Triptolide Induces the Expression of miR-142-3p: A Negative Regulator of Heat Shock Protein 70 and Pancreatic Cancer Cell Proliferation

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Abstract

Pancreatic ductal adenocarcinoma (PDAC), one of the deadliest malignancies, is resistant to current chemotherapies. We previously showed that triptolide inhibits PDAC cell growth in vitro and blocks metastatic spread in vivo. Triptolide downregulates HSP70, a molecular chaperone upregulated in several tumor types. This study investigates the mechanism by which triptolide inhibits HSP70. Because microRNAs (miRNA) are becoming increasingly recognized as negative regulators of gene expression, we tested whether triptolide regulates HSP70 via miRNAs. Here, we show that triptolide as well as quercetin, but not gemcitabine, upregulated miR-142-3p in PDAC cells (MIA PaCa-2, Capan-1, and S2-013). Ectopic expression of miR-142-3p inhibited cell proliferation, measured by electric cell-substrate impedance sensing, and decreased HSP70 expression, measured by real-time PCR and immunoblotting, compared with controls. We showed that miR-142-3p directly binds to the 3′UTR of HSP70, and that this interaction is important as HSP70 overexpression rescued miR–142-3p-induced cell death. We found that miR-142-3p regulates HSP70 independently of heat shock factor 1. Furthermore, Minnelide, a water-soluble produg of triptolide, induced the expression of miR-142-3p in vivo. This is the first description of an miRNA-mediated mechanism of HSP70 regulation in cancer, making miR-142-3p an attractive target for PDAC therapeutic intervention. Mol Cancer Ther; 12(7); 1266–75. ©2013 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), one of the leading causes of cancer-related death in the United States (1). At best, the 5-year survival rate for patients with localized PDAC, more likely candidates for surgical resection than those with metastatic disease, is 20%; however, when considering PDAC patients as a whole, including those with metastatic disease, the survival rate is only 5–6% (1). Over the past several decades, these survival rates have not improved due, in large part, to aggressive growth, metastasis, and drug resistance of PDAC cells (1). Efforts are ongoing to understand the pathobiology of PDAC and to develop innovative and effective therapies. An important part of this process is to understand the mechanism of action of potential chemotherapeutic agents.

Triptolide, a diterpene triepoxide extract from the Chinese herb *Tripterygium wilfordii*, has been shown to inhibit PDAC cell viability in vitro (2, 3) and to block growth and metastatic spread in vivo (4). To date, in vivo studies have shown that triptolide inhibits the growth of cholangiocarcinoma cells in hamsters (5) and xenografts of human melanoma, breast cancer, bladder cancer, and gastric carcinoma in nude mice (6). Previous data from our laboratory have shown that triptolide inhibits the growth of neuroblastoma cells in vitro (7) and prevents tumor growth in vivo (8).

Because triptolide was identified in a small-molecule screen to inhibit tumorigenic molecular chaperone HSP gene transcription (9), our laboratory has continued to show that triptolide, likewise, inhibits cancer cell proliferation while concurrently inhibiting HSP70 expression in PDAC cells (2–4, 10) or neuroblastoma cells (11). Some studies suggest that triptolide inhibits the transcription factor heat shock factor 1 (HSF1) and, in this way, suppresses HSP70 transcription (9); however, as microRNAs (miRNA) become increasingly recognized as major negative regulators of gene expression, we asked whether triptolide may regulate HSP70 expression via miRNAs.

In cancer cells, the dysregulation of miRNAs expression serves as an efficient means to rewire the cell gene expression map and generate a cancer phenotype. In particular, the loss of tumor-suppressive miRNAs upregulates oncogenic targets (12). No previous reports have examined...
the effect of triptolide on the PDAC miRNAome nor evaluated miRNA-mediated regulation of HSP70 in PDAC cells. Consequently, the aims of this study are to: (i) examine the effect of triptolide on the miRNAome of PDAC cells in vitro and verify this in vivo; (ii) evaluate whether miRNAs upregulated by triptolide play a tumor-suppressive role in PDAC; and (iii) validate that predicted miRNAs regulate HSP70 independent of the HSF1 pathway. We hypothesize that triptolide will upregulate tumor-suppressive miRNAs, which directly regulate HSP70. This is the first evaluation of an miRNA-mediated mechanism of HSP70 regulation in cancer.

