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Escherichia Coli-Lux Biosensor Used to Monitor the Cytotoxicity and Genotoxicity of Pharmacological Residues in Environment

Eliza Hawrylik[1*], Marzena Matejczyk[1]

[1] Department of Chemistry, Biology and Biotechnology, Faculty of Civil and Environmental Engineering, Bialystok University of Technology, Wiejska 45E, 15-351 Bialystok, Poland

[*] Corresponding author's e-mail: e.hawrylik@doktoranci.pb.edu.pl

ABSTRACT

The aim of the study was to evaluate the usefulness of *Escherichia coli* K-12 RFM 443 *recA::lux* for cytotoxicity and genotoxicity monitoring of metoprolol in the environment. Metoprolol is one of the most popular cardiac drug which belongs to the group of β-blockers. The drug was applied at the concentrations ranging from 0.01 μg/cm^3 to 100 μg/cm^3. The conducted research constitutes preliminary study aimed at validation of the *recA::lux* gene construct in order to determine its sensitivity to metoprolol. The drug concentrations were selected experimentally to obtain a positive luminescence response. The obtained data indicated the influence of metoprolol on *lux* gene expression and *recA* promoter activity based on the application of laboratory samples using PBS buffer. The results indicate a potential for using a bacterial biosensor *Escherichia coli* K-12 RFM 443 with *recA::lux* gene fusion in cytotoxicity and genotoxicity monitoring of the cardiac drugs residue in the environment.

Keywords: bacterial biosensors, reporter gene *lux*, β-blockers, cytotoxicity, genotoxicity

INTRODUCTION

The progressing civilization development as well as newer environmental threats, affect the growing scale of the population's morbidity. The obvious consequence of this situation is the increasing consumption of medicines [Szymonik and Lach 2012]. It is estimated, that nearly 30% of Poles suffer from hypertension, and less than 10% is affected by coronary heart disease [KPMG 2012]. Therefore, one of the most commonly used pharmaceuticals currently includes β-blockers. The medical readings for the use of β-blockers are: arterial hypertension, coronary heart disease, congestive heart failure and cardiac arrhythmia, and one of the most commonly used β-blockers is metoprolol potassium tartrate [Group work 2005]. Administration of metoprolol results in a reduced effect of catecholamines released during physical exertion or stress, which decreases the frequency of heart rhythm and its minute capacity, and as a result, there is a significant reduction in the blood pressure. Chemically, metoprolol tartrate is described by the molecular formula: $C_{15}H_{25}NO_3$, and its molecular weight amounts to 267.364 g/mol [Caban et al. 2012].

β-blockers, apart from a wide range of applications, are also characterized by incomplete metabolism; therefore, the efficiency of their removal in wastewater treatment plants is low. Research shows that it is in the case of β-blockers that one of the weakest purification effects is obtained at the level of 45–47%, despite the fact that according to the literature the maximum percentage of purification for metoprolol is 83% [Szymonik and Lach 2012]. The residues of β-blockers are therefore widely recognized in environmental samples. The conducted analyses indicate that metoprolol is one from the most often detected β-blockers in the aquatic environment [Nikolaou et al. 2007, Szymonik and Lach 2012], and its concentration in surface waters of Poland ranges from 0.05 to 0.15 μg/dm^3, while the maximum dose taken with long-term treatment of patients is 190 mg/day. Although the concentrations of metoprolol are not high, they pose a real threat

to the aquatic ecosystem and human health [Januszewicz 2011, Caban et al. 2012, Czech 2012]. This fact is confirmed by model QSAR studies (Quantitative Structure – Activity Relationship models), which show the toxicological effect of pharmaceuticals on some organisms (including *Daphnia magna*, *Synechococcus leopoliensis*) in the concentrations determined in the environment. In addition, it should be emphasized that drug metabolites and their degradation products, generated in the wastewater treatment processes or during the treatment of drinking water, can potentially increase their toxicological properties [Escher et al. 2006, Escher and Fenner 2011].

