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Synthetic progress and anti-leukemic research on indirubin analogues: a review since 2010

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Indirubin, an active component of the traditional Chinese medicine Indigo Naturalis, has drawn significant attention owing to its remarkable anti-leukemia activity. Nevertheless, it presents issues such as limited natural resources, poor solubility, and potential toxicity, which impede its clinical application. In recent years, within the research realm of indirubins, the efficient synthetic strategies and the exploration in the treatment of leukemia have remained the central research focuses in this field. This article comprehensively reviews the research advancements of indirubin derivatives in synthesis strategies and anti-leukemia effects since 2010s. Firstly, in this review, the synthetic methods of indirubin derivatives are categorized into two main types: chemical synthesis and bio & biomimetic synthesis. Among these, chemical methods assume a dominant position. These chemical synthesis approaches encompass acid-catalyze, organophosphorus-catalyzed, metal-catalyzed reactions, and one-step reduction reactions using KBH4. Secondly, based on the modification sites within the structure of indirubin, this paper undertakes a classified review of its derivatives and further delves deeply into the anti-leukemia activities of these derivatives. Additionally, we also discuss the future development directions of synthesizing indirubin derivatives and explore their structure–activity relationships in the context of anti-leukemia research. We firmly believe that this review can offer information support for scientific researchers engaged in the study of indirubin's anti-leukemia effects, thereby facilitating in-depth development.

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1 Introduction

Leukemia, a malignant clonal disorder of the hematopoietic system, is predominantly classified into two major types, namely acute and chronic forms, based on the disease course and pathological features.[1] Acute leukemia mainly encompasses acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and mixed linage leukemia (MLL). It has

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a relatively rapid onset, and the disease progresses swiftly.[1b,c] It is noteworthy that MLL is a high-risk subtype of leukemia characterized by specific gene rearrangements. It often exhibits features of both the lymphoid and myeloid linages simultaneously. This coexistence of features presents challenges to treatment and complicates the treatment plan.[1d,e] The common types of chronic leukemia are chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL), and the disease course is relatively slow.[1f,g] Different types of leukemia exhibit significant heterogeneity in pathogenesis and treatment responses, presenting a formidable challenge to clinical treatment.

Currently, the main treatment approaches encompass chemotherapy, targeted drugs, immunotherapy, and hematopoietic stem cell transplantation.[1c,f,2] Chemotherapy can rapidly eliminate malignant cells; however, it exhibits relatively high toxicity. Targeted drugs can act precisely on specific molecular targets. Immunotherapy combats tumors by mobilizing or modifying the patient's immune system. Hematopoietic stem cell transplantation can reconstruct normal hematopoietic function. The combined use of these methods has significantly enhanced the survival rate of patients.

However, the existing treatments still encounter numerous challenges. These include drug toxicity, drug resistance, limitations inherent in the therapies themselves, and difficulties in donor matching during hematopoietic stem cell transplantation. For instance, the five-year survival rate of adult acute myeloid leukemia remains less than 30%.[1c] Consequently, the development of novel drugs featuring high efficiency, low toxicity, and anti-drug resistance properties remains an urgent task in current research. Owing to this requirement, the exploration of anti-leukemia drugs is also being continuously and thoroughly advanced.

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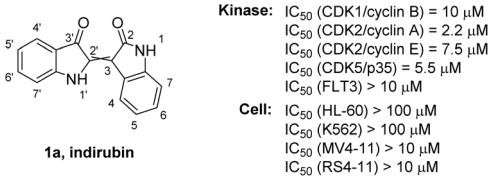

Fig. 1 The structure of indirubin and its typical anti-leukemia activity.

Indirubin (**1a**, Fig. 1), derived from the traditional Chinese medicine Indigo Naturalis, provides a brand-new idea for the treatment of leukemia. As early as the 1970s, relevant studies had confirmed that indirubin has a significant therapeutic effect on CML.[3] Indirubin exerts its anti-leukemia effect mainly through multiple mechanisms such as inhibiting cyclin-dependent kinases (CDKs), blocking the signal transducer and activator of transcription 3/5 (STAT3/STAT5) pathway, and inducing cell differentiation.[4]

However, indirubin has certain inherent drawbacks, such as poor water solubility, low bioavailability, and off-target effects. These factors have significantly limited its extensive application in clinical treatment.[5] To address these limitations, the structural modification of indirubin has become a research focus. Numerous studies have shown that the derivatives after structural modification exhibit broader application potential.[6] In addition, indirubin was mainly extracted from plants in the early stage. However, due to its low content in plants and poor water solubility, the extraction yield was low, and the production cost was high.[7] With the in-depth research, various synthetic strategies for indirubins have gradually emerged. Currently, indirubin and its derivatives are mainly prepared by chemical synthesis.

Although there are relatively abundant research achievements on indirubin and its derivatives at the current stage, there is a lack of systematic integration and analysis of leukemia-related information.[8] To a certain extent, this has restricted the comprehensive comparison and in-depth understanding of the relationship between structural modification and biological activity. Before 2010s, indirubin was mainly obtained through a single chemical synthesis method or separation and extraction from plants.[9] Since 2010s, biological methods and more diverse chemical synthesis methods have been developed. Meanwhile, the research on indirubin's anti-leukemia effect is no longer confined to chronic leukemia. Therefore, the year 2010 can be regarded as a watershed in the development of indirubin. Based on this, this paper focuses on the relevant literature published since 2010, comprehensively reviews the evolution process of the synthesis strategies of indirubin and its derivatives, their roles in leukem treatment, the structure–activity relationship, and explores their future development prospects.

2 Research on the synthetic strategy of indirubins

Prior to 2010, the synthesis of indirubins primarily depend on the condensation reaction between indoxyl acetates **2** and isatins **3** (Fig. 2a).[9] This approach features simple operation, mild reaction conditions, and relatively high yields. Since 2010, with the continuous advancement of technology, biosynthesis methods have been successively reported, and new chemical synthesis routes are also being constantly explored.

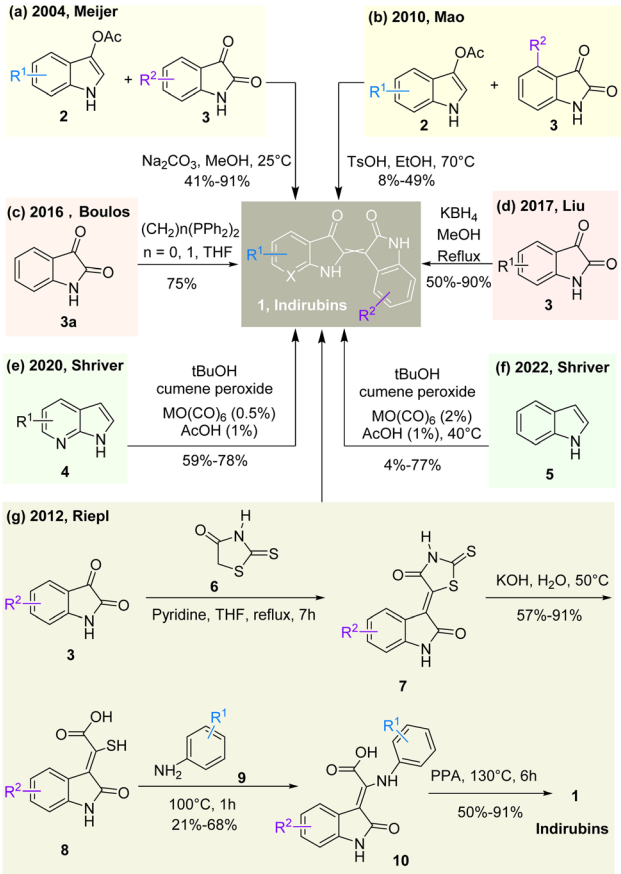

Fig. 2 The chemical synthesis strategies of indirubins since 2010.

2.1 The chemical synthesis strategies of indirubins

In 2010, Mao's group[10] conducted in-depth investigations and found that 4-substituted derivatives faced significant bottlenecks during the synthesis process. Specifically, under traditional basic conditions, their yields were extremely low. Taking 4-chloroindirubin as an example, the yield was only 5%. In view of this, Mao developed a novel acidic catalytic strategy (Fig. 2b). This strategy employed an acidic reaction system of *p*-toluenesulfonic acid/ethanol (at 70 °C). By means of the proton–hydrogen-bond coordination mechanism, it effectively overcame the problem of steric hindrance and successfully achieved the synthesis of 4-F/Cl/Br/CF_3/NO_2 indirubins, with yields ranging from 8% to 49%. It is worth emphasizing that this method achieved the efficient synthesis of 4-substituted indirubins for the first time. For example, the yield of 4-chloroindirubin was significantly increased to 49%, successfully breaking through the limitations of the traditional basic synthesis route and laying a solid synthetic foundation for the research and development of new anti-tumor drugs.

Considering the drawbacks of the traditional indirubin synthesis pathway, such as the generation of numerous by-products (*e.g.*, indigo) and the difficulty of introducing substituents into the indoxyl ring, Riepls[11] devised a completely novel strategy (Fig. 2g). The essence of this strategy lies in the design of a stable key intermediate **8**. The detailed steps are as follows: initially, rhodamine **6** and isatins **3** undergo a condensation reaction, and compound **7** is obtained through hydrolysis. Subsequently, the mercapto group is replaced with commercial aniline **7** to form the target intermediate **8**. Finally, intermediate **8** undergoes Nazarov cyclization (polyphosphoric acid, 110 °C) to yield indirubin derivatives. This method represents a breakthrough, enabling diverse substitutions (such as chlorine, methoxy, carboxyl) of the indoxyl ring. Moreover, it successfully synthesizes water-soluble indirubin derivatives with carboxylic acid substitution for the first time.

