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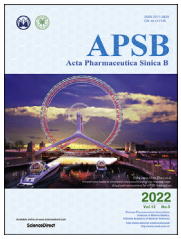

LETTER TO THE EDITOR

Discovery of ARF1-targeting inhibitor demethylzeylasteral as a potential agent against breast cancer

KEY WORDS

ADP-ribosylation factor 1;
Breast cancer;
Demethylzeylasteral;
Virtual screening

To the Editor:

ADP-ribosylation factor 1 (ARF1) plays a critical role in regulating vesicle formation and transport[1]. The dysregulation of ARF1 expression and/or activity is involved in many human cancers, such as breast cancer[2,3]. Therefore, ARF1 is one of the promising therapeutic targets for cancer treatment.

However, as a small G protein, ARF1 has a relatively smooth surface and lacks cavities suitable for small-molecule binding, thus being considered "undruggable"[4,5]. Nevertheless, many efforts have been made to develop pharmacological approaches to inhibit ARF1 activity. These reported modulators (listed in Supporting Information Table S1) block ARF1 activity mainly by disturbing the function of GEF or by disrupting the interaction between GEF and ARF1, and have not yet been developed to the pre-clinical stage. Hence, it remains an unmet medical need and highly challenging to discover more selective and more effective small molecules as ARF1 inhibitors.

Herein, starting with sequence-based AI virtual screening, followed by biochemical methods, we found that the natural product demethylzeylasteral (DMZ), a triterpenoid monomer extracted from *Tripterygium wilfordii* Hook F, has the potential to inhibit ARF1 activity. The highlights of the study are outlined as follows:

1. Sequence-based AI virtual screening and biochemical methods discovered DMZ as an ARF1 inhibitor

SPECS library and in-house natural product library were used for AI scoring and sorting (Fig. 1A). The top 0.5% candidates were subject to further validation, followed by clustering automatically. Then 51 candidates were tested by protein thermal shift (PTS) assay. Among them, Bavachin and DMZ induced Tm shifts for −0.5 and −3.5 °C, respectively (Fig. 1B and C). Besides, we found that DMZ has a better inhibitory potency on ARNO-catalyzed exchange of GDP for MANT-GTP (Fig. 1D) and its inhibition effect was concentration-dependent (Fig. 1E), thus DMZ was chosen for further research. Since the crystal structure of ARF1 is available in PDB database, we compared the traditional structure-based strategy with the sequence-based AI strategy and found that the latter can rapidly identify DMZ to be a hit compound with little computational time (Supporting Information Table S2).

2. Biophysical and biochemical experiments demonstrated the direct binding between DMZ and ARF1

We performed nuclear magnetic resonance (NMR) and surface plasmon resonance (SPR) experiments to investigate the interaction between ARF1 and DMZ. In the NMR experiment, the addition of ARF1 bring about a concentration-dependent attenuation of signal in CPMG spectra (Fig. 1F), SPR data showed that there was a binding between DMZ and ARF1 protein with kinetics K_D of 5.68 μmol/L and affinity K_D of 6.77 μmol/L (Fig. 1G). We also evaluated the binding ability of DMZ to other members of the ARF family (ARF5 and ARF6) using SPR assay. The results indicated that the binding ability of DMZ to these two ARF

