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Invited review

Leukemia, an effective model for chemical biology and target therapy[1]

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Abstract

The rapid rise of chemical biology aimed at studying signaling networks for basic cellular activities using specific, active small molecules as probes has greatly accelerated research on pathological mechanisms and target therapy of diseases. This research is especially important for malignant tumors such as leukemia, a heterogeneous group of hematopoietic malignancies that occurs worldwide. With the use of a chemical approach combined with genetic manipulation, great progress has been achieved over the past few decades on the biological, molecular and cytogenetic aspects of leukemia, and in its diagnosis and therapy. In particular, discoveries of the clinical effectiveness of *all-trans* retinoic acid and arsenic trioxide in the treatment of acute promyelocytic leukemia and the kinase inhibitors Imatinib and Dasatinib in the treatment of chronic myelogenous leukemia not only make target therapy of leukemia a reality, but also push mechanisms of leukemogenesis and leukemic cell activities forward. This review will outline advances in chemical biology that help our understanding of the molecular mechanisms of cell differentiation and apoptosis induction and target therapy of leukemia.

Introduction

It is increasingly clear that all biological processes, in principal, rely on chemical processes that are governed by the structure of the participating molecules and their interactions and, thus, the biological processes can be treated as chemical processes and studied in molecular detail. The Human Genome Program was a landmark event for life science and has uncovered a large number of novel genes and provided an unprecedented prospectus for target identification and the development of drugs. However, the function of these genes cannot be predicted merely from their structure, thereby demonstrating the need for the systematic collection of biological information in addition to the gene sequence to fully exploit the genomic data. As a result, the accumulation of genome sequence data has not resulted in the anticipated acceleration of novel therapeutic developments. We are now facing the exciting but daunting task of identifying and assigning functions and therapeutic potentials to all 30 000 human genes[1]. To address the latter issue, a myriad of strategies have emerged to facilitate genomics-driven target identification and validation. The "functional genomics" technologies, such as genetic-based knock-out and knock-in, RNAi and ectopic expression of targeted genes, high-throughput cellular profiling of gene and protein expression, systematic analyses of protein complexes and bioinformatics, have played active roles in the past few years. Many of these techniques are used in limited classes of gene products, but none of the techniques has proven to be the generally useful strategy that was anticipated[1,2].

Small molecules participate directly in many biological processes and function in diverse roles, including chemical

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triggers, inhibitors, stimulators and switches. In contrast, compared with genetic approaches, the effect of small molecules is generally fast and reversible because of the metabolism and excretion of the molecules, which enables transient study of the proteins. In addition, the effect of small molecules is tunable because a varying concentration can result in different degrees of phenotype expression[3]. Therefore, small active molecules, providing a common link between the fields of chemistry and biology, have long had an important role in deciphering answers to fundamental biological questions, especially in the exploration of signaling networks for cellular activities. In the past decade, chemical biology, aimed at studying signaling networks for basic cellular activities using specific, active small molecules as probes, has developed rapidly. This rise in chemical biology greatly accelerates research on molecular mechanisms and target therapy of diseases, especially in malignant tumors. Complementary to genetics and genomics, chemical biology has become an effective tool to study the functions of proteins/genes and important cellular activities[1,4,5]. The recent explosion of interest in chemical biology combined with genetic manipulation reflects a fascination with research at the interface of chemistry and biology, where chemical insights are brought to bear on exciting biological and biomedical problems[6].

Leukemia, a heterogeneous group of hematopoietic malignancies that occurs worldwide, includes acute/chronic and myeloid/lymphocytic leukemias. Despite many important advances in understanding the biological, molecular and cytogenetic aspects of leukemia, as well as in the diagnosis and therapy of leukemia, the most people will die of their diseases[7,8]. Obviously, it is imperative to understand the pathophysiological mechanisms of leukemia and to explore new therapeutic strategies. It has been widely understood that various kinds of leukemias present specific cytogenetic alterations, especially chromosome translocations, that generate abnormal oncogenic fusion proteins[9]. These alterations disrupt the normal signaling and cause uncontrolled proliferation, blocked differentiation and/or damaged apoptosis. Because of these genetic and cellular features, and because of the ease of capture of leukemia samples and the relatively convenient evaluation of therapeutic effects, small molecule-based chemical biology has been widely used with leukemia and important progress has been achieved over the past two decades. Based on several examples, mainly from our own work, this review will outline the advances of chemical biology in understanding molecular mechanisms of cell differentiation and apoptosis induction and target therapy of leukemia.

