A Novel Adenine Nucleotide Translocase Inhibitor, MT-21, Induces Cytochrome c Release by a Mitochondrial Permeability Transition-independent Mechanism*

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The release of cytochrome c from mitochondria is a critical step during apoptosis. In order to study this process, we have used a synthetic compound, MT-21, that is able to initiate release of cytochrome c from isolated mitochondria. We demonstrate that MT-21 significantly inhibits ADP transport activity in mitochondria and reduces binding of the adenine nucleotide translocase (ANT) to a phenylarsine oxide affinity matrix. These results suggest that ANT, one of the components of the mitochondrial permeability transition (PT) pore, is the molecular target for MT-21. In agreement with this, the MT-21-induced cytochrome c release was effectively inhibited in the presence of ANT ligands, and MT-21 could dissociate ANT from a complex with a glutathione S-transferase-cyclophilin D fusion protein. Interestingly, we also found that specific inhibitors of ANT such as MT-21 and atractyloside could induce cytochrome c release without mitochondrial swelling and that this event was highly dependent on the presence of Mg2+. These results suggest that although ANT resides in the mitochondrial inner membrane, specific ANT inhibitors can induce cytochrome c release without having an effect on inner membrane permeability. Therefore, MT-21 can be a powerful tool for studying the mechanism of PT-independent cytochrome c release from mitochondria.

Apoptosis is a programmed cell-suicide mechanism that plays important roles in development, homeostasis, and various diseases. In the beginning of apoptosis, death signals (anti-tumor compounds, superoxide, etc.) induce the release of apoptogenic factors such as cytochrome c and apoptosis-inducing factor from the mitochondrial intermembrane space into the cytosol (1–3). Once cytochrome c, a component of the mitochondrial respiratory chain, is released from mitochondria, it binds to Apaf-1 and promotes the assembly of a multiprotein complex that induces proteolytic processing and activation of the caspase cell death proteases (1, 4). Despite this level of knowledge, the mechanism underlying cytochrome c release from mitochondria is not fully understood. One hypothesis is that a mitochondrial protein complex called the permeability transition (PT) pore mediates cytochrome c release. The components of this pore include the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), cyclophilin D, and kinases such as hexokinase and creatine kinase (5). Evidence to support this hypothesis has come from the use of low molecular weight compounds such as cyclosporin A and bongkrekic acid that are known to interact with PT pore components and are able to inhibit both the opening of the PT pore and the release of cytochrome c (6–9). It is believed that cyclosporin A specifically binds cyclophilin D (6, 7).

ANT is located in mitochondrial inner membrane where it is a specific carrier for ADP and ATP. ANT has two conformational states, c-state and m-state, and ADP/ATP are transported via interconversion of these two states. In c-state a hydrophilic loop of the ANT, which is the nucleotide-binding site, faces toward cytoplasmic side of the inner membrane, and in m-state it is exposed to the matrix side (8, 9). Bongkrekic acid inhibits PT pore opening and the release of cytochrome c via binding to ANT and fixing its conformation in the m-state (10, 11). Conversely, a different ANT inhibitor, atractyloside, induces PT pore opening and cytochrome c release by changing the ANT conformation to its c-state (12, 13). ANT can form a lethal pore upon interaction with pro-apoptotic agents including Ca2+, atractyloside, –SH reagents, the human immunodeficiency virus, type 1, protein Vpr, and pro-oxidants (13–18). In a previous study, it was shown that overexpression of ANT-1, which is one of the ANT isoforms, induced apoptotic cell death and that this was suppressed by co-expression of cyclophilin D (19). Thus, a molecular interaction between ANT and cyclophilin D may be important for anti-apoptotic functions in mitochondria.

