Rokitamycin Induces a Mitochondrial Defect and Caspase-Dependent Apoptosis in Human T-Cell Leukemia Jurkat Cells

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Abstract. Macrolides are a well-known family of oral antibiotics whose antibacterial spectrum of activity covers most relevant bacterial species responsible for respiratory infectious disease. In recent years, it has been reported that macrolides have not only bactericidal activity but also direct immunomodulating activity in mammals. In this study, we observed new physiological activity of macrolides and examined whether various macrolides induce apoptosis in human leukemia cell lines. We investigated the effects of 13 different macrolides on the viability of Jurkat and HL-60 cells. Among all the macrolides used in this study, rokitamycin, a semi-synthetic macrolide with a 16-member ring, effectively induced cell death. Rokitamycin induced DNA fragmentation and caspase activation, resembling the progression of apoptosis. Moreover, rokitamycin reduced the mitochondrial transmembrane potential and released cytochrome c from mitochondria to the cytosol, suggesting that mitochondrial perturbation is involved in rokitamycin-induced apoptosis. These results suggest that rokitamycin possesses not only bactericidal activity but also pro-apoptotic activity in human leukemia cells.

Keywords: macrolide, rokitamycin, apoptosis, mitochondria, caspase

Introduction

Macrolides are antibiotics with bactericidal effects. In general, macrolides are much better known for their activity against Gram-positive bacteria and Mycoplasma pneumoniae than for their activity against Gram-negative bacteria. Macrolides bind to bacterial 50S ribosome subunits, thus inhibiting bacterial expansion (1). There is, however, an increasing body of evidence demonstrating that macrolides may have activities not related to their antibiotic properties.

Long-term therapy, with low doses (not sensitive to bacteria) of erythromycin (ERY), has been shown to be effective in treating patients with diffuse panbronchiolitis (DPB) (2 – 6). Similar clinical effects of other 14-member macrolides such as clarithromycin (CLR) and roxithromycin (RXM) or a 15-member macrolide, azithromycin (AZM), for patients with DPB have also been reported (7, 8). Several actions of 14-member macrolide antibiotics provide support for these clinical effects (2, 3, 6, 9, 10). A number of other studies have suggested that macrolides have direct immunomodulatory effects other than antibiotic effects (11).

This immunomodulating action of macrolides may contribute to its clinical effectiveness in the treatment of such inflammatory diseases as DPB (12, 13) and certain dermatologic disorders (14). Recent studies suggest that a direct immunomodulating role of macrolides can be assumed as follows: neutrophil chemotaxis inhibition (15 – 18), oxidant generation (15, 19, 20), phagocytosis progression (21, 22), and apoptosis induction (23).

Recently, apoptosis induction in activated lymphocytes by macrolides has been considered to play an important role in immunomodulation (24). Aoshiba et al. have proposed that cAMP is increased in ERY-induced apoptosis (23). Jun et al. have reported that Fas ligand expression is enhanced in ciprofloxacin- and RXM-induced apoptosis (25). However, details regard-
ing the overall apoptotic induction mechanism by macrolides remain obscure.

In the present study, we attempted to observe the apoptotic ability of various macrolides using human leukemia cell lines. We investigated the effects of stimulation by 13 different macrolides on the viability of human leukemia cell lines. Among all the macrolides, semisynthetic macrolides, with its 16-member rokitamycin (RKM), was found to effectively induce cell death in a dose-dependent manner. Therefore, the present study was undertaken to evaluate the effects of RKM and other macrolides on the apoptosis of leukemia and to determine what mechanism underlies this effect.

Materials and Methods

Macrolides
ERY, oleandomycin (OL), RXM, triacetyloleandomycin (TAO), and AZM (Sigma, St. Louis, MO, USA); CLR (Taisho Pharmaceutical, Tokyo); josamycin (JM), leucomycin (LM), spiramycin (SPM), midecamycin (MDM), and tylosin (TYL) (Wako Pure Chemical Industries, Osaka); miocamycin (MOM; Meiji Seika Kaisha, Tokyo); and RKM (Asahi Kasei, Tokyo) were dissolved in dimethyl sulfoxide (DMSO, Wako) and subsequently diluted in RPMI 1640 or DMEM medium.

