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SPECIAL ARTICLE

Overview on the current status of virtual high-throughput screening and combinatorial chemistry approaches in multi-target anticancer drug discovery; Part I

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Summary

Conventional drug design embraces the "one gene, one drug, one disease" philosophy. Nowadays, new generation of anti-cancer drugs, able to inhibit more than one pathway, is believed to play a major role in contemporary anticancer drug research. In this way, polypharmacology, focusing on multi-target drugs, has emerged as a new paradigm in drug discovery. A number of recent successful drugs have in part or in whole emerged from a structure-based research approach. Many advances including crystallography and informatics are behind these successes. Increasing insight into the genetics and molecular biology of cancer has resulted in the identification of an increasing number of potential molecular targets, for anticancer drug discovery and development. These targets can be approached through exploitation of emerging structural biology, "rational" drug design, screening of chemical libraries, or a combination of these methods. The result is the rapid discovery of new anticancer drugs. In this article we discuss the application of molecular modeling, molecular docking and virtual high-throughput screening to multi-targeted anticancer drug discovery. Efforts have been made to employ in silico methods for facilitating the search and design of selective multi-target agents. These computer aided molecular design methods have shown promising potential in facilitating drug discovery directed at selective multiple targets and is expected to contribute to intelligent lead anticancer drugs.

Key words: *combinatorial chemistry, computational molecular docking, computer aided drug design, multi-target drug discovery, signaling networks, virtual high-throughput screening*

Abbreviations: *CADD, computer aided dug design; DTome, Drug-Target Interactome; EGF, epidermal growth factor; HTS, high throughput screening; NMR, nuclear magnetic resonance; PBPK, physiologically-based pharmacokinetic; PD, pharmacodynamics; PK, pharmacokinetics; QSAR, quantitative structure activity relationship; RTKs, receptor tyrosine kinases; SAR, structure activity relationship; VEGFR, vascular endothelial growth factor receptor; vHTS, virtual high-throughput screening; VS, virtual screening*

Introduction

To understand and cure a disease is as difficult as ever. There is an urgent need to make sense of it all, going from sequence to information. Yet, there are no revolutionary insights, because it's a most complicated issue. Medicinal chemists today are facing a serious challenge because of the increased cost and enormous amount of time taken to discover a new drug, and also because of fierce competition among different drug companies. The hope lies in the rational design of new therapeutic agents. Conventional strategy for the discovery of new drugs is to take a lead structure and develop analog molecule to exhibit desired biological properties. The strategy for screening single-target and highly specific agents has been widely researched [1].

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The lead is generally found by chance or random screening. Old strategies for drug design and development involve laborious trial and error approaches. However, many effective drugs are found today by using this approach. The traditional processes are now supplemented by more direct approaches and are derived from understanding the pathogenetic molecular processes of the diseases.

New era in rational anticancer drug design: from single to multi-target drugs in cancer therapy

In the pharmacological industry in the era of full genome sequencing, there has already been a shift from symptomatic oriented drugs to pathology-based drugs whose targets are the genes and proteins involved in carcinogenesis. Drugs targeting the affected pathway have thus the potential to become therapeutic [2].

Pathology-based and target-based drug discoveries constitute two principal approaches in drug innovation, which are mutually complementary and collaborative. Recent developments in biological systems and overall clinical experience have revealed that the single-target drugs may not always induce the desired effect to the entire biological system even if they successfully inhibit or activate a specific target [3,4]. Researchers in efforts to find more effective antineoplastic treatments are looking toward the chemical industry as well as traditional herbal medicines to find multi-target interventions.

Three approaches have proven to be effective in seeking multi-target drugs: (1) designing drugs with multiple components; (2) discovering drugs through the study of synergistic compound-compound interactions in medicinal herbs or among chemical drugs and herbal components; and (3) developing drugs to tackle complex multi-component diseases [5]. Multi-target drugs against selective multiple targets improve therapeutic efficacy, safety and resistance profiles. One major target of multi-targeted agents is the human kinome and especially the receptor tyrosine kinases (RTKs) which are a vital element in regulating many intracellular signal transduction pathways involved in cancer growth.

We are witnessing an explosion of knowledge around signal transduction pathways, impacting virtually all areas of biology and medicine. The exploration of the most significant signaling pathways suitable for the development of new multi-target drugs that are categorized under chromatin modification, MAP kinase signaling, Akt signaling, energy metabolism related signaling, translational control, cell cycle control, immunology and others, is in progress. Therapeutic targets against cancer include tubulin, topoisomerases, various types of tyrosine kinases, mammalian target of rapamycin, phosphatidylinositol 3-kinase, histone deacetylases, poly (ADP-ribose) polymerase (PARP), focal adhesion kinase, AMP-activated protein kinase (AMPK), 26S proteasome complex, and cyclooxygenase, among others [6].

Dual or multiple-target drugs can modulate two receptors, inhibit two enzymes, act on an enzyme and a receptor, or affect an ion channel and a transporter. Thus the original concept of key and lock analogy is modernized into hand-glove analogy obtaining multiple functions at once. From the viewpoint of molecular design, a dual or multiple-target drug molecule can simply be created by combining two or more active molecules or their pharmacophores with a linker. The integrated molecule comes into an entity either by fusing or by merging the common structural or pharmacophoric features of two active molecules, depending on the extent of the common features (Figure 1a,b). Although multi-target action for an agent can be achieved in several ways, it is the coordinated effect at the set of targets that results in the biological and, hopefully, therapeutic effect [7]. This approach facilitates the reduction of molecular size and molecular weight and the consequent optimal overlap between the pharmacodynamic and pharmacokinetic properties certainly elevates its probability of being a marketed drug.