All-trans retinoic acid-based chemical biology of leukemia

An important feature of acute myeloid leukemia (AML), which accounts for 75% of all acute leukemias, is the blockage of the differentiation of myeloid cells at different stages. Over the past 20 years, many studies have explored the differentiation-inducing agents for leukemic cells. A typical and successful example is the discovery of *all-trans* retinoic acid (ATRA) as a differentiation-inducing drug for acute promyelocytic leukemia (APL)[10], a unique subtype of AML that is defined by blockage at the promyelocytic stage of myeloid cell maturation. Using cytogenetic analysis, it has been shown that over 95% of patients with APL carry specific chromosome translocation t(15,17), which leads to the fusion of the promyelocytic leukemia (PML) gene on chromosome 15 and the retinoic acid receptor-alpha (RARα) gene on chromosome 17, and the generation of PML-RARα fusion protein[11]. A series of *in vitro* and *in vivo* works, including those from transgenic mice[12], have shown that this fusion protein plays a critical role in the pathogenesis of APL via interfering functions of wild-type PML, RARα and its heterodimeric partner retinoid X receptor (RXR), as well as obtaining its new functions, as widely reviewed[13–15]. As widely confirmed, ATRA can induce complete clinical remission, although retinoid resistance and relapse frequently occur[16]. Because ATRA can cleave the PML-RARα fusion protein and, more importantly, it can potently drive AML cells to undergo differentiation, ATRA as a probe has greatly promoted our understanding of signaling pathways of leukemogenesis and leukemic cell differentiation. Using chemical and biological studies based on ATRA and other differentiation-inducing agents, such as phorbol 12-myristate 13-acetate (PMA) and vitamin D_3, many differentiation and leukemogenesis-related molecules, such as CCAAT/enhancer binding protein (C/EBP), nuclear corepressors (N-CoR), Sin3A and histone deacetylase (HDAC), have been examined[17,18]. Vice versa, knowledge of these differentiation-related molecules has also led to the development of new differentiation agents, such as HDAC inhibitor valproic acid[19].

As an example, phospholipid scramblase 1 (PLSCR1) is a multiply palmitoylated protein that is localized in either the cell membrane or nucleus depending on its palmitoylated state. Increasing evidence has demonstrated the biological role of PLSCR1 in cell signaling, maturation and apoptosis[20]. ATRA can elevate PLSCR1 expression in ATRA-sensitive APL cells NB4 and HL60, but not in maturation-resistant NB4-LR1 cells. Furthermore, PMA, but not dimethyl sulfoxide (DMSO), sodium butyrate or vitamin-D_3-

induced leukemic cell differentiation, is also accompanied by increased PLSCR1 protein[20]. Based on the fact that ATRA and PMA can activate protein kinase Cdelta (PKCδ) by phosphoryla-tion, PKCδ-specific inhibitor rottlerin is shown to nearly eliminate ATRA-induced and PMA-induced PLSCR1 expression, whereas ectopic expression of a constitutively active form of PKCδ directly increases PLSCR1 expression[21]. Thus, PKCδ is proposed to exert a critical role in ATRA-induced or PMA-induced PLSCR1 expression. Furthermore, interferon upregulates PLSCR1 by sequential activation of PKCδ, JNK and STAT1[22]. In contrast, decreasing PLSCR1 expression with small interfering RNA (siRNA) inhibits ATRA/PMA-induced differentiation[21], whereas inducible PLSCR1 expression induces growth arrest at the G_1 phase and granulocyte-like differentiation together with increased sensitivity to apoptosis induction[23]. All these data support the anti-leukemia role of PLSCR1, although its exact molecular mechanisms need to be further investigated. In agreement with this notion, M2, M5a, and M5b type AML cells present lower levels of PLSCR1 than normal bone marrow cells, and the PLSCR1 mRNA level is associated with significantly longer overall survival[24].