Preparation of mitochondria and cytosol

Mitochondria and cytosol fraction was collected as described before (27). Briefly, cells (1×10^7) were washed once with PBS, resuspended in 5 volumes of cell-free system buffer (CFS buffer) (220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM M_2Cl_2, 5 mM sodium pyruvate, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM HEPES-NaOH, pH 7.4) and swollen on ice for 20 min. Cells were disrupted by 10–15 aspirations through a 22-gauge needle and centrifuged at 750×g for 5 min at 4°C to remove nuclei. The supernatant was centrifuged again (10,000×g for 30 min at 4°C, the remaining supernatant was used as the cytosol fraction. Both samples were used immediately or stored at −80°C until immunodetection of cytochrome c.

Assessment of macrolide cytotoxicity

To assess the susceptibility to macrolides, the relative number of viable cells (percentage of viable cells) was determined by colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) according to the manufacturer’s protocol. In brief, cells were cultured at 2×10^4 cells/well in flat-bottomed, 96-well plates with macrolides. Twenty-four hours later, MTT was added to each well at the final concentration of 0.5 mg/ml and further incubated for 1 h. The net absorbance at 570 nm, which was proportional to the viable cell number, was then measured using a microplate reader. The absorbance of culture wells without macrolides was set as 100%.

Determination of mitochondrial transmembrane potential (∆Ψ_m) on intact cells

The ∆Ψ_m value was determined using 3,3′-dihexyloxacarbocyanide iodide (DiOC₆(3); Invitrogen, Carlsbad, CA, USA) as described (26).

DNA fragmentation

Apoptosis was determined by assaying DNA fragmentation by agarose gel electrophoresis. Cells were rinsed once with PBS and dissolved in 50 mM Tris-HCl buffer, pH 7.8, containing 10 mM EDTA, 0.5% sodium lauryl sarcocinate, and 0.5 mg/ml RNase A. After incubation at 50°C for 1 h, Proteinase K was added at a concentration of 1 mg/ml and then the mixture was further incubated at 50°C for 30 min. Chromosomal DNA was analyzed by 2% agarose gel electrophoresis.

Preparation of mitochondria and cytosol

Mitochondria and cytosol fraction was collected as described before (27). Briefly, cells (1×10^7) were washed once with PBS, resuspended in 5 volumes of cell-free system buffer (CFS buffer) (220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM M_2Cl_2, 5 mM sodium pyruvate, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM HEPES-NaOH, pH 7.4) and swollen on ice for 20 min. Cells were disrupted by 10–15 aspirations through a 22-gauge needle and centrifuged at 750×g for 5 min at 4°C to remove nuclei. The supernatant was centrifuged again (10,000×g, 10 min, 4°C) to recover mitochondria. The 10,000×g supernatant was ultracentrifuged at 100,000×g for 30 min at 4°C, and the remaining supernatant was used as the cytosol fraction. Both samples were used immediately or stored at −80°C until immunodetection of cytochrome c.

Western blots

Whole cell lysate, mitochondria and cytosol fractions were mixed in the same volume of SDS sample buffer (4% SDS, 125 mM Tris, pH 6.8, 10% glycerol, 0.02 mg/ml bromophenol blue, and 10% 2-mercaptoethanol) and heated at 100°C for 5 min. Proteins were separated by 4%–20% gradient SDS-PAGE and electrically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking the membrane using 5% skimmed milk, caspase-3, caspase-8, caspase-9, cytochrome c, Bax, phosphorylated (phos-
pho)-SAPK/JNK, and native-SAPK/JNK were immuno-detected using anti-caspase-3 Ab (1:1000; Cell Signal-ing Technology, Beverly, MA, USA), anti-caspase-8 Ab (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase-9 Ab (1:200, Santa Cruz), anti-cytochrome c Ab (1:1500; BD Bioscience, San Jose, CA, USA), anti-Bax Ab (1:1000, Santa Cruz), and anti-phospho-SAPK/JNK Ab and anti-native-SAPK/JNK Ab (each diluted 1:1000, Cell Signaling Technology). Thereafter, HRP-conjugated anti-mouse IgG or antirabbit IgG was applied as second Abs, and positive bands were detected by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK).

**Determination of isolate mitochondrial membrane potential and cytochrome c release**

Determination of isolated mitochondria was described before (27, 28). Briefly, to determine \( \Delta \Psi_m \), isolated mitochondria were washed and resuspended in CFS buffer at a concentration of approximately 25 mg mitochondrial protein in 100 ml CFS buffer. Protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA). After an incubation with or without macrolides for 30 min at 37°C, the mitochondrial suspensions were further incubated for 15 min with DiOC6 (3). Mitochondrial \( \Delta \Psi_m \) was examined by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA, USA). To immunodetect cytochrome c, isolated mitochondria were washed and resuspended in CFS buffer at a concentration of approximately 100 mg mitochondrial protein in 100 ml CFS buffer. After incubating with or without macrolides for 60 min at 37°C, reaction mixtures were centrifuged (10,000 \( \times \) g, 30 min, 4°C). The mitochondria pellet and supernatant were separated and immediately used or stored at –80°C until the immunodetection of cytochrome c.

