Bull Environ Contam Toxicol (2012) 89:234–239
DOI 10.1007/s00128-012-0695-7

Biochemical Effects of Veterinary Antibiotics on Proliferation and Cell Cycle Arrest of Human HEK293 Cells

Hyeon Young Kim · Ki-Tae Kim · Sang Don Kim

Received: 28 February 2012 / Accepted: 24 May 2012 / Published online: 7 June 2012

Abstract The purpose of this study was to examine the effects of veterinary antibiotics, including amoxicillin (AMX), chlortetracycline (CTC) and tylosin (TYL), on the biochemical mechanism of human embryonic kidney cells (HEK293). CTC and TYL inhibited HEK293 cell proliferation, in both time- and dose-dependent manners, and changed the cell morphology; whereas, AMX showed no cytotoxic effects. The cell cycle analysis of CTC and TYL revealed G1-arrest in HEK293 cells. Western blot analysis also showed that CTC and TYL affected the activation of DNA damage responsive proteins, as well as cell cycle regulatory proteins, such as p53, $p21^{Waf1/Cip1}$ and Rb protein, which are crucial in the G1-S transition. The activation of $p21^{Waf1/Cip1}$ was significantly up-regulated over time, but there was no change in the level of CDK2 expression. The results of this study suggest that veterinary antibiotics, even at low level concentrations on continuous exposure, can potentially risk the development of human cells.

Keywords Veterinary antibiotics · HEK293 cells · Cell damage · Cell cycle arrest

Veterinary antibiotics (VAs) are biologically active compounds, of natural or semi-synthetic origin, used to treat disease and promote the growth of animals (Boxall et al. 2001). Due to their excessive usage as feed additives for livestock, antibiotics are discharged into the environment via excretion. Generally, the route of veterinary pharmaceuticals into the aquatic environment is different from that of human pharmaceuticals; animal manure is a major source for water contamination due to veterinary medicines; whereas, human pharmaceuticals are released into watersheds mostly via wastewater treatment plants (Kim et al. 2008). Therefore, veterinary medicines are regarded as nonpoint source pollutants, which require more effort in their control than the management of human pharmaceuticals.

Recently, there has been growing concern regarding the presence of pharmaceutically active compounds (PhACs) in the aquatic environment (Hirsch et al. 1999). PhACs and their metabolites have frequently been detected at low levels, far below their therapeutic doses, in surface, drinking and ground waters (Kolpin et al. 2002), since even advanced water treatments are unable to achieve the perfect removal of the target chemicals. Therefore, the long term exposure to a low level of PhACs (e.g., via drinking water) and its chronic effects are significant issues in environmental health studies (Jones et al. 2005) and attract much greater attentions than acute toxicity studies (Ankley et al. 2007). However, little is known about the long-term adverse effects of VAs at sub-therapeutic levels on non-target organisms, such as humans (Erickson 2002). Thus, studying their chronic effects has been encouraged to uncover the environmental health risks caused by the presence of antibiotics at trace levels (Webb et al. 2003). Despite the need for such assessments, most existing in vitro toxicity tests at the cellular level have been conducted in the short term (e.g., 24–48 h) (Babín et al. 2005), with the aim of identifying their acute effects. This is mainly

H. Y. Kim · S. D. Kim (✉)
School of Environmental Science and Engineering,
Gwangju Institute of Science and Technology (GIST),
261 Cheomdan-gwagiro, Buk-gu, Gwangju 500-712, Korea
e-mail: sdkim@gist.ac.kr

K.-T. Kim
Department of Environmental and Molecular Toxicology,
Oregon State University, Corvallis, OR, USA

due to the exposure time limitation associated with reduced survival, growing cell density, nutrient depletion in media and lack of cell–cell interactions, etc. (Eisenbrand et al. 2002). As a consequence, effective concentrations in short term in vitro tests are much higher than those that are environmentally relevant. To compensate for these problems, the exposure period of in vitro toxicological tests using human cell lines should be extended for finding lower effect concentrations of antibiotics, which would be helpful in gaining an insight into the realistic risk of antibiotics imposed on human health.

