Radiol Oncol 2000; 34(1): 49-57.

Antitumor effectiveness of bleomycin on SA-1 tumor after pretreatment with vinblastine

Maja Čemažar, Marija Auersperg, Gregor Serša

Institute of Oncology Ljubljana, Slovenia

Background. *In our previous study, vinblastine (VLB) was shown to increase the plasma membrane fluidity. This effect of VLB might be exploited for better transport of drugs through the plasma membrane. The aim of the present study was to determine whether pretreatment with VLB can increase the cytotoxic effect of BLM on intraperitoneal SA-1 tumors in mice.*

Materials and methods. *BLM and VLB were used as single agents or in various combinations, i.e. BLM injected 24 h before VLB or vice-versa, VLB injected 24 h before BLM. Cell and animal survival together with DNA histograms were the end-points used to determine the effect of these combined treatments.*

Results. *Both drugs, either as single treatment or in different combined therapy schedules reduced significantly the number of cells in peritoneal lavage, compared to control, saline treated animals. The combination of VLB, followed by BLM after 24 h reduced significantly the number of cells in peritoneal lavage, compared to the treatment in which BLM was followed by VLB or to the treatment with single drugs alone. Median survival time of mice treated with VLB alone, BLM alone and combination of both drugs was significantly prolonged compared to the control untreated mice. When VLB and BLM were combined, both treatment combinations were more effective than monochemotherapies with VLB or BLM. The best results were obtained when VLB was followed by BLM after 24 h. The DNA histogram of cells treated with VLB showed a decreased number of cells in S phase and an increased number of cells with DNA values greater than in G2M compartment compared to the control untreated cells. BLM in the dosage used in these experiments did not affect the progression of cells through cell cycle. Both combinations of VLB and BLM produced similar cell kinetic effect as VLB alone.*

Conclusion. *From these results we can conclude that the underlying mechanisms for enhanced antitumor effectiveness of BLM when VLB was given 24 h before BLM could be attributed predominantly to an increased membrane fluidity and possibly a cell kinetic effect of VLB.*

Key words: vinblastine; bleomycin; sarcoma experimental; fibrosarcoma

Received 15 December 1999
Accepted 24 January 2000

Correspondence to: Maja Čemažar, Ph.D., Department of Tumor Biology, Institute of Oncology, Zaloška 2, SI-1000 Ljubljana, Slovenia. Tel.: +386 61 323 063 ext. 29-33; Fax.: +386 61 133 74 10; E-mail: mcemazar@onko-i.si

Introduction

The design of presently used combined chemotherapeutic schedules is based on the data derived from preclinical studies, phase I and II clinical studies. However, little attention is paid to timing of drugs or possible interaction of drugs in a particular combined schedule. Both these factors could be crucial for the clinical effect of chemotherapy. The increasing knowledge and understanding of molecular mechanisms of drug-induced cytotoxicity form the basis for rational planning of clinical chemotherapy. In our previous work, we studied mechanisms of action of vinblastine (VLB), an antimitotic alkaloid.[1-3] VLB exerts cytotoxic activity against various tumors and is, at present, used mainly in combined chemotherapeutic schedules for treatment of testis tumors, Hodgkin's and non-Hodgkin's lymphomas, breast carcinomas, gastric carcinomas, squamous cell carcinomas, and many others.[4-8] In our previous study, we have demonstrated that, beside other effects, VLB also increases the plasma membrane fluidity and, consequently, its permeability.[1]

Our rationale for the use of VLB in combined chemotherapeutic schedules is that pretreatment with VLB could facilitate the transport into the cell of another chemotherapeutic drug with a hampered transport through the plasma membrane. One of such drugs is bleomycin (BLM); therefore, studies combining BLM and VLB could be very interesting. BLM is highly cytotoxic drug when present inside the cells.[9,10] It was shown that as little as several thousand molecules of BLM present inside the cell induce cell death.[10] BLM is a water soluble glycopeptidic antibiotic with limited antitumor effectiveness, used only in combined chemotherapeutic schedules in cancer treatment. The major reason for its limited antitumor effectiveness is the hampered transport of BLM through the plasma membrane. However, once inside the cell, BLM is highly cytotoxic inducing single and double strand breaks of DNA. Therefore, the cytotoxicity of BLM is dependent on its intracellular concentration and also upon the membrane permeability, which influences the uptake of the drug.[9,10] In our previous study, we already demonstrated that pretreatment with VLB significantly prolonged the survival of mice bearing SA-1 tumors treated with BLM, compared to the control groups.[11] We presumed that the observed antitumor effectiveness was due to the increased uptake of BLM and possibly also to the cell kinetic effects of both drugs. Therefore, in this study, we further elaborated the effect of pretreatment with VLB on i.p. SA-1 tumors treated with BLM. Cell and animal survival together with DNA distribution pattern were the end-points used for determining the effect of this combined treatment.