**Results**

**Effects of macrolides on cell viability**

We investigated the effects of macrolides on Jurkat human T leukemia cell line and HL-60 human acute promyelocytic cell lines. Cells were exposed to 13 macrolides, respectively, for 24 h. One of the 16-ring member macrolides, RKM, exhibited strong cytotoxic activity in Jurkat, but the others did not (Fig. 1a). RKM also reduced cell viability in HL-60 (Fig. 1b). Furthermore, the dose-dependent effect of RKM was determined. Various leukemia cell lines (human B leukemia cell line BALL-1, human T leukemia cell line Jurkat, human acute promyelocytic cell line HL-60) showed dose-dependent decreases in cell viability, with a similar trends (Fig. 1c).

![Fig. 1. The cytotoxic effects of 13 macrolide antibiotics on human leukemia cell lines. Human T lymphoma cell line (Jurkat) (a) and human acute promyelocytic cell line (HL-60) (b) were treated with macrolides at 10 \( \mu \)M (open bars) or 40 \( \mu \)M (closed bars) for 24 h, respectively. The relative number of viable cells (percentage of viable cells) was determined by a MTT colorimetric assay, as described in Materials and Methods. Vertical lines indicate the S.D. (n = 3). c: Human B lymphoma cell line (BALL-1), T lymphoma cell line (Jurkat), and myelogenous leukemia cell line (HL-60) were each treated with the indicated doses of RKM for 24 h. The relative number of viable cells (percentage of viable cells) was determined by a MTT colorimetric assay, as described in Materials and Methods. Vertical lines indicate the S.D. (n = 3).](image)

**RKM-induced DNA fragmentation and caspase activation**

We investigated whether the observed leukemia cell death was from apoptosis or necrosis. The presence of DNA degradation of nucleosomal units and sub-G1 DNA content cells in 40 \( \mu \)M RKM–treated Jurkat cells indicates that the form of cell death was apoptosis (Fig. 2: a and b). The mass of sub-G1 DNA content cells was not significantly altered by MOM (another 16-member macrolide)-treatment (Fig. 2b). We then tested whether RKM can induce apoptotic-specific proteases,
caspases activation. In RKM-treated Jurkat cells, a large fragment of active caspase-3 (17, 19-kDa) was detected (Fig. 3a). Moreover, RKM cleaved one of the caspase-3–cleaving upstream caspases, caspase-9 (17-kDa fragment) (Fig. 3a). The pan-caspase inhibitor Z-VAD-fmk inhibited caspase-3 and -9 activation and DNA degradation in RKM-treated Jurkat cells, suggesting that RKM-induced DNA fragmentation is caspase-dependent (Fig. 3: a and b). Another caspase-3–cleavable upstream caspase, caspase-8, was also activated by RKM treatment (Fig. 3a). However, RKM-induced caspase-8 activation was inhibited by a caspase-3 specific inhibitor, Ac-DEVD-fmk, suggesting that mitochondria perturbation is the major pathway for RKM-induced apoptosis (Fig. 3a).

**RKM-induced apoptosis in intact cells via a mitochondrial pathway**

Because RKM induced caspase-9 activation, we examined the release of cytochrome c from mitochondria, which is the major event of mitochondria-mediated apoptosis. Similar to the results of cytotoxicity and DNA fragmentation assay (Figs. 1 and 2), treatment with 40 μM RKM induced cytochrome c release from
mitochondria to the cytosol, but treatment with 20 μM RKM did not (Fig. 4a). To determine whether RKM-induced mitochondrial cytochrome c release or RKM-induced caspase activation is the prior action, we used Z-VAD-fmk. After a 1-h preincubation with 10 μM Z-VAD-fmk, RKM-induced DNA fragmentation was completely blocked (Fig. 3b). However, Z-VAD-fmk did not inhibit the RKM-induced mitochondrial cytochrome c release (Fig. 4b). These results indicate that RKM-induced mitochondrial cytochrome c release precedes caspase activation, thus demonstrating that RKM-induced mitochondrial perturbation is caspase-independent.

