Transcriptome and Proteome Analyses of Drug Interactions with Natural Products

Hai Fang[1,4], Kankan Wang[2,*] and Ji Zhang[1,2,3,*]

[1]*Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences/Shanghai Jiao Tong University School of Medicine (SJTUSM), Shanghai, China;* [2]*Shanghai Institute of Hematology, Ruijin Hospital, SJTUSM, Shanghai, China;* [3]*Sino-French Laboratory of Genomics and Life Sciences, Ruijin Hospital, SJTUSM, Shanghai, China;* [4]*Graduate School of the Chinese Academy of Sciences, Shanghai, China*

Abstract: Advances in high-throughput technologies to measure genome-scale changes of genes, proteins, and other biomolecular components ('omics') in complex biological systems have dramatically revolutionized biomedical research. However, the benefits of utilizing omics information in drug development have not yet been fully realized. Fortunately, the integration of modern systems biology efforts with traditional medicine philosophies, together with integrative bioinformatics, has driven the development of a new drug discovery paradigm. Using leukemia as a disease model, therapeutic synergism between drugs and natural products has been investigated by incorporating transcriptomics and proteomics data into a network-like understanding. Here, these recent advancements will be discussed in detail, along with perspectives in the field of drug synergism.

Keywords: Transcriptome, proteome, component plane presentation integrated self-organizing map, drug synergism, leukemia.

INTRODUCTION

Three scientific breakthroughs have markedly accelerated our understanding and treatment of human diseases over the past half-century. The first is the progressive elucidation of the genetic basis of biological information [1], from information storage (DNA) to processing (RNA) and to execution (proteins and metabolites). The second is the evolution of high-throughput omics technologies [2], including genomics, transcriptomics, proteomics, metabolomics, etc., which quantify various genome-wide biological information in a simultaneous, parallel, and automated manner. The third are various conceptual advances in systems biology [3], allowing the integration of disparate omics data into a network-like understanding of the underlying pathogenesis of human diseases. These advances hold great promise for the identification and characterization of potential drugs, their modes of action, and their molecular targets, with the ultimate goal of predictive, preventive, and personalized medicine [4].

Drug discovery paradigms have experienced a shift from traditional, folk-medicine practices to target-based chemical screening, and now back again to a biology-driven approach [5]. Although target-directed drug discovery is effective for screening large libraries of candidate drugs for interactions with well-validated molecular targets, such a discovery approach has proven disappointing and has no guarantee of success. Drug candidates that are screened against less well-validated targets do not always survive the requirements of human drug metabolism, known as a "target-rich but lead-poor" environment. In contrast, biology-directed drug discovery, which uses principles of systems biology, is driven by insights into biological responses. Two factors make a biology-driven drug discovery approach practical. First, recent advances in omics technologies (e.g., pervasive transcriptomics and emerging proteomics) have propelled the rapid accumulation of genome-scale databases of existing drugs and other bioactive compounds. Integrative bioinformatics has facilitated the conversion of this extensive omics data into mechanisms of therapeutic action. Second, traditional drug discovery paradigms, such as the traditional Chinese medicine (TCM) system, provide a long history of successful experiences in disease treatment. The TCM system is thought to contain some of the most valuable bioactive compounds and combinatory therapeutic practices available [6]. According to a recent investigation, natural products still play the dominant role in modern drug discovery [7]. For instance, as many as 75% of the anti-tumor compounds discovered over the last 25 years have been natural products or natural product–derived mimics, while only rare *de novo* synthetic compounds have been approved as anti-cancer drugs. The TCM system contains nearly 100,000 formulae (combinations) of medicinal herbs/minerals (i.e., natural products), thus representing a rich source of bioactive compounds. Databases relevant to TCM have been developed worldwide; for instance, a database comprising almost 600 Chinese medicinal herbs and minerals was recently released in Germany [8].

Combinational therapies have proven powerful for combating major human diseases. They are necessitated by the complexity of disease-perturbed networks, and the occurrence of drug resistance and relapse after conventional regimens. For example, increasing evidence demonstrates that anti-cancer regimens containing multiple anti-tumor agents with distinct but related mechanisms always maximize the therapeutic efficacy while minimizing the adverse effects [9]. In this respect, TCM has advocated combinatory therapeutic strategies for thousands of years. Based on the symptoms of patients and guided by the theories of TCM (e.g., yin-yang, the five elements), medicinal herbs and/or minerals are combined to improve clinical efficacy [10].

With the availability of appropriate disease model systems, the next task is to explore the pharmacological mechanisms of natural products, and more importantly, to evaluate their synergistic therapeutic efficiencies with existing U.S. Food and Drug Administration (FDA)–approved drugs. Significant progress has been made in leukemia drug development and evaluation [11, 12]. Leukemia is a group of hematological malignancies characterized by uncontrolled proliferation, decreased apoptosis, and blocked differentiation. Based on the stage at which differentiation is blocked and on the hematopoietic lineage, leukemia can be characterized as acute or chronic, and lymphoid or myeloid, each of which has a number of subtypes. Acute myelogenous leukemia (AML) is a malignant disease of the bone marrow in which hematopoietic precursors are arrested at an early stage of myeloid development. According to the French-American-British (FAB) classification [13], AML can be divided into M0-M7 subtypes. The main advantage of using AML as a disease model for exploring therapeutic synergism is its subtype-specific sensitivity to various combinations of drugs and natural products, with responses that are prominently reflected at the gene expression level. Due to worldwide effort on AML treatment,

*Address correspondence to these authors at Institute of Health Sciences, 225 South Chong-Qing Road, Shanghai 200025, China; Tel: 86-21-6385-2742; Fax: 86-21-6415-2869; Email: jizhang@sibs.ac.cn and Ruijin Hospital, 197 Ruijin Rd. II, Shanghai 200025, China; Tel: 86-21-64370045; Email: kankanwang@shsmu.edu.cn

1389-2002/08 $55.00+.00

several of its subtypes have being turned from being highly fatal into highly curable [14].

