Nucleic Acids Research, 2018 **1**
doi: 10.1093/nar/gky202

ZYH005, a novel DNA intercalator, overcomes all-trans retinoic acid resistance in acute promyelocytic leukemia

Qingyi Tong[1,†], Huijuan You[1,†], Xintao Chen[1], Kongchao Wang[1], Weiguang Sun[1], Yufeng Pei[1], Xiaodan Zhao[2], Ming Yuan[3], Hucheng Zhu[1], Zengwei Luo[1,*] and Yonghui Zhang[1,*]

[1]Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China, [2]Mechanobiology Institute, National University of Singapore, 117411, Singapore and [3]School of Pharmacy, Hubei University of Chinese Medicine, Wuhan 430065, China

Received January 30, 2018; Revised March 02, 2018; Editorial Decision March 07, 2018; Accepted March 09, 2018

ABSTRACT

Despite All-trans retinoic acid (ATRA) has transformed acute promyelocytic leukemia (APL) from the most fatal to the most curable hematological cancer, there remains a clinical challenge that many high-risk APL patients who fail to achieve a complete molecular remission or relapse and become resistant to ATRA. Herein, we report that 5-(4-methoxyphenethyl)-[1, 3] dioxolo [4, 5-j] phenanthridin-6(5H)–one (ZYH005) exhibits specific anticancer effects on APL and ATRA-resistant APL in vitro and vivo, while shows negligible cytotoxic effect on non-cancerous cell lines and peripheral blood mononuclear cells from healthy donors. Using single-molecule magnetic tweezers and molecule docking, we demonstrate that ZYH005 is a DNA intercalator. Further mechanistic studies show that ZYH005 triggers DNA damage, and caspase-dependent degradation of the PML-RARa fusion protein. As a result, APL and ATRA-resistant APL cells underwent apoptosis upon ZYH005 treatment and this apoptosis-inducing effect is even stronger than that of arsenic trioxide and anticancer agents including 5-fluorouracil, cisplatin and doxorubicin. Moreover, ZYH005 represses leukemia development in vivo and prolongs the survival of both APL and ATRA-resistant APL mice. To our knowledge, ZYH005 is the first synthetic phenanthridinone derivative, which functions as a DNA intercalator and can serve as a potential candidate drug for APL, particularly for ATRA-resistant APL.

INTRODUCTION

Normally, cells are equipped with DNA damage response (DDR) pathways and damage to DNA is detected and repaired. However, most cancer cells have relaxed DDR pathways, and more importantly, they are capable of ignoring DNA damage and allowing cells to achieve high proliferation rates, increasing their susceptibility to DNA damage drugs compared to that of normal cells since replication of damaged DNA increases the possibility of cell death (1,2). Consequently, the concept of targeting DNA in cancer therapy has inspired the development of numerous anticancer drugs, particularly DNA-binding drugs such as cisplatin, carboplatin, oxaliplatin, mitoxantrone, amsacrine, temozolomide and anthracyclines (3–5). Despite dose-limiting side effects, the extensive use of these DNA-binding drugs in clinical practice has revealed their utility, and they will continue to be a staple in anticancer regimens. Meanwhile, the discovery of new DNA-binding drugs with improved effects and a high specificity for cancer cells is of great importance.

DNA-binding drugs include covalent binding ligands (alkylating agents) and non-covalent ligands (groove binders and intercalators) (5). DNA intercalators, which bind DNA by inserting aromatic moieties between adjacent DNA base pairs, have attracted considerable attention due to their potent anticancer activity. For example, several acridine and anthraquinone derivatives (i.e. anthracycline) are excellent DNA intercalators that are currently available on the market and widely used as anticancer agents (6,7). Acridine and anthraquinone represent

*To whom correspondence should be addressed. Tel: +86 27 83692868; Fax: +86 27 83692762; Email: zhangyh@mails.tjmu.edu.cn
Correspondence may also be addressed to Zengwei Luo. Email: luozengwei@hust.edu.cn
†The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

two of the main frameworks of DNA intercalators, and the other well-known framework is phenanthridine (6). For many decades, phenanthridine derivatives have been recognized for their efficient DNA intercalative binding capability (8) and have been applied as gold-standard DNA/RNA-fluorescent markers (ethidium bromide, EB) and probes for DNA (propidium iodide, PI); however, they are also considered disadvantageous due to their potential genotoxic and mutagenic effects. In the past decade, Amaryllidaceae alkaloids with a phenanthridinone rather than phenanthridine skeleton, such as narciclasine, *cis*-dihydronarciclasine, 7-deoxypancratistatin, lycoricidine and pancratistatine, have been reported to have potent and selective anticancer effects (9–12). Importantly, narciclasine and other natural phenanthridinone alkaloids are considered potentially useful drug leads for the treatment of apoptosis-resistant cancers and metastatic cancers (13–15). In addition, synthetic phenanthridinone derivatives which exhibited greater anticancer activity than several standard chemotherapeutics (Taxol, doxorubicin, gemcitabine and cisplatin) also have been reported (16). And some phenanthridinone derivatives have been proven to be potent PARP inhibitors (6(5H)-phenanthridinone, PJ34) (17,18), topoisomerase I inhibitors (ARC-111 and its analogues) (19,20), all of which exhibit pronounced and targeted antitumor activity. Therefore, phenanthridinone alkaloids and their derivatives have been under intense scrutiny and are currently being pursued as clinical candidates for cancer treatment (12,14,21,22). These findings prompted us to develop novel phenanthridinone derivatives with selective anticancer effects and to explore whether these derivatives act as DNA intercalators in the same manner as phenanthridines.

Acute promyelocytic leukemia (APL) is the M3 subtype of acute myeloid leukemia (AML), with 98% of patients harboring the t(15;17) chromosomal translocation, involving the fusion of the genes encoding PML (promyelocytic leukemia) and RARα (retinoic acid receptor alpha) (23–26). In addition, impaired homologous recombination (HR) (25,27,28), non-homologous end-joining (NHEJ) repair (25) and base excision repair (BER) (27) pathways have been found in APL, all of which are considered essential contributors to APL pathogenesis. All-trans retinoic acid (ATRA) and arsenic trioxide (ATO) are PML-RARα targeting drugs that bind to the RARα and PML moieties, respectively. These two drugs have transformed APL from the most fatal to the most curable hematological cancer (23,29,30). Despite the unprecedented success, many high-risk patients fail to achieve complete molecular remission or relapse and become resistant to ATRA (31,32). Therefore, alternative agents must be developed, particularly for relapsed APL with ATRA resistance.

We previously isolated and identified 24 new Amaryllidaceae alkaloids (33–35), and demonstrated that *N*-methylhemeanthidine chloride, which with a phenanthridine core, exhibited prominent anticancer effects on pancreatic cancer and AML in vitro and in vivo (36,37). Later, we developed an environmentally friendly and inexpensive procedure in which the phenanthridinone skeleton was synthesized through $Na_2S_2O_8$-promoted decarboxylative cyclization of biaryl-2-oxamic acid (38). This is of great significance because the development of phenanthridinone alkaloids is hampered by a lack of efficient supply, since total syntheses are complex and biotechnological approaches are completely missing. In this study, we synthesized 20 phenanthridinone-based alkaloids using this method and found that ZYH005 exerts specific anticancer effects on APL and ATRA-resistant APL models. Mechanistically, ZYH005 exerts its effects through intercalative binding to DNA, which subsequently triggers DNA double-strand breaks and the accumulation of DNA damage, causes G2/M cell cycle arrest and caspase-dependent degradation of PML-RARa. As a result, APL and ATRA-resistant APL cells underwent apoptosis upon ZYH005 treatment. Notably, this apoptosis-inducing effect of ZYH005 was found to be stronger than that of ATO and widely used anticancer agents (5-fluorouracil, cisplatin and doxorubicin). To the best of our knowledge, this is the first report of a synthesized phenanthridinone alkaloid that functions as a DNA intercalator and has potential therapeutic value for the treatment of both APL and relapsed APL with ATRA resistance.

MATERIALS AND METHODS

Synthesis of ZYH005 and its analogues

Briefly, the phenanthridinone was synthesized from a ketoacid through transition metal-free decarboxylative cyclization (38). Then, the appropriate side chain was introduced to obtain ZYH005 through a nucleophilic substitution reaction. The compounds ZYH001-ZYH004 and ZYH006-ZYH020 were obtained using the same procedure as that used to synthesize ZYH005, with different starting materials. A detailed description of the process by which these compounds were synthesized, as well as their ^{1}H NMR data (^{13}C NMR data for selected compounds), is included in the Supplementary Information.

Reagents

All-trans retinoic acid, nitroblue tetrazolium (NBT) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (MO, USA). Arsenic trioxide was obtained from Tongji Hospital (Wuhan, China). Cremophor® EL was purchased from Aladdin Chemicals (Shanghai, China). The cell lysis buffer, BCA Protein Assay Kit and ACK buffer were purchased from Beyotime Biotechnology (Shanghai, China). Matrigel was purchased from BD Biosciences (CA, USA). The Wright-Giemsa Staining Kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). Z-VAD-FMK, 5-fluorouracil and cisplatin were purchased from Selleck Chemicals (Shanghai, China). Doxorubicin, idarubicin, chloroquine and MG-132 were purchased from MedChemExpress (NJ, USA). Antibodies against PARP, cleaved-caspase-3, Bcl-2, Bax, Bak, γH2AX, β-actin, GAPDH and the appropriate secondary antibodies were purchased from Cell Signaling Technology (MA, USA). Polyclonal antibodies against RARα (C-20, sc-551) and PKCδ (C20, sc-937) were purchased from Santa Cruz Biotechnology (CA, USA).

Mice

BALB/c-nu/nu mice were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China), and FVB/N mice

were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed under specific pathogen-free (SPF) conditions and handled in accordance with the Guidelines for the Care and Use of Laboratory Animals of Tongji Medical College of Huazhong University of Science and Technology.

Cell lines and cell culture

The APL cell lines NB4, NB4-MR2 (39), NB4-LR2 (40), hPML-RARα and mutant hPML-RARα transgenic mouse-derived leukemic cells (41) were kind gifts from Professor Guoqiang Chen and Yingli Wu (Shanghai Jiao Tong University, Shanghai, China). The DND-41, KOPT-K1 and CUTLL1 cell lines were kindly provided by Dr Warren Pear (University of Pennsylvania, USA) and Professor Hudan Liu (Wuhan University, China) and maintained as previously described (42). The MOLT4, K562, Kg1a, THP-1, Kasumi, and HL60 cell lines were purchased from American Type Culture Collection (VA, USA). HPDE6-C7 and NCM460 cell lines were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air (v/v).The leukemia cells were cultured in RPMI 1640 medium (Hyclone, UT, USA), and the other cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, UT, USA), both types of media were supplemented with 10% (v/v) heat-inactivated FBS. All cell lines were cultured for fewer than 6 months after resuscitation and tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection kit (Lonza, Slough, UK).

Cell viability assay

Cell viability was measured with an MTS Kit (Promega, WI, USA). Briefly, the cells were seeded into 96-well plates at a density of 5000 cells/well and incubated with or without drugs. After 24 h, 20 μl of MTS was added to the wells. The cells were subsequently incubated in the dark for 3 h; then, the optical density values were measured at 490 nm. The 50% inhibitory concentration (IC_{50}) for each drug was calculated using the SPSS software.