Commonly, pro-apoptotic agents cause mitochondrial depolarization, swelling, and rupture of the outer membrane, resulting in the release of proteins from the intermembrane space (13–18). However, it has been reported that the pro-apoptotic Bcl-2 family protein, Bax, causes Mg2+-dependent cytochrome c release without altering inner membrane permeability and that this event was not inhibited by cyclosporin A (20–22). Interestingly, other investigators have reported that the Bax-mediated cytochrome c release is PT-dependent and inhibited by cyclosporin A (7, 23–25). Thus, even in a simple assay system using isolated mitochondria, the significance of the PT pore complex in cytochrome c release remains controversial.

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¶ The abbreviations used are: PT, permeability transition; ANT, adenine nucleotide translocase; VDAC, voltage-dependent anion channel; GST, glutathione S-transferase; BA, bongkrekic acid; Atr, atractyloside; PhAsO, phenylarsine oxide; PPase, peptidylprolyl-cis-trans-isomerase; PMSF, phenylmethylsulfonyl fluoride; MOPS, 4-morpholinepropanesulfonic acid.
We reported previously (26) that a synthetic compound, MT-21, induced apoptotic cell death in human promyelocytic leukemia HL-60 cells through induction of cytochrome c release from mitochondria. In this present study, we demonstrate that the molecular target of MT-21 is ANT and that MT-21 specifically inhibits the molecular interaction between ANT and cyclophilin D. We show that specific ANT inhibitors such as MT-21 and atractyloside cause cytochrome c release without mitochondrial swelling, and we therefore propose that modification of ANT, one of the PT pore components, can cause cytochrome c release by a PT-independent mechanism.

MATERIALS AND METHODS

Reagents—Monoclonal antibody against cytochrome c (clone 7H8.2C12) was purchased from BD PharMingen and polyclonal antibody against ANT (Q-18) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-porin (VDAC) monoclonal antibody (31H1, clone 7) and anti-cyclophilin D polyclonal antibody were from Calbiochem. Bongkrekic acid and cyclosporin A were purchased from Calbiochem. Atractyloside and phenylarsine oxide were obtained from Sigma. Succinyl-Ala-Ala-Pro-Phe-4-nitroanilide and α-chymotrypsin were from Peptide Inc. (Osaka, Japan).

Expression of Mitochondria—Mitochondria were isolated from human leukemia HL-60 cells as described previously (27). Mouse liver mitochondria were purified from male mice by Percoll gradient centrifugation (28).

Detection of Cytochrome c—Mitochondria were incubated with MT-21 at 30 °C in PT buffer (10 mM Tris-MOPS, pH 7.4, 250 mM sucrose, 5 mM succinate, 10 μM EGTA) and centrifuged at 13,000×g for 10 min at 4 °C. For detecting cytochrome c released from mitochondria, mitochondrial pellet and supernatant fractions were subjected to SDS-PAGE (15%) and Western blot analysis.

Effect of MT-21 on the Binding of ANT to a Phenylarsine Oxide-conjugated Agarose—Mitochondria (0.5 mg/ml) were treated with or without MT-21 in PT buffer. After a 30-min incubation, mitochondria were collected by centrifugation and solubilized in 100 μl of HEPES buffer, pH 7.4, containing 150 mM NaSO₄, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, and 0.25% (v/v) of Triton X-100. Each lysate was incubated with 4-aminophenylarsine oxide agarose (ThioBond Resin, Invitrogen) that had been equilibrated in the identical buffer. Samples were incubated at 4 °C for 30 min, and the resin was washed with equilibration buffer. Proteins bound to the resin were eluted with 50 μl of elution buffer that included 10 mM diithothreitol. Twenty-μl samples of the eluted fractions were subjected to SDS-PAGE (15%) followed by Western blot analysis.

Mitochondrial Swelling Assay—To measure the mitochondrial swelling that accompanies opening of the permeability transition pore, mitochondria (0.5 mg of protein/ml) were incubated in PT buffer at 30 °C in the presence of either CaCl₂ or MgCl₂. Changes in ΔΨ were followed by measuring changes in electrical potential of the mitochondria by a microphotometer (22).

