A competition-based assay for the screening of species-specific antibiotics

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Objectives: To develop a high throughput screening-compatible assay for the selection of species-specific antibiotics that do not harm human cells.

Methods: Staphylococcus aureus and human reporter cells continuously generating a fluorescence signal were competitively co-cultivated. The fluorescence signals were determined in the presence and absence of the specific antibiotic streptomycin and the toxic compound sodium azide. The results were compared with a standard cfu assay.

Results: In the absence of an effective antibiotic, S. aureus outgrew the human reporter cells and thus abolished the fluorescence signal. Conversely, the addition of streptomycin resulted in the growth of the reporter cells and a strong fluorescence signal. When sodium azide was added instead of streptomycin, only a very low background signal was obtained indicating toxicity and damage to the human reporter cells. The assay proved to be highly reliable (Z-factor >0.9) and high fluorescence signals correctly correlated with the efficient inhibition of S. aureus, as determined in comparative cfu assays.

Conclusions: In contrast to conventional cfu assays, the co-cultivation system allows the effects of a drug candidate on pathogens and human cells to be monitored simultaneously. Cytotoxic compounds can, therefore, be quickly ruled out during a primary screen. The nature of the screen also enables effective antibiotics to be identified without engineering the target pathogen to yield a fluorescence signal.

Keywords: Staphylococcus aureus, high-throughput assays, drug development, staphylococci, cytotoxicity

Introduction

Infectious and parasitic diseases are the second most frequent cause of death in the world after cardiovascular diseases. Respiratory infections alone account for 6.5% of all deaths worldwide. Even in countries with well-developed health systems, the emergence of multiresistant bacterial strains, such as methicillin-resistant Staphylococcus aureus (MRSA), is causing major problems. Some reports suggest that in 2005 more people in the USA died from an MRSA infection (18000 victims) than from AIDS (16500 victims). Hence, there is a huge demand for novel antibiotics and, by extension, the assay systems that allow the screening of large compound libraries for antimicrobial properties.

Assays for the screening of potential antibiotics can be divided into two main classes: biochemical and whole-cell assays. Biochemical assays make use of the isolated and purified proteins from the pathogen. However, this strategy requires well-characterized drug targets and may lead to the selection of compounds that do not show any significant activity towards the whole pathogen (rather than the purified target). For example, the selection of compounds that do not penetrate or persist in cells is a significant problem, especially since many bacterial strains express the so-called drug efflux pumps whose activities on small molecules are difficult to predict.

These limitations can be circumvented using whole-cell assays. In these systems, phenotypic changes of the pathogen (e.g. growth arrest) are monitored in response to a drug candidate. In the simplest case, bacterial growth directly results in an easily detectable signal. Such signals include changes in absorption, fluorescence, bioluminescence (Bio-Siv) or the release of radioactively labelled CO2 (BACTEC). More complex
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systems that allow conclusions to be drawn about the drug targets involved have also been established. For example, arrays of bacterial strains, each one hypersusceptible to the inhibition of one particular gene, have been generated.8 In addition, assays based on the mass spectrometric analysis of cell lysates have been used to screen compounds for their ability to specifically inhibit a given drug target.9 However, these kinds of assays require detailed knowledge of the pathogen and its potential drug targets as well as genetic modifications of the corresponding microorganisms.

One common limitation of both biochemical and whole-cell assays is the potential selection of compounds that harm human cells as well as the pathogens. Even when using purified pathogen-specific drug targets, potential cytotoxic side effects for human cells cannot be ruled out completely. Therefore, a primary screen for hits is usually followed by a secondary screen, which determines the degree of cytotoxicity towards human cells.

We describe here a novel assay system based on the co-cultivation of human cells with the pathogen of interest. In this way, cytotoxic side effects can be directly monitored during the primary screen. Furthermore, the readout signal for the assay is generated by the human cells (rather than the microorganisms), meaning that no genetic modification of the pathogen is necessary. In theory, the assay does not require any detailed knowledge of the pathogen employed, nor its potential drug targets.

