Novel AXL-targeted agents overcome FLT3 inhibitor resistance in FLT3-ITD$^+$ acute myeloid leukemia cells

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Abstract. AXL receptor tyrosine kinase (AXL) upregulation mediates drug resistance in several types of human cancer and has become a therapeutic target worthy of exploration. The present study investigated AXL antigen expression and the effects of novel AXL-targeted agents in acute myeloid leukemia (AML) cells. AXL antigen expression in drug-sensitive and drug-resistant human AML cell lines, and AML blast cells from 57 patients with different clinical characteristics, was analyzed by flow cytometry and compared. Furthermore, the effects of the novel AXL antibody DAXL-88, antibody-drug conjugate DAXL-88-monomethyl auristatin E (MMAE), AXL small molecule inhibitor R428 and their combination with FMS-like tyrosine kinase 3 (FLT3) inhibitor quizartinib (AC220) in AML cells were analyzed by Cell Counting Kit-8 assay, flow cytometry and western blotting. The present study revealed that AXL antigen expression was upregulated in FLT3-internal tandem duplication (ITD)/tyrosine kinase domain mutation-positive (TKD)$^+$ AML blast cells compared with FLT3-ITD/TKD$^-$ AML cells. Additionally, AXL antigen expression was markedly upregulated in the AC220-resistant FLT3-ITD$^+$ MV4-11 cell line (MV4-11/AC220) and in FLT3 inhibitor-resistant blast cells from a patient with FLT3-ITD$^+$ AML compared with parental sensitive cells. The AXL-targeted agents DAXL-88, DAXL-88-MMAE and R428 exhibited dose-dependent cytotoxic effects on FLT3-mutant AML cell lines (THP-1, MV4-11 and MV4-11/AC220) and blast cells from patients with FLT3-ITD$^+$ AML. Combinations of AXL-targeted agents with AC220 exerted synergistic cytotoxic effects and induced apoptosis in MV4-11/AC220 cells and FLT3 inhibitor-resistant blast cells. The antileukemic effect of DAXL-88 and DAXL-88-MMAE may rely on their ability to block AXL, FLT3 and their downstream signaling pathways. The present study demonstrated the association between AXL antigen expression upregulation and drug resistance in FLT3-ITD$^+$ AML, and proposed a method for overcoming FLT3 inhibitor resistance of FLT3-ITD$^+$ AML using novel AXL-targeted agents.

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Abbreviations: AXL, AXL receptor tyrosine kinase; AML, acute myeloid leukemia; K562/ADM, Adriamycin-resistant K562 cell line; U937/Ara-c, cytarabine-resistant U937 cell line; MV4-11/AC220, AC220-resistant MV4-11 cell line; FLT3, FMS-like tyrosine kinase 3; WT, wild-type; ITD, internal tandem duplication; TKD, tyrosine kinase domain mutations; MMAE, monomethyl auristatin E

Key words: anti-AXL receptor tyrosine kinase antibody, AXL receptor tyrosine kinase small-molecule inhibitor, acute myeloid leukemia, FLT3 mutations, synergistic cytotoxic effect, drug resistance

Introduction

Acute myeloid leukemia (AML) is a term used to describe a group of genetically highly heterogeneous malignant clonal diseases characterized by abnormal differentiation and proliferation of immature myeloid protocells in the bone marrow. With the continuous optimization of chemotherapy, hematopoietic stem cell transplantation and supportive treatment, the prognosis of AML has improved; however, the 5-year overall survival rate remains low at 15-30% (1). Drug resistance of leukemic cells is the main cause of relapsed and refractory AML; thus, it is necessary to identify resistance-related therapeutic targets (2).

AXL receptor tyrosine kinase (AXL), a member of the receptor tyrosine kinase Tyro3, AXL and Mertk (TAM) family, is activated by growth arrest-specific factor 6 (GAS6). GAS6 binding leads to AXL dimerization, autophosphorylation and activation of subsequent signaling pathways, such as the PI3K/AKT, MAPK, STAT and NF-κB cascades (3). Upregulation and activation of AXL have been demonstrated to promote cell proliferation, chemotherapy resistance, invasion and metastasis

in several types of human cancer, thus becoming a therapeutic target worthy of exploration (4). Ben-Batalla *et al* (5) reported that AXL-mRNA is expressed in 57% (64/112) of newly-diagnosed normal karyotype genetic medium-risk AML cases and is an independent adverse prognostic factor. Hong *et al* (6) revealed that AXL-mRNA expression is upregulated in relapse-resistant AML cases and mediates resistance to a variety of chemotherapy drugs in U937 cells. Park *et al* (7) demonstrated that AXL is constitutively activated in blast cells from patients with AML and FMS-like tyrosine kinase 3 (FLT3)-internal tandem duplication $(ITD)^+$ AML cells, and the levels of total AXL and phosphorylated (p-)AXL protein are markedly increased following treatment with FLT3 inhibitor midostaurin (PKC412) or quizartinib (AC220) (8). These studies suggested that AXL is associated with drug resistance of leukemic cells and may be used as a therapeutic target for AML.

AXL-targeted therapies mainly include small-molecule inhibitors, ligand decoy antibodies (9,10) and monoclonal antibodies (11,12). BGB324 (R428) is the first selective AXL small-molecule inhibitor to enter clinical research, and was found to effectively inhibit the phosphorylation of AXL in AML cells and AML blast cells, induce cell apoptosis, and increase sensitivity of AML cells to doxorubicin and cytarabine (also known as Ara-c) (5). A multicenter phase Ib/II clinical study of BGB324 as a single agent or in combination with cytarabine/decitabine for the treatment of high-risk myelodysplastic syndromes and relapsed/refractory leukemia is under way (NCT02488408). DAXL-88 is a novel human antibody targeting AXL, which was constructed by Duan *et al* (13) by analyzing the spatial pattern of the AXL-GAS6 interaction and panning through the entire human natural phage antibody library. DAXL-88 blocks the interaction of AXL-GAS6 by binding to human and mouse AXL protein with a high affinity, inhibits the migration and invasion of human ovarian cancer SKOV3 cells and non-small cell lung cancer A549 cells induced by GAS6, and reverses the upregulation of p-AXL, p-AKT and p-ERK activated by GAS6 (14). However, DAXL-88 has no cytotoxic effect on these tumor cells. Duan *et al* (13) further modified DAXL-88 by conjugating it to monomethyl auristatin E (MMAE), a small molecule microtubule interferant, to form an antibody-drug conjugate termed DAXL-88-MMAE. After DAXL-88-MMAE binds to AXL, the antibody is internalized, and MMAE is released by lysosomal protease cleavage, which prevents microtubulin polymerization, causes cell cycle arrest and induces apoptosis.