Adenine Nucleotide Transport Activity Assay—The effect of MT-21 on transport of ADP into mitochondria was assayed as described previously (29). Mitochondria (1 mg/ml) were pretreated in PT buffer for 10 min with various concentrations of MT-21. These samples were further incubated for 5 min at 0 °C prior to the addition of 20 μM [14C]ADP (Pharmingen Life Sciences), specific radioactivity, 185 kBq/μmol) and incubated for an additional 20 s at 0 °C. The reaction was terminated by the addition of 100 μl of 0.4 M atractyloside. The mitochondrial pellet was solubilized in 0.2 ml of 1% SDS, and the radioactivity of the lysate was determined by liquid scintillation counting (TRICARB 2000, Packard Instrument Co.).

Expression of the GST-Cyclophilin D Fusion Protein—Human cyclophilin D was amplified from cDNA of human leukemia HL-60 cells (kindly provided by Dr. S. Simizu). The cyclophilin D gene was amplified using specific primers (5’ primer, 5′-GGCGGATCCATGCTG-GCCGGATGCCTG-3’ and 3’ primer, 5′-GGCGGAATTCCTGGACCAACT-GCCGACACTGCT-3’). The matured form of cyclophilin D truncated by its N-terminal 29 amino acids was generated by PCR using specific primers (5’ primer, 5′-GGGCGAATTCCTGGACCAACT-GCCGACACTGCT-3’ and 3’ primer, 5′-GGCGGAATTCCTGGACCAACT-GCCGACACTGCT-3’). The PCR product was subcloned into pGEX-6P-2 (Amersham Biosciences) to obtain the N-terminal GST-cyclophilin D fusion. Escherichia coli BL21(DE3) cells transformed with the plasmid were grown in LB broth at 28 °C, and production of the recombinant protein was induced by the addition of 0.2 mM isopropylthio-β-galactopyranoside. The cells were harvested and disrupted by sonication in buffer A (100 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.5 mM EGTA, 1 mM PMSF). The soluble fraction was obtained by centrifugation and mixed with GSH-immobilized beads (glutathione-Sepharose 4B, Amersham Biosciences) to allow adsorption of the fusion protein. The affinity matrix was then washed with buffer B (150 mM NaCl, 10 mM HEPES, pH 7.3) and used for the binding assay. Peptidylprolyl-cis,trans-isomerase (PPIase) activity was determined using a synthetic peptide, succinyl-Ala-Ala-Pro-Phe-4-nitroanilide, as a substrate in a two-step reaction coupled to chymotrypsin (30).

Binding of Mitochondrial Protein to the GST-Cyclophilin D Fusion Protein—Mitochondria (0.5 mg/ml), pretreated with or without MT-21, were solubilized in 100 μl of HEPES buffer, pH 7.4, containing 150 mM NaSO₄, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, and 0.25% (v/v) of Triton X-100. Each lysate was centrifuged, and 100 μl of the supernatant was dialyzed with 100 μl of the HEPES buffer prior to the addition of 20 μl of GST-cyclophilin D affinity matrix (0.5 μg/μl beads) and incubated for 60 min at 4 °C. The affinity matrix was washed, and conjugated fusion proteins were eluted by the addition of 100 mM Tris-HCl, pH 8.0, containing 20 mM glutathione. Each elute was subjected to SDS-PAGE (15%) and analyzed by Western blotting.

