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Review

A review of high throughput technology for the screening of natural products

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Abstract

High throughput screening is commonly defined as automatic testing of potential drug candidates at a rate in excess of 10,000 compounds per week. The aim of high throughput drug discovery is to test large compound collections for potentially active compounds ('hits') in order to allow further development of compounds for pre-clinical testing ('leads'). High throughput technology has emerged over the last few years as an important tool for drug discovery and lead optimisation. In this approach, the molecular diversity and range of biological properties displayed by secondary metabolites constitutes a challenge to combinatorial strategies for natural products synthesis and derivatization. This article reviews the approach of High throughput technique for the screening of natural products for drug discovery.
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1. Introduction

Natural products represent a rich source of biologically active compounds and are an example of molecular diversity, with recognized potential in drug discovery and development [1—5]. Despite changing strategies in natural product research, in which concern sample selection and collection, isolation techniques, structure elucidation, biological evaluation, semisynthesis, dereplication, biosynthesis, as well as optimisation of downstream processing [6,7] the rate of discovery of truly novel natural product has actually decreased. Reasons for this fact are related to high costs and time consuming of conventional programs in natural products [8,9], which led to the exploitation of modern high-throughput screening by the pharmaceutical industry, to generate new drug [10—12]. However, far from being competitive, natural product chemistry should complement on a synergistic perspective, since nature continues to be the most diverse and active compound library known [13—15]. Table 1 describes the some traditional as well as recent drugs of natural origin.

High-throughput screening (HTS) is an approach to drug discovery that has gained widespread popularity over the last six or seven years. HTS is the process of assaying a large number of potential effectors of biological activity against targets (a biological event). Many large companies study hundred targets or more each year, and in order to progress these targets, lead compounds must be found. Increasingly, pharmaceutical companies are relying on HTS as the primary engine driving lead discovery. The HTS process is a subset of the drug discovery process that can be described as the phase from Target to Lead. This phase can be broken down in to different steps as shown in Fig. 1.

2. Technology enabling high-throughput screening

The fundamental requirements for high-throughput screening are the availability of chemical libraries for testing and the

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Table 1
Drugs based on natural products

Traditional	Recent examples
Atropine	Artemisin
Ephedrine	Atracurium
Morphine	Catropil
Physostigmine	Ivermectin
Quinine	Pravastatin
Tubocurarine	Taxol

capacity to test them in a rapid fashion. Pharmaceutical companies have developed large collections of chemical compounds. Some of these collections, developed over many years, and in some cases recently expanded through technology for many hundreds of thousands of samples. Synthesis of many analogs during lead optimization of successful products, in some instances, has been thought to bias the range of chemical diversity in these corporate collections. Computational tools offer a variety of means for assessing diversity in chemical libraries. Numerous early efforts were made to use selected molecular descriptors to develop diverse as well as focused libraries of compounds. The primary aim of these efforts was to define libraries that could be optimized for testing against a specific molecular target and by doing so improve screening efficiency. While these electronic descriptors are a convenient way of defining chemical structures, the selection of which method to use, and the underlying features coded in different molecular descriptors will critically determine the composition of a focused library [16]. An additional component of these analyses involves the statistical methods used to define structural classes. A large number of approaches are currently available that involve supervised and unsupervised clustering methods [17–22].