In 2016, Boulos[12] reported a method for the synthesis of indirubin (Fig. 2c). This method entails refluxing isatin **3** with bisphosphine in tetrahydrofuran (THF). Ultimately, indirubin was successfully obtained with a yield of 75%.

In 2017, Liu and coworkers[13] developed a novel and efficient synthetic strategy (Fig. 2d). Unexpectedly, the authors discovered that when isatin **3** was reduced by KBH_4 in methanol and refluxed for 1 hour under an air atmosphere, indirubin **1a** could be directly synthesized in a single step, instead of the anticipated 3-hydroxyindolin-2-one. Through systematic optimization, the optimal conditions were established as follows: KBH_4 (0.5 equivalents) served as the reducing agent, methanol was used as the solvent, and air participated in the oxidation process. This method is straightforward to operate, achieving a yield as high as 90%. Moreover, it has been successfully obtained 11 of substituted indirubin derivatives in 50% to 90% yields. Mechanistic investigations have revealed that the reaction involves the reduction of isatin to form 3-hydroxyindolin-2-one and its anion, followed by nucleophilic addition and air oxidation to yield indirubin. This approach offers a new avenue for the efficient preparation of indirubins.

In the traditional view, the indoxyl intermediate in the oxidation process almost invariably generates indigo. Contrarily, in 2020, Shriver's group[14] discovered that when 7-azaindole was employed as the raw material, the corresponding intermediate 7-azaindoxyl solely produced the indirubin analogue 7,7′-diazaindirubin, rather than the anticipated diazaindigo (Fig. 2e). Stringent experimental validation demonstrated that 7-azaindoxyl was more inclined to undergo an intermolecular aldol condensation reaction in the keto/enol form, followed by oxidation to form product **1**, rather than adhering to the classical radical dimerization reaction pathway. Additionally, through substrate expansion experiments, they further found that electron-deficient indoles were more likely to yield indirubin products during the reaction process.

Two years later, Shriver[15] then put forward a proposal that temperature could be harnessed to modulate the competitive reaction's pathway to design a general approach for indirubin synthesis (Fig. 2f). The research employed a molybdenum-catalyzed oxidation system ($Mo(CO)_6$/cumene peroxide). At a scale of 5 mmol, the effects of temperature (ranging from 23 to 86 °C) and substituents (electronic properties and positions) on product selectivity were systematically investigated. The author found that low temperature significantly promoted the formation of indirubin. For example, when indole was reacted at room temperature, the ratio of indirubin to indigo was 3 : 2 (corresponding to 60% selectivity), while only indigo was obtained at 86 °C. Uniformly, electron-deficient indoles had a distinct advantage: when the 5/7-position was substituted with nitro, cyano, or aldehyde groups, the selectivity of indirubin exceeded 90% at 40 °C. This strategy enabled the direct synthesis of indirubin derivatives from readily available indoles. It demonstrated tolerance to functional groups (–NO_2, –CN, –CHO, –CO_2H, –Hal) and complemented the potassium borohydride reduction method.

2.2 The biosynthetic or biomimetic synthetic strategies of indirubins

In 2014, Rebelo[16] reported a work focusing on a sustainable and cost-efficient biomimetic catalytic strategy to replace the conventional highly polluting indigo pigment synthesis process and the complex biocatalytic method (Fig. 3a). Three manganese(III) porphyrins with different electronic characteristics (*e.g.*, [Mn(TDCPP)Cl]) were utilized to mimic the activity of P450 enzymes. Hydrogen peroxide, a green oxidant, was employed to catalyze the oxidation of indole-3-carboxaldehyde **11a** in acetonitrile at room temperature. Through the optimization of the reaction time (30 minutes) and the amount of the oxidant (4 equiv.), an indole conversion rate of 85% was achieved, and 2-oxindole (64%) and indirubin (7%) were successfully synthesized.

In 2019, Magiats[17] reported that indole-3-carboxaldehydes (**11**), the principal tryptophan metabolite of Malassezia, could be transformed into alkaloids such as indirubin and tryptanthrin under oxidative conditions (Fig. 3b). Consequently, a biomimetic one-step oxidation reaction was devised. Using indole-3-carboxaldehydes **11** as the substrate, in an acetonitrile/water mixed solvent system, with environmentally benign 6%

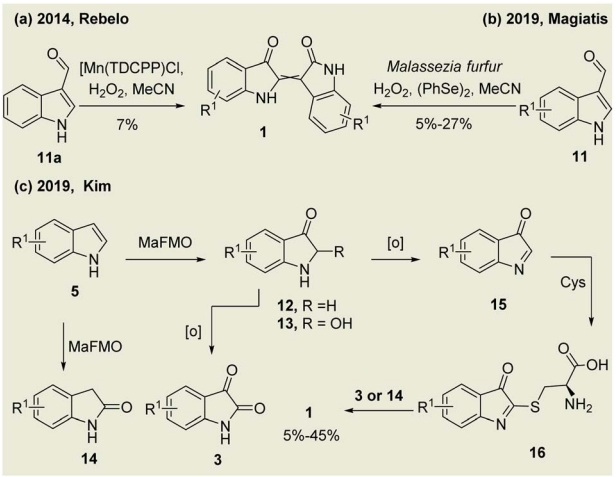

Fig. 3 The biosynthetic or biomimetic synthetic strategies of indirubins.

H_2O_2 and a catalytic quantity of diphenyldiselenide ($(PhSe)_2$), the reaction was carried out at room temperature for 72 hours to synthesize indirubins bearing halogen, methyl, or methyl ester groups in 5–17% yields.

In the same year, Kim's group[18] reported a novel route to solve the problem of low indirubin yields caused by severe dimerization side reactions during the microbial synthesis of indirubin (Fig. 3c). They found that the addition of cysteine during the catalytic synthesis by Flavin-containing monooxygenase (MaFMO) could remarkably increase the indirubin yield. Through a series of verification experiments, they revealed the triple-action mechanism of cysteine: (1) intermediate capture: cysteine reacts with indoxyl to form a stable 2-cysteinyl indolinones **15**, thus preventing its oxidative dimerization to indigo. (2) Promotion of secondary oxidation: the reducing property of cysteine slows down the auto-oxidation of indoxyl, allowing MaFMO to further hydroxylate indoxyl to form isatins **3**. (3) Activation of condensation: cysteine mediates the non-enzymatic condensation of **16** with **3** or **14**, resulting in the generation of indirubins. Based on this mechanism, Kim for the first time achieved the highly selective biosynthesis of various halogenated/nitro indirubin derivatives with 5% to 45% yields by precisely regulating different halogenated indoles **5** and the isolated 2-cysteinyl indolinone intermediates **15**.

In recent years, chemical synthesis approaches have still maintained their dominance and demonstrated relatively high reaction yields in the synthesis of indirubins. Specifically, for the newly reported one-step synthesis method (Fig. 2d), the yield can reach up to 91%. However, these methods are only applicable to the preparation of indirubin derivatives with identical substituents on the indole core and encounter limitation ns in the synthesis of indirubins with different substituents. Consequently, the chemical synthesis methods of indirubin still require further in-depth investigation.

Simultaneously, the biosynthesis method remains in the nascent stage of development. With a reaction yield of 45%, which is lower than that of chemical synthesis methods, it also calls for further in-depth exploration.

3 Research progress of indirubin in the treatment of leukemia

As early as 2001, Matsuda[19] identified indirubin as a potent endogenous aryl hydrocarbon receptor (AhR) ligand in human urine and serum. Its activity surpasses that of the classical ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In 2016, Julieson[20] discovered that indirubin, acting as an AhR agonist, could significantly induce the differentiation of acute myeloid leukemia-initiating cells. In 2022, Alshehri[21] performed a molecular docking simulation experiment involving the human FLT3 protein target (PDB ID: 6JQR) and 313 phytochemicals (including standard anti-cancer drugs such as sorafenib and gilteritinib and other anti-cancer agents). Thirteen optimal compounds, including indirubin, were screened out. Their binding affinity was superior to that of sorafenib and gilteritinib, indicating that indirubin can serve as a scaffold or lead compound for the discovery of new drugs for FLT3-induced acute myeloid leukemia. Recently, He[22] confirmed through methods such as network pharmacology, molecular docking, and experimental verification that indirubin can induce apoptosis of acute lymphoblastic leukemia (ALL) cells, arrest them at the G2/M phase, and downregulate the phosphorylation levels of proteins associated with the PI3K/AKT/mTOR pathway, thereby inhibiting the proliferation of ALL cells in a dose-dependent manner.

