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E. coli K-12 *recA::gfp* microbial biosensor used for screening of anticancer and antidiabetic pharmacist residues

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ABSTRACT

The autofluorescent reporter of *Escherichia coli* K-12 *recA::gfpmut2* strain containing a plasmid-borne transcriptional fusion between DNA-damage genotoxin-inducible *recA* promoter involved in the SOS regulon response and fast folding GFP (green fluorescent protein) variant reporter gene-*gfpmut2*, have been used. GFP-based bacterial biosensors allowed for detection of a cytotoxic and genotoxic anticancer drug—a cisplatin and antidiabetic drug—metformin in PBS buffer and surface water. Experimental data indicated that *recA::gfpmut2* genetic system was sensitive to applied drugs and the mixture of drugs. *RecA* promoter was a good bioindicator for cytotoxic and genotoxic effect screening of cisplatin, metformin, and the mixture of both drugs in PBS buffer and surface water. Stronger reactivity of *recA::gfpmut2* genetic system in surface water for the mixture of cisplatin (0.001 mg/ml) and metformin (0.3 mg/ml) treated samples and for prolonged incubation (up to 24 h) was obtained. The results showed that *E. coli* K-12 *recA::gfp mut2* strain could be potentially useful for first-step screening of cytotoxic and genotoxic effect of anticancer and antidiabetic pharmacist residues in water. The validation of *recA::gfpmut2* genetic system in *E. coli* K-12 demands further experimental analysis.

Keywords: SOS-*gfp* biosensor; Cytotoxicity; Genotoxicity; Cisplatin; Metformin

1. Introduction

Growing attention has been paid to study the occurrence, destiny, and potential risks of human pharmaceuticals (HPs) in the environment since their first detection in the aquatic environment in the 1970s. These compounds usually reach the environment in non-negligible amounts and because they are likely to exert biological and/or adverse effects on aquatic organisms. Thus, they can be regarded as environmental contaminants [1,2].

Several large-scale collaboration programs have been conducted in Europe and the United States, such as "Poseidon" (EU, 2001–2004), Norman (EU,

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2005–2008), PILLS (EU, 2007–2012) and Emerging Contaminants in the Environment (US Geological Survey, 2007–2011). The recent PHARMAS (EU, 2011–2013) and CytoThreat (EU, 2011–2013) projects have been specifically targeting the antibiotic and anticancer drugs. HPs and their metabolites were detected worldwide at ng/l to μg/l levels in surface water reservoirs in different areas [1–4]. In fact, HPs have been unrestrictedly discharged to the environment mainly via sewage treatment plants (STPs) for several decades.

It has been well recognized that conventional STPs carry some low removal content for many HPs as well as their metabolites excreted from human body after being administered in the hospitals. They are, therefore, continuously introduced into the environment, as the consumption of various HPs increases, as people live longer enjoying higher standards of living [1–8]. Household discharge by out-patients presents another pathway of cytostatics to the environment. Due to their highly potent mechanism of action (cytotoxicity, genotoxicity, mutagenicity, and teratogenicity), cytostatics could inflict adverse effects on any growing eukaryotic organism [1,9–15].

HPs' drugs used for cancer treatment—referred as anticancer or antineoplastic drugs—are suspected to represent a specific risk for aquatic non-target species [4]. Due to their intended function, the first ones (anticancer) could exert cytotoxic, genotoxic, mutagenic, carcinogenic, or teratogenic effects on aquatic species. However, due to their highly potent mechanism of action, this specific group of drugs is considered to be harmful to aquatic organisms and even human health [1,4,15–19].

cis-Diamminedichloroplatinum (II), commonly known in chemotherapy as cisplatin (CIS) is a major antineoplastic alkylating drug effectively used against various solid tumors such as ovarian cancer, non-small-cell lung carcinoma and head and neck cancer, both as a single agent or in the combination with other agents, radiotherapy and/or surgery [15–19].

The antidiabetic drug metformin is among the pharmaceuticals with the highest investigated numbers world-wide [20]. The measurements showed an almost ubiquitous presence of metformin in the aquatic environment—in sewage and surface waters. The significant reduction of metformin concentrations in sewage was mainly due to microbial degradation, but this drug was still found in all river waters. Concentration levels of metformin depend on the sewage fraction in the water and in most rivers are in the range of several to 100 ng/l [1], i.e. in the same order of magnitude (or even higher) than for other relevant pharmaceutical residues [20–24].