Arsenic trioxide-based chemical biology of leukemia

Following the successful practice of ATRA, several research groups in China, including ours, reported impressive clinical response rates in patients with APL who had received arsenic trioxide (As_2O_3), a common naturally occurring substance. Subsequently, clinical studies conducted in other nations confirmed earlier reports and As_2O_3, commercial name Trisenox (Cell Therapeutics, Seattle, WA, USA), was approved by the Food and Drug Administration for the treatment of relapsed/refractory APL[25–32]. Later, single-agent As_2O_3-induced durable remission with minimal toxicity in the newly diagnosed APL was also reported[33–35]. Following these successful experiences, the mechanisms of As_2O_3 action on APL cells and other cancer cells have been attracting wide interest and great progress has been made[36–38]. In brief, As_2O_3 can rapidly modulate the subcellular localization of PML and PML-RARα proteins and degrade PML-RARα protein[39–41]. In a cytological direction, As_2O_3 induces apoptosis in an impressive array of cancer cells in addition to APL[42], in which various kinds of mechanisms have been documented[43,44]. However, the clinical effectiveness of As_2O_3 appears to be restricted to APL, although clinical studies have shown that As_2O_3-based regimens are clinically active in patients with relapsed/refractory multiple myeloma (MM)[45]. In contrast, *in vivo* observations in APL patients and animal models show that As_2O_3 can induce partial differentiation of APL cells[27,41,46]. Hence, the *in vitro* differentiation-inducing effect of As_2O_3 was investigated in APL cells. Indeed, a relatively longer treatment of low-dose As_2O_3 (0.1–0.5 μmol/L) induces APL cells to undergo partial differentiation[41,47]. However, it appears that it is not more significant than its *in vivo* activity. Thus, we speculated that some factors in the bone marrow (BM) microenvironment could modulate the *in vivo* activity of As_2O_3. Towards this end, we considered that oxygen concentration may be one such factor that impinges on the *in vivo* action of As_2O_3 based on the following facts: leukemic cells are cultured *in vitro* at ambient oxygen, whereas *in vivo* cells are physiologically exposed to much lower oxygen levels ranging from 16% in pulmonary alveoli to less than 6% in most peripheral organs of the body. Oxygen levels of BM in AML patients may be decreased as a result of the fast growth of leukemic cells, and are possibly further aggravated by the anemia that often accompanies newly diagnosed AML patients, although leukemic cells do not form a well-circumscribed “mass” in BM like they do in solid tumors. More importantly, angiogenesis, which increases blood supply and oxygen tension, is also vital in the pathogenesis of different hematologic malignancies and provides an independent predictor of outcome in adults with AML, while the anti-angiogenic effects of chemotherapeutic and other novel drugs for the treatment of leukemia, such as ATRA and As_2O_3, might contribute to their therapeutic potentials. We investigated whether low oxygen tension impacts on the action of As_2O_3 on APL cells. Unexpectedly, cobalt chloride ($CoCl_2$)/desferrioxamine (DFO)-mimicked hypoxia or moderate hypoxia (2% and 3% O_2) can directly trigger AML cells to undergo differentiation both *in vitro* and *in vivo*[48–50]. The differentiation-inducing effect of As_2O_3 on APL cells is also enhanced by hypoxia mimetic agents[51]. Using chemical interference, inducible expression and siRNA technology, it is confirmed that hypoxia-inducible factor (HIF)1α exerts a critical role in this event, possibly in a transcription activity independent manner[49,52]. Furthermore, HIF-1α interacts with and increases the transcription activities of two known hematopoiesis transcription factors, AML1 and CCAAT/enhancer binding factor-alpha (C/EBPα)[49,53-55]. A low concentration of Tiron, a non-toxic chelator used to alleviate acute metal overload, has recently been shown to be a potent inducer of cell differentiation in human promyelotic HL-60 leukemia cells via increased HIF-1α and C/EBPα[56]. Intriguingly, HIF-1α is dysregulated in a murine APL model[57]. Nguyen-Khac also recently reported that hypoxia accelerates the differentia-

tion of normal $CD34^+$ cells, whereas TEL-ARNT fusion protein, which results from t(1;12)(q21;p13), inhibits hypoxia-induced normal progenitor cell differentiation through the interaction of TEL-ARNT with HIF-1α[58,59].