Materials and methods

Drug formulation

VLB (Vinblastine sulphate, Lilly France S.A.) was dissolved in 0.9% NaCl solution at a concentration 2.5 μg/ml. BLM (Bleomycin, Mack, Germany) was dissolved in 0.9 % NaCl solution at a concentration 500 μg/ml. Each animal was injected i.p. with 0.5 ml of the drug solution. According to Freireich *et al.*, the corresponding doses for VLB in humans would be 0.2 mg/m^2 (0.005 mg/kg) and for BLM 37 mg/m^2 (1.0 mg/kg).[12]

Animals

Inbred A/J mice were purchased from the Rudjer Bosković Institute (Croatia). Mice were maintained at a constant room temperature (22 °C) and natural day/night light cycle in a conventional animal colony. Before experiments, mice were subjected to an adaptation period of at least 10 days. Female mice in good condition, weighing 20-22 g, without

signs of infection, 10-12 weeks old, were included in the experiments.

Tumor model

Intraperitoneal (i.p.) SA-1 fibrosarcoma syngeneic to A/J mice was used in the study. The tumor was maintained i.p. as ascites by serial transplantation once a week. For induction of i.p. tumors, tumor cells from the donor mouse were harvested by peritoneal lavage with 4 ml of 0.9 % NaCl solution, washed and resuspended at a concentration of 6×10^5 cells/ml. Tumors were induced by i.p. injection of 3×10^5 viable SA-1 cells in 0.5 ml 0.9 % NaCl solution. Cell viability, determined by Trypan dye exclusion test, was over 95%.

Treatment protocol

Three days after tumor induction animals were randomly allocated into following groups: control (intraperitoneally treated with saline), VLB alone, BLM alone, VLB followed by BLM and BLM followed by VLB. The time interval between i.p. injection of the first and second drug was 24 h. In the case of monochemotherapy, 0.9 % NaCl was injected 24 h after the first drug. Each experimental group consisted of at least 6 mice and the data were pooled from 2-3 independent experiments.

Cell survival and flow cytometry

For the measurements, tumor cells were harvested by peritoneal lavage with 0.9 % NaCl 24 h after the completion of therapy *i.e.* 48 hours after the beginning of therapy. Tumor cells harvested from individual animals by peritoneal lavage were used for the determination of both, the cell survival and DNA distribution pattern. The effect of different treatments on cell survival was determined by counting the viable cells (Trypan dye exclusion test) in the peritoneal lavage of animals by means of hemocytometer. The results of cell survival were presented as a percentage of cells compared to the number of cells in the control animals. For flow cytometry measurements, cells were centrifuged, resuspended in citrate buffer solution and stored at -20 °C. Samples were then prepared according to the manufacturers protocol (CycleTEST PLUS, Becton Dickinson) and analysed using FACSCalibur flow cytometer (Becton Dickinson). The flow cytometer was operated at 488 nm and, after pulse shape analysis and gating on a cytogram of orthogonal vs. forward light scatter, a histogram of cell number against red (DNA-PI) fluorescence was recorded. Four different cell population were determined, cells in G_1, S, G_2M phases of cell cycle and cell with values of DNA greater than in G_2M compartment.

Animal survival assay

To determine the effect of BLM on the survival of mice pretreated with VLB, the mice were treated according to the treatment protocol and monitored for the day of death.

