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Chloramphenicol Arrests Transition of Cell Cycle and Induces Apoptotic Cell Death in Myelogenous Leukemia Cells

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Abstract Chloramphenicol is a broad-spectrum antimicrobial agent against Gram (+) and Gram (-) bacteria. Its clinical application has recently been limited, due to severe side effects such as bone marrow suppression and aplastic anemia. In the present study, the cytotoxic effects of chloramphenicol were investigated *in vitro* using chronic myelogenous leukemia K562 cells. Chloramphenicol inhibited the growth of K562 cells in a dose-dependent manner, but their growth was restored after the cessation of chloramphenicol, indicating reversible cytotoxic effects. The expression of cell cycle regulatory molecules, including E2F-1 and cyclin D1, was decreased at the translational and/or transcriptional level after being treated with a therapeutic blood level (20 μg/ml) of chloramphenicol. Chloramphenicol also induced apoptotic cell death through a caspase-dependent pathway, which was verified by Western blot analysis and the enzymatic activity of caspase-3. These results demonstrated that chloramphenicol inhibited the cell growth through arresting the transition of the cell cycle, and induced apoptotic cell death through a caspase-dependent pathway at therapeutic concentrations.

Key words: Chloramphenicol, cell cycle, apoptosis

Chloramphenicol is an effective antimicrobial agent for the treatment of a variety of bacterial infections, including invasive *Haemophilus influenzae* infections, serious anaerobic infections, ocular infections, salmonellosis, and rickettsioses in patients under the age of eight years [18, 26]. It reversibly binds to the 50S subunit of ribosomes and specifically inhibits protein synthesis in bacteria; however, it can also inhibit protein synthesis in mitochondria of eukaryotic cells by binding to the 70S subunit of mitochondrial ribosomes [11, 16]. This may induce serious side effects, including bone marrow suppression, aplastic anemia, and gray baby syndrome in humans [27]. Therefore, chloramphenicol is rarely used nowadays in many countries.

Hematologic toxicity is the most important adverse effect of chloramphenicol and is divided into two categories [24, 28, 29]. Dose-dependent reversible bone marrow suppression occurs when serum chloramphenicol exceeds 20 μg/ml. This toxic effect is reversible when administration of the drug is stopped. The second is fatal aplastic anemia, a life-threatening toxicity. This usually occurs at several weeks to months after chloramphenicol administration and is not related to drug dosage.

It has been known that chloramphenicol inhibits growth of cells and induces cell death, which might be associated with adverse hematologic toxicities [6, 15, 19, 20]. Chloramphenicol induces apoptosis of cells including monkey kidney cells, CFU-E from human neonatal cord blood, and rat brain cells [9]. However, the molecular mechanisms of inhibition of cell growth and induction of apoptotic cell death have not yet been fully elucidated. Therefore, we investigated the molecular mechanisms of the cytotoxic effects of chloramphenicol *in vitro*. The data showed that chloramphenicol inhibited the growth of K562 cells through arresting the transition of the cell cycle, and induced apoptotic cell death through a caspase-dependent pathway at therapeutic concentrations of the drug.

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MATERIALS AND METHODS

Cells and Culture Conditions

Human chronic myeloleukemia cells, K562 cells, were maintained in RPMI 1640 medium (HyClone, Logan, Utah, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone) and sodium bicarbonate (2 mg/ml), and were cultured in a 5% CO_2 incubator at 37°C.

Determination of Cell Growth

The growth inhibition of K562 cells was measured using a Premix WST1 cell proliferation assay system (TaKaRa Shuzo Co. Ltd., Shiga, Japan). WST1 is a type of tetrazolium salt that is cleaved to formazan dye by succinate-tetrazolium reductase, which exists in the mitochondrial respiratory chain and is active only in viable cells. K562 cells were seeded at a concentration of 1.0×10^4/ml in 96-well microplates. Wells of the microplates were treated with chloramphenicol at a range of 10 to 80 μg/ml. The growth of the cells was measured at 450 nm after 3 h of treatment with WST1. The same concentration of chloramphenicol was tested in four wells per experiment, and the growth of K562 cells was measured at 1, 2, 3, and 6 days.