The microbiological biosensors widely used in recent years can be applied with the full success for pricing the stair environmental pollutions with cardiological medicines and for the evaluation of their ecotoxicological risk. The technology of microbiological biosensors allows for a quick analysis of the biological activity of chemical compounds and drugs *in vivo* without disturbing the function of a cell. One of the most popular types of bacterial biosensors includes the systems with the fusion of bacterial SOS regulon (e.g. the *recA* promoter) with the reporter gene *gfp* or *lux*. Activation of the SOS regulation, which is a reaction to the DNA damage, finds its application in the measurement of cytotoxic and genotoxic effects of many chemical factors, such as antibiotics, chemotherapeutics, anti-cancer drugs and others on living cells [Matejczyk and Biedrzycka 2014].

The aim of this study was to evaluate the sensitivity and usefulness of *recA::luxCDABE* gene construct in *E. coli* K-12 RFM 443 as a bacterial biosensor for monitoring the metoprolol pharmaceutical residues in the environment.

MATERIALS AND METHODS

Chemicals and bacterial strains

Metoprolol was commercially obtained from a Bialystok pharmacy. This drug was dissolved in PBS buffer (1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 0.2gKCl, 8 g NaCl per 1000 cm^3 of distilled water, pH 7) at the determined experimental concentrations before their use. The research has been subjected to five concentrations of the pharmaceutical, ranging from 0.01 µg/cm^3 to 100 µg/cm^3. The conducted research constitutes preliminary study aimed at validation of the *recA::lux* gene construct in order to determine its sensitivity to metoprolol. The drug concentrations were selected experimentally to obtain a positive luminescence response. In the next stages of the experiment, we are planning to expand the range of concentrations of the studied drug and set the lowest level of detection for this gene construct. We will also check the reactivity of this genetic system to the metoprolol concentrations found in the environment.

The model organism for the cyto- and genotoxicity study were strains: *Escherichia coli* K-12 *recA::lux* and *Escherichia coli* K-12 *promoterless::DH5α/pBR2TTS-pless.* Biosensor RFM/*recA::lux* belongs to whole bacterial biosensors; the strains *Escherichia coli* K–12 RFM 443 with the genotype (*F-galK2lac74rpsL200*) were used for their construction. *Escherichia coli* RFM 443 contained plasmid transcriptional fusion between *recA* promoter which belongs to DNA damage genotoxin in ducible group of bacteria promoters, involved in the SOS regulon response. Bioluminescent bacterial sensor is based upon the fusion of bacterial bioluminescence gene – *luxCDABE* acting as a reporter element [Yagur-Kroll et al. 2010, Yagur-Kroll and Belkin 2011].

Bacteria growth condition

Escherichia coli K-12 RFM 443 strains from cultures on solid medium were transferred to LB agar medium and incubated at 30°C with 100 µg/cm^3 of ampicillin. The antibiotic was used to ensure selectivity of the medium used for incubation. Next, the colonies were carried to LB broth medium with the addition of the antibiotic and incubated for 24 hours at 37°C. After the incubation time, the cells were washed with PBS buffer.

Monitoring of bacteria growth and concentration

At the beginning of the experiment, the initial bacteria cells density standardized to OD = 0.2 (Optical Density) value by using a spectrophotometer at the wavelength of 600 nm. The concentration of bacteria cells was assessed by series dilutions system and expressed as CFU (colony forming units)/cm^3 values. Dynamic growth of the bacterial strains treated with metoprolol was monitored by the use of standard spectrophotometer analysis of Optical Density values at the wavelength of 600 nm. The values of bacteria growth stimulation (GS) were calculated according to the formula:

$$GS(\%) = ODDS(\%) - ODCS(\%)$$

where: *ODDS (%)* – Optical Density of bacteria samples treated with metoprolol
ODCS (%) – Optical Density of control sample = 100%.

Cyto- and genotoxicity testing of metoprolol

For cyto- and genotoxicity testing, an aliquot of 1 cm^3 bacteria cells (1×10^8 CFU/cm^3; OD = 0.2) was employed, which was suspended in 4 cm^3 of PBS buffer and the metoprolol at concentrations from 0.01 µg/cm^3 to 100 µg/cm^3. The experiment with the use of *Escherichia coli* K-12 *recA::lux* strains was carried out for a total of 24 hours. The cytotoxic and genotoxic effects of the medicine influence with reference to *E. coli* were recorded after 3 and 24 hours (to determine the sensitivity of the analyzed gene construct in rapid and late cellular response). *Escherichia coli* K-12 *promoter less::DH5α/pBR2TTS-pless* was used as the control for verification of the correct activity of *recA* promoter. The control sample was not treated with chemicals. Eight repetitions of each treatment were performed.

