The FTO inhibitor Rhein is a promising option for the treatment of multidrug resistance AML

Shuling Zhang
The First Clinical Medical College, Lanzhou University

Lanxia Zhou
Clinical Cellular Molecular Genetics and Immunology Laboratory, The First Affiliated Hospital of Lanzhou University

Jianle Lu
The First Clinical Medical College, Lanzhou University

Jincal Yang
The First Clinical Medical College, Lanzhou University

Lili Tao
The First Clinical Medical College, Lanzhou University

Youfan Feng
The First Clinical Medical College, Lanzhou University

Juan Cheng
Department of Hematology, The First Affiliated Hospital of Lanzhou University

Li Zhao (✉ zhaoli@lzu.edu.cn)
Clinical Cellular Molecular Genetics and Immunology Laboratory, The First Affiliated Hospital of Lanzhou University

Research Article

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Abstract

Purpose

Chemotherapy failure and resistance contribute to poor prognosis in Acute Myeloid Leukemia (AML) patients. The fat mass and obesity-associated protein (FTO) is dysregulated and plays crucial roles in AML. We aim to explore what role the FTO inhibitor Rhein played in multidrug resistance AML.

Methods

Bone marrow fluid was collected to clarify FTO expression in AML. Cell Counting Kit 8 reagent (CCK8) was used to detect the inhibition of proliferation. Migration assays were conducted using 24-well transwell chambers with 8-µm apertures. And flow cytometry and WB assays were used to clarify apoptotic effects of Rhein and proteins changes. The online SynergyFinder software was utilized to calculate drug synergy scores.

Results

We observed that FTO is overexpressed in AML, particularly in AML occurred multidrug resistance. Rhein significantly suppresses proliferation and migration in parent and multidrug-resistant AML cells in a dose- and time-dependent manner. In particular, multidrug-resistant AML cells did not show resistance to Rhein. Furthermore, Rhein promotes apoptosis and decreased the expression of Bcl-2 while increasing the expression of Bax. Additionally, Rhein suppressed FTO expression and inhibited the AKT/mTOR signaling pathways. We also identified that low-dose Rhein in combination with AZA could sensitize HL60 and HL60-ADR cells to AZA.

Conclusion

Rhein significantly suppresses proliferation and migration, promotes apoptosis, sensitive to multidrug-resistant AML cells, is a promising candidate for treating multidrug-resistant AML.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of primary hematopoietic neoplasms that primarily arise from myeloid lineage committed cells. Although significant progress has been made in AML treatment, relapse and multidrug resistance (MDR) still persist as leading causes of death (Shimony, Stahl, & Stone, 2023; Thol, Schlenk, Heuser, & Ganser, 2015). Urgent investigation into effective therapeutic methods to improve long-term survival and reduce recurrence is necessary. Treatment failures and chemoresistance consistently result in poor prognosis for AML patients, necessitating alternative or combination therapies to overcome these limitations (Thol & Ganser, 2020; Thol et al., 2015).
Epigenetic abnormalities have a significant impact on chromatin structure, gene expression, and cellular activities, playing a crucial role in the development of cancer. Among the more than 170 RNA modifications, N6-methyladenosine (m6A) is the most prevalent and abundant internal modification in mRNAs. It plays essential roles in regulating mRNA metabolism and fate, including stability, translation, splicing, and transport. The dynamic and reversible m6A modification is regulated by specific enzymes, known as 'writers' (METTL3, METTL14, WTAP), 'readers' (YTHDF1/2/3, YTHDC1/2, IGF2BP1/2/3), and 'erasers' (FTO and ALKBH5)(Mauer et al., 2016). FTO, the first discovered m6A demethylase, can remove m6A methylation from RRACH motifs in mRNAs and lncRNAs within cells(Yixing Li et al., 2019). Besides, FTO plays a vital role in cancer cell proliferation, apoptosis, cell cycle, migration, invasion, and drug resistance(Yangchan Li, Su, Deng, Chen, & Chen, 2022). In AML, FTO is upregulated and has an essential tumor-promoting role, enhancing leukemic oncogene-mediated cell transformation and leukemogenesis while inhibiting AML cell differentiation through the regulation of target genes ASB2 and RARA achieved by reducing m6A levels in these mRNA transcripts(Z. Li et al., 2017).

