In Vitro Impact of Macrolide Antibiotics on the Viability of Selected Mammalian Cell Lines

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Abstract

The aim of this study was to evaluate the in vitro cytotoxicity of different concentrations of macrolide antibiotics (tilmicosin-TILM, tylosin-TYL and spiramycin-SPI) on selected animal cell cultures. VERO cells (kidney cells from *Macacus rhesus*), FE cells (feline embryonal cells) and BHK 21 cells (cell line from young hamster kidneys) were used in the study and subjected to 50-1000 µg/mL macrolides, depending on the specific antibiotic and cell line. The cell viability was assessed using the metabolic mitochondrial MTT test. FE cells were the most sensitive to TILM with a significant decrease of viability at 100-150 µg/mL (P<0.001). VERO cells were the most resistant. FE cells showed the highest sensitivity to TYL, as 1000 µg/mL significantly reduced the cell viability (P<0.001) when compared to the untreated control. VERO cells exhibited the highest resilience, with no significant differences of viability in comparison to the control. BHK 21 cells exhibited the highest sensitivity to SPI, as all concentrations led to a significant decrease (P<0.001) of the viability. On the other hand, VERO cells revealed the highest resistance to SPI. Our data reveal that macrolides have a significant adverse effect on the cell viability.

Keywords: cell cultures, macrolides, mitochondrial activity, spiramycin, tilmicosin, tylosin

1. Introduction

Antibiotics play a significant role in treating and preventing diseases among animals in the contemporary livestock production. Antibiotics are usually administered to animals through feed however this practice often results in the release of antibiotic residues from the treated feed, animal feces, and even water runoff from manure-treated farmland that contaminates surrounding surface water [1]. These residues may exert a direct toxic effect on the microflora and microfauna [2], facilitate the development of antibiotic-resistant bacterial populations [3], and transfer antibiotic resistance to pathogenic microbes that may affect humans [4].

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Macrolides are a class of natural products consisting of a large macrocyclic lactone ring to which one or more deoxy sugars, usually cladinose and desosamine, may be attached. The lactone rings are usually 14-, 15-, or 16-membered. Macrolides belong to the polyketide class of natural products. Some macrolides, such as spiramycin, tilmicosin and tylosin have antibiotic or antifungal activity and are used as pharmaceutical drugs [5].

Antibiotic macrolides are used to treat infections caused by Gram-positive (e.g., *Streptococcus pneumoniae*) and limited Gram-negative (e.g. *Bordetella pertussis*, *Haemophilus influenzae*) bacteria, and some respiratory tract and soft-tissue infections [6, 7]. The antimicrobial spectrum of macrolides is slightly wider compared to penicillin, hence macrolides are a suitable substitute for subjects with a penicillin allergy. Beta-hemolytic streptococci, pneumococci,

staphylococci, and enterococci are usually susceptible to macrolides. Unlike penicillin, macrolides have been shown to be effective against *Legionella pneumophila*, mycoplasma, mycobacteria, some rickettsia, and chlamydia [7].
Despite the extensive use of macrolides in agriculture and veterinary medicine, information on the toxicity of such antibiotics to the eukaryotic cell is still insufficient. Furthermore, discrepancies in the toxicity of macrolides to organisms have been claimed – some studies have reported significant toxic effects [8-10], whereas other studies have not [11]. These discrepancies might be due to differences in the sensitivity of the tested organisms and criteria used for defining the toxic effects [4].
Therefore, the aim of this study was to evaluate the in vitro cytotoxicity of different concentrations of macrolide antibiotics (tilmicosin-TILM, tylosin-TYL and spiramycin-SPI) on selected mammalian cell cultures.

2. Materials and methods

Three different cell lines were used in the following experiments:

1. BHK 21 – cellular line from young hamster kidneys,
2. FE cells – feline embryonal cells,
3. VERO cells - kidney cells from *Macacus rhesus*.

All cell lines were obtained from cellular collections of the Department of Bio Preparations, Institute for State Control of Veterinary Biopreparations and Medicines in Nitra. The cells were revived according to relevant protocols. After revival the cells were transferred to sterile Roux flasks containing appropriate culture media (MEM containing 10% (v/v) FBS for BHK 21 cells; DMEM/F12 supplemented with 20% FCS, non-essential amino acids, glutamine, LIF, fibroblast growth factor-2, beta-mercaptoethanol and antibiotics for FE cells; and DMEM supplemented with 10% heat-inactivated fetal bovine serum FBS for VERO cells) and cultured in an incubator at 37°C. After 24 hours, the monoculture assessed and cell density was determined. Cell suspension was prepared by dilution of the cells using FBS enriched culture medium. The resulting suspensions were transferred into 48 well plates at 500 µl per well. After further incubation in FBS enriched culture media, the cells were assessed microscopically. When a single-layer was coherent, the medium was discarded and freshly prepared antibiotics were layered on cells. We chose tilmicosin, tylosin and spiramycin (macrolide antibiotics), which are used in veterinary medicine. The concentrations, used in our experiment were obtained on the basis of knowledge about their minimum inhibitory concentrations on bacteria and LD50 in laboratory animals. As these concentrations are non-toxic for eukaryotic cells, we raised them 1000-times. Subsequently we modified the concentrations to be toxic for all cell types (LD100). Such concentrations were used as zero dilutions, further titration was executed with a decimal dilution. Specific concentrations used for each cell type are displayed in Table 1. The resulting cell cultures were cultured for 24h.