Isolation and culture of peripheral blood mononuclear cells (PBMCs)

Experiments involving healthy volunteers (a healthy 31-year-old male, a healthy 31-year-old female, and a healthy 27-year-old male) donated blood were conducted with prior approval of Research Ethics Board of the Huazhong University of Science and Technology, with informed consent obtained from all subjects. Peripheral blood mononuclear cells (PBMCs) from healthy volunteers 1, 2 and 3 (PBMCs-V1, PBMCs-V2, PBMCs-V3) were collected and isolated by density gradient centrifugation (12). The isolated PBMCs were maintained in RPMI 1640 media supplemented and maintained in the same way as the APL cell lines. PBMCs were treated within 1 h of collection with various doses of ZYH005 for up to 24 h; the cell viability was measured with an MTS Kit.

Single-molecule magnetic tweezers assay

The 6618 bp dsDNA (∼50% GC) was prepared by PCR using TaKaRa Ex Taq DNA Polymerase (TaKaRa, Dalian, China) on a lambda phage DNA template (Thermo Scientific, MA, USA). The primers (Sangon Biotech, Shanghai, China) were labeled with 5′-biotin and 5′-thiol, respectively. The DNA was tethered between a streptavidin-coated paramagnetic bead (Dynabeads M-280, Thermo Scientific, MA, USA) and a (3-Aminopropyl) triethoxy silane (APTES, Sigma-Aldrich, MO, USA) -functionalized coverslip, through a biotin-streptavidin interaction and covalent bond (sulfo-SMCC crosslinker, Thermo Scientific, MA, USA). The height of the bead above the coverslip surface was measured based on the bead image using single-molecule magnetic tweezers (43). Extension changes in ds-DNA were measured based on force-jump measurements using single-molecule magnetic tweezers (43). At each force, the magnet was held for 5 s to measure extension changes at equilibrium from the average bead height.

The torsional constraint DNA for the supercoiling assay was prepared by ligating the 6573 bp DNA with a 510 bp dsDNA handle containing ∼50 biotinylated dUTP (Biotin-11-dUTP, Thermo Scientific, MA, USA) at one end and a 510 bp handle containing ∼50 digoxigenin-dUTP (Digoxigenin-11-dUTP, Roche, Basel, Switzerland) as reported in a previous study (44). dsDNA was tethered between a streptavidin coated 1-μm diameter paramagnetic bead (Dynabeads MyOne, Thermo Scientific, MA, USA) and an anti-digoxigenin antibody (Thermo Scientific, MA, USA) coated coverslip. The rotation of the magnetic bead was controlled by rotating a permanent magnet pair using a rotary motor as reported in a previous study (44). The rotation-extension curve data were recorded by measuring DNA extension for 20 s at different magnet turns.

Molecular docking

Compounds were subjected to molecular docking experiments using ICM 3.8.1 modeling software (45) on an Intel i7 4960 processor (MolSoft LLC, CA, USA). Molecules were built with Chemdraw and optimized at molecular mechanical and semiempirical levels using Open Babel GUI. The X-ray crystallographic structure of the DNA dodecamer d $(CGTACG)_2$ with doxorubicin was selected from the Protein Data Bank (PDB code: 2GB9) for the docking study (46). Ligand-binding pocket residues were selected using graphical tools in the ICM software to create the boundaries of the docking search. In the docking calculation, potential energy maps of the receptor were calculated using default parameters. Compounds were imported into ICM and an index file was created. Conformational sampling was based on the Monte Carlo procedure, and finally the lowest-energy and the most favorable orientation of the ligand were selected.

Immunofluorescence assay

Cells treated with or without ZYH005 were placed on slides and fixed in ice-cold 4% paraformaldehyde for 15 min. After permeabilization with 0.1% (v/v) Triton X-100 in PBS and blocking with 2% (w/v) bovine serum albumin (BSA)

in PBS, the cells were incubated with an antibody against γH2AX overnight at 4°C. Then, the cells were stained with the appropriate secondary antibody for 1 h, mounting medium with DAPI (Invitrogen, MA, USA) was added to the slides, and cover slips were mounted onto the slides in a manner that did not create bubbles. Fluorescence signals were detected with an LSM710 confocal microscope (ZEISS, Weimar, Germany). For the quantitative analysis, γH2AX foci were counted by eye during the microscopic examination and imaging process using a 100× objective. At least 100 cells per group were examined, and three independent experiments were performed.

Cell cycle, apoptosis assay and immunoblot analysis

Cell cycle, apoptosis assay and immunoblot analysis were done as previously describe d (47,48).

Xenograft tumor mouse model assay

A total of 5×10^6 NB4 cells in a PBS/Matrigel solution (1:1) were subcutaneously injected into the flanks of 5–6-week-old BALB/c-nu/nu mice. After the cells formed palpable tumors (100–150 mm^3), the mice were randomly assigned to groups treated with vehicle (5% DMSO, 5% Cremophor® EL, 90% Saline) or ZYH005 (10 mg/kg/day, intravenously) according to the preliminary toxicity test. Two weeks later, the animals were sacrificed; their tumors were removed, weighed and photographed. The visceral organs and tumors were collected and fixed in 10% formalin for hematoxylin and eosin (H&E) or immunohistochemistry (IHC) analysis.

Transplantation of ATRA-sensitive/-resistant leukemic mice and treatment

The female FVB/N mice (6–7 weeks old) were sublethally irradiated (4.5 Gy), then 2×10^5 viable leukemic cells isolated from spleens of leukemic hPML-RARα or mutant hPML-RARα transgenic mice were intravenously injected via tail vein (41,49,50). Four days after transplantation, the mice were randomly assigned to groups treated with vehicle (5% DMSO, 5% Cremophor® EL, 90% saline) or ZYH005 (10 mg/kg/day, intravenously). Normal mice (irradiated but not transplanted, no treatment) and mice treated with ATO (5 mg/kg/day, intraperitoneally) or ATRA (15 mg/kg/day, intraperitoneally, for ATRA-resistant APL model) were used as the controls. Cytological and histological analyses were performed as reported (41).

Statistical analysis

Comparisons between groups were performed using a standard two-tailed Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test and stated in the figure legends. All experiments were repeated at least three times. The data are presented as the mean ± S.D., and P values < 0.05 were considered significant.

RESULTS

Selection of ZYH005 for subsequent experiments

Alkaloids with N-phenylethyl phenanthridinone exhibited more potent cytotoxic activity (33). Therefore, we synthesized compounds with methoxyl, benzyl, phenylethyl, phenylpropyl and (4-methoxylphenyl) ethyl substituents at the hetero nitrogen atom of the phenanthridinone ring (ZYH001-ZYH005) (Supplemental Figure S1A). We preliminarily assessed their anti-proliferation effects on five cancer cell lines (HL60, SMMC-7721, A549, MCF-7, SW480), and found that ZYH005 inhibits the proliferation of all cancer cell lines at low concentrations after 48 h of treatment, especially the proliferation of the AML cell line HL60 ($IC_{50} = 0.037$ μM). Moreover, ZYH005 was more effective than the other *N*-alkyl derivatives and the positive control cisplatin (DDP) (Supplemental Figure S1B). Then, we focused on *N*-(4-methoxylphenyl) ethyl derivatives and synthesized compounds ZYH006–ZYH020 (Supplemental Figure S2A). Further investigation of the anti-proliferation effects of ZYH005–ZYH020 on HL60 cells showed that compounds ZYH006–ZYH020 exerted weaker inhibitory effects than ZYH005 (Supplemental Figure S2B). Therefore, ZYH005 was selected for subsequent pharmaceutical research experiments (Figure 1A, Supplementary methods). The ^1H spectral and ^{13}C NMR spectral data for ZYH005 are shown in Supplemental Figure S3.

ZYH005 treatment selectively inhibits the proliferation of APL and ATRA-resistant APL cells

To explore the anti-leukemia potential of ZYH005, we treated ten leukemia cell lines and two immortalized normal human epithelial cell lines with ZYH005 (0–0.16 μM) and then assessed their viability. As shown in Figure 1B, even after treatment for only 24 h, ZYH005 exerted significantly greater anti-proliferation effects on NB4 and HL60 cell lines than on the other cell lines. Furthermore, ZYH005 exerted minimal effects on the viability of the normal cell lines NCM460 and HPDE6-C7. The 24 h IC_{50} values for the NB4 and HL60 cell lines were 0.041 and 0.053 μM, respectively. We further assessed the effects of ZYH005 on ATRA-resistant cell lines. After a 24 h of treatment, high ATRA concentrations (12.5–50 μM) had almost no effect on the proliferation of the NB4-LR2 and NB4-MR2 cell lines. In contrast, ZYH005 at the concentrations of 0.04–0.06 μM inhibited the proliferation of these cell lines (Figure 1C). The effects ZYH005 on peripheral blood mononuclear cells isolated from blood samples of 3 healthy volunteers (PBMCs-V1, PBMCs-V2, PBMCs-V3) were also detected. Interestingly, the viability of PBMCs in the ZYH005-treated groups was nearly consistent with that in the non-treated groups, even at a ZYH005 concentration up to 0.64 μM (Figure 1D). Induction of promyelocytic leukemic cell differentiation plays an important role in the response of APL to both ATRA and ATO treatments (24). Therefore, we evaluated the differentiation-inducing effects of ZYH005. However, unlike ATRA and ATO, ZYH005 did not induce APL cell differentiation (Supplemental Figure S4A and B). These data demonstrate that ZYH005 is a novel molecule that is selectively cytotoxic to both APL and

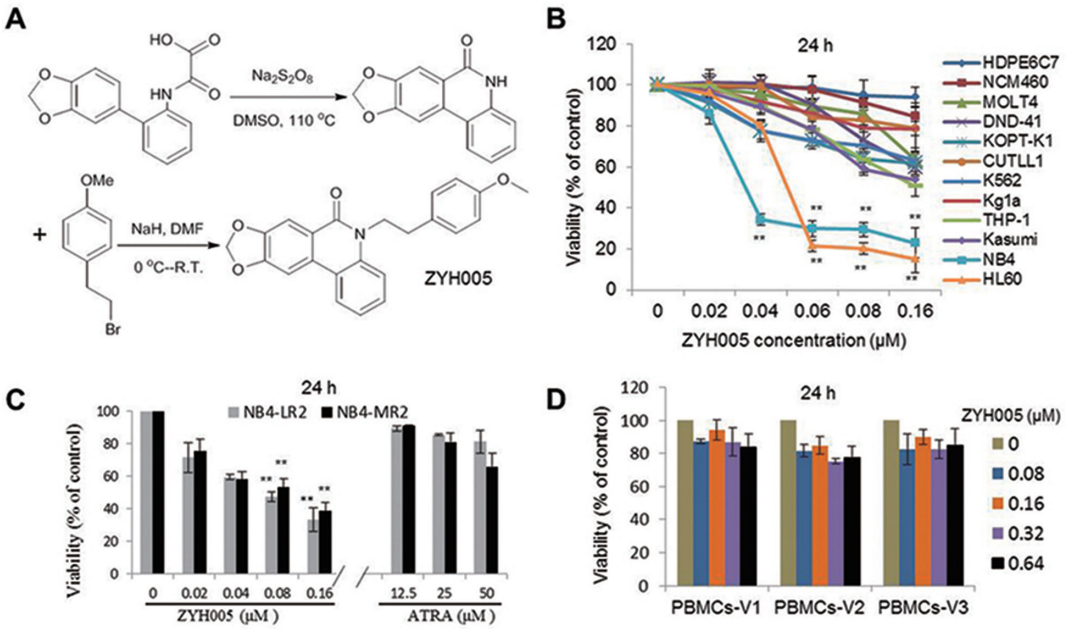

Figure 1. ZYH005 treatment specifically inhibits the proliferation of APL and ATRA-resistant APL cells. (**A**) The synthesis process of 5-(4-methoxyphenethyl)-[1, 3] dioxolo [4,5-j] phenanthridin-6(5H)–one (ZYH005). (**B**–**D**) The viability of cell lines was evaluated using an MTS kit after treated with drugs for 24 h. The effects of ZYH005 on two immortalized normal human epithelial cell lines (NCM460 and HPDE6-C7) and ten leukemia cell lines are shown in B. The effects of ZYH005 and ATRA on ATRA-resistant cell lines are shown in C. Effects of ZYH005 on peripheral blood mononuclear cells isolated from blood samples of 3 healthy volunteers (PBMCs-V1, PBMCs-V2 and PBMCs-V3) are shown in D. Data are shown as the mean ± S.D. of three independent experiments, unpaired two-tailed student's test was used for statistics, $^{**}P < 0.01$ compared to the control group (DMSO < 0.1%).