Trypan Blue Exclusion Assay

The viability of K562 cells was determined using the trypan blue exclusion assay [10, 17]. After cells were treated with various concentrations of chloramphenicol (10, 20, 40, and 80 μg/ml) for the time points indicated (3, 6, and 9 days), cells were harvested, washed with phosphate-buffered saline (PBS, pH 7.4), and stained with 0.4% trypan blue for 5 min at room temperature. Trypan blue-stained and unstained cells were counted using a hemocytometer.

Western Blot Analysis

Cells were lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, phenyl-methyl-sulfonyl fluoride (10 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), 5 mM phenanthroline, and 28 mM benzamidine-HCl] for 30 min on ice. The lysates were cleared by centrifugation at 12,000 ×*g* for 30 min. Protein concentration was determined using a Bradford assay kit with bovine serum albumin as a standard (Life Science Co., CA, U.S.A.). Supernatants containing 20 μg of proteins were separated by SDS-PAGE on 6.5% or 10% gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Millipore Co., Bedford, MA, U.S.A.), and the membrane was reacted with primary antibodies, followed by treatment with horseradish peroxidase-conjugated secondary antibody [13]. Protein bands were detected using an enhanced chemilluminescence kit (Amersham Life Scinece Co., Buckingghamshire, U.K.) according to the manufacturer's instructions. Primary antibodies against β-tubulin, cyclin D1 (mouse monoclonal; Santa Cruz Co., CA, U.S.A.), cyclin E (mouse monoclonal; Santa Cruz Co.), E2F-1 (mouse monoclonal; Santa Cruz Co.), caspase-3 (mouse monoclonal; Santa Cruz Co.), and poly[ADP-ribose] polymerase (PARP, mouse monoclonal; Santa Cruz Co.) were applied at optimized concentrations.

Table 1. Oligonucleotide primers used in this study.

Genes	DNA sequences (5' to 3')	PCR products (bp)
GAPDH	CGT CTT CAC CAC CAT GGA GA CGG CCA TCA CGC CAC AGT TT	300
Cyclin D1	ACC TGG ATG CTG GAG GTC TG GAA CTT CAC ATC TGT GGC ACA	402
Cyclin E	GGA AGG CAA ACG TGA CCG TT GGG ACT TAA ACG CCA CTT AA	638
E2F-1	ACG TGA CGT GTC AGG ACC TT GTT CTT GCT CCA GGC TGA GT	336

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

After cells were treated with chloramphenicol, they were harvested and homogenized with TRIzol reagent (Molecular Research Center Inc., U.S.A.) for 5 min at room temperature. Total RNA was washed once with 75% cold ethanol and dissolved in DEPC-treated water. The concentration and purity of extracted RNA were measured using a UV spectrophotometer. The extracted RNA was reversely transcribed to cDNA with MuLV reverse transcriptase (Takara Shuzo Co. Ltd.). The PCR reagent consisted of 5 μl of 10× buffer (15 mM $MgCl_2$, 100 mM Tris-HCl, 500 mM KCl, pH 8.3), 1 μl each of dNTP (10 mM), 1 μl each of primer (30 mM), 1 μl of cDNA, and 2.5 U of *Taq* polymerase. PCR products were electrophoresed in 1.5% agarose gel. Primer sequences for PCR are depicted in Table 1.

Measurement of DNA Fragmentation

After cells were treated with chloramphenicol, they were harvested and analyzed for DNA fragmentation [5, 10, 14]. Briefly, cells were collected by centrifuging at 1,000 ×*g* for 5 min at 4°C, washed with ice-cold PBS, and resuspended in lysis buffer (0.5% Triton X-100, 5 mM Tris, 20 mM EDTA, pH 7.4). The lysates were collected and kept on ice for 15 min. After centrifuging at 12,000 ×*g* for 15 min at 4°C, the supernatants were incubated with RNase A (5 μg/ml) at 37°C for 1 h, followed by 1 h of incubation at 50°C with proteinase K (200 μg/ml). After extraction with phenol/chloroform, DNA was precipitated with ethanol and 3 M sodium acetate. The fragmented DNA was analyzed on 1.5% agarose gel.

Enzymatic Cleavage Activity of Caspase-3

After K562 cells were treated with chloramphenicol, the cells were lysed in lysis buffer (50 mM Tris, 0.03% NP-40, 1.0 mM DTT, pH 7.5) for 30 min on ice. Following centrifugation at 12,000 ×*g* for 30 min at 4°C, the protein concentrations of supernatant were quantitated using a Bradford assay kit. To assay caspase-3 activity, the lysates were then incubated with 0.2 mM ac-DEVD-*p*NA (Enzyme System Product Co., CA, U.S.A.) in a total volume of 0.1 ml, in duplicate, and the results were presented as the average increase in absorbance at 405 nm.