Therapeutic agents directed at an individual target frequently show reduced efficacies, undesired safety profiles and drug resistances due to network robustness, redundancy, cross-talk, compensatory and neutralizing actions [8], anti-target and counter-target activities [9], and on-target and off-target toxicities [10]. Multi-target agents directed at selected multiple targets, have been increasingly explored, for achieving enhanced therapeutic efficacies, improved safety profiles, and reduced resistance activities by simultaneously modulating the activity of a primary therapeutic target and the counteractive elements and resistance activities [11], while limiting unwanted cross-reactivities via optimization of target selectivity [12]. Recent kinase drugs, such as imatinib mesylate and sunitinib [13], though perhaps designed for specificity,

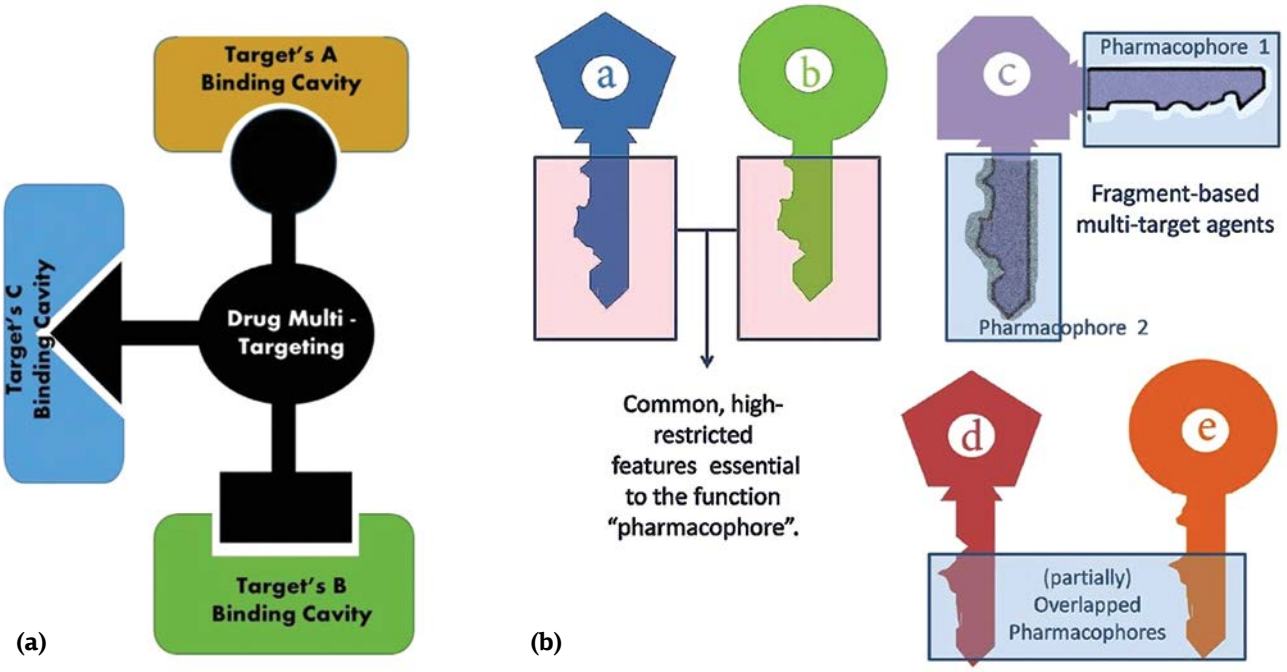

Figure 1. a. Illustration of fragment of the active molecules-based approach to multi-target drug discovery. A multiple-target drug can modulate e.g. three target proteins. A connective molecule (the drug multi-targeting), is combining three active molecules or their pharmacophore fragments (namely, circle, triangle, square) with a linker by fusing or merging the common structural or pharmacophoric features of the active molecules. **b.** Key (ligand) and lock (target) analogy in multi-targeted agents. The shaft of the key (pharmacophore) is more important in eliciting the biologic response than the base of the key which is less subject to structural restrictions. Frequently, different ligands share similar pharmacophores and thus exhibit similar activity (a and b) while some have less overlapping features (d and e). A multi-targeted ligand (c) can contain different pharmacophores that can have biologic effects on different targets. Adapted from Talevi (2015) [89].

modulate multiple targets and these "off-target" activities also may be essential for efficacy [14].

In silico methods have been widely explored for facilitating lead discovery against individual targets [15,16]. The conventional strategy for the discovery of new drugs is to take a lead structure, and develop analog molecules to exhibit desired biological properties. During the past years there has been a steady and exciting growth of novel inhibitors identified through computational analysis of target structure. A combination of more structures, advances in homology modeling, better docking and scoring tools, fragment-based methods, and advances in virtual screening have been fundamental in this progress. In particular, molecular docking [17], pharmacophore [18], SAR and QSAR [19], machine learning [20], and combination methods [21], have been extensively used for searching and designing active compounds against individual targets. Some of these methods have recently been explored for identifying and designing multi-target agents. Recently, a novel method was developed for the systematic *in silico* investigation of synergistic effects of currently available drugs on genome-scale metabolic networks [22]. Wei et al. have developed a computer-assisted strategy to screen for multi-target inhibitors using a combination of molecular docking and common pharmacophore matching [23].

Kinases in cancer

The kinase family is one of the largest target families in the human genome. It is estimated that there are more than 500 members of the major classes of protein serine/threonine, tyrosine, and dual specificity kinases within the human genome [24]. Protein phosphorylation is one of the most significant signal transduction mechanisms by which intercellular signals regulate crucial intracellular processes such as ion transport, cellular proliferation, and hormone responses [25]. Protein kinases represent as much as 30% of all protein targets under investigation by pharmaceutical companies [25,26].

The human protein kinase family is divided into the following groups: (1) AGC kinases - containing PKA, PKC and PKG; (2) CaM kinases - containing the calcium/calmodulin-dependent protein kinases; (3) CK1 - containing the casein kinase 1 group; (4) CMGC - containing CDK, MAPK, GSK3

and CLK kinases; (5) STE - containing the homologs of yeast Sterile 7, Sterile 11, and Sterile 20 kinases; (6) TK - containing the tyrosine kinases; and (7) TKL - containing the tyrosine-kinase like group of kinases.

The kinase dendrograms (http://kinase.com/human/kinome/ & http://www.cellsignal.com/reference/kinase/index.html) show the sequence similarity between protein's catalytic domains. The distance along the branches between two kinases is proportional to the divergence between their sequences. This may be exploited by the approach of multi-target drug design, aiming to intervene in multiple cytoplasmic signaling pathways, including the Ras/Raf mitogen-activated protein kinase (MAPK) pathway, the phosphoinositol 3'-kinase (PI3K)/Akt pathway, the signal transducer and activator of transcription 3 (STAT3) pathway, the protein kinase C (PKC) pathway and scaffolding proteins. The inhibition of multiple growth related kinases, especially tyrosine kinases, at the same time by one drug, might provide new therapies for diseases such as cancer [27].

Tyrosine Kinases: a major target

Protein Tyrosine Kinase Receptors (RTKs) have emerged as new promising targets for cancer therapy. They are essential enzymes in cellular signaling processes and signal transduction pathways that regulate cell proliferation and death, differentiation, migration and metabolism by catalyzing protein phosphorylation and dephosphorylation and tumor angiogenesis [28]. The advances in our understanding of the oncogenic activation of these receptors have been matched by the identification of new structural classes of kinase inhibitors with improved potency, specificity and efficacy [29].