Materials and methods

Cells

Reporter cells expressing a membrane-bound and HA-tagged form of tissue plasminogen activator (HEK293T-iPA) were obtained by retroviral transduction of HEK293T cells with MLV(VSV-G) pseudotype particles packaging the encoding vector (Patent WO2006082385). High-expression cells were selected with a fluorescence-activated cell sorter (MoFlo, BD) using goat polyclonal antibodies raised against tissue plasminogen activator (tPA, Abcam).

HEK293T-iPA cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL) supplemented with 10% (v/v) fetal bovine serum (GIBCO-BRL) and penicillin/streptomycin (105 U/L and 100 mg/L, respectively; GIBCO-BRL). Cells were incubated at 37°C in a 5% CO2 atmosphere saturated with water.

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In vitro co-cultivation assay

To set up the co-cultures, S. aureus was grown in DMEM supplemented with 10% (v/v) fetal bovine serum to an OD670 of 0.4 (corresponding to 3×107 cfu/mL, as determined by plating on agar). Subsequently, the samples were diluted 10-fold and 100 μL (≈30 bacterial cells) were added to each well of a 96-well plate. In parallel, HEK293T-iPA cells were washed twice with DMEM supplemented with 10% (v/v) fetal bovine serum and seeded onto the same wells at a density of 2×104 cells/well (determined with a Neubauer counting chamber: accurate counting of the cell density turned out to be crucial for reliable results). Antibiotics were added to the wells in the indicated concentrations. All samples were prepared in triplicate and incubated for 3 days at 37°C under a 5% CO2 atmosphere saturated with water. For the fluorescence readout, HDVLK-AMC (Bachem) and Plasminogen (Roche) were added to a final concentration of 1 mM and 1.67 μM, respectively. All measurements were performed with excitation and emission wavelengths of 370 and 450 nm, respectively, using a Spectramax M5 microplate reader (Molecular Devices).

For control purposes, supernatants of the co-cultures were plated on agar and incubated overnight at 37°C. The next day, surviving colonies were counted manually.

Antibiotics

Penicillin (Sigma) and streptomycin (Sigma) were added with sterile 1× PBS (Euromedex) to a concentration of 104 mg/L. For control purposes, sodium azide (Sigma) was diluted with sterile 1× PBS (Euromedex) to a concentration of 103 mg/L.

Determination of Z-factors

Z-factors were calculated using the following equation:10

\[ Z\text{-factor} = 1 - \left( \frac{3 \times (\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \right) \]

where \( \sigma \) is the standard deviation, \( \mu \) the mean signal, \( x_p \) the parameter of the positive control and \( x_n \) the parameter of the negative control.

Determination of the effect of bacterial metabolism on reporter cell survival

One day before starting an assay, HEK293T-iPA cells were seeded at a density of 2×104 cells/well into 6-well plates (VWR). In parallel, S. aureus was grown at 37°C in DMEM supplemented with 10% (v/v) fetal bovine serum to high density (72 h of incubation to mimic the conditions on the day of the assay readout). The bacterially conditioned DMEM was recovered by filtration through a sterile 1 μm sterile filter (Millipore) and its sterility was confirmed by plating samples on agar plates. Subsequently, 1 mL was added to each well hosting HEK293T-iPA cells (after removing the primary medium from those wells). For control purposes, the pH of the bacterially conditioned medium was adjusted to 7.7 (by adding 1 M HCl) and/or glucose was added to a final concentration of 4×103 mg/L. Fresh DMEM lacking glucose (pH 7.7 and 8) and fresh DMEM supplemented with glucose (pH 8) served as additional controls. Each sample was incubated with HEK293T-iPA cells for 3 days at 37°C under a 5% CO2 atmosphere and bright-field micrographs were taken daily (using a LEICA DMIRB microscope and a Guppy camera, Allied Vision Technologies). On the third day of incubation, the medium was removed from the cells and 1 mL of 0.25% (w/v) trypsin (GIBCO-BRL) was added. After detachment, the cells were centrifuged at 217 g for 5 min and resuspended in a live/dead staining solution (LIVE/DEAD Viability/Cytotoxicity Kit for animal cells, Invitrogen Kit L-3224). After 1 h of staining, the cells were manually counted using a microscope (Leica DM IRB) with a mercury-vapor lamp. For each sample, 1000 cells were counted to determine the fraction of living (green-stained) and dead (red-stained) cells.
Results