RESULTS

MT-21-induced Cytochrome c Release Is Enhanced by Mg²⁺—The precise requirement for divalent cations during mitochondrial cytochrome c release has previously been ambiguous. Therefore, we started this investigation by clarifying the effects of Mg²⁺ and Ca²⁺ on the induction of cytochrome c release promoted by various concentrations of MT-21. Because CaCl₂ at 50 μM induced cytochrome c release in the absence of MT-21 (data not shown), samples were treated with CaCl₂ at 20 μM. As shown in Fig. 1a, MT-21 did not induce cytochrome c release with this level of CaCl₂. MT-21 significantly induced cytochrome c release in the presence of 2.5 mM MgCl₂, and this concentration was similar to that shown to enhance Bax-mediated cytochrome c release in previous studies (20, 21). KCl did not enhance the cytochrome c release by MT-21 at concentrations of less than 10 mM (Fig. 1b). Although excess amount of KCl (40 mM) enhanced cytochrome c release by MT-21, this concentration was extraordinarily higher than that of MgCl₂.

![Fig. 1. Requirement of Mg²⁺ cation for the release of cytochrome c induced by MT-21. a, dose dependence and effects of divalent cations on cytochrome c release by MT-21. HL-60 mitochondria (0.5 mg/ml) were incubated with MT-21 (0–40 μM) in PT buffer with or without either 20 μM CaCl₂ or 2.5 mM MgCl₂. After 45 min, samples were centrifuged, and aliquots of the supernatants (sup.) and precipitates (ppt.) were subjected to Western blot analysis using antibodies specific for cytochrome c. b, the effect of MgCl₂ or KCl on cytochrome c release. Mitochondria were treated with MT-21 (40 μM) in the presence of either MgCl₂ (2.5 mM) or KCl (5–40 mM). Samples were analyzed as for a.](http://www.jbc.org/content/early/1998/09/25/jbc.273.183124/F1.large.jpg)
Therefore we concluded that the enhancing effect of MgCl₂ on the cytochrome c release by MT-21 was due to the Mg²⁺ cation rather than the Cl⁻ anion. Similar results were obtained when mouse liver mitochondria were used (data not shown).

**Effect of MT-21 on the Adenine Nucleotide Transport and ANT Conformation**—The VDAC regulates the transport of non-specific low molecular metabolites across the outer mitochondrial membrane, whereas ANT regulates the transport of adenine nucleotides across the inner membrane. In order to determine whether MT-21 inhibits ANT activity, we investigated if MT-21 affected mitochondrial [¹⁴C]ADP transport activity or not. Fig. 2a shows that MT-21 significantly inhibited the ADP uptake into mitochondria in a dose-dependent manner. One possible explanation for this observation is that MT-21 affects the ANT conformation thereby inhibiting its adenine nucleotide transport activity. ANT is a carrier protein that is able to form two conformational states, the c-state and the m-state. It has three reactive cysteines (Cys⁵⁶, Cys¹⁵⁹, and Cys²⁵⁶) that are all located on putative matrix-facing loops and whose thiol groups selectively react with thiol reagents when in the m-state (8, 9). Previous study showed that a derivative of atractyloside, carboxyatractyloside, fixes ANT in the c-state and greatly reduces the binding of ANT to phenylarsine oxide (PhAsO)-agarose, an affinity matrix that binds selectively with vicinal dithiols in proteins (15). In order to estimate whether MT-21 affects the conformational state of ANT, we investigated the effect of MT-21 on the capacity of ANT to bind to the PhAsO affinity matrix. Incubation of mitochondria with MT-21, atractyloside, or PhAsO inhibited binding of ANT to PhAsO matrix in a dose-dependent manner (Fig. 2b). In contrast to PhAsO that binds to the -SH moieties directly, the effect of MT-21 was abrogated by pretreatment with bongkrekic acid. It is known that bongkrekic acid fixes ANT conformation in the m-state (11). Moreover, when mitochondria were pretreated with physiological ligands of ANT such as ADP or ATP, the effect of MT-21 was also canceled (data not shown). These observations suggest that MT-21 and atractyloside both inhibit ANT binding through alteration of its conformation rather than by covalent binding to the ANT-SH groups. The effects of the ANT-ligands on the MT-21-induced cytochrome c release were also investigated. MT-21-induced cytochrome c release was partially or completely suppressed by the addition of bongkrekic acid and physiological ligands of the ANT such as ADP and ATP (Fig. 2c). These results suggest that the induction of cytochrome c release by MT-21 is related to the fixing of ANT in its c-state.

