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Schedule-dependency of doxorubicin and vinblastine in EAT tumours in mice

Marija Auersperg, Ana Pogačnik, Veronika Kloboves-Prevodnik, Gregor Serša, Maja Čemažar

Institute of Oncology Ljubljana, Ljubljana, Slovenia

Background. *Antitumour schedule-dependency of the doxorubicin and vinblastine combination was explored.*

Materials and methods. *Intraperitoneal Ehrlich ascites tumours (EAT) syngeneic to CBA mice were treated with vinblastine or doxorubicin alone, or in combined treatment schedules.*

Results. *Combinations of doxorubicin and vinblastine administered at 48-h, but not at 24-h interval, regardless of the sequence of drugs, significantly reduced the number of tumour cells in the ascites in comparison with all other treatments. In the combined treatment schedules, the predominant morphological changes as well as DNA distribution pattern were dependent on the first drug applied. Regardless of the sequence of the drugs, median survival times of animals did not significantly differ between the treatment groups.*

Conclusions. *The effect of combination of vinblastine and doxorubicin is schedule-dependent. The time interval, but not the sequence of drugs seems to be crucial for the observed effect. The data from preclinical studies are important for planning combined treatment schedules in clinical setting.*

Key words: carcinoma Ehrlich tumor – drug therapy; doxorubicin; vinblastine; drug administration schedule

Introduction

Doxorubicin (Doxo) and vinblastine (VLB) or their analogues are used in combined treatment schedules for a variety of malignant tumours, *i.e.* breast, ovarian, lung, urothelial cancer and Hodgkin disease.[1-6] The combination chemotherapy is used with the aim to enhance antitumour efficacy. In planning chemotherapy protocols in patients, drugs with different mechanisms of action, non–cross-resistant and with non-overlapping toxicities are used. In planning the intervals between sequential cycles of chemotherapy mostly the tolerance of bone marrow is taken into consideration. However, in clinical setting, attention is rarely paid to possible drug interference and schedule dependency of the drug combinations. There are usually no data on the sequence and exact timing of particular drugs in combined chemotherapy

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Correspondence to: Prof. Maja Čemažar, PhD, Institute of Oncology Ljubljana, Zaloška 2, SI-1000 Ljubljana, Slovenia. Phone: +386 (0)1 5879 544; Fax: +386 (0)1 5879 434; E-mail: mcemazar@onko-i.si

protocols. Schedule dependency and drug interference has been extensively studied in tumour models.[7-11] The implementation of results from preclinical studies could make planning of treatment schedules more rational and thus improve the effect of chemotherapy in patients.

In our clinical research on the combined treatment of anaplastic thyroid carcinoma, VLB or Doxo combined with radiation was a promising treatment for achieving local control of the primary tumour.[12] A logic further development of treatment of this very aggressive tumour would be a schedule combining both Doxo and VLB with radiation. In our previous preclinical and clinical studies, we demonstrated that, after pretreatment of tumour cells with VLB, the accumulation of bleomycin or cisplatin in the tumour cells was increased.[13,14] The increased cell membrane permeability and consequently a better penetration of the drug into the cell was the proposed mechanism of action. It would be clinically relevant to explore if pretreatment with VLB also influences the accumulation of Doxo in the tumour cells.

The primary objective of the current work was to explore in a preclinical study whether there is a schedule-dependency of the combination of VLB and Doxo. The second objective was to find out whether, after pretreatment with VLB, the accumulation of Doxo within the tumour cells could be increased.

Materials and methods

Drug formulation

VLB (Vinblastine sulphate, Lilly France S.A., Fagersheim, France) was dissolved in 0.9% NaCl solution at a concentration 2.5 μg/ml. Doxorubicin (Doxo; Doxorubin, Pharmachemie B.V., Haarlem, The Netherlands) stock solution (2 mg/ml) was further diluted in 0.9% NaCl solution to achieve doses of 0.9 to 3.6 mg/kg. Each animal was injected i.p. with adjusted volume (approx 0.5 ml) of drug solution to achieve VLB dose of 62.5 μg/kg and different Doxo doses ranging from 0.9 to 3.6 mg/kg. This low VLB dose was selected according to our previous studies where we demonstrated that this dose significantly affected cell membrane fluidity with a minimal effect on cell survival.[15,16] All doses used were far below the maximal tolerated dose level.

