Drug Discovery: Recent Progress and the Future

Review

Revolution of Small Molecule Drug Discovery by Affinity Selection-Mass Spectrometry Technology

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Affinity selection (AS)-MS is a label-free binding assay technology for the analysis of interactions between targets and small drug molecules, which does not require modification of targets or compounds. AS-MS technology has been used in drug discovery research for more than 10 years, and is currently one of the most important affinity-based screening techniques. As such, it may be the driving force for novel small molecule drug discovery. This review introduces the principles of AS-MS technology and its use in high-throughput screening (HTS), then discusses strategies for its use in drug discovery and its application in target identification.

Key words affinity selection-MS; label-free binding assay; high-throughput screening; target identification

1. Introduction

Hit compound identification is the first step for small molecule drug discovery, and most conventional high-throughput screening (HTS) assays such as enzymatics or G-protein coupled receptor (GPCR) signaling are based on the functional activity of the target. However, there are only a limited number of drug targets that can be developed using functional HTS screening, and there are many promising drug targets for which HTS assays cannot be developed. Affinity-based screening technologies have been developed to serve such situations.^{1–7)} In contrast to classical biochemical HTS techniques, these are applicable to any drug target. Affinity selection (AS)-MS, which uses size-exclusion techniques to separate target-compound complexes from unbound compounds and to identify bound compounds using LC/MS, is one of the most common approaches. Some pharmaceutical companies have in-house AS-MS technology, with each company evolving the technology independently.^{8–11)} SEEDSUPPLY's version, Binder Selection Technology that we have been developing for over 10 years, falls into this category.

In contrast to target-based drug discovery, phenotypic screening is an important strategy for drug discovery research. However, it can be difficult to identify the target protein of the bioactive compound. To enrich success probability of target identification, we and others have developed a novel label-free technology for target identification using AS-MS.^{10,12)}

2. Principles of AS-MS Screening

In AS-MS techniques (Fig. 1), compounds are reacted with a target protein, and the reaction mixture is subjected to size-exclusion chromatography. Small test compound molecules are retained in the column, while larger molecules such as the target protein pass through with bound compounds. Bound compounds are then dissociated and identified using LC/MS.

This final step renders labeling of compounds unnecessary, and even if compounds are mixed, each compound can then be analyzed individually. As such, AS-MS can be regarded as label- and immobilization-free. Compounds with a dissociation constant (K_d value) of 10 μ M or less can be identified,¹⁾ and soluble proteins can be used as targets. In addition, membrane proteins that are not solubilized from the membrane (*i.e.*, the microsomal fractions) can be used as targets for SEEDSUPPLY's technology.

3. Application of AS-MS Technology to HTS

At SEEDSUPPLY, we use approximately 400000 compounds, with 200 compounds per reaction mixture for HTS.

3.1. HTS for Unexplored Drug Targets AS-MS technology can be used in HTS for every kind of target, even those for whom measuring functional activity is difficult. By using AS-MS screening in addition to conventional drug targets, unexplored drug targets can be handled (Fig. 2). For example, if transporters are in a plasma membrane, assay systems for the uptake of RI-labeled substrates can be constructed, but it is difficult to measure functional activity if they are localized within intracellular organelles, which makes them impossible to screen using classical biochemical HTS technology. Using AS-MS screening, these transporters can be examined using HTS, and binding compounds can be identified. SEEDSUPPLY has identified binding compounds for various types of transporters localized in intracellular organelles. Functional assays such as cell phenotyping can be performed to validate these binding compounds. As such, the use of AS-MS screening means that functional assay throughput does not have to be high. Recently, the application of AS-MS screening to RNA strands has also been reported.¹³⁾ Furthermore, reaction conditions can be flexible, permitting the addition of substrates, ligands, cofactors, or other adjacent

compounds. This enables the identification of binding compounds with various properties. For example, if a substrate is added to a binding reaction mixture of enzymes, it can be expected that inhibitors with various modes such as competitive, non-competitive, and uncompetitive types will be identified as binding compounds. By adding substrates or ligands in excess, it will be also possible to selectively identify compounds

that bind to allosteric target sites.