cDNA Cloning and Expression of Human Cyclophilin D—Because MT-21 appeared to be acting by altering ANT conformation, it was therefore possible that it would also inhibit the binding between cyclophilin D and ANT. In order to test this hypothesis, we examined the effect of MT-21 on the binding activity of human cyclophilin D with ANT and VDAC. The cyclophilin D cDNA from human leukemia HL-60 was cloned and sequenced and found to be identical to that reported previously (GenBank™ accession number M80254). It is known that the amino acid residues 1–29 of the rat cyclophilin D function as a mitochondrial targeting sequence and that they are cleaved off during import into mitochondria (31). Thus, for the binding assay of cyclophilin D to mitochondrial proteins, GST was fused with the mature form of the cyclophilin D protein (amino acids 30–207). The ~46-kDa GST-cyclophilin D fusion protein was expressed in E. coli and purified from the soluble fraction using glutathione-Sepharose. Both the fusion protein and the 19-kDa cyclophilin D protein generated by cleavage of GST could be detected with anti-cyclophilin D antibody that recognize the N-terminal region of mature cyclo-

![Fig. 2. MT-21 induces cytochrome c release via inhibition of ANT.](http://www.jbc.org/)

**Fig. 2. MT-21 induces cytochrome c release via inhibition of ANT.** a, effect of different concentrations of MT-21 on the mitochondrial ADP transport activity. Mouse liver mitochondria were pretreated with MT-21 (0–80 μM), followed by incubation with 20 μM [¹⁴C]ADP at 0 °C for 20 s. The reaction was terminated by the addition of 100 μM atractyloside. The level of radioactivity in the mitochondrial fraction was determined using a liquid scintillation counter. In the absence of MT-21, the uptake of ADP was 246 pmol/mg protein. b, effects of MT-21 on the ANT thiol groups. Mitochondria (0.5 mg/ml) were incubated with atractyloside (Atr, 0–2 mM), MT-21 (0–80 μM), and phenylarsine oxide (PhAsO, 0–200 μM) with or without pretreatment with 20 μM bongkrekic acid (BA). The samples subsequently were lysed and incubated with phenylarsine oxide-agarose. Proteins bound to the resin were eluted and subjected to Western blotting using anti-ANT antibodies. c, protective effects of the ANT ligands on MT-21-induced cytochrome c release. Mitochondria (0.5 mg/ml) were pretreated in the presence or absence of the ANT ligands (1 mM ADP, 1 mM ATP, or 20 μM BA) for 10 min. Then MT-21 was added, and the samples further incubated for 45 min. After centrifugation, the presence of cytochrome c in the supernatant (sup.) was analyzed by Western blotting with anti-cytochrome c antibody.
pholin D (Fig. 3a). The peptidylprolyl-cis,trans-isomerase (PPIase) activity of the GST-cyclophilin D fusion protein was demonstrated to be similar to that of cyclophilin D purified by a conventional method (32) (data not shown). This activity was inhibited by the addition of cyclosporin A, a specific inhibitor of cyclophilin D (Fig. 3b). From these observations, it is likely that the enzymatic properties of cyclophilin D were largely preserved in the GST fusion protein. To detect the GST-cyclophilin D-binding proteins, we applied mitochondrial extracts to a cyclophilin D affinity matrix. Bound proteins were eluted by the addition of glutathione, and the eluted material was analyzed by Western blotting using anti-cyclophilin D antibody. Lanes 1–3 correspond to GST alone, the GST-cyclophilin D prior to cleavage, and the cleaved cyclophilin D protein, respectively. b, PPIase activity of the purified GST-cyclophilin D. PPIase activity of GST-cyclophilin D (200 ng/ml; ○), GST-cyclophilin D (100 ng/ml; ▲), GST (200 ng/ml; □), and GST-cyclophilin D (200 ng/ml + 1 μM cyclosporin A; ■) were measured. c, GST-cyclophilin D or GST (control) matrices were incubated with cyclophilin D complex prior to the addition of ANT inhibitors. When the GST-cyclophilin D fusion protein was pretreated with either GST or GST-cyclophilin D matrices, ANT bound to the GST-cyclophilin D affinity matrix was eluted with glutathione, and the eluted proteins were subjected to SDS-PAGE and analyzed by Western blotting using anti-ANT antibody. Lanes 1–3 correspond to GST alone, GST-cyclophilin D or GST matrices. ANT bound to the GST-cyclophilin D matrix was subsequently treated with either GST or GST-cyclophilin D matrices. ANT bound to the GST-cyclophilin D affinity matrix was eluted with glutathione, and the eluted proteins were subjected to SDS-PAGE and analyzed by Western blotting using anti-ANT antibody. b, effects of MT-21 on PPIase activity of GST-cyclophilin D fusion protein. PPIase activity of GST-cyclophilin D (500 ng/ml; ▲), GST-cyclophilin D (500 ng/ml + 160 μM MT-21; □), GST-cyclophilin D (500 ng/ml + 1 μM cyclosporin A; ○), and GST (500 ng/ml; ○) was measured. The fusion protein was pretreated with MT-21 or cyclosporin A for 10 min. c, mitochondrial extracts were preincubated with GST-cyclophilin D matrix, and the matrix was washed three times. The resulting ANT-GST-cyclophilin D protein complex was treated with ANT inhibitors MT-21 (0–160 μM), atracyloside (0–2 mM), or BA (0–40 μM) at 4 °C for 30 min. After washing, ANT was eluted and analyzed by Western blotting.