Statistical analysis

Data are represented as arithmetic means ± s.e.m.. The significance of the effect was determined using Student's t-test after one-way analysis of variance was performed; the levels of less than 0.05 were taken as indicative of significant differences. Survival curves were plotted by the Kaplan-Meier method. The differences between the survival curves were determined by Log Rank test.

Results

Cell survival

Cytotoxic effects of VLB and BLM as single treatments and in the combined treatment schedules on i.p. SA-1 tumors were deter-

Table 1. Comparison of the survival of mice with i.p. SA-1 tumors treated with combination of VLB and BLM and as a single drug treatment

		p compared to		
Group	Control	VLB	BLM	BLM-24h-VLB
VLB	<0.0001			
BLM	<0.0001	0.0051		
BLM-24h-VLB[1]	<0.0001	<0.0001	0.016	
VLB-24h-BLM[2]	<0.0001	<0.0001	0.003	0.0035

[1] BLM injected 24h before VLB

[2] VLB injected 24h before BLM

mined 24 h after the completion of therapy by counting the cells in the peritoneal lavage of the mice.

Both drugs, either as single treatment or in different combined therapy schedules reduced the number of cells in the peritoneal lavage significantly compared to the control, saline treated animals (Figure 1). VLB as single treatment was more effective compared to BLM; however, the difference in the mean value was not statistically significant.

The best results were obtained with combination of VLB followed by BLM after 24 h. The combination of pretreatment with VLB

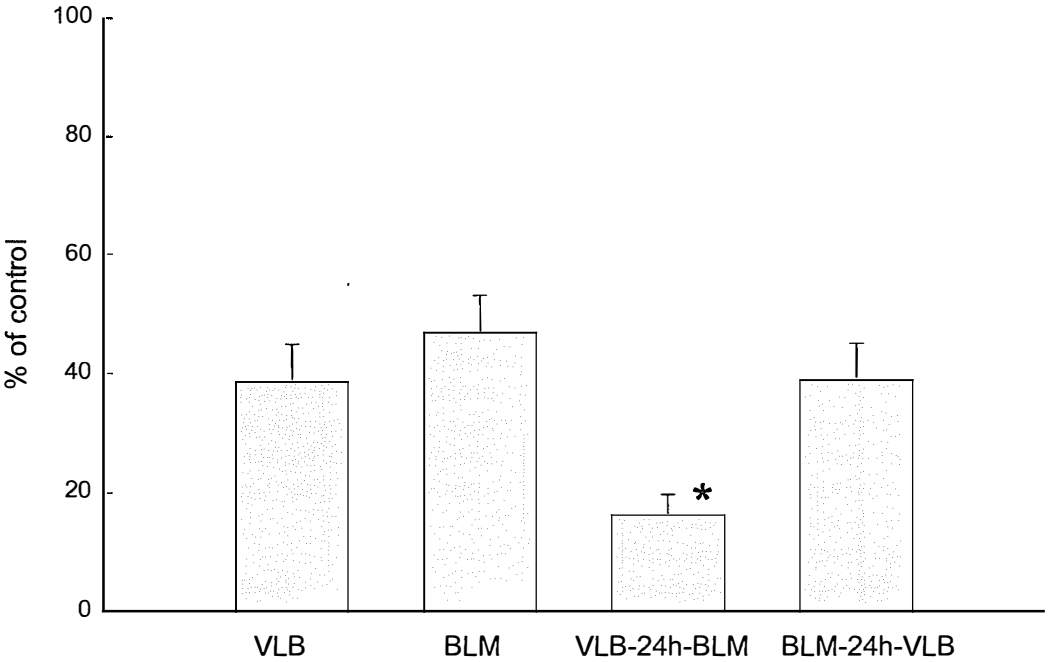

Figure 1. Number of cells in the peritoneal lavage of mice treated with VLB, BLM, VLB followed by BLM (VLB-24 h- BLM) and BLM followed by VLB (BLM-24h-VLB) presented as a percentage of number of viable cells in the peritoneal lavage of the control untreated mice. Bars are arithmetic means ± standard errors of the means. * $p<0.05$.

