Down-regulation of mitochondrial ATPase by hypermethylation mechanism in chronic myeloid leukemia is associated with multidrug resistance

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Background: To identify novel proteins involved in multidrug resistance in chronic myeloid leukemia (CML).

Materials and methods: Comparative proteomics was used to screen multidrug resistance-related proteins from K562 and K562/A02; the differently expressed proteins were further confirmed by western blot and real-time PCR. Short hairpin RNA (shRNA) assay was applied to determine the relationship between candidate protein and adriamycin resistance. Bisulfite sequencing was carried out to assess methylation status of candidate multidrug resistance-related gene promoter. K562/A02 was treated with 5-azacytidine or trichostatin A (TSA); multidrug resistance phenotype and corresponding protein or gene changes were detected.

Results: Seventeen proteins with altered abundances of more than twofold were detected, among which mitochondrial ATPase in K562/A02 was significantly down-regulated. Suppressing mitochondrial ATPase by shRNA could enhance adriamycin resistance and antiapoptosis activity of K562. The promoter hypermethylation in mitochondrial ATPase was found to be attributed to the adriamycin-resistant phenotype of both K562/A02 (methylation frequency 18.18%) and CML primary cells in accelerated phase (methylation frequency 7.95%) or blast crisis (methylation frequency 26.59%). Inhibition of hypermethylation increased adriamycin sensitivity of K562/A02. A synergistic effect on reversing adriamycin-resistant phenotype was obtained when 5-azacytidine was combined with TSA.

Conclusion: Down-regulation of mitochondrial ATPase can lead to adriamycin resistance in CML and the mechanism is associated with DNA methylation regulation.

Key words: chronic myeloid leukemia, methylation, mitochondrial ATPase, multidrug resistance, proteomics

Introduction

Adriamycin and its analogues daunomycin and mitoxantrone are widely used for treatment of hematologic malignancies, especially acute myeloid leukemia and chronic myeloid leukemia (CML) with blast crisis (BC). However, it is common for such patients to develop resistance to adriamycin. Although substantial improvement in patient survival may be obtained with the use of imatinib in some CML with BC cases, curative effect from imatinib is only minimal. It is mainly caused by the development of chemotherapy resistance, particularly multidrug resistance [1].

Various assumptions have been proposed to explain multidrug resistance. Some indicate that the mechanisms may involve P-glycoprotein (P-gp) [2], glutathione-s-transferase (GST) [3] and sorcin, which is a soluble resistance-related calcium-binding protein [4]. Obviously, elucidating the mechanisms at the molecular level is of great importance to the design of counter-resistance strategies.

Recently, down-regulation of mitochondrial ATPase expression has been reported in several solid tumors such as carcinomas of the liver, kidney, colon, esophagus, lung, breast and stomach [5-8]. These results not only provide compelling evidence that bioenergetic dysfunction of mitochondria is a hallmark of these types of tumors but also show that altered mitochondrial ATPase expression is attributed to 5-fluorouracil resistance in human colon cancer cells.

In searching for novel mechanisms involved in multidrug resistance and exploring the possible role of mitochondrial ATPase in the development of adriamycin resistance in leukemia, we carried out comparative proteomics and analyzed the differences in global protein expression in leukemia cell line K562 and its multidrug resistance counterpart, K562/A02 [9]. Our results indicate that mitochondrial ATPase down-regulation indeed plays an important role in adriamycin resistance in leukemia cells, possibly through DNA methylation regulation.
materials and methods

cell line and primary samples
Adriamycin-resistant CML cell line K562/A02 was provided by the Institute of Hematology, Tianjin, China, and K562 was from our laboratory. K562/A02 and K562 were maintained in RPMI-1640 (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (Gibco-BRL) in the presence and absence of 1 μg/ml adriamycin at 37°C in a humidified 5% CO2 atmosphere. Bone marrow samples were collected from 32 CML patients (10 for western blot and real-time PCR; 22 for methylation identification). Bone marrow mononuclear cells were isolated by Ficoll density gradient centrifugation. Confirmation of CML diagnosis was based on cell morphology and cytogenetic assays. All patients were positive on Philadelphia chromosome. In 22 patients for methylation assay, there were 9 in chronic phase (CP), 6 in accelerated phase (AP) and 7 in BC. The study was approved by our institutional review board and written informed consent was obtained from all patients.