RESULTS

Chloramphenicol Inhibits Growth of K562 Cells

Since chloramphenicol-induced cytotoxicity is strongly associated with suppression of precursor cells in bone marrow and also associated with iron homeostasis [16], we investigated the cytotoxic effects of chloramphenicol in human K562 cells. To determine whether chloramphenicol inhibited the cell growth, K562 cells were treated with 10 to 80 μg/ml of chloramphenicol for 6 days, and cellular proliferation was determined by WST1 assay. As shown in Fig. 1, the growth of K562 cells treated with chloramphenicol was not inhibited during the first 3 days, but the growth was inhibited at day 6 in a dose-dependent manner. Similar results were also obtained in the viability of K562 cells, when treated with chloramphenicol, which was verified by trypan blue exclusion assay (Fig. 2). Next, to determine whether cell growth was restored after the cessation of chloramphenicol, K562 cells were treated with 20 μg/ml of chloramphenicol for 3 and 6 days, and then recultured with fresh medium without chloramphenicol for another 6 and 3 days, respectively. The viability of K562 cells was increased after the cessation of chloramphenicol (Fig. 3). This finding suggested that chloramphenicol reversibly inhibited the growth of K562 cells.

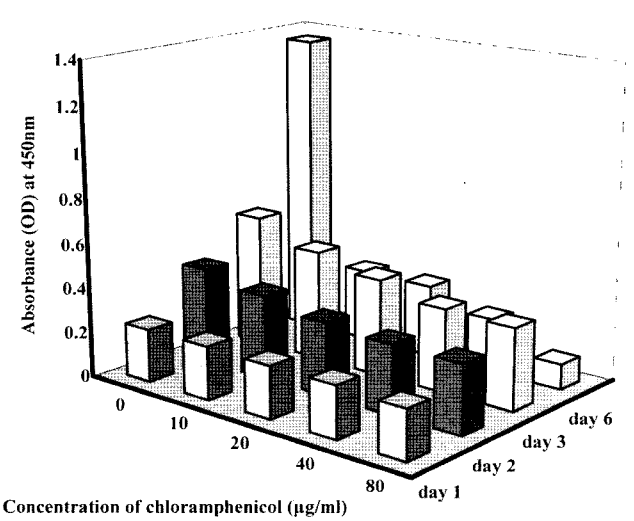

Fig. 1. Growth inhibition of K562 cells treated with chloramphenicol.
Growth inhibition of the cells was determined using the WST1 assay. K562 cells were treated with chloramphenicol for 6 days. Values represent the average of four culture-plate wells from a representative experiment.

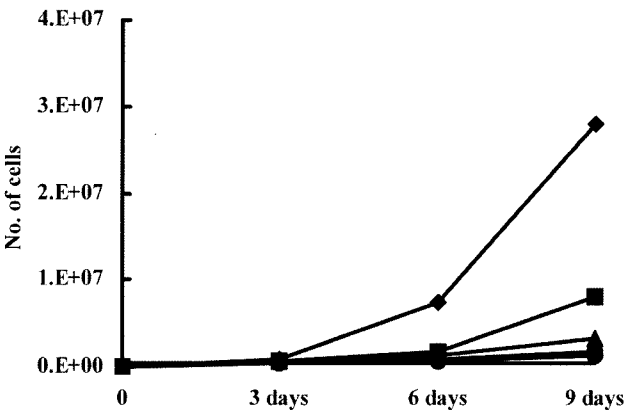

Fig. 2. Effect of chloramphenicol on the viability of K562 cells.
K562 cells were seeded at the concentration of 5×10^4 cells in 20 ml of RPMI 1640 medium and cultured for 3, 6, and 9 days with 0 (◆), 10 (■), 20 (▲), 40 (●), and 80 (*) μg/ml of chloramphenicol. Values are representative of three independent experiments.

