Combined in silico and in vitro Approach to Drug Screening*

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The purpose of High Throughput Screening (HTS) in pharmaceutical industry is to identify, as soon as possible, compounds that are good starting points for successful new drug development process. Experts from this area study the chemical structures of so called »hit« compounds that have been found to interact with the target protein, interfere with proliferation of different types of cells or stop bacterial or fungal growth. Hypotheses to design related structures with improved biological properties are then builded. Each idea is then tested by the iterative synthesis and testing of novel compounds in various biological assays, searching for hits with better properties and defining useful and promising »lead« molecules. In parallel, molecular modeling and chemoinformatics experts can increase efficiency and decrease experimental costs by using different database filtering methods. In such a way, hits from HTS may be assessed before committing significant resource for chemical optimization. Joint efforts of these HTS experimental and modeling groups are the best way to speed up the process of finding a new useful hits and promising leads.

Keywords
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INTRODUCTION

The new century has started with the characteristics of being landmark period for the science, medicine and drug discovery, as well. The drugs developed over the last four decades are targeted at about 500 different biological targets. With the sequencing of the human genome, over 100,000 new biological targets will be recognized. It has been estimated that at least 10 % of them could be used as targets for new medicines. Over the past decade, a variety of scientific advances and economic pressures have driven the need for improved drug discovery screening technology.2

To increase the number of new lead molecules (molecules with promising activities), many pharmaceutical companies made significant investment in combinatorial chemistry and High Throughput Screening (HTS) during 1990’s. Scientific and technological advance in the experimental technology, especially in solid-state synthesis, increased the number of synthesized molecules rapidly. Automation in HTS allows testing of hundreds or even thousands of compounds per day. However, in spite of the expectations, boost in the number of synthesized and tested molecules did not increase the number of candidates for lead optimization. Soon, it became obvious that additional efforts must be devoted to the design of compound libraries.2,3

Improvements in HTS efficiency can be achieved by applying different in silico methods. In silico screening

* Dedicated to Dr. Edward C. Kirby on the occasion of his 70th birthday.
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(virtual screening) is a relatively inexpensive method for identifying potential interactions among compounds and selected targets. By in silico screening, prior to any experimental work, subset of selected compounds can be enriched only with molecules likely to be hits for a considered target molecule.

METHODS

In Silico Approach

Starting compound database is usually reduced to the representative set (RS) in order to avoid redundancy in the chemical space, improve quality of screening library and thus increase screening efficiency. The RS of compounds is often selected through several consecutive steps as shown in Figure 1 and briefly described in the following text.

Preparation of Compound Database. – It is common that compounds are present in a starting database (regardless whether it is a company or commercially acquired database) in multiple forms, such as different acidic forms, tautomers, stereoisomers or mixtures. From the computational aspect this produces redundancy that can cause data management problems and incorrect results. Hence, all multicates should be removed at the beginning of the RS design. For this purpose, all original compounds are written in unique standardized manner, such as, for example, Daylight’s SMILES (Simplified Molecular Input Line Entry Specification) notation. Such a notation can be used in further filtering steps based only on two-dimensional (2D) information from connectivity tables of compounds. The »cleaning« procedure resulted in a prepared database (Figure 1).

Depending upon the type of the starting database and the purpose of designing RS, the prepared database can be filtered in various ways, according to physicochemical or pharmacokinetic properties, by using simple rule-based filters to complex neutral network models. In the case of databases composed of small molecules ($M_r < 500$) in the early phase of drug discovery, the prepared database is often filtered for drug-like compounds.

The commonly used drug-likeness filter is based on the 2D »Lipinski rule of 5« (LR5). According to the LR5, a compound that does not satisfy two or more of the four LR5 criteria: (i) molecular weight ($M_r \leq 500$); (ii) ClogP $\leq 5$; (iii) number of hydrogen bond donors (HBD) $\leq 5$; (iv) number of hydrogen bond acceptors (HBA) $\leq 10$; is likely to have poor solubility and/or permeability and thus poor oral bioavailability. In hit- and lead-discovery phase, the filtering borders in LR5 are put at a lower level (e.g. $M_r \leq 400$, ClogP $\leq 4$, HBD $\leq 4$, HBA $\leq 8$) because it is expected that during optimization phase additional functionalities will be added. Filtering based on a number of rotatable bonds in molecule can also be used in order to increase the share of drug-like molecules in RS.