Nowadays, bacterial-based genotoxicity biosensor systems display an important role in the rapid, first-step biological screening and detection of DNA damaging chemicals, drugs and potential drug candidates [25–30]. In such living cell systems, bacteria are especially attractive due to their rapid growth rate, low cost, and easy handling [31–35].

GFP fluorescence-based bacterial biosensors (fusion of *gfp*-reporter gene under control of the SOS dependent *recA* promoter) can detect the genotoxic mode of action of certain chemicals and are simple to apply, sensitive, and easy to measure—in the concentration of GFP and as a consequence the fluorescence signal [36–39].

The SOS regulon with *recA* promoter, is one of the most thoroughly studied genotoxic stress regulons for bacteria [40,41]. The genotoxin-sensitive *recA* promoter transcription is induced upon DNA damage (genotoxic and mutagenic effect). The application of *recA* promoter in order to create some effective genotoxicity bacteria biosensors is connected with broad involvement of *recA* protein 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 mechanism called SOS mutagenesis [31,39,40].

The pharmaceutical residues of cisplatin and metformin have been detected worldwide at ng/l to μg/l levels in environmental samples (influents and effluents, surface water). Due to their highly potent mechanism of action, (they directly or indirectly interact with the structure and functions of DNA) these specific groups of drugs are considered to be hazardous to living organisms and human health. There is a need to target HPs with environmental significance, quantify them, and assess their cytotoxic and genotoxic risk to living organisms [1–5]. Thus, the present study analyzes the potency of *E. coli.* K-12 *recA::gfp* microbial biosensor strain for cytotoxic and genotoxic effect screening of two commonly prescribed HPs: cisplatin —an anticancer drug and metformin—antidiabetic drug.

2. Materials and methods

2.1. Chemicals

Cisplatin (0.0001–1 mg/ml) and metformin (0.3–1 mg/ml) 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 1,000 ml of distilled water, pH 7) at determined experimental concentrations before they were used.

2.2. Bacteria strain and plasmid

In the experiment *E. coli* K-12 MG1655 stationary phase cells: *E. coli* K-12 *recA*::*gfpmut2* and *E. coli* K-12 *promoterless*::*gfpmut2*, genetically modified were used. They contained a pUA66 plasmid-borne transcriptional fusion between DNA-damage inducible, oxidative stress *recA* promoter involved in the SOS regulon response and fast folding GFP variant reporter gene-*gfpmut2*. The genetic structure of pUA66 plasmid is described in the work of Zaslaver et al. [38]. 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 [38].

2.3. Bacteria growth condition

E. coli K-12 MG1655 strains: *E. coli* K-12 *recA*::*gfpmut2* and *E. coli* K-12 *promoterless*::*gfpmut2* were cultured overnight in LB agar medium (Merck, Germany) at 30˚C supplemented with 100 μg/ml of kanamycin (Sigma–Aldrich, Germany). Colonies were carried to LB broth medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per 1,000 ml of distilled water) with 100 μg/ml of kanamycin and incubated overnight at 30˚C. Afterward the cells were washed with PBS buffer.

2.4. Monitoring of bacteria growth and concentration

At the beginning of the experiment, the initial bacteria cells density was standardized to optical density (OD) value by using spectrophotometer (Perkin Elmer Enspire 2300) at the wavelength of 600 nm. The concentration of bacteria cells per ml of PBS was assessed by series dilutions system and expressed as CFU/ml values.

Dynamic growth of bacteria strains treated with cisplatin and metformin was monitored by the use of standard spectrophotometer analysis of OD values at the wavelength of 600 nm.

The values of bacteria growth inhibition (GI) during the treatment with drugs at the start of bacteria incubation with drugs (time 0 and after 3 and 24 h) was calculated according to the formula:

$$\mathrm{GI}\ (\%) = \mathrm{OD_{CS}}\ (\%) - \mathrm{D_{ODTS}}\ (\%)$$

where $\mathrm{OD_{CS}}$ (%), OD of control sample = 100%; D_{ODTS} (%), the decrease in the value of OD of bacteria samples treated with drugs.

