

Overview of 17,856 Compound Screening for Translation Inhibition and DNA Damage in Bacteria

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Abstract Screening for new antibacterial compounds is an urgent need of medicinal chemistry. Understanding new antibiotics mechanism of action is needed for progression in the drug development pipeline. In the frame of the project supported by the Ministry of Science, we developed a reporter system which allows

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an express, cost-effective and high-throughput screening for simultaneous detection of antibacterial activity, protein synthesis inhibition and induction of DNA damage SOS response. Automation of the screening process developed in the frame of this project allowed to screen up to 17,856 compound chemical library, supplied by the industrial partner of the project, Research Institute of Chemical Diversity. Among the tested compounds, DNA damaging agents appeared almost sixfold more frequently than those that inhibited protein synthesis. Several new families of antibacterial compounds were found among the tested set.

Keywords Antibiotic · Inhibitor · Ribosome · Protein synthesis
Bacteria · Translation · Reporter strain

After initial success of antibiotics discovery [4, 21], the problem of bacterial infections appeared to be solved. In a golden period of antibiotics, lasted from 1940s to 1960s many diverse families of natural antibacterial compounds were discovered and applied in clinical practice [8]. However, the first antibiotic resistant bacteria were revealed shortly after [25]. Since that time, the frequency of new antibacterial compound discovery is decreasing dramatically [8]. At the same time, the antibiotic resistance isolates of pathogenic bacteria are spreading and even microbes resistant to multiple antibiotics and more recently totally resistant bacteria are found [1].

The standard pipeline of antimicrobial drug discovery starts with screening for antibacterial activity, most often in a culture broth of soil microorganisms. Following initial demonstration of the activity, an active compound needs to be purified to homogeneity and its mechanism of action should be studied *ab initio*. Often, and in the recent years even predominantly, application of this pipeline results in rediscovery of known antibacterials [3, 8, 16]. The time spent on the purification of the active compound and *ab initio* studies of the mechanism of action make the whole procedure lengthy, costly and inefficient.

The problems in the application of the standard pipeline noticed by both academic scientists and large pharmaceutical companies abandoned the area of new antimicrobials discovery [8]. However, the problem of antibiotic resistance remains

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and growing worse year by year. Fresh ideas are needed to speed up antimicrobial compounds discovery. One of the possibilities is to combine initial screening for antibacterial activity with the built-in procedure to assess the mechanism of action [22]. High-throughput automation combined with the cost-efficient mechanism of action testing creates a possibility to revive antibacterial compounds discovery pipeline.

Usually, determination of the mechanism of action is time-consuming, expensive and not suitable for automation [22]. For example, the standard way is to test for the efficiency of incorporation of radioactive precursor molecules into DNA, RNA, and proteins. Experiments of this type require specialized protective environment to grow bacteria in radioactive culture medium and could not be done in a high-throughput form. Replacement of the radioactive precursor molecules with fluorescent ones [13] creates some advantage, but it is hardly suitable for initial screening due to the multistep lengthy implementation and the cost of reagents per test. A set of *in vitro* tests for partial reactions of protein biosynthesis is valuable for the screening of inhibitors [9], but very costly if applied to the high-throughput screening, due to the cost of the reagents and multistep procedure of the reaction setup.

An alternative to these methods is application of the reporter strains that respond to particular functional type of the inhibitor by upregulation of the gene, whose product is easy to detect [14, 22]. Among the set of possible reporter genes, those coding for fluorescent proteins are preferable due to the lack of any reagent requirements, making application of other reporters, such as luciferases [18], much more expensive. Several reporter systems designed to detect particular classes of antibiotics are available. For example, there are reporters aimed in detection of beta lactams [24], tetracyclines [7], and macrolides [2, 11]. Detection of specific classes of known antibacterials is of use, but it is not applicable for the discovery of new antibacterials [14, 22].

