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A new set of assays for the discovery of aminoacyl-tRNA synthetase inhibitors

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ABSTRACT

Current biochemical methods available to monitor the activity of aminoacyl-tRNA synthetases (ARS) are ill-suited to high-throughput screening approaches for the identification of small-molecule inhibitors of these enzymes. In an attempt to improve the limitations of current assays we have developed a suite of new methods designed to streamline the discovery of new ARS antagonists. This set of assays includes approaches to monitor ARS activity *in vitro*, in human cells, and in bacteria. They are applicable to several ARSs from any given organism, can be easily adapted to very high-throughput set-ups, and allow for a multi-factorial selection of drug candidates.

1. Introduction

Aminoacyl-tRNA synthetases (ARS) are universally distributed enzymes, whose main function is to aminoacylate transfer RNAs (tRNA) with their cognate amino acids. This reaction takes place through two consecutive catalytic steps: the activation of the amino acid with ATP to form a transient amino acid adenylate, and the transfer of the activated amino acid to the ribose of the terminal adenosine of tRNA. In addition, some ARS can catalyze a third enzymatic reaction: the hydrolysis of misactivated amino acids or mischarged tRNAs to prevent the misincorporation of non-cognate amino acids into proteins.

These three reactions represent the house-keeping activities of ARS, and are all essential for life. This fact, combined with the extended evolutionary history of a family of enzymes that predates the appearance of the last common ancestor to all living species, makes ARS logical targets for the development of anti-infective drugs. This rational assessment is supported by several examples of naturally occurring small molecules synthesized by microorganisms as chemical aids against competing species. Possibly the clearest example is agrocin 84, a molecule produced by certain strains of *Agrobacterium tumefaciens* that effectively inhibits the activity of leucyl-tRNA synthetase (LRS) [1]. Agrocin 84-producing bacteria also synthesize an agrocin 84-resistant form of LRS, a fact that strongly suggests the use of the inhibitor to suppress the growth of other bacteria, and supports the possibility that other discriminating ARS-inhibitors may be developed and used as medicinal drugs.

Indeed, two different ARS inhibitors are currently available for antimicrobial treatment: mupirocin [2,3] and tavaborole [4]. These two molecules represent the successful end of a long list of ARS inhibitors that constitute the pipeline of drugs being developed with a similar mechanism of action. This list includes antibacterial, antifungal, and anti-parasitic drugs developed against isoleucyl-, leucyl-, tryptophanyl-, prolyl-, lysyl-, threonyl-, methionyl- and phenylalanyl-tRNA synthetases, among others [3–16].

The difficulty in the discovery of new ARS inhibitors is exacerbated by the lack of efficient biochemical assays that can be used in a high-throughput fashion. In addition, no available screening method exist that addresses, simultaneously, the efficiency and

Abbreviations: ARS, aminoacyl-tRNA synthetases; Cpd, compound; HTS, high throughput screening; IC_{50}, half maximal inhibitory concentration; K_m, Michaelis constant; LRS, leucyl-tRNA synthetase; MIC, minimum inhibitory concentration; OM, Omnia Molecular; RLU, relative light unit; RRS, arginyl-tRNA synthetase; RT, room temperature; SRS, seryl-tRNA synthetase; SD, standard deviation; tRNA, aminoacylate transfer RNA; YRS, tyrosyl-tRNA synthetase; WRS, tryptophanyl-tRNA synthetase; Wt, wild-type.

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specificity of potential inhibitors. Species-specificity is a crucial requirement for a lead compound acting as an ARS inhibitor, and is usually achieved thanks to the structural similarities that exist between the active sites of human and microbial ARS. For example, borrelidin derivatives are threonyl-tRNA synthetase inhibitors that hold promise as antimalarial drugs, but only when good species-selectivity is achieved through modifications in their original scaffold [10].

A third important aspect of drug discovery, independently of the target, is the need for candidate drugs to display, at the same time, good membrane permeability and target specificity. Molecular screenings based on biochemical assays typically ignore the first requirement. Whole cell assays where compounds are selected for their biocidal activity may filter out membrane impermeable compounds, but usually do not provide information on target selectivity or species specificity.

We set up to design new methods to monitor ARS activity that could be used to efficiently identify hit compounds combining both activity, membrane permeability, and species-selectivity. To design these assays we took advantage of the abundant data available on the construction of orthologous tRNA aminoacylation systems, which are most commonly used in synthetic biology for the incorporation of unnatural amino acids into proteins.

We reasoned that an orthologous system composed of an ARS from a pathogenic organism (pathogenic ARS) and a tRNA specifically aminoacylated by this enzyme could be expressed within a human cell, and used to generate a cell-autonomous phenotype dependent on the activity of the target. Once such cell line was constructed, its cultures could be used to screen small-compound libraries to identify molecules capable of eliminating the target-dependent phenotype without compromising the viability of the cell.