It is well known that in vitro test systems are suited for studying low molecular weight chemicals (Eisenbrand et al. 2002) and that the cell cycle is regulated by complex signal transduction pathways. In response to DNA damages, cell cycle regulatory proteins, such as p53, CKIs (CDK inhibitors) and CDKs (cyclin dependent kinases), are affected, which leads to cell cycle arrest (Sherr and Roberts 1995). In the present study, amoxicillin (AMX), chlortetracycline (CTC) and tylosin (TYL) were selected as target VAs, based on their usages, potentials for entering into the water environment and toxicity values. The human embryonic kidney cell line, HEK293, was chosen as the cellular model for normal embryonic cells. The purpose of this study was to confirm the effects of VAs on human embryonic cells, and the potential biochemical mechanism, by extending the exposure time up to 6 days and then observing cell proliferation, morphological changes, cell cycle regulatory protein activation and cell cycle progression.

Materials and Methods

Amoxicillin, Chlortetracycline hydrochloride and Tylosin tartrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared in deionized water and stored at 4°C. ß-actin, cyclin dependent kinase (CDK)2 (D-12) and phospho-Rb antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies of $p21^{Waf1/Cip1}$ and phospho-p53 (Ser15) were obtained from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated rabbit anti-Mouse IgG (H+L) was purchased from Jackson ImmunoResearch (West Grove, PA, USA).

HEK293 cells (human embryo kidney cells) were obtained from the KCLB (Korean Cell Line Bank) and cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, USA), containing 10 % fetal bovine serum (FBS) (GIBCO, USA) and 1 % Antibiotic–Antimycotic (GIBCO, USA). These cells were maintained at 37°C under a 5 % CO_2 atmosphere. For sub-culture, the cells were washed with phosphate buffer saline (PBS) and harvested using trypsin–EDTA (0.25 % Trypsin, EDTA•4Na) (GIBCO, USA).

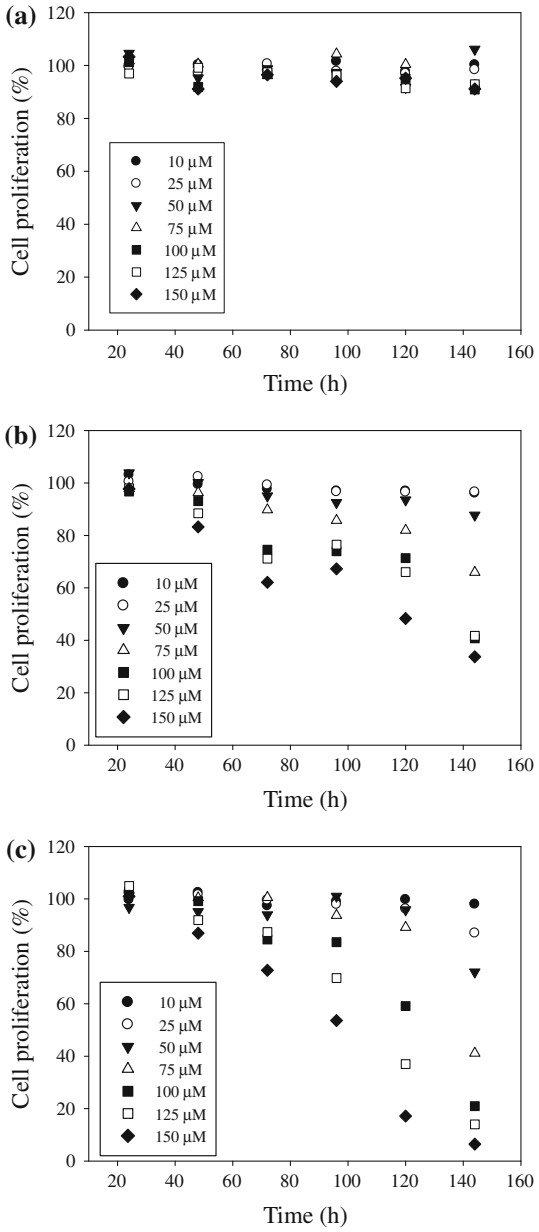

Fig. 1 Effects of veterinary antibiotics (VAs) on HEK293 cell proliferation, **a** amoxicillin, **b** chlortetracycline, and **c** tylosin. Values are presented as the OD ratio of treated to control cells