The present study aimed to solve the problem of drug resistance in the clinical treatment of AML, and proposed AXL as a therapeutic target. By comparing AXL antigen expression among drug-sensitive and drug-resistant human AML cell lines, and AML blast cells from patients with different clinical characteristics, FLT3-mutant AML with higher AXL antigen expression was selected for AXL-targeted therapy. Furthermore, in AML cell lines and blast cells, the cytotoxic effects of DAXL-88, DAXL-88-MMAE and R428, and their molecular mechanisms, were thoroughly explored.

Materials and methods

Cell culture, resistant cell induction and reagents. The human AML U937 (cat. no. TCHu-159), THP-1 (cat. no. TCHu-57) and MV4-11 (cat. no. SCSP-5031) cell lines were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, where they were characterized by mycoplasma detection, DNA fingerprinting, isozyme detection and cell vitality detection. The Adriamycin (ADM)-resistant K562 cell line (K562/ADM) was kindly provided by Dr Ming Xiong, Central Laboratory of the People's Liberation Army Navy General Hospital (Beijing, China). Cell lines were cultured in RPMI-1640 medium (cat. no. SH30809.01; HyClone; Cytiva) supplemented with 10% FBS (cat. no. 1997802C; Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin at 37˚C with 5% CO_2. The AC220-resistant MV4-11 cell line (MV4-11/AC220) was generated by continuous incubation of the AC220-sensitive MV4-11 cell line with increasing doses of AC220. The MV4-11 cell line was started in culture in the presence of 0.1 nM AC220 for ~3 weeks. Between days 22 and 79, the concentration of AC220 was gradually increased from 0.1 to 1 nM. Between days 79 and 96, the concentration of AC220 was gradually increased from 1 to 5 nM. The cytarabine-resistant U937 cell line (U937/Ara-c) was generated by short-range shock induction of the cytarabine-sensitive U937 cell line with increasing doses of cytarabine (cat. no. H20160403; Actavis Italy SpA). The culture started in the presence of 0.8 μM cytarabine for 24 h. Subsequently, cytarabine was removed by centrifugation at 45 x g for 5 min at room temperature, and shock was repeated at double the dose after cell activity recovery up to a maximum induced dose of 300 μM. DAXL-88, DAXL-88-MMAE and IgG1-MMAE were produced by Dr Yanting Duan. PKC412 (cat. no. S8064), AC220 (cat. no. S1526), R428 (cat. no. S2841) and MMAE (cat. no. S7721) were purchased from Selleck Chemicals.

Samples from patients with AML and clinical data. Cryopreserved leukemic blast cells were obtained from 57 patients with AML between May 2018 and January 2020, including 31 males and 26 females, with a mean age ± SD of 50±2.1 years (range, 16-88 years), who provided written consent with the approval of the Sixth Medical Center of PLA General Hospital Ethics Committee (research ethics no. HZKY-YJ-2020-1; Beijing, China). There were a total of 64 blast cell samples, including single samples from 53 patients, pre- and post-chemotherapy samples from 3 patients, and 5 dynamic samples from 1 patient. After thawing, AML blast cells were maintained in RPMI-1640 medium containing 20% FBS (8). The clinical data of patients with AML included in the present study are summarized in Table I.

Flow cytometry analysis of AXL antigen expression. AML cells ($1x10^6$) were incubated with 2 μl PE-conjugated anti-human AXL antibody (cat. no. FAB154P; R&D Systems, Inc.). AML blast cells ($1x10^6$) were incubated with 2 μl PE-conjugated anti-human AXL and 3 μl PerCP-conjugated anti-human CD45 (cat. no. Z6410013; Beijing Quantobio Biotechnology Co., Ltd.) in PBS for 60 min at room temperature. Cells were washed after staining and analyzed using a FACSCalibur flow cytometer (BD Diagnostics; Becton, Dickinson and Company) (15).

Cell Counting Kit-8 (CCK-8) assay of cytotoxicity and synergistic cytotoxicity. AML cells ($2x10^4$) and AML blast

Table I. AXL receptor tyrosine kinase antigen expression in 64 AML blast cell samples from 57 patients with different clinical characteristics.

Patient characteristics	AML blast cells (n)	AXL antigen expression (%), median (range)	P-value[a]
Disease status			0.637
De novo	29	1.98 (0.30-17.76)	
Relapsed/refractory	35	1.79 (0.27-54.16)	
FLT3-ITD/TKD			0.001
Positive	26	2.70 (1.03-54.16)	
Negative	38	1.51 (0.27-4.92)	
FLT3-ITD/TKD-positive			0.330
De novo	11	3.56 (1.18-17.76)	
Relapsed/refractory	15	2.03 (1.13-54.16)	
ELN2017 genetic risk stratification			0.923[b]
Favorable	7	2.23 (0.30-4.97)	
Intermediate	19	1.76 (0.44-6.79)	
Adverse	35	1.93 (0.27-54.16)	
Unknown	3	1.56 (1.45-2.15)	

[a]Mann-Whitney U test. [b]Kruskal-Wallis test. AXL, AXL receptor tyrosine kinase; AML, acute myeloid leukemia; FLT3, FMS-like tyrosine kinase 3; ITD, internal tandem duplication; TKD, tyrosine kinase domain mutations.

cells ($2x10^5$) were seeded in a 96-well tissue culture plates and exposed to different drug concentrations for 45 or 44 h at 37˚C in a 5% CO_2 atmosphere incubator. A total of 10 μl CCK-8 solution (cat. no. CK04; Dojindo Molecular Technologies, Inc.) was added to each well, followed by incubation for an additional 3 or 4 h. Subsequently, the absorbance was read at 450 nm using a Multiskan Mk3 microplate reader (16).

Flow cytometry analysis of apoptosis. Detection of apoptosis was performed using the Annexin V-FITC Apoptosis Detection Kit (cat. no. 130092052; Miltenyi Biotec GmbH). Following incubation of the cells with 10 μl FITC-conjugated Annexin V in the binding buffer for 15 min at room temperature, cells were washed, incubated with 5 μl PI and analyzed using a FACSCantoII flow cytometer (BD Diagnostics; Becton, Dickinson and Company) (15).