2.5. Bacteria cells treatment with cisplatin and metformin in PBS buffer

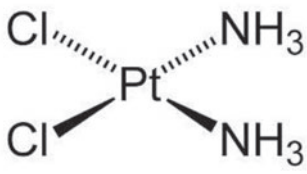
One milliliter of stationary phase bacteria cells (2×10^8 CFU/ml; OD = 0.2) were suspended in 4 ml of PBS buffer and the following drugs were used for genotoxicity testing: cisplatin (water solution of 5 mg/10 ml cisplatin) CIS, metformin (water solution of 800 mg of metformini hydrochloridum) (M) and cisplatin + metformin CIS + M in five different concentrations, for CIS: 0.0001; 0.001; 0.01; 0.1 and 1 mg/ml; for metformin: 0.3; 0.7 and 1 mg/ml and for CIS + metformin: (1) 0.0001 + 0.3; 0.001 + 0.3; 0.01 + 0.3; 0.1 + 0.3 and 1 + 0.3 mg/ml; (2) 0001 + 0.7; 0.001 + 0.7; 0.01 + 0.7; 0.1 + 0.7 and 1 + 0.7 mg/ml and (3) 0.0001 + 1; 0.001 + 1; 0.01 + 1; 0.1 + 1 and 1 + 1 mg/ml. The chemical structures of both drugs are presented in Fig. 1.

The concentration range of the drugs analyzed in research was selected experimentally from the minimum level of *recA::gfp* construct 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 and 24 h) was estimated for monitoring of sensitivity of *recA::gfp* genetic construct for quick (3 h) and later (24 h) response. The control sample—*E. coli* K-12 *recA*::*gfpmut2* strain in PBS buffer was not treated with drugs. For verification of the correct activity of *recA* promoter, *E. coli* K-12 strain containing pUA66 plasmid without the promoter—*E. 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 as genotoxin) as the positive control.

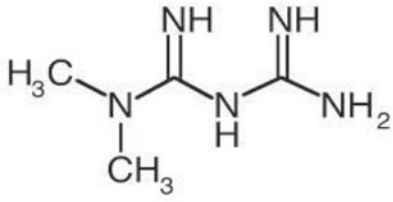
2.6. Bacteria cells treatment with cisplatin and metformin in surface water

Surface water samples were collected in sterile flasks from the Białka river. The samples were sterilized by filtration. 1 ml of stationary phase bacteria cells (2×10^8 CFU/ml; OD = 0.2) were suspended in 4 ml of surface water and the following drugs were used for genotoxicity testing: cisplatin (CIS), metformin (M), and cisplatin+metformin (CIS + M), for CIS at concentration of: 0.001 mg/ml; for metformin: 0.3; 0.7, and 1 mg/ml and for CIS + M: (1) 0.001 + 0.3; (2) 0.001 + 0.7, and (3) 0.001 + 1 mg/ml. Drug concentrations were selected for the highest stimulation of *gfp* gene expression in PBS buffer (for IF = 10.42).

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



Cisplatin



Metformin

Fig. 1. Chemical structure of cisplatin and metformin.

2.7. 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 1 ml of bacteria cells suspension (1×10^4 CFU/ml) in PBS buffer was measured with the spectrofluorometer (Perkin Elmer Enspire 2300). The measurements were done at excitation and emission wavelengths of 485 and 507 nm.

2.8. Assessment of SFI values

The specific fluorescence intensity (SFI) value, which is defined as the raw fluorescence intensity (IF) divided by the 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:.

$$\mathrm{SFI} = \frac{\mathrm{IF}}{\mathrm{OD}}$$

where SFI, specific fluorescence Intensity; IF, 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.

2.9. Detection of $S_{gfpexp.}$ value

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

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

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%.

2.10. Assessment of F_I values

For each concentration of tested drugs, the induction factors (F_I) were calculated. $F_I = (Fl_I/OD_0)/(Fl_0/OD_I)$, where Fl_I is the raw fluorescence of the culture treated with DNA-damaging compound; Fl_0 is the raw fluorescence of the control sample without genotoxin; OD_I is the optical density at 600 nm of treated culture and OD_0 is the optical density of the control sample.

The SFI, $S_{gfpexp.}$ and F_I, values express the potency of influence of both drugs on the sensitivity of oxidative stress *recA::gfp* construct.

2.11. Classification of tested drugs as genotoxins

The F_I values were calculated for classification of tested drugs as genotoxins. According to references Ptitsyn et al. [39] and Kostrzyńska et al. [31], a chemical was identified as a genotoxin if its induction factor was two or more ($F_I \geq 2$).

2.12. Statistical analysis

Statistical data obtained in this study are expressed as mean ± standard deviation (SD) for $n = 6$. 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.

3. Results

In the experiment, the positive fluorescence reactivity of *E. coli* K-12 *recA::gfpmut2* was obtained for each tested chemicals.