Chloramphenicol Arrests Cell Cycle Transition

To investigate whether the growth inhibition was caused by arresting the cell cycle transition, K562 cells were treated with 20 μg/ml of chloramphenicol, and the expression of cell cycle regulatory molecules was determined by Western blot analysis. The expression of E2F-1 was

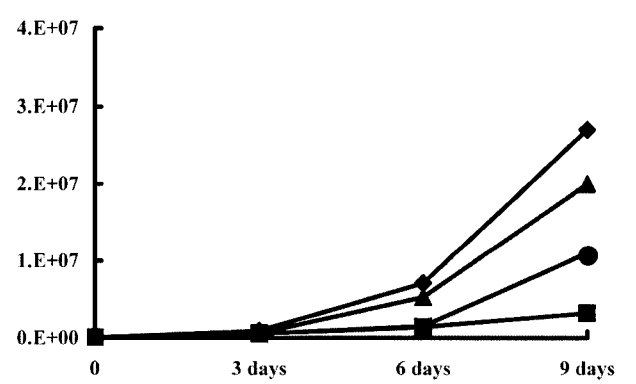

Fig. 3. Restoration of cellular viability after the removal of chloramphenicol.
K562 cells were seeded at the concentration of 5×10^4 cells in 20 ml of RPMI 1640 medium. Experiment I (▲), K562 cells were treated with 20 μg/ml of chloramphenicol for 3 days and cultured for another 6 days without chloramphenicol. Experiment II (●), K562 cells were treated with 20 μg/ml of chloramphenicol for 6 days and cultured for another 3 days without chloramphenicol. K562 cells were culture in 20 ml of RPMI1640 medium (◆) or in RPMI1640 medium with 20 μg/ml of chloramphenicol (■). Values are representative of three independent experiments.

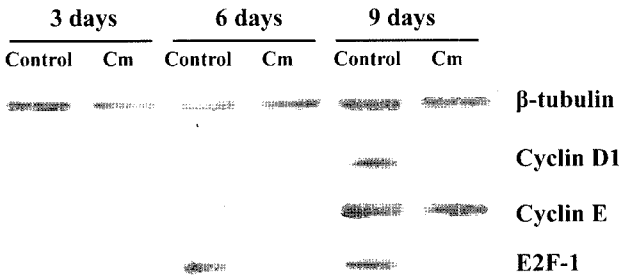

Fig. 4. Western blot of cell cycle-related proteins in K562 cells treated with chloramphenicol.
Cells were treated with 20 μg/ml of chloramphenicol (Cm) for 3, 6, and 9 days. Cell lysates were resolved on 6.5% or 10% polyacrylamide gel and immunoblotted with anti-β-tubulin, anti-cyclin D1, anti-cyclin E, and anti-E2F-1 antibodies.

greatly decreased as early as 6 days (Fig. 4), and the expression of cyclin D1 was decreased at 9 days, whereas cyclin E was not affected by chloramphenicol. It is well known that some metabolites of chloramphenicol induce cytotoxic effects at the transcriptional level [1, 15, 24, 30]. Therefore, to investigate whether the reduced expression of cyclin D1 and E2F-1 proteins was due to down regulation at transcriptional level, the mRNA expression of these molecules was determined by RT-PCR. As seen in Fig. 5, the mRNA expression of cyclin D1, cyclin E, and E2F-1 was slightly decreased, compared with the untreated control cells. These results suggest that chloramphenicol affects the expression of cell cycle regulatory molecules at the translational and/or transcriptional level, finally resulting in the inhibition of cell growth.

Chloramphenicol Induces Apoptosis of K562 Cells

To determine whether chloramphenicol induced apoptosis, K562 cells were treated with various concentrations of chloramphenicol for 3, 6, and 9 days, and a DNA fragmentation assay was performed. K562 cells treated with 10 or 20 μg/ml of chloramphenicol exhibited the features of typical DNA ladders at 9 days (Fig. 6). The induction of apoptosis is known to be mediated through an ordered series of events, such as the activation of caspases followed by its substrate degradation. Therefore, a question of whether

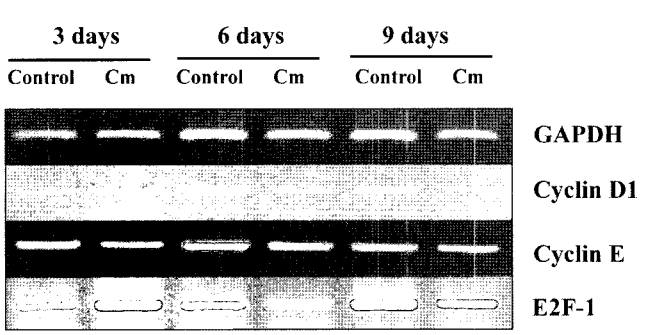

Fig. 5. The expression of mRNA of cell cycle-related genes in K562 cells treated with chloramphenicol.
K562 cells were treated with 20 μg/ml of chloramphenicol (Cm) for 3, 6, and 9 days. Total RNA was extracted and transcribed to cDNA. PCR for the genes encoding cell cycle regulatory proteins was performed as described in Materials and Methods.

