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Short communication

Antitumor effect of macrolides – erythromycin and roxithromycin in B16 melanoma-transplanted mice

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Abstract:
The aim of this study was to examine the effects of 14-membered ring macrolides administered intraperitoneally at two doses (10 or 50 mg/kg) on tumor growth in *B16F10* melanoma-transplanted mice. Erythromycin at the doses of 10 mg/kg or 50 mg/kg inhibited significantly the growth of *B17F10* melanoma in mice (1040.29 and 1026.53 *vs.* 2539.78 mm^3 respectively). Significant inhibition of tumor growth was also observed in the group receiving roxithromycin at a dose of 10 mg/kg compared to the control group (1334.12 *vs.* 2539,78 mm^3). No significant differences of the effect of roxithromycin administered at the dose of 50 mg/kg on the tumor volume compared to the control group was observed (2050.89 *vs.* 2539.78 mm^3).

Key words:
mouse B16 melanoma, macrolides, roxithromycin, erythromycin

Abbreviations: A – the longest diameter, B – perpendicular diameter, TGI – tumor growth inhibition, W_C – mean tumor volume in control group, W_T – mean tumor volume in treated groups

Introduction

In Europe, incidence of *melanoma malignum* is about 1% in men and 1.8% in women. The frequency of its occurence increases much faster than that of other cancers. Early detected melanoma can be effectively cured. However, in spite of the development of various treatment methods, they are still not effective enough in more advanced clinical stages [4]. Therefore, there is a great need to investigate new possibilities in order to achieve the greatest therapeutic benefits.

Macrolide antibiotics have been in clinical use since the early 1950s. Erythromycin is the prototypical antibiotic of this class. Macrolides are bacteriostatic agents which inhibit bacterial RNA-dependent protein synthesis. Macrolides are safe drugs and most of their adverse effects are not life threating [1, 2].

It has been reported that macrolides possess many additional properties, e.g. prokinetic on gastrointesti-

nal tract, immunomodulating and anti-inflammatory. Recently, it was shown that macrolides could also exert anticancer activity [3, 17].

The aim of this study was to examine the effects of 14-membered ring macrolides – erythromycin and roxithromycin – administered intraperitoneally on tumor growth in B16F10 melanoma-transplanted mice.

Materials and Methods

Chemicals

Minimal essential medium of Eagle (MEM, BioWhittaker, Belgium), 10% fetal calf serum (FCS, BioWhittaker, Belgium), L-glutamine, penicillin and streptomycin solution – 2 mM of L-glutamine, 100 UI/ml of penicillin and 100 μg/ml of streptomycin (Sigma, USA), trypsin and EDTA solution – 25 mg/ml of trypsin and 2 mg/ml of EDTA (Sigma, USA), 96% ethyl alcohol pure p.a. (Chempur, Poland), erythromycin (Sigma-Aldrich, Germany), roxithromycin (Sigma-Aldrich, Germany), thiopental (Biochemie, Austria), 36–38% formaldehyde solution (Chempur, Poland), *Aqua pro iniectione* (Polpharma S.A., Poland) were used in the study.

Cells culture

In the experiment, mouse B16F10 melanoma cell line was used. Cells were cultured in the Laboratory of Cell Culture of the Department of Histology and Embryology, Wroclaw Medical University. B16F10 cells grew in monolayers, adherent to the bottom of culture container filled with MEM, supplemented with 10% FCS and solution of L-glutamine, penicillin and streptomycin. The cultures were maintained at 37°C in 5% CO_2. A solution of trypsin and EDTA was used in order to recover cell lines from culture containers for the experiment. Cells at the stage of active growth were removed from the bottle, after which they were counted and the suspension of 2×10^6 cells/ml density was prepared.

Animals

Experiment was performed on C57BL/6 female mice bred in the animal house of the Department of Pathological Anatomy, Wroclaw Medical University. Average mice weight was 20.21 ± 2.67 g. Animals were housed up to five per cage, at 22°C and 40% humidity under 12 h light-dark cycle, with free access to water and standard food.

