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Natural products against hematological malignancies and identification of their targets

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Naturally occurring molecules derived from higher plants, animals, microorganisms and minerals play an important role in the discovery and development of novel therapeutic agents. The identification of molecular targets is of interest to elucidate the mode of action of these compounds, and it may be employed to set up target-based assays and allow structure-activity relationship studies to guide medicinal chemistry efforts toward lead optimization. In recent years, plant-derived natural compounds possessing potential anti-tumor activities have been garnering much interest and efforts are underway to identify their molecular targets. Here, we attempt to summarize the discoveries of several natural compounds with activities against hematological malignancies, such as adenanthin, oridonin, gambogic acid and wogonoside, the identification of their targets, and their modes of actions.

natural products, adenanthin, hematological malignancies, target identification

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The use of bioactive small molecules, either as single molecular entities or as mixtures, for treating disease is an integral part of human medicine [1,2]. Actually, the great reservoir of natural compounds derived from higher plants, animals, microorganisms and minerals demonstrate their important role in the discovery and development of novel therapeutic drugs [3,4]. According to a detailed analysis of new medicines approved by the US Food and Drug Administration (FDA) between 1981 and 2010, about 34% of these medicines based on small molecules were natural products or direct derivatives of natural products [5]. However, while there is an enormous diversity in the number of plant species (besides animals, microorganisms and minerals) worldwide, less than 10% have been screened for biological activity, and only 15% have been phytochemically evaluated. Information concerning the mechanisms of action of bioactive natural products at a molecular level, especially the molecular target(s) of a drug candidate, is of great sig-

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nificance, since it could help set up target-based assays and allow structure-activity relationship studies to guide medicinal chemistry efforts toward lead optimization [1,6]. However, achieving this in an effective, unbiased, and efficient manner is a significant challenge in this new era of drug discovery and optimization [7–9].

Hematological malignancies such as leukemia, lymphomas, and multiple myelomas are a large class of diseases that are characterized by the uncontrolled malignant proliferation, blockade of differentiation and inhibited apoptosis of blood cells [10]. Some of their prominent features include rapid progression and evasion to treatment, thus leading to resistance and relapse. In past decades, rapid development of molecular targeted therapy has been best illustrated by advances in the management of hematological malignancies [11]. For example, several generations of tyrosine kinase inhibitors including imatinib, dasatinib, nilotinib, as well as ponatinib and bosutinib have had a tremendous impact on the clinical outcomes of chronic myeloid leukemia (CML). These compounds act by selectively inhibiting the oncogenic breakpoint cluster region-Abelson (BCR-ABL) fusion protein [12], found in CML cells carrying the chromosome translocation t(9;22) (q34;11), known as the Philadelphia chromosome, which causes fusion between the *BCR* and the *ABL* genes resulting in the expression of the potent kinase activity by the BCR-ABL fusion protein [13–15].

As summarized by Meng and Huang [16] in this issue, Chinese scientists and physicians have also made great contributions to the fight against hematological malignancies, especially leukemia. A typical example is the effective applications of *all-trans* retinoic acid (ATRA, a vitamin A derivative) and arsenic trioxide (an ancient drug used in traditional Chinese medicine) in acute promyelocytic leukemia (APL, a subtype of acute myeloid leukemia (AML)), majority of which carry a chromosome translocation t(15;17) expressing the oncogenic promyelocytic leukemia retinoic acid receptor-alpha (PML-RARα) fusion protein [17]. Both drugs cleave or degrade the fusion protein, where arsenic trioxide was shown to bind directly to cysteine residues in zinc fingers located within the RBCC domain of PML-RARα and PML, inducing PML oligomerization and thus enhancing sumoylation and degradation of these two proteins [18]. On the other hand, ATRA selectively inhibits and degrades the active unique peptidyl-prolyl-isomerase Pin1 in cancer cells by directly binding to substrate phosphate- and proline-binding pockets in its active site, and thereby degrading degrades the oncogenic PML-RARα fusion protein [19].

Thalidomide, which was developed and sold as a sedative around 60 years ago but was soon banned because of its association with serious developmental defects, is being re-evaluated and is recognized as an effective treatment for myeloma [20,21]. Thalidomide derivatives including lenalidomide and pomalidomide have also been developed. More recently, thalidomide and its derivatives have been shown to directly target the cereblon (CRBN) protein, a substrate-recognition component of an ubiquitin E3 ligase enzyme complex, promoting the degradation of the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3), underlying the drugs' efficacy against myeloma [22,23]. Lenalidomide was also shown to be a highly effective treatment option for myelodysplastic syndrome (MDS) with deletions of chromosome 5q [24,25], a disorder of hematopoietic stem cells. Recently, Krönke et al. [26] reported that lenalidomide targets the CRBN protein to induce ubiquitination and degradation of casein kinase 1A1, which is encoded by a gene within the common deleted region in del(5q) MDS, providing a mechanistic basis for the therapeutic window of lenalidomide in del(5q) MDS.