ATRA-resistant APL cell lines, has a lesser effect on other leukemia cell lines, and has a negligible cytotoxic effect on non-cancerous cell lines as well as PBMCs from healthy donors.

ZYH005 intercalates double-stranded DNA

To determine whether ZYH005 could directly interact with double-stranded DNA (dsDNA), single-molecule magnetic tweezers were firstly used to investigate the effects of ZYH005 on dsDNA mechanical properties. The binding of small ligands to dsDNA alters its structure, causing lengthening or unwinding of the dsDNA (51,52). Figure 2A shows the single-molecule stretching experiment system for measuring the force-extension curves of a 6618 bp dsDNA tethered between a coverslip and a paramagnetic bead, as detailed in the methods section. Force was applied to the DNA molecule through the bead by a pair of magnets. Extension changes were measured based on the bead height using the diffraction pattern of the bead image. Figure 2B shows typical force-height curves of a dsDNA molecule in the presence of different concentrations of ZYH005 in buffer change experiments. The force-height curves of dsDNA showed significant elongation from 20 to 60 pN after adding ZYH005, which was concentration-dependent, suggesting that the binding of ZYH005 increased the length of dsDNA as is typically observed for DNA intercalators (51,52). In addition to lengthening dsDNA, the binding of ZYH005 also changed the behavior of the DNA overstretching transition. The naked dsDNA showed sudden elongation (Figure 2B, black curve) at a force of ∼65 pN, representing the transition of B-form DNA to S-DNA (stretched DNA), or the so-called B-to-S overstretching transition (53). At a low concentration of ZYH005 (1 μM), the overstretching transition force increased, suggesting that the binding of ZYH005 stabilizes B-form DNA. Increased overstretching transition forces and stabilization of B-form DNA have also been observed with other DNA intercalators such as EB (52). Taken together, ligand-induced dsDNA elongation and overstretching force alteration at a low concentration and ligand-induced dsDNA extension both suggest that ZYH005 binds to dsDNA as an intercalator.

To quantify the binding affinity of ZYH005 to dsDNA, titration curves of dsDNA elongation at different forces were fit using Hill equation (Figure 2C), $x_c - x_{nacked\ DNA} = (x_{saturate} - x_{nacked\ DNA})\frac{c^n}{K_d^n(F)+c^n}$, where the $x_c - x_{nacked\ DNA}$ is the elongation of dsDNA at different ligand concentration, $x_{satureate} - x_{nacked\ DNA}$ is saturated dsDNA elongation, $K_d(F)$ is the dissociation constant of ZYH005 with DNA at different forces, and $n = 1$ is the Hill efficiency. The dissociation constant for intercalation of ZYH005 with DNA at zero force K_{d0} is estimated to be 24 μM (binding constant $K_a = 1/K_{d0} = 4.1 \times 10^4\ M^{-1}$) by fitting the force-dependent binding constant (Figure 2D) with the exponential function, $K_d(F) = K_{d0} \times e^{-F\Delta x/k_BT}$, where k_B is the Boltzmann constant and T is temperature. The fitting parameter $\Delta x = 0.1$ nm indicates the dsDNA elongation upon a single ZYH005 molecule intercalation.

To further analyze the effects of ZYH005 on supercoiled dsDNA and to determine whether the binding of ZYH005 causes dsDNA unwinding, we performed a supercoiling assay using torsional constraint dsDNA and magnetic tweezers (Figure 2E). The preparation of torsional constraint ds-

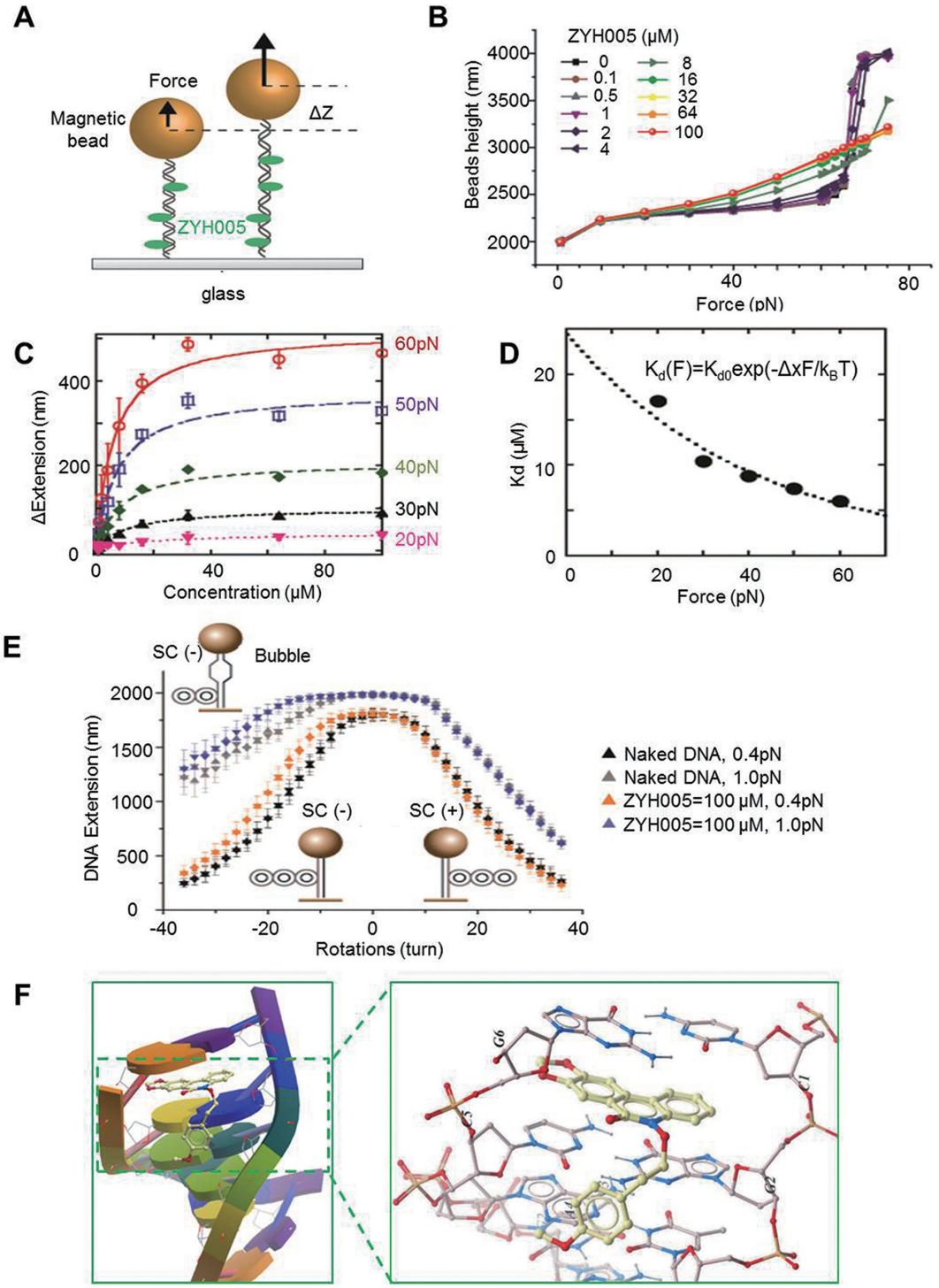

Figure 2. ZYH005 intercalates double-stranded DNA. (**A**) Binding of ZYH005 with dsDNA measured based on dsDNA stretching experiments. Schematic diagram of DNA stretching with magnetic tweezers. A 6618 bp dsDNA was tethered between a 2.8 μm-diameter paramagnetic bead and coverslip. (**B**) Typical DNA stretching curves in the presence of various ZYH005 concentrations. (**C**) Titration curves of dsDNA elongation measured at different forces were fit using the Hill equation. The elongation was measured from three independent measurements. (**D**) Dissociation constant dependent on force. (**E**) Effect of ZYH005 on DNA rotation-extension curves at low force (0.4 and 1.0 pN) and saturated ZYH005 concentration (100 μM). The 6573 bp DNA exhibits torsional constraint when rotation = 0; DNA is in relaxed conformation. The inserts depict the states of super coiled DNA under different tensional and torsional constraints. (**F**) Low-energy binding conformation of ZYH005 bound to DNA fragments generated by virtual ligand docking.

DNA and twisting of DNA by magnetic tweezers is detailed in the methods section. In the presence of a saturating concentration of ZYH005 (100 μM), evident elongation of DNA extension at 0.6 pN with negative rotations indicates that binding of ZYH005 causes DNA stiffen at negative supercoiled conformation, suggesting that ZYH005 binds to dsDNA at low- force (∼0.6 pN) regions. The shift of the buckling transition at 1 pN also indicates that ZYH005 directly interacts with dsDNA. Compared to other intercalative compounds such as YOYO-1 and EB, the peak of the supercoil curve did not show a significant shift, indicating that ZYH005 binds to dsDNA, but it did not induce significant unwinding of the dsDNA.

Finally, molecular docking simulations were carried out to elucidate the interaction of ZYH005 with DNA fragments under default conditions. ZYH005 was expected to show significantly higher binding affinity with a significant ICM score (–30.11). Furthermore, the lowest-energy binding conformation is shown in Figure 2F; the docked pose was stabilized electronically by π–π stacking between the aromatic group and the side chains of the DNA base pairs. The results obtained from molecular docking studies were consistent with the results of DNA binding studies, indicating that ZYH005 is a DNA intercalator.