Materials and Methods

Cell culture and drug treatment

Cells from the MIA PaCa-2, Capan-1, and HEK-293 lines were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies) containing 10% FBS (Life Technologies). S2-013 cells were kindly provided by Professor Masato Yamamoto (University of Minnesota at Minneapolis, Minneapolis, MN) and cultured in RPMI medium (Life Technologies) containing 10% FBS. Multiple aliquots of cells were cryopreserved when initially grown. All the cell lines were used within 6 months of resuscitation. No authentication was done by the authors, but ATCC authenticates cells using short months of resuscitation.

miRNA expression profiling

miRNA was isolated using the mirVana RNA Isolation Kit (Ambion) and quantified using a Nanodrop spectrophotometer (Thermo Fisher). RNA quality (RNA index number of ≥5) was verified by an Agilent 6000 nanochip (Agilent) before miRNA analysis using miRNA BeadArrays (Illumina). Arrays were imaged using an Illumina BeadArray Reader, and the fluorescent intensity of the electrode. MIA PaCa-2 or Capan-1 cells (6 × 10⁵ cells/well) and S2-013 (1 × 10⁵ cells/well) were plated in 8-well, gold-film electrode-coated 10E+ arrays (Applied Biophysics). Proliferation rates were normalized to 6 hours following transfection.

Dual-Luciferase reporter assay and 3’UTR binding-site mutagenesis

HEK-293 cells were seeded in 24-well plates (6 × 10⁴ cells/well). Mastermixes were diluted in serum-free media (Life Technologies) containing Attractene, pGL4.73 control vector expressing firefly luciferase (Promega), GoClone (HSPA1B) containing the wild-type HSPA1B 3’UTR expressing renilla luciferase (SwitchGear Genomics), miR-142-3p mimic, or NC (Thermo Scientific). Mutagenesis was done using the QuickChange Site-Directed Mutagenesis Kit (Agilent Stratagene). The Dual-Luciferase Reporter Assay System (Promega) was used on a Synergy2 luminometer (BioTek).

Transfection of HSPA1B (HSP70) or HSF1 ORF vector

Cells were seeded in 24-well plates (1.5 × 10⁴ cells/well) and were incubated overnight before transfection. Transfection mastermixes were diluted in Opti-MEM containing Attractene, HSPA1B (HSP70 isoform) ORF, HSF1 ORF, or NC (GeneCopoeia), together with miRIDIAN reagents (Thermo Scientific Dharmaco). To each well was added 400 ng of either plasmid together with transfection of miRIDIAN reagents.

Transfection of HSF1 siRNA

Cells were seeded in 6-well plates (8.0 × 10⁴ cells/well) and were incubated overnight before transfection. Transfection mastermixes diluted in Opti-MEM
containing HiPerFect (Qiagen), HSF siRNA, or non-silencing siRNA (Qiagen) at a final concentration of 25 nmol/L.

Measurement of miR-142-3p and HSPA1B (HSP70) levels from human xenograft PDAC tumor model

Three de-identified human pancreatic tumors were implanted subcutaneously into female severe combined immunodeficient (SCID) mice (The Jackson Laboratory, Bar Harbor, ME). When tumor volumes reached 500 mm³, tumors were dissected, cut into 10 mm³ pieces, and propagated in additional SCID mice (1 animal per tumor, n = 3 animals). Strict animal care procedures from the University of Minnesota Institutional Animal Care and Use Committee were followed. Animals were randomized and tagged before daily intraperitoneal (i.p) injections of Minnelide (0.42 mg/kg) or saline for 7 days. Mice were sacrificed, and tumors were stored in RNA-later (Qiagen). Samples were homogenized in 1 mL of TRIzol (Life Technologies). Gene levels were analyzed by RT-PCR.

Statistical analysis

Values are expressed as the mean ± SEM. All experiments with cells were repeated at least thrice. The significance of the difference between the control and each experimental test condition was analyzed by unpaired Student t test and a value of P < 0.05 was considered statistically significant.