Number of compounds can be further reduced by eliminating those with reactive functional groups and unsatisfied absorption, distribution, metabolism, excretion (ADME) and toxicity (Tox). In the ADME filtering, properties such as aqueous solubility, human intestinal absorption (HIA), Caco-2 permeability, and Blood-Brain Barrier (BBB) permeability are often considered. The filtering borders should be quite loose since this sifting is performed in very early stage of drug discovery process, and are highly dependant on the type of the project for which the filtering is being performed. There are many commercially available programs for estimating ADME properties of small molecules ($M_r < 500$), such as those in VolSurf, QikProp or C2.ADME.

Design of Representative Set. – Filtered database, obtained as described in the previous section (Figure 1), is enriched with drug-like molecules and is further reduced to RS for the purpose of screening, in silico or wet.

The RS can be selected in various ways using dissimilarity based methods, partitioning or clustering. Regarding clustering of structurally similar compound, various clustering techniques exist. In addition, numerous distances and coefficients have been proposed and used for similarity evaluation. Use of Daylight binary fingerprints as structural descriptors and Tanimoto coefficient (TC) as a similarity measure is a common practice, in spite of theirs limitations. TC has dem-

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onstrated its value in measuring intermolecular similarity for molecules represented by binary fingerprints:

\[ \text{TC}(a,b) = \frac{N_{ab}}{N_a + N_b - N_{ab}} \]  

(1)

where \(N_{ab}\) is the number of bits in common to both molecules \(a\) and \(b\) that are set to on (1); \(N_a\) represents number of bits in molecule \(a\) that are set to on (1), but which are off (0) in molecule \(b\); and \(N_b\) is the number of bits in molecule \(b\) that are set to on (1), but which are off (0) in molecule \(a\).

Two commonly used algorithms for clustering molecules represented by Daylight’s fingerprints and using TC as a measure of similarity between them are Jarvis-Patrick’s (J-P) algorithm\(^{25}\) and dbclus.\(^{26}\) Dbclus identifies dense clusters where similarity within each cluster reflects the TC value used for clustering. The cluster centroids, unveiled by this algorithm, will be at least similar, at the given TC value, to every other molecule within the cluster in a consistent and automated manner. The threshold for TC varies, dependent on the purpose of clustering performing.

In silico Screening. – Prior to any experimental work, the RS can be screened in silico in order to enrich it with molecules likely to be hits for a considered target macromolecule.\(^{27-29}\) By in silico screening, the limited subset of molecules is selected for biological testing to identify novel chemical entities affecting a selected target molecule. Such a selected subset contains significantly more hits than a randomly chosen set. Efficiency of in silico screening can be expressed in terms of true positive rate (hit-rate), false positive rate and enrichment factor.\(^{29}\)

The way of performing in silico screening depends upon the amount of structural and other knowledge about the target and ligands. In silico methods can be roughly divided into 2D and three-dimensional (3D) similarity searching approaches, ligand based pharmacophore mapping, target based pharmacophore mapping and molecular docking and scoring. Each approach has its virtues and shortcomings. No single in silico methods has confirmed generally accurate and superior to others. Hence, different methods are often combined together in a consensus way to improve efficiency of in silico screening.

Efficiency of most in silico methods depends upon preparing compounds. In the RS, each compound is usually written in unique, standardized 2D form. It is well-known that different ionic species, tautomeric forms and/or different stereoisomers of the same compound can have significantly different binding affinities for the same target macromolecule. Hence, prior to in silico methods, compounds should be transferred into proper ionization state(s), all possible tautomers or at least those that are energetically the most preferred (degeneracy)\(^{30,31}\) and all topologically and sterically possible or only the most stable stereoisomers.\(^{32}\) Furthermore, for each species, a reliable 3D structure(s) should be generated. The widely used programs for generation of 3D structures are CONCORDER\(^{33,34}\) and CORINA.\(^{35}\)

On the other hand, results in the case of structure-based VS such as molecular docking and scoring\(^{36,37}\) depend upon a quality of 3D structure of a target macromolecule. Selection of target macromolecule in pharmaceutical industry is based on several key points: (i) macromolecule should be recognized as a therapeutic target, and should be present in specialized databases, such as »Therapeutic target database«;\(^{38}\) (ii) its X-ray structure must be available; (iii) metabolic disorder connected to the protein function should be described.