ZYH005 treatment induces DNA damage and cell cycle arrest in APL and ATRA-resistant APL cells

DNA intercalators with anticancer effects lead to structural changes in DNA and subsequently cause DNA damage (54,55). Double-stranded DNA breaks (DSBs) are probably the most detrimental of the many types of DNA damage occurring within the cell (56). Accordingly, we detected γH2AX foci, which are considered indicative of an early response to DSBs (57). As shown in Figure 3A and B, NB4 and HL60 cells displayed significant levels of γH2AX foci after 0.05 μM ZYH005 treatment for 6 h. By immunoblot analysis, we found that ZYH005 treatment increased the levels of γH2AX in both APL and ATRA-resistant APL cell lines (Figure 3C). We further observed that ZYH005 treatment increased the expression levels of γH2AX in a time-dependent manner (Figure 3D). The cell cycle is a critical regulator of the processes of cell proliferation and division after DNA damage occurs. High levels of damage to DNA induce cell-cycle arrest to prevent the transmission of damaged DNA during mitosis. Therefore, we detected the cell cycle distribution in cell lines treated with or without low-dose ZYH005 for 24 h and found that ZYH005 induced a significant G2/M cycle arrest in both APL and ATRA-resistant APL cells (Figure 3E, Supplemental Figure S5A).

ZYH005 treatment induces apoptotic cell death in APL and ATRA-resistant APL cells

Damaged DNA accumulation and cell cycle arrest are activators of apoptotic signals (58), we also noted cell shrinkage and chromatin condensation in APL cells treated with ZYH005, and these findings prompted us to investigate whether ZYH005 could induce apoptosis in these cells. Therefore, we quantified the apoptosis cells by flow cytometer. With 24 h treatment, ZYH005 at 0.025 μM is sufficient to induce apoptosis in NB4 cells, and 0.05 μM ZYH005 could induce 57.7% and 49.9% (on average) of the cells underwent apoptosis in NB4 and HL60 cells, respectively (Figure 4A, Supplemental Figure S5B). Next, we evaluated the effects of ZYH005 on the expression of marker proteins of apoptosis (59). As shown in Figure 4B, ZYH005 treatment for 24 h altered the expression levels of Bcl-2, Bax, Bak, cleaved-caspase-3 and PARP in a dose-dependent manner. In addition, treatment with 0.05 μM ZYH005 could alter the expression levels of cleaved-caspase-3 and PARP within 6 h, indicating that ZYH005-induced apoptosis is also a time-dependent phenomenon (Figure 4C). We also found that ZYH005 treatment induced proteolytic cleavage of PKCδ, a cytoplasmic caspase-3 substrate, into a 41kDa catalytic fragment in a time- and dose-dependent manner (Supplemental Figure S5C). Moreover, 0.05 μM ZYH005 induced apoptosis in ATRA-resistant APL cell lines after 24 h of treatment, whereas treatment with a 200-times greater concentration of ATRA (10 μM) did not result in apoptosis induction (Figure 4D). Meanwhile, the immunoblot analysis showed that ZYH005 treatment altered the expression of apoptosis-related proteins in ATRA-resistant APL cell lines (Figure 4E). We compared the apoptosis-inducing activity of ZYH005 with that of ATO and other chemotherapeutics in NB4 and NB4-LR2 cells. As shown in Figure 4F, ATO, 5-fluorouracil, cisplatin and doxorubicin did not exhibit apoptosis-inducing activity at the indicated concentration and time (0.05 μM, 24 h). In contrast, the apoptosis induction effect of ZYH005 was observed close to that of idarubicin, the most effective anthracyclines in both in vitro assay (60) and in clinical trials (61). These results strongly support ZYH005 as an effective apoptosis inducer that induces apoptotic cell death in both APL and ATRA-resistant APL cell lines.

ZYH005 treatment induces caspase-dependent PML-RARα degradation

The PML-RARα fusion protein determines not only the phenotype of APL but also the response of APL to treatment (23), and several studies have shown that PML-RARa expression induces strong resistance to apoptosis (62). Therefore, we analyzed the effects of ZYH005 on PML-RARα. As shown in Figure 5A, the PML-RARα levels were significantly decreased in NB4, NB4-LR2 and NB4-MR2 cells after ZYH005 treatment for 24 h. Caspase family members have been recognized as key participants in apoptosis and play important roles in PML-RARα degradation (63,64). Therefore, we treated cells with ZYH005 in the presence or absence of the caspase inhibitor Z-VAD-FMK. As shown in Figure 5B, in both APL and ATRA-resistant APL cells, the ZYH005-induced apoptosis was significantly attenuated by the addition of Z-VAD-FMK. Additionally, the immunoblot results showed that Z-VAD-FMK blocked ZYH005-induced PML-RARα degradation and alterations of apoptosis-related proteins such as Bcl-2, Bax and cleaved-caspase-3; however, proteins related to the DNA damage response, such as PARP and γH2AX, were partially blocked by Z-VAD-FMK (Figure 5C), indicating that ZYH005-induced DNA damage is an event that occurs earlier than PML-RARα degradation and apoptosis.

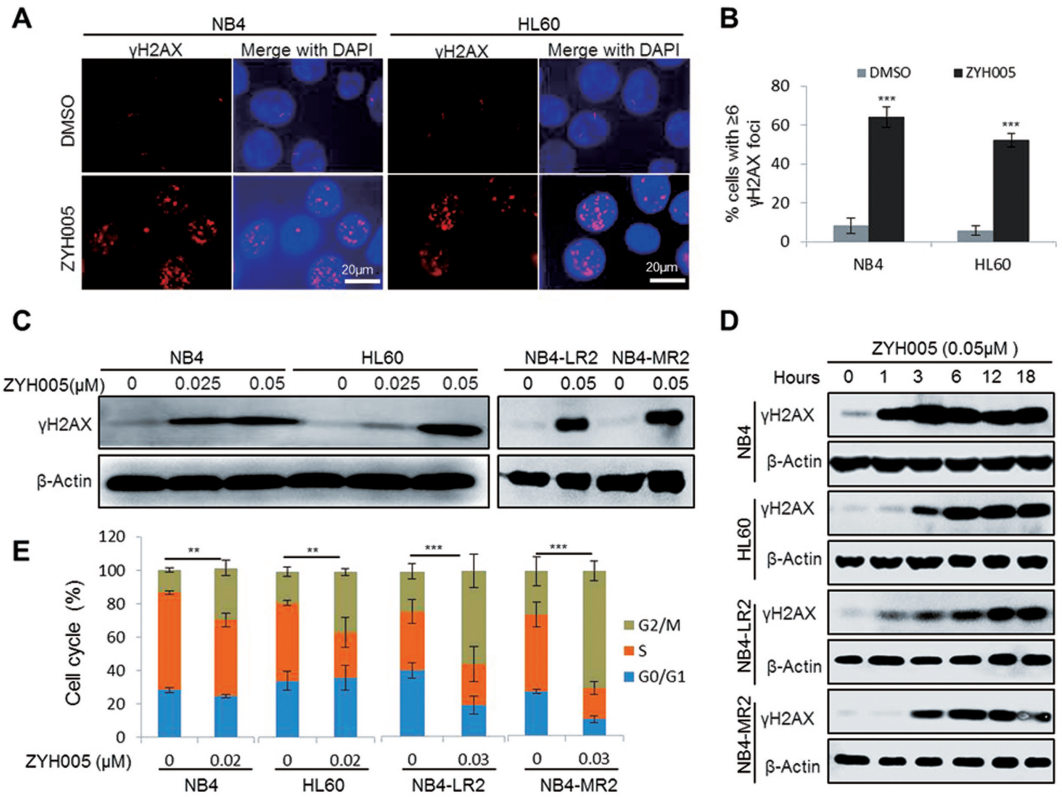

Figure 3. ZYH005 treatment induces DNA damage and cell cycle arrest in APL and ATRA-resistant APL cell lines. (**A**) Immunofluorescence microscopy analysis of γH2AX foci in NB4 and HL60 cells after 0.05 μM ZYH005 treated for 6 h. (**B**) Quantification of the percentage of cells with ≥ 6 γH2AX foci. At least 100 cells per group were examined. (**C**, **D**) Immunoblot analysis of γH2AX expression in cell lines after treated with ZYH005 at the indicated concentrations and times. (**E**) ZYH005 induces G2/M arrest in APL cells. After treated with DMSO (<0.1%) and ZYH005 for 24 h, cells were harvested, fixed and cell cycle distribution was analyzed by PI staining. (A, C and D), the data are representative of three independent experiments. (B and E) the data are expressed as the mean ± S.D. of three independent experiments, $^{**}P < 0.01$, $^{***}P < 0.001$ unpaired two-tailed Student's *t*-test. β-Actin was used as a loading control in immunoblot analysis.

A lysosome inhibitor (chloroquine), proteasome inhibitor (MG132) and RNF4 (the E3 ubiquitin ligase of PML) shRNA–silencing NB4 cells (65,66) were used to determine whether the autophagy-lysosome and proteasome-ubiquitin pathways had roles in the effect of ZYH005 on PML-RARα degradation, respectively. The results showed that all of them failed to attenuate the apoptosis and PML-RARα degradation induced by ZYH005 (Supplementary Figure S6A–E). Moreover, Z-VAD-FMK could block apoptosis and PML-RARα degradation in RNF4 shRNA-transfected NB4 cells (Supplementary Figure S6F and G). These results suggest that ZYH005-induced DNA damage activates caspase 3 and then induces caspase-dependent PML-RARa degradation and apoptosis.

ZYH005 treatment induces leukemia regression in APL and ATRA-resistant APL mice

To explore the anti-leukemia effect of ZYH005 in vivo, we first established NB4 xenograft mouse models and treated the mice with vehicle or ZYH005 (intravenously, 10 mg/kg/day according to the preliminary test). Two weeks later, we noted significant differences in the tumor weights between the ZYH005- and vehicle-treated mice, with a tumor inhibition rate of 62.3% ($P = 0.0048$) (Supplemental Figure S7A). Expression of Ki67, which is an important marker of cell proliferation (67), was significantly lower in tumor tissues following ZYH005 treatment (Supplemental Figure S7B). Moreover, ZYH005 injection did not affect the body weights of the tested animals (Supplemental Figure S7C). The H&E staining of visceral organs including heart, liver, spleen, lung and kidney were also relatively constant between ZYH005-treated mice and vehicle-treated mice (Supplementary Figure S7D), manifesting minimal toxicity of ZYH005 *in vivo*, which is the precondition to be a clinical candidate agent.