The reduction in the ΔΨm value, which is another major event in mitochondrial damage, was observed with DiOC6 (3) staining. The level of ΔΨm was dose-dependently reduced by RKM-treatment (Fig. 4b).

**RKM-induced apoptosis in p53-null Saos-2 cells**

Oncogene p53 is involved in mitochondria-mediated apoptosis by recruiting proapoptotic Bcl-2 family protein Bax to mitochondria (29). To determine whether p53 activation is required for RKM-induced apoptosis, we incubated Saos-2 (a p53-deficient human osteosarcoma cell line) cells with RKM at the indicated concentrations (Fig. 5). Exposure to RKM induced a dose-dependent cell death in Saos-2 cells. This finding indicates that p53 activation is not required for RKM-induced apoptosis.

**JNK/SAPK phosphorylation is not associated with RKM-induced apoptosis**

Proapoptotic MAPK, JNK/SAPK, and p38 induce mitochondria-mediated apoptosis (30–32). We further examined the possibility of JNK/SAPK activation by RKM. JNK/SAPK was phosphorylated within 2 h after exposure to RKM, and the phosphorylation status reached a maximum 4 – 6 h later (Fig. 6a). To determine whether JNK/SAPK phosphorylation is required for this apoptosis, we treated Jurkat cells with RKM after pre-treatment with JNK inhibitor. Phosphorylation of JNK/SAPK was significantly blocked by JNK inhibitor (Fig. 6b), but DNA fragmentation was not (Fig. 6c). Although RKM-induced JNK phosphorylation was not inhibited by pre-treatment with Z-VAD-fmk, RKM-induced apoptosis was significantly inhibited. These results indicate that RKM-induced JNK/SAPK phosphorylation is independent of RKM-induced apoptosis.
RKM directly affects mitochondria and induces $\Delta \Psi_m$ reduction and cytochrome c release

We considered whether RKM directly affects mitochondria, similar to several other reagents with direct effects (27, 33, 34). Mitochondria isolated from Jurkat cells were incubated with RKM and then stained with DiOC$_6$ (3) to assess $\Delta \Psi_m$ (Fig. 7a). After incubation with RKM, $\Delta \Psi_m$ was reduced. Moreover, mitochondrial cytochrome c was examined in isolated mitochondria. Isolated Jurkat mitochondria were incubated with RKM, and cytochrome c in isolated mitochondria and mitochondrial supernatant was then detected by Western blotting (Fig. 7b). RKM was found to induce a release of cytochrome c from the mitochondria into the mitochondrial supernatant in a dose-dependent manner, which was similar to the result in intact cells (Fig. 4a). Cytochrome c release was induced by treatment with a lower dose of RKM in isolated mitochondria than in intact cells. These findings indicated that RKM directly affects mitochondria and induces $\Delta \Psi_m$ reduction and cytochrome c release.
Discussion

In this study, the 16-member macrolide RKM was found to induce apoptosis in leukemia cells. Caspase-3 and caspase-9 were activated in Jurkat exposed to RKM in vitro. In addition, the pan-caspase inhibitor Z-VAD-fmk completely blocked this caspase activation and subsequent RKM-induced nuclear DNA fragmentation (Fig. 3). Thus, caspase activation appears to be essential for RKM-induced apoptosis.

Low et al. have previously reported that ionizing radiation, 5-fluorouracil, etoposide, and adriamycin induce p53-dependent apoptosis in mouse embryonic fibroblasts or thymocytes and that p53 tumor suppressor is required for efficient execution of the death program (35, 36). We therefore tested whether p53 tumor suppressor is required for RKM-induced apoptosis. Cell death was induced in saos-2 cells (p53-null) by exposure to RKM (Fig. 5). Moreover, in Jurkat cells, Bax (a pro-apoptotic Bcl-2 family protein affected by p53) expression was not altered after exposure to RKM (data not shown). These findings indicate that p53 tumor suppressor is not required for RKM-induced apoptosis.