Achieving effective and efficient drug development schemes requires the cooperation of multiple scientific disciplines, including pharmacology, genomics, transcriptomics, proteomics, and bioinformatics, and may also require the use of the TCM combinatory philosophy (Fig. **1A**). Accordingly, we will first address the current state of high-throughput transcriptomic and proteomic technologies relevant to drug studies. We will then discuss omics data mining and the challenges associated with the integration of heterogeneous omics data. The significance of high-throughput technologies in drug-related fields will be summarized based on recent applications of gene expression profiling in drug development. Next, we will focus on a detailed case involving transcriptome and proteome analyses of drug interactions with natural products, as represented by a synergistic therapy paradigm using *all-trans* retinoic acid (ATRA) and arsenic trioxide (ATO) in the treatment of acute promyelocytic leukemia (APL). Additional experiences with other hematological malignancies will also be discussed. Finally, we will close our discussion with our perspectives for the field of drug synergism.

PERVASIVE TRANSCRIPTOMICS AND EMERGING PROTEOMICS

The ability to analyze the genome-wide expression of genes has revolutionized biomedical research. Transcriptome profiling using microarrays was among the most successful high-throughput omics technologies [15, 16], and has been applied as pervasively as have RT-PCR and Western blots. Superior to these traditional techniques in terms of data amounts and acquisition, microarray technology permits the simultaneous and systematic monitoring of genome-wide gene expression levels. The two major microarray technology platforms, spotted arrays and oligonucleotide chips, differ in array fabrication and dye selection. In a typical microarray using a spotted array, tens of thousands of probes (cDNA clones) are robotically spotted onto a glass slide. Two different targets (mRNA samples) are then reverse transcribed into cDNA and simultaneously labeled with different fluorescent dyes (e.g., Cy3 and Cy5) for hybridization. After excitation by the appropriate wavelengths, the intensities of Cy3 and Cy5 fluorescence in each spot indicate the quantified levels of the targets. The normalized intensity ratio gives an estimate of the relative amounts of the targets hybridized to the same cDNA probe. As for oligonucleotide chips, long-oligonucleotide microarrays are similar to spotted arrays except for the genomic-derived probes used, while short-oligonucleotide microarrays involve high-density probe pairs (each consisting of a perfect-match and a mismatch probe) and only one target (mRNA sample) for hybridization. As a powerful high-throughput technology, the true potential of microarray technology has evolved beyond gene expression analysis [17].

Transcriptome profiles alone do not reflect all biological processes, as many biological functions are affected at the post-transcriptional (e.g., pre-mRNA splicing and export, microRNA regulation), protein synthesis, post-translational (e.g., protein location, modification, and protein-protein interactions), and/or metabolite levels. Each level is thought to be physically and functionally linked to the whole process, and subject to feedback controls by the other levels [18]. Therefore, other omics technologies, such as chromatin immunoprecipitation coupled promoter microarrays (ChIP-Chip) and ChIPSeq technologies [19, 20], microRNA [21], proteomic [22], and metabolomic profiles [23], and high-throughput two-hybrid screening [24], are needed to complete our knowledge of gene regulation.

Since proteins are the major executer of biological information, genome-wide analysis at the protein level provides a direct reflection of gene expression. Proteomics is defined as the genome-scale analysis of protein abundance, localization, modification, structure, and activity. There are several methods for proteomic profiling, including two-dimensional gel electrophoresis (2D) and mass spectrometry (MS), and liquid chromatography (LC) coupled with MS [25]. In many proteomics studies, 2D serves as the basis for high-resolution separation of the protein mixture [26]. It resolves protein mixtures into distinct spots by mass and charge, and then quantifies the protein intensity from the staining of the separated spots. Decoupled from separation and quantification in 2D, the quantified gel spots are subsequently identified by MS. Alternatively, in LC-MS, also known as gel-free proteomic profiling, peptide fractionation and quantification by high-performance LC is coupled with automated MS [27]. The LC-MS method improves the detection coverage and avoids the bias of 2D-MS towards soluble, high-abundance proteins, representing a powerful proteomics technology for detecting protein abundance, sub-cellular location, and post-translational states (e.g., phosphoproteomics [28]). However, due to the complexity of the proteome and current technical limitations [29], proteome characterization by these emerging technologies still lags behind transcriptome characterization.

TRANSCRIPTOMIC DATA MINING AND BEYOND

The conversion of the massive amounts of omics data into meaningful biological knowledge can bottleneck the application potentials of the high-throughput technologies. Most such efforts primarily use microarray technologies to handle transcriptomic data. In a static microarray design, the arrays are used irrespective of time to capture a snapshot of the expression profile. In contrast, temporal microarrays are collected over a time-series to measure the dynamic process of gene expression.