An appropriate amount of cells (1 ~ 32 × 10^4 cells) was seeded into each well of 24-well plates in 500 μL of DMEM. After incubation for 24 h, to allow for adhesion, 500 μL of DMEM 10 % FBS, containing various levels of the antibiotics, was added. The cells were exposed to the VAs treatments for between 24 and 144 h. A dye solution of tetrazolium salt (MTT) (Sigma, USA) was added, with the cells incubated for 4 h at 37°C. Culture media were aspirated, with dimethyl sulfoxide (DMSO) then added to each well. The absorbance was measured at 570 nm using a microplate reader (μQuant, BIO-TEK INSTRUMENTS INC., USA). The percentages of

cell proliferation were calculated by the follow formula: cell proliferation (%) = optical density (O.D.) of the treated cells/ O.D. of control cells.

To assess whether the inhibition of antibiotics-induced cell proliferation was related to the change in the cell cycle progression, the cell cycle was analyzed using a flow cytometer (Cell Lab Quanta^{TM} SC, Beckman Coulter, USA). Cells were treated with antibiotics and harvested by fixing 1×10^6 cells in 70 % ethanol and 30 % PBS for 1 h over ice. The fixed cells were centrifuged, with the cell pellets re-suspended with PBS. The cells were then treated with RNaseA (DNase free, 200 μg/mL in PBS) for 1 h at 37°C. The treated cells were stained with propidium iodide, at a final concentration of 40 μg/mL, in PBS. The samples were stored at −20°C until analyzed. Statistical significance was determined using the Student *t* test.

In order to determine whether the target antibiotics affect the cell cycle controlling protein activation levels, the HEK293 cells were exposed to 100 μM of the three antibiotics for 144 h, with the activation of proteins examined using a Western blot analysis. The cells were washed with ice-cold PBS, and extracted in Cell Extraction Buffer (Invitrogen, USA) with the addition of with a Protease Inhibitors Cocktail (Sigma, USA) and phenyl-methyl-sulfonyl-fluoride (Sigma, USA). The total protein concentration was measured using the Bradford method, with the sample lysate loaded onto a polyacrylamide gel. The proteins were separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a PVDF membrane (Pall Corporation, USA). The membranes were then blocked with 5 % skimmed milk in TBS-T (20 mM Tris, pH 7.6, 150 mM NaCl, 0.1 % Tween-20) and blotted with primary antibodies and horseradish peroxidase (HRP)-conjugated second antibodies. The blotted membranes were developed in a WEST-ZOL® plus Western Blot Detection System (iNtRon, Korea).

Results and Discussion

As indicated in Fig. 1, the treatment of HEK293 cells with various concentrations (10, 25, 50, 75, 100, 125, and 150 μM) of CTC (Fig. 1b) and TYL (Fig. 1c) caused time- and dose-dependent decreases in the cell numbers relative to the control cultures. However, AMX showed no cytotoxicity towards the cells (Fig. 1a). Similar outcomes were previously reported for different cell types (primary rainbow trout hepatocytes) (Laville et al. 2004). These results demonstrated that CTC and TYL can inhibit the proliferation of HEK293 cells; whereas, there was no evidence for