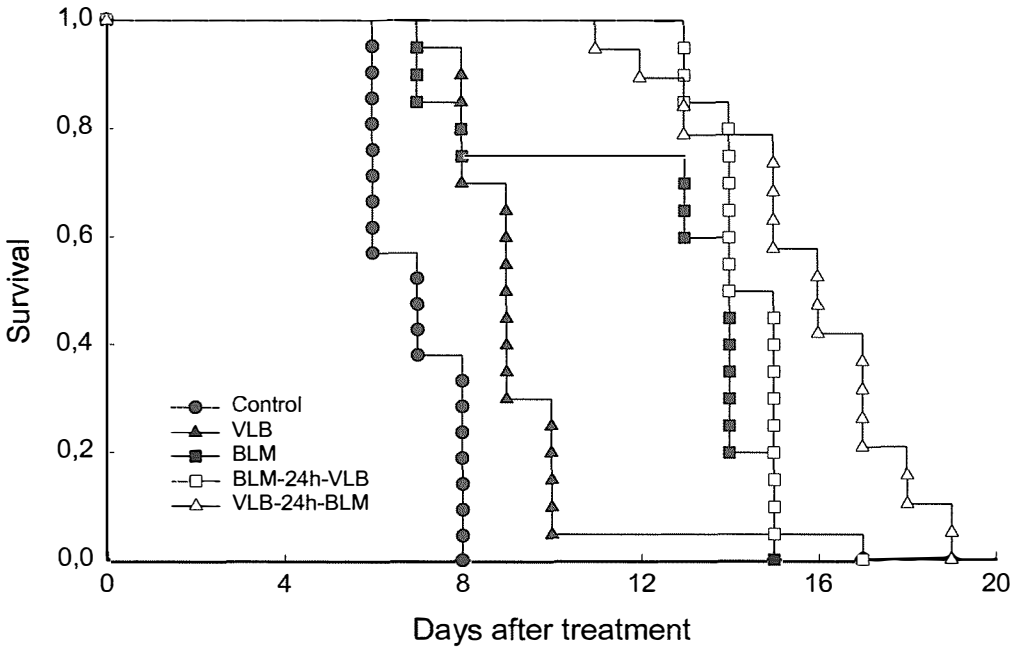

Figure 2. Survival of mice treated with VLB, BLM, VLB followed by BLM (VLB-24h-BLM) and BLM followed by VLB (BLM-24h-VLB). Survival curves were plotted by the Kaplan-Meier method.

followed by BLM reduced significantly the number of cells in the peritoneal lavage compared to the treatment in which BLM was followed by VLB or to the treatment with single drugs (Figure 1).

Animal survival

The antitumor effectiveness of VLB and BLM as single treatments and in the combined treatment schedules on i.p. SA-1 tumors were also determined on the survival of mice (Table 1, Figure 2). All treatments, VLB alone, BLM alone and combination of both drugs significantly prolonged median survival time of mice compared to the control untreated mice. When VLB and BLM were combined, both treatment combinations were more effective than monochemotherapies with VLB or BLM. The best results were obtained when VLB was followed by BLM after 24h. This treatment schedule resulted in the longest survival which was significantly better than in all other treatments (Table 1, Figure 1).

Flow cytometry

DNA histograms of the same samples that were used for measuring the cell survival were recorded by using FACSCalibur flow cytometer (Figure 3). The DNA histogram of cells treated with VLB showed a decreased number of cells in S phase and an increased number of cells with DNA values greater than in G_2M compartment groups compared to the control untreated cells.

BLM in the dosage used in these experiments did not affect the progression of cells through cell cycle. DNA histogram of the cells treated with BLM was in the same range as DNA histogram of the control untreated cells.