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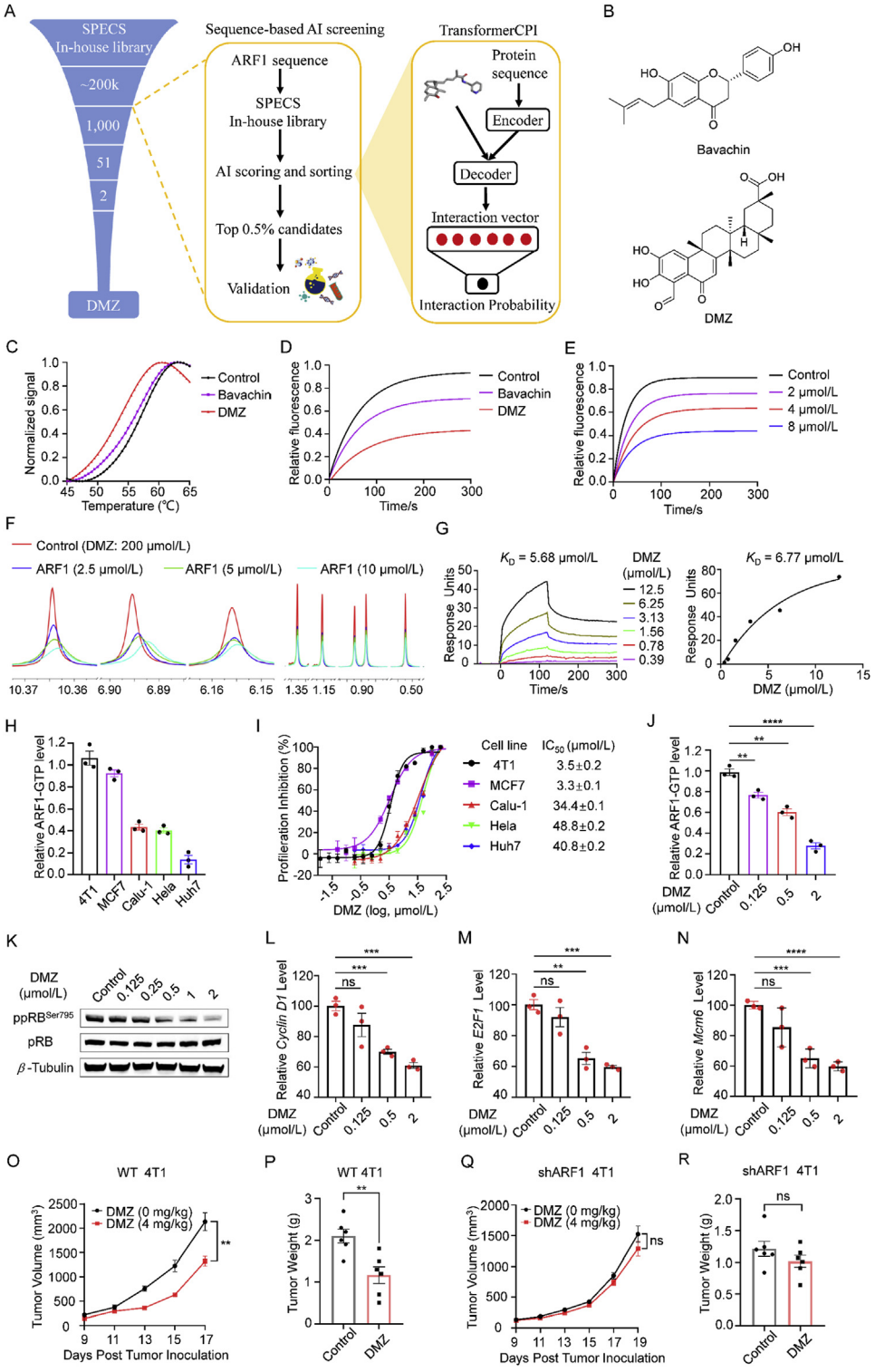

Figure 1 Discovering ARF1-targeting inhibitor demethylzeylasteral for breast cancer treatment with AI. (A) Flow chart for small molecule inhibitors screening of ARF1 protein in our drug center. (B) Chemical structures of bavachin and DMZ. (C) The thermal melting curve of ARF1 protein (2.5 μmol/L) treated with bavachin or DMZ (50 μmol/L). (D) The inhibitory effect of bavachin or DMZ on ARF1 nucleotide exchange at a concentration of 10 μmol/L. (E) The inhibition of DMZ on ARF1 nucleotide exchange was concentration-dependent. (F) NMR spectra for DMZ (red), DMZ in the presence of ARF1 at 2.5 μmol/L (blue), 5 μmol/L (green), and 10 μmol/L (cyan). (G) SPR measurement verified the direct binding of DMZ to ARF1 with kinetics K_D of 5.68 μmol/L and affinity K_D of 6.77 μmol/L. (H) Active levels of ARF1 in different cancer cells. (I) Inhibition of proliferation on different cancer cells by DMZ. (J) The levels of ARF1-GTP in 4T1 cells treated with DMZ. (K) Phosphorylated pRB (Ser795) levels in 4T1 cells treated with DMZ. (L–N) mRNA levels of *Cyclin D1*, *E2F1* and *Mcm6* in 4T1 cells treated with DMZ. (O) Tumor growth trend of BALB/c nude mice receiving 4T1 cells. (P) Tumor weight of BALB/c nude mice receiving 4T1 cells was measured on Day 17. (Q) Tumor growth trend of BALB/c nude mice receiving ARF1-knockdown 4T1 cells. (R) Tumor weight of BALB/c nude mice receiving ARF1-knockdown 4T1 cells was measured on Day 19. Bars, SEM; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

proteins was weaker than that to ARF1 protein (Supporting Information Fig. S1A and S1B). Taken together, these biophysical data validate that DMZ can directly binds to ARF1 with selectivity (to some extent).