Previously in our laboratory, a reporter plasmid was created that was based on the application of modified tryptophan attenuator as a sensor for translation inhibitors [12]. The attenuator of transcription preceding the genes coding for tryptophan biosynthesis pathway was discovered by C. Yanofsky group in 1970s [26]. Ribosome slows down on the doublet of tryptophan codons in a course of leader peptide synthesis results in the formation of antiterminator secondary structure in between the ribosome and RNA polymerase. In contrast, steady and quick translation in the presence of sufficient concentration of tryptophanyl-tRNA results in the formation of terminator hairpin in the nascent transcript, leading to premature termination of transcription preventing expression of tryptophan biosynthesis genes. We substituted tryptophan codons with those coding for abundant aminoacid alanine and introduced compensatory changes to the RNA in order to preserve a formation of alternative regulatory secondary structures [12]. These mutations made attenuator insensitive to the concentration of tryptophan. Ribosome interacting antibiotics may stall translation while the ribosome traverse leader region of attenuator. This stalling would cause folding of antiterminator RNA structure resulting in upregulation of the following gene.

In the original reporter construct, we introduced CER fluorescent protein [20] downstream from the genetically modified tryptophan attenuator. Unregulated RFP fluorescent protein [10] was used as a control. This reporter construct was previously successfully applied for the screening of soil microorganisms culture broths provided by Gauze Institute of new antibiotics search [12]. This work resulted in identification of the mechanism of action for antibiotic amicoumacin A [17]. Antibacterial activity of this antibiotic was described previously [6], however, its mechanism of action was revealed only in our study [17].

In the frame of the current project supported by the Ministry of science, we improved the reporter construct. Due to the high background fluorescence of the rich culture media in the spectral area of CER protein fluorescence, we replaced CER protein gene with the gene of far red fluorescent protein Katushka2S [5] under a control of modified tryptophan attenuator (Fig. 1a). To test for two potential mechanisms of action simultaneously, we inserted *Escherichia coli* *sulA* promoter [23] in front of the RFP protein (Fig. 1a). This promoter is regulated by LexA transcriptional repressor, which is inactivated upon induction of the DNA damage SOS response [19]. The cells transformed by the resulting plasmid pDualrep2 [15] became a sensor for translation inhibitors which might be monitored by induction of Katushka2S expression and DNA damaging agents, such as topoisomerase II inhibitors, monitored by induction of RFP expression. These proteins possess readily distinguishable spectral properties that allow their separate detection via fluorescence scanner. A typical translation inhibitor erythromycin induces Katushka2S expression, while levofloxacin, an inhibitor of topoisomerase, induces expression of RFP (Fig. 1b). A number of antibiotics with known mechanism of action, such as erythromycin, roxithromycin, azithromycin, sulfanilamide, polymyxin, rifampicin, chloramphenicol, kanamycin, tetracycline, streptomycin,

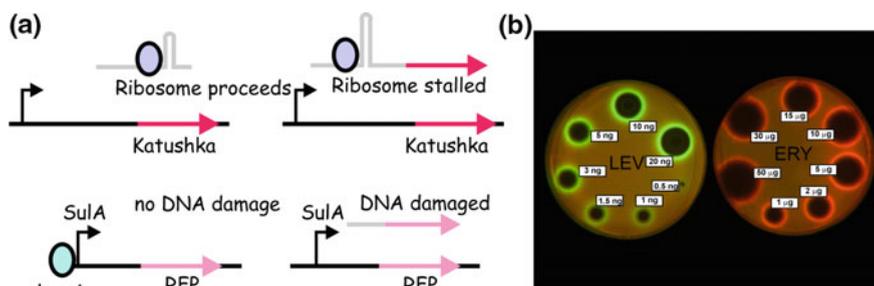


Fig. 1 Reporter system for detection of ribosome stalling and DNA damaging compounds. **a** Scheme of the reporter. *Upper panel* illustrates the organization of the sensor for translation inhibitors. On the *left* is the situation when ribosome is not inhibited. No Katushka2S expression is observed in this case. On the *right* is the situation when the ribosome is stalled by an antibiotic. In this case Katushka2S is upregulated. *Lower panel* corresponds to the sensor of DNA damage. On the *left* is situation when LexA represses transcription of the reporter in the absence of DNA damage. On the *right* is the situation when LexA is inactivated when DNA is damaged. In this case RFP is upregulated

