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SpeedScreen, a Label-Free, Affinity-Based High-Throughput Screening Technology for the Discovery of Orphan Protein Ligands

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Abstract: A high-throughput screening method, tailored to the discovery of ligands for both known and orphan proteins, was developed and implemented into Novartis' lead discovery process. Neither labeling of target proteins nor labeling of screening compounds is required, as the ligands are affinity-selected by incubation of the protein with mixtures of compounds in aqueous binding buffer. Unbound small molecular weight compounds are removed from the target protein:ligand complex by rapid size-exclusion chromatography in 96-well plate format. The protein fraction is then analyzed subsequently by liquid chromatography/mass spectrometry (LC/MS) for identification of the bound ligand(s). All sample handling steps and the analytics are rapid, robust, and largely automated, adopting this technology to the needs of present high-throughput screening (HTS) processes. This affinity-selection technology, termed SpeedScreen, is currently an integral part of our lead discovery process. In addition to the screening of conventional compound libraries this technology can also be applied for the de-convolution of libraries originating from combinatorial chemistry efforts as well as complex natural extracts.

Keywords: De-convolution of mixtures · Drug discovery · HTS · LC/MS-based affinity selection · Orphan targets

Introduction

Currently, a major route for the identification of novel drug candidates involves the systematic screening of historical compound collections, combinatorial and natural product libraries against a panel of biochemical and cell-based targets. Classical high-throughput screening (HTS) approaches are based on the knowledge of the properties and functions of the target protein, which can be exploited to monitor the activity of test compounds toward the target of interest. Deciphering the human genome [1] has generated a large number of new molecules with the potential of being pharmaceutically relevant drug targets. Obviously, target candidates belonging to known functional classes were the first to be channeled into the HTS programs. However, the large pool of new molecular targets with unknown function (orphan targets) or with non-tractable activities represent a promising source of new opportunities to be tapped in future. In order to successfully exploit the burst of orphan and non-tractable targets however, novel HTS strategies technologies need to be identified, developed and implemented.

Screening strategies addressing non-tractable targets need to identify compounds independent of the knowledge of the target's function. One minimal prerequisite such chemical compounds need to fulfill is binding to the molecular target. Thus, the most promising technology has to be based on affinity-selection, which identifies chemical ligands independent of functional information on the biological target. Furthermore, the process has to contain a step which discriminates between target-bound and target-unbound compounds.

We have developed and implemented a label-free, in-solution affinity selection-based screening technique, named SpeedScreen [2][3], particularly designed for high-throughput screening of orphan and non-tractable targets. The target protein and mixtures of compounds are incubated to form a protein:ligand complex which is then separated from non-binders for analysis. Size-exclusion chromatography (SEC) coupled to reversed phase liquid chromatography/mass spectrometry (LC/MS) is employed to isolate the target protein:ligand complex and to identify the free ligand after dissociation of the complex. The process was optimized to accommodate industrial scale high-throughput screening (HTS) in 96-well format, focusing on speed, robustness, and easiness of automation.

SpeedScreen offers the potential of a fast, cost-effective, and reliable approach applicable to any type of target protein in aqueous solution and independent of its functional knowledge. Therefore, SpeedScreen is currently among the first choice approaches for drug discovery on genomic targets with unknown biological function or unknown screening format.

The SpeedScreen Platform

Screening Process and the SpeedScreen 'Sandwich'

Key to the success of SpeedScreen is the compact integration of three fundamental steps into a process compatible with state-

