut2* and *E. coli* K-12 *katG::gfp mut2* treated with ketoprofen at concentration of 10^{-7} and 10^{-8} mg/dm^3 in comparison with the control sample (bacteria strain in surface water), *T* – time of bacteria strain incubation with drugs, F_I – induction factor values, $S_{gfpexp.}$ [%] – the percent of stimulation of *gfp* expression after treatment of bacteria cells with drug in comparison with the control sample [100%]

Strain	*C* [mg/dm^3]	*T* [h]	Control sample *SFI* ± SD	Ketoprofen *SFI* ± SD	F_I	$S_{gfpexp.}$ [%]
E. coli K-12 *recA::gfp*	10^{-8}	24	44.5 ± 5.4	403.0 ± 10$^{a\,A}$	9.06	806
E. coli K-12 *katG::gfp*	10^{-7}	24	39.8 ± 6.2	454.0 ± 12^{aB}	11.41	1041

Mean values ± SD; n = 8; a – significantly different from control ($p < 0.05$); A – significantly different from ketoprofen group in PBS buffer for *E. coli* K-12 *recA::gfp* ($p < 0.05$); B – significantly different from ketoprofen group in PBS buffer for *E. coli* K-12 *katG::gfp* ($p < 0.05$).

Discussion

The results of this study indicate that treatment of bacteria cells with ibuprofen, ketoprofen and paracetamol lead to over 6- and 8-fold stimulation (F_I = 8.01 in the case

of ibuprofen, F_I = 8.37 in the case of ketoprofen and F_I = 6.99 in the case of paracetamol) of bacteria genotoxin-sensitive *recA*, *katG* and *sodA* promoters and *gfp* gene expression.

The results obtained in the experiment are in agreement with the studies of Belkin et al [31]; Ptitsyn et al [45]; Albano et al [32]; Kostrzynska et al [39]; Norman et al [37]; Alhadrami and Paton [38] and some others [31–46], who presented data, that genetic constructs with *recA*, *katG* and *sodA* genes in transcriptional fusion with reporter gene systems (with *gfp* and *lux* reporters) were sensitive and useful for measurement of cyto- and genotoxicity of anticancer drugs and various chemicals in environmental studies.

According to the results obtained in this experiment IB, KP and PMC modulate and increase the reactivity of *recA*-, *katG*- and *sodA*-genotoxin sensitive, oxidative stress promoters in relation to control sample.

Most biotests have usually described the effects of medicine concentrations from micrograms per liter to milligrams per liter on aquatic organisms. Ibuprofen as a drug is very popular all over the world and has been detected in rivers [48].

Some laboratory studies on ecotoxicological influence of ibuprofen with use of tests with *Daphnia magna*, amphibians *Hyalille Azteca* and *O. niloticus* has revealed the genotoxic effect of ibuprofen at concentration of 300 ng/dm^3 [11, 49–50].

Medical studies established that nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of promising cancer chemopreventive agents and antineoplastic agents. It was observed that long-term use of NSAIDs has been shown to reduce the risk of cancer of the colon and other gastrointestinal organs as well as of cancer of the breast, prostate, lung, and skin. These very useful activity of NSAIDs is connected with its ability for restoring normal apoptosis and reducing cell proliferation in human adenomatous colorectal polyps, experimental colonic tumors, and in various cancer cell lines that have lost critical genes required for normal function. NSAIDs, particularly selective cyclooxygenase-2 (COX-2) inhibitors such as celecoxib, have been shown to inhibit angiogenesis in cell culture and in rodent models of angiogenesis. Probably, the main molecular mechanism of NSAIDs act is related to its down-regulation of pro-inflammatory cytokines and/or growth factors and its influence on transcription factors, the mechanism of which is not well understood [51–54].

The results of the above experiment provided the conformation of the possible influence of ibuprofen, ketoprofen and paracetamol on the genes expression, similarly as Kanwar et al [52]; Rayburn et al [53] and Vaish et al [51], In 40% for *recA* promoter, 43,33% for *katG* and 63,33% for *sodA* promoter of the cases there were significant differences (comparable to the control sample) regarding the level of promoters sensitivity and *gfp* expression after bacteria treatment with all applied concentrations of tested NSAIDs for both short (3 h) and longer time of incubation (up to 24 h).

Some authors [15, 17, 18, 21, 51–54] studies indicated that the activity of NSAIDs are dose- and time-dependent. It was also confirmed by data obtained in our studies. Longer bacteria exposure (up to 24 h) on IB, KP and PMC resulted in a progressive stimulation of promoters activity and *gfp* gene expression. Higher values of F_I factor were obtained for IB, KP and PMC after 24 hours incubation than after 3 h. Generally,

the strongest stimulation of *gfp* expression was possessed after bacteria treatment with IB and KP than PCM (F_1 = 8.01; F_1 = 8.37; and F_1 = 6.99, respectively).