Apoptosis induction-based chemical biology of leukemia

As an intrinsic cell death program, apoptosis plays a critical role in the tissue homeostasis of multicellular organisms, especially in organs, such as the hematopoietic system, where high rates of daily cell production are offset by rapid cell turnover. Indeed, the hematopoietic system provides numerous examples to show the importance of apoptotic cell death for achieving the control of homeostasis. Correspond-ingly, disorder of the apoptosis machinery would cause various diseases. For example, damaged apoptosis exerts an important role in the pathogenesis and resistance to chemotherapeutic drugs of cancers including leukemia[60]. Therefore, to understand the core molecular mechanisms of the apoptosis machinery has become an important and interesting research direction.

Over the past years, much progress has been achieved in understanding the molecular mechanisms of initiation and regulation of apoptosis. In brief, apoptosis is mainly caused by the activation of intracellular aspartate-specific proteases, known as caspases, with over 10 members (eg, caspase-8, 9 and 3). The mechanisms of caspase activation by apoptosis inducers have been the center of much attention in recent years. To date, two pathways of caspase activation and, thus, apoptosis have been elucidated in great detail, that is, the mitochondria-centered intrinsic and death receptor-mediated extrinsic pathways. As for the former, multiple signals cause dysfunction of mitochondria, especially disrupted mitochondrial transmembrane potentials, which leads to the release of apoptogenic proteins, such as cytochrome c, second mitochondrial activator of caspase (Smac) and apoptosis-inducing factor (AIF), into the cytosol. Cytochrome c binds the apoptotic protease-activating factor 1 (Apaf1) and forms the apoptosome together with procaspase-9. In this multiprotein structure, caspase-9 is activated and then activated caspase-9 activates downstream effector caspases, mainly procaspase-3. In the extrinsic apoptotic pathway, death receptors such as Fas, containing a cytosolic death domain (DD), cluster in membranes when they bind to their ligands, which recruits caspase-binding adaptor proteins, such as the Fas-associating protein with death domain (FADD) that contains both a death domain (DD) and a death effector domain (DED). The DED of FADD binds DED-containing procaspases (eg caspase-8 and caspase-10), forming a death-inducing signaling complex (DISC) and resulting in downstream effector caspase-3. The activated caspase-3 cleaves numerous cellular target proteins, which in aggregate produce an apoptosis-characteristic morphology and biochemical alterations. In contrast, apoptosis machinery is also regulated by many proteins, including bcl-2 family members, inhibitors of caspases, oncoproteins and tumor-suppressing protein, and other signaling molecules. Thus, a delicate balance between pro-apoptotic and anti-apoptotic regulators of apoptosis pathways is essential to ensure the survival of long-lived cells and the proper turnover of short-lived cells in a variety of tissues.