Targeting several RTKs can be done using single agents, the multi-target tyrosine kinase inhibitors (MTKIs), drugs that could dramatically affect the progression of cancer and decrease tumor resistance [28]. Two classes of compounds targeting RTKs are currently used in clinical practice: monoclonal antibodies and tyrosine kinase inhibitors. The first drugs with demonstrated clinical efficacy were mainly inhibitors of the ErbB family of receptors (i.e., EGFR and HER-2), either monoclonal antibodies (MAbs) or tyrosine kinase inhibitors (TKIs). The era of targeted therapy began with the approval of trastuzumab, a monoclonal antibody against HER-2 (human epidermal growth factor receptor 2), for treatment of metastatic breast cancer, and imatinib (STI–571), a small tyrosine kinase inhibitor targeting BCR-Abl, in chronic myeloid leukemia [30].

Despite the initial enthusiasm for the efficacy of single TKIs, clinicians had to face soon the problem of relapse, as almost invariably all cancer patients developed drug resistance, often due to the activation of alternative RTK pathways. In this view, the rationale at the basis of targeting drugs is radically shifting. Different strategies were pursued to inhibit multiple signaling pathways or multiple steps in the same pathway, either by the development of multi-targeted agents or with the combination of single targeted drugs. Now, there is a general agreement that molecules interfering simultaneously with multiple RTKs might be more effective than single target agents.

The approval by FDA (Food and Drug Administration) and EMEA (European Medicines Agency), of the successful anti-angiogenic multi-target tyrosine kinase inhibitors, sorafenib and sunitinib targeting VEGFR, PDGFR (plateled-derived growth factor receptor), FLT-3 (FMS-related tyrosine kinase 3) and c-Kit proteins and the dual tyrosine kinase inhibitors dasatinib against Abl and Src, and lapatinib against EGFR and ErbB-2 (HER-2/neu), marked the coming of age of this new generation of drugs [31]. These multi-target anticancer agents inhibit a primary therapeutic target that promotes tumor growth in a specific cancer patient group and block the alternative signaling or escape mechanism [32]

Many more similar multi-target drugs are undergoing clinical trials for a range of cancer types. Exelixis has developed the XL999 inhibitor by using their Spectrum Selective Kinase Inhibitor technology platform (SSKI) [33]. Each inhibitor developed with SSKI has a different spectrum of RTK inhibition, offering the potential to achieve efficacy through inhibition of multiple RTKs based on their established or potential involvement in cancer. SSKI's target both the tumor and its vasculature and each compound has the potential to maximize efficacy through simultaneous inhibition of multiple RTKs. In pre-clinical testing, XL999 inhibited KDR, FGFR1, PDGFR-beta and FLT3 with high potency.

Other Kinase targets: the options are ever expanding

Although TKIs are the most evaluated kinase inhibitors in clinical trials, other strong kinase target candidates studied for drug discovery in the treatment of cancer are the serine-threonine kinases (Ser/Thr kinase inhibitors, representing

a third of kinase inhibitors, the members of AGC kinase family, containing PKA, PKG and PKC kinase families), aurora kinases (playing a role in cell cycle regulation), CMGC (containing cyclin-dependent kinases (CDK), MAPK, GSK3, CLK), CDK (playing major role in cell cycle) and STE kinase families.

The National Human Genome Research Institute and the National Cancer Institute have launched The Cancer Genome Atlas (TCGA) [34] with an overarching goal of understanding the molecular basis of cancer, to improve our ability to diagnose, treat and prevent cancer. The development of genomic functional analysis software tools is important to human genome and cancer genome research. The aim of the scientists in this field is to develop next generation computational solutions for structural and functional genomics, *in silico* screening of leading compounds and designing new drugs, and annotation of genes for human cancer genomes through automated approaches. The effective utilization of very large databases combined with large-scale genomic computing needs significant computer science and engineering in order to tackle this biomedical problem. Scientists focus on the software development of new generation of computational intelligence for the personalized healthcare and translational medicine that will lead to new understanding of human diseases and future treatment. Hundreds of additional kinases that are coded in the human genome will be discovered, some of which may function as important regulators of signaling pathways potentially important in cancer.

Multi-target therapeutics and network analysis for cancer treatment

Drug design based on network analysis

The effect of a drug is studied in the context of relevant interactions (link) present in protein, regulatory, metabolic or signaling networks where the respective target is called an "element". Analyses based on genomic, proteomic, metabolism, etc. are methods to identify the systemic analysis of multi-target drug action [35]. A simple topological model is created by suppressing an element or a link and then its effect on the network is studied (Figure 2). It is found that multiple but partial attacks on various elements or links are more efficient than complete attack on a single element or link because the former affect more increased number of network links.

Multi-target approach in drug discovery (or multi-target lead discovery)

Current experience and the greater understanding of the disease network reveal that inhi-

Table 1. FDA approved tyrosine kinase inhibitors

Molecule	*Type of cancer*	*Molecular target*
Afatinib	NSCLC	EGFR
Axitinib	RCC	VEGFR
Bosutinib	CML	Bcr–abl
Cabozantanib	MTC	RET, VEGFR, MET, TRKB, TIE2
Cetuximab	Colon, NSCLC, HNC	EGFR
Crizotinib	NSCLC	EML4–ALK, ROS1, MET
Dabrafenib	Melanoma	BRAF V600E
Dasatinib	CML	Bcr–abl, SRC, cKIT, PDGFR
Erlotinib	NSCLC	EGFR (HER1)
Everolimus	Breast, RCC, pNET	mTOR
Gefitinib	NSCLC	HER1
Imatinib	CML, GIST, ALL	BCR-ABL, s-KIT, PDGFR
Nilotinib	CML	BCR-ABL
Sunitinib	GIST, RCC	VEGFR, PDGFR, c-KIT, FLT-3
Sorafenib	RCC, HCC, prostate	BRAF, VEGFR, PDGFR
Trastuzumab	Breast, stomach	HER2
Trametinib	Melanoma	MEK1, MEK2
Regorafenib	Colorectal	PDGFR, TIE2, VEGFR, c-KIT
Vandetanib	MTC	EGFR, VEGFR, RET