Western blot analysis. The anti-p-AXL antibody (Y799; cat. no. AF2228-SP) was obtained from BD Biosciences. Anti-AXL (cat. no. 8661), anti-p-FLT3 (cat. no. 3464), anti-FLT3 (cat. no. 3462), anti-AKT (cat. no. 4691), anti-p-AKT (cat. no. 4060), anti-ERK (cat. no. 4695) and anti-p-ERK (cat. no. 4695) antibodies were obtained from Cell Signaling Technology, Inc. MV4-11, MV4-11/AC220 and FLT3-ITD$^+$ AML blast cells were collected and lysed in ice cold RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with protease inhibitor cocktail (Roche Diagnostics) for 30 min. Protein concentrations were quantified using a BCA kit (Applygen Technologies, Inc.). Proteins (25 μg/lane) were separated via 8% SDS-PAGE, transferred onto a nitrocellulose filter membrane (EMD Millipore) and blocked with 5% TBST skim milk for 1 h at room temperature. The nitrocellulose membrane was first incubated with primary antibodies against the aforementioned proteins at a dilution of 1:1,000 (except for anti-p-AXL which was used at a dilution of 1:200), overnight at 4˚C, and then incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase (dilution, 1:5,000; cat. no. AS014; ABclonal Biotech Co., Ltd.) for 1 h at room temperature. An ImageQuant LAS4000 chemiluminescent imaging analyzer (GE Healthcare) was used for signal detection. GAPDH (cat. no. AC027; ABclonal Biotech Co., Ltd.) was used as a reference (13).

Statistical analysis. Differences between mean values of two groups were evaluated using an independent samples t-test, and that of multiple groups were evaluated using one-way analysis of variance followed by Dunnett's or Tukey's post hoc test. Differences between median values of two groups were evaluated using the Mann-Whitney U test, and that of multiple groups were evaluated using the Kruskal-Wallis test. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 17.0 (SPSS, Inc.), GraphPad Prism version 5.01 (GraphPad Software, Inc.), CalcuSyn version 2.1 (Biosoft) and ImageJ 1.51j8 (National Institutes of Health).

Results

AXL antigen expression is upregulated in drug-resistant AML cell lines and FLT3-ITD/tyrosine kinase domain mutation-positive (TKD)$^+$ AML blast cells. AXL antigen expression in drug-resistant K562/ADM, U937/Ara-c and MV4-11/AC220 cells was 43.31±1.78, 6.26±0.18 and 30.53±1.14%, respectively, revealing significant upregulation compared with that in drug-sensitive K562, U937 and MV4-11 cells (13.03±0.31, 1.12±0.06 and 5.03±0.04%, respectively; $P<0.001$; Figs. 1A and S1). AXL antigen expression in 64 blast cell samples from 57 patients with AML exhibited a skewed distribution with a median of 1.89% (range, 0.27-54.16%). The median AXL

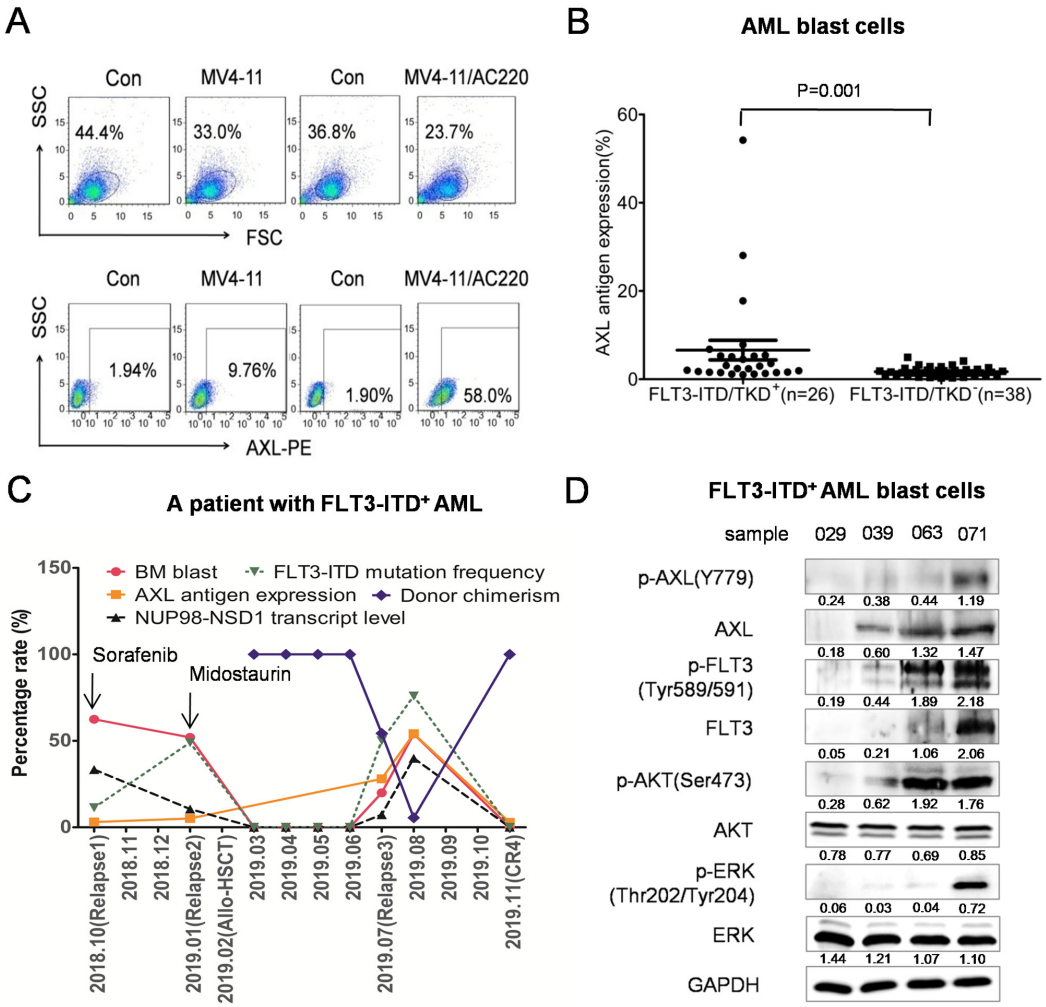

Figure 1. AXL antigen expression is upregulated in the MV4-11/AC220 cell line and FLT3-ITD/TKD$^+$ AML blast cells. (A) AXL antigen expression in the AC220-sensitive MV4-11 and AC220-resistant MV4-11/AC220 cell lines was detected by flow cytometry. (B) AXL antigen expression in FLT3-ITD/TKD$^+$ AML (n=26) and FLT3-ITD/TKD$^-$ AML (n=38) blast cells was detected by flow cytometry. (C) The dynamic changes of the bone marrow blast percentage, donor chimerism, AXL antigen expression, FLT3-ITD mutation frequency and NUP98-NSD1 gene transcript level (transcript copy number/housekeeping gene Abelson copy number) from a patient with relapsed/refractory FLT3-ITD$^+$ AML. (D) AML blast cells (samples 029, 039, 063 and 071) were subjected to western blot analysis to detect the levels of AXL, p-AXL, FLT3, p-FLT3, ERK, p-ERK, AKT, p-AKT and GAPDH. The intensity of the bands was analyzed using ImageJ 1.51j8 and denoted as intensity/GAPDH. BM, bone marrow; CR, complete remission; allo-HSCT, allogeneic hematopoietic stem cell transplantation; AXL, AXL receptor tyrosine kinase; AML, acute myeloid leukemia; FLT3, FMS-like tyrosine kinase 3; ITD, internal tandem duplication; TKD, tyrosine kinase domain mutations.