E. coli K-12 MG1655 *recA::gfpmut2* strain treatment with cisplatin and metformin showed that simultaneous administration of both drugs caused a

Table 1
SFI values for *E. coli* K-12 *recA::gfp mut2* treated with cisplatin (CIS), metformin (M), and combination of cisplatin and metformin (CIS + M) in PBS buffer in three different metformin concentrations (0.3; 0.7; 1 mg/ml) 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 CIS and CIS + M in comparison with the control sample (100%)

CIS (mg/ml)	M (mg/ml)	T	Control sample SFI±SD	M SFI ± SD	CIS SFI ± SD	F_I	$S_{gfpexp.}$ (%)	CIS+M SFI ± SD	F_I	$S_{gfpexp.}$ (%)
1	0.3	3	19.03 ± 1.36	16.11 ± 0.71^{a}	36.25 ± 1.93^{ab}	–	–	19.84 ± 1.82^{*bc}	–	–
		24	36.01 ± 2.46	32.08 ± 1.05^{a}	197.92 ± 5.15^{ab}	5.50	450	158.63 ± 4.13^{abc}	4.40	340
	0.7	3	19.03 ± 1.36	15.12 ± 2.01^{a}	36.25 ± 1.93^{ab}	–	–	32.0 ± 2.34^{abc}	–	–
		24	36.01 ± 2.46	29.87 ± 2.11^{a}	197.92 ± 5.15^{ab}	5.50	450	234.27 ± 8.61^{abc}	6.51	551
	1	3	19.03 ± 1.36	14.01 ± 1.72^{a}	36.25 ± 1.93^{ab}	–	–	19.08 ± 3.36^{**c}	–	–
		24	36.01 ± 2.46	26.15 ± 4.17^{a}	197.92 ± 5.15^{ab}	5.50	450	212.52 ± 4.42^{abc}	5.90	490
0.1	0.3	3	19.03 ± 1.36	16.11 ± 0.71^{a}	26.38 ± 1.52^{ab}	–	–	20.84 ± 2.45^{*bc}	–	–
		24	36.01 ± 2.46	32.08 ± 1.05^{a}	372.0 ± 7.86^{ab}	10.33	933	108.10 ± 2.60^{abc}	3	200
	0.7	3	19.03 ± 1.36	15.12 ± 2.01^{a}	26.38 ± 1.52^{ab}	–	–	16.80 ± 2.91^{**c}	–	–
		24	36.01 ± 2.46	29.87 ± 2.11^{a}	372.0 ± 7.86^{ab}	10.33	933	327.45 ± 7.31^{abc}	9.10	810
	1	3	19.03 ± 1.36	14.01 ± 1.72^{a}	26.38 ± 1.52^{ab}	–	–	9.64 ± 2.45^{a*c}	–	–
		24	36.01 ± 2.46	26.15 ± 4.17^{a}	372.0 ± 7.86^{ab}	10.33	933	225.50 ± 3.75^{abc}	6.26	526
0.01	0.3	3	19.03 ± 1.36	16.11 ± 0.71^{a}	25.30 ± 2.16^{ab}	–	–	21.30 ± 2.14^{*b*}	–	–
		24	36.01 ± 2.46	32.08 ± 1.05^{a}	243.3 ± 6.37^{ab}	6.76	576	214.64 ± 2.08^{**c}	6	500
	0.7	3	19.03 ± 1.36	15.12 ± 2.01^{a}	25.30 ± 2.16^{ab}	–	–	16.31 ± 2.70^{**c}	–	–
		24	36.01 ± 2.46	29.87 ± 2.11^{a}	243.3 ± 6.37^{ab}	6.76	576	237 ± 5.20^{ab*}	6.60	560
	1	3	19.03 ± 1.36	14.01 ± 1.72^{a}	25.30 ± 2.16^{ab}	–	–	15 ± 3.47^{**c}	–	–
		24	36.01 ± 2.46	26.15 ± 4.17^{a}	243.3 ± 6.37^{ab}	6.76	576	172.54 ± 7.72^{abc}	4.80	380
0.001	0.3	3	19.03 ± 1.36	16.11 ± 0.71^{a}	21.11 ± 2.64^{*b}	–	–	22.70 ± 2.10^{ab*}	–	–
		24	36.01 ± 2.46	32.08 ± 1.05^{a}	156.4 ± 2.10^{ab}	4.34	334	375.32 ± 3.06^{abc}	10.42	942
	0.7	3	19.03 ± 1.36	15.12 ± 2.01^{a}	21.11 ± 2.64^{*b}	–	–	18.72 ± 3.24***	–	–
		24	36.01 ± 2.46	29.87 ± 2.11^{a}	156.4 ± 2.10^{ab}	4.34	334	116.27 ± 6.37^{abc}	3.23	223
	1	3	19.03 ± 1.36	14.01 ± 1.72^{a}	21.11 ± 2.64^{*b}	–	–	9.0 ± 2.45^{abc}	–	–