Analytical method for the intensity of lux gene luminescence analysis

After exposure of bacteria cultures to the tested chemicals, the intensity of luminescence of *lux* gene was measured in the spectrofluorometer Glomax®, Multi Detection System, Promega. The measurements were performed at excitation and emission wavelengths, 478 and 505 nm, respectively.

Assessment of RLU values and the percentage inhibition of lux gene expression (Iluexp.) value

The Relative Luminescence Units (RLU) value measured during the experiment is defined as culture luminescence intensity divided by optical density (OD) at 600 nm for cell culture. RLU was calculated according to the formula:

$$RLU = \frac{Luminescence}{OD}$$

where: *RLU* – Relative Luminescence Units,
Luminescence – The raw fluorescence intensity of the strains at excitation and emission wavelengths of 478 and 505 nm,
OD – Optical Density at 600 nm.

For each of the analyzed concentrations of the pharmaceutical, the inhibition of the *lux* gene expression level was calculated according to the formula:

$$Iluxexp.(\%) = RLUCS\ (\%) - RLUDS\ (\%)$$

where: *RLUCS (%)* – RLU for the control sample = 100%,
RLUDS (%) – RLU values for tested compounds as a response to the level of *lux* inhibition in comparison with the control sample.

Assessment of F_I values

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

$$F_I = \frac{Fl_I/OD_0}{Fl_0/OD_I}$$

where: Fl_I – raw fluorescence of the culture treated metoprolol,
Fl_0 – raw fluorescence of the control sample without drugs,
OD_I – optical density at 600 nm of treated culture,
OD_0 – optical density of the control sample.

Statistical analysis

The data were analyzed using standard statistical analyses, including Student's test for multiple comparisons. The data obtained from eight measurements (n = 8) are expressed as a mean ± standard deviation (SD). P-values less than 0.05 were considered significant.

RESULTS

Escherichia coli K-12 RFM 443 *recA::lux* strains treatment with metoprolol showed a dose- and time-dependent increase in OD value, increased expression of *recA* promoter activity and intensified Iluexp. value compared to the control sample (Table 1). A significant increase in RLU values (above 30%) was observed after 3 hours of bacteria cells incubation, at the concentrations of 0.01 µg/cm^3, 0.1 µg/cm^3 and 10 µg/cm^3.

Expression of RLU values was obtained for almost whole applied concentration of metoprolol, comparing to the control sample. The maxi-

Table 1. OD and RLU values for *E. coli* K-12 RFM 443 *recA::lux* treated with metoprolol in comparison with the control sample

C (μg/cm^3)	T (h)	OD±SD	GS (%)	RLU±SD	Ilu exp. (%)
Control sample	3	0.150 ± 0.022	-	167920.48 ± 41271.83	-
	24	0.143 ± 0.016	-	20776.02 ± 7387.80	-
100	3	0.180 ± 0.019^{a}	19.78	211817.03 ± 38600.03^{a}	26.14
	24	0.214 ± 0.026^{abe}	49.23	24990.37 ± 10061.85	20.28
10	3	0.176 ± 0.019	17.62	233378.45 ± 24896.80^{d}	38.98
	24	0.187 ± 0,021^{b}	30.48	25787.45 ± 9162.70^{d}	24.12
1	3	0.187 ± 0.004^{c}	24.81	198599.91 ± 26338.09^{bc}	18.27
	24	0.204 ± 0.053^{b}	42.25	16763.29 ± 6749.62^{bc}	-19.31
0.1	3	0.175 ± 0.016	16.54	230478.08 ± 36002.43	37.25
	24	0.184 ± 0.018^{b}	28.2	26467.74 ± 10179.77	27.40
0.01	3	0.179 ± 0.013	19.37	230109.46 ± 26815.55	37.03
	24	0.157 ± 0.009	9.28	27427.74 ± 7501.77	32.02