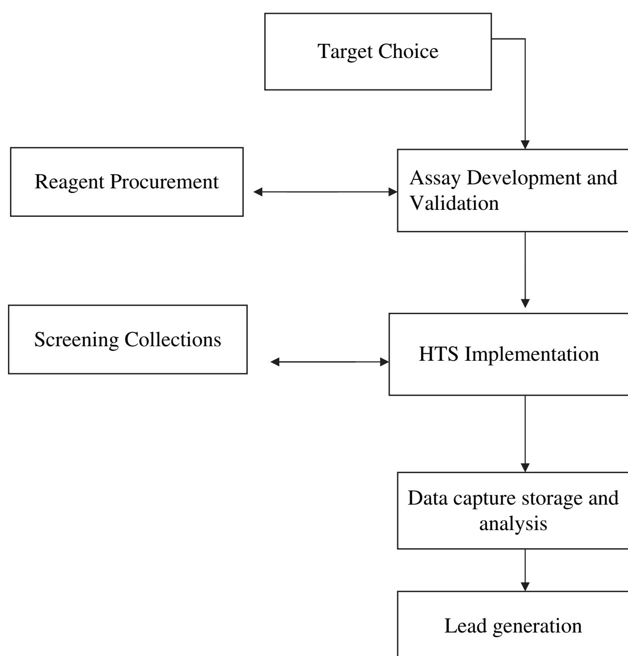

Fig. 1. Figure showing the Lead development from natural products. First phase of Lead generation is target choice then assay development and data capture storage and analysis and final phase is the Lead development.

2.1. Formats of HTS assay

Due to the need to process thousands of assays per day, HTS has revolved around the combined world of multiple-well microplates and robotic processing. For a number of years, HTS assays have been run in the standard 96-well microplate (working volume of up to 250 µL). The current goal of most companies is to move beyond this format to higher-density, lower-volume formats (e.g., 384 and 1536 well microplates). There are two primary advantages of these formats: increased throughput and lower volume, which translates into lower cost. At screening rates of 500,000 compounds/week, a cost of $1 per well is difficult for any company's budget to support on a weekly basis. The reduction of cost, rather than increase in throughput, is the primary driving force within most HTS groups to move to higher-density, lower-volume microplates. HTS is only one step in the early drug discovery process. Other steps include compound library construction, secondary screening, and compound library optimization through medicinal chemistry. Many companies that have developed automated HTS systems find their drug discovery rate-limiting step is often assay/target development or medicinal chemistry. The time frame for these steps often exceeds the 2–3 months it will take to screen a compound library. However, the increased throughput is obviously a welcome benefit of high-density plate formats.

3. Design of assay

The first-pass HTS assays (the primary screen) are less quantitative than traditional biological assays. Often, compounds are only tested in duplicate (an increasing number of companies are using singlets), and usually at one concentration (most often in the 1–10 µM range for combinatorial chemistry libraries). If a "hit" (positive) is discovered, more accurate secondary assays are used for follow-up, quantification. HTS uses standard assay types known to most biological and biochemical scientists (e.g., ELISA, proliferation/cytotoxicity assays, reporter assays, and binding assays). However, adaptations of these assays have emerged to facilitate throughput and relieve robotic complexity. Screeners define assays as either heterogeneous or homogeneous. Heterogeneous assays require steps that go beyond simple fluid additions, incubations, and reading (e.g., filtration, centrifugation, and plate washing steps). Table 2 summaries the procedure involved in natural products drug discovery.

3.1. Cell-free HTS assays

Among the simplest cell-free assays are those that directly assess interaction of proteins with peptides or nucleic acids. These can be conducted in simple aqueous buffers either as ELISA-style assays with target protein immobilized on assay plate surfaces or in solution, using fluorescence polarization

Table 2
Overview of procedure involved in natural products drug discovery

Sample/compound	Target identification development
High throughput	Screen technology, robotics
Primary hits	Multi-parametric criteria advanced informatics
Chemicals	Rapid selection quality
Chemical investigation	Biological profiling development and scale up elucidation
Quality drug leads	Chemical modification, Biological evaluation, clinical study
Lead optimization/drug development	

(FP) or other detection technology. As an example of the former approach fluorescence polarization technology is well suited to high-throughput screening since inhibition of macromolecular binding can be done rapidly, in solution, without any washing steps [20−22]. The availability of adequate supplies of well-characterized probe and target protein molecules is fundamental to development of HTS assays. Custom made, synthetic peptide or oligonucleotide probes labeled with fluorescein or other fluorescent dye molecule can be obtained commercially. Molecular target proteins produced by recombinant DNA technology, can be characterized by LC/MS to assure purity and identity of the reagents. Conventional modes of fluorescence detection can be very useful for HTS assays. However, application of fluorescence detection in assays utilizing crude natural product extracts can be problematic, due to the high degree of intrinsic fluorescence of many kinds of extracts, especially plant extracts. Use of Europium-labeled reagents and time resolved fluorescence detection, provides one approach to dealing with this problem [23].