Animals

Inbred CBA mice were purchased from the Institute of Pathology, Medical Faculty Ljubljana (Slovenia). 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. Mice of both sexes, in good condition, weighing 22-30 g, without signs of infection, 10-15 weeks old, were included in the experiments. Animal studies were carried out according to the guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia, permission No. 323-02-200/2004 and in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Bethesda MD).

Tumour model

Intraperitoneal (i.p.) Ehrlich ascites carcinoma (EAT) syngeneic to CBA mice was used in the study. The tumour was maintained i.p. as ascites by serial transplantations once a week. For the transplantation of i.p. tumours, the tumour 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 3×10^6 cells/ml. The tumours were transplanted by i.p. injection of 1.5×10^6

viable EAT cells in 0.5 ml 0.9% NaCl solution. Cell viability, determined by Trypan dye exclusion test, was over 95%.

Treatment protocol

In the first part of the study, different doses of Doxo (0.9, 1.8 and 3.6 mg/kg) were tested in order to determine the cell survival, accumulation of Doxo in the tumour cells and effects of Doxo on DNA distribution. Three days after tumour transplantation, the animals were treated with Doxo and thereafter sacrificed at different post treatment intervals (24, 48 and 72 h) to evaluate the treatment effectiveness.

In the combined treatment schedule, the animals were randomly allocated three days after tumour transplantation into the following groups: control (i.p. treated with 0.9% NaCl solution), VLB alone, Doxo alone, VLB followed by Doxo, Doxo followed by VLB and both drugs given simultaneously. The time interval between i.p. injection of the first and second drug was 24 or 48 h. When the chemotherapy with VLB or Doxo was tested alone, 0.9% NaCl was injected 24 h or 48 h afterwards, as a sham intervention (Table 1). The mice were sacrificed 24 h after the completion of therapy by cervical dislocation. Each experimental group consisted of at least 3 mice and the data were pooled from 2-3 independent experiments.

Cell number, flow cytometric analysis of DNA content, Doxo fluorescence and cell morphology

Twenty-four hours after the completion of therapy, the mice were sacrificed and tumour cells were harvested by peritoneal lavage with phosphate-buffered saline supplemented with 20% bovine serum albumin. The tumour cells harvested from individual animals were used for the measurement of cell number, flow cytometric DNA measurements and cell cycle analysis, Doxo accumulation and study of cell morphology. The effect of different treatments on cell survival

Table 1. Treatment protocol. A – 24 h interval between treatments; B. 48 h –interval between treatments.

A

Group	Day 0	Day 3	Day 4	Day 5
VLB 24 h	Tum. inoculation	VLB	Physiological saline	Harvesting
Doxo 24 h		Doxo	Physiological saline	Harvesting
VLB + Doxo 24 h		VLB + Doxo		Harvesting
VLB 24 h Doxo		VLB	Doxo	Harvesting
Doxo 24 h VLB		Doxo	VLB	Harvesting

B

Group	Day 0	Day 3	Day 5	Day 6
VLB 48 h	Tum. inoculation	VLB	Physiological saline	Harvesting
Doxo 48 h		Doxo	Physiological saline	Harvesting
VLB + Doxo 48 h		VLB + Doxo		Harvesting
VLB 48 h Doxo		VLB	Doxo	Harvesting
Doxo 48 h VLB		Doxo	VLB	Harvesting

was determined by counting the tumour cells in the peritoneal lavage of the animals by means of haemocytometer. The results of cell number were presented as the percent of cells compared to the number of cells in the control, saline treated animals. For the flow cytometric DNA measurements, the cells were prepared according to a modified Otto method.[17] In brief, the cells were treated for 20 min with a solution consisting of 0.2 M citric acid and 0.5% Tween 20, and then fixed with 70% ethanol for at least 24 h. After the treatment with 0.5% pepsin (Serva, Heilderberg, Germany) for 5 min, the cells were stained with 4'6-diamidino-2 phenylindole (DAPI, Serva) for DNA. The measurements of DNA content of cells were performed using a PAS III (Partec, Münster, Germany) flow cytometer. The results were presented in the histograms of cell number against fluorescence. The data were analyzed with Multicycle AV (Phoenix Floe Systems, San Diego, CA) program. For flow cytometric measurement of Doxo accumulation in the tumour cells, the cells were centrifuged and resuspended in phosphate buffered saline at a concentration of $5x10^5$ cells/ml. The samples were analysed on FacsCalibur flow cytometer (BD PharMingen, San Jose, California, USA) using 585/42 bandpass filter and the results were presented in the histograms of cell number against fluorescence. In addition, the cell samples counterstained with DAPI to distinguish between dead cells (DAPI positive) and the cells with Doxo accumulation were studied on fluorescence microscope. Cell morphology was studied on the cell smears stained with Giemsa (Merck, Darmstadt, Germany).