The preparation of target in molecular docking is influenced by the docking algorithm that is going to be used. Specific attention should be paid to hydrogen atom positioning (hydrogen atoms are not present in crystal structures), avoiding atomic clashes and correcting the orientation of active-site hydroxyl-groups. In some cases, water molecules that are buried in the active site play a role in ligand-binding, and they should be kept during the docking. As well, the metal ions can be crucial for the target biological function and should be taken into account in modeling of complexes between a target and ligand in molecular docking.

In addition, efficiency of 3D VS depends very much upon extension of structural flexibility taken into account. Most commercially available methods treat explicitly flexibility of ligands. Although important, target flexibility is an extremely complex problem and is usually not explicitly taken into consideration. The docking tools take the protein as being fixed and use its crystal structure conformation. In some docking software the hydroxyl groups are allowed to rotate.\(^{39}\) However, in most state-of-the-art docking software FlexE\(^{40}\) target flexibility is modeled by taking ensemble of various available X-ray structures.

In VS, based on molecular docking and scoring, by modeling complexes between a target and ligand, the binding mode and binding affinity are predicted. The widely used docking tools include GOLD\(^{39}\), FlexX\(^{41}\) and DOCK.\(^{42}\) The collection of various docking tools was compiled by van Leeuwen\(^{43}\) and can be used for further reference. It has been thought that the limitations of this VS approach mostly come from insufficient accuracy of scoring functions used as approximations of binding affinity.

In vitro Screening

Compounds Library. – The prerequisite of successful screening process is to have good compound library. Most drug discovery companies have created their own compound library, although it is also possible to acquire targeted libraries from different commercially available sources. The employees taking care about the library are
responsible for the compound administration (acceptance, sorting, registration and deposition) of new chemical compounds, acquisition of synthetic compounds and natural products (Figure 2). In PLIVA – Research Institute we have developed two brand new applications named ChemClient and BioClient designed with the capacity of having efficient data handling in relation to chemical compounds in general, as well as biological activity of the compounds.44

The strategy of HTS is to establish a successful screening of compound libraries for new therapeutic lead candidates and faster definition of potential targets. The complete integration of screening system is usually resolved and set up on large-scale pipetting platforms (such as TECAN Genesis – Figure 3). Through implementation of robotic system technologies laboratories are capable to increase drug-screening capacity, and to reduce consumption of reagents.

Microbiology Assays. – In the field of microbiology screening, research targets are bacterial microorganisms such as S. aureus, S. pneumoniae, S. pyogenes, E. coli, M. catarrhalis, H. influenzae etc. and fungi such as Saccharomyces cerevisiae, Candida spp. etc. Primary screening for novel compounds is done on a defined set of microorganisms which enables to detect basic activity as well as activity on resistant organisms carrying different resistance genes and exerting the resistance by several mechanisms.

Strains are cultured on Miller-Hinton agar (Merck, Germany) except strains belonging to genus Streptococcus and Haemophilus which are cultured on blood agar plates (Biomerioux, France) and chocolate agar plates (Biomerioux, France), respectively. Minimum inhibitory concentration (MIC) is determined by the microtitre liquid dilution method (Figure 4) as described is standard operation procedure,45 except that for Streptococcus medium. Read out is carried out by eye inspection and minimal inhibitory concentration (MIC) is expressed as lowest concentration showing 90 % of inhibition of control growth.

Cytotoxicity Screening. – Cytotoxicity screening of novel compounds with a defined set of standard cell lines (THP-1, COS, Hep G-2, CHO, A549, H 292, PLB 985, COR-L23, COR-L23R, COR-5010, HL-60, MIA-Pa-Ca-2, HeLa) enables to detect basic possible cytotoxicity effect of novel substances.46 Principle of testing is that cells in phase of growth are exposed to a drug. The cells are allowed to proliferate in order to distinguish between cells that remain viable and are capable of proliferation and those that remain viable but cannot proliferate.