It has been verified that the traditional Chinese medicine formula Realgar-Indigo Naturalis Formula (RIF), comprising four traditional Chinese herbs—Realgar, Indigo naturalis, Salvia miltiorrhiza, and Pseudostellaria heterophylla, exhibits therapeutic effects on human acute promyelocytic leukemia (APL).[23] It notably includes tetra-arsenic tetra-sulfide, indirubin, tanshinone IIa, and total saponins of *Radix Pseudostellariae* as its primary active components. In 2018, Ni's research[24] on the combined application of realgar and Indigo Naturalis revealed that As_4S_4 in Realgar and indirubin in Indigo Naturalis might form a complex *via* non-covalent interactions. These interactions facilitate the improvement of the two-way permeability of arsenic. Consequently, based on their synergistic effect in leukemia treatment, the area under the curve (AUC), half-life, and mean residence time (MRT) of arsenic in the blood of mice have been increased. In addition, the combined use of drugs can significantly inhibit the proliferation of K562 cells and induce apoptosis at low concentrations, while single-drug use fails to produce such an effect. Recently, Hou[25] indicated that, in comparison to the use of a single drug or two drugs, the use of three drugs (indirubin, tanshinone IIa, and total saponins of *Radix Pseudostellariae*) could enhance the effect of As_4S_4 in down-regulating PML^{A216V}-RARα, and the mechanism was suggested to be related to inhibiting the mTOR pathway to activate autophagy.

In summary, previous studies have demonstrated that indirubin exhibits antileukemia activity *via* multiple pathways. These pathways involve synergistically regulating drug permeability, inhibiting the activity of FLT3, suppressing the mTOR pathway to active the autophagic process, and downregulating the phosphorylation levels of proteins associated with the PI3K/AKT/mTOR pathway.

4 Research progress on structural modifications associated with the antileukemia activity of indirubin

4.1 Derivatives with N1 modifications

In 2017, Zhang[26] successfully synthesized five novel indirubin derivatives featuring hydrophilic amino side chain *via* an N1 substitution reaction in the presence of base (Fig. 4). Subsequently, the CCK-8 assay was employed to assess the effect of these derivatives on the proliferation of acute myeloid leukemia cells HL-60. The research revealed that compounds **17a** and **17c** demonstrated more remarkable cytotoxicity and their IC_{50} values were 3.564 ± 0.211 μM and 4.446 ± 0.459 μM, respectively. This effect was comparable to etoposide on HL-60 cells.

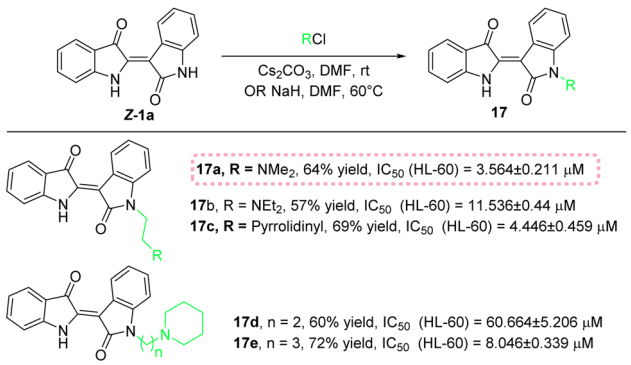

Fig. 4 The synthesis of N1-modified derivatives **17** and anti-leukemia activities of them.

However, the indirubin exhibit poor effects (IC_{50} > 100 μM). The structure–activity relationship analysis indicated that the hydrophilic amino side chain attached to indirubin effectively enhanced its solubility and anticancer activity. Compared with derivatives bearing other side chain, the dimethylaminoethyl side chain displayed more prominent anticancer activity. Additionally, compound **17a** could upregulate the level of cleaved caspase-3 and downregulate the expression of CDK1 and CDK2, thereby significantly inducing cell cycle arrest and apoptosis in HL-60 cells.

In the next year, Zhang incorporated podophyllotoxin **18** into the indirubin structure *via* two base-mediated substitution reactions, successfully synthesizing a series of novel hybrid molecules of podophyllotoxin and indirubin **21** (Fig. 5).[27] Subsequently, the CCK-8 assay was employed to assess the anti-cancer effects of these hybrid molecules in leukemia cell lines. Compound **21a** was demonstrated to be the most pronounced inhibitory effect on K562 cells and drug-resistant K562/VCR cells.

Its IC_{50} value was 0.076 ± 0.008 μM on K562/VCR cells, exhibiting evident multifunctional anti-drug resistance characteristics. Additionally, **21a** could promote cell apoptosis by inducing G2-phase cell cycle arrest, causing a reduction in mitochondrial membrane potential, activating PARP cleavage, and triggering intracellular ROS accumulation. **21a** also could significantly downregulate the expression levels of P-gp and MRP1 proteins, effectively inhibiting the function of drug efflux pumps, and simultaneously upregulate the expression levels of autophagy-related proteins Beclin1 and LC3-II. The result of molecular docking experiment indicated that **21a** could interact with the colchicine binding site of tubulin, thereby disrupting the microtubule network. Structure–activity relationship studies showed that the carbon chain length connecting indirubin and podophyllotoxin influenced its anti-cancer activity. Short-chain connections (such as **21a**) were the crucial factor in maintaining high anti-proliferative activity. Conversely, long chains might reduce the binding ability to the target because of steric hindrance.

In 2019, Van[28] synthesized indirubin derivatives **24** bearing 1,3,4-thiadiazole heterocycles *via* the ring-opening reaction between epoxy groups and thiols (Fig. 6). They selected 6-mercaptopurine, indirubin-3′-oxime, and etoposide as positive controls and performed anti-proliferative activity assays against four human cancer cell lines (SW480, LU-1, HepG2, and HL-60). These compounds demonstrated great cytotoxicity against all four cell lines, particularly showing remarkable inhibitory activity against the leukemia cell lines HL-60. Compounds **24a** and **24b** exhibited stronger cytotoxicity against the HL-60 cell lines, with IC_{50} values of 1.35 μM and 4.28 μM, respectively, which were considerably lower than those of 6-mercaptopurine and indirubin-3′-oxime.

Multiple reports have shown that IDO1 is overexpressed in various malignant tumors.[29] To develop novel IDO1 inhibitors, Wang and his team synthesized a novel indirubin derivatives **30** *via* the click reaction (Fig. 7).[30] The authors performed *in vitro* anti-leukemia activity assays on compounds **30**. Compounds **30a** and **30b** demonstrated moderate inhibitory activity against K562 cells, with IC_{50} values of 24.96 μM and 54.71 μM, respectively. Among them, **30a** exhibited the most pronounced inhibitory effect, far exceeding that of the control drugs lenalidomide and pomalidomide. Meanwhile, compounds **30a** and **30b** could effectively inhibit IDO1, with IC_{50} values of 41.69 μM

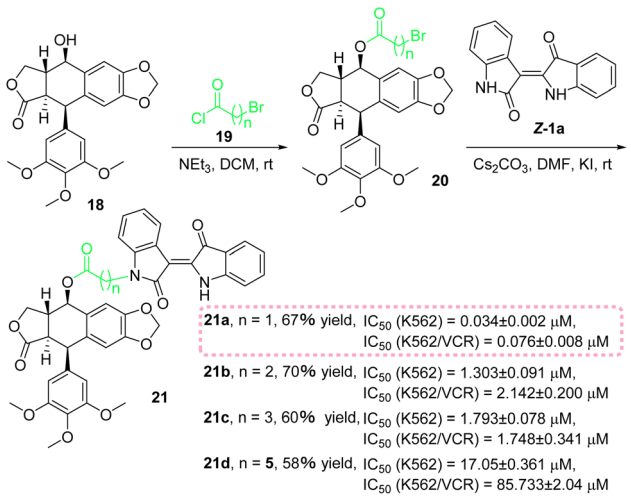

Fig. 5 The synthesis of N1-modified derivatives **21** and anti-leukemia activities of them.

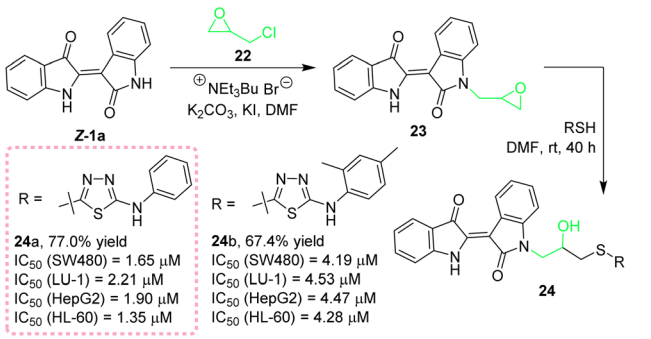

Fig. 6 The synthesis of N1-modified derivatives **24** and anticancer activities of them.

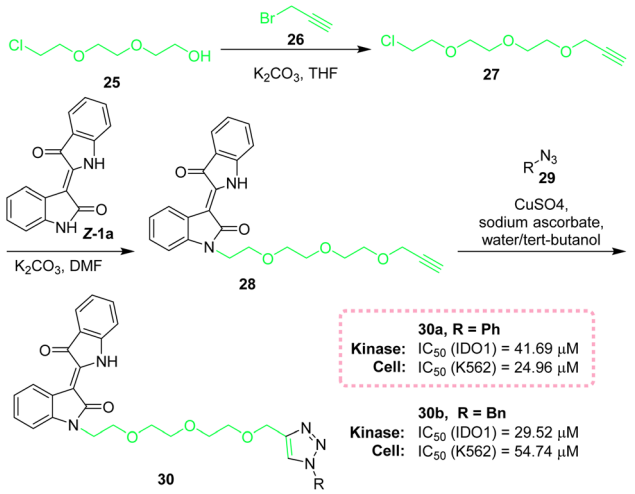

Fig. 7 The synthesis of N1-modified derivatives **30** and anti-leukemia activities of them.

and 29.52 μM, respectively, and **30b** was the most effective inhibitor among them.