General setup of the assay

The first requirement for establishing the novel assay system was the use of human reporter cells continuously generating a fluorescence signal. For this purpose, we chose HEK293T-derived cells constitutively expressing a membrane-bound and HA-tagged form of tissue plasminogen activator (HEK293T-tPA). These cells were originally developed for viral inhibition assays (Patent WO2006082385) and generate a strong fluorescence signal upon the addition of plasminogen and the fluorogenic substrate HDVLK-7-amino-4-methylcoumarin (HDVLK-AMC, Bachem). During this reaction, the membrane-bound tPA converts plasminogen into plasmin, which subsequently releases the 7-amino-4-methylcoumarin group of HDVLK-AMC, resulting in fluorescence (Figure 1a).

The next step was to find a way of competitively co-cultivating these cells with a metabolically active pathogen. In particular, we wanted to set up a system in which the absence of an antibiotic would result in the reporter cells being outgrown by the pathogen and dying, while remaining alive and generating a strong fluorescence signal in the presence of an antibiotic (Figure 1b). Using *S. aureus* (ATCC strain 52156) as a model organism, we determined whether reporter cells seeded onto the wells of 96-well plates indeed died due to the growth of the pathogen. We inoculated the samples with different amounts of *S. aureus* in the presence and absence of streptomycin (at concentrations of $10^{-2}$–$10^{-4}$ mg/L) and performed microscopic analyses during the following days. The biggest difference between the two samples (+/− antibiotics) and the lowest standard deviations were obtained when inoculating each well with ~30 bacterial cells: within 3 days, the absence of an antibiotic resulted in the detachment and fragmentation of the reporter cells and the formation of large bacterial aggregates. In contrast, the addition of the antibiotics inhibited bacterial growth and resulted in viable, proliferating reporter cells (data not shown).

Determinants for the viability of the human reporter cells within the co-cultures

To elucidate the mechanism by which the reporter cells were ultimately killed, we performed experiments using sterile, filtered supernatants of *S. aureus* cultures grown in DMEM for 72 h (as it is the case for the co-cultivation assay). We incubated human reporter cells with this bacterially conditioned medium and determined the survival rate by microscopic analysis and a commercially available live/dead stain (Figure 2a and b). We observed that the death of the reporter cells was not dependent on the presence of viable bacteria. In fact, the reporter cells appeared to have died due to the presence of toxic metabolic products or the lack of nutrition. To test this, we repeated the experiments with additional samples. In particular, we included bacterially conditioned medium whose pH was adjusted to 7.7 (instead of pH 8 without any treatment), bacterially conditioned medium supplemented with $4 \times 10^3$ mg/L glucose and bacterially conditioned medium with glucose and a pH of 7.7. Adjustment of the pH as well as the addition of glucose to the bacterially conditioned medium significantly increased the survival rates of the reporter cells (with the addition of glucose having the stronger effect). Performing both modifications at the same time resulted in survival rates almost identical to the control samples with fresh DMEM (>97% viable cells). This indicated that within the co-cultures, the reporter cells ultimately died due to the lack of nutrition and changes in the pH.

As further proof for this hypothesis, we also performed experiments using fresh (non-bacterially conditioned) DMEM lacking glucose (pH 7.7 and 8) and fresh DMEM supplemented with glucose (pH 8). In agreement with our earlier experiments,

![Figure 1](image-url)
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Figure 2. Survival of human reporter cells exposed to bacterially conditioned DMEM. (a) HEK293T-tPA cells were grown either in fresh DMEM (black bars) or in sterile, bacterially conditioned DMEM (white bars). For control purposes, the pH was modified (by the addition of 1 M HCl or 1 M NaOH) and/or the glucose concentration was changed (with and without 4\% C2HCl or 1 M NaOH) and/or the glucose concentration was changed (with and without 4\% C2HCl or 1 M NaOH). Survival rates were determined after 3 days of incubation using a live/dead stain. (b) Microscopic images of the HEK293T-tPA cells after 3 days of incubation.

Fluorescence signals using streptomycin and sodium azide as model compounds

Next, we focused on the fluorescence readout of the assay and characterized its suitability for high-throughput screening of drug candidates. In 96-well plates, we seeded reporter cells, cultures of S. aureus and co-cultures of both species in the presence and absence of streptomycin (an antibiotic active against bacteria) and sodium azide (a compound which kills both species).