3.2. Cell-based HTS assays

Cell-based assays can be utilized to assay drug effects on generalized pathways or more specific targets [24]. Utilizing commercially available reagents (Homogeneous Caspases Assay, Roche Molecular Biochemicals, Indianapolis, IN). Shoemaker et al. [25] have implemented a high-throughput, homogeneous screening assay for the detection of apoptosis applicable to a variety of human tumor cell lines. Activated caspase activity (aggregate activity of cellular caspases) is assessed as liberation of free rhodamine following incubation of treated cells with rhodamine-labeled (quenched) substrate solution. Unquenched rhodamine fluorescence is measured on a plate reader. Parallel assay plates were assayed for cytotoxicity using a SRB assay [24]. Compounds producing a five fold or greater increase in fluorescence intensity were defined as ‘‘active’’ inducers of caspase activity. A true high-throughput screening campaign would evaluate cytotoxicity in secondary testing. Cellular reporter constructs have found wide application in basic cell and molecular biological research. Non-radioactive versions have largely supplanted the classic chloramphenicol acetyl transferase (CAT) assays done on TLC plates and other reporters such as green fluorescent protein or luciferase have grown in popularity. These latter assays can be very sensitive and convenient for HTS.

3.3. A successful HTS operation

There are two central components that will predict success of an HTS operation: screening assays and compound collections. The screening assay determines the type of biological activity we are searching. Generally speaking, bad assays create bad hits, and good assays do not necessarily create good hits. This problem is usually directly related to the assay’s clinical relevance. Most high-throughput screening is concerned with simple biochemical screening-testing a chemical compound’s ability to interact with a given protein target. These approaches are inherently distinct from activity in a living organism, and our ability to reach conclusions about a compound’s activity and relevance in a clinical setting are limited. Firstly, we need to consider the issue of specificity. Even if a compound interacts as desired with a target, this does not preclude its interaction in an undesirable manner with other targets, once inside the complex human body. Secondly, the problem of inherent complexity of cells and the human body needs to be considered. It is entirely possible that the biochemical interaction with a target can no longer be repeated once the interaction takes place within the environment of a living cell where all enzymes, salts, cofactors and nucleotides are present and located at their correct positions. Thirdly, most diseases are the result of a number of molecular aberrations and although our understanding of these complex interactions is constantly advancing, it is still only partial for most diseases. Of course, basing HTS screening on partial information is likely to affect the quality and relevance of the screening models chosen and thus the hits discovered. The second component, the compounds, is potentially even more important than the assays. A chemical library can be anything from an historical collection of molecules synthesised over the years in the course of different drug programs, through combinatorial compound libraries deliberately synthesised with the purpose of reflecting a certain chemical diversity or likeliness, to collections of plant and other nature-derived materials. In most cases, the number of compounds contained therein and the breadth of chemical diversity can be used as proxies for library quality. However, when looking at a library, one should not ignore factors such as drug likeliness (good drug properties), the number of related clusters in the library, IP considerations and availability of larger quantities of the compounds for follow-up studies.

3.4. HTS trends

There are a number of trends that are worthwhile following at present. Firstly, there has been a vast increase in the use of cell-based assays, allowing the testing of compounds directly in living cells. Secondly, smarter screens that incorporate both efficacy and specificity in the same assay have been developed. Thirdly, there has been the utilization of high-throughput approaches for functional genomics. Fourthly, there are assays and approaches that start to take into account the industry’s biggest problem, which remains the high attrition rates throughout the development process. Finally,

systems that bridge between post-genomic activities and drug discovery are likely to quickly penetrate the market in the future, as the gene-to-drug link becomes a bottleneck in many companies.

4. Problems of the natural compounds for drug screening?

The estimation of many synthetic chemists regarding natural compounds is based on a variety of problems in drug screening: The availability of compounds from their biological source, the chemical complexity and stereochemistry, compound stability, unreliability in assay systems and the most named reason among others: natural product research is very cost intensive. These arguments led to the unilateral trend towards Combinatorial Chemistry as an option to reduce research costs at an early stage of the drug discovery process also with regard to applicability in high-throughput screening (HTS). Natural products many times have been taken out from high-throughput screenings, because the required purity, availability and behavior in these screens did not suffice the afforded requirements. Furthermore, natural product research has been cut down or even discontinued in many firms. However, structural diversity of natural products still surpasses that from synthetic compounds and is far beyond any imagination of experts in the field. Wider access to previously untapped biodiversity and application of modern biological and chemical techniques for producing natural compounds is the key for the aimed structural diversity.