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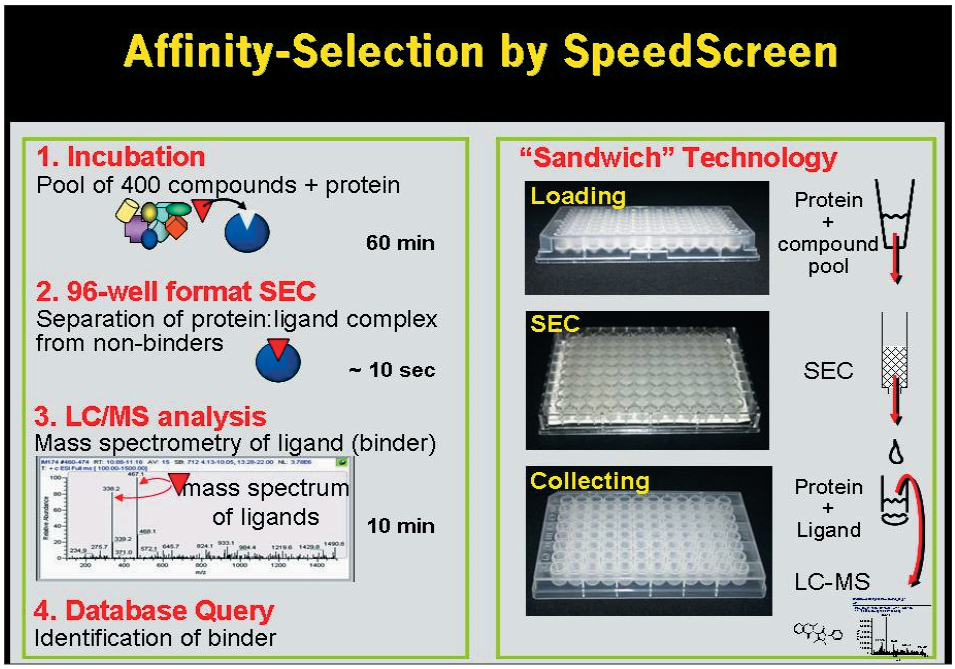

Fig. 1. The concept (left) and the technical realization (right) of the SpeedScreen technology. Pools of compounds are incubated with the target protein (1. Loading) prior to SEC separation of unbound compounds (2. SEC) and LC/MS analysis of ligands (3. Collecting) simultaneously in the 96-well plate sandwich format. Compounds are then identified using the mass signal(s) for database query (4.).