Inspired by these successful practices, many natural compounds were evaluated for their potential activities against hematological malignancies in the past decades [27,28]. Here we attempt to focus on several natural products that have shown anti-hematological malignancy activity and the identification of their potential targets.

1 Adenanthin

Diterpenoids, a large class of secondary metabolites isolated from plants, possess a wide spectrum of biological activities such as anti-tumor, anti-inflammation and significant cardiovascular effects [29,30]. Structurally, they can be classified into four groups including the ent-kauranes, ent-6,7-secokauranes, ent-8,9-secokauranes and others (for more detail, see reference [30]). More than 600 diterpenoids have been identified in China thus far, some of these are abundant in plants such as Isodon (Rabdosia). Through our cooperation with professor Hang-Dong Sun's group in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, we screened up to 400 natural ent-kaurene diterpenoids [31] for their potential anti-leukemic activities. We demonstrated that pharicin B, a natural ent-kaurane diterpenoid from the leaves of *Isodon pharicus* (a type of Chinese herbal plant found mainly in Tibet), can rapidly stabilize and enhance ATRA-dependent transcriptional activity of the RARα protein, through which the natural product exhibits a synergistic or additive differentiation-enhancing effect with ATRA in several AML cell lines and some primary leukemic cells [32]. We also reported that pharicin A (Figure 1B), another ent-kaurene diterpenoid originally extracted from *Isodon pharicus* leaves, directly binds to BubR1 to induce mitotic arrest in leukemia and solid tumor-derived cells including chemotherapeutic drug paclitaxel-resistant malignant cells, and cells with unaligned chromosomes, aberrant BubR1 localization and deregulated spindle checkpoint activation [33]. Here we focus on the anti-leukemic activity of adenanthin

and the identification of its targets. Adenanthin (Figure 1), a diterpenoid isolated from the leaves of *Rabdosia adenantha* [34], significantly decreases cell viability at a concentration of more than 4 μmol L^{-1}, while it also induces APL cells to undergo differentiation, independent of their sensitivity to ATRA [35]. Our further investigations showed that the adenanthin-induced differentiation can also be seen in primary leukemic blasts from the bone marrow of APL patients and in four of six newly diagnosed non-APL AML patients [36], suggesting that its differentiation-inducing effect is not restricted to APL. Applying isogenic B strain of Friend leukemia Virus (FVB/N) mice models with intravenous transplantation of ATRA-sensitive and ATRA- resistant leukemic blasts from transgenic mice expressing human PML-RARα and its mutated form, we revealed that the intravenous administration of adenanthin (5 mg kg^{-1} body weight, each day for five consecutive days a week) significantly induces differentiation and tumor regression, and prolongs the survival of these two kinds of leukemic mice in spite of their sensitivity to ATRA [35]. Furthermore, adenanthin also significantly eliminates APL-initiating progenitor cells ($CD34^+$, c-kit^+, $Fc\gamma RIII/II^+$, and $Gr1^{int}$) in ATRA-sensitive leukemic mice [35]. All these results point to the potential therapeutic efficacy of adenanthin against AML.

Thus, we attempted to identify the potential target(s) for adenanthin's differentiation-inducing effect via a chemical proteomic approach [35,36], similar to that summarized by Ziegler et al. [6]. For this purpose, a biotin-tagged adenanthin probe, which retained the ability to induce leukemic cell differentiation, was synthesized after establishing the structure-activity relationships of adenanthin. It was assumed that the α,β-unsaturated moiety in adenanthin is a Michael acceptor that potentially captures nucleophiles such as cysteine (Cys) at its targeted binding site and forms covalent adducts, thus allowing for efficient protein recovery by the pull-down probe. Actually, reduction of the Michael acceptor double bond of adenanthin to a single bond abolished its *in vitro* and *in vivo* differentiation-inducing activity in leukemic cells (Figure 1), making it a suitable negative control. Thus, the NB4 APL cell line lysates were incubated with biotin-tagged adenanthin or free biotin, and the bound proteins were isolated with streptavidin-coated agarose beads, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. The biotin-tagged adenanthin clearly precipitated only one detectable band at