flow cytometry analysis
P-gp expression on K562/A02 and K562 was evaluated using polyethylene-conjugated anti-P-gp antibody (BD Biosciences, San Jose, CA). Cell cycle progression was detected with BD FACScan flow cytometer (BD Biosciences) and analyzed with Cell Quest software (BD Biosciences).

two-dimensional gel electrophoresis-based comparative proteomics
Two-dimensional gel electrophoresis (2-DE) was carried out as previously described [10]. Briefly, 1 mg protein of K562/A02 or K562 was applied to 24-cm immobilized pH gradient strip (pH3-10L) (Amersham Biosciences, Uppsala, Sweden). The second-dimension separation used sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Blue silver staining was used to visualize the protein spots in gels [11]. PDQuest system (Bio-Rad Laboratories, Hercules, CA) was used for image analysis. Proteins with altered abundances of more than twofold were subjected to matrix-associated laser desorption ionization-mass spectrometry analysis for identification [10]. Briefly, protein spots were excised and gel pieces were incubated in 10-μl digestion solution with 20 μg/ml trypsin (Promega, Madison, WI) for 10–12 h at 37°C. The trypptic peptide mixture was mixed with t-cyano-4-hydroxyamic acid matrix solution (Sigma-Aldrich, St Louis, MO). One microliter of the mixture was analyzed by Voyager System DE-STR 4307 matrix-assisted laser desorption/ionization time of flight mass spectrometer (Applied Biosystems, Foster City, CA). Database searches with monoisotopic peptide masses were carried out against the SwissProt or NCBInr database with Mascot search engine. Mass spectrometer (Applied Biosystems, Foster City, CA). Database searches with monoisotopic peptide masses were carried out against the SwissProt or NCBInr database with Mascot search engine.

RNA isolation and real-time PCR
Total RNA was isolated from 5 × 106 cells using Trizol (Gibco-BRL) following manufacturer’s protocol. Five micrograms of RNA was reverse transcribed into complementary DNA using A3500 Reverse Transcription System (Promega). Real-time PCR was carried out on Roche LightCycler system (Roche Diagnostics, Mannheim, Germany) using DyNaMo SYBR® Green qPCR Kit (FINNZYMES, Espoo, Finland). Primer for mitochondrial ATPase β subunit was as follows: sense, 5′-TGGTTGGTGTGCTGGACTTGG-3′; antisense, 5′-GTGCCTGCTGCTGATGACTTG-3′; the following primers were used—round I: sense, 5′-ACCCAATAAACCTACTCTACCAAG-3′; antisense, 5′-AGATAGGGTAAAATATATTTAGGAAAT-3′; and round II: sense, 5′-TTTTTTTGTAGTTTGTATTTG-3′; antisense, 5′-CCTCTATAAAAGGCTTCATCTTCTTC-3′. The PCR product containing 13 potential methylatable CG pairs was cloned into pGEM-T Vector System (Tiangen Biotech, Beijing, China) and probed with antibodies against GST (Genesil Biotechnology, Wuhan, China). The scrambled sequences (control short hairpin RNA (shRNA)), composed of same nucleotides as in shRNA but in randomized order, were used as negative control. The shRNA duplexes were transfected into K562 via electroporation as previously described [8]. The effects of shRNA on mitochondrial ATPase expression were determined by western blot at 48 h after transfection. The chemosensitivity of transfected K562 to adriamycin was evaluated by methylthiazoltetrazolium (MTT) assay [14].

apoptosis analysis
K562 cells transfected by shRNA were treated with 0.5 mg/l adriamycin for 48 h. The apoptotic morphology of K562 was evaluated by Hoechst 33342 (Genesil Biotechnology) [15]. Early apoptosis was evaluated using Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) following manufacturer’s protocol.