Experiment

Mice were inoculated subcutaneously into the left flank region with 0.2 ml of viable tumor cell suspension of 2×10^6 cells/ml density on day 0 of the study. The next day mice were randomized into control group (C, n = 12) and four experimental groups: receiving erythromycin at a dose of 10 mg/kg (E10, n = 8), receiving erythromycin at a dose of 50 mg/kg (E50, n = 12), receiving roxithromycin at a dose of 10 mg/kg (R10, n = 8) and receiving roxithromycin at a dose of 50 mg/kg (R50, n = 10). The studied substances were administered intraperitoneally in a volume of 5 ml/kg, control group received 0.5% ethanol in saline in the same volume. The studied substances in experimental groups or vehicle in the control group were administered once a day at 9 a.m. from 1st to 22nd day of the study. Mice were weighted and observed every day. First tumors appeared on 13th day of the experiment. From that moment tumors were measured every day and tumor volume was assessed according to the formula $(A \times B^2)/2$ (A – the longest diameter, B – perpendicular diameter) [13]. On the basis of tumor volume, tumor growth inhibition (TGI [%]) index was calculated according to the formula $W_T/W_C \times 100–100$ (W_T – mean tumor volume in treated groups, W_C – mean tumor volume in control group). The animals were sacrificed under deep thiopental narcosis (100 mg/kg, *ip*) on day 23rd after tumor cell inoculation. The primary tumors were excised and prepared for histological examination.

The experiment was carried out after approval of the First Local Ethics Commission in Wrocław (license no. – 32/05).

Histology

Primary tumors were fixed in 4% solution of formaldehyde, prepared *ex tempore* from the original solution, embedded in paraffin, sliced through the greatest diameter and stained with eosin/hematoxylin. The percentage of necrosis in relation to the greatest area of cross-section was assessed.

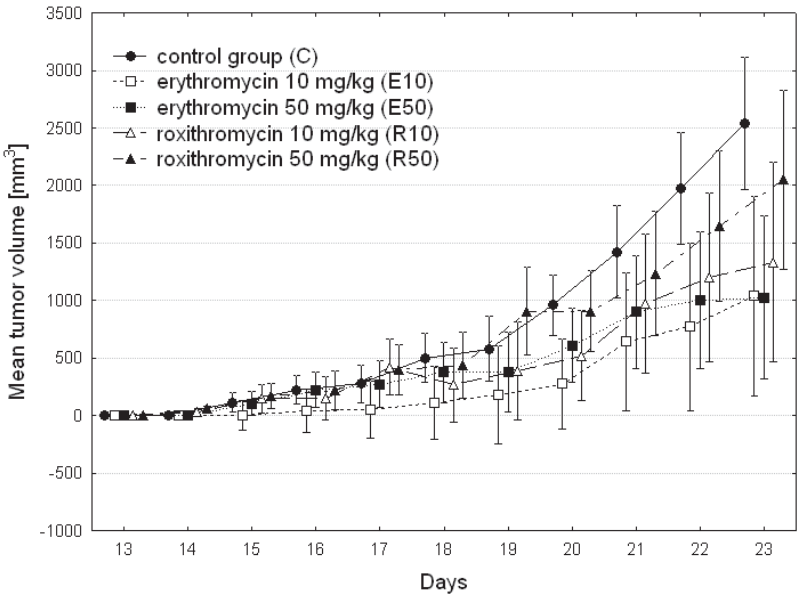

Fig. 1. Differences in the mean tumor volume [mm^3] (mouse B16F10 melanoma) as a function of time of drug administration in all experimental groups: control group (C, n = 18), group receiving erythromycin 10 mg/kg (E10, n = 8), group receiving erythromycin 50 mg/kg (E50, n = 12), group receiving roxithromycin 10 mg/kg (R10, n = 8) and group receiving roxithromycin 50 mg/kg (R50, n = 10). Values are presented as the mean ± SD

Statistical analysis

Data were shown as the mean and standard deviation (X ± SD). Differences between control group and experimental groups were evaluated using one-way analysis of variance (ANOVA) followed by a NIR *post hoc* test. The STATISTICA 6.0 software was used, $p < 0.05$ was considered to be statistically significant.

Results

Subcutaneous inoculation of 2×10^6 B16F10 cells induced tumor growth in C57BL/6 mice, with an average tumor volume of 2539.78 ± 1751.15 mm^3 in control group (C) at 23 days after inoculation.

In the present study, we observed a significant influence of erythromycin administered *ip* at the doses of 10 mg/kg (E10) and 50 mg/kg (E50) on the growth of mouse B16F10 melanoma (Fig. 1).