(*Continued*)

Table 1 (*Continued*)

CIS (mg/ml)	M (mg/ml)	T	Control sample SFI±SD	M SFI ± SD	CIS SFI ± SD	F_I	$S_{gfpexp.}$ (%)	CIS+M SFI ± SD	F_I	$S_{gfpexp.}$ (%)
		24	36.01 ± 2.46	26.15 ± 4.17^{a}	156.4 ± 2.10^{ab}	4.34	334	59.27 ± 3.20^{abc}	–	–
0.0001	0.3	3	19.03 ± 1.36	16.11 ± 0.71^{a}	24.60 ± 2.74^{ab}	–	–	27.82 ± 1.45^{ab*}	–	–
		24	36.01 ± 2.46	32.08 ± 1.05^{a}	134.40 ± 5.67^{ab}	3.73	273	188.20 ± 3.0^{abc}	5.23	423
	0.7	3	19.03 ± 1.36	15.12 ± 2.01^{a}	24.60 ± 2.74^{ab}	–	–	14.64 ± 2.44^{a*c}	–	–
		24	36.01 ± 2.46	29.87 ± 2.11^{a}	134.40 ± 5.67^{ab}	3.73	273	133.72 ± 4.80^{a*c}	3.71	271
	1	3	19.03 ± 1.36	14.01 ± 1.72^{a}	24.60 ± 2.74^{ab}	–	–	14.14 ± 3.52^{a*c}	–	–
		24	36.01 ± 2.46	26.15 ± 4.17^{a}	134.40 ± 5.67^{ab}	3.73	273	50 ± 4.51^{abc}	–	–

Notes: Mean values ± SD; $n = 6$; a—significantly different from control ($p < 0.05$); b—significantly different from metformin (M) group ($p < 0.05$); c—significantly different from cisplatin (CIS) group ($p < 0.05$); *—no significantly different.

significant increase ($P \leq 0.05$) in SFI, F_I and $S_{gfpexp.}$ values compared to non-treated cells (Table 1).

Longer treatment with CIS and CIS + M (up to 24 h) intensified SFI, F_I, and $S_{gfpexp.}$ values. Bacteria cells incubation in PBS buffer under each concentration of metformin treatment caused a decrease in SFI values compared to the control sample. A sustained decrease in SFI values was observed after 24 h.

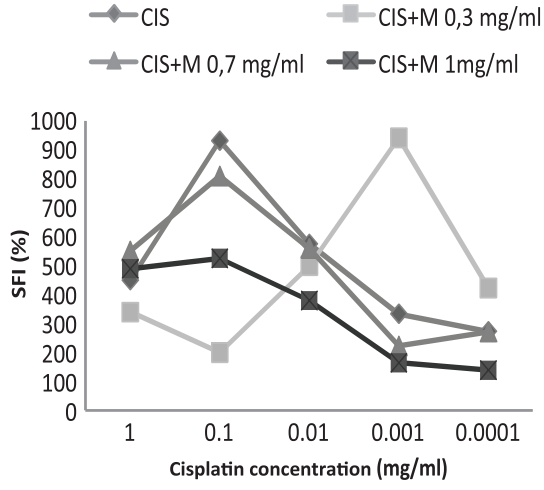


Fig. 2. The dynamic of the SFI values for *E. coli* K-12 *recA::gfp mut2* treated with CIS and CIS with three concentrations of metformin (CIS + M) after 24 hours incubation with drugs compared to control sample. SFI values are expressed as the percent of *gfp* gene IF stimulation of bacteria cells normalized by dividing by OD value. Mean values ± SD; $n = 6$.

Fig. 2 shows the dynamic of the SFI values for *E. coli* K-12 *recA::gfp mut2* treated with CIS and CIS with combination with three concentrations of metformin (CIS + M) after 24 h incubation with drugs compared to the control sample not-treated with drugs.

Bacteria treatment with cisplatin resulted in a progressive significant stimulation of SFI values for 1; 0.1; 0.01 and 0.0001 mg/ml for 3 and 24 h incubation compared to the control sample and metformin treated cells. For 0.001 mg/ml, a significant increase for SFI values was observed for 24 h. The maximum point for SFI value ($S_{gfpexp.} = 933\%$) was for cisplatin in the concentration of 0.1 mg/ml and 24 h of incubation time.