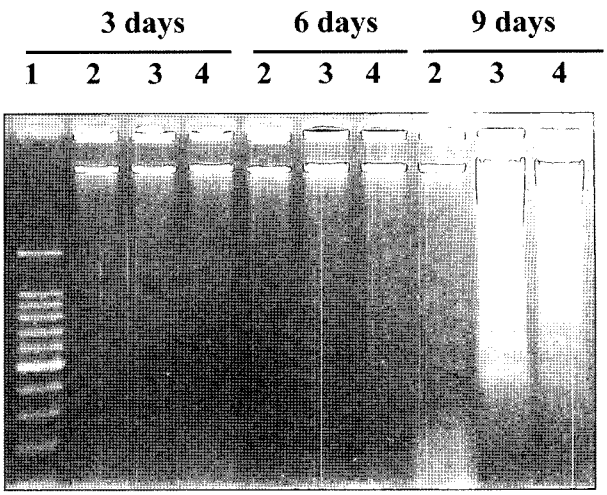

Fig. 6. Analysis of internucleosomal DNA fragmentation of K562 cells treated with chloramphenicol.
Cells were treated with culture medium alone (lane 2), 10 μg/ml (lane 3), and 20 μg/ml (lane 4) of chloramphenicol for 3, 6, and 9 days. DNA was extracted from the cells. Extracted DNA was electrophoresed in 1.5% agarose gel. Lane 1 is 100 bp size marker.

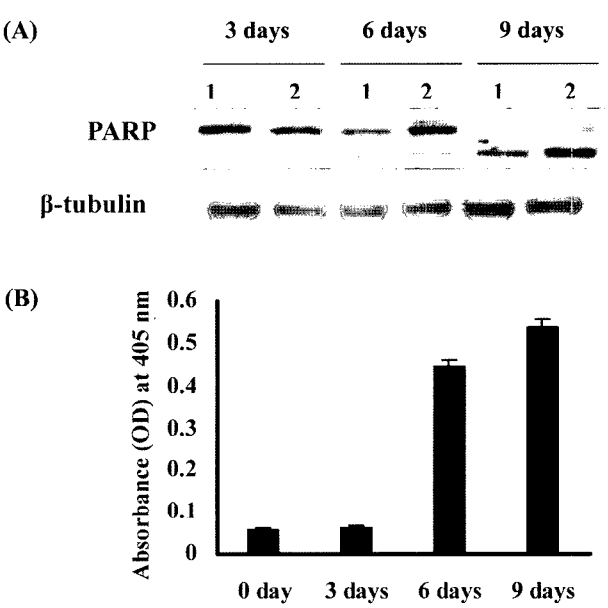

Fig. 7. K562 cells treated with chloramphenicol underwent apoptosis through caspase-dependent pathway.
(A), Western blot analysis of PARP. K562 cells were treated with 10 μg/ml (lane 1) and 20 μg/ml (lane 2) of chloramphenicol for 9 days, and proteins were extracted from the cells. (B), The caspase-3-dependent activity was determined using the synthetic tetrapeptide substrate ac-DEVD-*p*NA. Cells were treated with 20 μg/ml of chloramphenicol for 9 days.

chloramphenicol-treated apoptosis occurred through a caspase-dependent pathway was investigated by Western blot analysis of PARP and caspase-3. The 116 kDa nuclear enzyme PARP, a common substrate for multiple caspases, was cleaved to an 85-kDa subunit as early as 6 days (Fig 7A). Caspase-3 is synthesized as a 32 kDa precursor, which is cleaved to 17 kDa and 12 kDa subunits on activation. The total amount of procaspase-3 was decreased in K562 cells treated with chloramphenicol for 6 and 9 days; however, the cleaved forms of caspase-3 could not be detected (data not shown). To confirm whether caspase-3 is involved in chloramphenicol-induced apoptosis, the enzymatic cleavage activity of caspase-3 was investigated. Consistent with Western blot analysis of PARP upon treatment with chloramphenicol, the enzymatic activity of caspase-3 sharply increased after 6 days (Fig. 7B), indicating that K562 cells treated with chloramphenicol underwent apoptosis through a caspase-dependent pathway.