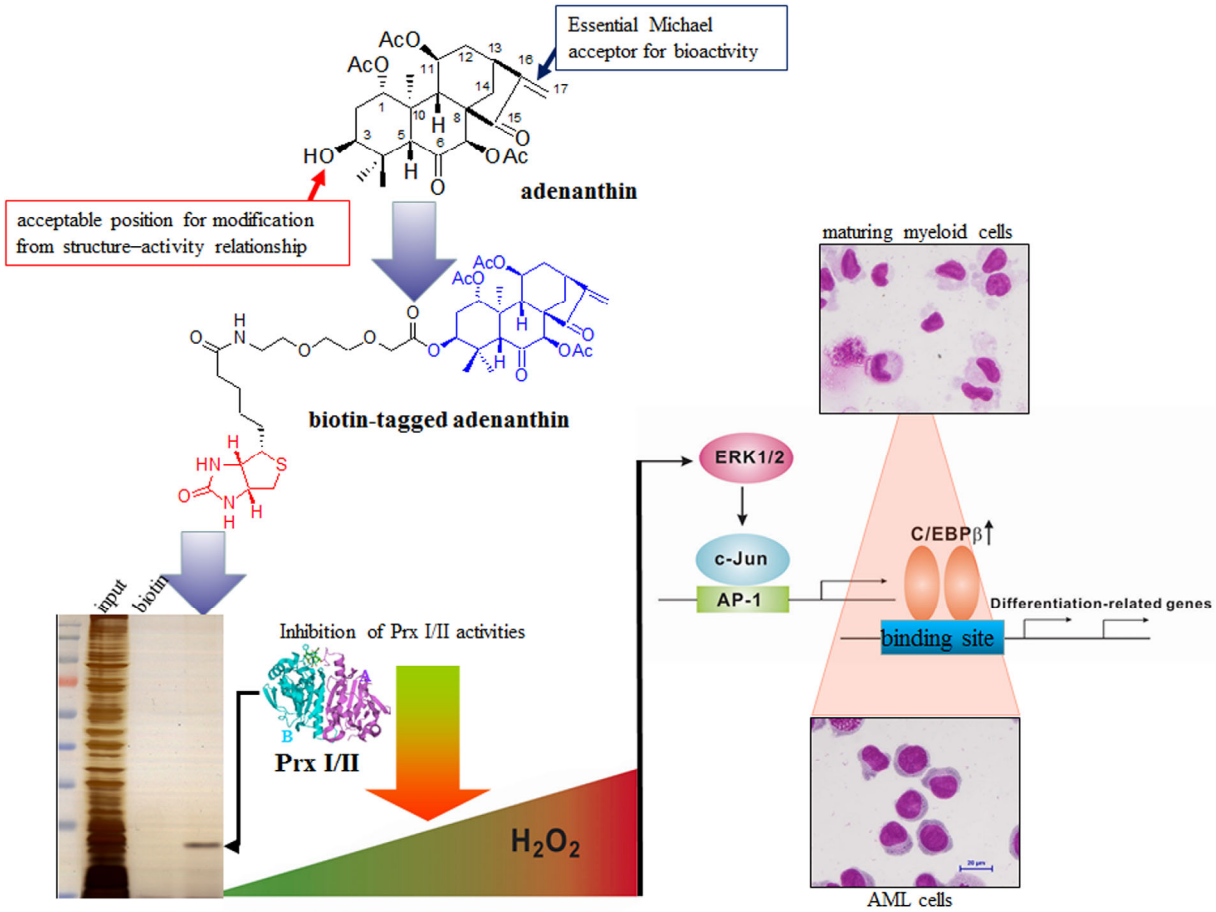

Figure 1 Structural formula of adenanthin and adenanthin-based pulldown probes as well as identification of its targets and molecular mechanism [6,35,36].

approximately 23 kD (Figure 1), which was completely inhibited by high concentrations of unlabeled adenanthin, indicating a specific interaction. Mass spectrometry revealed that the adenanthin-bound protein is peroxiredoxin I and II (Prx I–II) [35].

Prxs, a family of small non-seleno peroxidases that catalyze peroxide reduction of reactive oxygen species (ROS, the most significant of which is hydrogen peroxide), have drawn much interest because of their role as second messengers in cellular signaling pathways [37,38]. They have a conserved Cys residue (the peroxidatic Cys, C_P) that serves as the site of oxidation by peroxides. Peroxides oxidize the C_P-SH to C_P-SOH, which reacts with another Cys residue typically named resolving Cys (C_R) to form a disulfide that is subsequently reduced by an appropriate electron donor. According to the location or absence of the C_R, the mammalian Prxs include 2-Cys (Prx I-IV), atypical 2-Cys (Prx V), and 1-Cys Prx (Prx VI) subfamilies [39]. Thus, we further blotted the precipitates with antibodies against all these six Prxs, all of which were expressed in NB4 cells, and demonstrated that biotin-tagged adenanthin pulled down cellular Prx I and II but not Prx IV-VI. Adenanthin also effectively bound to the *in vitro* recombinant Prx I and II proteins, an interaction that was competitively inhibited at high concentrations of unlabeled adenanthin but not by the negative control compound mentioned above. Moreover, biotin-tagged adenanthin co-localized with Prx I and II in the cytoplasm and nuclei of NB4 cells. Further investigations of mass spectrum-fragmentation patterns revealed selectivity for the Cys residues modified in Prx I and II, that is, the conserved C_R of Prx I (Cys^{173}) or II protein (Cys^{172}) is the specific binding site for adenanthin. It remains to be investigated how adenanthin selectively binds to the specific Cys residues. Such a selective Cys binding action was recently reported for other natural compounds. For instance, a complex natural product ainsliadimer A selectively binds to the conserved Cys^{46} residue of IκB kinase α/β, leading to the inhibition of both canonical and non-canonical nuclear factor kappa-B (NF-κB) pathways [40].