4.2 Derivatives with C-3′ modifications

4.2.1 Indirubin-3′-oxime. Indirubin is a rigid planar structure. Due to its limited flexibility, it has relatively low water solubility, poor cell activity, and unsatisfactory druggability. Meijer[31a] found that indirubin and its derivatives are potent inhibitors of CDKs. Research has indicated that, compared with indirubin, indirubin-3′-oxime **31** (ref. **31b**) synthesized *via* the carbonyl-amine condensation reaction demonstrated stronger inhibitory activities against CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, CDK4/cyclin D1, and CDK5/p35. Their IC_{50} values are 0.18, 0.44, 0.25, 3.33, and 0.1 μM respectively, and the water solubility is relatively improved as well.

Prior to 2010s, several research teams had carried out studies on the outstanding anti-cancer activity and anti-cancer mechanism of indirubin-3′-oxime **31** against leukemia cell lines. For instance, indirubin-3′-monoxime could promote the apoptosis of human leukemia KBM-5 cells induced by tumor necrosis factor by regulating the NF-κB signaling pathway.[32] Compound **31** could block the autophosphorylation process of FGFR1 kinase in KG-1a cells, thus inhibiting the proliferation of myeloid leukemia KG-1a cells cultured *in vitro* (Fig. 8). Additionally, indirubin-3′-monoxime **31** inhibited Notch1 signal

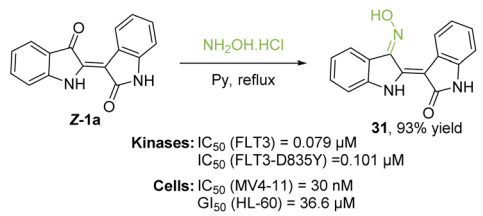

Fig. 8 The synthesis of indirubin-3′-oxime **31** and anti-leukemia activities of it.

transduction in a GSK-3β-dependent manner.[33] It can inhibit the growth of TALL-1 cells at a concentration of 8 μM and the growth of K562 cells at a concentration of 20 μM.[4b]

In the past fifteen years, research on the anti-leukemia effect of indirubin-3′-oxime **31** continued to progress (Fig. 8). Choi[34] found that indirubin-3′-oxime exerted extremely potent inhibitory effects on the kinase activities of FLT3 and FLT3-D835Y. The IC_{50} values were 0.079 μM and 0.101 μM, respectively. Moreover, indirubin-3′-oxime **31** could inhibit the growth of leukemia cells MV4-11 (the activated form of FLT3) by inducing cell cycle arrest at the G1 phase and promoting cell apoptosis. The IC_{50} value was 30 nM, but it had no effect on RS4-11 cells (wild-type FLT3). In the same year, Kang and his colleagues[35] confirmed that indirubin-3′-oxime **31** could inhibit the proliferation of HL-60 cells, with the half-maximal growth inhibitory concentration (GI_{50}) value being 36.6 μM. Subsequently, Chuang[36] reported that indirubin-3′-oxime could significantly induce G2/M phase arrest in leukemia JM1 and K562 cells, and trigger cell apoptosis and autophagic death. Additionally, indirubin-3′-oxime showed relatively low cytotoxicity to healthy lymphocytes and granulocytes.

4.2.2 Indirubin-3′-oxime derivatives with substituents. Many researches had confirmed that converting the C3′ carbonyl group into an oxime during the synthesis of indirubin-3′-oxime **31** can enhance the water solubility and selectivity. Based on this, numerous studies have introduced substituents at other positions and conducted research on anti-leukemia. Therefore, this section will further summarize the research on C3′-oxime derivatives of substituted indirubin, which feature a 3′-oxime and substituents at other positions.

Firstly, Quan and coworkers[37] successfully synthesized a novel indirubin-3′-oxime derivative **36** *via* the click reaction (Fig. 9). Subsequently, the *in vitro* cytotoxic activity of this derivative against four cancer cell lines was screened by SRB assay. The research revealed that compound **36** exerted growth inhibitory effects on all cell lines. Moreover, compounds **36a–36c** demonstrated stronger cytotoxic activity against the HL-60 cell line, with IC_{50} values of 0.96 ± 0.12, 1.28 ± 0.34, and 1.28 ± 0.1 μM, respectively. These values suggested that their activities were superior to those of the parent indirubin-3′-oxime and comparable to those of the reference compound ellipticine. Structure–activity relationship studies indicated that the oxime group significantly enhanced the molecule's cytotoxic activity. Molecular docking showed that the OC_2H_5 substituent of compound **36a** interacted with the active site region of thr138 of CSK-3β protein, indicating that this residue plays an important role in constructing the binding affinity of the ligand. Additionally, the hydrophobic pocket composed of iso62, asp64, val70, ala83, lys85, asp 133, arg141, and cys199 also enhanced the interaction between **36a** and the protein target.

Kim[38] discovered that indirubin-3′-oxime derivatives could potentially be a class of FLT3 inhibitors. Through the screening of indirubin derivatives with substitutions at the 5- and 5′-positions, the researchers noted that compounds **37a** and **37b** were effective FLT3 tyrosine kinase inhibitors (Fig. 10). The IC_{50} values of these two compounds were 0.062 μM and 0.015 μM, respectively. Simultaneously, these two compounds also

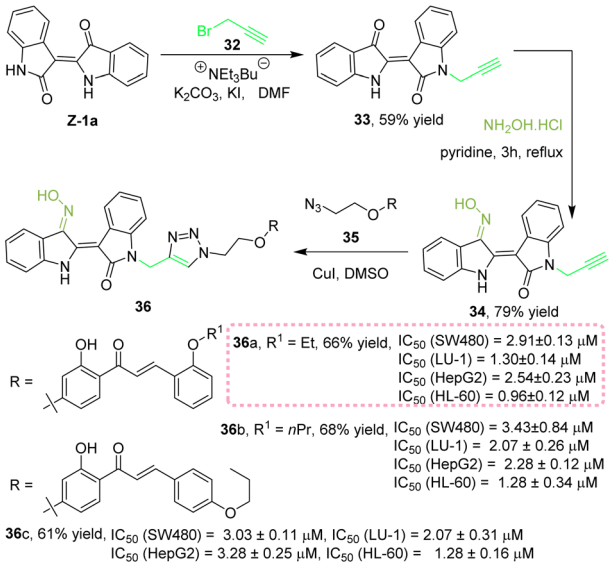

Fig. 9 The synthesis of indirubin-3′-oxime derivatives **36** and anti-cancer activities of them.

demonstrated significant cytotoxic activities against acute myeloid leukemia RS4-11 and MV4-11 cells. Among them, compound **37a** exhibited the highest inhibitory potency against RS4-11 cells (bearing the FLT3/ITD mutation), with an IC_{50} value of 0.56 μM. Compound **37b** showed the strongest inhibitory potency against MV4-11 cells (also with the FLT3/ITD mutation), with an IC_{50} value of 0.072 μM. Furthermore, compound **37b** was confirmed to possess selective and potent FLT3 kinase inhibitory activity and was capable of inducing cell cycle arrest of MV4-11 cells in the G0/G1 phase. Structure–activity relationship studies indicated that small-volume substituents at the 5-position (such as F and CH3) exhibited stronger inhibitory effects on FLT3 kinase and cancer cells. Likewise, large-volume substitutions like 5′-Br or 5,7-di-substitution diminished their inhibitory effects on FLT3 and anti-cancer activities. Notably, although the 5-sulfonate substitution had the strongest kinase inhibitory activity (IC_{50} = 0.009 μM), it lost its cellular activity because of poor membrane permeability.

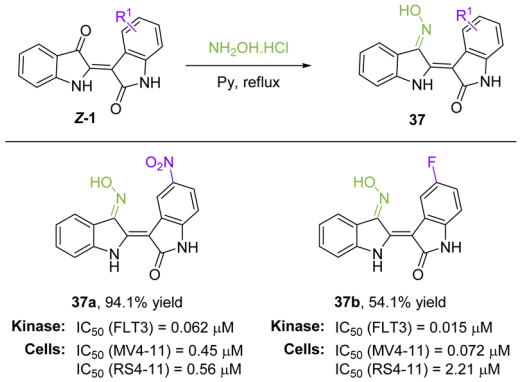

Fig. 10 The synthesis of indirubin-3′-oxime derivatives **37** and anti-AML activities of them.

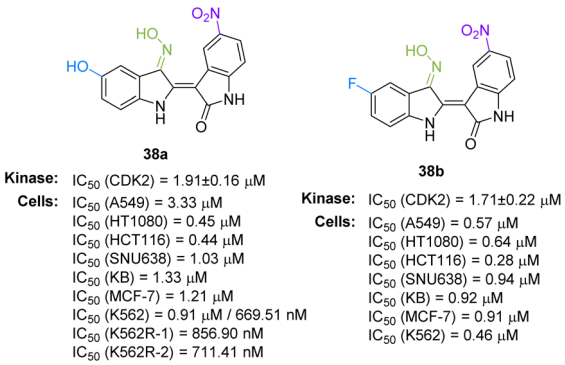

Fig. 11 The anticancer activities of **38**.

