Midazolam induces apoptosis in MA-10 mouse Leydig tumor cells through caspase activation and the involvement of MAPK signaling pathway

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Introduction
Midazolam (Dormicum®; F. Hoffmann-La Roche Ltd, Basel, Switzerland), a benzodiazepine-derivative drug, has powerful anxiolytic, amnestic, hypnotic, and sedative properties by modulating the γ-aminobutyric acid (GABA) receptor in the central nervous system.1,2 The putative receptor of midazolam, the peripheral-type benzodiazepine receptor (PBR), as a small 18 kDa protein, is organized in clusters of four to six molecules on the outer mitochondrial membrane.3,4 Studies have illustrated that the binding of PBR ligand to PBR results in the cholesterol movement from the outer mitochondrial membrane to the inner mitochondrial membrane, which could stimulate steroidogenesis.5 In fact, we have previously demonstrated that midazolam could significantly stimulate steroidogenesis in MA-10 mouse Leydig tumor cells by activating protein kinase A and protein kinase C pathways with the expression of PBR and steroidogenic acute regulatory proteins.6 Interestingly, we also observed that higher dosage with long treatment of midazolam could significantly induce MA-10 cell apoptosis.

Apoptosis is a process of program cell death, and plays an important role in physiological processes such as embryonic development and tissue homeostasis.7,8 Apoptosis can be induced by various stimuli, and two major signaling pathways leading cell...
apoptosis have been studied intensively: the extrinsic and intrinsic pathways. The extrinsic pathway is initiated through death ligands binding to death receptors, and subsequently activates downstream death-inducing signaling complex (DISC).10–12 DISC then activates caspase-8 and -3 through the cleavage of these enzymes from proenzymes and results in the cleavage of poly (ADP-ribose) polymerase (PARP), which induces apoptosis.11,12 In the other way, the intrinsic pathway is initiated by mitochondrial damage where it releases cytochrome-c and activates caspase-9 to associate with Apaf-1 to form apoptosome, and activates caspase-3 to induce apoptosis.10,13 Extensive evidence indicates that during apoptosis, mitochondrial outer membrane becomes permeable, and this permeability transition of mitochondrial membrane is regulated by the Bcl-2 family.14 The Bcl-2 family includes two groups, antiapoptotic and proapoptotic proteins, which share one or more homologous domains called BH domains. The antiapoptotic family members include Bcl-xl, Mcl-1, and Bcl-2, which contain BH1 to BH4 domains. The proapoptotic family members, such as Bax and Bak, are redundant promoters of cell death.15 The BH3-only proteins, such as Bid, however, are usually kept inactivated by different mechanisms, and these proteins are activated to function as effectors of apoptosis upon various death stimuli.16,17 The activation of caspase cascade is required in both extrinsic and intrinsic pathways.

Besides caspase cascades, mitogen-activated protein kinases (MAPKs) are also involved in apoptosis regulation.18 MAPKs consist of three family members: extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 proteins.18 Studies have reported that stress signals can activate the SAPK/JNK protein kinases to mediate cellular steps in apoptosis on some cell types.18,19 It has been shown that ERK is responsive to growth stimuli as the important signal for antiapoptosis.18,19 However, the involvement of p38 in apoptosis is diverse. Phosphorylation of p38 can be initiated by MKK3 and MKK6 at threonine and tyrosine regions, which can control many transcriptional factors and kinases to enhance cell survival or prompt apoptosis.18,19 In fact, studies have also shown that the PI3K/Akt/mTOR signaling pathway could promote cell growth and survival.20 Akt, a serine–threonine kinase that is directly activated in response to PI3K, is a major effector of PI3K and leads to increased cellular growth and survival in cancers.21 Accordingly, caspase and Akt and MAPKs pathways may play important roles in apoptosis of tumor cells activated by chemotherapy agents.