T – time of bacteria strain incubation with drugs, GS (%) – the percent of bacteria growth stimulation after treatment of metoprolol with the control sample (100%), Iluexp.(%) – the percent of stimulation of *lux* expression after treatment of bacteria cells with drug in comparison with the control sample (100%)
Mean values ±SD; n=8; a – significantly different from control ($p<0.05$); b – significant difference from concentration 0.01 μg/cm^3, ($p<0.05$); c – significant difference from concentration 0.1 μg/cm^3, ($p<0.05$); d – significant difference from concentration 1 μg/cm^3, ($p<0.05$); e – significant difference from concentration 10 μg/cm^3, ($p<0.05$).

mum point for *recA* promoter expression was observed for the 10 μg/cm^3 concentration of drugs, after 3 hours of incubation. For the concentration of metoprolol equal to 1 μg/cm^3, the inhibition of the *lux* gene (24-hour incubation) was observed. The 3-hour incubation at all applied concentrations showed a more intensive effect of *lux* expression than with 24 hours of incubation. A statistically significant increase in RLU values is shown below (Table 2).

The monitoring of bacteria cultures growth expressed as OD indicated an increase in GS (growth stimulation) values for whole applied concentration of metoprolol, comparing to the control sample. A significant increase in OD values (above 30%) was observed after 24 hours of incubation of bacteria cells, at the concentrations of 100 μg/cm^3, 10 μg/cm^3 and 1 μg/cm^3. Statistically significant increase in the OD values is shown below (Table 3).

F_I values for all tested concentrations of metoprolol were calculated for evaluation of genotoxic sensitivity of *E. coli* K-12 RFM 443 *recA::lux*. According to references, the chemical was identified as a genotoxin if its induction factor was two or more (FI≥2). During 3 hrs and 24 hrs of incubation, Metoprolol was used as a positive control. For concentration of drugs equal to 100 μg/cm^3, 10 μg/cm^3, 0.1 μg/cm^3 during 24-hrs incubation and 0.01 μg/cm^3 during 3-hrs incubation F_I (induction factor) values above 2 were obtained. These results showed the sensitivity of *E. coli* K-12 RFM 443 *recA::lux* biosensing system for the tested β-blocker.

DISCUSSION

Many drugs are capable of damaging DNA and triggering the genotoxic impact on the living cells. Microbiology biosensors based on fusing of the *lux* or *gfp* gene reporter have been widely applied to assay the cytotoxicity and genotoxicity of chemical compounds and drugs and they have been investigated by numerous authors [Ptitsyn et al. 1997, Kostrzyńska et al. 2002, Zaslaver et al., 2004, Matejczyk 2010, Alhandrami and Paton 2013, Park et al., 2013, Quinn et. al. 2013]. The obtained results indicate that the treatment of bacteria cells with metoprolol causes genotoxin-sensitivity (F_I – induction factor – above 2) and *lux* gene expression.

The authors of the tested gene construct, i.e. Dr Sharon Yagur-Kroll and prof. Shimshon Belkin, conducted numerous analyses using bacterial sensors capable of detecting the cytotoxicity or genotoxicity of specific compounds. They have examined, among others, the luminescence intensity issued by strains containing the *lux* reporter gene in combination with the *sulA or recA* promoters. These promoters respond to SOS-inducing genotoxicants such as nalidixic acid (NA)

Table 2. Statistically significant increase in RLU values

Statistically significant increase in RLU values was observed in relation to the following concentrations:		
After 3 hours of incubation:		
100 µg/cm³	to	Control sample
10 µg/cm³		1 µg/cm³
1 µg/cm³		0.1 µg/cm³
1 µg/cm³		0.01 µg/cm³
After 24 hours of incubation:		
10 µg/cm³	to	1 µg/cm³
1 µg/cm³		0.1 µg/cm³
1 µg/cm³		0.01 µg/cm³

Table 3. Statistically significant increase in OD values

Statistically significant increase in OD values was observed in relation to the following concentrations:		
After 3 hours of incubation:		
100 µg/cm³	to	Control sample
1 µg/cm³		0.1 µg/cm³
After 24 hours of incubation:		
100 µg/cm³	to	Control sample
100 µg/cm³		10 µg/cm³
100 µg/cm³		0.01 µg/cm³
10 µg/cm³		0.01 µg/cm³
1 µg/cm³		0.01 µg/cm³
0.1 µg/cm³		0.01 µg/cm³