Furthermore, Kim[39] presented evidence of the inhibitory effect of indirubin-3′-oxime derivatives with combined substitutions at the C5 and C5′ positions on CDKs. The majority of the synthesized derivatives displayed an inhibitory potency exceeding 90% against CDK2 at a concentration of 1 μM. Compounds **38a** and **38b** not only demonstrated satisfied selective inhibitory activities against CDK1 and CDK2 but also exhibited favorable anti-cancer activities against various cancer cell lines, including the leukemia cell lines K562 (Fig. 11). Docking studies showed that the 5′-OH of compound **38a** formed a novel hydrogen bond with Asp 86 in the solvent-accessible region of CDK2, and the 3′-oxime moiety formed a hydrogen bond with Ile10 instead of Gln131. Structure–activity relationship studies indicated that the anti-proliferative activity of compound **38** against tumor cells is contingent upon the substituents at the C5 and C5′ positions. At the C5 position, in the order of electron-withdrawing ability, it is NO_2 > F > Cl > OCF_3, Me. Small substituents at the C5′ position, such as –OH or –F, can notably enhance the inhibitory potency against CDK2.

Subsequently, Park and his co-workers[40] further reported that compound **38a** could significantly decrease the viability of leukemia K562 cells, with an IC_{50} value of 669.51 nM. For two imatinib-resistant K562R cell lines, the IC_{50} values of the inhibitory effect of **38a** were comparable, being 856.90 nM and 711.41 nM, respectively. This suggested that compound **38a** could be utilized for the treatment of imatinib-resistant chronic myeloid leukemia. When compared with the single-drug treatment using either compound **38a** or imatinib, the combined administration of **38a** and imatinib can further increase the number of apoptotic K562R cells. Intriguingly, in *in vivo* experiments, compound **38a** at a dose of 15 mg kg^{-1} can significantly inhibit the growth of K562R tumors, while imatinib fails to show such an effect.

Additionally, 5-diphenylacetamido indirubin-3′-oxime **39** (LDD398), screened by Kim from 28 5-substituted indirubin-3′-oxime derivatives, was verified as a novel mitochondria-targeted anti-leukemia drug (Fig. 12).[41] LDD398 can effectively inhibit the growth of leukemia K562 cells by activating caspases, arresting cells at the G2/M phase, and disrupting the mitochondrial membrane potential. Significantly, LDD398 not only exerts a marked inhibitory effect on the growth of primary

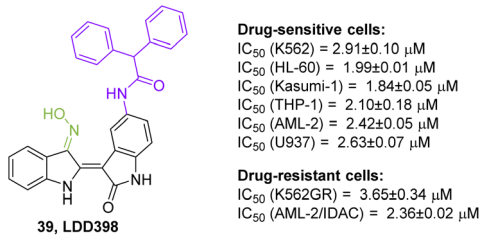

Fig. 12 The anti-leukemia activities of **39**.

leukemia cells (K562 and AML-2), but also demonstrates a notable inhibitory effect on drug-resistant leukemia cells (K562GR and AML-2/IDAC). The IC_{50} values are 3.65 ± 0.34 μM and 2.36 ± 0.02 μM, respectively.

Subsequently, Han[42] made additional discoveries of two 5-substituted indirubin-3′-oxime derivatives, namely LDD1075 (**40a**) and LDD1076 (**40b**) (Fig. 13). *In vitro*, LDD-1076 exhibited potent inhibitory activity against FLT3 kinase, with an IC_{50} of 7.89 ± 1.38 nM. Conversely, LDD-1075 displayed relatively weaker anti-FLT3 activity, with an IC_{50} of 3190 ± 538 nM. Contrary to the results of the FLT3 kinase activity inhibition assay, although the compound LDD1075 had lower anti-FLT3 activity, it demonstrated stronger cytotoxicity against MV4-11 cells (GI_{50} = 54.1 ± 2.06 nM) and LDD1076 had weaker cytotoxicity (GI_{50} > 1 μM). When LDD-1075 was incubated with MV4-11 cell lysates, the formation of LDD-1076 could also be detected. Further investigations indicated that LDD1075 could inhibit the phosphorylation of downstream STAT5 in MV4-11 cells, promote PARP cleavage, induce apoptosis of MV4-11 cells, and arrest the cell cycle at the G1 phase.

4.2.3 Indirubin-3′-oxime ether derivatives. In the structural modification of indirubin-3′-oxime, aside from preparing the aforementioned substituted indirubin-3′-oximes, a certain proportion of relevant research focuses on constructing substituted indirubin-3-oxime ethers with high anti-leukemia activity. This is achieved by introducing other groups (such as polyols, alkylamines, amide chains, or their salts) onto the hydroxyl group of 3′-oxime through substitution reactions. In this section, we will summarize such compounds.

As early as 2005, Jove successfully synthesized a series of indirubin-3′-oxime ether compounds **42** bearing hydrophilic substituents *via* substitution reaction (Fig. 14). Research verified that **42a** (E804) could directly inhibit the activity of Src kinase (IC_{50} = 0.43 μM).[43] This led to the downregulation of the

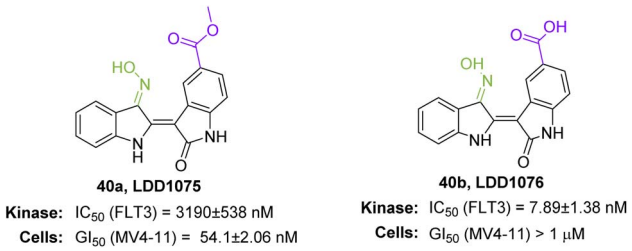

Fig. 13 The anti-AML activities of **40**.

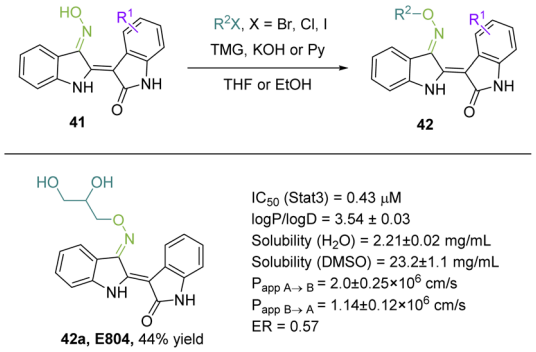

Fig. 14 The synthesis of **42** and the performance demonstration of **42a**.

expression of the anti-apoptotic proteins Mcl-1 and Survivin, which are encoded by Stat3 target genes, thereby inducing the apoptosis of tumor cells.

Subsequent research by Jove and coworkers[44] demonstrated that E804 could significantly inhibit the tyrosine phosphorylation of Stat5 in human K562, KCL-22 M cell lines, and primary chronic myeloid leukemia cells. Consequently, it inhibited the DNA-binding activity of Stat5. At a concentration of 5 μM, **42a** markedly inhibits the tyrosine phosphorylation of Stat5 (at the Y694 site) and its DNA-binding activity, thus downregulating the expression levels of Bcl-XL and Mcl-1. Moreover, at a concentration of 5 μM, E804 could also impede the autophosphorylation of Src or Src family kinases (SFKs) in K562 and KCL-22M cells. At a concentration of 10 μM, E804 could gently decrease the autophosphorylation of Src or SFKs in primary patient CML cells. Nevertheless, E804 exhibits a relatively weak direct inhibitory effect on Bcr-Abl. In K562 cells, when the concentration of E804 exceeds 20 μM, the level of Bcr-Abl decreases. This suggests that E804 exerts its anti-leukemia effect primarily by interfering with the SFK/Stat5 signaling pathway rather than directly targeting Bcr-Abl. Significantly, E804 also demonstrated notable activity against imatinib-resistant KCL-22M cells and exhibited a more potent pro-apoptotic effect than imatinib in primary chronic myeloid leukemia (CML) cells. In 2013, Fricker[45] characterized the physicochemical properties of E804. E804 did not ionize within the pH range of 2–12 (pK_a not detected); both log *P* and log *D* were 3.54. It had high lipophilicity but extremely low water solubility (2.21 μg mL^{-1}). After being solubilized with the surfactant Poloxamer 188, E804 showed good permeability in the Caco-2 cell model (Papp A → B = 2.0×10^{-6} cm s^{-1}). The efflux ratio (ER = 0.57) and the calcein-AM experiment confirm that it is not a substrate of P-glycoprotein, indicating the potential for oral absorption of E804.