Statistical analysis

The data are presented as arithmetic means ± SE (standard error of the mean). The significance of the effect was determined using post-hoc Tukey'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 using Dunn's method after Kruskal-Wallis One way analysis of variance on ranks was performed. Statistical analysis was carried out using SigmaStat statistical software (SPSS, Chicago, USA).

Results

Effect of different Doxo doses in EAT tumour cells

The growth curves of the cells in the ascites of CBA mice treated with different Doxo doses did not differ up to 48 h post treatment. Only at 72 h post treatment, already the lowest dose of Doxo (0.9 mg/kg) induced a significant reduction of the number of cells in the ascites (Figure 1). The increase in Doxo dose resulted in an increased cytotoxicity at this time interval. However, there was no statistical difference between the two higher doses tested (1.8 and 3.6 mg/kg). Based on these results, the lowest, relatively non-cytotoxic Doxo dose (0.9 mg/kg) was chosen for the subsequent experiments combining VLB and Doxo. In order to detect a possible potentiation of Doxo or VLB cytotoxicity in combined therapy schedules, an excessive cell kill caused by higher dose of Doxo alone would not be desirable.

Beside cytotoxicity of different doses of Doxo, intracellular accumulation of Doxo, morphological changes and DNA distribution in EAT tumour cells were also studied. From the flow cytometric measurements of number of cells with internalized Doxo it was evident that the number of fluorescent cells did not differ between the doses tested at 24 and 48 h post treatment, whereas at 72 h post treatment, significantly less fluorescent cells were observed at two

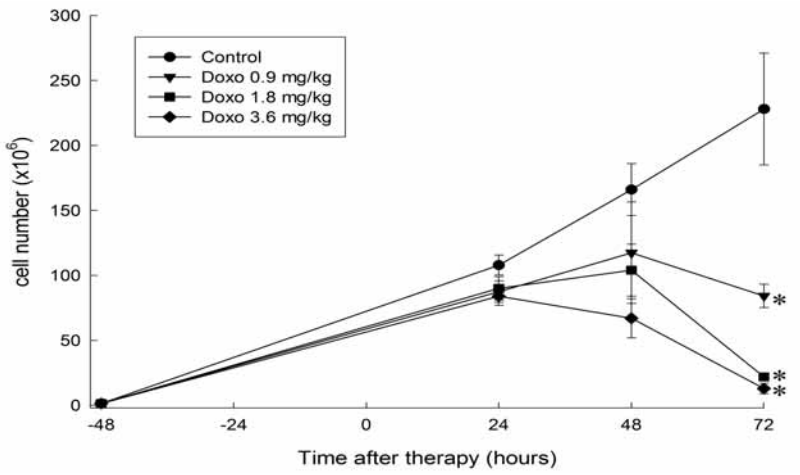

Figure 1. Cytotoxic effect of Doxo to EAT tumour cells in ascites. EAT tumour cell number as a function of time in the ascites of mice treated with different doses of Doxo injected i.p. Points are mean of 9 mice per group. *p<0.05 compared to control group.

higher doses because of significant cell kill induced by these doses (Figure 2).

However, the amount of Doxo in the tumour cells increased in a dose-dependent manner, as evident from the median value of the peak of fluorescence intensity of cells and fluorescence microscopy (Figure 3). Median value of the peak of fluorescence intensity at the lowest dose was 167 and increased to 320 at 3.6 mg/kg Doxo 24 h post treatment (data not shown).The cell cycle phase distribution in EAT tumour cells 48 h post treatment demonstrated that Doxo greatly reduced the number of cells in G_1 phase of cell cycle and caused a block in G_2M compartment (see Figure 6). The