**Effects of MT-21 on the Association of ANT with Human Cyclophilin D**—When mitochondria were pretreated with inhibitors of ANT such as atracyloside, bongkrekic acid, or MT-21, binding of ANT to the cyclophilin D affinity matrix was inhibited in a dose-dependent manner (Fig. 4a). However, MT-21 did not inhibit the PPIase activity of the GST-cyclophilin D fusion protein at concentrations that inhibited ANT binding to GST-cyclophilin D (Fig. 4b). To investigate further the conformational change of the ANT by MT-21, the cyclophilin D affinity matrix was preincubated with mitochondrial protein extracts, thus allowing the formation of the ANT-GST-cyclophilin D complex prior to the addition of ANT inhibitors. When the ANT-GST-cyclophilin D complex was subsequently treated with either MT-21 or atracyloside, it was found that both could dissociate the ANT from GST-cyclophilin D matrix effectively (Fig. 4c). In contrast, bongkrekic acid, which is known as an inhibitor of cyclophilin D release, did not dissociate the ANT from GST-cyclophilin D matrix.

**MT-21-induced Cytochrome c Release Is Independent of a Permeability Transition**—When the PT pore is opened, there is an alteration in the permeability of the inner membrane and the mitochondria become swollen. It is known that atracyloside causes mitochondrial swelling (14), and we wished to determine whether MT-21 has the same effect. As expected, when
mitochondrial swelling (Fig. 5, porin A-insensitive manner that was not accompanied with MT-21 were able to induce cytochrome c (0
important role in mitochondrial cytochrome c release or not. ANT inhibitors in the presence of 20 μM CaCl₂, Mitochondria were incubated in the presence of none (○), 2 mM atractyloside (●), 40 μM MT-21 (□), Atr + 2 μM cyclophilin A (▲), and Atr + 20 μM of bongkrekic acid (▼)), respectively. Mitochondrial swelling was assessed by monitoring the absorbance at 540 nm. b, mitochondria (0.5 mg/ml) were treated with ANT inhibitors in the presence of 2.5 mM MgCl₂. Mitochondria were incubated in the presence of none (○), 2 mM atractyloside (●), 40 μM MT-21 (□). c, the effect of cyclophilin A on cytochrome c release induced by Atr or MT-21 was analyzed in the presence of 20 μM CaCl₂ or 2.5 mM MgCl₂. Mitochondria (0.5 mg/ml) were pretreated in the presence or absence of 2 μM cyclophilin A prior to the addition of MT-21 (0–40 μM) or atractyloside (0–2 mM). After a 45-min incubation, the samples were centrifuged, and the presence of cytochrome c in the supernatant was assessed by Western blotting.