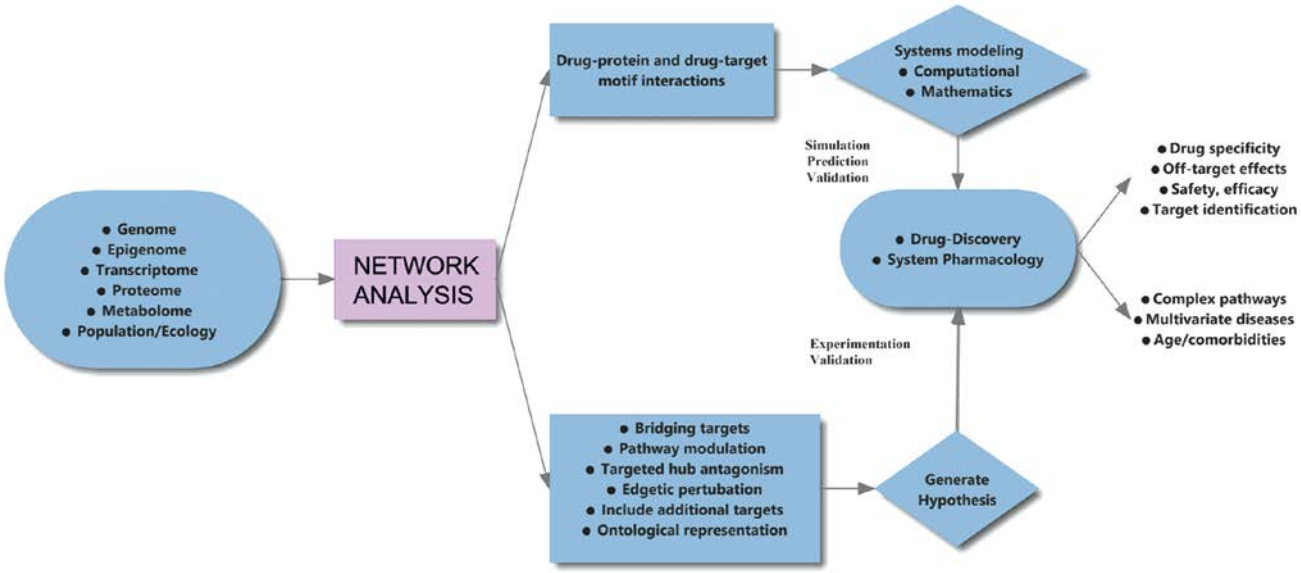

Figure 2. Drug design based on network analysis

bition of an individual target is - in many cases - insufficient to restore the system to the previous "healthy state" thus, modulating the activity of multiple targets might be required to achieve optimal therapeutic benefit [36]. A new generation of multi-targeted agents is currently emerging from clinical development. There are several categories of multi-target therapeutics that can be defined on the basis of target relationship. Multi-target drugs multiply the number of pharmacologically relevant target molecules by introducing a set of indirect, network-dependent effects. Parallel with this, the low-affinity binding of multi-targeted drugs eases the constraints of druggability and significantly increases the size of the druggable proteome. These effects tremendously expand the number of potential drug targets and introduce novel classes of multi-targeted drugs with lower side effects and toxicity. Compared to single-targeted agents, the wider effect on a network leads to a higher efficacy.

Therefore, the currently followed drug-development paradigm can be summarized as to: (1) find a target of clinical relevance; and (2) identify the "best-binder druggable molecule" by rational drug design based on the three-dimensional structure of the target [37]. Multi-target drugs offer a magnification of the "sweet spot" of drug discovery, meaning the overlap between pathways, which are interesting from the pharmacological point of view, and the hits of chemical proteomics, which represent those proteins, that can interact with druggable molecules (meaning small, hydrophobic molecules with a good bioavailability). The "sweet spot" represents those few hundred proteins, which are both parts of interesting pathways and are druggable. Multi-targeting lead discovery is a promising tool for the identification of unexpectedly novel effects of drug combinations [37,38].

The modern drug discovery has shifted its focus from the concept of one gene, one drug, and one disease, i.e. single-targeted strategy to multi-targeted strategy, which is based on network biology [39]. Single-targeted agents suffer from lack of efficacy, toxicity, drug resistance and huge investment. A multi-targeted agent possibly circumvents the problems regarding equitable pharmacokinetics and bio-distribution. The formulation of an individual active agent is easier compared with that of a mixture [40].

To manufacture polypharmacological drugs (like sunitinib and sorafenib described above), one has to identify a lead compound which has activity against multiple targets without becoming non-selective and then this should be transformed into a drug with a good pharmaceutical profile. Optimizing the potency at both targets is the challenging part.

To predict a multi-target compound requires high level computational methods to explore a vast number of possible drug and target combinations which are compatible with each other. The traditional approach to drug discovery of "one drug-one target-one disease" is insufficient, especially for complex diseases, like cancer. This inadequacy is partially addressed by accepting the notion of polypharmacology-one drug is likely to bind to multiple targets with varying affinity.

Identifying multiple targets

Identifying multiple targets for a drug is a complex and challenging task. A promising approach is to develop a structural proteome-wide off-target determination pipeline, by integrating computational methods for high throughput ligand binding site comparison and binding free energy calculations to predict potential off-targets for known drugs [39,40].The goal then is to perturb multiple relevant targets. Perturbation may be achievable through the use of drug cocktails, or possibly through a single drug that has the appropriate polypharmacological effect [3,40,41]. To rationally design such a drug is a very complex problem that begins by identifying the targets to which that drug binds. Available computational tools that quantitatively study protein-ligand interactions are based predominantly on protein-ligand docking and free energy calculations for the protein-ligand complex [42,43].

Multi-target models in clinical trials

In the literature, there is a plethora of publications dealing with the rationale underlying the multi-targeted approach. As a consequence, a whole new wave of multi-targeted compounds is moving into clinical trials. As previously mentioned, network models suggest that partial inhibition of a small number of targets can be more efficient than the complete inhibition of a single target [3]. The success stories of multi-target drugs and combinatorial therapies suggest that systematic drug-design strategies should be directed against multiple targets.

Combining kinase-focused chemistry, kinome-wide profiling and cancer genetics provides a powerful system of polypharmacological approach towards developing kinase-inhibitor drugs with a maximal anticancer therapeutic index [44]. The complexity of cancer and the unpredictability of optimal kinase-inhibition profiles led to Ret-kinase-driven model for the mechanistic basis of efficacy and dose-limiting toxicity and the design of a drug that hits multiple targets through "rational polypharmacology". The developed lead drugs, AD57 and AD80, suppressed several cancer signals emanating from Ret. These signals include some of the best-known cancer proteins such as Raf, Src, and Tor. Ret itself was not entirely shut down, which suggested to scientists that a patient would experience fewer side effects. On the other hand, reducing Tor made AD57 more toxic, so researchers christened Tor an "anti-target", a new concept in drug discovery. This approach represents a new concept which is believed to have great success in suppressing tumors.