Following binding to Prx I and II, adenanthin effectively inhibits the peroxidase activity of the recombinant Prx I/II protein. Accordingly, adenanthin treatment induced a moderate increase in intracellular H_2O_2 level. The concept has been proposed that modulation of the abundance of H_2O_2 is critical in regulating the balance between self-renewal and differentiation of hematopoietic stem cells [41]. Indeed, pre-treatment with N-acetyl-l-cysteine, which effectively inhibits adenanthin-induced H_2O_2 accumulation, completely abrogated adenanthin-induced growth arrest and differentiation of APL cells. Our results further propose that the adenanthin-elevated H_2O_2 levels activate extracellular signal-regulated kinase (ERK) 1/2 and subsequently leads to increased transcription of CCAAT/enhancer binding protein beta, which has been proven to induce differentiation of immature cells to granulocytes and monocytes (Figure 1).

Notably, we also showed that the Prx I protein is essential for the survival of hepatocellular carcinoma cells, and that adenanthin can kill these malignant liver cells *in vitro* and in xenografts by targeting Prx I/II [42]. However, the possibility could not be excluded that adenanthin might also bind with other protein(s). Actually, we showed that adenanthin exerts effective preventive and therapeutic effects on experimental autoimmune encephalomyelitis, which might be mediated via its inhibitory action on NF-κB signaling pathway [43]. More recently, it was also shown that, next to Prx I/II, adenanthin also targets and inhibits the activity of additional proteins including the thioredoxin-thioredoxin reductase (TrxR) system and protein disulfide isomerase [44], as well as glutathione (GSH) [45]. However, Siernicka et al. [46] recently report that adenanthin only slightly reduces the total GSH in natural killer (NK) cells, which are considered critical components of the innate and adaptive immune responses as reviewed in this issue [47]. In the Siernicka et al.'s [46] report, they also show that adenanthin induces intracellular oxidative stress in human NK cells, with greater effects towards Prxs-related antioxidants rather than GSH-related defences. In spite of this, the interactions between adenanthin and Prx are crucial for proposing Prx as a therapeutic target for cancer [38]. Adenanthin and the recently identified Prxs-targeting compound theonellasterone [48], a steroidal metabolite isolated from a Theonella sponge, warrant further investigation for their anti-leukemia and anti-cancer activity.

2 Oridonin

Oridonin is also a natural ent-kaurane diterpenoid which was isolated in the 1970s from the Isodon plant *Rabdosia rubescens* (Donglingcao named in Chinese), a Chinese traditional medicinal herb commonly used for the treatment of sore throat, inflammation, and gastrointestinal problems in central China [49,50]. A series of *in vitro* studies showed that oridonin can inhibit the proliferation and/or induce apoptosis of cancer cells of various origins such as prostate cancer, breast cancer, non-small cell lung cancer, glioblastoma and human melanoma [51–54]. The cytokinetic effects of oridonin on murine leukemia cells were reported in the middle of the 1980s, furthering our understanding of its properties [55,56]. For the past ten years, the anti-leukemic effects of oridonin have been attracting greater interest. Zhou et al. [57] reported that oridonin induces apoptosis of AML cells carrying the chromosome translocation t(8;21), which expresses the AML1-ETO fusion protein, and prolongs the lifespan of C57 mice bearing AML1-ETO-expressing leukemic cells. It also inhibits tumor growth in nude mice inoculated with

t(8;21)-harboring leukemic Kasumi-1 cells. They further demonstrated that oridonin cleaves AML1-ETO to a catabolic fragment, which can be blocked by pre-incubation with caspase inhibitors, and that the ectopic expression of AML1-ETO enhances the apoptotic effect of oridonin in the non-AML1-ETO-carrying AML cell line U937. Mechanistically, oridonin interacts with glutathione and TrxR to increase intracellular ROS, which in turn activates caspase-3 in t(8;21)-carrying AML cells. In addition, oridonin binds to the Cys^{347} of AML1-ETO, directing enzymatic cleavage at its aspartic acid 188 via caspase-3 to generate a truncated AML1-ETO (ΔAML1-ETO) and preventing the protein from further proteolysis. The ΔAML1-ETO interacts with AML1-ETO and interferes with the trans-regulatory functions of the remaining AML1-ETO oncoprotein, thus acting as a tumor suppressor that mediates the anti-leukemia effect of oridonin [58]. These results show that oridonin is a potential lead compound for molecular target-based therapy of leukemia.