antigen expression in FLT3-ITD/TKD$^+$ AML blast cells was 2.70% (range, 1.03-54.16%), which was increased compared with that in FLT3-ITD/TKD$^-$ AML blast cells (1.51%; range, 0.27-4.92%; P=0.001; Fig. 1B). However, there was no statistical difference in AXL antigen expression between the *de novo* and relapsed/refractory groups, among the favorable, intermediate and adverse genetic risk groups, and between the FLT3-ITD/TKD$^+$ AML *de novo* and relapsed/refractory groups (Table I).

A 19-year-old male patient was diagnosed with FLT3-ITD$^+$ AML (M4), and experienced three relapses and four complete remissions during clinical treatment between June 2018 and November 2019 (Fig. S2). The dynamic changes of AXL antigen expression in blast cells were consistent with the clinical resistance to the FLT3 inhibitors sorafenib and PKC412 (Fig. 1C). Western blotting was performed on AML blast cells and revealed that, with the upregulation of p-AXL, the downstream molecules p-AKT and p-ERK were also upregulated (Fig. 1D). These data suggested that increased AXL activation by FLT3 inhibitors may mediate resistance of leukemic cells in patients with FLT3-ITD$^+$ AML.

AXL-targeted agents inhibit the growth of FLT3-mutant AML cell lines and FLT3-ITD$^+$ AML blast cells in a dose-dependent manner. AXL antigen expression was upregulated in the FLT3-ITD$^+$ MV4-11/AC220 resistant cell line, FLT3-ITD/TKD$^+$ AML blast cells and FLT3 inhibitor-resistant blast cells from a patient with FLT3-ITD$^+$ AML, suggesting that AXL antigen upregulation was associated with FLT3-ITD/TKD$^+$ AML, particularly with FLT3 inhibitor-resistant FLT3-ITD$^+$ AML, and that targeting AXL may have clinical value. DAXL-88 exerted a dose-dependent cytotoxic effect on FLT3-wild type (WT)$^+$ THP-1, FLT3-ITD$^+$ MV4-11 and MV4-11/AC220 cells, as well as FLT3-ITD$^+$ AML blast cells, but had no effect on the proliferation of FLT3-ITD$^-$ U937 and FLT3-ITD$^-$ AML blast cells (Fig. 2A and B).