In the active area for the search of more potent anti-breast cancer drugs, the use of approaches based on chemo-informatics has played a very important role. Speck-Planche et al. [45] have introduced the first chemo-informatics multi-target approach for the *in silico* design and virtual screening of anti-breast cancer agents against 13 cell lines. Multi-target QSAR discriminant model was developed using a large and heterogeneous database of compounds. Several fragments extracted from the molecules were identified as potential substructural features responsible for anti-breast cancer activity and new molecules designed from those fragments with positive contributions were suggested as possible potent and versatile anti-breast,brain, colorectal and prostate cancer agents [46-48].

Wei et al. [23] have developed a computer-assisted strategy to screen for multi-target inhibitors using a combination of molecular docking and common pharmacophore matching. This strategy was successfully applied to screen for dual-target inhibitors against both the human leukotriene A(4) hydrolase (hLTA4H) and the human non pancreatic secretory phospholipase A2 (hnps-PLA2). With the aid of this pharmacophore model, a number of compounds screened from the chemical database MDL Available Chemical Directory, were found to inhibit these enzymes in nanomolar concentrations. Similarly, combining molecular docking and pharmacophore filtering has been employed in a way to identify chemical compounds that can simultaneously inhibit hLTA4H and the human leukotriene C4 synthase (hLTC4S) enzymes [49]. A huge set of 4966 drug-like compounds from the Maybridge database (http://www.maybridge.com/) were docked into the active site of hLTA4H using the GOLD program (http://www.ccdc.cam.ac.uk/products/life_sciences/gold/). Common feature pharmacophore models were developed from the known inhibitors of both the targets using Accelrys Discovery Studio 2.5 (http://accelrys.com/events/webinars/discovery-studio-25/abstracts.html). The hits from the hLTA4H docking were filtered to match the chemical features of both the pharmacophore models. The compounds that resulted from the pharmacophore filtering were docked into the active site of hLTC4S and those hits bound well at both the active sites and matched the pharmacophore models were identified as possible dual inhibitors for hLTA4H and hLTC4S enzymes.

Rational drug discovery via "computer-assisted combinatorial method" may also be used to develop hybrid drug molecules. Hybrid compounds are defined as chemical entities with two or more structural domains having different biological functions and dual activity, indicating that a hybrid molecule acts as two distinct pharmacophores [50].

Curcumin (diferuloylmethane), the active ingredient in turmeric (Curcuma longa), with the aid of molecular docking techniques, has been shown to bind by multiple forces directly to numerous signaling molecules, such as inflammatory molecules, cell survival proteins, protein kinases, protein reductases, histone acetyltransferase, histone deacetylase, glyoxalase I, xanthine oxidase, proteasome, HIV1 integrase, HIV1 protease, sarco (endo) plasmic reticulum Ca(2^+) ATPase, DNA methyltransferases 1, FtsZ protofilaments, carrier proteins, and metal ions. Curcumin can also bind directly to DNA and RNA [51].

Huang and his colleagues [52], with a view to better integrate three dimensional protein structural information in cancer systems biology, have constructed a Human Cancer Pathway Protein Interaction Network (HCPIN) by analysing several classical cancer-associated signaling pathways and their physical protein-protein interactions. Many well-known cancer-associated proteins play central roles as "hubs" or "bottlenecks" in the HCPIN. At least half of HCPIN proteins are either directly associated with or interact with multiple signaling pathways. Recently, a large number of biological pathway and network databases have been developed to capture the expanding knowledge of protein-protein interactions (e.g. Human Protein Reference Database (HPRD) [53] and Database of Interacting Proteins [54] and of metabolic and/or signaling pathways (e.g. KEGG [55], Reactome [56], Signal Transduction Knowledge Environment (STKE), and BioCarta). A few databases are specifically focused on cancer-associated signaling pathways, such as The Cancer Cell Map and the Rel/NF-κB Signal Transduction Pathway. Pathguide [57] provides an overview of more than 200 Web-based biological pathway and network databases.

Fragment-based approach to multi-target drug discovery

Fragment-based approaches combine multiple structural fragments that bind to each individual target to design compounds that bind to multiple targets, which have been introduced as tools for the design of multi-target agents (Figure 1b) [58]. In one approach, the structure-activity relationships against individual targets are analyzed to find molecular fragments and essential binding features which are either combined or incorporated into active agents against selected multiple targets. In another approach, molecular fragment libraries are created to find the fragments with certain levels of activity against selected multiple targets, and the identified fragments are further optimized into more potent, bigger-sized multi-target active agents. A fragment-based approach to multi-target drug discovery could lead to a new generation of compounds with improved physicochemical and pharmacokinetic properties [59].

Optimizing fragments with weak multiple activities into potent multi-target, drug-like agents can be more easily achieved for targets sharing a conserved binding site [59]. As binding sites become more dissimilar, it is increasingly difficult to improve and adequately balance the high binding affinities needed to achieve acceptable *in vivo* efficacy and safety. One way to reduce this difficulty is to explore synergistic targets, such that multi-target agents with modest activity at one or more of the relevant targets may still produce similar or better *in vivo* effects compared with higher affinity, target-selective compounds [41].

In another approach, the incorporation of multi-target or species variations of binding-site features into the multi-target dependent molecular descriptors [60] or species-dependent molecular descriptors has driven to the development of multi-target QSAR models [48] with successful binding of the drug to multiple targets.

The attack approach in multi-target drug design

Another useful strategy in multi-target drug design is the attack approach with examination of the effects of drugs in the context of cellular networks [61]. In this model, a drug-induced inhibition of a single target means that the interactions around a given target are eliminated, whereas partial inhibition can be modeled as a partial knockout of the interactions of the target. Several classes of drugs, such as anticancer drugs, are designed to destroy the normal function of cellular networks. Networks have a number of vulnerable points and, therefore, can be attacked in many ways.

Drug design and discovery

Drug discovery and development is an in-

tense, lengthy and an interdisciplinary endeavor. Drug design, sometimes referred to as rational drug design or more simply rational design, is the inventive process of finding new medications based on the knowledge of a biological target [62].