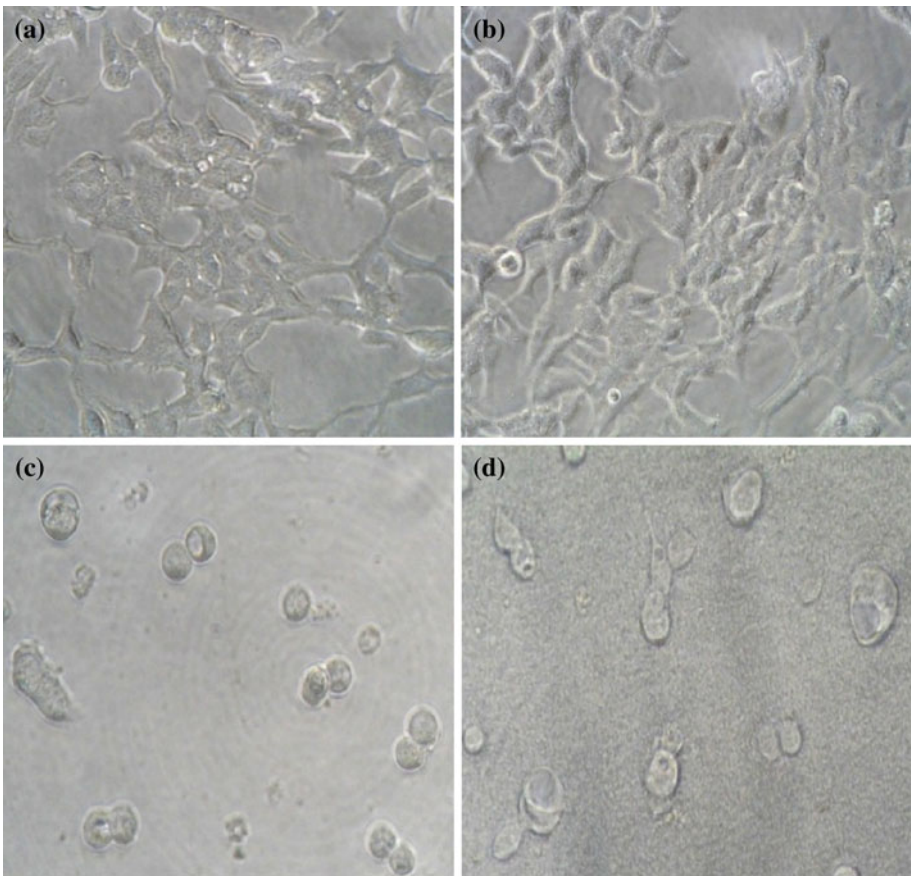

Fig. 2 Light microscopic images (×40 magnification) of HEK293 cells treated by 100 μM of the three target antibiotic for 48 h. **a** Control, **b** amoxicillin, **c** chlortetracycline, and **d** tylosin

the cytotoxicity of AMX in the present study. HEK293 cells treated with 100 μM of CTC showed about 60 % inhibition of cell proliferation relative to the control after 144 h of exposure. The effective concentration of CTC determined in the test was lower than that of other tests using other human cell types (Bacon et al. 1990). Other mammalian kidney cell lines exhibited no effects at 0.1 mg/mL.

Figure 2 shows light microscope images of HEK293 cells exposed to 100 μM of the three antibiotics for 48 h. As shown in the microscopic observation of the treated HEK293 cells (Fig. 2a, b), AMX showed no differences to the control cultures, which were characterized by an elongated star-shaped cell morphology. Conversely, the cells exposed to CTC (Fig. 2c) and TYL (Fig. 2d) displayed not only morphological changes, such as rounding up and losing contact between cells, but also numerical changes, such as smaller proliferative colonies compared to the control cultures.

The cell cycle distribution of HEK293 cells with 100 μM of the three antibiotics are shown in Fig. 3. A cell cycle analysis revealed that, with the exception of AMX (Fig. 3a), that the VAs treatments caused differences from the controls observations with respect to the cell populations in the different phases of the cell-cycle. In the case of the treatments with 100 μM of CTC (Fig. 3b) and TYL (Fig. 3c), the percentages of cells in the G1 phase increased, while those in the S and G2/M phases decreased. These results confirmed that CTC and TYL can cause G1-arrest in a time-dependent manner, suggesting a possible growth inhibitory effect of antibiotics due to retardation of DNA replication in the cell cycle.

The exposure to 100 μM AMX showed no evident effect on protein activation (Fig. 4a). The level of $p21^{Waf1/Cip1}$ was increased slightly after 48 h of exposure, but underwent no change thereafter. Conversely, the activation of $p21^{Waf1/Cip1}$ increased and the phosphorylation of the Rb decreased in time-dependent manners as a result of the treatment with 100 μM of CTC (Fig. 4b). Phosphorylation of the DNA damage responsive protein, p53, was up-regulated after 24 h of exposure compared to the control level. Similarly, TYL induced increased activation of $p21^{Waf1/Cip1}$ and decreased phosphorylation of the Rb (Fig. 4c). Phosphorylation of p53 was also up-regulated after 24 h of exposure. CTC and TYL induce no significant changes in the levels of CDK2 over time.