There are reports indicating that general anesthetic drugs could regulate apoptosis in the developing brain in rats and rabbits.22,23 We have previously demonstrated that midazolam could induce apoptosis in MA-10 mouse Leydig tumor cells but not in normal mouse Leydig cells. In the present study, we investigated the mechanism of midazolam-induced MA-10 Leydig tumor cell apoptosis. In fact, this study first demonstrated the cytotoxic/apoptotic effect of midazolam in the reproductive system.

### Materials and methods

#### Chemicals

Midazolam (Dormicum) was purchased from Roche Products Ltd (Auckland, New Zealand). Waymouth MB 752/1 medium, fetal bovine serum, Dulbecco’s phosphate buffered saline (PBS), and lyophilized trypsin-ethylenediaminetetraacetic acid were purchased from Life Technologies (Carlsbad, CA, USA). Bovine serum albumin, propidium iodide (PI), and ribonuclease A were purchased from Sigma-Aldrich (St Louis, MO, USA). Tissue culture grade sodium bicarbonate (NaHCO3), sodium carbonate (Na2CO3), sodium chloride (NaCl), sodium dihydrogen phosphate (NaH2PO4), and potassium chloride (KCl) were purchased from Riedel-deHaen (Seelze, Germany). Sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium dodecyl sulfate (SDS), sucrose, and Tween-20® were purchased from Merck KGaA (Darmstadt, Germany). Tris base was purchased from Calbiochem (San Diego, CA, USA). HEPES was purchased from Mallinckrodt Baker, Inc. (Paris, KY, USA). Acrylamide was purchased from JT Baker (Phillipsburg, NJ, USA). The enhanced chemiluminescence detection kit was purchased from Amersham Biosciences (Piscataway, NJ, USA). Donkey antirabbit immunoglobulin G conjugated with horse-radish peroxidase and sheep antimouse immunoglobulin G conjugated with horseradish peroxidase were purchased from Amersham Life Science Inc. (Arlington Heights, IL, USA). Polyclonal antibody against β-actin, cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, phosphor-ERK and phosphor-JNK, and monoclonal antibody against Bid, cytochrome-c, Akt, phosphor-Akt, and phosphor-p38 were purchased from Cell Signaling (Beverly, MA, USA). PARP was purchased from Oncogene (San Diego, CA, USA).

#### Cell line and cell culture

The MA-10 mouse Leydig tumor cell line was a gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA), and was maintained in Waymouth medium (Life Technologies) containing 15% fetal bovine serum and incubated in a humidified atmosphere containing 95% air and 5% CO2 at 37°C. Cells were starved for 24 hours to be synchronized before the incubations of midazolam for various experiments.
Flow cytometry analysis
To determine whether midazolam could induce apoptosis in MA-10 cells, different phase distribution of the cell cycle was assessed by flow cytometry analysis using PI staining. MA-10 cells (5 x 10^3) were seeded in a 6 cm dish (Techno Plastic Products AG, Trasadingen, Switzerland) with 3 mL serum-containing medium. After 70%–80% confluence, cells were treated with serum-free medium containing various concentrations of midazolam for 1 hour, 3 hours, 6 hours, 12 hours, and 24 hours, respectively. Midazolam-treated cells were harvested by trypsinization, washed with PBS, and fixed in 75% ethanol for 2 hours at -20°C. After fixation, cells were washed with cold PBS, spun down, mixed with 100 µg/mL RNase, and stained with PI (40 µg/mL). The stained cells were analyzed using a fluorescence activated cell sorter (FACScan, Becton-Dickinson, Mountain View, CA, USA) at λ=488 nm using Cell-Quest™ software (Becton-Dickinson). The DNA content distribution of normal growing cells is characterized by two peaks, G1/G0 and G2/M phases. The hypodiploid cells were defined as apoptotic cells, where hypodiploid DNA content represents DNA fragmentation.