Three are the basic questions that should be answered, concerning the many disciplines related to the drug design and discovery process: (1) what is the state-of-the-art in drug discovery today?; (2) what are the latest tools used in the drug discovery process; and (3) where is drug discovery going in the new millennium? Drug discovery is mostly portrayed as a linear, consecutive process that starts with target and lead discovery, followed by lead optimization (Figure 3) and pre-clinical *in vitro* and *in vivo* studies (Figure 4) to determine if such compounds satisfy a number of pre-set criteria for initiating clinical development (detailed in part II).

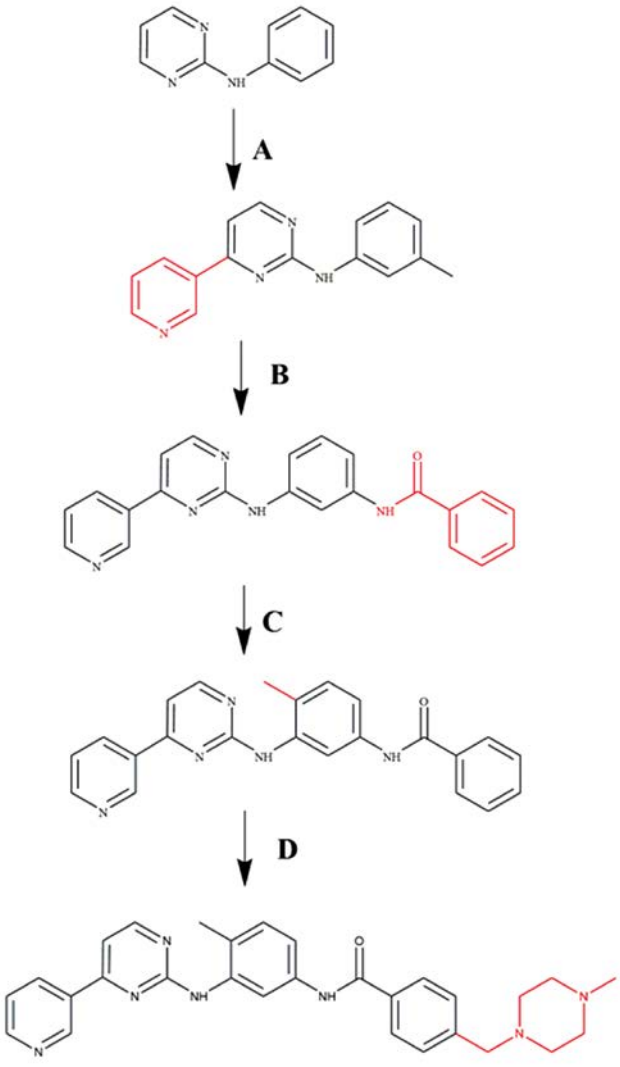

Figure 3. Imatinib lead optimization from a 2-phenylaminopyrimidine backbone. **(A)** introduction of a 3'pyridyl group (red) at the 3'- position of the pyrimidine improved activity in cellular assays. **(B)** Activity against tyrosine kinases enhanced by addition of a benzamide group (red) to the phenyl ring. **(C)** Attachment of a flag-methyl group (red) ortho to the diaminophenyl ring strongly reduced activity against protein kinase C. **(D)** Addition of an N-methylpiperazine (red) increased water-solubility and oral bioavailability. Adapted from Deininger et al (2005) [89].

Despite remarkable advances in technology and understanding of biological systems (progress in genomic, proteomic and high-throughput screening methods, the rational drug design, and the massive drug-development efforts), drug discovery is still a lengthy task with the number of novel, single-target drugs to have fallen much behind expectations during the past decade [62], "expensive, difficult, and inefficient process" with low rate of new therapeutic discovery [3,63]. Currently, the research and development cost of each new molecular entity (NME) is approximately 1.8 billion US$ [64].

Traditionally, drugs were discovered by synthesizing compounds in a time-consuming multi-step processes against a battery of *in vivo* biological screens and further investigating the promising candidates for their pharmacokinetic properties, metabolism and potential toxicity. Such a development process has resulted in high attrition rates with failures attributed to poor pharmacokinetics (39%), lack of efficacy (30%), animal toxicity (11%), adverse effects in humans (10%) and various commercial and miscellaneous factors. Today, the process of drug discovery has been revolutionized with the advent of genomics, proteomics, bioinformatics and efficient technologies, like combinatorial chemistry, HTS, virtual screening, *de novo* design, *in vitro*, *in silico* ADMET screening and structure-based drug design [65].

Rational drug discovery

In contrast to traditional methods of drug discovery, which rely on trial-and-error testing of chemical substances on cultured cells or animals, and matching the apparent effects to treatments, rational drug design begins with a hypothesis that modulation of a specific biological target may have therapeutic value. In order for a biomolecule to be selected as a drug target, two essential pieces of information are required. The first is evidence that modulation of the target will have therapeutic value. This knowledge may come from, for example, disease linkage studies that show an association between mutations in the biological target and certain disease states. The second is that the target is "druggable". This means that it is capable of binding to a small molecule and that

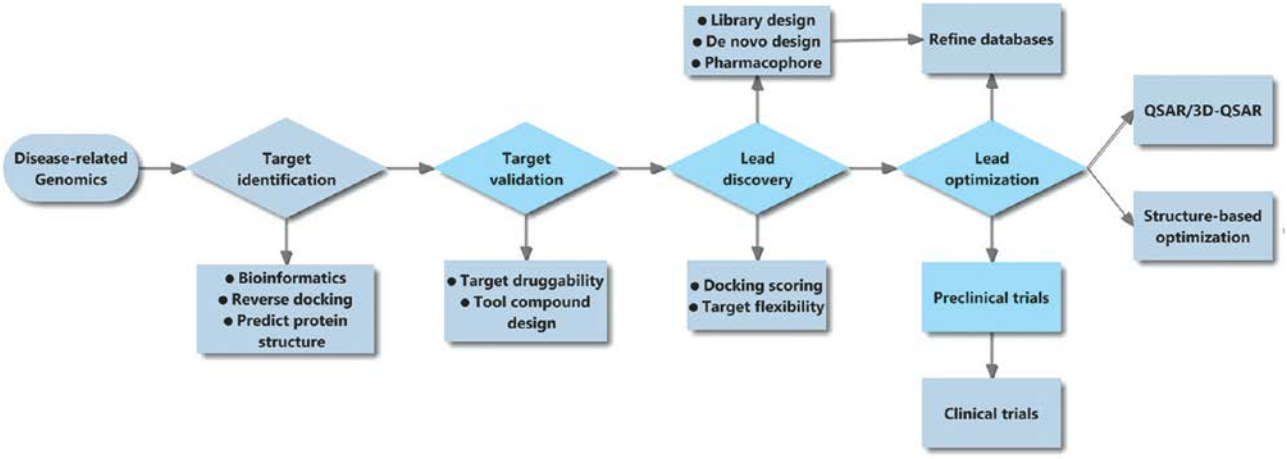

Figure 4. Computer aided drug design (CADD) and lead optimization.

its activity can be modulated by the small molecule. Once a suitable target has been identified, the target is normally cloned and expressed. The expressed target is then used to establish a screening assay. In addition, the three-dimensional structure of the target may be determined. The search for small molecules that bind to the target is begun by screening libraries of potential drug compounds. This may be done by using the screening assay (a "wet screen"). In addition, if the structure of the target is available, a virtual screen of candidate drugs may be performed. Ideally the candidate drug compounds should be "drug-like", that is they should possess properties that are predicted to lead to oral bioavailability, adequate chemical and metabolic stability, and minimal toxic effects.