Meijer' group successfully achieved the synthesis of 15 6-bromo indirubins.[46a] Then Skaltsounis[46b] found these compounds were verified to be notable inhibitors of mutant leukemia cells, and they could effectively suppress the activities of c-Src and Abl kinases (Fig. 15). Specifically, compounds **46a**, **46b** and **47** displayed distinct cytotoxicity against both wild-type KCL-22 cells and T315I mutant KCL-22 cells, with IC_{50} values

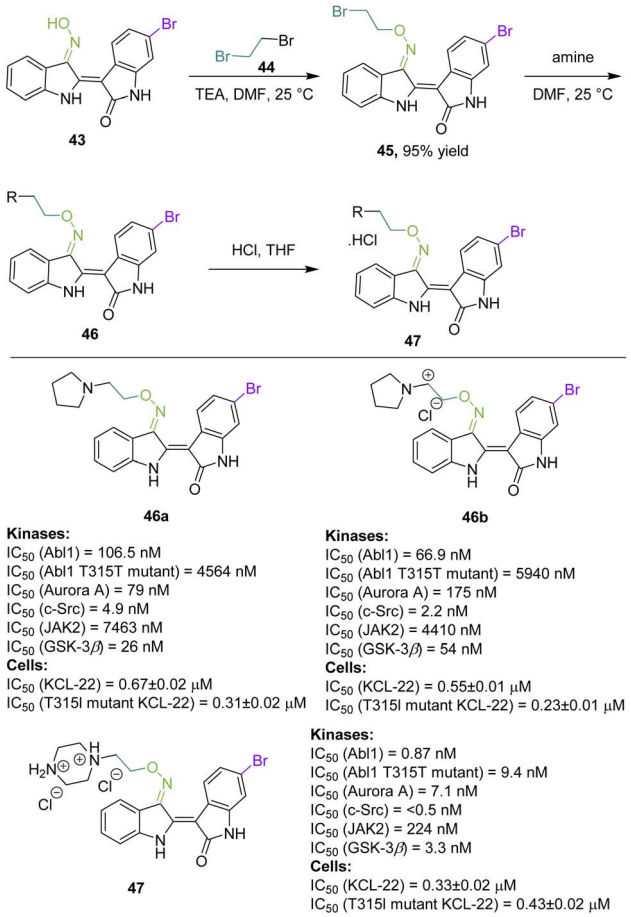

Fig. 15 The chemical structures and anticancer activities of **46** and **47**.

ranging from 0.23 to 0.67 μM. More significantly, the IC_{50} values of compound **47** against Abl1 and T315I mutant Abl1 kinases were 0.87 nM and 9.4 nM, respectively, and the IC_{50} value against c-Src kinase was less than 0.5 nM. Structure–activity relationship analyses indicated that the introduction of a bromine atom at the 6-position and a flexible alkylamino chain at the 3′-oxime group could enhance the cytotoxicity of the compounds. However, the introduction of a trifluoromethyl or bromine atom at the 7-position was not favorable for the manifestation of cytotoxic activity. Docking studies revealed that compounds **46** and **47** inhibited the T315I Abl kinase by binding to both the active and Src-like inactive conformations in an unprecedented way.

Kim's research[47] verified that indirubin derivative **48** (LDD1937) was a highly effective FLT3 inhibitor *in vitro* (Fig. 16). Its IC_{50} value was 3 ± 0.525 nM, and it could inhibit the phosphorylation of downstream Stat5. Related research has demonstrated that LDD1937 exhibited selective anti-cancer activity against leukemia MV4-11 cells *in vitro*, with an IC_{50} value of 1.2 nM. Its mechanism of action was achieved through G2/M phase arrest and induction of cell apoptosis. *In vivo* experiments have shown that LDD1937 (10 mg kg^{-1}) can suppress tumor growth in the MV4-11 tumor xenograft model without causing changes in the body weight of mice.

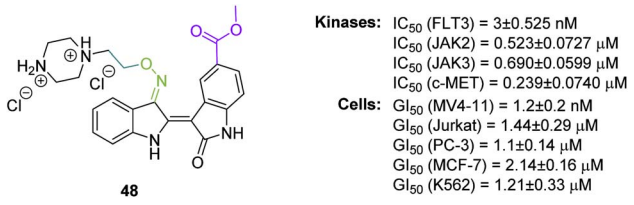

Fig. 16 The chemical structures and anticancer activities of **48**.

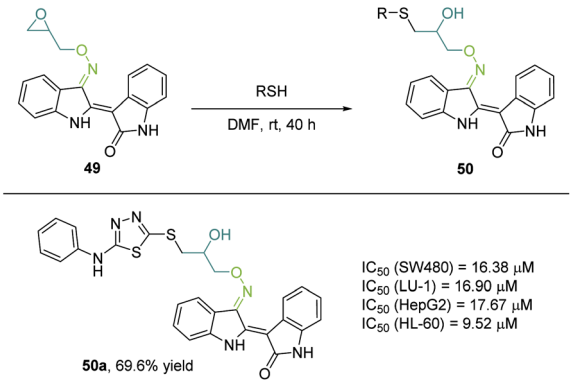

Fig. 17 The synthesis of 46 and the anticancer activities of **50a**.

Van[28] synthesized a series of novel indirubin-3′-oxime derivatives **50** and evaluated their anti-cancer activity against human cancer cell lines. Among these, the IC_{50} values of compound **50a** against several cancer cell lines ranged from 9.52 to 17.67 μM, and it exhibited the highest activity against the leukemia cell line HL60 (Fig. 17). Structure–activity relationship analyses indicated that the 3′-oxime group could enhance the anti-cancer activity. However, the introduction of large-volume substituents into this group through chemical manipulation mostly led to a reduction of anti-cancer activity.

In 2020, Kim[48] reported the synthesis of a series of indirubin-3′-oxime ethers and evaluated their anti-tumor activities both *in vitro* and *in vivo* (Fig. 18). Most of the synthesized compounds displayed good inhibitory effects against FLT3 kinase and MV4-

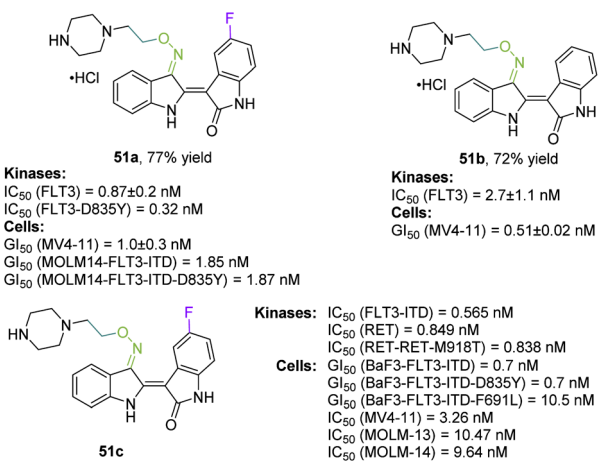

Fig. 18 The chemical structures and anticancer activities of **51**.

11 cells. Among these compounds, **51a** and **51b** exhibited more potent activities. Specifically, compound **51a** demonstrated a stronger FLT3 kinase inhibitory activity ($IC_{50} = 0.87 \pm 0.2$ nM), while compound **51b** showed stronger cytotoxicity ($IC_{50} = 0.51 \pm 0.02$ nM). Furthermore, the pharmacodynamic characteristics of **51a** indicated that its IC_{50} value against FLT3/D835Y was 0.32 nM. Simultaneously, **51a** exerted potent inhibitory effect on MOLM14 cells expressing FLT3/D835Y, with GI_{50} value of 1.87 nM. Compound **51a** has a high oral bioavailability of 42.6%. In a mouse xenograft model, when administered orally at a dosage of 20 mg kg^{-1} once daily for 21 days, **51a** exhibited significant *in vivo* anti-tumor activity. The molecular docking study of **51a** was performed in the DFG-in conformation homology model of FLT3. In type 1 kinases, its binding mode was rational and resembled the binding modes of the previously reported type 1 FLT3 inhibitors, crenolanib and gilteritinib. Compound **51a** successfully docked into the ATP binding pocket, and its aromatic portion fit snugly into the pocket. The N1′–H and C2′-carbonyl on the indirubin scaffold of **51a** form two crucial hydrogen bonds with the main chain carbonyl and amino group of the residue of cys694. Additionally, the tertiary amine of the piperazine moiety of **51a** might form ion-dipole or ionic interactions with the main chain carbonyl of asn816 and the side chain of asp698; the amino group at the end of the piperazine could act as a hydrogen bond donor and interact with the side chains of asn816 and asp829. Recently, Kim[49] also made a discovery: the free compound **51c** (PLM-101) of **51a** serves as a novel dual-target inhibitor of RET/FLT3 kinases. *In vitro* kinase experiments demonstrated that **51c** exhibited more potent inhibitory activity against FLT3 ($IC_{50} = 0.565$ nM) and RET ($IC_{50} = 0.849$ nM) compared with gilteritinib. Moreover, it proved effective against drug-resistant mutations (such as D835Y and F691L). When tested against FLT3-ITD positive AML cell lines (MV4-11, MOLM-14, and MOLM-13), **51c** showed stronger anti-leukemia activity than gilteritinib, with IC_{50} values of 3.26 nM, 10.47 nM, and 9.64 nM respectively. Mechanistic investigations revealed that **51c** could potently inhibit FLT3 kinase and trigger its autophagic degradation by inhibiting RET. Its mechanism of action is superior to that of drugs targeting only FLT3. *In vivo* studies indicated that oral administration of PLM-101 in mice significantly suppressed tumor growth (MV4-11 and MOLM-14 cells). Additionally, no obvious drug-related adverse reactions were detected in the single and multiple-dose toxicity tests conducted during the study (NOAEL >160 mg kg^{-1}). The oral bioavailability of **51c** was 17.6%, which is lower than that of the salt form (**51a**).

In 2021, He and his colleagues[50] employed the pharmacophore combination strategy to design and synthesize a series of novel indirubin derivatives (Fig. 19). These newly developed derivatives not only maintained the high inhibitory activity of indirubin against CDKs but also conferred the targeting ability towards histone deacetylases (HDACs) on the compounds. As such, they could serve as dual inhibitors of CDKs and HDACs. Among them, the lead compound **53a** demonstrated inhibitory activities against CDK2/4/6 and HDAC6, with IC_{50} values of 60.9 $\pm$ 2.9 nM, 276 $\pm$ 22.3 nM, 27.2 $\pm$ 4.2 nM, and 128.6 $\pm$ 0.4 nM, respectively. It was able to effectively induce apoptosis and S-phase arrest in multiple cancer cell lines. In the K562 leukemia cell's model, this compound displayed excellent anti-proliferative activity ($IC_{50} = 2.5 \pm 0.1$ μM), which was significantly superior to that of the parent compound indirubin.