lincomycin, clindamycin, nalidixic acid, levofloxacin, ciprofloxacin, tobramycin, neomycin, etoposide, furagin, microcin B17, spectinomycin, etamycin A, hygromycin B, griseoviridin, tylosin, amicoumacin A, fusidic acid, puromycin, and gentamicin were tested against the created reporter strain [15]. It appeared that induction of RFP reporter is caused by all known topoisomerase inhibitors and in addition DNA precursor biosynthesis inhibitors. All substances that induced expression of *Katushka2S* belonged to translation inhibitors, although some ribosome-targeting antibiotics do not induce expression of the reporter gene. This result is expected, since some ribosome targeted antibiotics, such as aminoglycosides induce translation misreading, but not ribosome stalling.

The cells transformed by the pDualrep2 reporter were used for automated high throughput screening of the collection of 17,856 compounds provided by the industrial partner of the project, Research Institute of Chemical Diversity. The set was assembled from the core collection of Research Institute of Chemical Diversity aiming at maximization of chemical diversity. The screening was performed by automated liquid handling station Janus (Perkin Elmer). We spread the lawn of the *E. coli* reporter strain on top of 245 mm × 245 mm square agar plate. The tested compounds were dissolved in DMSO to the concentration 17 mg/ml. Total amount of 2 μl volume of each obtained solution were spotted on agar plates by a 96-channel pipetting head of Janus liquid handling station (Perkin Elmer). After overnight growth at the 37 °C, the plates are scanned by the fluorescence scanner (e.g., ChemiDoc, Bio-rad) at the wavelengths 553/574 nm (RFP) and 588/633 nm (*Katushka2S*). An example of a resulting plate scan is presented in Fig. 2a.

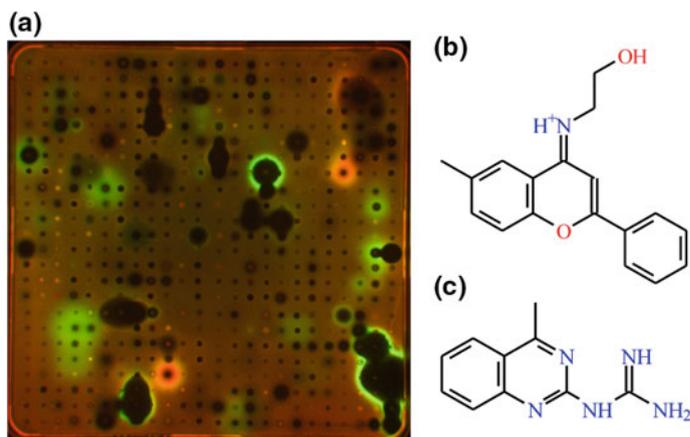


Fig. 2 Results of the screening with the developed reporter system. **a** A sample of the screening plate with the 576 compounds applied onto the *square* agar plate with the reporter strain. The image is the superposition of the same plate scanned at 553/574 nm (RFP) presented as *green* pseudocolor and at 588/633 nm (*Katushka2S*) presented as *red* pseudocolor. **b** An example of the lead compound identified in the screening, 2-hydroxyethyl-(6-methyl-2-phenylchromen-4-ilyden)azaniam. **c** Another lead compound identified in the screening, 4-methyl, 2-guanidino quinazoline

In total, 184 compounds strongly inducing RFP and 32 compounds strongly inducing Katushka2S were found among the set of 17,856 compounds. Notably, DNA damaging agents are almost sixfold more abundant relative to translation inhibitors. Among the new lead compounds, we identified 2-hydroxyethyl-(6-methyl-2-phenylchromen-4-ylidene) azanium (Fig. 2b) and 2-guanidino quinoxaline derivatives (Fig. 2c). These lead structures will be further optimized aiming at the design of new more potent and selective antimicrobial substance.

Conclusions

The system for high throughput screening of antimicrobial compounds was created. The system allows classification of antibacterial compounds into stalling protein biosynthesis, inducing DNA damage and the compounds with the mechanism of action unrelated to these two. The system was successfully tested on a large set of the compounds. Several new classes of translation inhibitors were found.

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