After 3 days of incubation, we added the components of the fluorescence readout (plasminogen and HDVLK-AMC) and determined the fluorescence signal (Figure 3). While the samples with the reporter cells alone generated a strong fluorescence signal, the S. aureus cultures and the co-cultures of both species showed very low background fluorescence (Figure 3a). This changed when adding different concentrations of streptomycin (10^{-3}–10^{4} mg/L): for all concentrations \( \geq 1 \text{ mg/L} \), a significant increase in fluorescence was observed (Figure 3b). A concentration of exactly 1 mg/L resulted in the strongest signal, with an intensity corresponding to that of the positive control (reporter cells without S. aureus). Higher concentrations of streptomycin yielded continuously decreasing fluorescence intensities, most likely due to the increasing cytotoxicity. In fact, when incubating the reporter cells with streptomycin in the absence of bacteria, we observed decreasing fluorescence intensities for concentrations of the antibiotic \( > 10^{-3} \text{ mg/L} \) (Figure 3b). As a further demonstration that our assay system takes cytotoxic effects into account, we analysed the results for the samples containing the highly toxic compound sodium azide (killing both bacterial and human cells). As expected, even very low concentrations (10^{-2}–1 mg/L) of the compound abolished the fluorescence signals of the reporter cells when they were alone as well as when they were co-cultured (Figure 3c). The sodium azide was effective at killing the S. aureus (as determined by plating the supernatants on agar; Figure 3c), but its toxicity towards human cells prevented a fluorescence signal being generated.

In summary, these results show that the assay can be used to identify compounds that specifically kill a given pathogen without harming human reporter cells.

Correlation between fluorescence signals and growth inhibition of S. aureus

To benchmark our fluorescence readout, we repeated our co-cultivation experiments, and determined the effect of the antibiotic by plating the co-culture supernatants on agar and counting any resulting colonies. In the absence of streptomycin, we determined an increase in the bacterial titre from \( 10^2 \) to \( 10^3 \) cfu/mL after 3 days (data not shown). In the presence of streptomycin, however, final bacterial titres were observed to decrease with increasing streptomycin concentrations (Figure 3b). At concentrations of 1 mg/L (the concentration mediating the strongest signal in the fluorescence readout) and above, no surviving colonies were counted.

A similar effect was observed when using sodium azide as the antimicrobial agent (Figure 3c). As for streptomycin, increasing concentrations resulted in decreasing bacterial titres. In the absence of the results from the novel fluorescence assay, these results would lead to the false conclusion that sodium azide might be a good antibiotic. Hence, the assay based on the co-cultivation of pathogens with human reporter cells is highly preferable.

Reliability of the assay

To further characterize the quality of the novel fluorescence assay, we determined its Z-factors\(^{10} \) using the fluorescence
values for the co-cultures in the presence (positive control) or absence (negative control) of streptomycin (Table 1). For all concentrations $\geq 1$ mg/L, the Z-factor exceeded 0.79 with the best value being 0.95 when using 10 mg/L streptomycin. These values mean that the assay clearly fulfils the requirements for being an 'excellent' assay.\textsuperscript{10} The signal to background values (fluorescence of the positive control divided by the fluorescence of the negative control) for the assayed samples were in the range 0.73–25.24.

As the next step, we determined the reproducibility of the assay by performing two completely independent sets of experiments (Run1 and Run2) on two different days. Each experiment contained triplicates of all samples for which fluorescence was determined (using streptomycin concentrations of $10^{-3}$–$10^{4}$ mg/L; 54 samples per run). Subsequently, the values obtained for Run1 were plotted against the corresponding values for Run2 (Figure 4). The resulting data points in the dot plot show a linear correlation, demonstrating a high degree of reproducibility for the assay. The fitted trend line has a coefficient of determination ($R^2$) of 0.881. In parallel, we determined the (mean) relative standard deviation (RSD) between the replicates in one run (intra-comparison) and between the two runs (inter-comparison). The obtained values of 9.41% and 9.91% demonstrate the reliability and robustness of the assay, respectively.