bisulfite sequencing
Five micrograms of purified DNA from K562/A02, K562 or primary CML cells was treated with sodium bisulfite (Sigma-Aldrich), which only converted unmethylated cytosine to uracil, and then the desired sequence [217 bp (~278 to ~62) mitochondrial ATPase β subunit promoter] was amplified using nested PCR [16]. Primers were designed according to MethPrimer (http://www.urogene.org/methprimer/). The following primers were used—round I: sense, 5′-ACCCAATAAACCTACTCTACCAAG-3′; antisense, 5′-AGATAGGGTAAAATATATTTAGGAAAT-3′; and round II: sense, 5′-TTTTTTTGTAGTTTGTATTTG-3′; antisense, 5′-CCTCTATAAAAGGCTTCATCTTCTTC-3′. The PCR product containing 13 potential methylatable CG pairs was cloned into pGEM-T Vector System (Tiangen Biotech, Beijing, China) and probed with antibodies against GST (Genesil Biotechnology, Wuhan, China) and mitochondrial ATPase β subunit protein was synthesized as previously described [17].

effect of 5-Aza and/or trichostatin A on mitochondrial ATPase β subunit
K562/A02 was treated with 5-Aza (DNA methyltransferase inhibitor) or trichostatin A (TSA) [histone deacetylase (HDAC) inhibitor]. The concentrations of 5-Aza (Sigma-Aldrich) were 0, 10, 20 and 40 μM; the concentrations of TSA (Sigma-Aldrich) were 0, 200 and 400 nM. K562/A02 was also treated with low-dose 5-Aza (10 μM) and TSA (200 nM) in combination. After 48 h, total RNA and protein were extracted. Primer for mitochondrial ATPase β subunit (328 bp) was as follows: sense, 5′-GGCTCTGAGTTGATTCTTGC-3′; antisense, 5′-GCCTGAGGATTTGTAAG-3′. Western blot
For protein extraction, cells were homogenized on ice in lysis buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1 mM phenylmethanesulphonylfluorid; 0.1 % sodium dodecyl sulfate; 1% Nonidet P-40; 5 μg/ml aprotinin) and cellular debris was pelleted at 10 000 g for 10 min at 4°C.
statistical analyses
We carried out statistical analyses using Student’s t-test or analysis of variance. Significance was set at $P < 0.05$.

results
up-regulation of P-gp on K562/A02
Flow cytometry results showed that P-gp-positive cells in K562/A02 were 20.45%, which was ~10-fold higher than that of K562 (2.32%), confirming the multidrug resistance character of K562/A02. Cell cycle analysis showed that K562/A02 and K562 were almost in the same cell cycle progression. These results ensure the comparability of these two cell lines with regard to the following proteomics approaches.

altered protein profiles in K562/A02 and K562
In the pH range 3–10, 2-DE of K562/A02 and K562 displayed ~1000 spots each. We found 17 protein spots of different density between K562/A02 and K562 (Figure 1). Compared with K562, four proteins (two protein spots were identified as the same protein), namely GST, creatine kinase-B and 40S laminin receptor 1, exhibited increased expression in K562/A02, while the other 13 proteins (two protein spots were identified as the same protein), including premature ovarian failure 1B protein, cofflin-1, human leukocyte antigen-DRB sigma antigen DRB3-0202-Dw25, α-enolase, aminoacylase, inorganic pyrophosphatase, HSPC115, mitochondrial ATPase β subunit, adenine phosphoribosyltransferase, protein tyrosine phosphatase receptor pi, PIG48 and heterogeneous nuclear ribonucleoprotein H3 isoform, were down-regulated in K562/A02.

up-regulation of GST and down-regulation of mitochondrial ATPase β subunit on K562/A02
To further confirm the chemoresistance-related proteins identified by comparative proteomics, western blot and real-time PCR were carried out in two candidate proteins. Changes of GST and mitochondrial ATPase β subunit protein were consistent with 2-DE. Compared with K562, GST protein

![Figure 1. Representative two-dimensional gel electrophoresis maps of K562 and adriamycin-resistant K562/A02 cellular lysates. (A) Protein profiles of extracts from K562 (left) or K562/A02 (right). Seventeen differential proteins were marked with arrows and were chosen for further characterization by matrix-associated laser desorption ionization-mass spectroscopy. (B) Close-up images of partial differentially expressed protein spots between K562/A02 and K562.](image-url)
exhibited an obvious up-regulation and a marked down-regulation for mitochondrial ATPase β subunit on K562/A02 (Figure 2). Messenger RNA (mRNA) changes were consistent with protein expression. Compared with K562, mRNA level of GST was higher 3.72-fold and that of mitochondrial ATPase with protein expression. Compared with K562, mRNA level of (Figure 2). Messenger RNA (mRNA) changes were consistent independent of Bcr-Abl signal pathway.