During these great advances on apoptosis, chemical approaches with small-molecule apoptosis inducers and inhibitors as probes provided a huge help. In fact, a lot of information on cell apoptotic processes was contributed by chemical biological investigations[61]. For example, wild p53 was found to actively mediate the apoptosis of the hematopoietic cells by depriving the cells of specific growth factors, such as IL-3, and p53 might be involved in the response of myeloid precursors to environmental cytokines for the maintenance of hematopoietic homeostasis[62]. Recently, we reported that nanomolar concentrations of NSC606985, a rarely studied camptothecin analog, induces apoptosis in AML cells through the rapid proteolytic activation of PKCδ, followed by the loss of mitochondrial transmembrane potential and caspase-3 activation[63]. Furthermore, the potential therapeutic effect of this agent on AML mice generated by syngenic grafts of leukemic blasts from transgenic mice with PML-RARα was also shown[64]. Moreover, inducible expression of AML1-ETO, a fusion gene of the AML1 gene on chromosome 21 with the ETO (eight-twenty-one) gene on chromosome 8, endows leukemic cells with susceptibility to NSC606985 and other insult-induced apoptosis[65]. Using subcellular and quantitative proteomic analyses, moreover, we found a set of unique deregulated proteins in NSC606985-induced apoptotic AML cell line NB4 cells, including 16 compartment-compartment translocated ones. These proteins contributed to multiple functional activities, such as DNA damage repair, chromosome assembly, mRNA processing, biosynthesis, modification and degradation of proteins (Figure 1)[66]. These discoveries shed new insights for systematically understanding the mechanisms of camptothecin-induced apoptosis.

In contrast, the application of chemical biology in understanding the core components of the apoptosis machinery at the molecular and structural levels aids the discovery of many new potential therapies for leukemia, as reviewed by