Next, we transplanted leukemic cells isolated from spleens of leukemic hPML-RARα or mutant hPML-RARα transgenic mice intravenously into sub-lethally irradiated isogenic FVB/N recipients to generate APL mice or ATRA-resistant APL mice (41,49,50). On day 4 after transplantation, mice were treated intravenously with vehicle, ZYH005, ATO (for APL mice) or ATRA (for ATRA-resistant APL mice). All mice were sacrificed for histological analyses when the first vehicle-treated mouse was moribund. In both APL and ATRA-resistant APL leukemic mouse models, results of Wright-Giemsa staining showed that mice in ZYH005-treated group had near-normal peripheral blood (PB), and leukemic blasts were rarely observed in their bone marrow (BM); results of histologi-

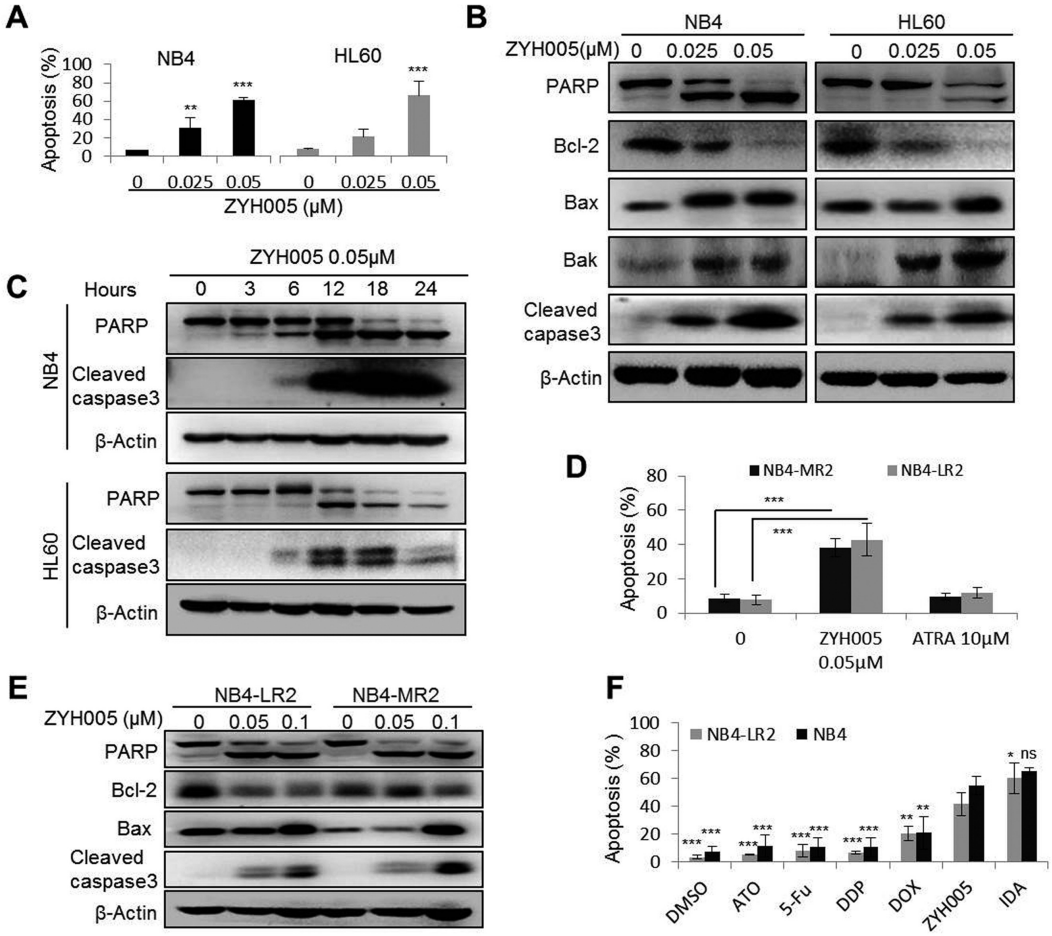

Figure 4. ZYH005 treatment induces apoptotic cell death in APL and ATRA-resistant APL cell lines. (**A**) NB4 and HL60 cells were treated with ZYH005 for 24 h, and then cell apoptosis was determined. (**B**) Immunoblot analysis of the expression of apoptosis-related proteins in NB4 and HL60 cells after 24 h of ZYH005 treatment. (**C**) Immunoblot analysis of the expression of PARP and cleaved-caspase-3 in NB4 and HL60 cells after 0.05 μM ZYH005 treated for different time. (**D**) ATRA-resistant cells were treated with 0.05 μM ZYH005 and 10 μM ATRA for 24 h, and then cell apoptosis was determined. (**E**) Immunoblot analysis of apoptosis-related proteins in ATRA-resistant cells after ZYH005 treated for 24 h. (**F**) NB4 and NB4-LR2 cells were treated with ZYH005, arsenic trioxide (ATO), 5-fluorouracil (5-Fu), cisplatin (DDP), doxorubicin (DOX) and idarubicin (IDA) at 0.05 μM for 24 h, and then cell apoptosis was determined. (A, D and F), for DOX and IDA treatment, cell apoptosis was determined by an Annexin V-APC/7-AAD staining kit; for other treatments, cell apoptosis was determined by an Annexin V-FITC/PI staining kit. Data are expressed as the mean ± S.D. of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns, not significant compared to ZYH005-treated group; unpaired two-tailed Student's *t*-test in A and D; one-way ANOVA analysis followed by Tukey's post hoc test in F.β-Actin was used as a loading control in immunoblot analysis.

cal examination with H&E staining revealed that leukemic cell infiltration into spleens and livers was inhibited by ZYH005 (Figure 6A and B). Moreover, vehicle-treated mice exhibited enlarged spleens and livers, which could be alleviated by ZYH005 treatment (Figure 6C and Supplementary Figure S8A). ZYH005 was also found to facilitate recovery of swollen spleens and livers, even in ATRA-resistant leukemic mice (Figure 6D and Supplementary Figure S8B). Finally, the effect of ZYH005 on the survival of leukemic mice was also evaluated using the same treatment procedure as described above but using at least six mice for each treatment group. All vehicle-treated leukemic mice developed leukemia within 24–28 days post-transplantation and died within the following 4 days. When the first vehicle-treated mouse died, we stop the treatments. Both APL and ATRA-resistant APL mice showed longer survival following treatment with ZYH005 (Figure 6E and F). These results indicate that ZYH005 has significant therapeutic efficacy against APL and ATRA-resistant APL *in vivo*.

Myeloid cells positive for four markers ($CD34^+$, c-kit^+, $Fc\gamma RIII/II^+$, $Gr1^{int}$) in APL mice have been identified as leukemia initiating cells (LIC), and PML-RARa degradation triggers LIC clearance (68,69). Therefore, we analyzed these cells in APL mice and found that the numbers of these cells were decreased in the BM of ZYH005-treated mice, result similar those noted in ATO-treated mice (Supplementary Figure S8C), indicating that ZYH005 can eliminate LIC in leukemic mice, which mirrors PML-RARα degradation *in vivo*. In addition, we conducted immunohistochemical analysis of cleaved caspase-3 expression in the spleens of APL and ATRA-resistant APL mice, and the results showed that ZYH005 treatment induced apoptosis in the APL mice similarly to ATO treatment (70) (Supplementary Figure S8D). We also performed immunoblot analyses us-

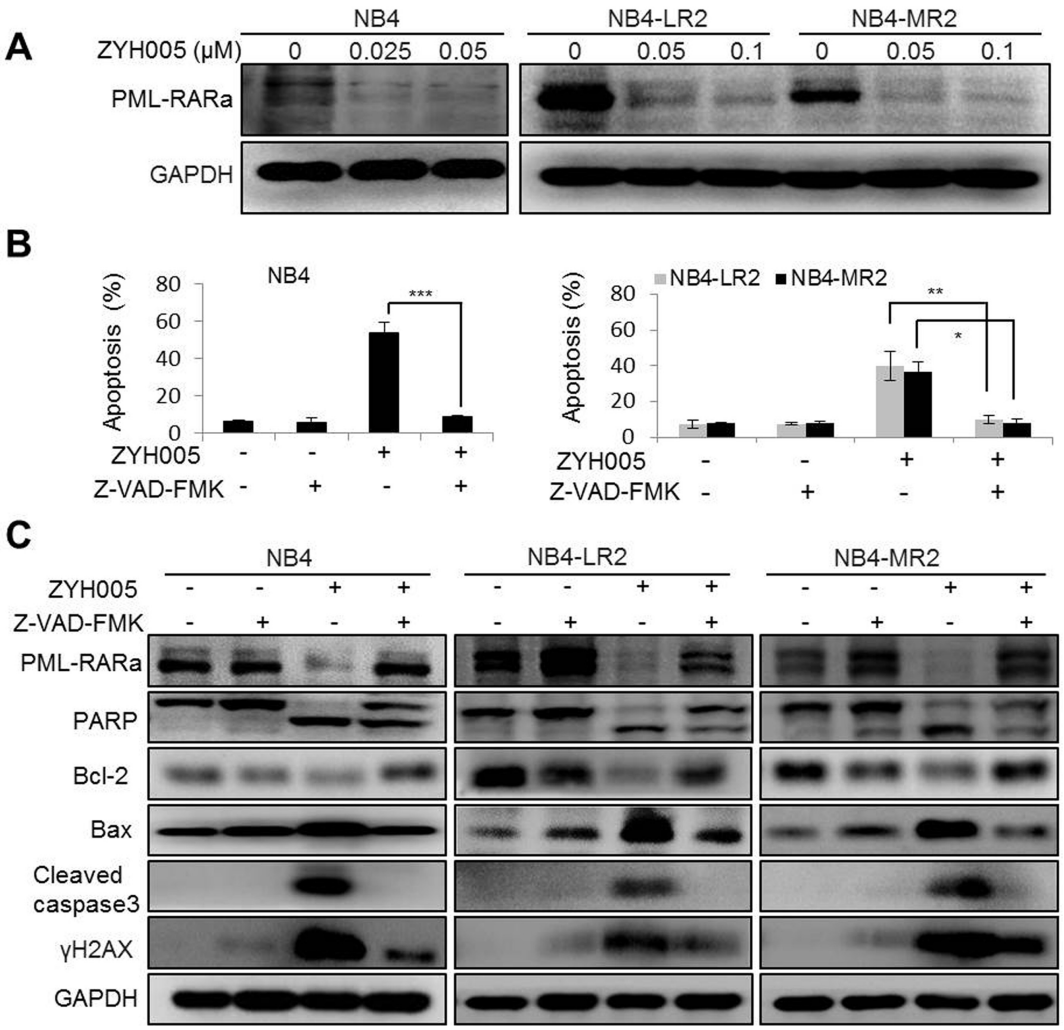

Figure 5. ZYH005 induces caspase-dependent PML-RARα degradation and apoptosis. (**A**) Immunoblot analysis of PML-RARα expression in NB4 and ATRA-resistant cells treated with ZYH005 for 24 h. (B and C) NB4 and ATRA-resistant cell lines were treated with 80 μM Z-VAD-FMK for 1 h, and then 0.05 μM ZYH005 was added. 24 h later, cell apoptosis was determined by an Annexin V-FITC and PI staining kit (**B**). Data are expressed as the mean ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, unpaired two-tailed Student's *t*-test. The expression levels of related proteins were evaluated by immunoblot analysis (**C**). GAPDH was used as a loading control.

ing NB4 xenograft tumor lysate, and the results showed that ZYH005 treatment activated caspase-3 and induced PML-RARα degradation in vivo (Supplementary Figure S8E).

Collectively, these data provide evidence of the mechanisms of ZYH005 treatment, which mainly mediate intercalative binding to DNA, subsequently trigger DNA double-strand breaks and accumulation of DNA damage, induce G2/M cell cycle arrest, caspases 3 activation and ultimately cause PML-RARα degradation and apoptosis in both APL and ATRA-resistant APL models (Figure 6G).