**inhibition of mitochondrial ATPase β subunit leads to decreased chemosensitivity and increased apoptotic resistance by shRNA on K562**

To uncover a causative relationship of mitochondrial ATPase for adriamycin resistance, K562 were transfected with shRNA specific to mitochondrial ATPase β subunit. The expression of mitochondrial ATPase β subunit was significantly down-regulated on K562 transfected with shRNA (Figure 3A). MTT assay showed that shRNA transfection led to decreased adriamycin sensitivity and the concentration that causes 50% inhibition of growth (IC₅₀) was 0.114 mg/l, compared with that of 0.038 mg/l in control shRNA, representing a threefold decrease toward drug sensitivity (Figure 3B).

Effects of apoptotic resistance to adriamycin were observed by Hoechst 33258 and Annexin V assay. The Hoechst 33258 assay showed that the percentage of apoptosis on K562 of transfected shRNA was 16.8%, which was significantly lower than that on K562 of transfected control shRNA (29.5%) and phosphate-buffered saline (PBS) buffer only (26.7%). The result of Annexin V assay was consistent with Hoechst 33258, in which the percentage of apoptosis on K562 of transfected shRNA (12.4%) was lower than that on K562 of transfected control shRNA (30.5%) and PBS buffer only (32.7%) (Figure 3C). These findings indicate that mitochondrial ATPase plays an important role in regulating K562 apoptosis and is involved in the adriamycin resistance of K562/A02.

We also analyzed the expressions of Bcr-Abl transcripts before and after K562 transfection. The results showed that shRNA had no influence on Bcr-Abl levels, indicating that chemoresistance mediated by mitochondrial ATPase may be independent of Bcr-Abl signal pathway.

**mitochondrial ATPase β subunit methylation status on cell line and CML primary cells**

Through bisulfite sequencing, we found 26 (18.18%) and 0 (0%) of totally analyzed 143 CG pairs to be methylated in K562/A02 and K562, respectively (P = 0.003); the analyzed sequence and methylation status of each CG pair was shown in Figure 4A and B. K562/A02 displayed a higher methylation status on mitochondrial ATPase β subunit compared with K562.

We also analyzed the methylation sequence in primary cells from 22 CML patients. The results showed that 121 (26.59%) of analyzed 455 CG pairs for CML-BC and 31 (7.95%) of analyzed 390 CG pairs for CML-AP were methylated (P = 0.009), and the average methylation frequency of each CG pair is showed in Figure 4C and D. More interestingly, we did not detect any methylated CG pair in 585 CG pairs for CML-CP.

**hypermethylation status of mitochondrial ATPase β subunit promoter is related to adriamycin resistance**

Mitochondrial ATPase β subunit transcription or translation levels were significantly increased in 5-Aza-treated K562/A02 both in mRNA abundance and protein level (Figure 5A); on K562/A02, it was also up-regulated slightly by TSA (Figure 5B). While K562/A02 cells were exposed simultaneously to 5-Aza and TSA, a synergic effect was observed. Compared with treatment with either agent alone, relatively low concentration of 5-Aza combined with TSA led to increased expressions of both mRNA and protein of mitochondrial ATPase β subunit on K562/A02 (Figure 5C).

We then carried out chemosensitivity assay to determine the contribution of hypermethylation of mitochondrial ATPase to adriamycin resistance. MTT assay demonstrated that 3-Aza treatment led to increased adriamycin sensitivity on K562/A02: the IC₅₀ of K562/A02 (10 μM 5-Aza) was 0.394 mg/l, compared with untreated K562/A02 (IC₅₀ was 4.16 mg/l); chemosensitivity increased by ~10-fold (Figure 5D).