Data generated from either microarray design can be tabulated in a matrix form, i.e., a gene expression matrix of expression levels (rows) under different experimental conditions (columns). However, a number of problems are inherent in these data, including a low signal/noise ratio, large numbers of missing values, and small sample size *vs.* huge gene volume. Various data mining methods have evolved, which range from simple fold-change approaches [30] to statistical inference based on differentially-expressed gene selection (e.g., SAM [31], QVALUE [32], EDGE [33]), from the commonly applied hierarchical clustering [34] to artificial intelligence algorithms based on a self-organizing map (SOM) for gene clustering and visualization [35, 36], and from functional enrichment analyses [37, 38] to data integration–based network reconstruction [39, 40]. Since temporal microarray experiments characterizing the genome-wide dynamic regulation of gene expression can be more abundant in information relevant to real biological processes (e.g., responses of cancer cells to drug treatment), the development of computational methodologies specifically designed for time-series gene expression data will be of great interest.

Accordingly, we have proposed a novel framework for time-series gene expression data mining and visualization. Briefly, gene expression data are first subjected to robust gene selection that integrates SOM for data pre-processing [41] and singular value decomposition (SVD) for pattern recognition [42, 43]. Using multiple testing procedures for false-discovery rate estimation [44], our hybrid SOM-SVD bases the entire gene selection process on statistical inference, allowing the maximum retention of information inherent to the primary microarray data. For gene clustering and visualization, the selected gene expression data are analyzed by component plane presentation (CPP)-integrated SOM [36]. As demonstrated in our previous publications [45-47], each presentation illustrates a treatment-specific transcriptome (or proteome) map and permits the direct comparison of expression changes within/between different treatment series. Our approach results in much more accurate and more complete gene clustering than traditional approaches. Furthermore, it facilitates the in-depth mining of biological information, such as hypergeometric distribution–based enrichment analyses of biological themes. Annotated biological themes are collected

in various databases; for example, Gene Ontology [48] is a database of gene annotations where a controlled vocabulary describes genes in terms of biological processes, molecular functions, and cellular localizations. Gene Ontology is an ideal resource for functional enrichment analyses, which can also be applied to databases of pathway-relevant genes (e.g., KEGG [49], GenMAPP [50], and Biocarta), transcription factor–targeted genes (e.g., TRANSFAC [51] and JASPAR [52]), or microRNA target genes (e.g., miRBase [53]). The power of systematic enrichment analyses has also been demonstrated by the interpretation of large-scale static cancer transcriptome [54-58]. These studies revealed that the gene-expression signatures of specific cancer types/subtypes are specifically enriched with functional modules and regulatory profiles, and therefore decode distinct biological processes and regulatory programs. As the accuracy and coverage of biological theme databases improves, integrative enrichment analyses will be able to extract greater biological insights from time-series gene expression data and collective cancer microarray databases (e.g., NCBI GEO [59], ArrayExpress [60], Oncomine [61], and Stanford SMD [62]). Furthermore, the principles of data mining observed with transcriptomic data are also applicable to other large-scale omics data (e.g., proteomics).

CHALLENGES OF TRANSCRIPTOME AND PROTEOME INTEGRATION

While single omic data mining continuously improves, comparing data from different high-throughput omics platforms remains challenging, as indicated by the integration of transcriptomic and proteomic analyses. For instance, pair-wise correlations between mRNA transcript and protein levels are quite weak, which may result from technical limitations and/or biological factors. Information coverage by proteomic technologies is rather narrow, so that focusing only on overlaps results in a high false-negative rate; the correlation is relatively stronger as proteomic coverage increases, such as with the use of gel-free proteomic profiling [63]. Differential lifetime between mRNA and protein further complicates their relationships, necessitating the characterization of temporal changes in transcript and protein expression levels.

However, the primary challenge associated with integrating transcriptomic and proteomic analyses is to distinguish true mRNA-protein concordance/discordance from those falsely discovered. While a high concordance between a transcriptome and a proteome can increase the confidence, the true discordance is of greater scientific interest for revealing post-transcriptional regulatory mechanisms. The use of the multi-dimensional visualization system of CPP-SOM allows the comparison of transcriptomic and proteomic data in a straightforward, easy-to-interpret manner [46]. Finally, integration at the functional or higher network level is more informative than at the transcriptome-proteome level, and enables a greater understanding of the relationships between the transcriptome and proteome. Substantial agreement in functional enrichment has been revealed between the transcriptome and proteome levels [64], highlighting the value of expanding mere correlation analyses to a systemic level of integration.

SIGNIFICANCE OF GENE EXPRESSION PROFILING IN DRUG DEVELOPMENT

Gene expression profiles have successfully classified disease states [65] and predicted disease survival [66], metastatic progression [67], and treatment response [68-70]. Another promising application for such profiles is in drug discovery [71]. General strategies based on biological response profiling represent a more practical approach for drug discovery than purely target-based approaches [5]. High-throughput gene expression profiling holds great promise for facilitating this process.

The gene expression–based strategy was first demonstrated in screening candidate compounds capable of inducing leukemia differentiation [72]. Using a gene expression–based high-throughput screening (GE-HTS) approach, Stegmaier *et al.* established the drug-specific gene expression signature of a therapeutic state in AML cells (i.e., differentiation). They then screened a library of 1,739 compounds for their ability to induce the target phenotype, and validated their findings with additional assays. Eight compounds were identified that reproducibly triggered the differentiation signature, including an epidermal growth factor receptor (EGFR) kinase inhibitor. To evaluate EGFR inhibitors as a potential differentiation therapy for leukemia patients, they tested the preclinical efficacy of the FDA-approved EGFR inhibitor gefitinib. Gefitinib induced myeloid differentiation in AML cell lines and in primary patient-derived AML blasts at pharmacological concentrations [73]. Designed for the systematic discovery of compounds capable of modulating biological processes, the GE-HTS strategy is solely based on the gene expression signatures of the compounds and does not require any prior knowledge of key targets in the biological process of interest.