The anti-leukemic effects of oridonin do not appear to be restricted to t(8;21)-carrying AML cells. Actually, oridonin has also been shown to enhance leukemia cell differentiation or induce cell death in several other types of AML cells. Oridonin was also shown to kill some lymphoid malignancies, including multiple myeloma, adult T-cell leukemia and non-Hodgkin's lymphoma (NHL), possibly mediated by inhibition of the DNA-binding activity of NF-κB [59]. The natural compound also dose-dependently induced apoptosis of the OCI-AML3 cell line harboring nucleophosmin (NPM) 1 mutations [60]. This is the most common genetic lesion in adult AML patients, accounting for 60% of cases with a normal karyotype and causes the skewed cytoplasmic accumulation of the NPM mutant protein ($NPM1c^+$), which is thought to be responsible for leukemia pathogenesis. The oridonin-induced apoptosis is accompanied by activation of caspase-3 and nuclear translocation of the $NPM1c^+$ protein [60]. However, the direct target of oridonin responsible for the alteration of NPM1 mutant localization is currently unknown. Similar to pharicin B [32], oridonin enhances ATRA-induced cell differentiation in ATRA-sensitive NB4 and ATRA-resistant NB4-R1 cell lines [61], together with stabilization of RARα, which might be dependent on the mildly increased ROS and the subsequent activation of NF-κB [62]. Besides AML, Guo et al. [63] investigated the potential effects of oridonin on Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph^+ ALL), which is triggered by constitutively activated BCR-ABL and Src family tyrosine kinases. They found that oridonin inhibits activation of Lyn (one of Src family kinases) and ABL and their downstream effectors in the Akt/mammalian target of rapamycin, Raf/mitogen-activated protein kinase kinase/ERK, and signal transducer and activator of transcription (STAT) 5 pathways. Oridonin also downregulates Bcl-2 but upregulates Bax protein, leading to apoptosis of Ph^+ ALL cell line SUB-B15 cells and primary specimens from Ph^+ ALL patients.

Although oridonin appears effective in treating various kinds of malignant hematologic cancers, the underlying mechanism of oridonin is very complicated and its direct target is largely unclear. Similar to bioactive naturally occurring products such as eriocalyxin B [64] and adenanthin [35,45], oridonin also contains an α,β-unsaturated ketone in the D-ring, destruction of which abolishes its anticancer activity. The α,β-unsaturated ketone, as a Michael acceptor, is an electrophilic center susceptible to nucleophilic attack (Michael addition) by a sulfhydryl group of reduced glutathione or cysteine residues in proteins, leading to adducts at the β-position. Thus, alkylation of crucial cysteine residues can result in a loss of function or activation of the target proteins. As mentioned above, oridonin can directly bind cleaved AML1-ETO at Cys^{347} [58] and TrxR [57,65]. TrxRs are essential proteins for regulating cellular redox balance and mitigating the damage caused by ROS. Inactivation of TrxR is a strategy to treat cancer through increasing the intracellular ROS level. In fact, a series of investigations have demonstrated that oridonin-induced cell death is associated with increased ROS production.

Dal Piaz et al. [66] recently used a chemical proteomic approach to identify the molecular chaperone heat shock protein (HSP) 70 1A as an oridonin target in leukemic Jurkat cells. In detail, oridonin covalently binds to Cys^{267} of HSP70 1A and inhibits its activity. HSP70 is an ATP-dependent molecular chaperone that plays a key role in refolding misfolded proteins and promoting cell survival following stress. The chaperone is marginally expressed in nontransformed cells, but is greatly elevated in cancer cells. Silencing of Hsp70 is cytotoxic to tumor but not to normal cells, thus suggesting a mechanism of action for the diterpene consistent with its the multiple biological activities. HSP70 inhibition by oridonin might indeed result in impairment of some host proteins, thus affecting several molecular pathways. In particular, HSP70 inhibition could account for the effects of oridonin on the activity of mitogen-activated protein kinases, Akt, BAX, NF-κB and caspases, since the functionality of these proteins depends on HSP70. Of note, Ye et al. [67] reported that vibsanin B, a novel macrocyclic diterpenoid isolated from *Viburnum odoratissimum* Ker-Gawl preferentially binds to HSP90β to inhibit interstitial leukocyte migration, thus ameliorating experimental autoimmune encephalomyelitis in mice.

Notably, leukemia cells resistant to chemotherapy-induced apoptosis have been found to be sensitive to oridonin. As an example, compared with HL60 cells, K562 and K562/ADR cells were resistant to apoptosis stimulated by Ara-C and VP-16, but were sensitive to the effects of oridonin. Mechanistic investigations revealed that oridonin upregulated BIM-S by diminishing the expression of miR-17 and miR-20a, leading to mitochondria-dependent

apoptosis. In contrast, neither Ara-C nor VP-16 can reduce miR-17 and miR-20a expression or trigger BIM-S-mediated apoptosis. Notably, silencing miR-17 or miR-20a expression by treatment with microRNA inhibitors or oridonin restored sensitivity of K562 cells to VP-16. The synergistic effects of oridonin and VP-16 were documented in cultured cells as well as mouse tumor xenograft assays [68].