Rhein (4, 5-dihydroxyanthraquinone-2-carboxylic acid), a natural anthraquinone derivative predominantly found in the rhizome of rhubarb, is a well-known traditional Chinese herb with various medicinal uses. It possesses pharmacological activities such as anti-cancer, anti-bacterial, anti-fungal, anti-oxidant, anti-atherogenic, anti-angiogenic, anti-fibrosis, anti-inflammatory, hepatoprotective, and nephroprotective properties(He et al., 2011). Baoen Chen's team discovered Rhein as the first small-molecule inhibitor of FTO through structure-based virtual screening and biochemical analyses. Rhein competitively binds to the FTO active site in vitro and exhibits significant inhibitory activity on m6A demethylation within cells(Chen et al., 2012).

In conclusion, FTO plays an oncogenic role in AML, and further investigation is needed to understand its involvement in drug resistance. Targeting FTO might hold promise and reveal new strategies for future clinical therapeutic approaches. Rhein, a small molecule inhibitor, shows promise as a potential novel anticancer agent. Therefore, we aimed to study the function of the FTO inhibitor Rhein in AML and AML with MDR.

Materials and methods

Extraction of AML monocytes

Add 3ml separation solution into a 15ml centrifuge tube, carefully draw the bone marrow sample with a straw and gently add it to the liquid surface of the separation solution, then centrifuged at 600 g for 30 min, resulting in the separation of the contents into four layers from top to bottom: the plasma layer, a ring-shaped milky white monocyte layer, a transparent separation liquid layer, and the red blood cell layer. The monocyte layer was aspirated using a pipette and transferred to another 15mL centrifuge tube. Subsequently, 10mL of PBS solution was added, and the tube was centrifuged at 200g for 10 min. This process was repeated twice to obtain the final precipitate of AML bone marrow monocytes.

Cell lines and cell culture
AML cell lines K562, MV4-11, MOLM-13 were purchased from ATCC, HL60, THP1, the human adriamycin-resistant AML cell line HL60-ADR was purchased from Winter Biotechnology, the human adriamycin-resistant AML cell lines K562-ADM was obtained from the Central Laboratory of the First Hospital of Lanzhou University, and OCI-AML3 was purchased from Fu Heng Biology. THP1 and OCI-AML3 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 15% fetal bovine serum (Excell Bio) and 1% penicillin-streptomycin (Solarbio Life Sciences), while the others cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Excell) and 1% penicillin-streptomycin (Solarbio Life Sciences). All cells were maintained in a humidified incubator at 37°C with 5% CO₂.

**Protein extraction and western blot analysis**

Collected and washed cells twice with ice-cold PBS, and lysed on ice for 20 min using RIPA buffer (Beyotime) with proteinase inhibitors (Beyotime) and a protein phosphatase inhibitor complex (Beyotime). After centrifugation at 14,000 g for 15 min, the supernatant was collected and the protein concentration was measured using the BCA protein assay kit (Solarbio Life Sciences). 20 µg of protein was loaded onto 10% SDS-PAGE gels (NCM Biotechnology), the separated proteins were subsequently transferred to PVDF membranes (Merck Millipore). Blocked the membranes with 5% skimmed milk powder and incubated with primary antibody at 4°C overnight. Membranes were washed with TBST after primary antibody incubation, and secondary antibody was added for 1 h at room temperature. Finally, ECL chemical color development solution was used for color development, and a chemiluminescence imaging system (Amersham Imager 680) was used for photography.