Our hypothesis was that 'VAs interfere with DNA synthesis in HEK293 cells, prompting the activation of DNA damage responsive proteins and retardation of the cell cycle'. The cell cycle is the fundamental process by which cells reproduce, which underlies the differentiation and development in all living organisms (Nurse 2000). The DNA damage response checkpoint is a mechanism that

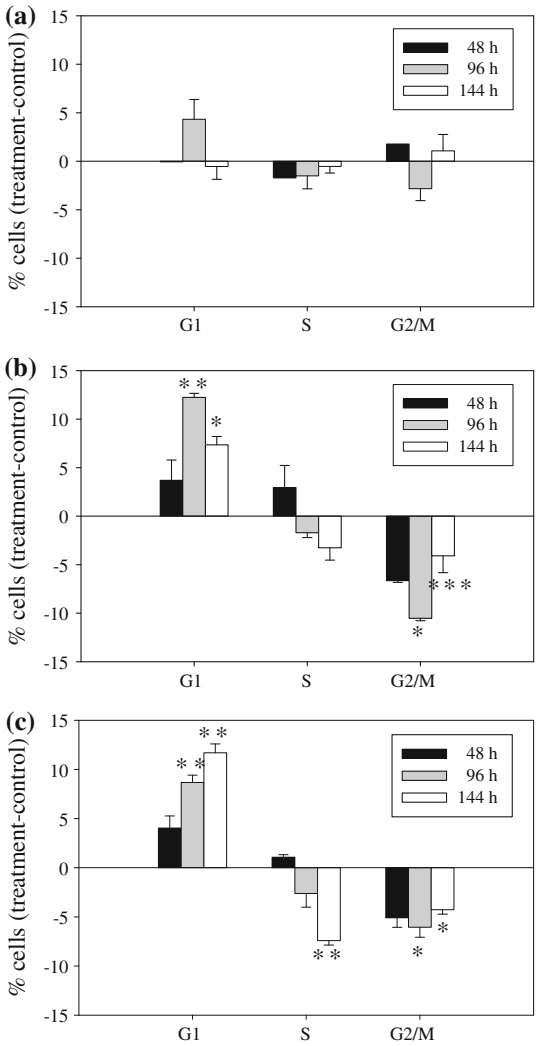

Fig. 3 Effects on cell cycle progression of HEK293 cells treated with 100 μM of the three target antibiotics, **a** amoxicillin, **b** chlortetracycline, and **c** tylosin. Quantification refers to the difference between the samples and controls. Values are presented as mean ± SE (N = 3). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student *t* test)

recognizes damaged DNA and generates a signal that arrests cells in the G1 phase of the cell cycle, slows down the S phase (DNA synthesis), providing time for repair, and arrests cells in the G2 phase, inducing the transcription of repair genes (Elledge 1996). The tumor suppressor gene, p53, induces the expression of p21 in response to DNA damage. Transcriptional activation of p21 inactivates the CDK2 activity, which results in cell cycle arrest (Sherr and Roberts 1995). Pomati et al. (2006) previously observed that pharmaceutical mixtures stimulated the expressions of p16 and p21 in HEK293 cells, with slight accumulation of cells in the G2/M phase of the cell-cycle. No evidence was found for p53 and CDK2. Although the target materials in the present study were different from those in the study of Pomati et al. similar results were obtained.