Results

Triptolide alters the miRNAome of PDAC cells, in particular, inducing miR-142-3p expression

Previous data from our laboratory have shown that triptolide induces PDAC cell death in vitro and in vivo, while concurrently downregulating HSP70 expression (2–4, 10). Triptolide was identified as an HSP70 inhibitor by virtue of its ability to inhibit its main transcription factor, HSF1 (9). Given that miRNAs are becoming increasingly recognized as major negative regulators of gene expression (12), triptolide-induced upregulation of HSP70-targeting miRNAs was studied. We treated MIA PaCa-2 and S2-013 cells with triptolide and screened the collected miRNAs by microarray. These cell lines were selected because triptolide induces different mechanisms of cell death in these primary and secondary (metastatic) tumor types, respectively (3). A 100 nmol/L concentration of triptolide was selected, for all studies discussed in this article, because this concentration has been shown to inhibit cell viability of both MIA PaCa-2 and S2-013 by 40% or 70% after 24 or 48 hours of treatment, respectively (3). Time points as early as 6 hours following triptolide treatment were selected to observe early miRNAome changes linked to proliferative pathways. Later time points were evaluated to verify that changes were sustained. In MIA PaCa-2 cells, 15 miRNAs significantly changed (10 upregulated and 5 downregulated; P < 0.05; Fig. 1A). In S2-013 cells, 14 miRNAs significantly changed (10 upregulated and 4 downregulated; P < 0.05; Fig. 1A). Principal component analysis of the resulting dataset shows that miRNA transcript levels changed linearly in both cell lines (Supplementary Fig. S1). Over the time-course of triptolide treatment, upregulation of miR-142-3p was 1 of the most significant changes in both cell lines (8–9-fold at 24 hours; Supplementary Table S1). This induction of miR-142-3p was validated in a third cell line, Capan-1 (Fig. 1B). Furthermore, independent validation of miRNA microarray was done by RT-PCR (Supplementary Fig. S2). In addition, the levels of those miRNAs known to play a role in PDAC, such as miR-155, miR-21, and miR-221 (16–18), were not observed to change in response to triptolide (Supplementary Fig. S3 and Supplementary Table S2). These results show that triptolide alters the miRNAome of PDAC cells, notably by increasing miR-142-3p.

Quercetin, but not gemcitabine, also induces miR-142-3p expression

To evaluate whether the induction of miR-142-3p was unique to HSP70-inhibiting compounds such as triptolide, we tested whether other chemotherapeutic agents upregulated miR-142-3p. As studies in our laboratory have previously shown that quercetin (100 µmol/L) inhibited HSP70 protein levels and decreased MIA PaCa-2 cell viability by 50% after 24 hours (13), we tested whether quercetin likewise induced miR-142-3p. To address this, we treated PDAC cells with quercetin and assayed for miR-142-3p expression via RT-PCR. Expression of miR-142-3p was upregulated 3-fold by quercetin in MIA PaCa-2 and S2-013 cells and upregulated 8-fold in Capan-1 cells (Fig. 1B). To test whether the induction of miR-142-3p may be common among other chemotherapeutic agents, we measured miR-142-3p levels following gemcitabine treatment. We selected 1 µmol/L gemcitabine treatment because it has been shown to inhibit MIA PaCa-2 (14, 19) viability by at least 50% after 72 hours. This concentration allows us to evaluate early changes in the miRNAome directly linked proliferative pathways. Gemcitabine treatment did not alter miR-142-3p levels (Fig. 1B). These results show that miR-142-3p induction is common among HSP70 inhibitors triptolide and quercetin but is not present with the nucleoside analogue gemcitabine.

Triptolide and ectopic expression of miR-142-3p inhibit PDAC cell proliferation

Because triptolide induced the expression of miR-142-3p (Fig. 1) and inhibited cell viability in MIA PaCa-2, Capan-1, and S2-013 cells (3), we verified whether triptolide inhibits proliferation in these cell lines using the ECIS assay, an established method to test cancer cell proliferation in real time (20). Due to the insulating properties of cell membranes, the measured impedance increases with accumulating coverage of the electrode. We found that triptolide treatment inhibited proliferation of MIA PaCa-2, Capan-1, and S2-013 as early as 15 hours following treatment (Fig. 2A). To test whether ectopic expression of miR-142-3p was
playing a tumor-suppressive role in PDAC cells, we measured PDAC cell proliferation rates following miR-142-3p overexpression. Likewise, we found that overexpression of miR-142-3p inhibited proliferation of MIA PaCa-2, Capan-1, and S2-013 as early as 15 hours following transfection (Fig. 2B). These data show that miR-142-3p and triptolide both suppress PDAC cell proliferation.