3 Gambogic acid

Gambogic acid (GA) is a natural compound derived from the Chinese herb gamboges, which has been approved by the Chinese FDA for the treatment of different cancers in clinical trials [69]. Most recently, Liu's group reported that GA overcomes the drug-resistance of hematological malignancies, including CML with imatinib resistance and diffuse large B-cell lymphoma (DLBCL) resistant to R-CHOP (rituximab combined with cytoxan, hydroxyrubicin, oncovin, and prednisone). It is well known that the BCR-ABL T315I mutation is the predominant cause of resistance of CML cells to imatinib [70]. The emergence of imatinib resistance in patients with CML les to the search for novel approaches for its treatment. Liu's group [71] has investigated the antineoplastic effects of GA in CML cell lines, mononuclear cells from CML patients and in imatinib-resistant xenograft murine models. The results indicated that GA induces apoptosis together with cell proliferation inhibition in CML cells, including those cells harboring the BCR-ABL-T315I mutation, and primary mononuclear cells from CML patients resistant to imatinib. It also inhibits the growth of imatinib-resistant BCR-ABL-T315I-carrying CML cell xenografts in nude mice.

DLBCL is an aggressive form of NHL. Resistance to the R-CHOP therapy regimen poses a great challenge to improving the survival of patients with DLBCL, especially those with activated B-cell-like DLBCL (ABC-DLBCL) [72]. Therefore it is urgent to search for novel agents for the treatment of DLBCL. The Liu group [73] investigated the effect of GA on cell survival and apoptosis in DLBCL cells including both germinal center B cell (GCB-)- and ABC-DLBCL cells. The results collectively demonstrated that GA has a significant effect against the GCB and ABC subtypes of DLBCL cells both *in vitro* and *in vivo*. The research provided the first demonstration that GA may have clinical benefit in patients with DLBCL, particularly in patients with ABC subtypes DLBCL.

Several potential molecular targets of GA have been reported, including binding to the transferrin receptor and suppressing the NF-κB signaling pathway [74], as well as inhibiting vascular endothelial growth factor receptor 2 [75]. However, its molecular targets have not been thoroughly studied. Liu's group [76] reported that GA inhibits tumor proteasome activity, with potency comparable to bortezomib, the first proteasome inhibitor approved for the treatment of multiple myeloma, but with less toxicity. Their results showed that GA gains the proteasome-inhibitory function after being metabolized by intracellular CYP2E1, that GA induced proteasome inhibition is a prerequisite for its cytotoxicity and anticancer action without off-target effects, and that the expression of the CYP2E1 gene is very high in tumor tissues but low in many normal tissues, and thus GA can produce tissue-specific proteasome inhibition and tumor-specific toxicity (Figure 2). Further, they showed that GA-induced proteasome inhibition and caspase activation are required for GA-induced BCR-ABL downregulation and cell apoptosis [71]. Judging by present reports, most current studies are mainly focused on finding tyrosine kinase inhibitors to either downregulate BCR-ABL transcription or inhibit its tyrosine kinase activity. Here, the Liu group proposes an alternative strategy to enhance proteasome inhibition-induced BCR-ABL downregulation by activating the caspase system. Caspase-dependent BCR- ABL cleavage is effective both in KBM5-T315I cells and in the cancer cells from imatinib-resistant patients with CML, indicating that this strategy has a great promise in overcoming imatinib resistance.

4 Wogonin and wogonoside

Huang-Qin (*Scutellaria baicalensis* Georgi), one of the 50 fundamental herbs used in traditional Chinese medicine, is rich in flavonoids including wogonin (5,7-dihydroxy-8-

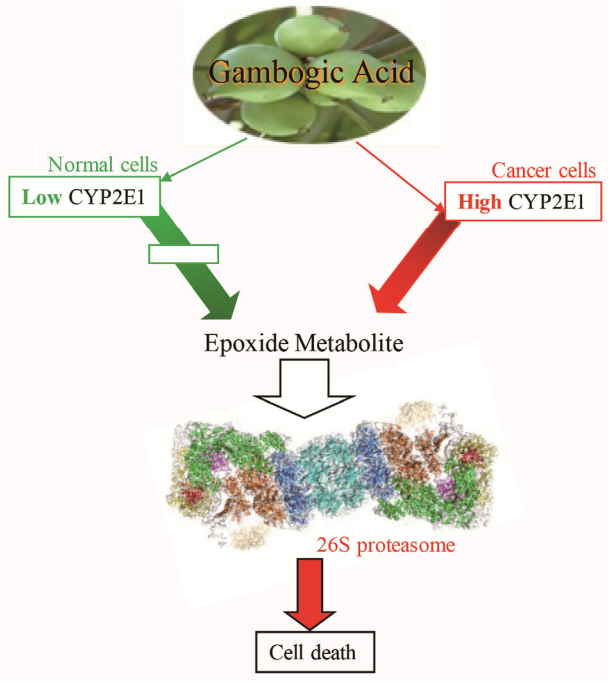

Figure 2 A schematic illustration of the mechanism for gambogic acid to induce cytotoxicity of cancer cells [76,77].