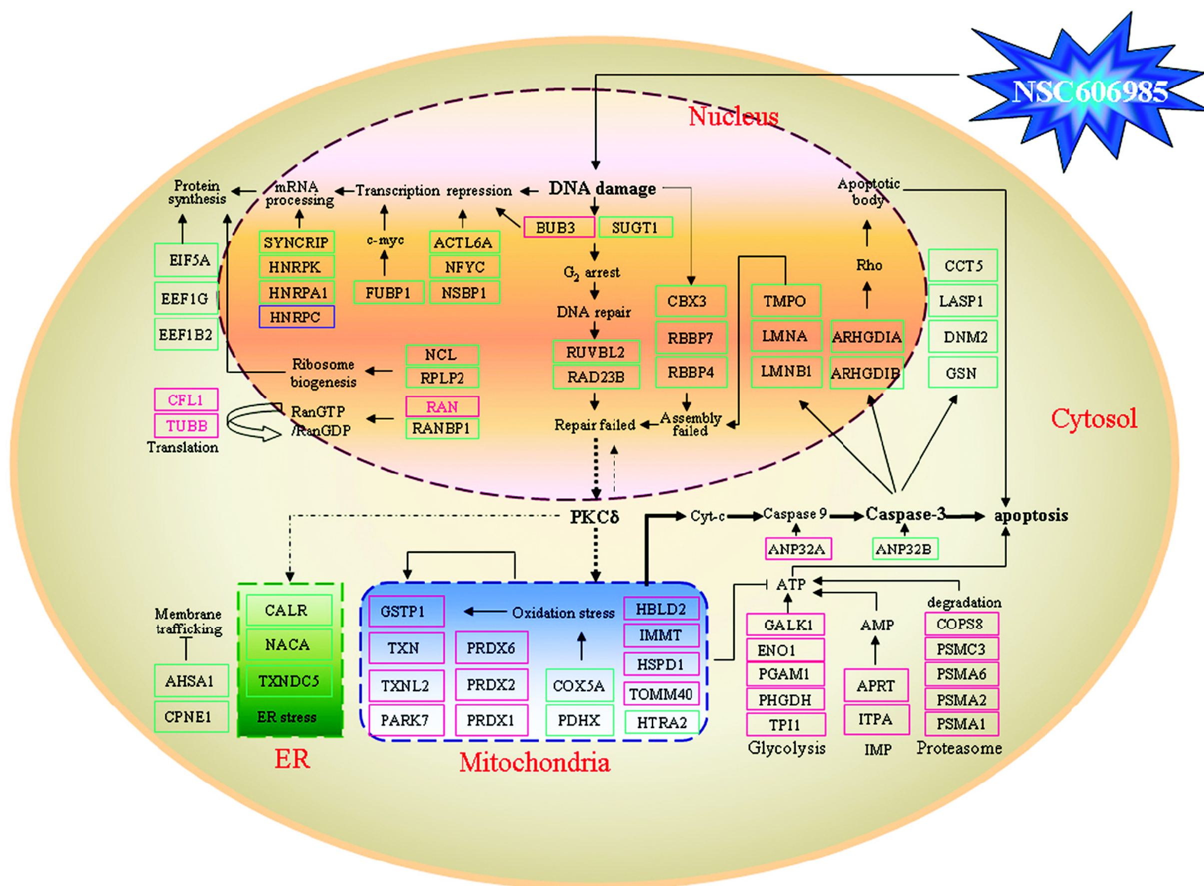

Figure 1. Ideogram illustration of the proteins involved in NSC606985-induced NB4 cell apoptosis. With NSC606985, a camptothecin analog, as an apoptosis-inducing probe, we analyzed protein expression profiles of fractionated nuclei, mitochondria, raw endoplasmic reticula and cytosols of NSC606985-induced apoptotic AML cell line NB4 cells using two-dimensional electrophoresis combined with MALDI-TOF/TOF tandem mass spectrometry. A total of 90 unique deregulated proteins, including 16 compartment–compartment translocated proteins, were identified. These proteins contributed to multiple functional activities, such as DNA damage repair, chromosome assembly, mRNA processing, biosynthesis, modification and degradation of proteins. With their functional analyses, the possible roles of these deregulated proteins in NSC606985-induced apoptosis were demonstrated using an ideogram illustration. Upregulated proteins are marked in red and the downregulated proteins are marked in green. Modified proteins are marked with blue. All abbreviations are derived from the Swiss-Prot database.

Reed and Pellecchia[67]. For instance, Bcl-2 family was recently raised up as the drug target for leukemia. The common aberrant expression of Bcl-2 in chronic lymphocytic leukemia (CLL) was proposed to confer CLL B-cell the unusual feature of resistance to undergoing apoptosis and dying properly, which is associated with leukemogenesis, poor response to chemotherapy and decreased overall survival of CLL. Consequently, selective apoptosis induction of malignant cells by interfering Bcl-2 is growing as a potential basic therapeutic strategy. In the past years, various apoptosis inducing and regulating drugs or lead compounds, including anti-apoptotic Bcl-2 or Bcl-X_L expression regulating drugs (such as synthetic retinoic acid and inhibitor of histone deacetylase) and drugs directly binding to Bcl-2 or other anti-apoptosis proteins (such as Gossypol isolated from cottonseed, HA14-1, BH3I-1, and BH3I-2, GX15-070) have been discovered.

AML cells are also characterized by constitutive and abnormal activation of the nuclear factor-kappaB (NF-κB) transcription factor. In addition, NF-κB also plays a crucial role in the pathogenesis, survival and resistance to apoptosis of HTLV-I-infected leukemic cells[68], B-cell chronic lymphocytic leukemia (B-CLL)[69], which is also supported by the fact that the PML can enhance the sensitivity of TNF-α induced apoptosis through the repression of the NF-κB survival pathway[70], while transfection of a dominant-negative

mutant NF-κB inhibitor represses p53-dependent apoptosis in acute lymphoblastic leukemia cells (ATL)[71]. Taken together, NF-κB is a suitable target for the prevention and treatment of ATL[68]. Interestingly, a specific pharmacological inhibitor of AS602868 could block NF-κB activation and lead to apoptosis of human primary AML cells. Moreover, AS602868 potentiated the apoptotic response induced by the current chemotherapeutic drugs doxorubicin, cytarabine and etoposide (VP16). In addition, NF-κB inhibition did not affect normal $CD34^+$ hematopoietic precursors, suggesting that it could represent a new adjuvant strategy for AML treatment[72]. At the same time, indole-3-carbinol, found in brassica species vegetables (such as cabbage, cauliflower and brussels spouts), can inhibit NF-κB and NF-κB-regulated gene expression and this mechanism may provide the molecular basis for its ability to suppress tumorigenesis and clinical application[73].