DISCUSSION

In this study, we report that ZYH005, a novel synthesized phenanthridinone alkaloid, has specific and effective cytotoxic effects in APL and ATRA-resistant APL models. In contrast, ZYH005 showed negligible toxic effect on PBMCs from healthy volunteers as well as non-cancerous cells. Single-molecule force-spectroscopy experiments have been used to characterize the binding mode of drug-DNA interactions (51), the binding affinity (52), binding kinetics (71) and the effects of drugs on the supercoiled dsDNA (72). Using single-molecule magnetic tweezers, we show that ZYH005-binding increases DNA extension and twists DNA, which suggesting that ZYH005 is a DNA intercalator. It should be noted that ZYH005 is a moderate intercalator when comparing with classical intercalators such as YOYO-1 and EB, as ZYH005 only causes moderate change of the extension and twists of DNA at low force. We further demonstrate that ZYH005 exerts its effects by triggering DNA damage, cell cycle arrest, caspase-dependent degradation of PML-RARa and apoptosis. These findings highlight the potential of ZYH005 for APL therapy and for overcoming ATRA resistance.

Cells respond to DNA damage through DDR signalling networks, which determine cell fate and promote not only DNA repair and cell survival but also cell cycle arrest and

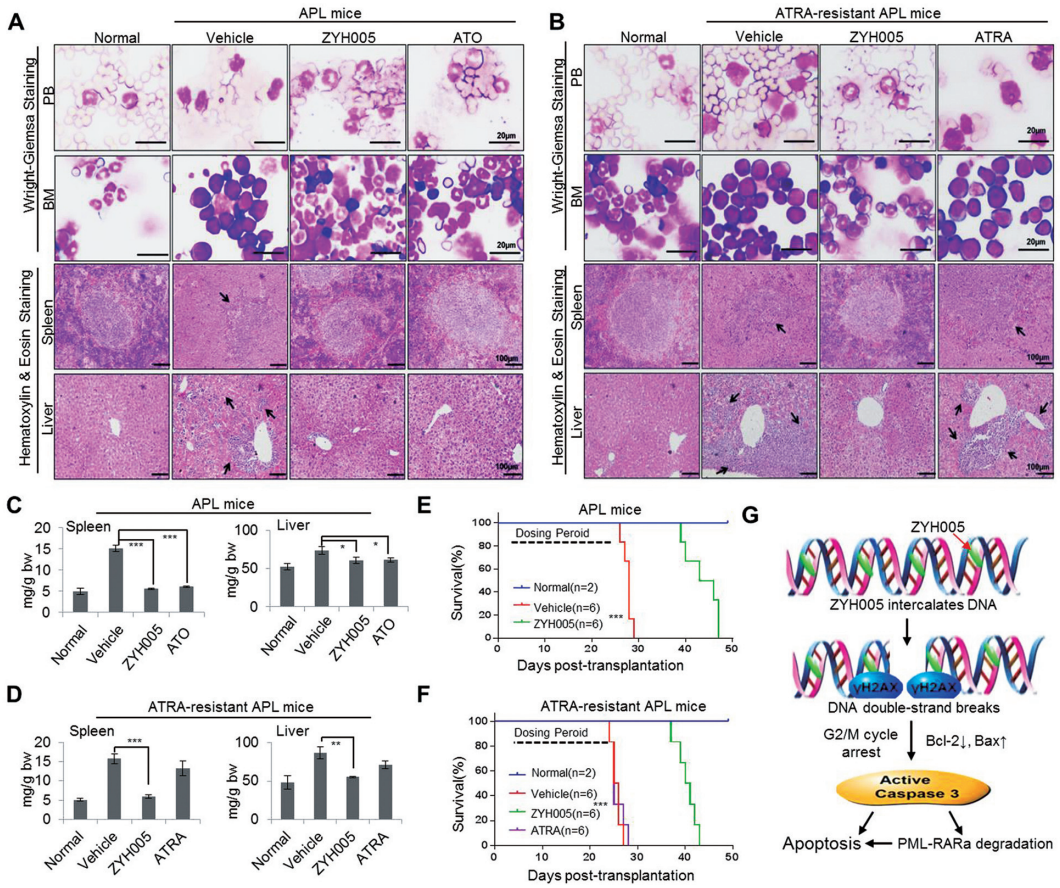

Figure 6. Effects of ZYH005 on APL and ATRA-resistant APL mouse models. On day 4 after transplantation, APL and ATRA-resistant APL mice were treated with vehicle (5% DMSO, 5% Cremophor® EL, 90% Saline) or ZYH005 (10 mg/kg/day, intravenously). Normal mice (irradiated but not transplanted, no treatment) and mice treated with ATO (5 mg/kg/day, intraperitoneally) or ATRA (15 mg/kg/day, intraperitoneally, for the ATRA-resistant APL model) were used as the controls. (**A**–**D**) When the first vehicle-treated leukemic mice were moribund, all mice were sacrificed and analyzed. Peripheral blood (PB) and bone marrow (BM) were subjected to Wright-Giemsa staining. Invasion of the spleen and liver by leukemic cells was detected by hematoxylin and eosin (H&E) staining, the arrowheads indicate infiltrating cells (A and B). The spleens and livers were weighed (C and D), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, unpaired two-tailed Student's *t*-test. (**E**–**F**) Kaplan–Meier survival curves of APL (E) and ATRA-resistant APL (F) mice. The numbers of mice are indicated in parentheses, and $***P < 0.001$ compared to the vehicle-treated mice. Every experiment was repeated at least three times with the similar results. (**G**) Diagram of signaling pathways regulated by ZYH005 in APL and ATRA-resistant APL.

cell death, thus determining the outcome of cancer therapy with DNA-binding drugs (1). Therefore, defects in the DDR have been exploited therapeutically in the treatment of cancer with DNA-binding chemotherapies and PARP inhibitors (2,73). Compromised DDR has been found in APL (25,27,28), indicating that APL cells may be sensitive to DNA-binding drugs. Associated with this, before the introduction of ATRA and ATO for APL, the first-line therapy for APL was DNA intercalators (daunorubicin, doxorubicin, idarubicin) plus the DNA synthesis inhibitor cytarabine (Ara-C), and the complete remission rate was as high as 75% (23,74). Until now, ATRA in combination with these DNA binding chemotherapies remained standard practice for APL treatment (23,31,74). Recently, Esposito et al. reported that APL is extremely sensitive to DDR target drugs (PARP inhibitors) due to their compromised DDR (28). These findings suggest that the impairment DDR not only determines APL pathogenesis but also plays an important role in the therapy response of APL. Moreover, molecular mechanism underlying ATRA-resistant is genetic mutations in the RARa ligand binding domain (75) which is not related to the DDR, and impaired DDR are still observed in ATRA-resistant cells (26). ZYH005 was identified according to the results of specific cytotoxic activity to APL and ATRA-resistant APL cells. Moreover, in ZYH005-treated APL cells, γH2AX were increased sharply even at 1 h and cleaved PARP were detectable at 3 h, but caspase-3 weakly activated at 6 h, indicating that DNA damage is the earliest event in the cell after ZYH005 treatment. These findings suggest that impairment of the DDR determined the response of APL and ATRA-resistant APL cells to ZYH005 and reflecting a new strategy to overcome ATRA resistance with DNA-binding drugs.

Several therapeutics have been tested to overcome ATRA resistance, such as ATO, Am80 (76) and histone deacetylase inhibitors (77), among these, ATO is considered the most active agent (78). The PML-RARa fusion protein is clearly the central player driving APL, which also induces strong resistance to apoptosis in APL cells (62). ATO could directly target the PML component of PML-

RARα and degrade it and then led to apoptosis of APL cells. Similarly, ZYH005 treatment induces PML-RARα degradation in APL and ATRA-resistant APL cells. The difference is ATO degrade PML-RARα mainly through ubiquitin proteasome system (79,80), while ZYH005 induces caspase-dependent PML-RARα degradation. Moreover, ZYH005 induces apoptosis in both APL and ATRA-resistant APL cells, and its apoptosis-inducing effect is more potent than those of ATO and other widely used anticancer drugs such as 5-fluorouracil, cisplatin and doxorubicin. On the other hand, the caspase inhibitor Z-VAD-FMK blocked ZYH005-induced PML-RARα degradation and apoptosis, but proteins related to the DNA damage such as PARP and γH2AX, were partially blocked by Z-VAD-FMK. Therefore, we conclude that ZYH005-induced DNA damage activates caspase 3, the activated-caspase 3 cleaves the PML/RARa fusion protein subsequently facilitates APL and ATRA-resistant APL cells undergoing apoptosis. The precise mechanism of ZYH005-induced degradation of PML-RARα remains unclear; yet the apoptosis inducing effect of ZYH005 offers a potent therapeutic advantage of ZYH005 and its role in overcoming ATRA resistance warrants further validation in patient-derived samples in future studies.

Although ATO is used as the best salvage therapy agent for ATRA-resistance patients, these patients subsequently resistant to ATO have also been reported (23,81). Furthermore, ATRA is a polyene compound whose sensitivity to light and heat may render it unstable, and the use of ATO is associated with many acute adverse effects and clinical complications, such as differentiation syndrome (7–35%), pseudotumour cerebri, hyperleukocytosis, leukocytosis (32–73%) and hepatic toxicity (74,82). Notably, ZYH005 possesses some advantages over the above agents, as the drug has a simple, stable structure, inexpensive synthesis procedure, and exerts negligible toxicity in vitro and in vivo. Additionally, we observed that of the series of N-(4-methoxylphenyl) ethyl derivatives, ZYH006 and ZYH007 showed activity similar to that of ZYH005 in the preliminary anticancer evaluation, indicating that the heteroatom substituent in the core may determine the anticancer activity of these compounds; these findings as well as the potential of intercalation in double-stranded DNA provide a valuable resource for the field of biologically active phenanthridinone alkaloids.

In conclusion, our findings indicate that the novel compound ZYH005 may be a promising candidate drug for newly diagnosed APL and relapsed APL with ATRA resistance. The potential therapeutic value of ZYH005 as well as that of some of its analogues, in APL and other cancers warrants further study.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Professor Yingli Wu of Shanghai Jiao Tong University for generously providing cells regarding this study. We also thank Professor Hudan Liu of Wuhan University, Professor Jie Yan of National University of Singapore for discussions and comments.

FUNDING

National natural Science Foundation of China [81503305 to Q.T. and 21708009 to H.Y.]; China Postdoctoral Science Foundation funded project [2015M582227 to Q.T.]; Program for Changjiang Scholars of Ministry of Education of the People's Republic of China [T2016088 to Y.Z.]; National natural Science Foundation for Distinguished Young Scholars [81725021 to Y.Z.]; Innovative Research Groups of the National Natural Science Foundation of China [81721005 to Y.Z.]; Academic Frontier Youth Team of HUST; Integrated Innovative Team for Major Human Diseases Program of Tongji Medical College, HUST; Fundamental Research Fund for the Central Universities [2017KFYXJJ153 to H.Y and 2017KFYXJJ152 to Z.L.]; Singapore Ministry of Education Academic Research Fund Tier 3 [MOE 2012-T3-1-001 to J.Y.]. Funding for open access charge: National natural Science Foundation of China [81503305 to Q.T.].
Conflict of interest statement. None declared.