The main molecular mechanisms of action of NSAIDs act by suppressing cyclo-oxygenase (COX)-1 and COX-2 enzymes or tumour necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS). Phospholipase A_2 (PLA_2) or phospholipase C (PLC) catalyses liberation of arachidonic acid from membrane phospholipids, which is further oxygenated into PGs by the action of lipoxygenase (LOX) and COX enzymes. By blocking the action of COX, NSAIDs can relieve inflammation by reducing vasodilatation and pain, which is produced mainly by prostaglandin E (PGE)2 and prostacyclin (PGI2). Most of the NSAIDs block synthesis of PGs by inhibiting COX enzymes nonselectively [4–9, 18, 21, 55].

Our results showed that IB and KP *E. coli* K-12 longer (up to 24 h) treatment significantly inhibited bacteria cells growth. Bacteria incubation with IB and KP importantly, dose-dependently intensified their cytotoxic effect on living bacteria cells after 24 hours incubation. Our results are in agreement with earlier empirical studies of other authors who demonstrated cyto- and genotoxic effect of IB and KP on living cells. Vaish et al [51], Kanwar et al [52] and Rayburn et al [53] have demonstrated that NSAIDs reduced cell proliferation in human cells and in various cancer cell lines. This group of drugs influenced the activity of DNA of the cells and genes expression and it could be the main mechanisms of its cyto- and genotoxicity.

We obtained stronger reactivity of *E. coli* K-12 *recA::gfp mut2* and *E. coli* K-12 *katG::gfp mut2* in surface water for ketoprofen at concentration of 10^{-8} and 10^{-7} mg/dm^3 treated samples. It was possibly due to the different chemical composition and pH value of PBS buffer and tested sample of surface water. Additionally the presence of the other chemicals in surface water which could influence *gfp* expression in bacteria strains maybe occured. It is important, therefore, to check all river's water (specially in the hospital's surroundings) for the presence of drugs belonging to these groups of chemicals.

The cyto- and genotoxicity studies of NSAIDs are being conducted for some years on bacteria, human cells and other organisms [51–53]. In above experiment we applied *E. coli* K-12 bacteria cells with three different genetic constructs with *recA*, *katG* and *sod*A promoters and *gfp* gene as reporter as a model organism for genotoxic studies. Obtained data, are generally in agreement with other results which were previously obtained in *in vivo* and *in vitro* tests of higher organisms, including human cells.

Conclusions

1. The results of the presented study indicated that *recA::gfpmut2*, *katG::gfpmut2* and *sodA::gfpmut2* genetic systems were sensitive to IB, KP and PCM applied in the experiment.

2. Experimental data indicated that three promoters fusions with *gfp* gene as reporter were differently sensitive to applied drugs. For applied drugs the highest sensitivity was observed for *sodA* promoter and IB and KP.

3. The results indicated that *gfp E. coli* strains with *recA*, *katG* and *sodA* could be potentially useful for monitoring of cyto- and genotoxic effect of pharmacist residues in water.

4. The validation of used in this work genetic systems in *E. coli* demands more experimental analysis, which should be focused on the assessment of their sensitivity on drugs with different chemical structure and mechanisms of biological activity.

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MONITOROWANIE POZOSTAŁOŚCI FARMACEUTYCZNYCH NIESTERYDOWYCH LEKÓW Z ZASTOSOWANIEM *Escherichia coli-GFP* BIOSENSORA

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Abstrakt: W pracy wykorzystano szczepy *Escherichia coli* zawierające plazmidowe, trzy różne konstrukty genowe indukowalnych genotoksynami promotorów *recA, kat G* i *sodA* pochodzących z regulonu SOS oraz szlaków bakteryjnej odpowiedzi stresowej w fuzji z genem reporterowym *gfp*. GFP-bakteryjny biosensor pozwolił na detekcję cyto- i genotoksycznej aktywności ibuprofenu, ketoprofenu i paracetamolu – konwencjonalnych niesterydowych leków przeciwzapalnych w buforze PBS oraz wodzie powierzchniowej. Leki stosowano w stężeniach 10^{-6}; 10^{-7}; 10^{-8}; 10^{-9} and 10^{-10} mg/dm^3, z czasem inkubacji bakterii 3 i 24 godziny. Wyniki eksperymentu wykazały zróżnicowaną wrażliwość trzech różnych konstrukcji genowych na badane leki. Szczepy bakterii oraz *RecA, kat G* i *sodA* promotory okazały się dobrymi bioindykatorami monitorowanego cyto- i genotoksycznego efektu testowanych leków w buforze PBS i wodzie powierzchniowej. Uzyskane rezultaty wskazują na potencjalną użyteczność stosowanych w pracy bakteryjnych biosensorów w monitorowaniu pozostałości farmaceutycznych leków w środowisku.

Słowa kluczowe: monitoring środowiskowy, niesterydowe przeciwzapalne leki, cytotoksyczność, genotoksyczność, *gfp* biosensory