In our laboratory we have screened several plant products for its immunomodulatory activities [26–31]. It takes a lot of time and man power to screen a single plant product. By applying HTP technology we can screen several plant products in a short period of time. The conventional methods used for the screening of natural products for its immunomodulatry activity is described in Table 3.

5. Summary

The HTS field continues to be dynamic and very competitive. However, there is also a good deal of information sharing, even between competing companies. The somewhat open technology transfer meetings and publications have greatly accelerated the development of the industry. It is an industry that

Table 3
Conventional *In-vitro* and *In-vivo* tests for evaluation of immunomodulators

The agent can be added to *in-vitro* cultures or administered to experimental animals (with or without specific antigen) and thereafter the following tests can be performed.

1. Immunosuppressant activity:

***In-vitro*:** a) Inhibition of lymphocyte proliferation and cytokine production of either Th1 (IL-2, IFN-γ and TNF-α) or Th2 (IL-4, IL-5 and IL-10) type following stimulation with mitogen or specific antigen.
b) Inhibition of antibody forming cells in Jerne's plaque assay and antibody production.
c) Inhibition of IL-1 and TNF-α production by macrophages in response to LPS.
d) Inhibition of NK cell activity in absence or presence of IFN-γ.

***In-vivo*:** a) Suppression of antibody production to specific antigens.
b) Suppression of DTH response to specific antigens.
c) Suppression of clearance by reticuloendothelial cells.
d) Suppression of induction/progression of autoimmune disease in experimental animal models.
e) Prevention of allograft (or xenograft) rejection.

2. Immunostimulant activity:

***In-vitro*:** a) Stimulation of lymphocyte proliferation and cytokine production of either Th1 (IL-2, IFN-γ and TNF-β) or Th 2 (IL-4, IL-5 and IL-10) type following stimulation with sub-optimal dose of mitogen or specific antigen.
b) Quantitation of the expression of cell activation markers on cell surface viz. CD25, CD69, CD80 and CD86 by fluorescence activated cell sorter (FACS).
c) Quantitation of different lymphocyte sub populations by FACS- CD3, CD4, CD8, CD16/CD56, CD19, CD20 and CD45.
d) Augmentation of NK cell cytotoxicity.
e) Stimulation of IL-1 and TNF-α production by macrophages in response to LPS.
f) Stimulation of antibody plaque forming cells and antibody production *in-vitro*.

***In-vivo*:** a) Stimulation of antibody titer to specific antigens
b) Stimulation of DTH to specific antigens.
c) Evaluation in rodent malaria and Japanese encephalitis virus models etc. for animal protection against infections with or without chemotherapy.
d) Spleen foci (colony formation) assay as a measure of radioprotection.

3. Immunoadjuvant activity:

The agent is administered in combination with potential vaccine and the effect is estimated by:
a) Specific antibody profile and titer in immunized animals (*in-vivo*).
b) DTH response to the specific antigens (*in-vivo*).
c) Jerne's plaque assay using spleenocytes from immunized animals (*in-vitro*).
d) Lymphocyte proliferation test using splenocytes from immunized animals against T-cell epitopes of the immunizing antigen (*in-vitro*).
e) Th1/Th2 cytokine profile (*in-vivo*).

4. Effector arm of the immune response:

a) Chemokine levels in the treated animal and *in-vitro* assay by chemotaxis.
b) Inhibition/stimulation of phagocytosis (*in-vitro*/*in-vivo*).
c) Free radical production (*In-vitro*).
d) Nitric oxide production (*in-vitro*).

has been significantly driven by implementing technologies from vendor companies rather than through developments occurring within drug research companies. The future will certainly hold change as the industry strives to reach such high-density formats as the 1536-well plate, and such technologies as microassay systems using nanoscale capillaries. However, for the moment, these technologies are still a few years away from routine in-house screening.

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