Table 1. Concentrations of macrolides used for the experiments

	Tilmicosin	**Tylosin**	**Spiramycin**
Cell type	**Concentration [µg/mL]**		
BHK 21	0	0	0
	100	750	150
	200	900	200
	300	1500	300
FE	0	0	0
	75	800	250
	100	900	350
	150	1000	540
VERO	0	0	0
	50	100	200
	75	200	450
	200	300	500

Viability of the cells exposed to selected antibiotics in vitro was evaluated using the metabolic activity (MTT) assay [12]. This colorimetric assay measures the conversion of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. The resulting formazan can be measured spectrophotometrically at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, Thermo Fisher Scientific, Finland). The data are expressed in percentage of control (i.e. optical density of formazan from cells not exposed to the antibiotic). Results from the

analysis were collected during three repeated experiments at each concentration.

All data were subjected to statistical analysis using the GraphPad Prism program (version 3.02 for Windows, GraphPad Software incorporated, San Diego, California, USA, http://www.graphpad.com/). Results are quoted as arithmetic mean ± standard error (SEM). To compare the results, Scheffe´s and Student´s t-test were used. The level of significance for the comparative as well as correlation analysis was set at ***(P<0.001); **(P<0.01); *(P<0.05).

3. Results and discussion

Dose-responses of the selected macrolides measured by the MTT test are shown in Tables 2, 3 and 4, respectively.

Table 2. The effect of tilmicosin (TILM) on the viability of selected mammalian cells

TILM applied [µg/mL]	Cell viability [%]
BHK 21 cells	
0 (Ctrl)	100.00±0.17
100	88.24±0.15
200	88.24±0.20
300	76.47±0.13***
FE cells	
0 (Ctrl)	100.00±0.14
75	78.57±0.11
100	64.29 ±0.09***
150	50.00 ±0.07***
VERO cells	
0 (Ctrl)	100.00±0.09
50	100.00 ±0.12
75	100.00 ±0.09
200	88.89 ±0.07

Mean ± SEM.
***(P<0.001); **(P<0.01); *(P<0.05).

A dose-dependent toxic effect of timlicosin (TILM) was observed after its administration to all mammalian cell lines. The mitochondrial activity of the BHK21 cell was significantly (P<0.001) decreased after the administration of 300 µg/mL TILM when compared to the Control. Lower TILM concentrations decreased the BHK 21 cell viability as well, however with insignificant statistical differences. The FE cell line exhibited the highest degree of susceptibility to the antibiotic, as 100 and 150 µg/mL TILM led to significant decline (P<0.001) of the FE viability when compared to the Control. On the other hand, VERO cells were the most resistant to the macrolide, as no significant decrease of viability was observed at any TILM dose (Table 2).

Table 3. The effect of tylosin (TYL) on the viability of selected mammalian cells

TYL applied [µg/mL]	Cell viability [%]
BHK 21 cells	
0 (Ctrl)	100.00±0.17
750	88.24±0.15
900	82.35±0.14*
1500	76.47 ±0.13***
FE cells	
0 (Ctrl)	100.00±0.14
800	85.71 ±0.12
900	78.57 ±0.11
1000	57.14 ±0.08***
VERO cells	
0 (Ctrl)	100.00±0.09
100	100.55 ±0.19
200	88.89 ±0.08
300	77.78 ±0.07

Mean ± SEM.
***(P<0.001); **(P<0.01); *(P<0.05).

Toxic effects of tylosin (TYL), although to a lower extent in comparison to TILM were observed particularly on the BHK-21 and FE cell lines. FE cells showed the highest sensitivity to TYL, as 1000 µg/mL reduced the cell viability to a half (P<0.001) when compared to the untreated control. Similarly, a decreased viability of BHK 21 cells was observed following the supplementation of 1500 (P<0.001) and 900 (P<0.05) µg/mL TYL. VERO cells exhibited the highest resilience to the TYL treatment, with no significant differences of viability in comparison to the control (Table 3).

Spiramycin (SPI) supplementation revealed to have the similar toxic effects on the mammalian cell lines as the other selected macrolides had. In this case, BHK 21 cells exhibited the highest sensitivity to the antibiotic, as all SPI concentrations (150, 200, 300 µg/mL) led to a significant decrease (P<0.001) of the mitochondrial activity. Similarly, the viability of FE cells significantly (P<0.05) decreased after the administration of 350 and 540 µg/mL SPI. On the other hand, VERO cells revealed the highest resistance to the antibiotic, with no significant effects in comparison to the control (Table 4).