Upregulation of miR-142-3p inhibits HSPA1B (HSP70) expression

We tested whether ectopic expression of miR-142-3p inhibited HSPA1B (HSP70) expression for several reasons: first, our lab has previously shown that triptolide inhibited HSPA1B (HSP70) mRNA expression by 74% or more (Fig. 3A); and third, miR-142-3p is predicted to target the HSPA1B isoform of HSP70 by 3 independent computational prediction programs (miRDB updated 4.2012, TargetScanHuman Release 6.2, MicroCosm Targets Version 5). Overexpression of the miR-142-3p mimic (5 nmol/L) inhibited at least 31% of HSPA1B (HSP70) mRNA expression following 24 hours of transfection (Fig. 3A). Similarly, overexpression of miR-142-3p inhibited total HSP70 (both HSPA1B and HSPA1A) protein expression by at least 50% following transfection for 72 hours (Fig. 3B). These results show that ectopic expression of miR-142-3p decreases HSPA1B (HSP70) mRNA and protein levels. Further, pancreatic cancer cells do not
compensate for the loss in HSPA1B expression, induced by miR-142-3p, by upregulating the HSPA1A isoform of HSP70 (Supplementary Fig. S4).

**MiRNA-142-3p directly binds to the 3'UTR of HSPA1B (HSP70)**

Three independent programs (miRDB updated 4.2012, TargetScanHuman Release 6.2, MicroCosm Targets Version 5) predict that the miR-142-3p binding site is located within the HSPA1B (HSP70) 3'UTR (Fig. 4A). To verify this interaction, a double-point mutation (C–G and T–A) was inserted into the predicted binding site of HSPA1B (HSP70), preventing the miR-142-3p seed sequence from interacting with this region (Fig. 4A). Overexpression of miR-142-3p decreased the renilla-to-firefly ratio to 43% of control (Fig. 4B). Upon mutating the miR-142-3p binding site of the renilla-expressing construct, the renilla-to-firefly ratio was rescued to 92% of control (Fig. 4B). These results support the prediction that miR-142-3p regulates HSPA1B (HSP70) transcript levels by binding to its 3'UTR.

**HSPA1B (HSP70) is a functional target of miR-142-3p in PDAC cells**

Because 1 miRNA may control the expression of many targets within the cell (12), we evaluated whether miR-142-3p was targeting HSPA1B (HSP70) as a means to control PDAC cell viability. To test this, we measured whether the loss in cell viability induced by miR-142-3p could be rescued by HSPA1B (HSP70) ORF overexpression. This construct was used because it lacks the miR-142-3p binding site. Following 24 hours of transfection, HSPA1B (HSP70) was upregulated 13 ± 10^3-fold in MiaPaCa-2 cells overexpressing miR-142-3p and upregulated 8 ± 10^3-fold upregulation in control cells (Fig. 4C). Following 48 hours of transfection, miR-142-3p caused cell viability to decrease to 56% of control, but cotransfection of HSPA1B (HSP70) ORF rescues this level to 77% of control. Because a significant, but not complete, rescue was observed, miR-142-3p may be targeting other predicted downstream targets (Supplementary Table S3). These results show, however, that miR-142-3p is targeting HSPA1B (HSP70) in PDAC cells and that this interaction is important in regulating cell viability.

**HSF1 and miR-142-3p independently regulate HSPA1B (HSP70)**

We tested whether miR-142-3p regulation of HSPA1B (HSP70) was independent of HSF1 because our laboratory has shown that HSF1 inhibition decreases HSP70 expression (21). This was evaluated by measuring HSF1...
levels by RT-PCR in PDAC cells following ectopic expression of miR-142-3p. Although triptolide inhibited HSF1 mRNA expression (Fig. 5A), as well as downstream transcriptional targets HSPA1B (HSP70) [Fig. 3A] and HSP27 (Supplementary Fig. S5A), ectopic expression of miR-142-3p had no effect on either HSF1 or HSP27 levels (Fig. 5A; Supplementary Fig. S5A). These data show that miR-142-3p does not regulate HSF1, but these results do not address whether HSF1 controls miR-142-3P levels. To test this, we silenced HSF1 and measured miR-142-3p levels. Both HSF1 siRNA sequences independently inhibited HSF1 mRNA expression (Supplementary Fig. S5B). Levels of miR-142-3p did not change, compared with the control cells, following 24 hours of transfection (Fig. 5B).