methoxy-2-phenyl-4H-chromen-4-one) [78–80]. In fact, these naturally-derived biologically active polyphenolic compounds are widely distributed in plants and relatively common in human diets. In the past decades, a series of *in vitro* and *in vivo* investigations showed that several structurally related flavonoids such as wogonin, luteolin, nobiletin and baicalein have multiple biological effects including anti-oxidant, anti-inflammatory, anti-viral, neuroprotective, anxiolytic, and anti-cancer activities [81–83]. Here we focused on the potential anti-leukemia effects of wogonin. It was shown that wogonin potently induces apoptotic cell death in leukemic HL-60 cells by increasing the expression of pro-apoptotic Bax protein and decreasing that of the anti-apoptotic myeloid cell leukemia 1 (Mcl-1) protein [84] and inhibiting the expression of human telomerase reverse transcriptase, telomerase-associated protein 1 and Myc mRNA, thereby down-regulating telomerase activity [85]. Recently, wogonin was also reported to induce the expression of certain members of the endoplasmic reticulum (ER) stress pathway and the activation of multiple branches of ER stress transducers, which are possibly mediated by the inhibition of the phosphoinositide 3-kinase/Akt signaling pathway [86]. Wogonin was also shown to induce cell cycle arrest at the G_2/M phase and apoptosis of human monocytic leukemia cell line THP-1 but not that of the normal human fetal lung diploid cell line Tokyo Metropolitan Institute of Gerontology (TIG)-1 [87]. The following studies demonstrated that wogonin has almost no toxicity on T lymphocytes from healthy donors but that it induces apoptosis in malignant T cells *in vitro* and suppresses growth of human T-cell leukemia xenografts *in vivo* [88]. Mechanistically, wogonin induces prolonged activation of phospholipase Cγ1 via H_2O_2 signaling, which leads to cytosolic Ca^{2+} overload and thus disruption of the mitochondrial membrane in malignant but not normal T cells [88]. Furthermore, wogonin and the structurally related natural flavones apigenin, chrysin and luteolin, bind directly to cyclin-dependent kinase 9 (CDK9) presumably to the ATP-binding pocket, and block phosphorylation of the carboxy-terminal domain of RNA polymerase II, leading to reduced RNA synthesis and subsequently rapid downregulation of the short-lived Mcl-1. Furthermore, wogonin preferentially inhibits CDK9 in malignant lymphocytes compared with normal lymphocytes [89,90]. More recently, an *in vivo* study with intraperitoneal injection of murine leukemia WEHI-3 cells into normal BALB/c mice demonstrated that wogonin increases the survival rate and the body weight of leukemic mice by increasing the populations of T- and B-cells [91]. More surprisingly, wogonin displayed little apoptosis-inducing effect on human promyelocytic leukemia cell line NB4 cells, but continuous treatment of wogonin efficiently induces NB4 cells to undergo differentiation [92].

Flavonoid aglycones, which undergo rapid and extensive metabolism after either oral or intravenous administration, enter the bloodstream in the form of glucuronide or sulfate conjugates. Very little unchanged aglycone can be found in the plasma, although there are high levels of glucuronic acid conjugates. Glucuronidation is thought to affect the biological activity of aglycones by altering the physicochemical properties of flavonoids, in what is considered a detoxification process [93]. Wogonin undergoes rapid metabolism and enters the bloodstream mainly in the form of the glucuronide wogonoside (Figure 1). As a metabolite of wogonin, wogonoside can also be derived from *Scutellaria baicalensis* Georgi. This wogonin derivative has been known to possess anti-inflammatory activity [94] and to inhibit lipopolysaccharide-induced angiogenesis *in vitro* and *in vivo* via toll-like receptor 4 signal transduction [95]. Wogonoside appears to have less toxicity to AML cells than wogonin does *in vitro*, in spite of no obvious difference in terms of acute *in vivo* toxicity. Therefore, it is interesting to investigate the potential effects of wogonoside on leukemic cells. Indeed, wogonoside exerts an *in vitro* antiproliferative activity in the AML cell lines U937, HL-60, and primary AML cells, and it inhibits the proliferation of U937 xenografts and prolongs survival in AML-bearing mice *in vivo*. Wogonoside-induced cell cycle arrest is associated with the downregulation of cyclin D1 and CDK4, and it also induces the differentiation of AML cell lines and primary AML cells [96].