The Connectivity Map (CMap) approach applies the gene expression–based strategy to the systematic discovery of functional connections between human diseases and potential drugs [74]. Lamb *et al.* created a reference database of gene expression signatures for cultured human cancer cell lines treated with more than 160 drugs or other bioactive compounds. They then used pattern-matching software to classify and compare the various signatures, and more importantly, to query the referenced database with users' own gene-expression profiles of interest. CMap showed promise in identifying: (1) additional compounds of similar action but different chemical structure (when querying with a drug of a known mechanism of action), (2) potential mechanisms of action (when querying with an uncharacterized compound), and (3) potential disease therapeutics (when querying with a disease state–derived profile, e.g., drug resistant *vs.* sensitive). The third possibility assumes that compounds/drugs that reverse the gene expression profile of the disease state could serve as new therapeutic agents for that disease.

To date, several applications of gene expression–based strategies (e.g., GE-HTS and CMap) have been successfully reported for screening modulators of various biological processes. Based on the gene expression signature of glucocorticoid (GC) sensitivity/resistance in acute lymphoblastic leukemia, Wei *et al.* identified the mTOR inhibitor rapamycin as a modulator of GC resistance, indicating the rapamycin/GC combination in treating lymphoid malignancies [75]. Based on the defined gene expression signature of androgen receptor (AR) signaling in prostate cancer, Hieronymus *et al.* screened celestrol and gedunin as novel inhibitors for AR signaling through modulation of the HSP90 pathway [76]. To identify candidate drugs of previously intractable tumor-associated oncoproteins (e.g., EWS/FLI in Ewing sarcoma) with traditional screening approaches, Stegmaier *et al.* utilized the gene expression signature of EWS/FLI inactivation to screen a small molecule library enriched in FDA-approved drugs [77]. They identified cytosine arabinoside (Ara-C) as the top-scoring EWS/FLI modulator, thereby demonstrating that the GE-HTS of existing drugs represents a powerful discovery platform for screening oncoprotein-modulating candidate drugs in a more clinical setting. Similarly, to identify drugs acting through the transcriptional co-activator PGC-1alpha, Arany *et al.* performed gene expression–based screening and identified microtubule and protein synthesis inhibitors as PGC-1alpha inducers [78]. A recent study demonstrated that GE-HTS may serve as a general approach to discover modulators of any signaling pathway of interest [79]. Another recent study extended the CMap principle to allow the use of any publicly-available gene expression database (e.g., GEO), and discovered two previously unknown compounds for eradicating leukemia stem cells (LSCs) [80].

TRANSCRIPTOMICS AND PROTEOMICS OF ATRA/ATO COMBINATION THERAPY IN APL AS A PARADIGM FOR SYNERGISTIC THERAPY

The Advent of the ATRA/ATO Combination in APL Treatment

Formerly considered to be the most fatal but now the most curable malignancy, APL is the M3 subtype of AML according to the FAB classification. Morphologically, APL is characterized by the accumulation of immature promyelocytes in the bone marrow. APL exhibits a balanced reciprocal chromosome translocation t(15,17) [81], which results in fusion between the promyelocytic leukemia (*PML*) and the retinoic acid receptor alpha (*RARA*) genes. Since the discovery of the ATRA/ATO combined therapy, the APL model has provided a striking paradigm of synergistic therapy [82, 83].

The vitamin A (retinol) derivative ATRA represents the typical class of differentiation-inducing anti-leukemia drugs. Rather than inhibiting or killing proliferating malignant cells by chemotherapy or radiotherapy, malignancy reversion by inducing cellular differentiation is an alternative treatment approach for cancer cells [84]. Zhen-Yi Wang and his team from the Shanghai Institute of Hematology (SIH) introduced ATRA as a remission-inducing treatment for APL, which marked the dawn of differentiation-induced cancer therapies [85] and soon became a routine treatment against APL [86-89]. Although ATRA-based differentiation therapy was unprecedented in its ability to improve the complete remission rate and five-year disease-free survival rate, APL patients refractory to ATRA remained common. Alternative bioactive compounds were therefore urgently needed to overcome the limitations of ATRA in relapsed or refractory APL patients. The TCM system, with its combinatory therapeutic strategies and large number of recorded natural products (i.e., medicinal herbs and minerals), was approached to meet this need. Arsenic, a naturally occurring toxic metalloid in organic and inorganic forms, is frequently used in TCM to cure various diseases, with the ancient philosophy of taming an evil with a toxic agent [90]. At the end of the past century, Chinese scientists first reported the effective use of arsenic compounds in treating APL, where it induces apoptosis at high concentrations and partial differentiation at low concentrations. Controlled clinical trials using white arsenic (ATO, As_2O_3) showed that ATO is an effective and relatively safe drug for APL patients refractory to ATRA and conventional chemotherapy [91-93]. In September 2000, ATO (Trisenox™) was approved for the treatment of relapsed or refractory APL by FDA. In addition, other inorganic forms of arsenic, including red arsenic (realgar, As_4S_4) and yellow arsenic (orpiment, As_2S_3), were also reportedly effective in APL treatment [94].