Immunoblotting analysis
Cells (3 x 10^3) were seeded in a six-well plate. After treatment, cells were rinsed with cold PBS and then harvested by 40 µl lysis buffer (50 mM Tris-base, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% deoxycholate, acid, and 1 mM phenylmethylsulfonyl fluoride). Cell lysate was centrifuged at 32,000 rpm for 12 minutes at 4°C. The pellet was collected by 20 µl lysis buffer and was centrifuged again at 12,000 x g. The supernatant, which contained total protein, was collected and stored at -20°C. Protein concentration of the cell lysate was determined by the Lowry method. Lysate (30–50 µg) was separated in 12% SDS–polyacrylamide gel and electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk, washed by Tris-buffered saline and Tween-20, and subsequently incubated with primary antibodies against cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, cleaved PARP, Bax, cytochrome-c, Akt, phosphor-Akt, phosphor-ERK, phosphor-p38, and phosphor-JNK. After washing, the membrane was incubated with horseradish peroxidase-conjugated sheep antimouse antibody or donkey antirabbit antibody, and the immunocomplex was then visualized by an enhanced chemiluminescence detection kit. The optical density of each protein band was quantified by a Quantity One (PDI, Huntington Station, NY, USA) computer-assisted image analysis system. Due to the huge number of samples, two gels were exploited to hold 16 protein samples (one gel for eight samples) for electrophoresis, simultaneously. The amount of β-actin in each lane was performed as an internal control to normalize the expression of each protein.

Statistics
Each result represents mean ± standard error of the mean (SEM) of at least three separate experiments. Statistically significant differences between treatments and controls were determined by analysis of variance and then a least significant difference comparison procedure. Statistical significance was set at P<0.05.

Results
Midazolam induced apoptosis in MA-10 mouse Leydig tumor cells
In the present study, MA-10 cells were treated without or with midazolam (0 µM, 6 µM, 30 µM, and 150 µM) for different time points, and the cell cycle distribution was then analyzed by flow cytometry to confirm the apoptotic phenomenon. Results showed that there was no difference in sub-G1 or G2/M phase between control and 6 µM and 30 µM midazolam treatments before 12 hours (Figure 1A–E, P>0.05), whereas the G2/M phase significantly decreased at 6 hours with 150 µM midazolam treatment (Figure 1A–D and F, P<0.05). After 12-hour and 24-hour treatments, the cell population in the sub-G1 phase significantly increased with 150 µM midazolam (Figure 1A–E, P<0.01), whereas the G2/M phase significantly decreased with 150 µM midazolam (Figure 1A–D and F, P<0.001). The increasing sub-G1 phase percentage with the decreasing G2/M phase percentage in 12-hour and 24-hour treatments strongly suggests midazolam-induced apoptosis in MA-10 mouse Leydig tumor cells.

Midazolam activated caspase cascade in MA-10 mouse Leydig tumor cells for apoptosis
To further elucidate the mechanism of apoptosis induced by midazolam in MA-10 cells, caspase cascade was investigated by Western blotting. Results showed that pro-caspase-8 significantly decreased with 150 µM midazolam after 12-hour and 24-hour treatments (Figure 2A and B, P<0.05), whereas cleaved caspase-8 significantly increased after 12-hour treatment with 150 µM midazolam (Figure 2A and C, P<0.05). In addition, midazolam significantly induced caspase-9 cleavage at a dosage of 150 µM after 24-hour treatment (Figure 2A and D, P<0.05). Moreover, cleaved caspase-3 decrease...
significantly increased after 12-hour treatment with 150 µM midazolam (Figure 2A and E, $P<0.01$). It is well known that PARP is one of the downstream substrates of activated caspase-3, which could be cleaved into 85 KDa degraded product of PARP. We also examined the cleavage of PARP upon midazolam treatment, and the results showed that cleavage of PARP activated by caspase-3 significantly increased after 6-hour treatment with 150 µM midazolam, and this phenomenon was sustained for 24 hours (Figure 2A, $P<0.05$). These results suggested that midazolam could stimulate the cleavage of caspase-8, -9, and -3 and PARP to induce apoptosis in MA-10 mouse Leydig tumor cells.
Midazolam modulated mitochondria pathways in MA-10 mouse Leydig tumor cells for apoptosis