Xia et al. demonstrated that activation of JNK/SAPK and p38 are critical for the induction of apoptosis after withdrawal of nerve growth factor from rat PC-12 pheochromocytoma cells (37). Recent studies have revealed that JNK/SAPK and p38 MAP kinase are activated by cytotoxic stresses such as UV radiation, X-ray, heat shock, and osmotic shock, as well as by proinflammatory cytokines such as tumor necrosis factor and interleukin-1 (38, 39). However, we demonstrated here that the JNK/SAPK-induced apoptosis pathway is independent of the RKM-induced apoptosis pathway. Activation of JNK/SAPK was significantly blocked by the JNK inhibitor, but DNA fragmentation was not (Fig. 6). Although RKM-induced activation of JNK/SAPK was not inhibited by pre-treatment with Z-VAD-fmk, RKM-induced apoptosis was significantly inhibited. These results indicate that RKM-induced activation of JNK/SAPK is independent of RKM-induced apoptosis. Phosphorylation of p38 MAP kinase was not detected after exposure to RKM (data not shown).

In this study, cytochrome c was released from the mitochondrial intermembrane into the cytosol, and \( \Delta \Psi_m \) was reduced in RKM-treated Jurkat cells (Fig. 4). Cytochrome c release was not inhibited by pre-incubating the cells with Z-VAD-fmk. This evidence suggests that releasing cytochrome c from mitochondria and reducing \( \Delta \Psi_m \) are upstream events before caspase activation. Furthermore, studies using a cell-free system revealed that RKM directly triggers the reduction of \( \Delta \Psi_m \) and the release of cytochrome c from mitochondria (Fig. 7). Moreover, overexpression of Bcl-2 inhibits apoptotic cell death induced by RKM (data not shown). These results suggest that RKM-induced mitochondria perturbation is essential for RKM-induced apoptosis. The macrolide derivatives antimycin, oligomycin, and rapamycin influence mammalian mitochondrial activity by binding to cytochrome b (40), F1F0-ATPase (41), and mammalian TOR (42, 43), respectively. Since macrolide derivatives retain structural similarity, RKM (and other macrolides) may influence mitochondria by binding to mitochondrial proteins directly. So why does RKM induce mammalian cell death specifically among the other macrolides? Ishiguro et al. revealed that the 16-membered macrolide RKM penetrated into human polymorphonuclear leukocytes in a greater amount than the other macrolides that are 14- or 15-membered (44). Taking this into consideration, RKM’s higher potency to induce mammalian mitochondria perturbation, compared to the other less effective macrolides, may result from its greater ability to penetrate into the intracellular space. Further analysis is necessary to elucidate the targeting molecule of RKM in mammalian cells and the mechanism underlying mitochondrial defects.

The RKM concentrations that we used in these studies were higher than the serum drug concentration, which are achieved in serum clinically after oral ingestion. In staphylococci, the \( IC_{50} \) of macrolides used in this study are about 0.06 – 1 \( \mu g/\text{ml} \), which are less than the respective concentrations that we used in this investigation (40 \( \mu M \) RKM is about 32 \( \mu g/\text{ml} \)). However, tissue/cellular drug concentrations might be much higher than the serum drug concentrations. It has been reported that ERY concentration has been concentrated up to 50-fold in neutrophils (45). As previously mentioned, since RKM tended to penetrate into human polymorphonuclear leukocytes more effectively than 14-membered ERY (44), RKM might affect not only bacteria but also to mammalian cells in vivo. A previous study reported that RKM induced human polymorphonuclear chemotaxis at doses over 20 \( \mu g/\text{ml} \) (about 25 \( \mu M \)). Similarly, we determined that RKM was toxic to leukocytes at doses similar to those toxic to leukemia cells. The \( IC_{50} \) dose in leukocytes was 35 \( \mu M \) (data not shown) and that in Jurkat cells was 25 \( \mu M \). However, the human normal fibroblast cell line MRC-5 was resistant to RKM (\( IC_{50} \) = 70 \( \mu M \)) (data not shown). Further in vivo studies estimating the clinical relevance of RKM are warranted.

In summary, RKM induces apoptosis more effectively than the other 12 macrolides used in this study. RKM directly induces mitochondrial defects and cytochrome c release. The released cytochrome c then binds to Apaf-1 and activates further caspases, including caspase-9 and caspase-3. Activated caspases then execute apoptotic
cell death. Tumor suppressor p53 and the MAPK family are independent of this mechanism. This above-mentioned mitochondrial perturbation mechanism by RKM seems to occur without cell specificity in leukemia cells (Fig. 1c). Moreover, RKM may be effective not only for DBP but also for tumor therapy because RKM can significantly and independently induce apoptosis p53 and it can remove tumors that have a mutation in p53. Future studies will assess whether the induction of apoptosis by RKM has clinical efficacy as an anti-tumor drug and/or immunosuppressant drug.

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