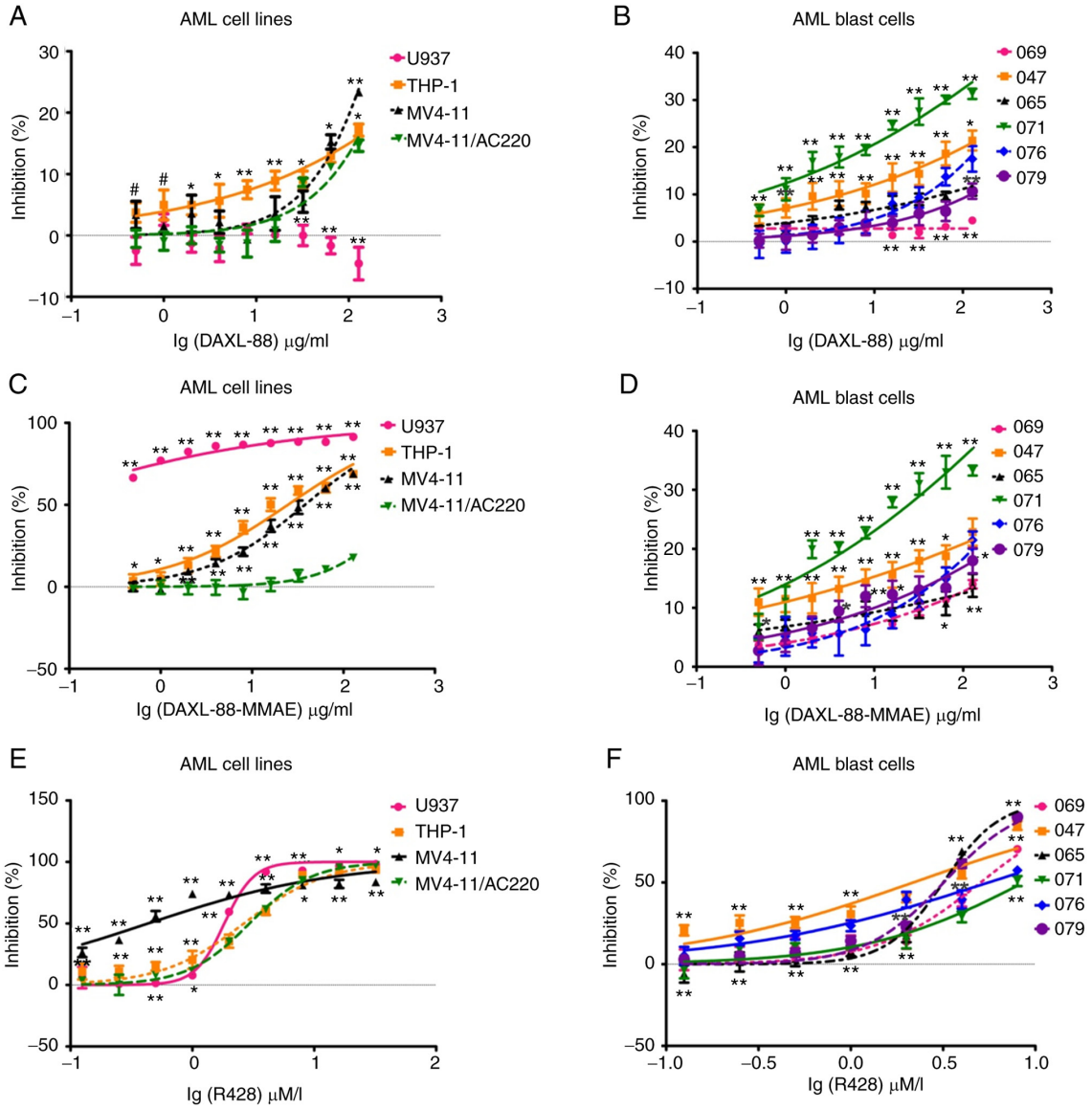

Figure 2. AXL-targeted agents inhibit the proliferation of FLT3-mutant AML cell lines and FLT3-ITD$^+$ AML blast cells in a dose-dependent manner. (A) DAXL-88 inhibited the proliferation of FLT3-WT$^+$ THP-1, FLT3-ITD$^+$ MV4-11 and MV4-11/AC220 cells. (B) DAXL-88 inhibited the proliferation of FLT3-ITD$^+$ AML blast cells. (C) DAXL-88-MMAE inhibited the proliferation of AXL-expressing AML cells. (D) DAXL-88-MMAE inhibited the proliferation of AXL-expressing AML blast cells. (E) R428 inhibited the proliferation of AXL-expressing AML cells. (F) R428 inhibited the proliferation of AXL-expressing AML blast cells. Cells were treated with DAXL-88, DAXL-88-MMAE and R428 at the indicated concentrations for 48 h. Cell viability was measured using a Cell Counting Kit-8 assay. $^{\#}$P<0.05, *P<0.01, and **P<0.001 (ANOVA and Dunnett test; AML cells vs. MV4-11/AC220 cell line; AML blast cells vs. patient's sample 076). Data are presented as the mean ± SEM. AXL, AXL receptor tyrosine kinase; AML, acute myeloid leukemia; FLT3, FMS-like tyrosine kinase 3; ITD, internal tandem duplication; WT, wild-type.

Considering the non-optimal cytotoxic effect of DAXL-88, the antibody-drug conjugate DAXL-88-MMAE was further prepared to improve the cytotoxic effect on these cells. DAXL-88-MMAE exerted a dose-dependent cytotoxic effect on AXL-expressing U937, THP-1, MV4-11 and MV4-11/AC220 cells (Fig. 2C), and AML blast cells (Fig. 2D). The cytotoxic effect of DAXL-88-MMAE was markedly enhanced in U937, THP-1 and MV4-11 cells compared with that of DAXL-88, and this was independent of the AXL antigen expression intensity. However, it was associated with the sensitivity of cell lines to MMAE (Table SI). The cytotoxic effect was not significantly enhanced in the MV4-11/AC220 cell line (Fig. 2C; Table SI) and AML blast cells (Fig. 2D; Table SII). In order to exclude the cytotoxic effect caused by free MMAE from DAXL-88-MMAE, an IgG1-MMAE antibody was also synthesized as an isotype control, and this exerted no cytotoxic effect on AML cell lines or blast cells (data not shown). These data suggested that the cytotoxic effect of DAXL-88-MMAE was AXL-targeted MMAE cytotoxicity.

R428 also exerted a dose-dependent cytotoxic effect on AXL-expressing U937, THP-1, MV4-11 and MV4-11/AC220 cells (Fig. 2E), and AML blast cells (Fig. 2F). The IC_{50} at 48 h for FLT3-ITD$^-$ AML (sample 069) and relapsed/refractory FLT3-ITD$^+$ AML (samples 071 and 076) blast cells was 5.59±0.84 μM/l, which was higher compared with that for the *de novo* FLT3-ITD$^+$ AML samples (2.84±0.60 μM/l; samples 047, 065 and 079; P=0.012; Table SII).