Several methods are available to estimate druglikeness such as Lipinski's Rule of Five and a range of scoring methods such as Lipophilic efficiency. The prediction of drug metabolism has also been proposed in the scientific literature, and a recent example is SPORCalc concerning ligand-based drug design (discussed later) [66]. Due to the complexity of the drug design process, two terms of interest are still serendipity and bounded rationality. Those challenges are caused by the large chemical space describing potential new drugs without side-effects.

The drug is most commonly an organic small molecule that activates or inhibits the function of a biomolecule such as a protein, which in turn results in a therapeutic benefit to the patient. In the most basic sense, drug design involves the design of small molecules that are complementary in shape and charge to the biomolecular target with which they interact and therefore will bind to it.

Drug design, frequently but not necessarily, relies on computer modeling techniques [67]. This type of modeling is often referred to as computer-aided drug design. Finally, drug design that relies on the knowledge of the three-dimensional structure of the biomolecular target is known as structure-based drug design. What is really meant by drug design is ligand design (i.e., design of a small molecule that will bind tightly to its target) [68]. Although modeling techniques for prediction of binding affinity are reasonably successful, there are many other properties, such as bioavailability, metabolic half-life, lack of side effects, etc., that must first be optimized before a ligand can become a safe and efficacious drug. These other characteristics are often difficult to optimize using rational drug design techniques.

Drug targets

Many diseases can be linked to a human protein, referred to as the target. Typically, a drug target is a key molecule involved in a particular metabolic or signaling pathway that is specific to a disease condition or pathology or to the infectivity or survival of a microbial pathogen [69]. Some approaches attempt to inhibit the functioning of the pathway in the diseased state by causing a key molecule to stop functioning. Drugs may be designed that can bind to the active region and inhibit this key molecule. Another approach may be to enhance the normal pathway by promoting specific molecules in the normal pathways that may have been affected in the diseased state. In addition, these drugs should also be designed so as not to affect any other important "off-target" molecules or anti-targets that may be similar in appearance to the target molecule, since drug interactions with off-target molecules may lead to undesirable side effects.

The definition of "target" itself is something argued within the pharmaceutical industry. Gene-

rally, the "target" is the naturally existing cellular or molecular structure involved in the pathology of interest that the drug-in-development is meant to act on. However, the distinction between a "new" and "established" target can be made without a full understanding of just what a "target" is. This distinction is typically made by pharmaceutical companies engaged in discovery and development of therapeutics. In an estimate from 2011, 435 human genome products were identified as therapeutic drug targets of FDA-approved drugs [70].

"Established targets" are those for which there is a good scientific understanding, supported by a lengthy publication history, of both how the target functions in normal physiology and how it is involved in human pathology. This does not imply that the mechanism of action of drugs that are thought to act through a particular established target is fully understood. Rather, "established" relates directly to the amount of background information available on a target, in particular functional information. The more such information is available, the less investment is (generally) required to develop a therapeutic directed against the target. The process of gathering such functional information is called "target validation" in pharmaceutical industry parlance. Established targets also include those that the pharmaceutical industry has had experience mounting drug discovery campaigns against in the past; such a history provides information on the chemical feasibility of developing a small molecular therapeutic against the target and can provide licensing opportunities and freedom-to-operate indicators with respect to small-molecule therapeutic candidates.

In general, "new targets" are all those targets that are not "established targets", but which have been or are the subject of drug discovery campaigns. These typically include newly discovered proteins, or proteins whose function has now become clear as a result of basic scientific research.

During the recent 10 years it was observed an increase in the number of targets, collected from the Drugbank database in 2011and associated with new molecular entities (NMEs), giving rise to drug-target pairs [71,72]. Moreover, an increase in the average target number of blockbuster drugs is also documented. This indicates that multi-target drug discovery is indeed a status over the past decade and a possible trend in the future, although many single-target drugs are still used today.

Cellular networks

Cellular networks offer a lot of possibilities to point out their key elements as potential drug targets. As an example of these possibilities, signaling networks have inter-digitized pathways and multiple layers of cross-talks [73]. Cellular networks, contain hubs, i.e. elements, which have a large number of neighbors. These networks can be dissected to overlapping modules, which form hierarchical communities [74-76]. Also, Ye et al. [77] studied the drug function based on similarity of pathway fingerprint. According to Ye et al. drugs sharing similar therapeutic function may not bind to the same group of targets. However, their targets may be involved in similar pathway profiles which are associated with certain pathological process. Pathway fingerprint was introduced to indicate the profile of significant pathways being influenced by the targets of drugs. This method may be useful to further study on the potential function of known drugs, or the unknown function of new drugs.

Target–target and drug–drug networks

Two networks, namely, the target–target and drug–drug networks may be visualized using network analysis tools [3,78]. The target–target and drug–drug networks were to make a realistic visualization of information and directly determine the connections between targets and drugs, thereby providing important information on the current status of drug discovery.

Target–target networks

In target–target networks, the interacting molecules (drugs) are considered as the elements, and their interactions form the weighted links of the respective structural network. The targets of the anticancer drugs have been effectively separated to some extent. Targets for cancer treatment were relatively more scattered than those for other diseases, indicating the complex mechanism involved in cancer development and the diverse methods for cancer chemotherapy [78]. From target-target network visualization it is deduced that most of the targets are bound to others by links (in many cases with more than one multi-drug), confirming the importance of multi-target drugs in many diseases including cancer. The between target links, may also be thought as representations of signaling or metabolic processes for the encountered disease [74]. Links usually have a weight, which characterizes their strength (affinity or propensity). Thicker lines indicate that more drugs affect the connecting targets. A typical agg-

regation is that of tyrosine kinases. In fact, several anti-cancer drugs target MCSF1R (macrophage colony-stimulating factor 1 receptor), MSCGFR (mast/stem cell growth factor receptor), POTP-KABL1 (proto-oncogene tyrosine-protein kinase ABL1) and VEGFR2, among others. Special signaling elements, such as the PI-3-kinase, the Akt-kinase, or the insulin-receptor substrate family have been called "critical nodes". These "critical nodes" have multiple isoforms, and are important junctions of signaling pathways [79]. Both the bridge-elements of signaling networks providing cross-talks and the "critical nodes" can be important targets of network-based drug development. Domain-specific targets offer a larger flexibility and may actually reflect a family of multiple targets due to frequent "re-use" of domain-variants as a result of modular evolution [80,81].