DISCUSSION

This study demonstrated that chloramphenicol inhibits the progression of cell cycle through the suppression of cyclin D1 and E2F-1, which play an important role in G1/S transition of cells, and induces apoptotic cell death through a caspase-dependent pathway. Although the inhibition of cell growth and apoptotic cell death were not observed at the wide range of concentrations of chloramphenicol during the first 3 days, they appeared as early as 6 days. It is well known that bone marrow suppression generally occurs when the serum chloramphenicol level exceeds 20 μg/ml [26]. However, our results suggest that adverse side effects can be induced by low concentrations of chloramphenicol (less than 20 μg/ml) *in vitro*. The growth of K562 cells treated with chloramphenicol was restored after removal of the drug from the culture medium. This indicates that the cytotoxic effects of chloramphenicol are reversible, suggesting that bone marrow suppression can be removed when administration of the drug is stopped *in vivo*.

The G1/S transition of the cells is regulated by cyclin-dependent kinase 2 (Cdk2), Cdk4, and Cdk6 in association with cyclin D and E. E2F and Rb are key regulatory proteins in G1/S transition [2, 7, 8, 12, 17, 21–23]. The Rb/E2F complex suppresses transcription of S phase genes. Hyperphosphorylation of Rb protein by cyclin D/Cdk 4 and 6 complexes results in dissociation of E2F from Rb, and free E2F activates transcription of its target genes essential to enter the S phase. In the present study, the expression of cyclin D1 and E2F-1 proteins in K562 cells when treated with 20 μg/ml of chloramphenicol was decreased, compared with the untreated control cells. This indicates that chloramphenicol arrests G1/S transition through suppression of cell cycle regulatory proteins. The expression of cyclin D1, cyclin E, and E2F-1 mRNA was also slightly decreased at 9 days (Fig. 5), indicating that chloramphenicol may have genotoxic effects as well as inhibiting protein synthesis in mitochondria. Chloramphenicol itself is not genotoxic in human cells; however, some metabolites induce toxic effects [3, 4]. Chloramphenicol is metabolized and produces six metabolites in the body [25]. Three metabolites, including nitroso-chloramphenicol, dehydro-chloramphenicol, and dehydro-chloramphenicol base, have been shown to produce breaks of single DNA strand, whereas chloramphenicol itself, chloramphenicol-G, chloramphenicol base, and alcoholic derivative are totally devoid of genotoxic effects on human cells [1, 15, 24, 30].

K562 cells treated with 10 and 20 μg/ml of chloramphenicol showed apoptotic cell death at 9 days. Apoptosis is an active process of gene-directed cellular self-destruction after the cells come in contact with certain stimuli. It is induced by a variety of stimuli including chemicals, chemotherapeutic agents, and radiation. Several pathways transduce the apoptotic signals to the cell death machinery. Mitochondria play a central role in apoptosis of the cells and chloramphenicol induces mitochondrial damage. Caspases are a family of cysteine proteases that play a central role in the apoptotic pathway. On apoptotic stimulation, multiple caspases are sequentially activated, and ultimately break the cellular structure by the recognition and cleavage of specific substrate proteins, leading to apoptotic morphologies. In the present study, the enzymatic activity of caspase-3 was found to increase in the cells treated with chloramphenicol, thus indicating that chloramphenicol activates the apoptotic cascade through a caspase-dependent pathway.

Chloramphenicol has been used in both human and veterinary medicines; however, in rare cases, this drug induces hematologic adverse effects. In the present study, chloramphenicol inhibited the cellular growth through arresting the transition of the cell cycle and induced apoptotic cell death through a caspase-dependent pathway *in vitro*. Short exposure (less than 3 days) of K562 cells to chloramphenicol inhibited the cellular growth, but did not induce apoptosis at the molecular and morphological levels. However, long exposure (more than 6 days) to chloramphenicol inhibited the cell growth and induced apoptosis in K562 cells. These results suggested that hematologic toxic effects such as bone marrow suppression and aplastic anemia could be induced by a therapeutic blood level of chloramphenicol. Therefore, it is concluded that an appropriate antimicrobial therapy and careful monitoring of patients are necessary to prevent the adverse side effects of chloramphenicol.

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