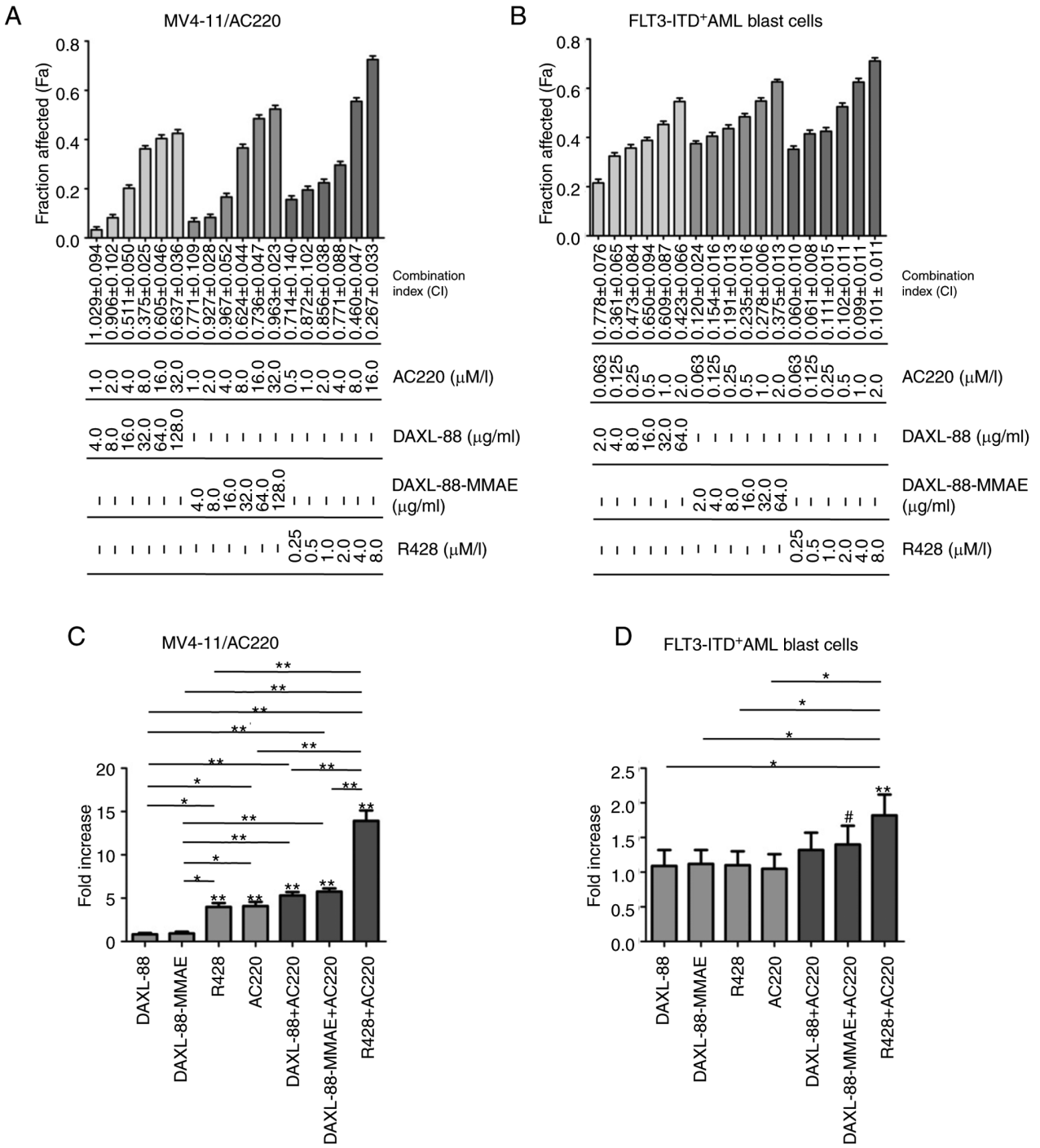

Figure 3. AXL-targeted agents in combination with AC220 synergistically inhibit the proliferation and induce the apoptosis of MV4-11/AC220 cells and FLT3 inhibitor-resistant AML blast cells. (A) MV4-11/AC220 and (B) AML blast cells (sample 071) were treated with DAXL-88, DAXL-88-MMAE, R428 and AC220, and their combinations, at a constant ratio and the indicated concentrations for 48 h. Cell viability was examined using a Cell Counting Kit-8 assay. The Fa and CI values for each pair of drugs were calculated using Calcusyn2.1 software. CI<1 indicates a synergistic effect; CI=1 indicates an additive effect; and CI>1 indicates an antagonistic effect. (C) MV4-11/AC220 cells treated without (control culture) or with DAXL-88 (64 μg/ml), DAXL-88-MMAE (64 μg/ml), R428 (3 μM), AC220 (16 μM) and their combination for 48 h. (D) AML blast cells (sample 071) treated without (control culture) or with DAXL-88 (5 μg/ml), DAXL-88-MMAE (5 μg/ml), R428 (3 μM), AC220 (1 μM) and their combination for 24 h. Cells were stained with PI and Annexin V-FITC, and analyzed by flow cytometry. The fold increase (relative to control untreated cultures of each cell group) of apoptosis is presented as the mean ± SEM of three experiments. $^{\#}$P<0.05, *P<0.01 and **P<0.001 (ANOVA and Dunnett test, vs. control untreated cells; ANOVA and Tukey's test, vs. different treatment groups). CI, combination index; Fa, fraction affected; MMAE, monomethyl auristatin E; FLT3, FMS-like tyrosine kinase 3; ITD, internal tandem duplication; AXL, AXL receptor tyrosine kinase; AML, acute myeloid leukemia.

AXL-targeted agents in combination with AC220 synergistically inhibit proliferation and induce apoptosis of MV4-11/AC220 and FLT3 inhibitor-resistant AML blast cells. The AXL-targeted agents DAXL-88, DAXL-88-MMAE and R428 were less effective in killing MV4-11/AC220-resistant cells and relapsed/refractory FLT3-ITD^{+} AML blast cells (Tables SI and SII), suggesting that it is necessary to target both AXL and FLT3 to overcome drug resistance. DAXL-88,