Such a human cell-based screening assay may accelerate and improve the discovery of anti-infective drugs targeting pathogenic ARS. To be positively selected in this assay (*In Omnia* assay) compounds would be required to cross the lipid membrane of the human cell, selectively inhibit the ARS from the pathogen, and be relatively innocuous to the cell's metabolism. This 'positive selection' approach, in contrast to the usual biocidal assays used in the discovery of antimicrobials, has the advantage of combining several selection parameters simultaneously.

In addition to the *In Omnia* assay, our screening tests (Fig. 1) include complementary approaches to further characterize the screened compounds. An *in vitro* aminoacylation assay (Biothema assay) suitable for use in high-throughput assays was developed to directly address if the aminoacylation reaction performed by the pathogenic ARS is inhibited. Also, a complementation assay based on bacterial strains overexpressing the human orthologue of the ARS target was developed to confirm the specificity and selectivity of initial hits. Active compounds are also tested in a classical biocidal test with the pathogen of interest to determine their actual antimicrobial activity.

Here we will describe the details of these assays in the hope that they can be of use to ongoing or future programs dedicated to the discovery of new ARS inhibitors.

2. Cellular assay: *In Omnia*

2.1. Principle of the In Omnia assay

The *In Omnia* assay was designed to monitor a pathogenic ARS activity inside human cells (Fig. 2A). The approach is based on the use of a gene coding for a luminescent reporter (luciferase) whose sequence is interrupted by a stop codon. This stop codon is translated by a modified tRNA that contains a suppressor anticodon (complementary to the stop codon that interrupts the gene coding for the reporter protein). The suppressor tRNA is specifically aminoacylated by the pathogenic ARS. Thus, the luminescence

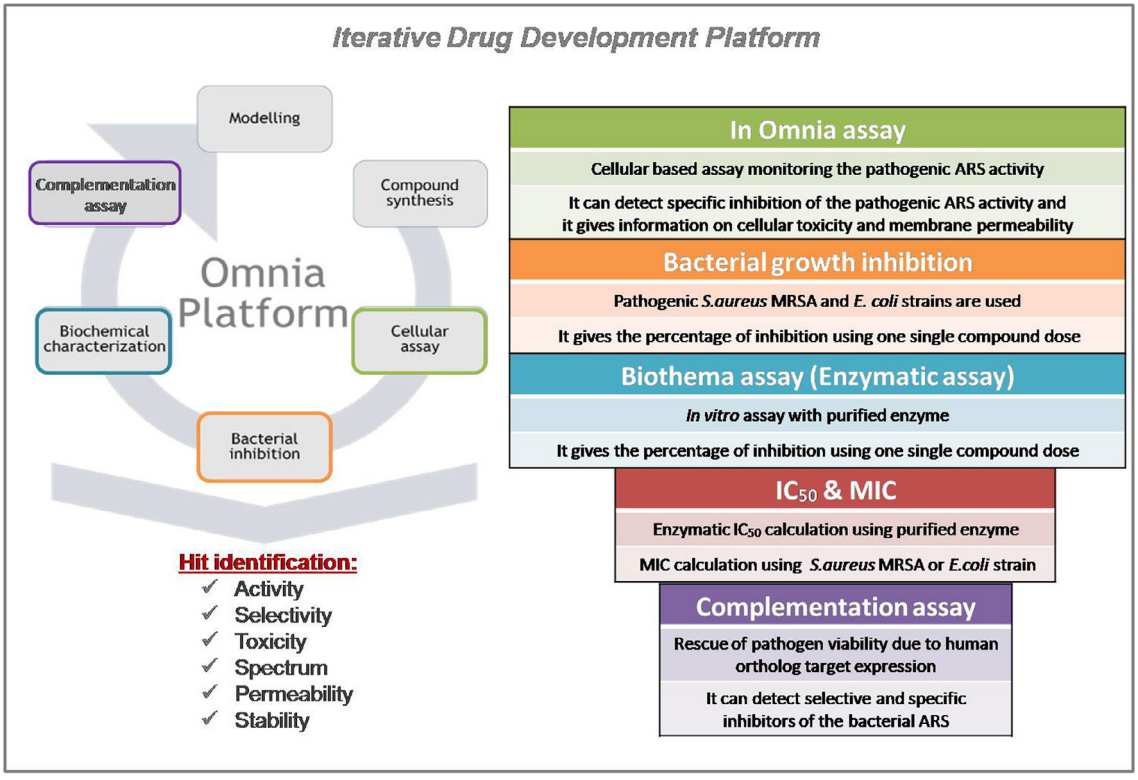

Fig. 1. The complete set of assays developed by OM to monitor ARS activity *in vitro*, in human cells, and in bacteria.