HSF1 and miR-142-3p mediate triptolide-induced suppression of PDAC proliferation

HSF1 and miR-142-3p have each been shown to independently regulate HSP70 and to be inhibited or induced by triptolide, respectively. For this reason, we tested whether HSF1 and miR-142-3p were important in mediating triptolide-induced suppression of PDAC proliferation via HSP70 modulation. We found that simultaneous overexpression of HSF1 and inhibition of miR-142-3p significantly rescued from triptolide-induced cell death. The HSF1 ORF vector yielded sufficient overexpression (Fig. 5C). Triptolide deceases cell viability to 42% of control, but overexpression of HSF1 and inhibition of miR-142-3p rescues this to 63% of control (Fig. 5D). In addition, we tested whether these conditions could rescue from triptolide-induced loss of HSPA1B (HSP70) expression. Triptolide suppresses HSPA1B (HSP70) levels to 26% of control, but while concurrently overexpressing of HSF1 and inhibiting miR-142-3p, this is increased to 35% of control. Although this was expected, the difference was not statistically significant (Supplementary Fig. SSC). These data corroborate our results showing that HSPA1B (HSP70) is an important, although not the only, target of miR-142-3p (Figs. 3 and 4). Likewise, HSPA1B (HSP70) is one of several transcriptional targets of HSF1 (22). Both HSF1 and miR-142-3p play an important role in mediating triptolide-induced suppression of cell proliferation, each independently regulate HSPA1B (HSP70) expression but also regulate other targets (Fig. 5E).
Figure 4. miR-142-3p modulates HSPA1B (HSP70) expression by binding to its 3' UTR. A, schematic of HSPA1B (HSP70) mRNA showing predicted miR-142-3p interaction site (top). Seven-nucleotide interaction sequence between wild-type (wt) HSPA1B (HSP70)-3'UTR and miR-142-3p and mutant HSPA1B (HSP70)-3'UTR construct is shown (bottom). B, luciferase reporter assay using HEK-293 cells to show the direct interaction of miR-142-3p and the 3'UTR of HSPA1B (HSP70). After 24 hours, miR-142-3p mimic (10 nmol/L) reduced the ratio of renilla-to-firefly expression but not when the 3'UTR bears 2 point mutations in the miR-142-3p binding site. C, HSPA1B (HSP70) ORF (lacking the 3'UTR containing the miR-142-3p binding site) transfection causes overexpression of HSPA1B (HSP70) expression (as assessed by RT-PCR) in MIA PaCa-2. D, HSPA1B (HSP70) ORF overexpression rescued loss in cell viability caused by miR-142-3p (5 nmol/L) overexpression for 48 hours in MIA PaCa-2 cells. The bars represent mean ± SEM, n = 3; * or **, P < 0.05 (t test).
Among more than 200 reports evaluating the role of miRNAs in PDAC, only 2 evaluate the role of the mir-142 precursor, and none study miR-142-3p. In one study, SUIT-2, the cell line from which S2-013 was derived (26), and Capan-1 were studied to find miRNAs that were altered upon developing gemcitabine resistance. This report showed that gemcitabine-treated patients with high miR-142-5p and miR-204 expression had longer survival times than those with low expression (27). Interestingly, triptolide induces miR-204 expression, although not to the degree that it induces the expression of miR-142-3p (Fig. 1A). Another RT-PCR profiling study found that 100 miRNA precursors were aberrantly expressed in PDAC, including miRNAs previously reported as upregulated in other human cancers such as miR-155, miR-21, and miR-221 (16–18). The mir-142 precursor was found to be 15-fold downregulated in PDAC compared with normal pancreas (28). These findings support our hypothesis that miR-142-3p plays a tumor-suppressive role in PDAC cells. Although studying the biological role of miRNAs dysregulated in PDAC has proven informative, our findings support the importance of evaluating miRNA changes in PDAC following chemotherapy treatment to better understand key miRNAs that regulate proliferation.