Bacteria cells co-administrated simultaneously with cisplatin and metformin exerted some influence on SFI and the parameters with the maximum point for SFI ($S_{gfpexp.} = 942\%$) were for 0.001 mg/ml of CIS and 0.3 mg/ml of M after 24 h incubation with both drugs. Metformin almost at each concentration significantly modulated (in 86.67% of cases) cisplatin activity. It is seen, mainly at the 1 mg/ml concentration of M and after 24 h incubation. Only in four cases, (in 13.33%) no significant differences in SFI between CIS and CIS + M were observed. The most frequent inhibition of SFI of cisplatin was detected after metformin administration at the concentration of 0.7 and 1 mg/ml (Fig. 2).

Simultaneous treatment of bacteria cells with cisplatin and metformin (at the lowest concentration 0.3 mg/ml of metformin) compared to bacteria treated only with cisplatin significantly inhibited the SFI value of cisplatin at 0.1 mg/ml concentration and significantly stimulated at CIS concentration of 0.001 mg/ml.

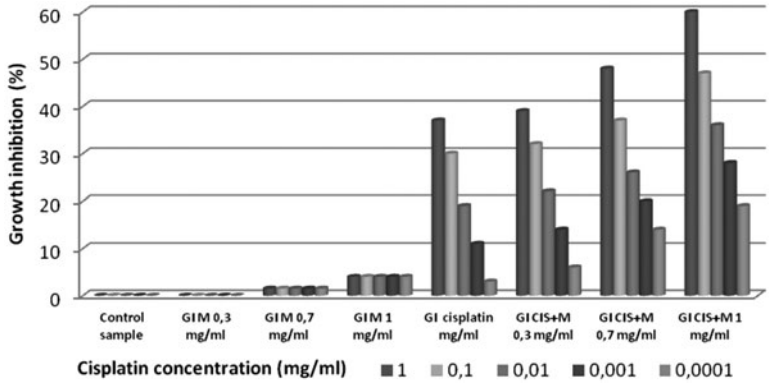


Fig. 3. The comparison of GI values of *E. coli* K-12 *recA::gfp* cells after 24 h of incubation with cisplatin, metformin, and simultaneous incubation with cisplatin and metformin compared to control sample not-treated with drugs. Mean values ± SD; $n = 6$.

F_I values ≥ 2 were obtained for all cisplatin concentrations after 24 h incubation. For co-administration of CIS + M for the combination of CIS (0.001 mg/ml) + M (1 mg/ml) and CIS (0.001 mg/ml) + M (1 mg/ml), the F_I values were below 2. $F_I \geq 2$ for metformin co-administrated with cisplatin in 86.67% of cases enhanced inhibition of SFI and decreased F_I values were observed as compared to the bacteria cells treated only with cisplatin. Bacteria cells incubated 24 h with the combination of CIS (at the concentration of 0.001 and 0.0001 mg/ml) with 1 mg/ml of metformin resulted in a progressive decrease in F_I (below 2) and SFI values. For all applied concentrations of cisplatin, there were no linear correlations between SFI values and CIS concentration. In the case of CIS, CIS + M (0.7 mg/ml), and CIS + M (1 mg/ml) the highest stimulation of SFI value was observed for 0.1 mg/ml of CIS and minimum for 0.001 and 0.0001 mg/ml. A different reaction was noticed for CIS + M (0.3 mg/ml), where the strongest inhibition of SFI value was registered for 0.1 mg/ml of CIS and the highest stimulation of SFI for 0.001 mg/ml of CIS.

Table 2
SFI values for *E. coli* K-12 *recA::gfp mut2* treated with cisplatin (CIS), metformin (M), and combination of cisplatin (0.001 mg/ml) and metformin (CIS + M) in surface water in three different metformin concentrations (0.3; 0.7; 1 mg/ml) 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 CIS and CIS + M in comparison with the control sample (100%)