Alone, ATRA or ATO makes a remarkable contribution to APL treatment. It is therefore logical to speculate that synergistic therapy could be attained if a combination of the two drugs were used. Using an *in vitro* cell-line model and an *in vivo* mouse model, several studies showed that this combination accelerated disease regression and prolonged survival [95-97]. Furthermore, a clinical trial with the ATRA/ATO combination demonstrated superiority in treating APL over mono-therapy, in terms of the quality of disease-free survival [82, 83]. However, to be useful, clinically effective dual therapy requires an understanding of the underlying molecular mechanisms accounting for the synergism between the differentiation-inducing drug and the TCM-derived natural product.

Synergistic Mechanism of ATRA/ATO Determined by Transcriptomics and Proteomics

Using a reductionist approach, i.e., individually studying the function of each gene/protein, researchers obtained essential information on the actions of ATRA and ATO in APL therapy. PML-RARA was identified as the primary APL leukemogenesis, and ATRA and ATO were found to directly target the RARA and PML moieties, respectively [98]. However, many fundamental questions remain to be elucidated concerning the actions of these anti-cancer agents, particularly with respect to the dynamic changes they evoke, their target properties, and their underlying synergism. Since cellular drug response systems are thought to be complex networks of interconnected genes/proteins, the behavior of the drug response network should therefore be studied as a whole.

Unlike a single omics approach [99, 100], we performed a comprehensive analysis of ATRA, ATO, and ATRA/ATO treatments of the APL cell line NB4 using a systems biology approach that integrated transcriptomics, proteomics, and computational biology with robust data mining tools [46]. By comparing transcriptomic and proteomic data by CPP-SOM (Fig. **1B**), we identified three novel features that indicated coordinated networks with a temporal-spatial relationships and reflected the synergistic effects of ATRA and ATO (left panel of Fig. **1C**).

First, the vertical (cross-platform) view of the transcriptome-proteome comparison revealed distinct target properties for ATRA and ATO, which exerted their effects mainly at the transcriptome and proteome levels, respectively. This is consistent with the observation that the transcriptomic and proteomic data of ATRA/ATO combination therapy are generally complementary rather than duplicative. ATRA-induced differentiation mainly involved nuclear receptor–mediated transcriptional remodeling, with modulation of a large number of genes involved in the initiation and promotion of granulocytic differentiation, such as the granulopoiesis-associated transcription factors *C/EBPs* and *bHLHs*, and cytokines/cytokine receptors and their corresponding downstream effectors. In contrast, ATO enhanced post-transcriptional and translational modifications, modulating proteins involved in metabolism, nuclear and cytoplasmic structures, and translational machinery. These results provide the molecular foundation at a global scale for elucidating the synergism between ATRA and ATO.

Second, the horizontal (within-platform) comparison of the transcriptome/proteome level revealed the synergistic target properties of ATRA and ATO. At the transcriptome level, these properties were highlighted by an enhanced ubiquitin-proteasome system (UPS) and by the repression of events related to various chromosomal translocations in human malignancies. ATRA specifically up-regulated genes encoding components of the typical immunoproteasome, whereas ATO significantly induced those encoding subunits of the conventional UPS. Enhanced UPS can account for degradation of PML-RARA oncoprotein, indicating that this degradation by ATRA or ATO is through the same pathways with distinct mechanisms. ATRA targets the RARA moiety and recruits the 19S proteasome regulatory complex, while ATO targets the PML moiety and recruits the 11S proteasome activator, both of which lead to PML-RARA degradation [101]. Enhanced UPS is also consistent with data suggesting that the extent of PML-RARA degradation is positively associated with better recovery from APL [82]. Translocation-related genes were synergistically down-regulated, suggesting that ATRA/ATO co-treatment is more effective in eliminating oncogenic properties and reducing cell survival potentials than treatment by ATRA or ATO alone. At the proteome level, synergistically-targeted properties were characterized by inhibited translational factors. Since translational regulation helps coordinate tumor proliferation, the inhibition of translational factors suggests that tumor growth arrest is favored in co-treated APL cells.

Third, the temporal view of the information gathered at both the transcriptome and proteome levels revealed that the target properties of ATRA and ATO were integrated into a functional network, thereby contributing to the underlying ATRA/ATO synergistic effect on APL. At the early stage of co-treatment (0-6 h), ATRA/ATO modulated nuclear receptor signaling molecules and transcription factors/cofactors associated with myeloid-specific gene expression. At the intermediate stage (12-24 h), ATRA/ATO regulation of genes/proteins seemed to be amplifying retinoic acid signaling, as indicated by its effects on the interferon, calcium, cAMP/PKA, and MAPK/JNK/p38 pathways. Another prominent

event occurring at this stage was the enhanced activation of UPS, which might facilitate the PML-RARA degradation. At the late stage (48-72 h) of co-treatment, the expression of the differentiation markers and functional molecules reached a maximum, while genes/proteins promoting cell cycle or enhancing cell proliferation were significantly repressed. As the cells approached terminal differentiation, the apoptotic potential was gradually restored, as indicated by the recovery of the nuclear body and the up-regulation of the caspase cascades.