**Cell proliferation assay**

Cells were seeded in 96-well plates (Nest) at a density of 2 × 10⁴ cells per well and treated with varying doses of Rhein obtained from MedChemExpress. Rhein was dissolved in DMSO, stored at −20°C, and prepared at a concentration of 40 mM by ultrasound and heat treatment for dissolution. HL60 and K562 cells were exposed to Rhein at concentrations of 0, 10, 20, 50, 100, and 200µM, while adriamycin-resistant cell lines K562-ADM and HL60-ADM were treated with Rhein at concentrations of 0, 10, 20, 50, 100, 200, and 400µM. Subsequently, 10µL of Cell Counting Kit 8 reagent (CCK8) from NCM Biotechnology was added to each well at 0, 24, 48, and 72 h and incubated at 37°C for 3 h to prevent bubble formation, followed by measurement of the absorbance at 450 nm using a microplate reader.

**Transwell cell migration assay**

Migration assays were conducted using 24-well transwell chambers (Nest) with 8-µm apertures. HL60 and HL60-ADR cells were treated with 50 and 100 µM Rhein for 24 h, respectively. After treatment, cells were resuspended in serum-free RPMI 1640, adjusted to a density of 2 × 10⁶/mL, and seeded onto the upper chambers of the transwell inserts. The lower chamber was filled with 600 µL of culture medium containing 10% FBS. The transwell chambers were washed twice with PBS after 24 h incubation, then fixed with 4% paraformaldehyde for 15 min, and stained with 0.1% crystal violet for 20 min. Carefully removed non-migrating cells from the upper chambers with a cotton swab. The migrated cells on the undersides of the upper chambers were then observed and photographed in five random fields under a
microscope, the number of migrated cells was quantified using Image J software. Significant differences in absorbance values were detected by CCK8 for cells that had migrated into the lower chamber.

Flow cytometric analysis

Cells were exposed to 0, 50, and 100 µM Rhein for 48 h. Subsequently, 5×10^5 cells were collected and resuspended in 500 µL of 1×Binding Buffer diluted in double-distilled water. Annexin V-APC (5 µL) and 7-AAD (10 µL) were added to each tube for staining, followed by gentle vortex mixing. The cells were then incubated at room temperature in the dark for 5 min before undergoing flow cytometric analysis using a BD instrument. Data analysis was performed using NovoExpress software.

Synergy determination with Synergy Finder

HL60 and HL60-ADR cells were seeded into 96-well plates at a density of 2 × 10^4 cells per well and treated according to the following protocol. Azacitidine(AZA) obtained from MedChemExpress and dissolved in DMSO at a concentration of 100 mM. Either single drug (AZA, Rhein) or combinations (AZA and Rhein) were analyzed at specified concentrations determined from the cytotoxicity assay mentioned earlier. The concentration gradient of AZA and Rhein was based on their respective IC50 values. Cell viability was assessed at a constant dilution ratio of the two inhibitors: Rhein concentrations (10, 20, 40, and 80 µM) and AZA concentrations for HL60 (0.062, 0.125, 0.25, and 0.5 µM) and HL60-ADR (10, 100, 200, and 500 µM). After 48 h of treatment, cell viability was measured using the CCK-8 Kit and a plate reader. The online SynergyFinder software (https://synergyfinder.fimm.fi) was utilized to calculate drug synergy scores using the response surface model and zero interaction potency (ZIP) calculation method. Drug combinations with ZIP Synergy scores greater than 0 were considered synergistic (red regions) (Aittokallio, Giri, & Ianevski, 2020; Ianevski, Giri, & Aittokallio, 2022). Heatmaps were generated to assess the therapeutic significance of the drug combinations based on their response patterns.

Statistical analyses

All data were analyzed using GraphPad Prism 8.0 software. Results are presented as mean ± SD, and statistical significance was determined using non-linear regression or one-way ANOVA. The standard t-test was utilized to evaluate the differences between two groups. All experiments were performed at least three times. A p-value of less than 0.05 was considered statistically significant, and the levels of significance are indicated as follows: ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05.