CIS (mg/ml)	M (mg/ml)	T	Control sample SFI ± SD	M SFI ± SD	CIS SFI ± SD	F_I	$S_{gfpexp.}$ (%)	CIS + M SFI ± SD	F_I	$S_{gfpexp.}$ (%)
0.001	0.3	3	20.31 ± 2.13	18.25 ± 1.83^{a}	23.15 ± 2.64^{*b}	–	–	30.85 ± 3.20^{ab*}	1.52	52
		24	40.44 ± 3.55	35.44 ± 2.65^{a}	166.3 ± 5.10^{ab}	4.14	311	450.43 ± 6.23^{abcA}	11.13	1013
	0.7	3	20.31 ± 2.13	17.32 ± 2.33^{a}	23.15 ± 2.64^{*b}	–	–	22.62 ± 3.54***	–	–
		24	40.44 ± 3.55	32.91 ± 3.12^{a}	166.3 ± 5.10^{ab}	4.11	311	125.32 ± 7.23^{abc}	3.09	209
	1	3	20.31 ± 2.13	16.31 ± 2.82^{a}	23.15 ± 2.64^{*b}	–	–	14.32 ± 2.85^{abc}	–	–
		24	40.44 ± 3.55	29.33 ± 3.67^{a}	166.3 ± 5.10^{ab}	4.11	311	65.55 ± 4.15^{abc}	1.62	62

Notes: Mean values ± SD; $n = 6$; a—significantly different from control ($p < 0.05$); b—significantly different from metformin (M) group ($p < 0.05$); c—significantly different from cisplatin (CIS) group ($p < 0.05$); A—significantly different from cisplatin + metformin (CIS + M) group in PBS buffer ($p < 0.05$); *—no significantly different.

The monitoring of bacteria cultures growth OD at the start of bacteria treatment (time 0) and after 3 and 24 h of incubation with drugs indicated a significant increase in GI values for all tested concentrations of metformin and cisplatin for 24 h treatment. Addition of metformin to cisplatin and simultaneous action of both drugs on bacteria cells significantly enhanced the GI values for 24 h incubation (Fig. 3).

There were no statistical differences in the case of a shorter time (3 h) of drug influence on bacteria cells.

Prolonged treatment (up to 24 h) of bacteria cells with metformin at the concentrations of 0.7 and 1 mg/ml significantly influenced the GI of bacteria. For 3 h of incubation, there were no significant changes in OD values.

Bacteria incubation with PBS buffer (the control sample) without any drug addition resulted in no statistical differences in OD value from 0 to 24 h continuous cultivation.

Treatment of *gfp* biosensor bacteria strain in surface water enhanced the sensitivity of *recA::gfpmut2* genotoxic system and increased the stimulation of *gfp* expression and SFI value in comparison to incubation in PBS buffer (Table 2). Prolonged treatment (up to 24 h) of bacteria cells with the combination of cisplatin (0.001 mg/ml) and metformin (0.3 mg/ml) significantly influenced *gfp* expression with the maximum values for IF = 11.13 and 1,013% of $S_{gfpexp.}$ values comparable to the control sample.

In this experiment, for assessment of genotoxic sentivity of a *recA::gfp* genetic biosensing system, 4% acetone was tested as the negative control. In the case of this chemical, there was no increase in F_I values for 3 and 24 h of incubation. Methylnitronitrosoguanidine (MNNG)—known as 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 3 h (data not shown). These results proved stronger sensitivity of a *recA::gfp* biosensing system for MNNG than for an acetone stressor.

4. Discussion

Various drugs are capable of damaging DNA and triggering the genotoxic and mutagenic impact on the living cells. If not repaired, or if produced in excessive amounts, damaged DNA can initiate a cascade of biological cellular, organic, or individual effects which is as a consequence carcinogenesis [20]. The results of this study indicate that treatment of bacteria cells with cisplatin and the mixture of cisplatin and metformin lead to over 10-fold stimulation ($F_I = 10.33$ in the case of cisplatin and $F_I = 10.42$ in the case of simultaneous coadministration of cisplatin and metformin) of bacteria genotoxin-sensitive *recA* promoter and *gfp* gene expression. The variable levels of efficiency of *gfp* expression are bioindicators of cisplatin genotoxic effect generation potency. Present study with the use of *E. coli* K-12 with a genetic construct *recA::gfp* indicate cisplatin and metformin dose- and time-dependent ability to react with *recA* promoter.

The results obtained in the experiment are in agreement with the studies of Kostrzyńska et al. [31], Norman et al. [29], Ptitsyn et al. [39] and some others, who presented data, that reporter gene systems (with *gfp* and *lux* reporters) were sensitive and useful for measurement of genotoxins, drugs, and various chemicals in environmental studies [26–34,40].

Previous studies showed that *recA* promoter was induced by selected anticancer drugs [30,31]. In living cells during metabolism of cisplatin—an alkylating drug increased ROS and it can lead to oxidative stress formation that modulates different cellular processes (46–49). Consequently, oxidative stress generation is one of the most important mechanisms involved in cisplatin induced cytotoxicity, very dangerous to normal, healthy cells during patients' chemotherapy [15,16,18,19].