Although this study is the first to evaluate the role of miR-142-3p in PDAC, more than 20 studies have evaluated the tumor-suppressive role of miR-142-3p in other cancer types. In miRNA profiling studies of acute myeloid leukemia patients, miR-142-3p was downregulated (29) and shown to regulate tumorigenic targets: CCNT2 and TAB2 (30). In hepatocellular carcinoma, miR-142-3p was downregulated as compared with normal liver and shown to target RAC1, a GTPase involved in cell growth, migration, and the activation of protein kinases (31). Because HSPA1B (HSP70) significantly, although not completely, rescued from miR-142-3p–induced cell death (Fig. 4D), and the miR-142-3p inhibitor somewhat, although not significantly, rescued from triptolide-
induced suppression of HSPA1B (HSP70) (Supplementary Fig. S5C), miR-142-3p may bind to additional targets in PDAC cells. There are a 9 other targets predicted by multiple algorithms (Supplementary Table S3). Testing whether mir-142-3p targets RLF rearranged L-myc fusion protein merits further investigation because it has been established as an oncogene in small-cell lung carcinoma (32). CCNT2, TAB2, RAC1 or RLT may be important miR-142-3p targets in PDAC, and this is worthy of future study. Our data support the hypothesis that miR-142-3p plays a tumor-suppressive role by regulating HSPA1B (HSP70) in PDAC (Figs. 3 and 4).

Although there are several cardiovascular and pulmonary studies evaluating miRNA regulation of HSP70, none have focused on HSPA1B isoform of (HSP70). Hsp70.3 (HSP2A isoform) has been shown to possess general cytoprotective properties in preventing ischemic damage. One study has shown that the Hsp70.3 (HSP2A) gene product is subject to miRNA regulation via miR-378 and miR-711 (33). In lung tissue, it has been shown that HSP70 is regulated by miR-146a and miR-146b-5b. These miRNAs were found to increase greatly, and inversely correlate with HSP70 levels, following treatment with gefitinib; this may contribute to pulmonary fibrosis (34). The results obtained in this study support an miRNA-mediated mechanism of HSP70 regulation independent of HSF1 (Fig. 5). Although HSF1 regulation of HSP70 has been well-documented (9, 21, 22), miRNA regulation of HSP70 merits further investigation.

Because miR-142-3p negatively regulates protumorigenic genes, it holds promise as a target for future PDAC therapeutic development. As with many miRNAs, miR-142-3p may play an opposite role in different cancer types. For example, miR-142-3p has been reported to be oncogenic and upregulated in human T-cell acute lymphoblastic leukemia (35). Although the majority of studies evaluating miR-142-3p in cancer show its tumor-suppressive role (29–31), those who further develop miR-142-3p as a therapy in PDAC will need to verify its tumor-suppressive role in prospective patients. Restoring repressed miRNA levels in patients holds promise because it has been shown to be feasible via systemic delivery of lipid nanoparticles carrying miR-34a, miR-143, and miR-145 in treating orthotopic PDAC tumors in vivo (36). This study was especially important as blood flow to the pancreas is thought to be low (37). Understanding an miRNA-mediated mechanism of triptolide action, especially the induction of miR-142-3p, will be useful as Minnelide moves into clinical trials. Moreover, miR-142-3p can be a target for future PDAC therapeutic development.

Disclosure of Potential Conflicts of Interest
The University of Minnesota has filed a patent for Minnelide, which has been licensed to Minneamrita Therapeutics LLC. Inventors on this patent include Selwyn Vickers and Ashok K. Saluja. Selwyn Vickers and Ashok K. Saluja have financial interests in this company. Minneamrita synthesis has been filed under patent WO/2010/129918. Selwyn Vickers and Ashok K. Saluja have an ownership interest (including patents) with Minneamrite (minor: $10,000 or less) and are associated in a consultant/advisory board capacity with Minneamrita Therapeutics LLC, relationship (major: $10,000 or more). No conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.N. MacKenzie, N. Mujumdar, V. Sangwan
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