Lessons Learned from Using an Integrative Omics Approach to Understand Synergism

The successful application of transcriptome and proteome analyses to understanding the synergistic action of ATRA and ATO in APL justifies the power of integrative omics approaches in characterizing synergism. Genome-wide assessment represents an efficient strategy for studying the whole effects of multi-agent combination therapy. Furthermore, the integration of two or more genome-wide platforms can overcome the intrinsic technological and biological limitations of each individual approach. While routine transcriptomic profiling allows the monitoring of transcriptional regulation in response to drug treatment, emerging proteomic profiling also provides insights into gene translation and post-translational modifications. Promisingly, recent evidence suggests that microRNA expression profiling contributes to post-transcriptional regulation for the clinical response of APL to ATRA [102]. Capturing the temporal changes in gene/protein expression makes the integration of such multi-layer information feasible and reliable. Finally, a functional or higher level integration may reveal additional mode-of-action information, as exemplified by the functionally-related yet distinct ATRA- and ATO-induced UPS genes. Such integration is particularly useful when gene-protein matching is difficult. For example, performing individual functional enrichment analyses at the transcriptome or proteome level may reveal biological functions shared by genes and proteins. These lessons may benefit other APL combination therapies, such as retinoic acid plus cAMP, arsenic plus cAMP, or retinoic acid plus the HDAC inhibitor [98].

Several lessons were also learned with respect to the synergistic action of the drugs and the natural products. First, the interactions of combination treatment regimens can be more complex than previously recognized. Second, these agents interact in a functional manner to amplify the therapeutic efficacies of each agent. This can be achieved through physically binding to the same molecular defects (e.g., PML-RARA), targeting the same pathways with distinct mechanisms (e.g., ATRA and ATO both targeted UPS, but via recruitment of different proteasomes), or targeting multiple pathways at various levels (e.g., transcriptome and proteome) to achieve the common goal of therapeutic synergism. Consistent with the observation that multiple agents can hit multiple targets to exert synergistic therapeutic efficacies, TCM has long advocated combination therapies where one agent represents the principal element and the other agents assist the effects or facilitate the delivery of the principal element. Recent studies have shown that ATRA assists ATO uptake through modulating the transmembrane arsenic channel aquaglyceroporin 9 [103], adding another aspect to the synergistic therapeutic efficacy. Finally, these studies showed us that efforts to identify of all the possible components of the interactions between drugs and/or natural products should be taken as a priority to direct combinations of therapeutics.

IMATINIB/ATO COMBINATION THERAPY FOR CHRONIC MYELOGENOUS LEUKEMIA (CML)

The transcriptome and proteome analyses of ATRA/ATO synergism in APL led us to use functional genomics to understand combination therapy synergism in other hematologic malignancies. Specifically, we analyzed imatinib/ATO combination therapy in the treatment of CML. Imatinib is a specifically-designed molecule (tailored drug) for CML (Gleevec™, approved by FDA in May 2001), and was one of the first therapeutic strategies to target molecules critical to the pathogenesis of a human malignancy. In contrast, ATO has long been used as an ancient remedy for CML, with known efficacy [104, 105]. Imatinib/ATO combination therapy has shown promising synergistic potential in inducing the apoptosis of CML cells [106, 107].

To understand the mechanisms underlying this synergism, we obtained a time-series of the transcriptome changes of the CML cell line K562 in response to treatment with imatinib, ATO, or imatinib/ATO. Numerous response features displayed temporal-spatial relationships, indicating apoptotic synergy at the transcriptome level [47] (right panel of Fig. **1C**). Compared with monotherapy, combination therapy led to more profound or earlier suppression of genes involved in cell cycle, BCR-ABL oncogenic signal transduction, anti-apoptotic/survival PI3K/AKT pathways, RNA processing, and/or protein synthesis. This synergistic suppression may augment pro-apoptotic/apoptotic activities compared to monotreatment. Indeed, data further suggest that imatinib may induce the intrinsic pathway of cell apoptosis, ATO may possibly induce the ER stress mediated pathway of cell apoptosis and the combination of these two agents may activate the intrinsic, extrinsic and ER stress mediated pathway of cell apoptosis, resulting in a more effective and efficient apoptosis in CML.

Although the expression levels of a wider variety of proteins can be determined through proteomic profiling, it is fairly straightforward to focus on protein levels of those involved in triple apoptotic pathways to validate synergistic mechanisms revealed by transcriptome analysis. We therefore used a series of protein biochemistry assays to identify the levels of caspases and markers involved in the extrinsic, intrinsic, and ER stress-mediated apoptotic pathways. Activity assays and Western blot analysis indicated that the expression and activity of CASP8, a key factor in the extrinsic apoptotic pathway, was markedly enhanced in imatinib/ATO co-treated samples. Similarly, Western blotting analyses of mitochondria-associated intrinsic apoptotic caspase cascades revealed the expression of strongly activated forms of CASP9, CASP3, and PARP in co-treated samples. The protein levels of the ER stress markers GRP78/HSPA5 and DDIT3 were more prominently expressed in co-treated samples and correlated well with mRNA levels. Taken together, these protein biochemical data provide additional evidence for the involvement of the three apoptotic pathways in imatinib/ATO co-treated K562 cells. We also found that the fresh bone marrow cells from the CML patients further supported the notion that imatinib/ATO combination therapy can effectively and efficiently induce more programmed cell death through the coordinated engagement of these apoptotic pathways.

The synergism revealed by transcriptomics can be immediately validated by small-scale techniques routinely used in protein biochemistry studies, and can be further confirmed *in vivo*. Ongoing research, from data-driven discovery to hypothesis-driven validation, allows us to gain detailed information on the mechanisms underlying synergism in an efficient, effective, and reasoned framework. Furthermore, the knowledge obtained in such research can assist us in eventually developing more sophisticated protocols for the treatment of leukemia and other human malignancies. In particular, the use of combination therapies that mechanistically target distinct apoptotic pathways can result in the synergistic induction of apoptosis in malignant cells.