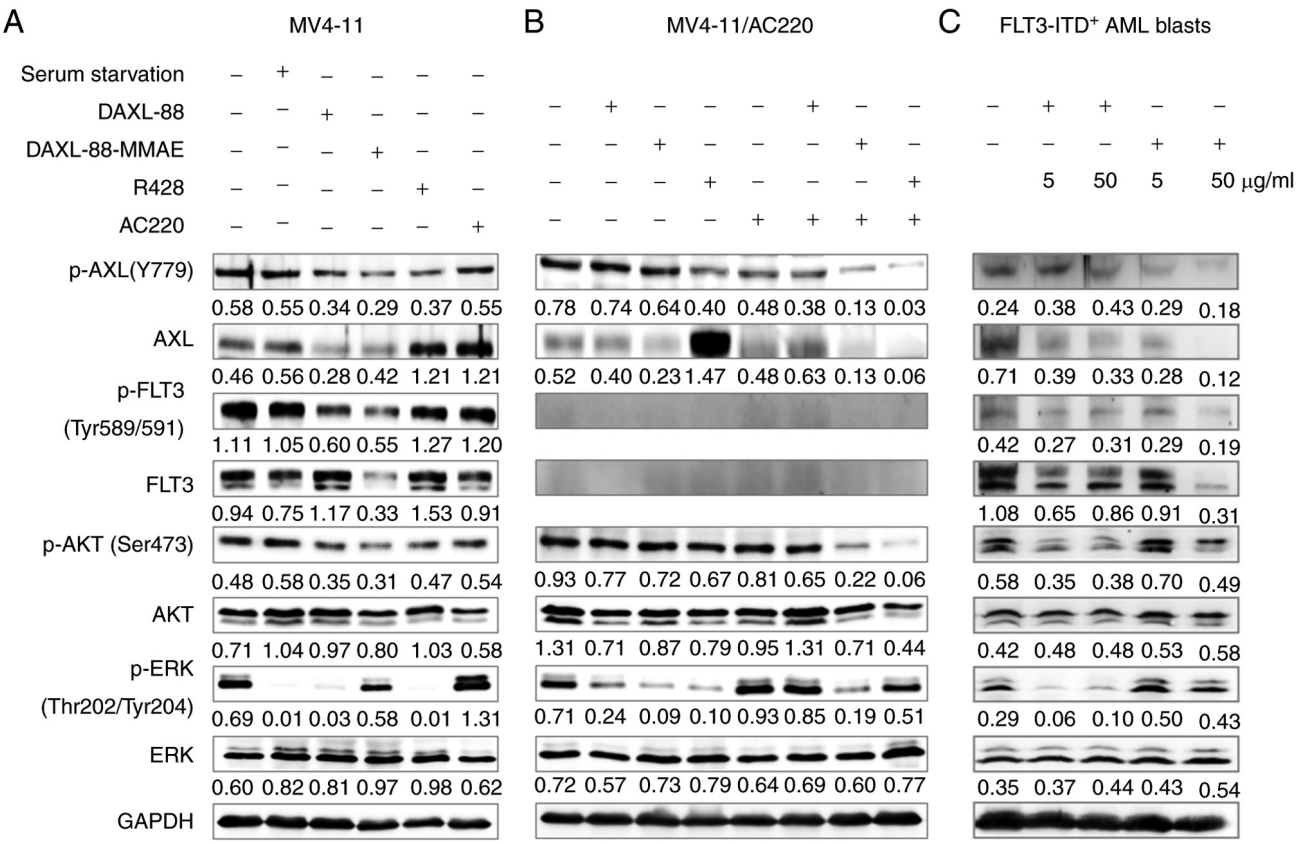

Figure 4. DAXL-88 and DAXL-88-MMAE effectively block AXL, FLT3 and their downstream signaling pathways. (A) MV4-11 cells treated without (control culture) or with serum starvation (2% FBS), DAXL-88 (64 μg/ml), DAXL-88-MMAE (64 μg/ml), R428 (0.4 μM) or AC220 (2 nM) for 48 h. (B) MV4-11/AC220 cells treated without (control culture) or with DAXL-88 (64 μg/ml), DAXL-88-MMAE (64 μg/ml), R428 (3 μM), AC220 (16 μM) and their combinations for 48 h. (C) AML blast cells (sample 071) treated without (control culture) or with DAXL-88 (5 or 50 μg/ml) and DAXL-88-MMAE (5 or 50 μg/ml) for 48 h. Cells were subjected to western blot analysis to detect the levels of AXL, p-AXL, FLT3, p-FLT3, ERK, p-ERK, AKT, p-AKT and GAPDH. The intensity of the bands was analyzed using ImageJ 1.51j8 and denoted as intensity/GAPDH. AXL, AXL receptor tyrosine kinase; AML, acute myeloid leukemia; FLT3, FMS-like tyrosine kinase 3; ITD, internal tandem duplication.

DAXL-88-MMAE and R428 in combination with AC220 at the indicated ratio (Fig. 3A and B) exerted synergistic effects on the MV4-11/AC220 cell line and FLT3 inhibitor-resistant AML blast cells (sample 071).

DAXL-88 (64 μg/ml) and DAXL-88-MMAE (64 μg/ml) could not effectively induce apoptosis in MV4-11/AC220 cells; however, the percentage of apoptotic cells was significantly increased when combined with AC220 ($P<0.001$; Fig. 3C). The apoptosis of FLT3 inhibitor-resistant AML blast cells (sample 071) was not elevated following 24-h sublethal single-dose treatment with DAXL-88 (5 μg/ml), DAXL-88-MMAE (5 μg/ml), R428 (3 μM) or AC220 (1 μM), but was increased following combination treatment with DAXL-88-MMAE and AC220 ($P<0.05$; Fig. 3D), and with R428 and AC220 ($P<0.001$; Fig. 3D).

DAXL-88 and DAXL-88-MMAE effectively block AXL, FLT3 and their downstream signaling pathways. To explore the mechanism underlying the antileukemic effect, the changes in the signal transduction pathways of MV4-11, MV4-11/AC220 and FLT3 inhibitor-resistant AML blast cells (sample 071) following treatment with DAXL-88, DAXL-88-MMAE, R428, AC220 and their combinations were analyzed by western blotting. In the MV4-11 cell line, the levels of p-AXL and p-FLT3, and those of their downstream molecules p-AKT and p-ERK, were downregulated by DAXL-88 and DAXL-88-MMAE. Furthermore, the expression levels of AXL and FLT3 were also downregulated by DAXL-88-MMAE. AXL expression was upregulated, and the levels of p-AXL, p-AKT and p-ERK were downregulated by R428 (Fig. 4A). In FLT3 inhibitor-resistant AML blast cells (sample 071), the levels of AXL, FLT3 and its downstream target p-AKT were also downregulated by treatment with DAXL-88 and DAXL-88-MMAE (Fig. 4C). Compared with those in MV4-11 cells, the AXL and p-AXL levels in MV4-11/AC220 cells were increased (data not shown), and the expression of FLT3 and p-FLT3 was almost completely inhibited (Fig. 4B). p-AXL and p-AKT levels were not downregulated by DAXL-88 (64 μg/ml), DAXL-88-MMAE (64 μg/ml) or R428 (3 μM). When DAXL-88, DAXL-88-MMAE and R428 were combined with AC220, the p-AXL, AXL and p-AKT levels were decreased compared with those in the single-agent groups (Fig. 4B).

Discussion

The present study selected daunorubicin, ADM and cytarabine to induce the resistance of AML cell lines with reference to the clinical AML standard '3+7' induction chemotherapy (1). Additionally, the FLT3 inhibitors PKC412 and AC220 were

selected as the resistance-inducing medications for the following reasons: i) 20-30% of patients with AML harbor the FLT3-ITD/TKD mutation; ii) FLT3-ITD and FLT3-TKD are high-risk AML biomarkers (17); and iii) FLT3-targeted therapies have been widely used in the clinical treatment of FLT3-ITD/TKD$^+$ AML, and resistance to FLT3 inhibitors is a recent problem. Only three stable drug-resistant AML cell lines (K562/ADM, U937/Ara-c and MV4-11/AC220) were obtained, and AXL antigen expression in these cells was markedly upregulated compared with that in drug-sensitive cell lines, indicating that upregulation of AXL antigen expression may be associated with resistance in leukemic cells (Fig. 1A and Fig. S1). In particular, K562/ADM and MV4-11/AC220 cells may be transformed into semi-adherent cells due to the upregulation of the adhesion molecule AXL (18).

Compared with that in FLT3-ITD/TKD$^-$ AML, AXL antigen expression level in FLT3-ITD/TKD$^+$ AML blast cells (7 cases of FLT3-ITD$^+$ and 2 cases of FLT3-TKD$^+$) was increased (Fig. 1B). The dynamic upregulation of AXL antigen expression (Fig. 1C) and p-AXL protein levels (Fig. 1D) in blast cells from a typical patient with relapsed/refractory FLT3-ITD$^+$ AML was consistent with clinical resistance to FLT3 inhibitors, which is characterized by increased FLT3 mutation frequency and new inserted fragments (Fig. S2). These data suggested that AXL antigen upregulation was associated with FLT3-ITD/TKD$^+$ AML, particularly with drug-resistant FLT3-ITD$^+$ AML. Therefore, targeting AXL has clinical value in FLT3-mutant AML. The present study further compared AXL antigen expression in *de novo* FLT3-ITD/TKD$^+$ AML blast cells with that in relapsed/refractory FLT3-ITD/TKD$^+$ AML, and no significant difference was observed (P=0.330; Table I). This may be associated with the insufficient sample size and the insufficient cases using FLT3 inhibitors. Additional clinical specimens will be collected in the future to confirm higher AXL antigen expression in relapsed/refractory FLT3-ITD/TKD$^+$ AML.