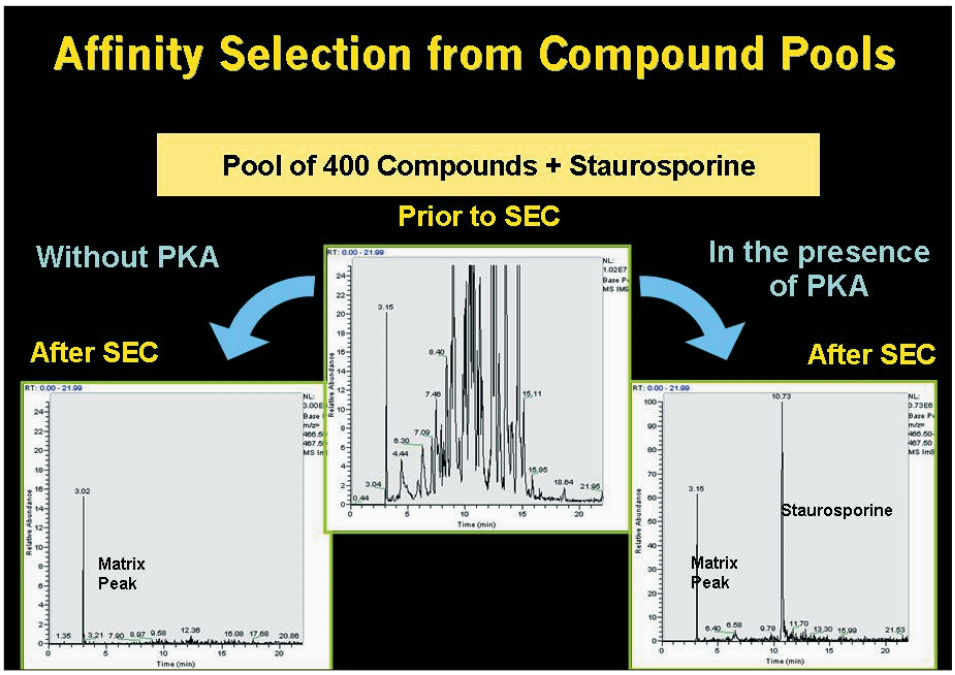

Fig. 2. Validation of the SpeedScreen technology using known protein:ligand model systems. The ligand staurosporine was added to individual pools of compounds and incubated with (right) and without (left) protein kinase A (PKA) prior to size-exclusion separation. The SEC eluates from the incubation mixtures were then analyzed to identify the ligand. An additional mass signal, detected in the eluate containing PKA, was identified as a false positive (matrix peak) as the same signal showed up also in absence of the target protein.

of-the-art HTS requirements. These steps are i) isolation of ligands from assayed compound pools by means of affinity-selection, ii) elimination of unbound compounds by means of SEC and iii) chemical identification of compounds bound to the target of interest by means of LC/MS. The outline of this process as well as the technical realization are shown in Fig. 1.

Screening compounds from various sources were pooled to 400 compounds per pool and incubated with the target of interest for one hour at room temperature. Incubation was carried out in a 96-well plate containing tiny pinholes in the bottom of the wells. After binding equilibrium was reached, the so-called SpeedScreen 'sandwich', consisting of the incubation plate stacked on a equilibrated size exclusion chromatography (SEC) plate and a collection plate beneath the SEC plate, was assembled (Fig. 1, right panel). By centrifugation of the sandwich the 96 samples are simultaneously passed through the pinholes of the incubation plate onto the SEC plate where unbound compounds are retained in the SEC matrix and potential ligands complexed to the target are eluted into the collection plate. Strongly binding ligands are resistant to various separation methods whereas low affinity ligands are often sensitive to the applied separation procedure. Therefore the fast size exclusion step reached by centrifugation was found to be critical for the identification of high as well as low affinity ligands. After SEC the samples in the collection plate were analyzed by LC/MS using a standard LCQ DECA XP mass spectrometer. Identification of binders is achieved by comparing the mass signals of the eluted protein:ligand complexes with the masses of the compounds from the corresponding pools. An example is given in Fig. 2, where a compound pool was spiked with protein kinase inhibitor staurosporine and screened against protein kinase A (PKA). Hit candidates from this first round of screening are subsequently assayed as single compounds in presence and absence of the target protein to confirm binding and to eliminate false positives interacting with the SEC matrix.

Instrumental Requirement and Set-up

SpeedScreen is conceptually a simple, but effective screening technology for non-tractable targets based on a 96-well plate format. Besides having a library of pooled test compounds of known molecular masses, the minimal instrumental requirements to implement this method are a plate centrifuge and an LC/MS system consisting of a gradient HPLC pump with an online eluent degasser, a temperature-controlled automated sample injector and an ion trap mass spectrometry detector (Fig. 3). Since the analysis of the screening plates is performed on a well by well basis, and not by imaging the whole plate, pooling of compounds is required to satisfy high-throughput screening requirements. Pool complexities of 100 to 600 compounds were examined using various control proteins and respective reference compounds. Mixtures of 400 compounds per pool were found to be optimal for SpeedScreen. Lowering pool complexity would decrease throughput, whereas increasing pool complexity can lead to interference of compounds and thus to a large number of false positives.