CONCLUSIONS AND PERSPECTIVES

Understanding the complexity and dynamics of combination therapy requires data- and hypothesis-driven, quantitative, high-throughput measurements of genes and proteins at both spatial and temporal levels. Using transcriptomics, proteomics, computational biology, protein biochemistry, and routine validation techniques,

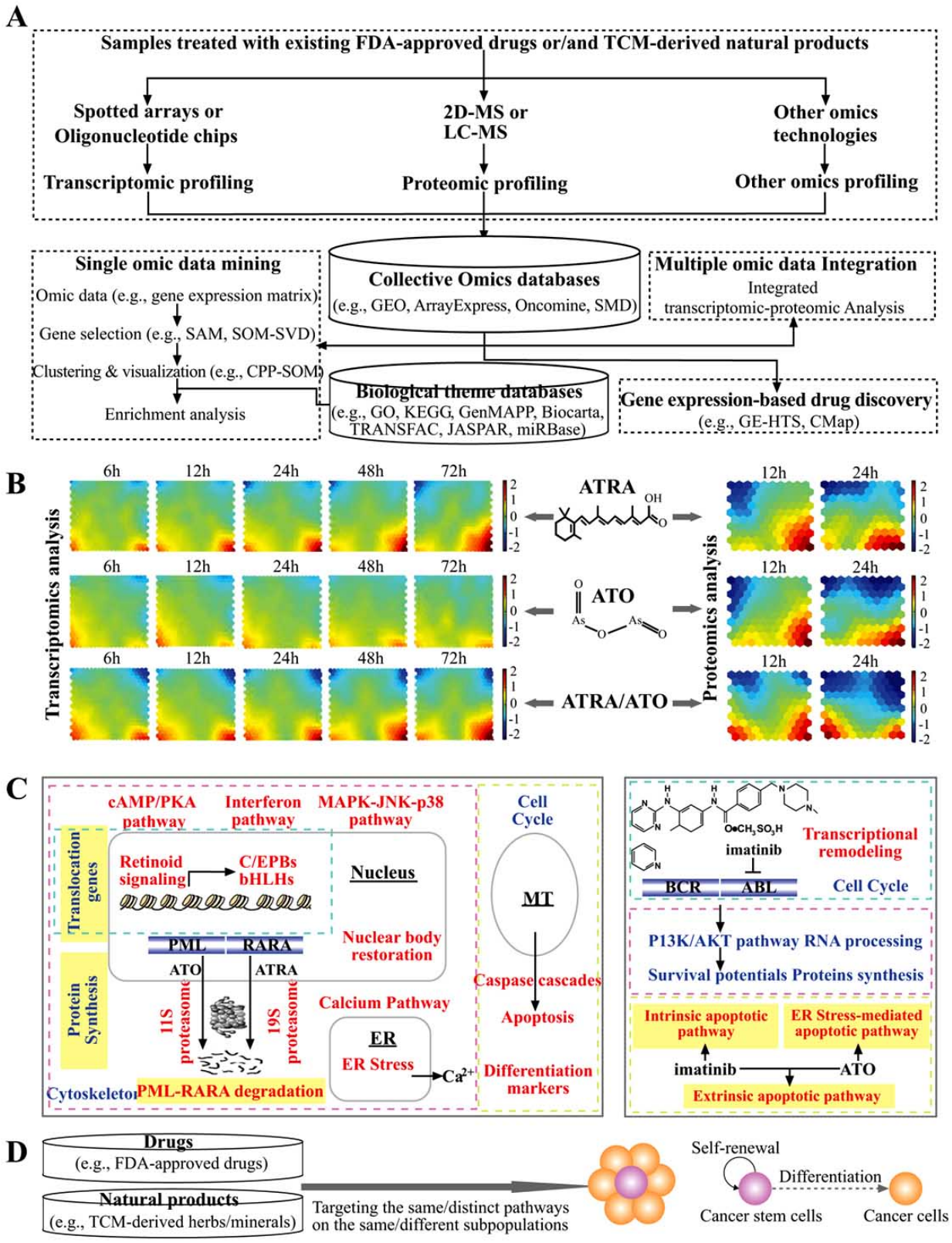

Fig. (1). Transcriptome and proteome analyses of the synergism between drugs and/or natural products. **(A)** Framework of high-throughput omics technologies in achieving effective and efficient drug development. Cultured human cancer cells (e.g., leukemia cells) are exposed to a single or a combination of agents, including existing FDA-approved drugs and TCM-derived natural products. The treated samples are subjected to diverse omics analyses in a simultaneous, parallel, automated manner, such as spotted arrays or oligonucleotide chips for transcriptome profiling, and 2D-MS or LC-MS for proteomic profiling. Gene-expression profiles are submitted to collective omics databases. These data warehouses serve as the hub for data mining, ranging from a typical pipeline of single omic data mining (e.g., transcriptomic data analysis procedures, such as gene selection, gene clustering, and visualization, and enrichment analysis of biological themes), to multiple omics data integration (e.g., integrated transcriptomic-proteomic analyses), and to GE-HTS and CMap for drug discovery.

(Legend Fig. 1) contd….