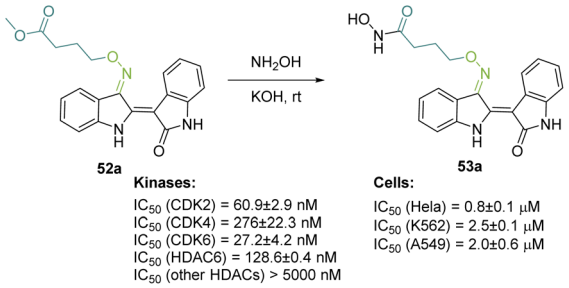

Fig. 19 The synthesis and the anticancer activities of **53a**.

In 2022, Kim[51] substituted the ethyl linker between the piperazine and oxime groups of compounds **51** with acyl or sulfonyl groups to enhance its performance in the phosphate-binding region. Consequently, acetyl compounds **56a** and **56b**, which possess excellent FLT3 inhibitory activity, were designed and synthesized (Fig. 20). Structure–activity relationship studies indicated that the size of the 5-position substituents significantly affected the activity: hydrogen, chlorine, and bromine substituents showed high activities (FLT3/D835Y $IC_{50} \approx 0.18$–0.26 nM), while larger substituents such as iodine, methoxy, or trifluoromethoxy groups exhibited lower activitives. Moreover, the piperazine group could stably bind to asp829 and arg815 *via* hydrogen bonds. After the calculation of the flexible docking protocol, the introduced carbonyl group could act as an additional hydrogen bond acceptor and interact with the guanidyl group of arg815. Lengthening the carbon chain or replacing the piperazine group with a piperidinyl group would decrease the activities. Finally, compound **56b** was chosen as the more promising candidate molecule. Its IC_{50} values for FLT3 and FLT3/D835Y kinase were 0.26 nM and 0.18 nM, respectively, surpassing those of the clinical drug gilteritinib. In addition, compound **56b** demonstrated nanomolar anti-proliferative

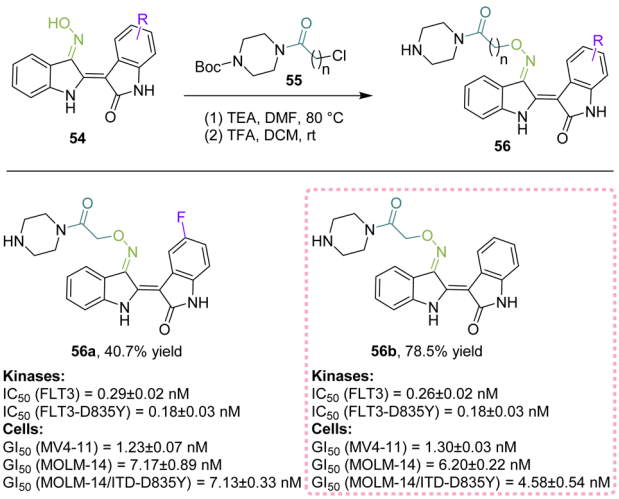

Fig. 20 The chemical structures and anticancer activities of **56**.

Table 1 Summary of the synthesis methods

Methods	Reactants	Yield (%)	Features	Literature
Coupling reaction catalyzed by base	Isatins, indoxyl acetates	41–91	The traditional methods have significant limitations	9
Coupling reaction catalyzed by acid	Isatins, indoxyl acetates	8–49	Improve the yield of the synthesis of indirubins with C4 substituent	10
Sequential construction	Isatins, rhodamine, substituted aniline	50–91	It demonstrated tolerance to functional groups ($-NO_2$, –CN, –CHO, $-CO_2H$)	11
Organophosphorus reagent-mediated reaction	Isatin	75	The product was synthesized *via* the Wittig/carbene mechanism	12
Reductive coupling reaction catalyzed by KBH_4	Isatins	63–91	One-pot method, high yield and green	13
Oxidation-condensation catalyzed by $Mo(CO)_6$	Indoles	4–78	Regulatable selectivity	14 and 15
Bionic oxidation by $Mn(TDCPP)Cl/H_2O_2$	Substituted indole-3-carboxaldehyde	5–27	It has strong controllability and belongs to the type of green catalysis	16
Oxidative coupling reaction catalyzed by $H_2O_2/(PhSe)_2$	Indole-3-carboxaldehyde	7	Two products can be obtained in one pot reaction, enabling biomimetic synthesis	17
Enzymatic-cysteine reaction	Indoles	5–45	Green, highly selective, and capable of derivatization	18

activity in AML cell lines and was effective against the FLT3/F691L mutation kinase. Kinase selectivity analysis revealed that **56b** primarily targeted the receptor tyrosine kinase family and had a relatively weak inhibitory effect on c-KIT. This might potentially reduce the side effect of myelosuppression. *In vivo* experiments have confirmed that compound **56b** significantly suppressed tumor growth, with a slightly less extent compared to that of gilteritinib.

5 Summary and discussion

5.1 Summary and discussion of the synthesis method

The statistical details of the synthesis methods of indirubins are presented in Table 1. The data in Table 1 suggest that chemical synthesis methods remain the primary approach for the efficient synthesis of indirubin. Specifically, the KBH_4 reduction coupling method can produce indirubin derivatives with a high yield of up to 91%. The biosynthetic method began relatively late, yet its significance cannot be overlooked. Currently, the product can be obtained in an environmentally friendly manner with a yield of 45%. Additionally, the derivatization of indirubin typically mainly involves reaction methods such as *de novo* synthesis, condensation, and substitution. Substituent groups are introduced onto the N1, C3′ or aromatic rings of indirubin to construct the target indirubin derivatives, such as indirubin-3′-oxime, 5-F-indirubin or 5-F-indirubin-3′-oxime.

Although numerous outstanding synthetic methods have been reported, particularly for the synthesis of indirubin

Table 2 Summary of leukemia-related interacting proteins

Compound		Interacting protein	IC_{50}	Literature
Indirubin	**1a**	CDK1, CDK2, CDK5	2.2–10 μM	4*b*, 38
		FLT3	>10 μM	
Indirubins with N1 modifications	**30b**	IDO1	29.52 μM	30
Indirubin-3′-oxime	**31**	CDK1, CDK2, CDK4, CDK5	0.1–3.3 μM	4*b*, 34
		FLT3	0.079–0.101 μM	
Indirubin-3′-oxime derivatives with substituents	**37b**	FLT3	0.015 μM	38
	38b	CDK2	1.71 ± 0.22 μM	39
	40b	FLT3	7.89 ± 1.38 nM	42
Indirubin-3′-oxime ether derivatives	**42a**	c-Src	0.43 μM	43
	47	Abl1, Abl1 T315T mutant	0.87–9.4 nM	46
		Aurora A	7.1 nM	
		c-Src	<0.5 nM	
		JAK2	224 nM	
		Gsk-3β	3.3 nM	
	48	FLT3	3 ± 0.525 nM	47
		JAK2, JAK3	0.523–0.690 μM	
	51c	FLT3	0.565 nM	49
		RET	0.849 nM	
	53a	CDK2, CDK4, CDK6	27.2–276 nM	50
		HDAC6	128 nM	
	56b	FLT3, FLT3-D835Y	0.18–0.26 nM	51

derivatives featuring the same substituents on the B-ring and D-ring, which exhibit remarkable reaction tolerance, for the preparation of indirubin derivatives with different substituents, only traditional base-mediated coupling reactions are currently available. Due to the instability of indole acetates, it is challenging to successfully prepare some specific target indirubins. Thus, there remains a need for in-depth research on the chemical synthesis methods of indirubin derivatives. In the future, it is advisable to consider achieving the coupling of two different indole derivatives through the regulation of base catalysis, metal catalysis, photocatalysis, or radical catalysis to construct the target derivatives. Simultaneously, green biosynthesis methods also require further in-depth exploration.

5.2 Summary and discussion of leukemia-related interacting proteins

The effects of indirubin and its derivatives on leukemia-related interacting proteins and the anti-proliferative activity at the cellular level are shown in Tables 2 and 3. The data in Table 2 indicates that the structurally modified indirubin derivatives not only exhibit a more significant inhibitory effect on CDK but also show potential inhibitory activity against other kinases (such as FLT3 and IDO1). Notably, these derivatives demonstrate excellent inhibitory activity against CDK2, GSK-3β, c-Src, and FLT3, as well as other interacting proteins like RET, Aurora, Abl, JAK, and HDAC6 kinases. Among them, the inhibitory effect on FLT3 is more prominent.