Results

High expression of FTO is associated with drug resistance in AML

We obtained bone marrow specimens from normal bone marrow transplant donors, as well as from AML individuals who were newly diagnosed, achieved complete remission (CR) after chemotherapy, and those with relapsed/refractory. These bone marrow specimens were all used secondarily after the completion of
clinical testing. Bone marrow mononuclear cells were extracted for Western blot analysis, revealing elevated FTO expression in AML patients, particularly in those with relapsed/refractory AML compared to normal samples (Fig. 1A). Clearly, relapsed/refractory cases are frequently associated with the development of multidrug resistance. In comparison to normal controls, AML cells including THP1, HL60, MV4-11 (with FLT3-ITD mutation), MOLM13 (with FLT3-ITD mutation), OCI-AML3 (with NPM1 and DNMT3A mutation), and K562 exhibited elevated FTO expression, with the lowest in THP1 and the highest in K562. Additionally, we compared FTO expression in HL60/K562 and HL60-ADR/K562-ADM, revealing higher FTO expression in the drug-resistant cell lines (Fig. 1A). Our findings demonstrate that FTO plays a pivotal oncogenic role in AML and occupies a significant position in AML drug resistance.

**Rhein inhibits AML cell proliferation by inhibiting FTO**

As depicted in Fig. 1B, Rhein was administered to HL60/K562 cells at concentrations of 0, 10, 20, 50, 100, and 200 µM, and to HL60-ADR/K562-ADM cells at concentrations of 0, 10, 20, 50, 100, 200, and 400 µM for 24, 48, and 72 h. The findings revealed that Rhein dose-dependently and time-dependently suppressed AML cell proliferation, including drug-resistant cells. Additionally, the IC50 values of Rhein exhibited comparable inhibitory effects on HL60 (IC50 = 60.99 µM)/K562 (IC50 = 40.54 µM) and their drug-resistant counterparts HL60-ADR (IC50 = 55.38 µM)/K562-ADM (IC50 = 69.2 µM) (Fig. 1C). Furthermore, we confirmed that both HL60-ADR and K562-ADM cell lines exhibited multidrug resistance, rather than being solely resistant to adriamycin. The results suggested that higher concentrations of Rhein could enhance the inhibition of FTO. The concentration gradient of Rhein for subsequent experiments will be determined based on the IC50 value obtained.

**Rhein inhibits the migration of AML cells**

The migration ability of AML cells, which greatly increases the likelihood of extramedullary invasion, is associated with poor therapeutic outcomes. HL60 and HL60-ADR cells were treated with Rhein at concentrations of 0, 50, and 100 µM for 24 h, resulting in a significant inhibition of AML cell migration. Staining with crystal violet revealed a dose-dependent reduction in the number of cells migrating to the underside of the chamber membrane as Rhein concentration increased. Subsequently, we collected the cells that had migrated to the lower chamber and performed CCK-8 analysis, revealing a gradual decrease in absorbance values with increasing Rhein concentration. As shown in Fig. 2A-D, the results confirm that Rhein effectively reduces the migration ability of both HL60 and HL60-ADR cells.

**Rhein induces apoptosis in AML cells by inactivating the AKT/mTOR pathway**

Following treatment with varying concentrations of Rhein for 48 h, flow cytometric analysis revealed that Rhein effectively induces apoptosis in AML cells, with the rate of apoptosis increasing in a dose-dependent manner (Fig. 3A). We conducted Western blot analysis to investigate the impact of Rhein on apoptosis in AML cells, and found that Rhein significantly reduced Bcl-2 protein expression while
increasing Bax levels in a dose-dependent manner (Fig. 3B). Additionally, Western blot analysis showed that Rhein inhibits FTO and suppresses the activity of the AKT/MTOR pathways in both HL60 and HL60-ADR cells (Fig. 3C).