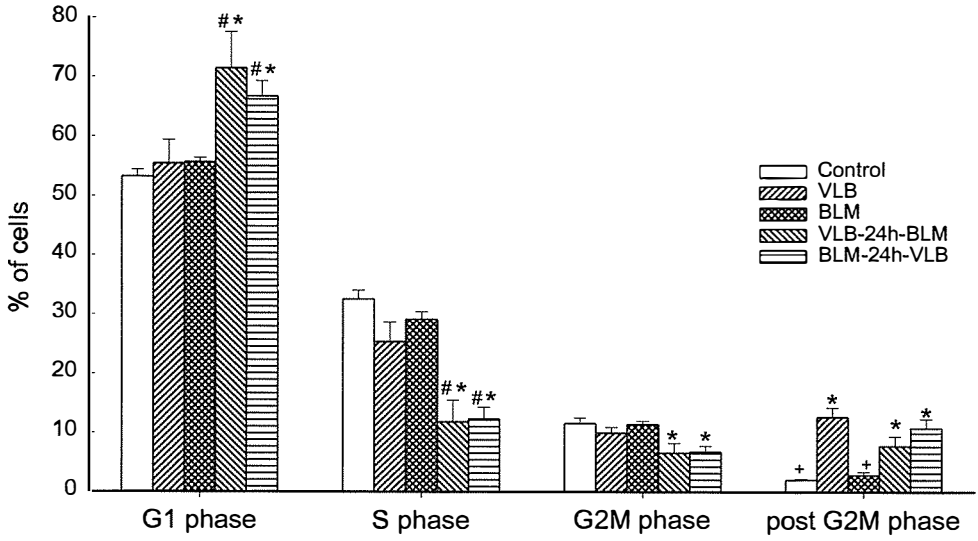

Figure 3. Percentage of cells in different compartment of cell cycle after the treatment of mice with VLB, BLM, VLB followed by BLM (VLB-24h-BLM) and BLM followed by VLB (BLM-24h-VLB). Bars are arithmetic means ± standard errors of the means. *p<0.05 compared to control and BLM treated animals, #p<0.05 compared to VLB treated animals, + p<0.05 compared to VLB treated animals and mice treated with combinations of VLB and BLM.

Both treatment combinations of VLB and BLM increased thge number of cells in G_1 phase, decreased the number of cells in S and G_2M phases of the cell cycle. In addition, both treatment combinations increased the number of cells with DNA values greater than in G_2M compartment, which was in the same range as the number of cells treated with VLB alone (Figure 3).

Discussion

Our study showed that pretreatment with VLB injected 24h before BLM enhanced antitumor effectiveness of BLM as determined by cell and animal survival of SA-1 tumor bearing mice. In spite of being widely used in various multidrug chemotherapy settings,[4,8,13,14] to our best knowledge, there is no studies exploring the role of timing of VLB and BLM.

VLB and BLM have different mechanisms of action. VLB interferes with the polymerization of tubulin, a protein which is involved in the formation of mitotic spindle microtubules and also an important component of cytoskeleton. In accordance with its effect on mitotic spindle microtubules, VLB blocks the cells in the metaphase of mitosis and thus acts as a cell synchronizing agent.[15] In addition, Madoc-Jones *et al.* reported on lethal action of VLB in interphase with the concentrations higher than those producing mitotic arrest.[17] The effect on cytoskeleton, however, might influence, beside the structures necessary for cell division, also the structures in the cell membrane. In our previous experiments using the same tumor model as in this study, we demonstrated that VLB increased plasma membrane fluidity as measured by electron paramagnetic resonance.[1] The same phenomenon was demonstrated in patients with squamous cell carcinoma and soft tissue sarcoma.[2,3] The results of both, experimental and clinical studies, suggested that VLB might be used for the enhancement of drug transport into the tumor cells. To prove this hypothesis, we tested the effectiveness of

VLB and BLM alone and in different combinations on the same tumor model as in the previous study.[11] The end-point used was animal survival. The results of that study showed that the best survival was obtained in mice treated with VLB 24 h before BLM. The observed effect was attributed to the increased plasma membrane fluidity and cell kinetic effect.[11] In the present study, we wanted to elucidate the mechanisms of this combination on the same tumor models, same drug combinations and at the same dosage of the drugs used by studying, beside animal survival, also cell survival and DNA distribution pattern. The animal survival experiments in the present study confirmed the results of previous experiments.[11] Again, the longest survival was obtained when VLB was given 24 h before BLM. In addition, this combined schedule also significantly reduced the cell survival compared to all three other treatments and to the control untreated tumors. In contrast, the survival of cells when BLM was followed by VLB did not significantly differ from the cell survival after treatment with single drugs. Therefore, the survival of cells did not match the survival of animals in this group. The survival of animals treated by BLM followed by VLB was better than the survival after monochemotherapies with VLB or BLM and worse than the survival of animals after treatment with VLB followed by BLM. One possible explanation for the discrepancy between the cell and animal survival after BLM followed by VLB is that cell death occurred later than 24 h after the completion of therapy, which was the time point for measuring cell survival in our experiments.