We next investigated the role of mitochondrial pathway in midazolam-induced MA-10 cell apoptosis. Studies have demonstrated that while cells undergo apoptosis through mitochondrial pathway, activation of the proapoptotic BH3-only protein, Bid, will induce the release of cytochrome-c from mitochondria to form apoptosome with caspase-9, which could link the interaction between intrinsic and mitochondrial apoptotic pathway.

Figure 2 Midazolam activated caspase cascade in MA-10 mouse Leydig tumor cells. MA-10 cells were treated without or with different concentrations of midazolam (6 µM, 30 µM, and 150 µM) for 3 hours, 6 hours, 12 hours, and 24 hours, respectively. The levels of procaspase-8 protein (57 KDa), cleaved caspase-8 (47 KDa), cleaved caspase-9 (17 KDa), cleaved caspase-3 (19 KDa), and cleaved poly (ADP-ribose) polymerase (PARP) (90 KDa) were analyzed by Western blot. Immunoblots represent the observations from one single experiment repeated three times (A). The integrated optical densities of procaspase-8 (B), cleaved caspase-8 (C), cleaved caspase-9 (D), cleaved caspase-3 (E), and cleaved PARP (F) proteins were analyzed after normalization with β-actin (43 kDa) in each lane. Data in (B–F) represent the mean ± standard error of the mean of three separate experiments.

Note: *Indicates significant difference between control and midazolam-treated groups at the same time (P<0.05).
extrinsic caspase pathways.²⁵,²⁶ Our results showed that total Bid was significantly decreased by 150 µM midazolam after 24-hour treatment (Figure 3A and B, \( P<0.05 \)), which implied the increase of t-Bit that might be associated with caspase-9. However, midazolam did not change the cytosolic levels of cytochrome-c, which was expected to be released from mitochondria due to the activation of Bid (Figure 3A and D, \( P>0.05 \)). Furthermore, we also examined the proapoptotic protein Bax protein, and the results showed that midazolam did not change levels of Bax (Figure 3A and C, \( P>0.05 \)). These results suggested that midazolam might activate caspase-8 to downregulate Bid without affecting Bax and cytochrome-c expressions in MA-10 mouse Leydig tumor cells.

Midazolam downregulated Akt pathway in MA-10 mouse Leydig tumor cells for apoptosis

We further investigated whether midazolam would affect the expression of Akt and phosphor-Akt in MA-10 mouse Leydig tumor cells. Results showed that midazolam (6 µM, 30 µM, and 150 µM for 3 hours and 6 hours, respectively) did not have any effect on total Akt protein expressions (Figure 4A and B, \( P>0.05 \)). However, 150 µM midazolam

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**Figure 3** Midazolam regulated mitochondria pathways in MA-10 mouse Leydig tumor cells. MA-10 cells were treated without or with different concentrations of midazolam (6 µM, 30 µM, and 150 µM) for 3 hours, 6 hours, 12 hours, and 24 hours, respectively. Protein levels of Bid (22 KDa), Bax (20 KDa), and cytochrome-c (14 KDa) were analyzed by Western blot. ImmunobLOTS represent the observations from one single experiment repeated three times (A). The integrated optical densities of Bid (B), Bax (C), and cytochrome-c (D) proteins were analyzed after normalization with \( \beta \)-actin (43 kDa) in each lane. Data in (B-D) represent the mean ± standard error of the mean of three separate experiments.