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Upon analyzing the data in Table 3, it becomes evident that the structurally modified compounds not only augment the

Table 3 Summary of the anti-proliferative activity at the cellular level

Compound		Cell lines	IC_{50} or GI_{50}	Literature
Indirubin	**1a**	HL-60	$IC_{50} > 100$ μM	26–28
		K562	$IC_{50} > 100$ μM	
		MV4-11	$IC_{50} > 10$ μM	
		RS4-11	$IC_{50} > 10$ μM	
Indirubins with N1 modifications	**17a**	HL-60	$IC_{50} = 3.56 \pm 0.211$ μM	26
	21a	K562	$IC_{50} = 0.034 \pm 0.002$ μM	27
		K562/VCR	$IC_{50} = 0.076 \pm 0.008$ μM	
	24a	HL-60	$IC_{50} = 1.35$ μM	28
	30a	K562	$IC_{50} = 24.96$ μM	30
Indirubin-3′-oxime	**31**	MV4-11	$IC_{50} = 30$ nM	34–36
		HL-60	$GI_{50} = 36.6$ μM	
Indirubin-3′-oxime derivatives with substituents	**36a**	HL-60	$IC_{50} = 0.96 \pm 0.12$ μM	37
	37a	RS4-11	$IC_{50} = 0.56$ μM	38
	37b	MV4-11	$IC_{50} = 0.072$ μM	
	38a	K562	$IC_{50} = 669.51$ nM	39
		K562R-1	$IC_{50} = 856.90$ nM	
		K562R-2	$IC_{50} = 711.41$ nM	
	39	K562	$IC_{50} = 2.91 \pm 0.10$ μM	41
		HL-60	$IC_{50} = 1.99 \pm 0.01$ μM	
		AML-2	$IC_{50} = 22.42 \pm 0.05$ μM	
		K562GR	$IC_{50} = 3.65 \pm 0.34$ μM	
		AML-2/IDAC	$IC_{50} = 2.36 \pm 0.02$ μM	
	40a	MV4-11	$GI_{50} = 54.1 \pm 2.06$ nM	42
Indirubin-3′-oxime ether derivatives	**42a**	K562, KCL-22M	Not given	44
	43c	KCL-22	$IC_{50} = 0.55 \pm 0.01$ μM	46
		T3151 mutant KCL-22	$IC_{50} = 0.43 \pm 0.02$ μM	
	44	MV4-11	$IC_{50} = 1.2 \pm 0.2$ nM	47
		K562	$IC_{50} = 1.21 \pm 0.33$ μM	
	46a	HL-60	$IC_{50} = 9.52$ μM	27
	47a	MV4-11	$IC_{50} = 1.0 \pm 0.3$ nM	48
	47c	MV4-11	$IC_{50} = 3.26$ nM	49
		MOLM-13	$IC_{50} = 10.47$ nM	
		MOLM-14	$IC_{50} = 9.64$ nM	
	49a	K562	$IC_{50} = 2.5 \pm 0.1$ μM	50
	50b	MV4-11	$IC_{50} = 1.30 \pm 0.03$ nM	51
		MOLM-14	$IC_{50} = 6.20 \pm 0.22$ nM	
		MOLM-14/ITD-D835Y	$IC_{50} = 4.58 \pm 0.54$ nM	

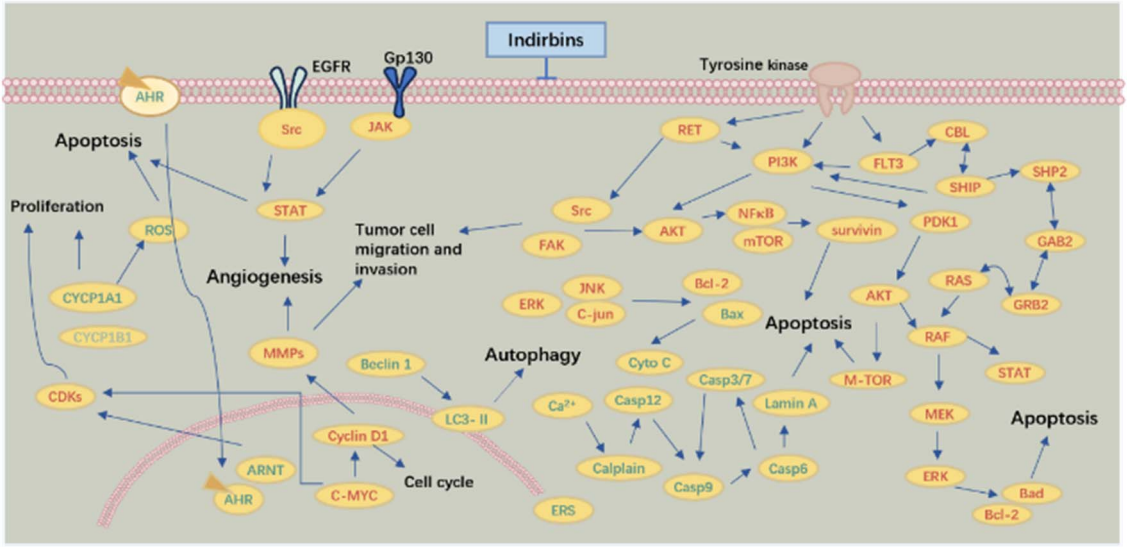

Fig. 21 Schematic illustration of several pathways involved in the anti-leukemia mechanisms of indirubins.

cytotoxicity against chronic leukemia cells K562 but also significantly enhance the cytotoxicity against acute leukemia cells such as KL-60, MV4-11, and RS4-11. Additionally, from the currently presented activity results, most indirubin derivatives after oximino ether derivation can readily reach the nanomolar level in terms of their toxicity to various acute leukemia cells, while the cytotoxicity to K562 remains at the micromolar level. This seemingly suggests that indirubin derivatives may be more readily developed into a class of specialized therapeutic drugs for acute leukemia.

By integrating the information in Tables 2 and 3, it is found that for compounds demonstrating good anti-proliferation activity, the proteins interacting with them are seldom single proteins. Instead, most of these compounds exert their therapeutic effects through the synergistic action of multiple targets, and some of the involved mechanisms are depicted in Fig. 21.[52] In future research on the anti-leukemia effect of indirubin compounds, it may be necessary to perform kinase profiling analysis and combine it with structural modification. This approach aims to enhance the activity of proteins with beneficial therapeutic effects while eliminating or reducing the activity of proteins with no effect or negative impacts.

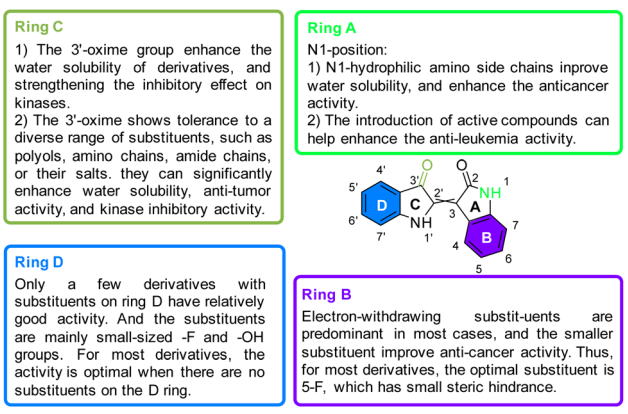

Fig. 22 Graphical depiction of the structure–activity relationship of indirubin derivatives derived from the available anti-leukemia results.

5.3 Summary and discussion of the structure–activity relationship

Based on the existing indirubin derivatives and their anti-proliferative activity results against leukemia, a highly significant structure–activity relationship (SAR) analysis was performed. The specific details are presented in Fig. 22. Briefly, modifications to ring B and ring C often result in more favorable outcomes. Modifying the N1 position of ring A by introducing hydrophilic amino side chains or bioactive compounds typically enhances the anti-tumor activity. Ring B is highly amenable to structural modification, particularly at the 5-position. Introducing small electron-withdrawing groups (such as F, Cl, Br, and $-NO_2$) chains at the 5-position can improve kinase inhibitory activity and cytotoxicity, while large substituents are not conducive to these effects. Kim[48] indicated through molecular docking experiments that C=O in C2′ and N1′–H tend to form intramolecular hydrogen bonds or hydrogen bonds with the target. Thus, the N1′ and C2′ of ring C is generally not modified. In contrast, the 3′-position of ring C appears to have better tolerance. Introducing an oxime group substituent at this position may enhance the anti-cancer activity and water solubility. Furthermore, the 3′-oxime group demonstrates good tolerance. The incorporation of hydrophilic substituents (such as polyols, amide chains, or their salts) can remarkably enhance water solubility, kinase inhibitory effects, as well as anti-cancer activities both *in vitro* and *in vivo*. Finally, chemical modification of the D-ring with small groups is viable. Nevertheless, based on the current modification findings, even without modifying the D-ring, high anti-tumor activity and kinase inhibitory activity can still be attained. In summary, the

structure–activity relationship studies conducted offer practical and advantageous modification strategies for the further development of indirubin derivatives with enhanced anti-leukemia properties.

6 Conclusions

In the contemporary era, leukemia persists in presenting a substantial threat to human health. There exists an urgent necessity to develop anti-leukemia drugs with promising outlooks and high efficacies. The indirubin skeleton is acknowledged as a privileged scaffold within the domain of drug research and development. A plethora of indirubin-based compounds have demonstrated remarkable anti-leukemia activities in both *in vitro* and *in vivo* experimental settings. Historically, indirubin was mainly extracted from plants. However, owing to its low content in plants and poor water solubility, the extraction yield was constrained, and the production cost remained high. Gradually, various synthetic strategies for indirubin derivatives have been developed. Currently, indirubin and its derivatives are predominantly synthesized *via* chemical methods. This review comprehensively covers the latest progress in the synthesis, anti-leukemia potential, structural modification, and structure–activity relationship of indirubin derivatives since 2010. The objective is to lay a foundation for the rational development of anti - leukemia drugs based on indirubin molecules.

Conflicts of interest

There are no conflicts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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