REFERENCES

1. Roos,W.P., Thomas,A.D. and Kaina,B. (2016) DNA damage and the balance between survival and death in cancer biology. *Nat. Rev. Cancer*, **16**, 20–33.
2. Pearl,L.H., Schierz,A.C., Ward,S.E., Al-Lazikani,B. and Pearl,F.M. (2015) Therapeutic opportunities within the DNA damage response. *Nat. Rev. Cancer*, **15**, 166–180.
3. Puigvert,J., Sanjiv,K. and Helleday,T. (2016) Targeting DNA repair, DNA metabolism and replication stress as anti-cancer strategies. *FEBS J.*, **283**, 232–245.
4. Palchaudhuri,R. and Hergenrother,P.J. (2007) DNA as a target for anticancer compounds: methods to determine the mode of binding and the mechanism of action. *Curr. Opin. Biotechnol.*, **18**, 497–503.
5. Sirajuddin,M., Ali,S. and Badshah,A. (2013) Drug–DNA interactions and their study by UV–visible, fluorescence spectroscopies and cyclic voltametry. *J. Photochem. Photobiol. B: Biol.*, **124**, 1–19.
6. Wheate,N.J., Brodie,C.R., Collins,J.G., Kemp,S. and Aldrich-Wright,J.R. (2007) DNA intercalators in cancer therapy: organic and inorganic drugs and their spectroscopic tools of analysis. *Mini Rev. Med. Chem.*, **7**, 627–648.
7. Ferguson,L.R. and Denny,W.A. (2007) Genotoxicity of non-covalent interactions: DNA intercalators. *Mut. Res./Fundam. Mol. Mech. Mutagen.*, **623**, 14–23.
8. Tumir,L.-M., Stojković,M.R. and Piantanida,I. (2014) Come-back of phenanthridine and phenanthridinium derivatives in the 21st century. *Beilstein J. Org. Chem.*, **10**, 2930.
9. Nair,J.J., van Staden,J. and Bastida,J. (2016) Apoptosis-inducing effects of amaryllidaceae alkaloids. *Curr. Med. Chem.*, **23**, 161–185.
10. Wu,Z.-p., Chen,Y., Xia,B., Wang,M., Dong,Y.-F. and Feng,X. (2009) Two novel ceramides with a phytosphingolipid and a tertiary amide structure from Zephyranthes candida. *Lipids*, **44**, 63–70.
11. McNulty,J., Thorat,A., Vurgun,N., Nair,J.J., Makaji,E., Crankshaw,D.J., Holloway,A.C. and Pandey,S. (2010) Human cytochrome P450 liability studies of trans-dihydronarciclasine: a readily available, potent, and selective cancer cell growth inhibitor. *J. Nat. Prod.*, **74**, 106–108.
12. Griffin,C., Hamm,C., McNulty,J. and Pandey,S. (2010) Pancratistatin induces apoptosis in clinical leukemia samples with minimal effect on non-cancerous peripheral blood mononuclear cells. *Cancer Cell Int.*, **10**, 6.
13. Van Goietsenoven,G., Andolfi,A., Lallemand,B., Cimmino,A., Lamoral-Theys,D., Gras,T., Abou-Donia,A., Dubois,J., Lefranc,F. and Mathieu,V. (2010) Amaryllidaceae alkaloids belonging to different structural subgroups display activity against apoptosis-resistant cancer cells. *J. Nat. Prod.*, **73**, 1223–1227.

14. Van Goietsenoven,G., Mathieu,V., Lefranc,F., Kornienko,A., Evidente,A. and Kiss,R. (2013) Narciclasine as well as other amaryllidaceae isocarbostyrils are promising GTP-ase targeting agents against brain cancers. *Med. Res. Rev.*, **33**, 439–455.
15. Luchetti,G., Johnston,R., Mathieu,V., Lefranc,F., Hayden,K., Andolfi,A., Lamoral-Theys,D., Reisenauer,M.R., Champion,C. and Pelly,S.C. (2012) Bulbispermine: a crinine-type amaryllidaceae alkaloid exhibiting cytostatic activity toward apoptosis-resistant glioma cells. *ChemMedChem.*, **7**, 815–822.
16. Ma,D., Pignanelli,C., Tarade,D., Gilbert,T., Noel,M., Mansour,F., Adams,S., Dowhayko,A., Stokes,K. and Vshyvenko,S. (2017) Cancer cell mitochondria targeting by pancratistatin analogs is dependent on functional complex II and III. *Scientific Rep.*, **7**, 42957.
17. Yamamoto,H., Mukoyoshi,K. and Hattori,K. (2003) Google Patents.
18. Wahlberg,E., Karlberg,T., Kouznetsova,E., Markova,N., Macchiarulo,A., Thorsell,A.-G., Pol,E., Frostell,Å., Ekblad,T. and Öncü,D. (2012) Family-wide chemical profiling and structural analysis of PARP and tankyrase inhibitors. *Nat. Biotechnol.*, **30**, 283–288.
19. Li,T.-K., Houghton,P.J., Desai,S.D., Daroui,P., Liu,A.A., Hars,E.S., Ruchelman,A.L., LaVoie,E.J. and Liu,L.F. (2003) Characterization of ARC-111 as a novel topoisomerase I-targeting anticancer drug. *Cancer Res.*, **63**, 8400–8407.
20. Zhu,S., Ruchelman,A.L., Zhou,N., Liu,A., Liu,L.F. and LaVoie,E.J. (2006) 6-Substituted 6H-dibenzo [c, h][2, 6] naphthyridin-5-ones: reversed lactam analogues of ARC-111 with potent topoisomerase I-targeting activity and cytotoxicity. *Bioorg. Med. Chem.*, **14**, 3131–3143.
21. Kornienko,A. and Evidente,A. (2008) Chemistry, biology, and medicinal potential of narciclasine and its congeners. *Chem. Rev.*, **108**, 1982–2014.
22. Griffin,C., Karnik,A., McNulty,J. and Pandey,S. (2011) Pancratistatin selectively targets cancer cell mitochondria and reduces growth of human colon tumor xenografts. *Mol. Cancer Ther.*, **10**, 57–68.
23. Coombs,C., Tavakkoli,M. and Tallman,M. (2015) Acute promyelocytic leukemia: where did we start, where are we now, and the future. *Blood Cancer J.*, **5**, e304.
24. Li,J., Zhu,H., Hu,J., Mi,J., Chen,S., Chen,Z. and Wang,Z. (2014) Progress in the treatment of acute promyelocytic leukemia: optimization and obstruction. *Int. J. Hematol.*, **100**, 38–50.
25. Voisset,E., Moravcsik,E., Stratford,E.W., Jaye,A., Palgrave,C.J., Hills,R.K., Salomoni,P., Kogan,S.C., Solomon,E. and Grimwade,D. (2016) Pml Nuclear Body Disruption Cooperates in APL Pathogenesis, Impacting DNA Damage Repair Pathways. *Blood*, **128**, 742.
26. Di Masi,A., Cilli,D., Berardinelli,F., Talarico,A., Pallavicini,I., Pennisi,R., Leone,S., Antoccia,A., Noguera,N. and Lo-Coco,F. (2016) PML nuclear body disruption impairs DNA double-strand break sensing and repair in APL. *Cell Death Dis.*, **7**, e2308.
27. Casorelli,I., Tenedini,E., Tagliafico,E., Blasi,M., Giuliani,A., Crescenzi,M., Pelosi,E., Testa,U., Peschle,C. and Mele,L. (2006) Identification of a molecular signature for leukemic promyelocytes and their normal counterparts: Focus on DNA repair genes. *Leukemia*, **20**, 1978.
28. Esposito,M.T., Zhao,L., Fung,T.K., Rane,J.K., Wilson,A., Martin,N., Gil,J., Leung,A.Y., Ashworth,A. and So,C.W.E. (2015) Synthetic lethal targeting of oncogenic transcription factors in acute leukemia by PARP inhibitors. *Nat. Med.*, **21**, 1481–1490.
29. Griffin,J.D. (2016) Blood's 70th anniversary: arsenic—from poison pill to magic bullet. *Blood*, **127**, 1729.
30. Nichol,J.N., Garnier,N. and Miller,W.H. (2014) Triple A therapy: the molecular underpinnings of the unique sensitivity of leukemic promyelocytes to anthracyclines, all-trans-retinoic acid and arsenic trioxide. *Best Pract. Res. Clin. Haematol.*, **27**, 19–31.
31. Norsworthy,K.J. and Altman,J.K. (2016) Optimal treatment strategies for high-risk acute promyelocytic leukemia. *Curr. Opin. Hematol.*, **23**, 127–136.
32. Altman,J.K., Rademaker,A., Cull,E., Weitner,B.B., Ofran,Y., Rosenblat,T.L., Haidau,A., Park,J.H., Ram,S.L. and Orsini,J.M. (2013) Administration of ATRA to newly diagnosed patients with acute promyelocytic leukemia is delayed contributing to early hemorrhagic death. *Leuk. Res.*, **37**, 1004–1009.
33. Luo,Z., Wang,F., Zhang,J., Li,X., Zhang,M., Hao,X., Xue,Y., Li,Y., Horgen,F.D. and Yao,G. (2012) Cytotoxic alkaloids from the whole plants of Zephyranthes candida. *J. Nat. Prod.*, **75**, 2113–2120.
34. Zhan,G., Zhou,J., Liu,R., Liu,T., Guo,G., Wang,J., Xiang,M., Xue,Y., Luo,Z. and Zhang,Y. (2016) Galanthamine, plicamine, and secoplicamine alkaloids from Zephyranthes candida and their anti-acetylcholinesterase and anti-inflammatory activities. *J. Nat. Prod.*, **79**, 760–766.
35. Zhan,G., Qu,X., Liu,J., Tong,Q., Zhou,J., Sun,B. and Yao,G. (2016) Zephycandidine A, the first naturally occurring imidazo [1, 2-f] phenanthridine alkaloid from Zephyranthes candida, exhibits significant anti-tumor and anti-acetylcholinesterase activities. *Scientific Rep.*, **6**, 33990.
36. Guo,G., Yao,G., Zhan,G., Hu,Y., Yue,M., Cheng,L., Liu,Y., Ye,Q., Qing,G. and Zhang,Y. (2014) N-methylhemeanthidine chloride, a novel Amaryllidaceae alkaloid, inhibits pancreatic cancer cell proliferation via down-regulating AKT activation. *Toxicol. Appl. Pharmacol.*, **280**, 475–483.
37. Ye,Q., Jiang,J., Zhan,G., Yan,W., Huang,L., Hu,Y., Su,H., Tong,Q., Yue,M. and Li,H. (2016) Small molecule activation of NOTCH signaling inhibits acute myeloid leukemia. *Scientific Rep.*, **6**, 26510.
38. Yuan,M., Chen,L., Wang,J., Chen,S., Wang,K., Xue,Y., Yao,G., Luo,Z. and Zhang,Y. (2015) Transition-metal-free synthesis of phenanthridinones from biaryl-2-oxamic acid under radical conditions. *Org. Lett.*, **17**, 346–349.
39. Rosenauer,A., Raelson,J.V., Nervi,C., Eydoux,P., DeBlasio,A. and Miller,W.J. (1996) Alterations in expression, binding to ligand and DNA, and transcriptional activity of rearranged and wild-type retinoid receptors in retinoid-resistant acute promyelocytic leukemia cell lines. *Blood*, **88**, 2671–2682.
40. Gu,Z.-M., Wu,Y.-L., Zhou,M.-Y., Liu,C.-X., Xu,H.-Z., Yan,H., Zhao,Y., Huang,Y., Sun,H.-D. and Chen,G.-Q. (2010) Pharicin B stabilizes retinoic acid receptor-α and presents synergistic differentiation induction with ATRA in myeloid leukemic cells. *Blood*, **116**, 5289–5297.
41. Liu,C.-X., Yin,Q.-Q., Zhou,H.-C., Wu,Y.-L., Pu,J.-X., Xia,L., Liu,W., Huang,X., Jiang,T. and Wu,M.-X. (2012) Adenanthin targets peroxiredoxin I and II to induce differentiation of leukemic cells. *Nat. Chem. Biol.*, **8**, 486–493.
42. Weng,A.P., Millholland,J.M., Yashiro-Ohtani,Y., Arcangeli,M.L., Lau,A., Wai,C., del Bianco,C., Rodriguez,C.G., Sai,H. and Tobias,J. (2006) c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev.*, **20**, 2096–2109.
43. Chen,H., Fu,H., Zhu,X., Cong,P., Nakamura,F. and Yan,J. (2011) Improved high-force magnetic tweezers for stretching and refolding of proteins and short DNA. *Biophys. J.*, **100**, 517–523.
44. Zhao,X., Peter,S., Droge,P. and Yan,J. (2017) Oncofetal HMGA2 effectively curbs unconstrained (+) and (-) DNA supercoiling. *Scientific Rep.*, **7**, 8440.
45. Totrov,M. and Abagyan,R. (1997) Flexible protein–ligand docking by global energy optimization in internal coordinates. *Proteins: Struct. Funct. Bioinformatics*, **29**, 215–220.
46. Hopcroft,N.H., Brogden,A.L., Searcey,M. and Cardin,C.J. (2006) X-ray crystallographic study of DNA duplex cross-linking: simultaneous binding to two d (CGTACG) 2 molecules by a bis (9-aminoacridine-4-carboxamide) derivative. *Nucleic Acids Res.*, **34**, 6663–6672.
47. Zhu,H., Chen,C., Tong,Q., Yang,J., Wei,G., Xue,Y., Wang,J., Luo,Z. and Zhang,Y. (2017) Asperflavipine A: a cytochalasan heterotetramer uniquely defined by a highly complex tetradecacyclic ring system from Aspergillus flavipes QCS12. *Angew. Chem.*, **129**, 5326–5330.
48. Zhu,H., Chen,C., Xue,Y., Tong,Q., Li,X.N., Chen,X., Wang,J., Yao,G., Luo,Z. and Zhang,Y. (2015) Asperchalasine A, a cytochalasan dimer with an unprecedented decacyclic ring system, from Aspergillus flavipes. *Angew. Chem. Int. Ed.*, **54**, 13374–13378.
49. Brown,D., Kogan,S., Lagasse,E., Weissman,I., Alcalay,M., Pelicci,P.G., Atwater,S. and Bishop,J.M. (1997) A PMLRARα transgene initiates murine acute promyelocytic leukemia. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 2551–2556.
50. Kogan,S.C., Hong,S.-h., Shultz,D.B., Privalsky,M.L. and Bishop,J.M. (2000) Leukemia initiated by PMLRARα: the PML domain plays a critical role while retinoic acid–mediated transactivation is dispensable. *Blood*, **95**, 1541–1550.