Fig. 5. MT-21 does not induce a mitochondrial permeability transition. a, mouse liver mitochondria (0.5 mg/ml) were treated with ANT inhibitors in the presence of 20 μM CaCl₂. Mitochondria were incubated in the presence of none (○), 2 mM atractyloside (●), 40 μM MT-21 (□), Atr + 2 μM cyclophilin A (▲), and Atr + 20 μM of bongkrekic acid (▼), respectively. Mitochondrial swelling was assessed by monitoring the absorbance at 540 nm. b, mitochondria (0.5 mg/ml) were treated with ANT inhibitors in the presence of 2.5 mM MgCl₂. Mitochondria were incubated in the presence of none (○), 2 mM atractyloside (●), 40 μM MT-21 (□). c, the effect of cyclophilin A on cytochrome c release induced by Atr or MT-21 was analyzed in the presence of 20 μM CaCl₂ or 2.5 mM MgCl₂. Mitochondria (0.5 mg/ml) were pretreated in the presence or absence of 2 μM cyclophilin A prior to the addition of MT-21 (0–40 μM) or atractyloside (0–2 mM). After a 45-min incubation, the samples were centrifuged, and the presence of cytochrome c in the supernatant was assessed by Western blotting.

mitochondria were incubated in CaCl₂-containing buffer, atractyloside induced cytochrome c release and mitochondrial swelling in a cyclophilin A-sensitive manner (Fig. 5, a and c). Because bongkrekic acid was able to abrogate the swelling, it is likely that atractyloside induced PT pore opening via a specific interaction with ANT. Interestingly, when mitochondria were incubated in MgCl₂-containing buffer, both atractyloside and MT-21 were able to induce cytochrome c release in a cyclophilin A-insensitive manner that was not accompanied with mitochondrial swelling (Fig. 5, b and c). These results indicate that atractyloside can cause both PT-dependent and PT-independent cytochrome c release, whereas MT-21 induced only PT-independent cytochrome c release.

DISCUSSION

We have reported previously that MT-21, a compound having a γ-lactase ring and an alkyl chain (n-C₈H₁₇) at N-1 (33), can induce apoptotic cell death via a direct effect on mitochondria (26). In this present study, we have investigated the mechanism of cytochrome c release induced by MT-21 and have identified the target molecule for MT-21 as ANT, one of the PT pore components. The PT pore is believed to play an important role in mitochondrial cytochrome c release during apoptosis. Indeed, the PT pore components, ANT and VDAC, have been proposed to be key molecules for regulation of this release (34–36).