Leukemogenic fusion proteins-based chemical biology

Different types of leukemia usually have specific chromosome translocations that cause the activation of oncogenes and, in particular, the formation of abnormal fusion genes, such as AML1-ETO[74–76], a fusion protein generated by t(8,21) translocation that occurs in approximately 12% of all AML and 40% of M2-type AML. These fusion proteins play a pivotal role in leukemogenesis. Thus, using some small molecules to destroy them or to inhibit their activities may become an effective method to treat the corresponding type of leukemia, while these active small molecules would be more useful for understanding leukemogenic mechanisms of these fusion proteins. The destruction of PML-RARα by ATRA and As_2O_3 is a good model. Another typical example is the discovery of Bcr-Abl kinase inhibitor imatinib mesylate (also called Gleevec, STI571)[77]. Bcr-Abl is a constitutively active, cytoplasmic tyrosine kinase that is produced by t(9;22) translocation in more than 95% of chronic myelogenous leukemia (CML) and about half of patients with adult-onset acute lymphoblastic leukemia (ALL). The central role of Bcr-Abl kinase in leukemogenesis promoted it as an ideal target for drug screen to treat CML. Hence, imatinib was discovered as a tyrosine kinase inhibitor and was introduced into the armamentarium of drugs for the treatment of CML and has since revolutionized its management[77]. Imatinib has a high affinity for the ATP-binding site of Abl, and clinical trials have validated the promise of this molecular targeted therapy. In the more advanced phases of CML, imatinib was able to induce a major (complete or partial) cytogenetic response in 16%–60% of CML patients. In a phase III trial comparing imatinib with interferon-α plus cytosine arabinoside in newly diagnosed chronic phase CML, 85% of patients treated with imatinib attained a major cytogenetic response after a median follow-up of 19 months, compared to 22% in patients treated with the latter combination. A recent update has shown a further increase in major cytogenetic response of up to 92% of patients in the imatinib arm after a median follow-up of 54 months. In view of its high efficacy and low toxicity, imatinib has now replaced interferon-α as the frontline treatment for CML patients who are not eligible for allogeneic stem cell transplantation. Meanwhile, the application of imatinib as a probe greatly improved our understanding of Bcr-Abl-targeted signaling pathways, and has revealed a complex web of signals that promote cell division and survival[78].

Imatinib is now frontline therapy for CML, but resistance is increasingly encountered, which has dampened the initial enthusiasm for this much heralded “magic bullet”. It has been shown that resistance to imatinib in CML occurs through the selection of tumor cells harboring Bcr-Abl kinase domain point mutations that interfere with drug binding. To date, at least 73 different point mutations leading to the substitution of 50 amino acid residues in the Abl kinase domain have been isolated from CML patients resistant to imatinib. Crystallographic studies revealed that imatinib binds to the ATP-binding site of Abl only when the activation loop of the kinase is closed and stabilizes the protein in this inactive conformation. Thus, most imatinib-resistant mutants should remain sensitive to inhibitors that bind Abl with less stringent conformational requirements. Based on this notion, an orally bioavailable Abl kinase inhibitor called Dasatinib (BMS-354825) has been examined[79], which presents a two-log increased potency relative to imatinib that retains activity against 14 of 15 imatinib-resistant Bcr-Abl mutants. Clinical trials of Dasatinib in imatinib-resistant and imatinib-intolerant CML and Ph chromosome-positive ALL are currently in progress[80–82].

Conclusion

The rapid rise of chemical approaches, that is, studying signaling networks using basic cell activities with specific, active small molecules as probes, greatly accelerates research on the molecular mechanisms of disease and target therapy, especially in malignant tumors such as leukemia. With the application of chemical approaches combined with genetic manipulation, significant progress on the basic knowledge, diagnosis and therapy of leukemia has been achieved over the past few decades. In particular, discoveries of the clinical effectiveness of ATRA and As_2O_3 in the treatment of APL

and of Bcr-Abl kinase inhibitor Imatinib and Dasatinib in the treatment of CML not only make target therapy of leukemia a reality, but also push mechanisms of leukemogenesis and leukemic cell activities forward. These successful practices are attracting wide interest on the application of a chemical approach in leukemia and other cancers.

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