DAXL-88 only exerted a dose-dependent cytotoxic effect on FLT3-WT$^+$ THP-1, FLT3-ITD$^+$ MV4-11 and MV4-11/AC220 cells, as well as on FLT3-ITD$^+$ AML blast cells, and this was independent of AXL antigen expression intensity (Fig. 2A and B; Tables SI and SII). Duan *et al* (14) constructed a three-dimensional model of AXL and DAXL-88 Fv fragments, and identified the interaction sites as Q^{122}-E^{129} and H^{201}-G^{205}. The interaction sites are not the key binding sites for GAS6, suggesting that DAXL-88 may rely on steric hindrance to block the binding of GAS6 to AXL. DAXL-88 exerted no cytotoxic effect on the SKOV3 human ovarian cancer cell line, the A549 non-small cell lung cancer cell line, the MDA-MB-231 breast cancer cell line with high AXL antigen expression (data not shown) or the U937 AML cell line with low AXL antigen expression, indicating that the cytotoxic mechanism of DAXL-88 is not mediated via blocking of the GAS6/AXL signaling pathway or the AXL self-activation signaling pathway (19). Park *et al* (7) demonstrated that, in the FLT3-ITD$^+$ MV4-11 cell line, there is a physical interaction between AXL and FLT3, and AXL can regulate FLT3 phosphorylation by affecting this interaction. In the present study, DAXL-88 may have blocked the physical interaction between AXL and FLT3 in FLT3-mutant AML cells by spatial steric hindrance, blocked the formation of AXL heterodimer, and inhibited the phosphorylation of AXL, FLT3 and their downstream molecules AKT and ERK, thus inducing cell apoptosis and inhibiting cell proliferation. DAXL-88-MMAE exerted stronger growth inhibitory and apoptosis-inducing effects on the FLT3-ITD$^+$ MV4-11 cell line (Fig. 2C; Table SI), which was associated with the downregulation of the levels of p-AXL, AXL, p-FLT3, FLT3 and their downstream molecules p-AKT and p-ERK (Fig. 4A).

The cytotoxic effect of DAXL-88-MMAE was not significantly enhanced in the MV4-11/AC220 cell line (Fig. 2C; Table SI) and AML blast cells (Fig. 2D; Table SII) compared with that of DAXL-88 (Fig. 2A and B; Tables SI and SII), which differed from U937, THP-1 and MV4-11 cell lines (Fig. 2A and C; Table SI). The sensitivity of cells to free MMAE is an important factor (12); however, there are other potential factors involved in the difference in cytotoxic effects, such as antigen expression (20), somatic mutations (21) and p-glycoprotein (p-gp)-related multidrug resistance. Gemtuzumab ozogamicin (CMA-676) (22,23), an anti-CD33 antibody conjugate, is actively pumped out by resistant leukemic cells and blast cells expressing p-gp, thereby reducing its intracellular accumulation and cytotoxic effect. The drug resistance mechanism of MV4-11/AC220 cells and the optimized modification of DAXL-88-MMAE will be explored in future studies.

R428 could effectively inhibit the proliferation (Fig. 2E and F; Tables SI and SII) and induce the apoptosis of MV4-11 and MV4-11/AC220 cells (Fig. 3C), revealing upregulation of AXL and slight downregulation of p-AXL (Fig. 4A and B). Mild inhibition of p-AXL was insufficient to explain the effective cytotoxicity of R428, suggesting that there may be other mechanisms independent of AXL to be explored, such as blocking of lysosomal acidification and recycling (24).

In addition to its role in driving drug resistance, recent studies have revealed that AXL serves an important role in the regulation of leukemic stem cells and the bone marrow hematopoietic niche. Leukemic stem cells, which are responsible for leukemia initiation, progression and relapse, are considered to be a key factor in eliminating leukemia. Jin *et al* (25) identified that the GAS6/AXL paracrine loop is a critical regulator of the self-renewal capacity of chronic myelogenous leukemic stem cells conferring imatinib resistance. Wang *et al* (26) reported that alkB homolog 5 RNA demethylase (ALKBH5) is specifically required to maintain the function of AML stem cells, and regulates AXL stability in leukemic cells in an N^6-methyladenosine-dependent manner. These findings indicate that targeting AXL/GAS6 or AXL upstream molecule ALKBH5 could eliminate leukemic stem cells. Dumas *et al* (27) demonstrated that the bone marrow hematopoietic niche enhances AXL expression through canonical ligand GAS6, STAT5-activating soluble factors and the local hypoxic environment, thus providing protection for FLT3-ITD$^+$ AML cells against AC220. The study suggested that dual inhibition of AXL and FLT3 not only diminished the AML burden, but also prevented AXL expression from alleviating protection against the leukemia-initiating cells provided by the hematopoietic niche.

DAXL-88, DAXL-88-MMAE and R428 in combination with AC220 exerted synergistic cytotoxic effects in the MV4-11/AC220 cell line and FLT3 inhibitor-resistant AML

blast cells (sample 071; Fig. 3A and B). Flow cytometry revealed that their combination was more effective in inducing apoptosis than treatment with a single agent (Fig. 3C and D). Furthermore, western blotting demonstrated that the levels of p-AXL, AXL and p-AKT were downregulated by combination treatment compared with single-agent treatment (Fig. 4B). Therefore, AXL-targeted agents could overcome the resistance of MV4-11/AC220 and FLT3 inhibitor-resistant AML blast cells to AC220. The efficacy of the AXL-targeted agents in combination with FLT3 inhibitors would be expected in FLT3-ITD$^+$ AML mouse xenotransplantation models, considering the possible triple inhibition of leukemic cells, leukemic stem cells and the bone marrow hematopoietic niche.

In conclusion, upregulation of AXL antigen expression was associated with FLT3-ITD/TKD$^+$ AML, particularly drug-resistant FLT3-ITD$^+$ AML. Therefore, targeting AXL has clinical value in FLT3-mutant AML. The AXL-targeted agents DAXL-88, DAXL-88-MMAE and R428 could effectively inhibit the growth of FLT3-mutant AML cells and FLT3-ITD$^+$ AML blast cells, and overcome resistance in the AC220-resistant MV4-11/AC220 cell line and FLT3 inhibitor-resistant AML blast cells.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YL and JW conceptualized the project, performed the experiments, analyzed the data and wrote the article. JL and WM performed the experiments. YD produced the anti-AXL antibody DAXL-88 and DAXL-88-MMAE. DL conceptualized the project, oversaw the experiments and edited the article. YL, JW and DL confirm the authenticity of the raw data. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Sixth Medical Center of PLA General Hospital. The patients provided written informed consent for the use of peripheral blood or bone marrow samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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