**Synergistic anti-leukemia effects of Rhein and azacitidine**

AZA is utilized for the treatment of relapsed/refractory (RR) AML patients due to its incorporation into RNA, which leads to disruption of RNA metabolism and inhibition of DNA methylation (Müller & Florek, 2010). Previous studies have demonstrated that combining epigenetic manipulation with other novel drugs can increase the efficacy of hypomethylating agents (Thol & Ganser). We demonstrated that Rhein and AZA have synergistic effects against both HL60 and HL60-ADR cells in vitro experiments. Initially, we utilized a CCK-8 assay to measure the biological effect of AZA on both HL60 and HL60-ADR cells, resulting in dose-response curves (Fig. 4A). The results indicated that HL60-ADR was resistant to AZA, whereas the IC50 of HL60 was 0.9764 µM and the IC50 of HL60-ADR was 385 µM. Utilizing the new concentration gradient and corresponding inhibition index, we calculated the ZIP synergy scores using the online SynergyFinder software. The results indicated that the average proportion (and maximum) of the antitumor response attributable to the drug interaction were 15.45 (21.86) in HL60 cells and 12.29 (20.67) in HL60-ADR. As shown in Fig. 4B, the white rectangle indicates the region of maximum synergistic effect. Our analysis revealed that the combined treatment of Rhein and AZA demonstrated synergistic effects in inhibiting the proliferation of both HL60 and HL60-ADR cells. The concentration of Rhein used in the study was 10µM, which was found to be the lowest concentration encompassing the region of highest synergy. Our findings indicate that Rhein can synergistically enhance the cytotoxicity of AZA against AML cells, including MDR-AML cells, even when used at concentrations well below its IC50 value.

**Discussion**

The primary objective of AML therapy is to eliminate minimal residual disease, with patients achieving complete remission (CR) after initial therapy followed by consolidation and/or maintenance therapy. However, 10–40% of newly diagnosed patients fail to attain CR after conventional chemotherapy, and approximately 50% of those who initially achieve CR will subsequently develop relapsed AML (Thol & Ganser, 2020). Overcoming drug resistance remains a prominent challenge in AML treatment.

FTO, known as an obesity risk-associated gene and the first identified m6A eraser, has been found related to cancer drug resistance. Its high expression often leads to increased resistance and poor prognoses, with the Wnt/β-catenin pathway, NF-κB pathway, and STAT3 signaling being activated by FTO, thereby promoting tumor resistance to chemotherapy, radiation therapy, and anti-PD1 therapy (Takeshi Fukumoto et al., 2019; Yang et al., 2019; Zhou et al., 2018). Additionally, FTO can enhance resistance by reducing m6A levels in mRNA transcripts (T. Fukumoto et al.; Lin et al.; Ou et al.). In contrast, Takeshi Fukumoto found that downregulation of FTO and ALKBH5 was sufficient to increase FZD10 mRNA m6A modification and reduce PARPi sensitivity, which correlated with an increase in homologous
recombination activity (T. Fukumoto et al.). Our study demonstrates the critical oncogenic role of FTO in leukemogenesis and chemotherapy resistance. We observed that compared to normal patients, higher expression of FTO in the bone marrow of AML patients, particularly those with relapsed/refractory AML, and these results were further confirmed in AML cell lines.

The conventional cytotoxic chemotherapy-based treatment regimen in AML is associated with numerous adverse reactions, and approximately 50% of patients experience recurrence. Therefore, there is an urgent need to develop new targeted therapy drugs with low toxicity and high efficacy for the treatment of relapsed AML patients. Rhein, a small molecule inhibitor of FTO, effectively reduces the proliferation of AML cells in a dose- and time-dependent manner. We observed that the IC50 of Rhein in both parental and resistant AML cells was close, While adriamycin-resistant cell HL60-ADR were about 400-fold more resistant to azacitidine than the parental cell HL60. The resistant AML cells are characterized by high expression of the first member of the ABC transport family ABCB1, that exhibits a broad substrate spectrum and can transport various drug molecules (Holohan, Van Schaeybroeck, Longley, & Johnston, 2013). We speculate that this may be due to the competitive binding of Rhein to the active site of FTO, which prevents ABCB1 from pumping out Rhein like it does with other drugs. Furthermore, FTO has been shown to be correlated with the expression of ABCC10 (Xiao et al.). Our study demonstrates the benefits of Rhein in relapsed and refractory AML, which further supports its potential use in the treatment of relapsed and refractory AML.