Table 4. The effect of spiramycin (SPI) on the viability of selected mammalian cells

SPI applied [µg/mL]	Cell viability [%]
BHK 21 cells	
0 (Ctrl)	100.00±0.17
150	76.47±0.13***
200	76.47 ±0.13***
300	70.59 ±0.12***
FE cells	
0 (Ctrl)	100.00±0.14
250	92.86 ±0.13
350	71.43 ±0.10*
540	71.43 ±0.10*
VERO cells	
0 (Ctrl)	100.00±0.09
200	133.33 ±0.12
450	100.12±0.08
500	88.89 ±0.08

Mean ± SEM.
***(P<0.001); **(P<0.01); *(P<0.05).

There is still a lack of complex data in the available literature about the behavior of macrolide antibiotics on cell morphology in prokaryotic or eukaryotic cells [5, 13, 14]. The assessment of antibiotic toxicity is critical with respect to the increasing bacterial resistance to antimicrobial agents. The increasing resistance can be partly regulated by increasing the dose to the treated organism. It is however possible to increase the specific dose only to a certain extent, as the toxic effect may prevail over the therapeutic one. If the toxicity of the treating substance is not significant, the possibility of bacterial resistance to high doses of such drug increases. In the case of macrolide antibiotics, such event may occur due to their mode of action, which depends on time, instead of the dose [5].

Assessments of the cytotoxic effects of macrolides have been previously performed on human liver cell lines. Viluksela et al. [15] used Chang liver cells to compare the cytotoxicity of three new semisyntetic macrolide antibiotics (roxithromycin, clarithromycin and azithromycin) with three older macrolides (erythromycin carbonate, erythromycin estolate and erythromycin base). Culture plates at a concentration of 5000 or 10 000 cells in 100 microliters were subjected to the MTT assay. The results showed that cytotoxicity of macrolides is significantly dose-dependent [15, 16].

Tilmicosin was originally developed as a one-off injection for the treatment of bovine pneumonia caused by *Pasteurella sp*. The effectiveness of TILM was tested on rats, where 50, 250 and 1000 mg TILM/kg body weight were applied for three months. Signs of toxicity were observed at doses of 250 and 1000 mg/kg, reflected in a reduced viability, reduced food intake, weight loss and organ weight change. Significant mortality was observed at 1000 mg/kg [17].

Tylosin toxicity in rodents is low. In a 17-month study, rats were administered food supplemented with 0, 0.1, 0.3 and 1% tylosin, equal to 1, 1000, 3000 and 10000 ppm. Enlargement of ovaries and uterus in some females were observed, however major biochemical, haematological, macroscopic or microscopic changes were not significant compared to the control [18].

Baguer et al. [19] tested the effects of tylosin on the growth and reproductive ability of selected soil fauna species (earthworms, springtails), and reported an overall low toxicity of this macrolide. Significant detrimental effects of TYL on the reproductive performance were reported only at a dose range higher than 4000-5000 mg/kg.

Spiramycin has been shown to exhibit low acute toxicity in animals after oral or subcutaneous administration. In a short-term dietary study in which rats were given the equivalent of up to 3900 mg/kg body weight for 13 weeks, the only major effects noted were a reduction in neutrophil counts in some mid- and high-dose animals, and dilatation of the caecum, which was attributed to the antibiotic effects on the rodent gut flora. In another dietary study in the rat, animals were given up to the equivalent of 720 mg/kg body weight/day for one year. The only notable effects were reductions in the body weights of females receiving the higher doses, and increases in relative liver, kidney, and adrenal weights at high dose levels in animals of both sexes. The genotoxic potential of spiramycin was investigated in a range of studies. Negative results were obtained in mammalian cells in vitro [20].

Knowledge of the specific chemical structure of natural antibiotics allows their modification, usually by binding side chains in order to provide agents with fewer side effects. Such modified semi-synthetic antibiotics, including macrolides do not have many natural disadvantages in comparison to the original erythromycin, which has been linked to nausea, vomiting, and interferes with other prescription drugs [21].

4. Conclusions

The cytotoxicity of natural antimicrobial substances assessed in mammalian cell cultures allows us to provide more knowledge to their specific in vitro and in vivo properties in real life circumstances. At the same time we have to be aware that any biologically active substance behaves differently in a living organism in comparison to sterile laboratory conditions. Toxic effects of specific antibiotics may vary according to their concentration, time of exposition and mode of action, along with the morphology and behavior of cells affected by such antimicrobial molecules. A direct comparison of results obtained in this study with already available reports is therefore quite complicated, as most toxicological studies are usually performed in vivo and examine the impact of substances on complex tissue and organ systems, rather than single purified cells.

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