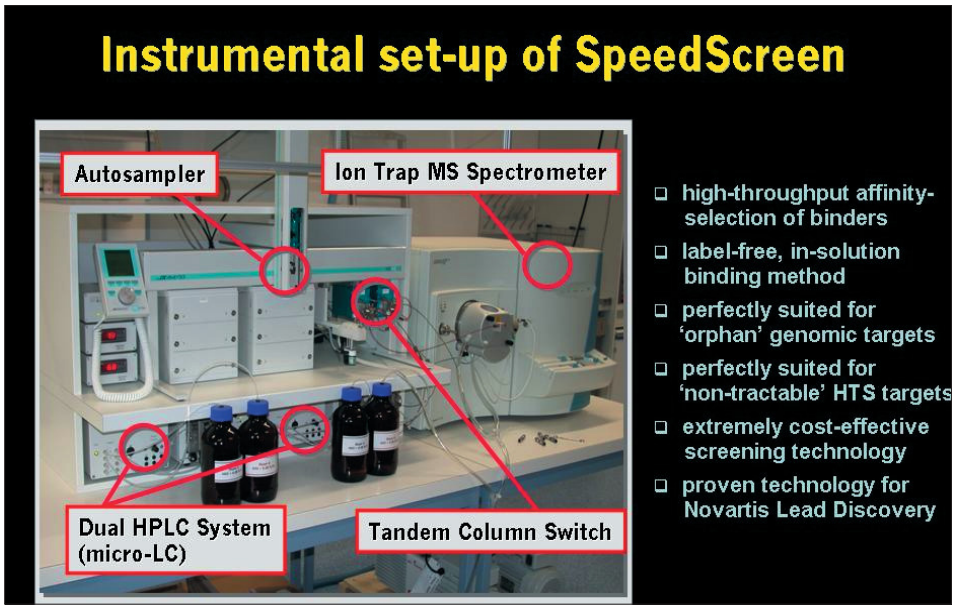

Fig. 3. Instrumental requirements of SpeedScreen. The current SpeedScreen set-up consists of an LC/MS system consisting of a gradient dual HPLC pump (micro LC) with an online eluent degasser, a temperature controlled automated sample injector Autosampler) and an ion trap mass spectrometry detector. In addition, to increase the throughput of the system, a tandem column switch was added. While one column is used for reversed phase chromatography, the second is equilibrated. The panel on the right summarizes the most important key features of SpeedScreen.

Discussion

SpeedScreen was developed to cope with increasing numbers of non-tractable HTS targets, as well as orphan proteins from functional genomic projects. It represents a cheap, simple but highly effective novel screening technology. The discovery of small molecular weight ligands by affinity selection facilitates the chemical validation of these disease targets. Orphan target proteins lack information about functional activity or mode of action and are therefore difficult to funnel into standard HTS programs. With our screening method, there is no need for known inhibitors or reference compounds, as this screening methodology does not rely on a competitive binding assay. This setup not only facilitates the identification of binders, which are then assayed for functionality in secondary assays or are utilized as tools to dissect activity of orphan targets or pathways, but also allows us to simultaneously identify binders to several binding sites on the target protein. As we detect the ligand by its chromatogram and mass spectrum (LC/MS), there is no need to label the protein or the compounds, broadening the applicability of SpeedScreen to nearly all compound sources (*e.g.* compounds from medicinal chemistry, combinatorial chemistry, natural products), as well as nearly all classes of target molecules (*e.g.* proteins, RNA, DNA, glycolipids, virus particles, *etc.*). Incubation conditions can be chosen according to the requirements of the target protein and therefore, optimal binding conditions can be easily adjusted. In addition to the screening of pooled archive compound samples, this technology is also applied for the de-convolution of complex mixtures like natural extracts or combinatorial synthesis mixtures.

In contrast to various reports about affinity selection of ligands combined with hyphenated analytical techniques [4–8] we see the strength of our methodology in the simultaneous processing of the incubation mixtures by using the 96-well plate-based sandwich format. SpeedScreen has been integrated productively into the lead discovery process of Novartis for the identification of binders to targets from genomic origin as well as to targets where conventional bioassays did not fulfill high throughput requirements. This technology is beginning to deliver ligands for both orphan targets (tools for target characterization and validation) as well as conventional targets (compounds entering medicinal or combinatorial chemistry programs).

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