Then, docking based molecular simulation was performed and the results showed that DMZ may unexpectedly occupy the GDP/GTP binding pocket (Supporting Information Fig. S2A). DMZ can make hydrogen bonds and three salt bridges in the binding pocket (Figs. S2B and S2C) with the docking score −7.744 kcal/mol. We further mutated key residues (GLY29, LYS30 and LYS127) of the binding pocket into ALA for analysis, and found that the docking score significantly dropped to −5.619 kcal/mol (Fig. S2D). Besides, we conducted molecular dynamics (MD) analysis for 100 ns. As a result, DMZ and ARF1 can form stable conformation with ΔRMSD<1 Å, while the RMSD of DMZ and mutated ARF1 increased gradually, suggesting the unstable binding between them (Fig. S2E). These results provide evidence that the binding pocket we proposed is reasonable. To confirm the binding site of DMZ, we attempt to purify the mutated ARF1 protein but unfortunately failed. We then turned to analyze the impact of DMZ on wild type ARF1 thermostability in present or absence of GTP. Preincubation with GTP attenuated the impact of DMZ on ARF1 thermostability, suggesting that DMZ competes with GTP, which is in line with our docking results (Fig. S2F). Combining the above conclusions with the sequence alignment of ARF family proteins (Supporting Information Fig. S3), we speculated that the high sequence identities of ARF family members and the conservation of GDP/GTP binding pocket might be the main reasons why DMZ is not particularly high selective for ARF1.

3. DMZ inhibits the proliferation of breast cancer cells in an ARF1 activation-dependent manner

Studies have shown that elevated ARF1 activity plays a key role in the hyper-proliferative behaviors of breast cancer cells[2]. GLISA result revealed that there was a much higher active status of ARF1 in 4T1 and MCF7 breast cancer cells compared with other tested cancer cells (Fig. 1H). In cytotoxicity experiment, 24 h DMZ treatment resulted in remarkable growth suppression of 4T1 and MCF7 cells with approximately 10 times stronger inhibitory effect than other cancer cell lines, indicating its great selectivity (Fig. 1I; Supporting Information Fig. S4). Therefore, 4T1 cells were selected in subsequent studies considering it has the highest ARF1-GTP level among these cancer cells. DMZ dose-dependently reduced the ARF1-GTP levels in 4T1 cells (Fig. 1J) that is consistent with its anti-proliferative effect. In summary, these data suggest that DMZ has obvious inhibitory activity toward the proliferation of breast cancer cells in an ARF1 activation-dependent manner.

4. DMZ inhibits hyperphosphorylation of pRB and its downstream pathway by targeting ARF1

As previously reported[2], the activation status of ARF1 is of great significance for breast cancer cell cycle progression, and ARF1 depletion or inactivation results in breast cancer cell growth arrest by modulating hyperphosphorylation of pRB and its association with E2F1, so that E2F1 fails to act on promotion of target genes transcription and initiation of cell-cycle progression. As expected, DMZ remarkably decreased phosphorylation of pRB (ppRB) and E2F target genes mRNA level in a concentration-dependent manner (Fig. 1K−N). Taken together, DMZ inhibited pRB hyperphosphorylation and its downstream pathway by blocking ARF1 activity, which resulted in proliferation inhibition of breast cancer cells.

5. DMZ inhibits tumor growth in the 4T1 cell transplanted tumor mouse model in an ARF1-dependent manner

We established an animal model by subcutaneously injecting 4T1 cells into BALB/c nude mice to evaluate the therapeutic effects of DMZ in breast cancer *in vivo*. Breast tumor growth was significantly inhibited in DMZ-treated mice (Fig. 1O and P, Supporting Information Fig. S5A). Remarkably, DMZ could not suppress tumor growth in ARF1-knockdown 4T1 cell transplanted tumor mouse model (Fig. 1Q and R, Figs. S5B and S5C). There was no noticeable difference of body weight change between the two groups (Figs. S5D and S5E), indicating that DMZ did not cause serious side effects for antitumor efficacy studies at a dose of 4 mg/kg. Taken together, these results suggested that DMZ could suppress 4T1 cell transplanted tumor growth in an ARF1-dependent manner.

In conclusion, our study demonstrates that DMZ remarkably suppresses breast cancer cell proliferation both *in vitro* and *in vivo* by directly targeting ARF1 and possesses the potential to be further developed. This fills a knowledge gap in the understanding of the DMZ target and ARF1 mediated breast cancer cell proliferation.

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Author contributions

Conception of the hypothesis: Mingyue Zheng, Sulin Zhang; Study supervision: Kaixian Chen, Hualiang Jiang, Mingyue Zheng and Sulin Zhang. Development of methodology: Jie Chang, Ruirui Yang and Lifan Chen. Acquisition of data: Jie Chang, Ruirui Yang, Lifan Chen, Zisheng Fan, Hao Guo, Yinghui Zhang, and Yadan Liu. Analysis and interpretation of data: Jie Chang, Ruirui Yang, Lifan Chen, Zisheng Fan, Hao Guo, Yinghui Zhang, Jingyi Zhou, Guizhen Zhou, and Keke Zhang. Writing, review, and/or revision of the manuscript: Jie Chang, Ruirui Yang, Lifan Chen, and Sulin Zhang. All authors discuss the study. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

All authors declare no competing interests.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2022.02.011.

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