**Note:** *Indicates significant difference between control and midazolam-treated groups at the same time \( P<0.05 \).
Midazolam induces MA-10 cell apoptosis

Figure 4  Midazolam downregulated Akt pathway in MA-10 mouse Leydig tumor cells. MA-10 cells were treated without or with different concentrations of midazolam (6 µM, 30 µM, and 150 µM) for 3 hours, 6 hours, 12 hours, and 24 hours, respectively. The protein levels of total Akt protein (60 KDa) and phosphor-Akt (60 KDa) were analyzed by Western blot. Immunoblots represent the observations from one single experiment repeated three times (A). The integrated optical densities of total Akt protein (B) and phosphor-Akt (C) proteins were analyzed after normalization with β-actin (43 kDa) in each lane. Data in (B and C) represent the mean ± standard error of the mean of three separate experiments.

Note: *P<0.05 and **P<0.01 indicate significant differences between control and midazolam-treated groups at the same time.

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Levels of total Akt (normalized with β-actin)

Levels of pAkt (normalized with β-actin)

at 12 hours and 24 hours did significantly reduce total Akt protein levels (Figure 4A and B, *P<0.05). In addition, 150 µM midazolam from 6 hours to 24 hours significantly reduced phosphor-Akt (Figure 4A and C, **P<0.01). These results suggest that midazolam could downregulate Akt pathway to induce apoptosis in MA-10 mouse Leydig tumor cells.

Midazolam regulated MAPK pathway in MA-10 mouse Leydig tumor cells for apoptosis

Recent studies have illustrated that MAPKs pathway could affect cell proliferation, differentiation, migration, and apoptosis.18,19,27 Thus, we sought to investigate the possible regulation of MAPKs pathway activated by midazolam in MA-10 cell apoptosis. Data showed that the expressions of phosphor-JNK1 and phosphor-p38 were significantly increased by 150 µM midazolam after 24-hour treatment (Figure 5A, B, and D, *P<0.05). However, the protein levels of phosphor-ERK and phosphor-JNK2/3 were not changed by various midazolam treatments (Figure 5A, C, and E, **P>0.05). These results suggest that midazolam could activate JNK1 and p38, but not ERK and JNK2/3, to induce MA-10 cell apoptosis.

Discussion

Early studies on sedative drug effects mainly focus on its clinical dosages and pharmacokinetic properties.28 Previous studies have indicated that midazolam has a possible neurotoxic effect in young animals and rabbits.22,23,29–31 One study did illustrate that midazolam could activate the intrinsic pathway of apoptosis independent of benzodiazepine and death receptor signaling in human lymphoma and neuroblastoma cell lines.31 Our earlier study showed that midazolam had cytotoxic effects on MA-10 cells.6 However, the underlying mechanism remains unknown. In the present study, we observed that midazolam could induce apoptosis in MA-10 cells in a dose- and time-dependent manner through the activation of caspase and MAPKs pathways. Thus, our findings are parallel to other studies,31,32 suggesting a similar mechanism of the cytotoxic side effect of midazolam upon neural, immune, and reproductive cells.

In general, there are extrinsic and intrinsic pathways for cells undergoing apoptosis.3 The extrinsic pathway is initiated through death receptor to activate downstream DISC and caspase-8. In the other way, intrinsic pathway is initiated at the mitochondrial outer membrane permeability to activate caspase-9 through cytochrome-c and Bcl-2 proteins.33 Caspase-8 and -9 will then stimulate caspase-3 cleavage.10,13
In the present study, we found that midazolam could activate extrinsic and intrinsic caspase cascades to induce apoptosis in MA-10 cells. The cleavage of PAPR after midazolam treatment further confirmed this phenomenon. In fact, studies have also illustrated that galectin-1 could simultaneously activate caspase-8 and -9 pathways to induce apoptosis in MA-10 cells and rat isolated Leydig cells.\(^34,35\) Thus, our findings, that midazolam induced MA-10 cell apoptosis through the activation of caspase cascades, are parallel to other studies in different cell types.