51. Eckel,R., Ros,R., Ros,A., Wilking,S.D., Sewald,N. and Anselmetti,D. (2003) Identification of binding mechanisms in single molecule-DNA complexes. *Biophys. J.*, **85**, 1968–1973.
52. Vladescu,I.D., McCauley,M.J., Nunez,M.E., Rouzina,I. and Williams,M.C. (2007) Quantifying force-dependent and zero-force DNA intercalation by single-molecule stretching. *Nat. Methods*, **4**, 517–522.
53. Cluzel,P., Lebrun,A., Heller,C., Lavery,R., Viovy,J.L., Chatenay,D. and Caron,F. (1996) DNA: an extensible molecule. *Science*, **271**, 792–794.
54. Ross,W.E. and Bradley,M.O. (1981) DNA double-strand breaks in mammalian cells after exposure to intercalating agents. *Biochim. Biophys. Acta (BBA)-Nucleic Acids Protein Synth.*, **654**, 129–134.
55. Yang,F., Kemp,C.J. and Henikoff,S. (2015) Anthracyclines induce double-strand DNA breaks at active gene promoters. *Mut. Res./Fundam. Mol. Mech. Mutagen.*, **773**, 9–15.
56. Khanna,K.K. and Jackson,S.P. (2001) DNA double-strand breaks: signaling, repair and the cancer connection. *Nat. Genet.*, **27**, 247–254.
57. Mah,L., El-Osta,A. and Karagiannis,T. (2010) γH2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia*, **24**, 679–686.
58. Rello,S., Stockert,J., Moreno,V., Gamez,A., Pacheco,M., Juarranz,A., Canete,M. and Villanueva,A. (2005) Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis*, **10**, 201–208.
59. Elmore,S. (2007) Apoptosis: a review of programmed cell death. *Toxicol. Pathol.*, **35**, 495–516.
60. Pearlman,M., Jendiroba,D., Pagliaro,L., Keyhani,A., Liu,B. and Freireich,E.J. (2003) Dexrazoxane in combination with anthracyclines lead to a synergistic cytotoxic response in acute myelogenous leukemia cell lines. *Leuk. Res.*, **27**, 617–626.
61. Wouters,K.A., Kremer,L., Miller,T.L., Herman,E.H. and Lipshultz,S.E. (2005) Protecting against anthracycline-induced myocardial damage: a review of the most promising strategies. *Br. J. Haematol.*, **131**, 561–578.
62. Zhu,J., Zhou,J., Peres,L., Riaucoux,F., Honoré,N., Kogan,S. and de Thé,H. (2005) A sumoylation site in PML/RARA is essential for leukemic transformation. *Cancer Cell*, **7**, 143–153.
63. Nervi,C., Ferrara,F.F., Fanelli,M., Rippo,M.R., Tomassini,B., Ferrucci,P.F., Ruthardt,M., Gelmetti,V., Gambacorti-Passerini,C. and Diverio,D. (1998) Caspases mediate retinoic acid–induced degradation of the acute promyelocytic leukemia PML/RARα fusion protein. *Blood*, **92**, 2244–2251.
64. Gianni,M. (1999) In acute promyelocytic leukemia NB4 cells, the synthetic retinoid CD437 induces contemporaneously apoptosis, a caspase-3-mediated degradation of PML/RARα protein and the PML retargeting on PML-nuclear bodies. *Leukemia (08876924)*, **13**, 739–749.
65. Lallemand-Breitenbach,V., Jeanne,M., Benhenda,S., Nasr,R., Lei,M., Peres,L., Zhou,J., Zhu,J., Raught,B. and de Thé,H. (2008) Arsenic degrades PML or PML–RARα through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nat. Cell Biol.*, **10**, 547–555.
66. Tatham,M.H., Geoffroy,M.-C., Shen,L., Plechanovova,A., Hattersley,N., Jaffray,E.G., Palvimo,J.J. and Hay,R.T. (2008) RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. *Nat. Cell Biol.*, **10**, 538–546.
67. Saito,Y., Uchida,N., Tanaka,S., Suzuki,N., Tomizawa-Murasawa,M., Sone,A., Najima,Y., Takagi,S., Aoki,Y. and Wake,A. (2010) Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nat. Biotechnol.*, **28**, 275–280.
68. Guibal,F.C., Alberich-Jorda,M., Hirai,H., Ebralidze,A., Levantini,E., Di Ruscio,A., Zhang,P., Santana-Lemos,B.A., Neuberg,D. and Wagers,A.J. (2009) Identification of a myeloid committed progenitor as the cancer-initiating cell in acute promyelocytic leukemia. *Blood*, **114**, 5415–5425.
69. Nasr,R. (2010) Eradication of acute promyelocytic leukemia-initiating cells by PML/RARA-targeting. *Int. J. Hematol.*, **91**, 742–747.
70. Lallemand-Breitenbach,V., Guillemin,M., Janin,A., Daniel,M., Degos,L., Kogan,S. and Bishop,J. (1999) Retinoic acid and arsenic synergize to eradicate leukemic cells in a mouse model of acute promyelocytic leukemia. *J. Exp. Med.*, **189**, 1043–1052.
71. Paramanathan,T., Vladescu,I., McCauley,M.J., Rouzina,I. and Williams,M.C. (2012) Force spectroscopy reveals the DNA structural dynamics that govern the slow binding of Actinomycin D. *Nucleic Acids Res.*, **40**, 4925–4932.
72. Lipfert,J., Klijnhout,S. and Dekker,N.H. (2010) Torsional sensing of small-molecule binding using magnetic tweezers. *Nucleic Acids Res.*, **38**, 7122–7132.
73. Lord,C.J. and Ashworth,A. (2017) PARP inhibitors: synthetic lethality in the clinic. *Science*, **355**, 1152–1158.
74. Li,J., Zhu,H., Hu,J., Mi,J., Chen,S., Chen,Z. and Wang,Z. (2014) Progress in the treatment of acute promyelocytic leukemia: optimization and obstruction. *Int. J. Hematol.*, **100**, 38–50.
75. Tomita,A., Kiyoi,H. and Naoe,T. (2013) Mechanisms of action and resistance to all-trans retinoic acid (ATRA) and arsenic trioxide (As2O3) in acute promyelocytic leukemia. *Int. J. Hematol.*, **97**, 717–725.
76. Tobita,T., Takeshita,A., Kitamura,K., Ohnishi,K., Yanagi,M., Hiraoka,A., Karasuno,T., Takeuchi,M., Miyawaki,S. and Ueda,R. (1997) Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-trans retinoic acid. *Blood*, **90**, 967–973.
77. Ungewickell,A. and Medeiros,B.C. (2012) Novel agents in acute myeloid leukemia. *Int. J. Hematol.*, **96**, 178–185.
78. Goto,E., Tomita,A., Hayakawa,F., Atsumi,A., Kiyoi,H. and Naoe,T. (2011) Missense mutations in PML-RARA are critical for the lack of responsiveness to arsenic trioxide treatment. *Blood*, **118**, 1600–1609.
79. Lallemand-Breitenbach,V., Zhu,J., Chen,Z. and de Thé,H. (2012) Curing APL through PML/RARA degradation by As 2 O 3. *Trends Mol. Med.*, **18**, 36–42.
80. Zhang,X.-W., Yan,X.-J., Zhou,Z.-R., Yang,F.-F., Wu,Z.-Y., Sun,H.-B., Liang,W.-X., Song,A.-X., Lallemand-Breitenbach,V. and Jeanne,M. (2010) Arsenic trioxide controls the fate of the PML-RARα oncoprotein by directly binding PML. *Science*, **328**, 240–243.
81. Chendamarai,E., Ganesan,S., Alex,A.A., Kamath,V., Nair,S.C., Nellickal,A.J., Janet,N.B., Srivastava,V., Lakshmi,K.M. and Viswabandya,A. (2015) Comparison of newly diagnosed and relapsed patients with acute promyelocytic leukemia treated with arsenic trioxide: insight into mechanisms of resistance. *PLoS One*, **10**, e0121912.
82. Cicconi,L. and Lo-Coco,F. (2016) Current management of newly diagnosed acute promyelocytic leukemia. *Ann. Oncol.*, **27**, 1474–1481.