Phospholipid scramblase 1 (PLSCR1) is a calcium-binding, multiply palmitoylated type II endofacial plasma membrane protein, which is localized in either the cell membrane or nucleus depending on its palmitoylated state. In the nuclei, the unpalmitoylated PLSCR1 protein can bind to genomic DNA. Early research showed that PLSCR1 contributes to the trans-bilayer movement of phospholipids, and the following studies revealed that PLSCR1 expression can be induced by some cytokines such as interferon, epidermal growth factor, and leukemic cell differentiation-inducing agents such as ATRA and phorbol 12-myristate 13-acetate [97,98]. Moreover, the induction of PLSCR1 transcription depends upon sequential activation of protein kinase C delta (PKCδ), c-Jun N-terminal kinases, and the phosphorylation of STAT1 at Ser-727 [99]. PLSCR1 was also shown to interact with several protein kinases including c-Abl, c-Src, PKCδ and onzin, suggesting a role of PLSCR1 in cell signaling [100–102]. Employinging an inducible PLSCR1-expressing myeloid leukemia U937 cell line, we showed that inducible PLSCR1 expression arrests the proliferation at the G_1 phase and induces granulocyte-like differentiation with increased sensitivity to etoposide-induced apoptosis of the genetically engineered cells [103]. Of great interest is the fact that PLSCR1 expression can also be induced during wogonin and wogonoside-induced differentiation of AML-derived cell lines via dramatically increased PKCδ phosphorylation at Ser^{643} [92,96]. Moreover, $p21^{waf1/cip1}$ expression in wogonoside-treated cells increases at 48 h

post-treatment, while conversely, the levels of c-Myc protein decrease upon exposure to wogonoside. The silencing of PLSCR1 by its sequence-specific siRNA blocks the wogonoside-induced increase in $p21^{waf1/cip1}$ and decrease in c-Myc [96]. PLSCR1 was found inside the nuclei in U937 and HL-60 cells after treatment with wogonoside. It specifically binds to a segment of the 5′-promoter of inositol 1,4,5-trisphosphate receptor 1 (IP3R1: $^{-101}$GTAACCATGTGGA^{-89}), enhancing the transcription of this gene [104]. IP3R1 is known to play a key role in IP3-mediated mobilization of intracellular Ca^{2+} stores from the ER in a variety of cells and tissues, and it is also essential for cell growth and differentiation [105,106]. Wogonoside promotes nuclear PLSCR1 binding to the transcriptional activation domain of IP3R1 and increases IP3R1 protein expression. SiRNA against PLSCR1 effectively blocked the increased expression of IP3R1 when cells were cultured with wogonoside. Wogonoside thus appears to augment PLSCR1 expression at the transcriptional level and promotes its localization in the nucleus and its binding to the IP3R1 promoter, thus influencing the expression of this receptor. Cumulatively, wogonin and its metabolite wogonoside appear to be of therapeutic potential in leukemia, and their clinical effectiveness deserves to be further investigated.

5 Conclusions

Numerous naturally occurring small molecules from the plant kingdom have interesting properties against cancers, especially hematological malignancies. They might target different molecular regulatory pathways to interfere with the proliferation, differentiation, and death of hematological malignant cells or malignant hematopoietic stem/progenitor cells [8]. To date, the Dictionary of Natural Products has recorded approximately 200000 plant secondary metabolites, with about 170,000 unique structures. Approximately 15% of the drug interventions in the ClinicalTrials.gov database are plant-related, while about 60% of these drugs are sourced from plants [107]. In spite of these successes, the vast majority of plant species remain to be systematically investigated in drug discovery campaigns. In particular, bioactive compounds from herbs that are used in our traditional medicine still need to be more thoroughly explored, and the constant development of high-throughput functional assays and phenotypic screens in the *omics* era provides new opportunities for revival of interest in natural products for drug discovery.

Besides drug discovery, the use of bioactive small molecules will help to decipher and control life's processes, thus promoting our understanding of the molecular mechanisms of normal and abnormal cellular activities. Based on this, the term "chemical biology" has rightly been given to this thriving area at the interface of chemistry and biomedicine. In spite of its definition, the core element of chemical biology is the use of exogenous chemistry (chemicals) to interrogate, modify, and manipulate biological systems at the cellular and organismal level in a highly controlled manner. From this view, chemicals offer opportunities usually not shared by classical genetic methods. On one hand, chemicals act rapidly such that the consequences of treatment with them can often be recorded after only minutes or seconds. Their effects are tunable and their influence is reversible, providing temporal control of protein function because the small molecules usually can be washed out or are metabolized. On the other hand, small bioactive molecules allow the targeting of proteins whose mutations, knock-out, or knock-down would be lethal. Moreover, chemicals can be used at any time point chosen for application. It is therefore crucial to identify and confirm the targets of bioactive small-molecules. In conclusion, scientists from biological, medicinal and chemical fields should come together to find more active compounds against hematological malignancies and to identify their targets.

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