We also analyzed the effect of 5-Aza on mitochondrial ATPase gene methylation. K562/A02 and primary cells from three CML-BC and three CML-AP patients were treated separately with 10 μM 5-Aza for 48 h and bisulfite sequencing analysis was carried out. Compared with untreated cells, 5-Aza had significant effect on the methylation status of mitochondrial ATPase. Before 5-Aza treatment, the methylated frequencies of K562/A02, CML-BC and CML-AP were 18.18%, 21.54% and 9.62%, respectively, which all decreased to 0% after 5-Aza treatment.

**mitochondrial ATPase β subunit expressive pattern on primary CML cells**

We investigated the expression of mitochondrial ATPase β subunit on primary CML cells both in mRNA and protein level (10 samples). Western blot documented that mitochondrial ATPase β subunit expression in CML-BC was obviously reduced than those in CML-CP or CML-AP (Figure 6A). Consistent with protein changes, the mRNA expression, assessed by real-time PCR, was significantly suppressed in CML-BC (Figure 6B).

**discussion**

A common mechanism of leukemia chemoresistance is the overexpression of plasma membrane drug efflux proteins, such
Figure 3. Suppressed mitochondrial ATPase β subunit expression by shRNA transfection (A) and its effect on adriamycin sensitivity (B and C). (A) Representing pattern of western blot detection obtained from K562 at 48 h after transfection (top panel); β-actin was used as control (bottom panel). (B) (i) After treatment of mitochondrial ATPase β subunit shRNA, K562 cells were incubated with different concentrations of adriamycin (0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 mg/l) and the cell viability was determined by MTT assay. (ii) Columns 1, 2 and 3 corresponded to the IC₅₀ of K562 transfected with shRNA specific to mitochondrial ATPase β subunit (0.114 mg/l), control shRNA (0.038 mg/l) and phosphate-buffered saline (PBS) buffer only (0.033 mg/l) to adriamycin, respectively. (C) Apoptosis resistance on K562 cells transfected with mitochondrial ATPase β subunit shRNA (Annexin V assay). The percentage of apoptosis on K562 cells transfected with mitochondrial ATPase β subunit shRNA was 12.4%, which was significantly lower than the percentage of apoptosis on K562 cells transfected with control shRNA (30.5%) and PBS buffer only (32.7%).
Figure 4. Mitochondrial ATPase β subunit methylation patterns on cell lines and primary cells from chronic myeloid leukemia (CML) patients. (A) Promoter and 5′-flanking region: sequence and structural features. The predicted transcription start site for mitochondrial ATPase β subunit promoter is shown. The potentially methylatable CG pairs are enclosed in box. (B) Mitochondrial ATPase β subunit promoter methylation patterns in K562/A02. The dots on the x axis represent the location of each CG pair, and the lines represent the mean fraction methylated of each dC base in these pairs. (C) DNA from bone marrow cells of seven CML-blast crisis (BC) patients was analyzed by bisulfite sequencing. The results represent the mean methylation frequencies of 35 determinations for seven patients. (D) DNA from bone marrow cells of six CML-accelerated phase (AP) patients was analyzed by bisulfite sequencing. The results represent the mean methylation frequencies of 30 determinations for six donors.
as P-gp [2]. Actually, blasts of CML-BC exhibit a high expression of P-gp, whereas little expression is found during CML-CP [18]. While various P-gp inhibitors are applied, only limited clinical benefits are achieved [19], indicating that P-gp is not the major or only mechanism of chemoresistance. Therefore, the development of effective multidrug resistance reversal agents is becoming urgent and depends greatly on our understanding to multiple molecular events involved in the generation of multidrug resistance.

By proteomics approaches, we detected 17 differentially expressed proteins; 4 of which were overexpressed and 13 were down-regulated in K562/A02. These proteins might be classified into four groups in the functional sense: (i) growth-associated proteins; (ii) metabolic enzymes; (iii) cytokine-related proteins and (iv) signal transduction-associated proteins. Among these proteins, a few have been proven to be related to chemoresistance, such as GST and mitochondrial ATPase β subunit. Looking at the protein expression profile, we found that mitochondrial ATPase β subunit, which is known to have an important role in bioenergetic metabolism and development of chemoresistance of cancer cells [20, 21], displays lower expression in K562/A02, and the phenomenon was further confirmed by western blot and quantitative PCR.
ATPase defect had a link to chemoresistance. Interestingly, we demonstrated down-regulation of mitochondrial ATPase not only in K562/A02 but also in primary cells from CML-BC. Consistent with clinical chemoresistance phenotype, the expressive level of mitochondrial ATPase β subunit was significantly lower than those in CML patients in CP or AP, indicating that there is a certain link between adriamycin resistance in CML-BC and mitochondrial ATPase down-regulation.