**Figure 3.** Fluorescence signals of the tPA-based readout system and corresponding bacterial titres. (a) The fluorescence was determined for reporter cells (C), S. aureus (B) and co-cultures of both (C+B). (b) and (c) Streptomycin (S) or sodium azide (N) was added at the indicated concentrations (in mg/L). Subsequently, the fluorescence signals (scale according to the y-axis on the left-hand side) were determined for the co-cultures (grey bars), as well as for the reporter cells alone (black bars). In parallel, the supernatants of the co-cultures were plated on agar and surviving colonies were counted the next day (white bars, scale according to the y-axis on the right-hand side).
Table 1. Z-factors and signal to background ratios for different concentrations of streptomycin

<table>
<thead>
<tr>
<th>Concentration of streptomycin (mg/L)</th>
<th>Z-factor</th>
<th>Signal to background</th>
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<tbody>
<tr>
<td>$10^4$</td>
<td>0.68</td>
<td>10.25</td>
</tr>
<tr>
<td>$10^3$</td>
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<tr>
<td>$10^{-2}$</td>
<td>-2.00</td>
<td>0.80</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>-2.11</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Figure 4. Reproducibility of the co-cultivation assay. Two completely independent sets of experiments were performed on two different days (using the same sample compositions). The fluorescence signals for all samples of Run1 were plotted against the corresponding fluorescence signals of Run2. $R^2$ = determination coefficient.

Discussion

We have developed a generic fluorescence assay to measure the inhibition of the growth of metabolically active pathogens. The system is based on the competitive co-cultivation of human reporter cells with the pathogen of interest. Using S. aureus as a model organism, we obtained Z-factors of up to 0.95 and a RSD of 9.41% for all samples within one run and 9.91% when comparing equal samples between two runs. According to our experiments, the minimum concentration of streptomycin required to efficiently inhibit the growth of S. aureus is in the range of 0.1–1 mg/L. This is in good agreement with our own plating experiments and other studies that found MICs (in liquid medium) of 0.065, 0.4 and 2 mg/L for different strains of S. aureus.\cite{11,12}

Hence, the results obtained with our novel assay are comparable to existing screens that are not based on the co-cultivation of two species. It is noteworthy that we also performed individual experiments using penicillin as a model antibiotic and obtained similar Z-factors (0.81–0.91 when using concentrations of $10^3–10^4$ mg/L; data not shown). The novel assay is, therefore, both generic and reliable.

The main advantage of this system is the fact that it allows the identification of specific antibiotics effective against metabolically active pathogens, even if they are not well characterized. It requires neither detailed knowledge about potential drug targets nor genetic modification of the pathogen (e.g., to establish a reporter system). Using live/dead stains, we were able to show that without adding effective antibiotics to the co-cultures, human reporter cells eventually die due to the lack of nutrition and, to a lesser extent, changes in the pH. This mechanism should, in theory, be adaptable to any metabolically active pathogen.

Even if the pathogen of interest has been well characterized and potential drug targets are known, it seems advantageous to use a reporter system based on human cells. This way, adverse side effects of the screened compounds are taken into account directly: we have shown here that sodium azide, a toxic compound harming both the pathogen and the human reporter cells, does not yield a positive fluorescence signal. Furthermore, we observed decreasing fluorescence signals for streptomycin concentrations $\geq$1 mg/L. This is likely due to its cytotoxic side effects at high concentrations (as demonstrated in our control experiments). It is well known that exposure to streptomycin affects the viability of human cells and can result in chromosome aberrations and cell death.\cite{13,14}

In summary, the assay system described here allows compounds with non-specific inhibitory properties to be immediately excluded in the primary screen and the determination of the optimal concentration of specific antibiotics. A secondary screen to probe for cytotoxicity against human cells is not required, thus saving time and money. Even though the screening system does not elucidate the mode of action of the screened molecules (e.g., it does not discriminate among compounds which do not penetrate the cell membrane), it might be useful for the identification of novel drug targets. Since the assay is compatible with poorly characterized pathogens, it might also lead to the discovery of compounds that act on previously uncharacterized targets, which could ultimately be identified using labelled or immobilized molecules.\cite{15}

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Transparency declarations

None to declare.
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