This increased effect of chemotherapy could be the result of either an increased plasma membrane fluidity or a cell kinetic effect caused by VLB or a combination of both effects. In our previous study, we found that VLB increases membrane fluidity of SA-1 tumor cells; we therefore assumed that this could be exploited to facilitate BLM uptake into the cells. To prove that an increased plasma membrane fluidity facilitates better accumulation of BLM in the cells, a measurement of BLM concentration in the cells after VLB treatment would be necessary. In our previous clinical studies using ^{99m}Tc labeled BLM (Tc-BLM), we showed that an increased accumulation of Tc-BLM was found in the tumors from approximately 24-48 h after the infusion of VLB.[8,16] In addition, our preliminary experiments using absorption spectroscopy for the quantification of cisplatin within the tumor cells showed a 4-fold increase of cisplatin after the pretreatment with VLB, compared to the treatment with cisplatin alone or when cisplatin was followed by VLB (Čemažar *et al.*, unpublished data).

A cell kinetic effect of VLB was proven by DNA single cell measurement. Cell kinetic effect of VLB seems to be dose dependent. Higher doses prolong the transition of cells through S phase, whereas lower doses, as used in the present study, increased the number of cells with DNA values greater than in G_2M phase of cell cycle.[17-19] BLM is reported to be the most effective in G_2M and G_1 and less in S phase of the cell cycle.[20] However, in the present study, the effect of BLM on the cell cycle was not demonstrated. One possible explanation for that could be high BLM dose used in our experiments which caused cell death in all phases of the cells cycle and therefore did not have the effect on the accumulation of the cells in a particular phase of the cell cycle. Both treatment combinations of VLB and BLM were effective in reducing cell survival which was reflected in DNA histograms as a decreased number of cells in S and G_2M phases of the cells cycle and a relative increase in the number of cells in the G_1 compartment of the cell cycle.

In conclusion, based on the known properties of VLB and BLM, we can assume that in the present study, predominantly an increased membrane fluidity and, possibly to a lesser extent, an accumulation of cells in

BLM-sensitive phases of cell cycle induced by VLB is responsible for the best effect of VLB and BLM combination in which VLB preceded BLM for 24 h. Our study also shows that understanding the interactions of agents in combined chemotherapeutic schedules could lead to a better planning and timing of drugs in clinical chemotherapy.

Acknowledgment

This work was supported by the Ministry of Science and Technology of the Republic of Slovenia. The authors thank Mira Lavrič, Karmen Zajc, Brigita Šturbej and Marjana Matič for their excellent technical assistance.

References

1. Serša G, Čemažar M, Šentjurc M, Us-Kraševec M, Kalebić S, Drašlar K, Auersperg M. Effects of vinblastine on cell membrane fluidity and the growth of SA-1 tumor in mice. *Cancer Lett* 1994; **79**: 53-60.

2. Šentjurc M, Schara M, Auersperg M, Jezernik M, Kveder M. Characterization of malignant tissues by EPR. *Stud Biophys* 1990; **136**: 201-8.

3. Šentjurc M, Schara M, Auersperg, M. Influence of vinblastine containing chemotherapy on tumor tissue membrane fluidity; an EPR study. *Arch Geschwulstforsch* 1988; **58**: 35-42.

4. Haskell CM. Drugs used in cancer chemotherapy. In: Haskell CM ed. *Cancer treatment*. WB Saunders Company; 1990. p. 69-70.

5. Van Tellingen O, Sips JMH, Beijnen JH, Bult A, Nooijen WJ. Pharmacology, bio-analysis and pharmacokinetics of the Vinca alkaloids and semi-synthetic derivates (review). *Anti-Cancer Res* 1992; **12**: 1699-716.

6. Auersperg M, Šoba E, Vraspir-Porenta O. Intravenous chemotherapy with synchronization in advanced cancer of oral cavity and oropharynx. *Z Krebsfotsch* 1977; **90**: 149-59.