Measuring the succinate dehydrogenase mitochondrial activity of living cells by specific reagent 3-(4,5-
dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) the amount of viable cells can be detected (Scheme 1). The enzyme turns the MTT into a brown color by cutting the tetrazolium ring. The amount of produced MTT-Formazan can be determined spectrophotometrically at 490 nm.47

Another approach is used in order to establish anti-proliferation assays of human or animal blood cells and other primary cell lines – bioluminescent adenosine triphosphate (ATP) cytotoxicity screening. This type of screening is intended for the rapid and safe detection of proliferation and cytotoxicity of mammalian cells and cell lines in culture by determination of their ATP levels. ATP can be used to assess the functional integrity of living cells since all cells require ATP to remain alive and carry out their specialized functions.48 The bioluminescent method utilizes an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin according to the following reaction (Scheme 2). The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer or beta counter. The assay is conducted at ambient temperature.49

Gene Expression Analysis (Microarrays). – Transcriptional profiling DNA microarray technique (gene chips) is a relatively new technology. It promises to monitor the whole genome on a single chip. In that way researchers can have a »global« picture about the interactions between the compound (lead) and thousands of genes relevant for the noteworthy disease. The remarkable power of DNA microarrays already has a strong impact on medicine: in the molecular characterization of diseases, drug discovery and evaluation of results. It is to be expected that quantitative applications will soon spread through all fields of life sciences.52

Data Processing and Visualization. – As a systematic response to the challenges of data explosion in biomedical sciences, pharmaceutical research should establish its own informatics solution that will be capable of harnessing the power behind the tidal waves of information sweeping through the drug discovery process. Clustering and dimensional reduction techniques are nowadays used for additional representation of specific and mostly complex relationships among chemical properties and biological activity. Such techniques are putting an emphasis on novel visualizations tools with the attempt to create a system for the automated deduction of important drug-target relationships.

CONCLUSION

In the past four decades about 500 therapeutically relevant targets have been recognized. Today, with the completion of the human genome it is expected that about 100,000 new protein structures will be recognized. Not all of them will be used in developing new therapies, but experts estimate that at least 10 % will be most likely declared as promising therapeutic targets, meaning that their activity can be modulated by external compounds.
and used in the treatment of different diseases. Such an increase of proteins is accompanied by extreme increase of synthesized small molecules, coming from commercial or proprietary databases (which is partially a result of the efforts in combinatorial synthesis).

This results in a combinatorial problem of many targets to which many molecules can be applied and a fast and effective solution is required to solve it. The results obtained by implementing an multidisciplinary approach, combining in silico screening with in vitro experiments (HTS), have shown that it represents the best way to get accurate results in a very short time period, in reagent-saving manner.

REFERENCES

SAŽETAK

Kombinirani in silico i in vitro pristup pronalaženju spojeva s mogućim ljekovitim djelovanjem

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Svrha HTS-a u farmaceutskoj industriji je identifikacija spojeva koji mogu poslužiti kao dobre polazne molekule u procesa razvoja lijeka iz novih kemijskih entiteta. Proučavanjem kemijske strukture takvih »hit« spojeva koji interagiraju s proteinom – metom, stručnjaci iz tog područja tragaju za strukturama poboljšanih bioloških svojstava. Svaka se ideja kasnije provjerava iterativnim postupkom sinteze i testiranja novih spojeva uporabom različitih metoda bioloških probira, kako bi se došlo do hitova s boljim svojstvima i do uporabivih i obećavajućih »lead« molekula. Istovremeno, molekularno modeliranje i kemoinformatika mogu povećati učinkovitost i smanjenja troškova eksperimenta uporabom različitih metoda filtriranja baza spojeva. Na taj način, »hitovi« iz HTS-a mogu virtualno biti procijenjeni prije značajnog ulaganja resursa u kemijsku optimizaciju. Udruženi napori eksperimentalnih HTS grupa i grupa koje se bave molekularnim modeliranjem najbolji su način ubrzavanja procesa pronalaženja novih, uporabivih »hitova« i obećavajućih »leadova«.