Mean tumor volume in the group receiving erythromycin at a dose of 10 mg/kg (E10) was significantly lower than in the control group (C) on 20th, 21st, 22nd and 23rd day of the experiment ($p = 0.005$, $p = 0.03$, $p = 0.008$, $p = 0.006$, respectively) (Tab. 1). The difference between these two groups on 18th day was of a borderline significance ($p = 0.05$). The increase in

Tab. 1. Effect of drug administration on the mean tumor volume [mm^3] (mouse B16F10 melanoma) on 23rd day of experiment in all experimental groups: control group (C), group receiving erythromycin 10 mg/kg (E10) or 50 mg/kg (E50) and group receiving roxithromycin 10 mg/kg (R10) or 50 mg/kg (R50). Values are presented as the mean ± SD

Group	No. of animals	Tumor volume [mm^3]	TGI [%]	Necrosis [%]
C	18	2539.78 ± 1751.14	–	32.78 ± 10.6
E10	8	1040.29 ± 648.9*	–59.04	29.38 ± 15.45
E50	12	1026.53 ± 706.22**	–59.58	29.17 ± 19.75
R10	8	1334.12 ± 541.98***	–47.47	30.63 ± 20.6
R50	10	2050.89 ± 1223.07	–19.25	29.44 ± 18.62 (N = 9)

* $p < 0.01$; ** $p < 0.005$; *** $p < 0.05$ *vs.* the control group (C)

tumor volume in the E10 group between 16th and 23rd day of the study was significantly lower than in the control group ($p = 0.006$). In the E10 group tumors appeared significantly later than in the control group ($p = 0.002$) (Tab. 2).

Tab. 2. Influence of drug administration on the mean day of tumor appearance in all experimental groups: control group (C), group receiving erythromycin 10 mg/kg (E10) or 50 mg/kg (E50) and group receiving roxithromycin 10 mg/kg (R10) or 50 mg/kg (R50). Values are presented as the mean ± SD

Group	No. of animals	Mean day of tumor appearance
C	18	15.1 ± 1.8
E10	8	17.9 ± 1.4*
E50	12	16.1 ± 3.0
R10	8	14.1 ± 1.4
R50	10	14.6 ± 1.4

* $p < 0.005$ *vs.* the control group (C)

Erythromycin at the dose of 50 mg/kg also decreased tumor growth (Fig. 1). Mean tumor volume in the E50 group was significantly lower than in the control group (C) on 22nd and 23rd day of the experiment ($p = 0.01$ and $p = 0.002$, respectively) (Tab. 1). The increase in tumor volume in the E50 group between 16th and 23rd day of the study was significantly lower than in the control group ($p = 0.0005$).

Inconsiderable differences in mean tumor volume between the group receiving roxithromycin at a dose of 10 mg/kg (R10) and the control group was observed (Fig. 1). Only on the last day of the study, mean tumor volume in the R10 group was lower than in the control group ($p = 0.02$) (Tab. 1). However, differences in the increase in mean tumor volume between 21st and 23rd day in these groups were more pronounced ($p = 0.005$).

In this experiment, no significant influence of roxithromycin administered at a higher dose of 50 mg/kg (R50) on the growth of mouse B16F10 melanoma compared to the control group was observed (Fig. 1). Mean tumor volume in the R50 group was significantly larger than in the E10 group on 19th and 20th day ($p = 0.01$ in both cases) and in the E50 group on 19th day ($p = 0.04$). Additionally, the increase in mean tumor volume in R50 group was greater than in E10 and E50 groups ($p < 0.02$).

Daily administration of 10 or 50 mg/kg of the studied substances over experimental period did not affect the body weight of the examined mice.

In histological studies, no significant differences in the area of the necrosis were observed between the groups (Tab. 1).

Discussion

In the present study, we have demonstrated that erythromycin and roxithromycin attenuated growth of mouse B16F10 tumor cells *in vivo* (Fig. 1).

It was reported that 14-membered ring macrolides have antitumor effect and might be useful agents for therapeutic application. Erythromycin increased survival time in Ehrlich ascites carcinoma-ddY and P388 leukemia-CDF1 mouse system [5]. Clarithromycin modified biological response resulting in a beneficial therapeutic antitumor effect in the 13762NF mammary adenocarcinoma and F-344 rat system [11] and also retarded the growth of subcutaneously inoculated Lewis lung carcinoma and increased mean survival time of inoculated C57BL/6 mice [6]. The number of adenomas and carcinomas was decreased in rats followed by diet containing erythromycin plus *sho-saiko-to* for 8 weeks [15].