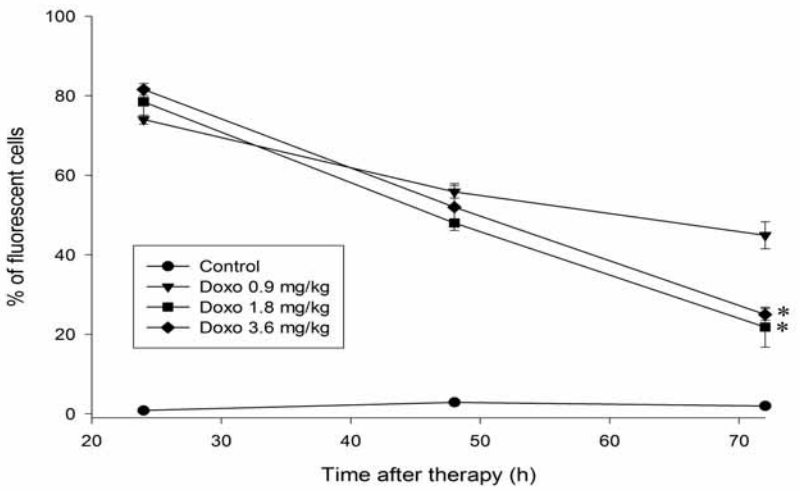

Figure 2. Percentage of EAT tumour cells with Doxo accumulation as determined from fluorescent histograms obtained by flow cytometer. Points are mean of 9 mice per group .* p<0.05 compared to treatment with Doxo dose of 0.9 mg/kg.

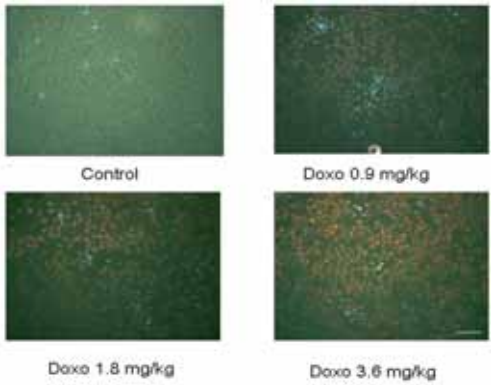

Figure 3. Fluorescence intensity in EAT tumour cells 24 h after treatment demonstrated increased amount of Doxo within the cells with increasing Doxo doses. Doxo positive cells – red, DAPI positive (dead) cells –blue (bar 100 μm).

morphological changes observed in the cell smears prepared from the same samples corresponded to the changes in cell cycle phase distribution. Enlarged cells with enlarged nuclei and nucleoli were observed at all doses tested compared to the untreated control cells. In addition, mitoses were rare and degenerative changes, such as poto-

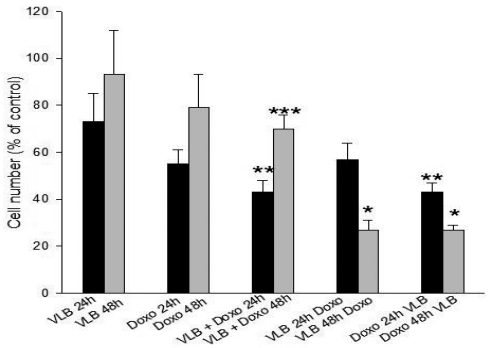

Figure 4. Survival of EAT tumour cells after treatment with 24-h (black bars) or 48-h (grey bars) interval between the treatments with Doxo and VLB. Pertinent control groups, *i.e.* treatment with either of the drugs alone and VLB + Doxo given simultaneously, are included. For protocol see Table 1. Cells from ascites were harvested 24 h after completion of treatment. Bars are mean of at least 6 mice per group. * p<0.05 compared to all treatment groups; ** p<0.05 compared to treatment with VLB, but not to treatment with Doxo; *** p<0.05 compared to control.

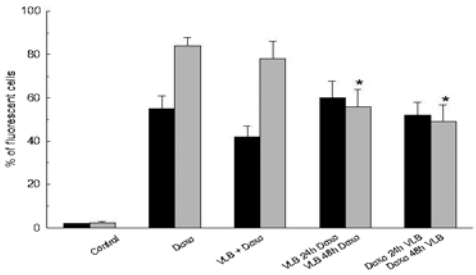

Figure 5. Accumulation of Doxo in EAT tumour cells. Percentage of Doxo positive EAT tumour cells after treatment with 24-h (black bars) or 48-h (grey bars) time interval between Doxo and VLB administration. Pertinent control groups, *i.e.* treatment with either of the drugs alone and VLB+Doxo given simultaneously, are included. For protocol see Table 1. Cells from ascites were harvested 24 h after completion of therapy. Bars are mean of at least 6 mice per group.* p<0.05 compared to treatment with Doxo and VLB + Doxo.

cytosis and fragments of cytoplasm, were observed at all doses tested (see Figure 7).