7. Auersperg M, Us-Kraševec M, Lamovec J, Erjavec M, Benulič T, Porenta Vraspir O. Chemotherapy-a new approach to the treatment of verrucous carcinoma. *Radiol Iugosl* 1989; **23**: 387-92.

8. Auersperg M, Šoba E, Porenta O, Erjavec M, Us-Kraševec M, Bergant D, Furlan L. Rational scheduling of multidrug chemotherapy with synchronization in advanced squamous cell carcinoma of the oral cavity and oropharynx. In: Čupar I and Padovan I eds. *Currents concepts of head and neck cancers*. Jugoslavenska Akademija Znanosti i Umjetnosti; 1981. p. 286-99.

9. Lazo JS, Sebti SM, Schellens JHM. Bleomycin. In: Pinedo HM, Longo DL, Chabner BA, eds. *Cancer chemotherapy and biological response modifiers annual 16*. Elsevier Science B.V.; 1996. p. 39-47.

10. Tounekti O, Pron G, Belehradek J, Mir LM. Bleomycin, an apoptosis-mimetic drug that induced two types of cell death depending on the number of molecules internalized. *Cancer Res* 1993; **53**: 5462-9.

11. Čemažar M, Auersperg M, Serša G. Vinblastine increases antitumor effectiveness of bleomycin. *Radiol Oncol* 1997; **31**: 364-7.

12. Freireich EJ, Gehan EA, Rall DP, Schmidt LH, Skipper HE. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. *Cancer Chemother Rep* 1966; **50**: 219-45.

13. Jaffe N, LeeYY, Auersperg M, Us-Kraševec M, Porenta O, Gohde W, Lamovec J. Novel therapeutic strategies for the treatment of rhabdomyosarcoma and soft tissue sarcomas: Observations with arterial infusion chemotherapy. In: Maurer HM, Ruymann FB, Pochedly CE. eds. *Rhabdomyosarcoma and related tumors in children and adolescents*. Boca Raton, CRC Press; 1991. p. 243-60.

14. Pecorelli S, Wagenaar HC, Vergore IB, Curran D, Beex LVA, Wiltshaw E, Vermorken JB. Cisplatin (P), vinblastine (V) and bleomycin (B) combination chemotherapy in recurrent or advanced granulosa(-theca) cell tumors of the ovary. An EORTC gynaecological cancer cooperative group study. *Eur J Cancer* 1999; **35**: 1331-7.

15. Jordan MA, Thower D, Wilson L. Mechanism of inhibition of cell proliferation by Vinca alkaloids. *Cancer Res* 1991; **51**: 2212-22.

16. Auersperg A, Erjavec M, Us-Kraševec M. Accumulation of ^{99m}Tc-Bleomycin in human squamous cell carcinoma in vivo after synhronisation by vinblastine. *IRCS Medical Science: Cancer; Clinical Pharmacology and Therapeutics; Radiology and Nuclear Medicine* 1975; 3: 560.

17. Madoc-Jones H, Mauro F. Interphase action of vinblatine and vincristine: differences in their lethal action through the mitotic cycle of cultured mammalian cells. *J Cell Physiol* 1972; **72**: 185-96.

18. Auersperg M, Us-Kraševec M, Bešič N, Pogačnik A, Stanič K, Jezeršek B. Flow-cytophotometric DNA measurements for monitoring and planning chemotherapy in anaplastic thyroid carcinoma. In: Pimpl W, Galvan G, Kogelnik HD, Manfreda D, Niederle B, Schlag P, Waclawiczek HW eds. *Struma maligna*. Springer-Verlag; 1993. p. 107-13.

19. Auersperg M, Us-Kraševec M, Pogačnik A, Stanič K, Hočevar M, Jezeršek B. Flow-cytophotometric DNA measurements for planning chemotherapy in differentiated thyroid cancer *Radiol Oncol* 1993; **27 Suppl 6**:192S-7S.

20. Olive PL, Banath JP. Detection of DNA double strand breaks through the cell cycle after exposure to X-rays, bleomycin, etoposide and 125IdUrd. *Int J Radiat Biol* 1993; **64**: 349-58.