Intraperitoneal administration of roxithromycin or clarithromycin at 50 mg/kg/day reduced the tumor size of B16BL6 to about 41% and 56%, respectively, of that in the control group and significantly suppressed pulmonary metastasis formation [18]. In our experiment, tumor growth was inhibited to a lesser extent (by 19.25%) by roxithromycin administered at the same dose (50 mg/kg) However, in our present work roxithromycin at the dose of 10 mg/kg exerted stronger action and reduced tumor volume by 47.47% (Tab.1). It was also reported that roxithromycin (100 mg/kg /day *ip)* significantly reduced tumor size of mouse B16BL6 melanoma and pulmonary metastasis formation in a spontaneous system compared to control [20].

Roxithromycin and clarithromycin may potentiate the inhibition of tumor growth (mouse B16BL6 melanoma) induced by cyclophosphamide, cis-diamine-

dichloroplatinum(II), adriamycin and vindesine *in vivo* [19].

One of the postulated mechanisms of antitumor effect of macrolides might involve inhibition of angiogenesis, i.e. the process of proliferation and migration of endothelial cells what leads to the formation of new blood vessels. The development of blood vessels within tumor tissues may be correlated with invasion and metastasis of malignant neoplasms. Inhibition of angiogenesis may lead to reduction of tumor growth and formation of metatasis [18]. The inhibition of tumor growth caused by macrolides was accompanied by a decrease in vascularity and increase in apoptosis of mouse B16BL6 cells *in vivo*. 14-Membered ring macrolides, roxithromycin (20 or 50 mg/kg/day, *ip*) and clarithromycin significantly reduced the dense capillary network area in a mouse dorsal air sack angiogenesis model. On the other hand, azithromycin (15-membered ring macrolide) and josamycin (16-membered ring macrolide) administered at the same dose did not show any inhibitory effect on angiogenesis [18, 20].

Other mechanisms could also play an important role in antitumor activity of macrolides. *In vitro* treatment with clarithromycin inhibited the expression of the matrix metalloproteinase-9, transforming growth factor beta and tumor necrosis factor alpha genes in 13762NF rat mammary adenocarcinoma cells [12]. Some data suggested that macrolide antibiotics might overcome anticancer drug resistance by inhibiting the binding of vinblastine or cyclosporine to P-glycoprotein in P388/ADR cells. Erythromycin, josamycin, clarithromycin increased intracellular accumulation of vinblastine or cyclosporine *in vitro* and enhanced antitumor activity of vinblastine *in vivo* using mouse leukemia P388 cells and anticancer drug-resistant (P388/ADR) cells [16].

Some chemicals may potentiate therapeutic efficiency of other drugs in human neoplastic cells [9]. Influence of macrolides on human neoplastic cell lines in animal models was also investigated. The inhibitory effect of roxithromycin and clarithromycin on the human lung squamosus cell line H157 by using a mouse dorsal air sack model was dose-dependent. Both agents (200 mg/kg/d, *ip*) reduced the dense capillary area to about 20 and 30% of that of the control, respectively [21, 22].

Beneficial effects of macrolides as anticancer therapy were also observed in clinical trials. Long-term treatment with clarithromycin prolonged the median survival of patients with unresectable non-small-cell lung cancer. Median survival time for the clarithromycin group was 535 days, compared to 277 days in the non-clarithromycin group [8]. Clarithromycin may reduce the progression of cancer-associated cachexia and significantly prolonged survival in patients with non-small cell lung cancer through its ability to suppress IL-6 production [10]. Incidence of lung cancer metastasis was lower in clarithromycin-treated patients than in untreated ones. The expression of interleukin-12 and interferon gamma mRNAs was increased by clarithromycin, while the expression of interleukin-6 and interleukin-10 mRNAs was decreased [7]. Regression of Hodgkin's disease in the lung of a 33-year-old woman was also observed upon prolonged treatment with ciprofloxacin and clarithromycin [14].

Conclusions

The results of our study suggest that 14-membered ring macrolides erythromycin and roxithromycin have antitumor effects and might have potential therapeutic applications as anticancer agents. Further studies are required to understand the exact mechanism by which 14-membered ring macrolides exhibit antitumor activity.

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