ANT has six transmembrane helices, and under physiological conditions it regulates adenine nucleotide transport across the inner membrane. Recently, ANT has been regarded as one of the targets for the pro-apoptotic Bcl-2 family protein such as Bax (38), and several chemical agents that bind to ANT are known to induce cytochrome c release from mitochondria (37, 38). Cyclophilin D, a PPIase, is also considered to be a PT pore component, and it has been shown that high Ca²⁺ or oxidative stress enhances cyclophilin D binding to mitochondrial inner membrane resulting in PT pore opening. These events are prevented by cyclosporin A, a specific cyclophilin inhibitor (15). However, it has also been reported that ANT tightly binds to GST-cyclophilin D fusion proteins in the absence of Ca²⁺ and oxidants (39, 40). We have shown that cyclosporin A did not inhibit ANT-cyclophilin D binding, a result that is consistent with the initial observation (39) (Fig. 3c). Moreover, when the ANT-VDAC-GST-cyclophilin D protein complex was reconstituted into liposomes, the Ca²⁺-stimulated pore formation could be observed, and this pore formation was indeed cyclophilin A-sensitive (39). Thus, how cyclophilin D regulates the PT pore conformation remains unclear. A previous study (40) showed that the ANT inhibitors interfered with binding of ANT to GST-cyclophilin D, and we also found that both atractyloside and bongkrekic acid could inhibit the interaction of ANT with a GST-cyclophilin D fusion protein (Fig. 4a). These results may suggest that the molecular interaction between ANT and cyclophilin D is not important for cytochrome c release, because atractyloside is a cytochrome c release inducer whereas bongkrekic acid is an inhibitor. However, once the ANT-GST-cyclophilin D protein complex is formed, only the inducers of cytochrome c release, MT-21 and atractyloside, could dissociate ANT from the GST-cyclophilin D fusion protein (Fig. 4c). This observation implies that in intact mitochondria ANT is associated with cyclophilin D and forms part of PT pore complex. Thus, the ANT/cyclophilin D binding may indeed be important for regulation of cytochrome c release.

It was reported that Bax, a pro-apoptotic Bcl-2 family protein, is able to interact with VDAC (34, 35) and ANT (36) and that Bax induces cytochrome c release from isolated mitochondria in vitro. However, there are controversial papers on Bax-induced cytochrome c release. Some papers showed that Bax-induced cytochrome c release was promoted by Mg²⁺, and it was PT-independent (20, 21). In contrast, other papers have reported that Bax-induced cytochrome c release occurs in a cyclophilin A-sensitive manner (23, 24) and is accompanied by mitochondrial swelling (7). A recent study (41) has shown that superoxide is able to induce cytochrome c release without mitochondrial swelling and that this event is not inhibited by cyclophilin A. It still remains controversial whether the PT pore participates in cytochrome c release or not.

Because ANT resides in the inner membrane of mitochondria, it was considered that pro-apoptotic factors that affect the ANT should induce an alteration in inner membrane permeability and result in mitochondrial swelling followed by outer membrane rupture (25). Therefore, apoptogenic stimulators that cause PT-independent cytochrome c release were considered to interact with VDAC because this resides in the outer membrane. Our present study demonstrates that specific inhibitors of ANT such as MT-21 and atractyloside can induce cytochrome c release by a PT-independent mechanism (Fig. 5, b and c). Thus, we propose that modification of the ANT, a PT pore component in the mitochondrial inner membrane, can result in PT-independent cytochrome c release. As for Bax, this type of cytochrome c release is thought to be highly dependent
on the presence of Mg$^{2+}$ cations. It has been suggested that divalent cations such as Mg$^{2+}$ and Ca$^{2+}$ bind VDAC or ANT and affect their conformation (42). Therefore, there is a possibility that these cations themselves cause a conformational change in the PT pore complex. Recently, it was reported that hexokinase interacts with ANT by a direct or indirect mechanism (43) and associates with VDAC, regulating BAX-mediated cytotoxic c release (44). Consequently, studies that focus only on VDAC or ANT may not be sufficient to explain the mechanism of induction of cytotoxic c release. It will be necessary to consider molecular interactions of each PT pore component and the conformational changes of the entire PT pore complex in order to precisely define the molecular events underlying release of cytotoxic c. Because superoxide induced release of cytotoxic c without a major change of mitochondrial structure in intact cell (41), a PT-independent cytotoxic c release is considered to be important. A compound that specifically induces a PT-independent cytotoxic c release is required. MT-21 did not induce Ca$^{2+}$-dependent mitochondrial swelling, which is different from atracysptide. Therefore, MT-21 should prove to be a useful tool in understanding the mechanism of PT-independent cytotoxic c release.

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