Notably, one disadvantage of AS-MS screening is that the functional activity (inhibitory or activating) of binding compounds are unknown when the compounds are identified. There is possibility that binding compounds have no functional activity or that binding compounds adsorb non-selectively to targets. However, in our variation studies, most of the identified binding compounds had functional activity. At SEEDSUPPLY, more than 70% of the binding compounds of various channels and almost all binding compounds of GPCRs, transporters, and enzymes had functional activity. Moreover, binding compounds have functional activity with a high probability since compounds identifiable by AS-MS technology have an affinity of about $10\mu\text{M}$ or less, and are likely to bind to the pocket of targets. These pockets are likely to be ligand binding sites for GPCRs and substrate binding sites for transporters and enzymes, and compounds that bind to these pockets will modulate the function of targets. Compounds that bind to allosteric sites can also be identified by AS-MS screening.^{1,14)}

3.2. Drug Target Prioritization Using AS-MS Screening After assay development during AS-MS screening, HTS can proceed quickly. At SEEDSUPPLY, HTS is initiated as soon as a target protein has been prepared, with about 3 months required for identifying binding compounds and calculating binding affinities. Next, in AS-MS screening, it is not necessary to develop an assay system for each target, so numerous types HTS campaigns can be performed in parallel. At SEEDSUPPLY, we can perform more than 100 HTS campaigns per year with limited resources. The speed and processing capacity of AS-MS screening can contribute to drug target prioritization strategies. By using AS-MS screening, it is possible to perform comprehensive HTS against a number of candidate targets, and prioritize them based on results and functional assays of the hit compounds. A study of target prioritization has been reported using the nuclear factor- κB (NF- κB) pathway.¹⁵⁾ This enables strategic allocation

Fig. 1. Schematic Diagram of AS-MS Technology
(Color figure can be accessed in the online version.)

Fig. 2. Development of Unexplored Drug Targets by AS-MS Screening
(Color figure can be accessed in the online version.)

Fig. 3. Schematic Diagram of Target Identification Technology Using AS-MS
(Color figure can be accessed in the online version.)

of resources on promising drug targets, saving considerable money and time.

4. Application of AS-MS Technology in Target Identification

Phenotypic screening is useful in drug discovery as an alternative to target-based screening, and many drugs on the market have been discovered using this approach. However, it is still challenging to identify real targets for bioactive compounds. Traditional technologies of target identification, such as affinity pull-down and photoaffinity labeling, require modification of compounds and considerable input of resources. AS-MS technology does not require such modification, and can identify both soluble and membrane proteins as targets.

We and others have developed novel label-free based target identification technology for small molecules by combining AS-MS technology and an *in vitro* expressed human protein library,^{10,12} referred to as Open reading frame (ORF) expression/AS-MS (Fig. 3). The human protein library of >18000 proteins was prepared by using a wheat germ cell-free protein synthesis system and a mammalian cell expression system. In the mammalian cell expression protein library, target proteins are expressed and cells are physically disrupted. Using this method, membrane proteins are maintained *in situ*, enabling the ORF expression/AS-MS platform to handle membrane proteins as well as soluble proteins. These protein libraries contain one known protein per well. In ORF expression/AS-MS, compound binding assays are performed for all proteins in a library. If the compound binds to the protein in a well, it will pass through chromatography in complex with the protein, and its signal can be detected. Since the protein in each well is known, it is possible to conclude which proteins bound the compound examined in this system. The macrolide immunosuppressants FK506 (Tacrolimus) and rosiglitazone, a peroxisome proliferator-activated receptor- γ (PPAR γ) agonist, were used to validate this technology platform, and FK506 binding protein family (FKBPs) and PPAR γ were identified as their binding proteins (Fig. 3). In addition, as an example of an anti-cancer agent target, GLUT8 (SLC2A8) has been identified as a target.¹²⁾

There are major advantages of ORF expression/AS-MS platform compared to popular pull-down type experiments. First, ORF expression/AS-MS platform does not require any modification of the compound to experiment. This makes target identification easier for any types of small molecule compounds. Second, unlike pull-down type experiments using cell extracts, ORF expression/AS-MS platform does not depend on the expression level of the target protein in native cell because individual proteins are overexpressed. Third, ORF expression/AS-MS works very well for identification of membrane proteins of native forms.

5. Conclusion

AS-MS technology is a simple, accurate, and versatile label-free binding assay technology, which can be applied to HTS and target identification. Furthermore, AS-MS technolo-

gy can be used to classify compound binding sites¹⁾ and determine the dissociation rate constant of compounds.¹⁶⁾ Recently, in addition to classical small molecule approaches, various therapeutic modalities such as antibodies, peptides, and gene and cell therapies have come under development, and small drug molecules are being investigated for target protein degradation and small molecule drug targeting of RNA chains. In this environment, the value of small molecule drug discovery will refine and likely increase, and AS-MS technology can contribute to these efforts.

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Conflict of Interest I am director and Chief Technology Officer of SEEDSUPPLY INC.

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