It has been shown that the induction of caspase-9 could be mediated by converging at the mitochondria, which can be activated by cytochrome-c, Bax, reactive oxygen species, and JNK pathways to induce cell apoptosis. Release of cytochrome-c from the mitochondria to the cytoplasm initiates a caspase-9 cascade.\(^36\) Our data showed that the expression of Bax and cytochrome-c did not significantly change with midazolam treatment. Orser et al.\(^37\) have found that sedative drugs such as midazolam and propofol could bind to different types of GABA receptors to achieve sedative effects. It is possible that midazolam could also bind to GABA receptors to activate specific signal pathway, inducing MA-10 cell apoptosis without activating cytochrome-c release. The report has also shown that factors could induce apoptosis without the existence of Bax.\(^38\) Thus, midazolam could induce MA-10 cell apoptosis without the expression
of Bax and cytochrome-c. In fact, the increasing trends of Bax expression at 24 hours and cytochrome-c release at 6 hours by 30 µM midazolam treatments could be observed in the present study. Further investigation will be executed to dissect the changes of Bax and cytochrome-c between cytoplasmic and mitochondrial portions under midazolam treatments, which could truly reveal whether or not midazolam could induce both proteins to activate caspase-9 and then apoptosis in MA-10 cells.

A previous study indicated that mitochondrial intrinsic pathway and extrinsic pathway could be linked by Bid, a proapoptotic protein, which could be activated by caspase-8 in the cytoplasm to regulate the mitochondrial membrane potential by forming a complex with Bax. In the present study, the amount of total Bid significantly decreased by 150 µM midazolam at 4-hour treatment, which indicates that t-Bit was significantly stimulated. This observation suggests that midazolam might activate caspase-8 and -9 pathways through the linkage of t-Bit. However, our study also illustrated that the level of total Bax did not significantly change after midazolam treatment on MA-10 mouse Leydig tumor cells. This phenomenon that upregulation in t-Bit without significant change of Bax protein will be further investigated to understand the detail mechanism.

It is well documented that induction of cell apoptosis by different cellular stresses is involved with JNK, ERK, and p38 MAPK pathways. Mitogen-activated protein kinases are signaling components that are important in converting extracellular stimuli into a wide range of cellular responses. It is reported that JNK signaling pathway is essential for neuronal apoptosis in response to excitotoxic stress, but the role of JNK in the apoptotic responses of other cell types is unclear. On the other hand, many studies demonstrate that JNK and p38 pathways are activated to promote cell survival. In addition, the ERK signaling pathway plays a key role in several steps of tumorigenesis, including cancer cell proliferation, migration, invasion, and apoptosis. As well, PI3K/Akt pathway has been reported to crosstalk with MAPK signaling pathway by autocrine signaling through Ras for cell survival. In fact, a study has demonstrated that constitutive activation of PI3K/Akt could be observed in many cancers, and the targeting of the PI3K/Akt pathway is of great importance in treating prostate cancer. In the present study, we observed that midazolam significantly activated p38 and pJNK1. However, midazolam had no effect on ERK activity. Moreover, midazolam significantly decreased the levels of Akt and phosphor-Akt proteins. These results demonstrate that midazolam could upregulate MAPK pathway and downregulate Akt survival signal to induce MA-10 cell apoptosis.

Conclusion

In summary, midazolam could inhibit cell viability and arrest cell cycle to induce apoptosis in MA-10 mouse Leydig tumor cells. Midazolam did induce t-Bit, JNK, and p38, but not ERK, with the activation of caspase-8, -9, and -3 and PARP plus downregulation of Akt, to induce apoptosis. The current observations regarding the apoptotic effect of midazolam on MA-10 mouse Leydig tumor cells could highlight a potential antitumor therapeutic event in clinical application upon the reproductive system. Taken together, midazolam induced MA-10 cell apoptosis through the activation of caspase cascade, the inhibition of Akt pathway, and the stimulation of p38 and JNK pathways.

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Disclosure

The authors report no conflicts of interest in this work.

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