To further document the relationship between mitochondrial ATPase and adriamycin resistance, shRNA approach was applied on K562—the parental cell line of K562/A02. shRNA transfection was able to suppress mitochondrial ATPase expression and decrease sensitivity to adriamycin. This observation strongly supports the fact that down-regulation of mitochondrial ATPase may be a single molecular event to regulate the response to adriamycin in human leukemia cells. Accordingly, K562 transfected with shRNA exhibited an obviously apoptotic-resistance effect to adriamycin. While a direct correlation between mitochondrial ATPase defection and impairment of apoptosis pathway cannot be clearly established in our experiments, there have been literature indicating that acquired chemoresistance in cancer cells could involve increased BCL-2 and BCL-XL expressions or XIAP overexpression [27, 28], indicating an interconnection for chemoresistance and antiapoptotic effect. Because the oxidative phosphorylation capability of cancer cells is diminished, the apoptotic potential of the cells, including mitochondrial-mediated apoptosis, is hampered [20, 21]. These data probably provide an explanation for down-regulated mitochondrial ATPase and apoptotic resistance in K562/A02. However, the exact mechanisms remain to be elucidated in future studies.

It has been recognized that epigenetic factors occur in tumor formation, progression and chemoresistance [29]. Segura-Pacheco et al. [30] described a global DNA hypermethylation-related adriamycin resistance in MCF-7 cells and found that the multidrug resistance phenotype of MCF-7/Adr cells was accompanied by global DNA hypermethylation, whereas hydralazine, a specific demethylating agent, was applied, the resistant phenotype could be reverted. Our data showed that K562/A02 cells displayed hypermethylation status on mitochondrial ATPase β subunit promoter. When hypermethylation was inhibited by 5-Aza, it not only up-regulated the expression of mitochondrial ATPase but also enhanced the adriamycin sensitivity of K562/A02, indicating that the epigenetic regulation on mitochondrial ATPase may reverse the resistant phenotype of K562/A02. We also observed that TSA could slightly up-regulate mitochondrial ATPase level, and no evident effect was found for adriamycin resistance reversal; combined interference of 5-Aza and TSA has a synergistic effect on K562/A02. These findings indicate that preclinical treatment strategies combining DNA methylation inhibitor and HDAC inhibitor should be evaluated to reduce the potential development of adriamycin resistance in CML patients.

Furthermore, we investigated the methylation status of mitochondrial ATPase on primary cells from 22 CML patients. Noticeably, among CML patients of different stages, there were significant differences in the frequencies of methylated cytosine. The changes were basically correlated with the status of chemoresistance in CML patients of various stages with hypermethylation. Our results reflect that hypermethylation of mitochondrial ATPase gene existed in the primary CML cells with chemoresistance, which was responsible for the drug-resistant phenotype of CML-AP or CML-BC. These observations would prove to be pertinent and useful in the development of counter-chemoresistance strategies.

In summary, our study illustrates that down-regulation of mitochondrial ATPase occurs in CML cells with induced adriamycin resistance, and DNA hypermethylation of mitochondrial ATPase promoter may be responsible for the

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**Figure 6.** Mitochondrial ATPase β subunit expression on primary cells from chronic myeloid leukemia (CML) patients. (A) Protein expression of mitochondrial ATPase β subunit (western blot). Lanes 1 and 2 stand for CML-chronic phase (CP), lanes 3 and 4 stand for CML-accelerated phase (AP) and lanes 5–10 stand for CML with blast crisis (BC). (B) Relative quantification of messenger RNA for mitochondrial ATPase β subunit (real-time PCR).
down-regulation. A demethylating agent can partly reverse the resistant phenotype and exhibits a synergistic effect with a HDAC inhibitor, thus indicating that inhibition of hypermethylation may be an effective strategy to overcome drug resistance associated with mitochondrial ATPase expression.

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disclosure
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references