In our investigation of drug-resistant AML cells, we observed rapid migration of these cells within the bone marrow following drug administration. Recent studies have confirmed that drug-resistant cells "run fast rather than hide", this finding challenges the prevailing theory that drug-resistant cells remain hidden in the bone marrow, suggesting that migration was closely increased in drug-resistant cell (Hawkins et al.). We confirm the migration is inhibited by Rhein with a dose-dependent manner in parent and resistant AML cells, this may be a novel target for reversing drug resistance. Furthermore, recent reports have indicated the upregulation of AKT/mTOR in AML, highlighting its potential as a therapeutic target. Activation of the mTOR pathway has been shown to enhance the invasive ability of AML cells(Deng et al., 2016) and reduce apoptosis (Rahmani et al., 2018; Wang et al., 2023). Therefore, we performed migration experiments and discovered that Rhein inhibits migration and increases apoptosis in leukemia cells, including MDR-AML cells.

AZA is commonly used for the treatment of relapsed, refractory, and elderly AML patients (Thol et al., 2015). Combining AZA with histone deacetylase inhibitors can potentially improve treatment efficacy by modulating the methylation and acetylation states of silenced genes (Topper et al., 2017). Our findings indicate that once leukemia cells become resistant to one drug, they are likely to develop multidrug resistance. For instance, HL60-ADR cells, which are resistant to adriamycin, were also found to be resistant to the DNA demethylation drug AZA. The treatment of AML is a complex process, and combination therapy has been shown to offer greater benefits to patients. Our study examined the combination of Rhein and a DNA methylation drug and found that it had a similar inhibitory effect on both HL60 and HL60-ADR cells. By using Rhein at low concentrations, it can enhance the anti-tumor
effect of azacitidine. Furthermore, our results indicate that Rhein is effective even in resistant cells, suggesting its potential as an ideal therapeutic option for AML patients, especially for patient who can't benefit from intensive regimen therapy.

Our findings suggest that Rhein is a potent inhibitor for studying the effects of RNA methylation on cancer progression and multidrug resistance. In the future, Rhein may be further modified or combined with other chemotherapy drugs to enhance its efficacy. Furthermore, preclinical primary safety evaluations and pharmacokinetic studies of Rhein are urgently needed. Combination therapy using targeted FTO inhibitors and other AML chemotherapy drugs may also hold great promise for the treatment of AML, and is thus a direction worth exploring.

Declarations

Author Contribution

All authors contributed to the research conception and design. Experimental operations, data collection and analysis were performed by Zhang S, Zhou L, Zhao L and Cheng J. The first draft of the manuscript was written by Zhang S and all authors commented on previous versions of the manuscript. Zhang S, Lu J, Yang J, Tao L, and Feng Y prepared figures 1-4. All authors read and approved the final manuscript.

References


**Figures**
Expression of FTO in AML and Rhein inhibits AML cell proliferation. (A) Expression of FTO in AML bone marrow and different cell lines. CR: achieved complete remission after chemotherapy. RR-AML: Relapse and refractory AML (B) Rhein inhibits proliferation of AML cells: After AML cells were treated with different concentrations of Rhein for 24 h, 48 h and 72 h. (C) IC50 of Rhein on HL60/K562 and HL60-ADR/K562-ADM after treatment of Rhein for 48h.
Figure 2

Rhein inhibits the migration and induces apoptosis in AML cell. (A, B) HL60 were incubated for 24 h with Rhein (0, 50 and 100 μM) and inhibits cell migration in a dose-dependent manner. (C, D) HL60-ADR were incubated for 24 h with Rhein (0, 50 and 100 μM) and also inhibits cell migration in a dose-dependent manner. The number of cells migrating to the under-side of up chamber membrane and absorbance values detected by CCK8 for cells falling into the lower chamber.
Figure 3

Rhein induces AML cell apoptosis, suppresses AKT/mTOR signaling. (A) Rhein (0, 50 and 100 μM) induces AML cell apoptosis. (B) Rhein have a concentration dependence inhibition of FTO expression, reduce BCL-2 expression and increase BAX expression. (C) Rhein suppresses AKT/mTOR signaling.
Figure 4

Rhein and AZA have synergistic effects. (A) IC50 of AZA on HL60 and HL60-ADR cells after treatment with different concentrations for 48h. (B) Heatmaps of drug combination responses. Rhein and AZA act synergistically on HL60 and HL60-ADR cells.