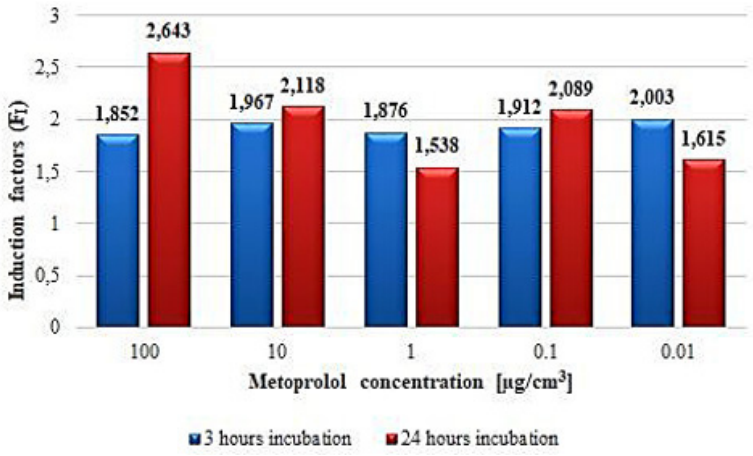

Figure 1. The induction factors (F_I) of bacterial culture *E. coli recA::lux* after 3 and 24 hours treatment of metoprolol

used in research [Yagur-Kroll et al. 2010]. In another work, Yagur-Kroll and Belkin [2011] show that bacterial biosensors with *lux CDABE* genes can be split into two smaller functional units: *luxAB*, that encodes for the luciferase enzyme, which catalyzes the luminescence reaction, and *lux CDE* that encodes for the enzymatic complex responsible for synthesis of the reaction's substrate. Results indicate that the split combinations proved to be superior to the *lux CDABE* configuration and demonstrate that an improved biosensor performance may be achieved by rearrangement of the *lux* operon genes.

Other researchers also dealt with the practical use of bacterial biosensors. Matejczyk et al. [2014] and Matejczyk and Rosochacki [2015] examined the cytotoxic and genotoxic properties of anticancer drugs with the use of *E. coli* K-12 *recA::gfpmut2* biosensor strain. Those studies indicated that the activity of anticancer drugs

were dose- and time-dependent. In another work, Matejczyk et al. [2015] showed the cytotoxic and genotoxic sensitivity of bacterial biosensors using the *recA* promoter to antibiotics.

The results obtained in this work show the possibility of using *E. coli* bacterial strains with *recA* promoter for cyto- and genotoxicity studies of another group of drugs, i.e. cardiac drugs.

According to the results obtained in this experiment, a β-blocker – metoprolol, modulated the reactivity of genotoxin sensitive promoters of *recA* in relation to the control sample. The results of the above-mentioned experiment provided the confirmation of the possible influence of this drug on the genes expression, as it was shown by Hawrylik and Matejczyk [2017]. In both studies, there were significant differences (for *recA* and *katG* promoter of the cases comparable to the control sample) regarding the level of promoters sensitivity and *lux* expression after bacteria treatment with all applied concentrations of metoprolol for short (3 hrs) and longtime of incubation (24 hrs).

The strongest reactivity of metoprolol at the concentrations of 10 μg/cm^3, 0.1 μg/cm^3 and 0.01 μg/cm^3 in RLU value were obtained in *E. coli recA::lux* bacterial strains. In the case of strains *E. coli katG::lux*, the highest RLU values have been achieved for metoprolol concentrations equal to 100 μg/cm^3 and 10 μg/cm^3 [Hawrylik and Matejczyk 2017].

These differences illustrate that the same drug may have different effects on bacterial strains containing various promoters (*recA* and *katG*); however, both are useful for susceptibility testing of pharmacological residues found in the environment.

CONCLUSIONS

1. Results of the present study indicated that *recA::lux* genetic system was sensitive to metoprolol applied in the experiment.
2. Metoprolol, in all tested concentrations, as well as in variable incubation time (3 and 24 hours), is a modulator of expression of the *lux* gene in *E. coli recA::lux*.
3. The results obtained in this study indicate a potential possibility of using a bacterial biosensor *Escherichia coli* K-12 RFM 443 with *recA::lux* gene fusion in cytotoxicity and genotoxicity monitoring of the pharmacological residues in the environment.

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