According to the results obtained in this experiment, metformin together with cisplatin modulate and decrease the reactivity of *recA*-oxidative stress promoter in relation to cisplatin in *E. coli* K-12 *recA::gfp mut2* living bacteria cells.

The mechanisms of metformin on DNA molecules is still unknown. Results, from *in vivo* and *in vitro* studies which have been undertaken remain controversial. While some reports indicated no genotoxic effects [42], some others [43] assumed that metformin could produce oxidative stress due to DNA fragmentation. The results of the above experiment provided the conformation of the possible influence of metformin on the genes, similarly as Anedda et al. [23] and Amador et al. [24]. In 100% of the cases, there were significant differences (comparable to the control sample) regarding the level of *recA* promoter sensitivity and *gfp* expression after bacteria treatment with all applied concentrations of metformin and for both short (3 h) and longer time of incubation (up to 24 h).

Kefas et al. [44] studies indicated that the activity of metformin is dose- and time-dependent. It was also confirmed by data obtained in our studies. Longer metformin exposure (up to 24 h) resulted in a progressive inhibition of cisplatin influence on *recA* promoter and *gfp* gene expression. F_I values ≥ 2 were obtained for CIS + M after 24 h incubation, but not for 3 h. Generally, the strongest inhibition of *gfp* expression by cisplatin was noticed when 1 mg/ml of metformin was

added rather than in the case of lower concentrations of an antidiabetic drug. The highest concentrations of cisplatin (1; 0.1 mg/ml) and metformin (1 mg/ml) inhibited SFI values. It suggests the possible repression of transcription of *recA::gfp* genetic construct by higher concentration of the drugs.

According to some authors, the mechanisms of metformin effects on DNA molecules might possibly be mediated through its activation of AMPK, thereby increasing nitric oxide synthase [45]. Zou et al. [46] speculated that mitochondria-derived reactive-nitrogen-species mediate AMPK activation due to metformin. Depending on the dose, nitric oxide is capable of inducing beneficial effects by playing a role in the gene regulation and signal transduction pathways possibly involved in defensive mechanisms against the oxidative stress [46].

In the light of earlier reports and results of above experiment, during metformin metabolism in living cells, direct or indirect protective mechanisms against cisplatin-induced oxidative stress may occur.

Our results showed cisplatin *E. coli* K-12 longer (up to 24 h) treatment significantly inhibited bacteria cells growth. Either metformin has cytotoxic effect by inhibition of bacteria cells growth for the highest applied concentrations and 24 h treatment. Co-administration of metformin with cisplatin importantly, dose-dependently intensified cisplatin cytotoxic effect on living bacteria cells after 24 h incubation. Our results are in agreement with earlier empirical studies of other authors who demonstrated that co-administration of metformin with chemotherapeutic agents intensified the inhibition of cancer cells proliferation and significantly improved cisplatin-induced cytotoxicity [21,23,24]. Janjetovic et al. [43] have assumed that metformin can produce oxidative stress due to DNA fragmentation. DNA damage can initiate a cascade of cellular biological effects including cell death. The direct and indirect metformin influence on DNA could be the main mechanisms of its cyto- and genotoxicity.

We obtained stronger reactivity of *recA::gfpmut2* genetic system in surface water for drug treated samples. It was possibly due to the presence of different chemicals in surface water which could influence *gfp* expression in bacteria strain. 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 toxicity of cisplatin and metformin is well-known and established in experimental

studies on bacteria, human cells, and other organisms. In the above experiment, we applied *E. coli* K-12 bacteria cells 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.

5. Conclusions

(1) The results of the presented study indicated that *recA::gfpmut2* genetic system was sensitive to a particular drug and the mixture of drugs applied in the experiment.
(2) *RecA* promoter was a good bioindicator for cytotoxic and genotoxic effect screening of cisplatin, metformin, and the mixture of both drugs in PBS buffer and surface water.
(3) The results indicated that *E. coli* K-12 *recA::gfp mut2* strain could be potentially useful first-step screening of cytotoxic and genotoxic effect of anticancer and antidiabetic pharmacist residues in water.
(4) The validation of *recA::gfpmut2* genetic system in *E. coli* K-12 demands more experimental analysis, which should be focused on the spectrofluorometry fluorescence screening of *recA::gfpmut2* sensitivity after treatment of bacteria cells with anticancer drugs (or drugs candidate) belonging to different groups of its biological mechanisms activity.

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