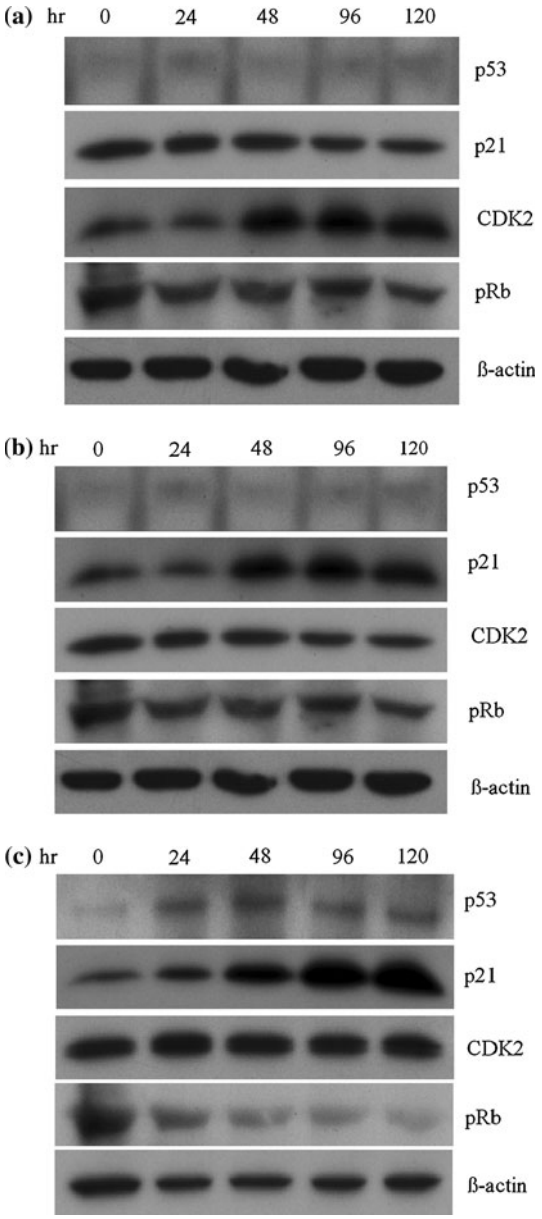

Fig. 4 Effects on the expressions of G1/S phase regulatory proteins in HEK293 cells (100 μM exposure), **a** amoxicillin, **b** chlortetracycline, and **c** tylosin. ß-actin was used as an internal control

Therefore, the p53/p21$^{Waf1/Cip1}$ DNA damage responsive pathways played important roles in regulating the G1-S transition in response to cellular stresses, which was associated with the observed reduction of cell proliferation in this study. As shown in Fig. 4, phospho-p53, p21$^{Waf1/Cip1}$ and phospho-Rb were affected by CTC and TYL; whereas, there was no difference in the quantity of CDK2 before and after exposure to either of the target chemicals. Despite the lack of evidence of a quantitative change in CDK2, it is likely that the target chemicals would induce DNA damage, resulting in the retardation of cell cycle progression. CDK2 complexes with cyclin E advance the G1-S transition by causing further phosphorylation of pRb (Hengstschläger et al. 1999). However, the western blot analysis did not seem to be an appropriate test method for measuring the complexation between CDK2 and cyclin E, or its activity, as it was designed only for quantifying the amount of protein. Therefore, on the basis that CDK2 is a key regulator of the G1-S transition, the G1 arrest of the cells observed in the present study was possibly associated with inhibition of the activity of the cyclin E/CDK2 complex, but it is strongly recommended that this be confirmed by further study. Consequently, the effective concentrations of VAs determined from the 6-day exposure test were lower than those in a previous study (Bacon et al. 1990), suggesting the possibility of potential adverse effects of VAs on human health. Nevertheless, the effective concentrations were still higher than environmental levels and; thus, are not likely to show any relationship between the presences of VAs at trace levels and ecotoxicological disruptions. The use of laboratory cell lines may overestimate the effects of drugs at the organism level, since long-term consequences on adult animal survival can be balanced by homeostatic processes (Pomati et al. 2006). Nevertheless, the results observed on cellular physiology suggest that the investigated drugs could interfere with mitogenic and antimitogenic signals, such as those involved in differentiation and development. This indicates that organisms in the early stage of growth may be sensitive to pharmaceutical contamination of aquatic environments. The identification of a cell cycle mechanism is critical to disease, especially cancer. The measurement of the transcription and translation products of a gene's expression can reveal valuable information about the potential toxicity profile of chemicals.

Acknowledgments This research was supported by "Innovative Technology of Ecological Restoration" project at GIST.

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