Drug-drug networks

Identifying drug-drug interactions (DDIs) is a critical process in drug administration and drug development. A novel approach that integrates text mining and automated reasoning to derive DDIs has been presented by Tari et al. [82]. This approach can uncover potential DDIs with scientific evidences explaining the mechanism of the interactions. Drug interaction information has been extensively compiled into large databases. For this reason, network analysis tools can give information and directly determine the connections between drugs. Hu et al. [83] have constructed drug-drug interaction networks in which the interacting drugs were treated as nodes and were connected with links that represent interactions. They determined the number of interactions of each drug in the network and prepared histograms to show the frequency distribution.

In drug-drug network it is obvious that anticancer drugs cluster relatively closer compared with other drugs. For example, DNA, DNA synthesis-related enzymes, different types of tyrosine kinases, histone deacetylase inhibitors, and proteasome inhibitors, among others, are all anti-cancer targets, which lead to the development of anti-drugs in different clusters. In particular, arranon, dacogen, eloxatin, and vidaza target DNA; Tarceva, tykeerb, and iressa target EGFR (epidermal growth factor receptor); Sorafenib, sunitinib, imatinib, and dasatinib target other tyrosine kinases; vorinostat targets histone deacetylases and velcade targets proteasome.

As previously pointed out, it seems that using single-targeted agents to cure these complex diseases is almost impossible. The multiple tyrosine kinase inhibitor imatinib induces better anticancer effects compared with that of gefitinib, which involves a single target [84], further indicating that drugs with multiple targets may exhibit a better chance of affecting the complex equilibrium of whole cellular networks, than drugs that act on a single target.

The major problem is that we cannot definitely pre-determine which targets should be combined to design better anticancer drugs. Combinatorial therapy as an alternative, applied for decades in clinical practice, is unable to simultaneously affect all known targets associated with cancer. On the other hand, the increased side effect toxicity, due to off-target aiming of the drug combination, is a limiting factor for the combinatorial anticancer therapy. Thus, a better solution is to combine the targets selectively according to the developing knowledge on the mechanism of tumor growth and screen the compounds for rational drug discovery. Therefore, rapid development about genomics, proteomics, metabonomics, may enhance our understanding of the nature of cancer, effectively find possible therapeutic targets, and generate computer models that will identify the correct multi-fitting and further make this novel drug design paradigm successful.

Drug target networks

Predicting potential drug-target interactions from heterogeneous biological data is critical not only for better understanding of the various interactions and biological processes, but also for the development of novel drugs and the improvement of human-intended medicines [85]. Chen and his colleagues have developed a method of Network-based Random Walk with Restart on the Heterogeneous network (NRWRH) to predict potential drug-target interactions on a large scale under the hypothesis that similar drugs often target similar target proteins and the framework of Random Walk. In comparison with traditional supervised or semi-supervised methods, NRWRH makes full use of the tool of the network for data integration to predict drug-target associations. An approach to fill in the existing gap between chemical genomics and network pharmacology and thus accelerate the drug discovery processes has recently appeared in the literature [86].

Yamanishi et al. [87] have investigated the relationship between the chemical space, the pharmacological space and the topology of drug-target interaction networks, and showed that drug-target

interactions are more correlated with pharmacological effect similarity than with chemical structure similarity. They developed a new method to predict unknown drug-target interactions from chemical, genomic and pharmacological data on a large scale.

Drug-Target Interactome (DTome) provides a computational framework to effectively construct drug target networks by integrating the drug-drug interactions, drug-target interactions, drug-gene associations and target/gene-protein interactions. DTome also provides the network analysis illustration using the available network analysis software (http://bioinfo.mc.vanderbilt.edu/DTome/) [88]. In this study, Sun et al. designed a drug-target interactome to include drugs, targets, and proteins and their interactions. The targets included the drug primary targets and drug-associated genes with known PK and PD evidence. DTome network was designed to include three types of nodes and four types of relationships. The three types of nodes referred to drugs, proteins and genes. The four types of relationships included drug-drug interactions, drug-target interactions, drug-gene associations, and target-/gene-protein interactions. For drug-drug interactions, the compiled drug pairs were based on DrugBank interaction annotation. To obtain drug target/gene-protein interactions, target/gene's direct interactors were retrieved from human protein-protein interaction (PPI) data from the PINA (Protein Interaction Network Analysis) database. In the drug-gene interactions, the connection was defined between a given drug and its associated genes based on the evidence extracted from PharmGKB (The Pharmacogenomics Knowledge Base) database (databases reported here will be reviewed in Part II to be published in J BUON).

The DTome tool provides a computational workflow to integrate candidate drugs with their adverse drug interactions, primary targets, and associated genes in the context of human PPIs. The workflow includes three main steps: (1) dataset preparation and database construction (this step includes parsing the data from multiple databases and creation of a database); (2) generation of user-specified data and network (the user-specified data include a candidate drug or a list of drugs and four types of interactions. After merging the interactions, a DTome network is formed; and (3) network analysis and visualization via Cytoscape software (http://www.cytoscape.org/). This tool is computationally efficient and represents a promising strategy to investigate the molecular mechanisms of drug actions. Drug efficacy can be affected by the complexity of biological networks, of which targets are only a part. A user-friendly web interface for the DTome tool allows users to refine searches.

Conclusion

In this first part, we described the rationale behind the need for search of novel inhibitors targeting important cellular molecular pathways or cascades, as well as the fundamentals behing the design and discovery of targeted agents. In part II we'll describe in detail the methods used in designing and manufacturing novel molecules and specifically computer-aided drug design (CADD), virtual high throughput Screening (vHTS), Homology modeling, details of combinational chemistry and pharmacophores and we'll briefly dissert on the role of informatics in this exciting field of medicinal chemistry.

Conflict of interests

The authors declare no confict of interests.

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