(B) CPP-SOM illustrates the dynamic changes in the transcriptome and proteome of NB4 leukemia cells treated with ATRA, ATO, or ATRA/ATO. Each map presentation illustrates treatment-specific transcriptome (or proteome) changes, in which up- (red), down- (blue), and moderately-regulated (yellow and green) genes are well delineated. The color bar represents the expression level (log ratio with base 2). **(C)** Ideogram illustrating the temporal-spatial relationships among major molecular events occurring during ATRA/ATO-induced differentiation/apoptosis in APL (left panel) or during imatinib/ATO-induced apoptosis in CML (right panel). Molecular events enriched with up-regulated genes/proteins are marked in red, whereas those enriched with down-regulated genes/proteins are marked in blue. Synergistically-regulated events are highlighted with yellow background. Events occurring at the early, intermediate, and late stages are outlined by cyan, pink, and green dotted lines, respectively. Also indicated as necessary are the intracellular compartments, including the nucleus, ER, and mitochondria (MT). **(D)** Synergistic therapeutic actions of drug/natural product combinations. Drug synergism can be achieved by targeting the same pathway (e.g., ATRA/ATO combination in APL) or distinct pathways (e.g., imatinib/ATO combination in CML). Synergism can also be achieved by targeting different subpopulations of cancer cells in a hierarchical fashion, i.e., one agent targets cancer cells, while the other targets cancer stem cells that have limitless self-renewal capacities and the potential to differentiate into multiple lineages of cancer cells.

we performed a systems analysis of ATRA/ATO-induced differentiation/apoptosis in APL and imatinib/ATO -induced apoptosis in CML. These studies provide enormous insights into the molecular networks underlying differentiation-induced and apoptosis-induced therapies, and into combination therapy synergism. Our results justify the power of an integrative approach in drug discovery, and dramatically expand our understanding of the components of synergism between drugs and/or natural products.

The continued application of high-throughput technologies and the broad evaluation of known drugs, and uncharacterized natural products from TCM will be abundantly useful in cancer stem cell (CSC) research. In the past decade, accumulating knowledge in CSC biology has tremendously impacted our understanding of the genesis of human malignancy and the development of CSC-targeted therapies [108-113]. In leukemia, drug resistance and relapse after conventional chemotherapy are largely thought to be attributed to the persistence of surviving LSCs, which are unresponsive to chemotherapeutics such as cytosine arabinoside that mainly kill more mature dividing leukemic blasts [114-116]. There is an urgent need for therapeutic strategies that specifically ablate LSCs while sparing normal hematopoietic stem cells (HSCs), not only for overcoming resistance and relapse, but also for preventing and ensuring complete disease remission. Thus, CSC-specific targeted therapies are a promising approach for designing, or identifying therapeutic strategies and agents, as evidenced in emerging LSC-specific and LSC-related combination therapies.

Experimental and clinical data have shown that the LSC populations display unique molecular characteristics that are distinct from their normal counterparts [114], enabling the design of drugs specifically targeting LSCs. For example, NF-κB is constitutively active in most AML LSCs but not in normal HSCs [117]; therefore, drugs that inhibit NF-κB-mediated survival signals, such as the proteasome inhibitor MG-132 and the natural bioactive product parthenolide (PTL), may induce selective apoptosis in AML stem cells [118]. Similar efforts are being made to identify attractive therapeutic targets of LSCs and to develop targeted drugs, including rapamycin for the PI3K/Akt/mTOR pathway in AML [119, 120], a BCR-ABL tyrosine kinase inhibitor (e.g., imatinib, dasatinib, or nilotinib) combined with myeloid cytokines in CML [121], and antibodies against LSC cell-surface molecules combined with conventional chemotherapy in AML [122-126].

The LSC-specific and LSC-related combination therapies will greatly benefit from known FDA-approved drugs and uncharacterized natural products in TCM. The synergism of combined therapeutic agents can be achieved by targeting either the same and/or distinct pathways on the same and/or different cancer cell subpopulations, or by combination of these mechanisms (Fig. **1D**). Advances in transcriptomics, proteomics, and other functional genomics, along with integrative bioinformatics, hold significant promise in the identification and understanding of the underlying synergistic components in combination therapeutics.

ACKNOWLEDGEMENTS

This study was supported in part by the Knowledge Innovation Program of Chinese Academy of Sciences (KSCX2-YW-R-19) and the 100-Talent Program (J.Z.), Ministry of Science and Technology of China Grants (2006CB910405, 2007AA02Z335, 2006CB 910700, 2006AA02Z302, 2006AA02Z332 and 2009CB825607), National Natural Science Foundation Grants (30730033, 30670436, 30600260 and 30500201), Shanghai Commission of Science and Technology (06XD14012 and 07SP07002).

ABBREVIATIONS

2D-MS	=	Two-dimensional gel electrophoresis and mass spectrometry
AML	=	Acute myelogenous leukemia
APL	=	Acute promyelocytic leukemia
Ara-C	=	Cytosine arabinoside
ATO	=	Arsenic trioxide
ATRA	=	*All-trans* retinoic acid
ChIP-Chip	=	Chromatin immunoprecipitation coupled microarrays
CMap	=	Connectivity Map
CML	=	Chronic myelogenous leukemia
CPP-SOM	=	Component plane presentation integrated self-organizing map
CSCs	=	Cancer stem cells
ER	=	Endoplasmic reticulum
FDA	=	U.S. Food and Drug Administration
GE-HTS	=	Gene expression-based high-throughput screening
HSCs	=	hematopoietic stem cells
LC-MS	=	Liquid chromatography coupled with mass spectrometry
LSCs	=	Leukemia stem cells
PML-RARA	=	The fusion between the promyelocytic leukemia gene and retinoic acid receptor alpha
RT-PCR	=	Real-time reverse transcription-polymerase chain reaction
SVD	=	Singular value decomposition
TCM	=	Traditional Chinese medicine
UPS	=	Ubiquitin-proteasome system.

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Received: July 15, 2008 **Revised: September 22, 2008** **Accepted: October 30, 2008**