Effects of different VLB and Doxo treatment combinations delivered with a 24-h interval between the administration of the two drugs in EAT tumour cells

To determine the effect of VLB and Doxo combinations delivered with a 24-h interval between the two drug administrations on the survival of EAT tumour cells, the cell number was determined in the ascites. All the treatments significantly reduced the EAT tumour cell number in the ascites compared to the untreated control animals. However, there was no significant reduction in cell number following the treatment with different treatment combinations compared to the treatment with Doxo alone. The treatment combinations in either of the schedules reduced the survival only to the level of the survival induced by Doxo alone (Figure 4). In contrast, compared to the treatment with VLB alone, the treatment combinations with two drugs, injected simultaneously or with Doxo preceeding

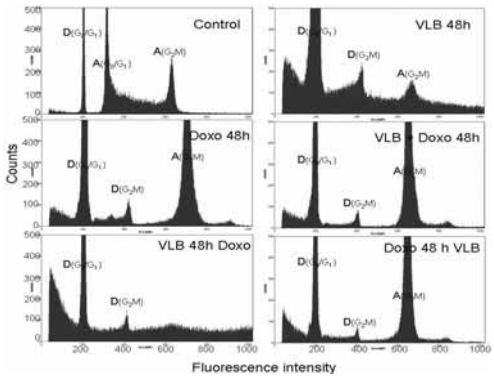

Figure 6. The DNA distribution in EAT tumour cells after treatment with different VLB+Doxo treatment schedules, with the 48-h interval between the injections of drugs. D-diploid value of DNA (inflammatory cells); A – aneuploid value of DNA (tumour cells).

VLB resulted in significantly reduced cell number (Figure 4).

The percentages of fluorescent (Doxo positive) cells measured 24 h after the completion of treatment were the same in all groups regardless of the treatment schedule (Figure 5).

The DNA distribution measurement of tumour cells from the animals treated with Doxo alone showed an increased G_2M compartment of the cell cycle. In the cytological smears (Doxo 24 h) prepared from the same samples as for the DNA measurements, enlarged cells with enlarged nuclei and nucleoli were observed. Mitoses were very rare. The same effects on the cells were seen also in the samples when Doxo preceded VLB for 24 h as well as when the drugs were given simultaneously. Rare mitoses in cytological smears together with enlarged G_2M compartment in DNA histograms speaks for a block in G_2 phase of the cell cycle.

The DNA distribution measurements of the cells treated with VLB alone (VLB 24 h) or when VLB preceded Doxo for 24 h showed the cells with very high DNA values, but with no distinctive peaks (data not shown). The cells taken from the same samples were enlarged, but to the lesser degree than the

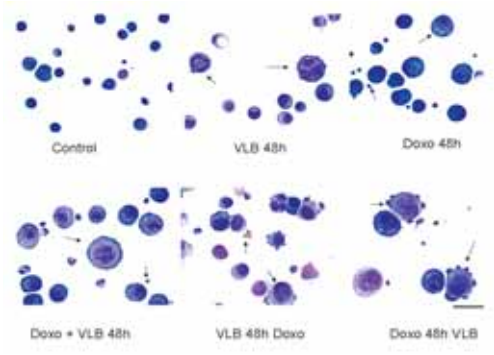

Figure 7. Morphological changes in EAT tumour cells after treatment with different VLB+Doxo treatment schedules, with the 48-h interval between the injections of drugs. (Giemsa staining, bar 50 μm). Arrows indicate enlarged cells, potocytosis and fragments.

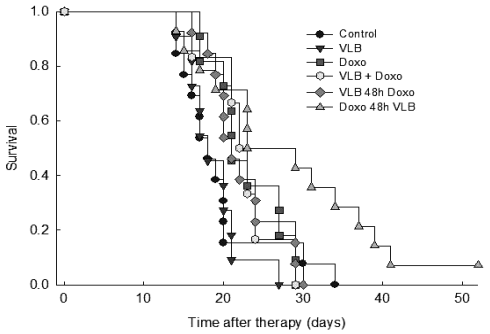

Figure 8. Animal survival after treatment with different VLB+Doxo treatment schedules, with the 48-h interval between the drugs injections.

cells treated with Doxo. Multinucleated cells and cells with irregular mitoses were also observed (data not shown).

In cytological smears of all treatment groups, a high percentage of inflammatory cells was observed, which was also shown in DNA histograms.

Effects of different VLB and Doxo treatment combinations delivered with a 48-h interval between the administration of the two drugs in EAT tumour cells

The cytotoxic effects of VLB and Doxo combinations delivered with a 48-h interval

between the applications of the two drugs were also tested. In contrast to the 24-h interval between the drugs, at the 48-h interval, both treatment combinations (VLB preceding Doxo or VLB following Doxo) resulted in a significantly reduced cell survival in comparison to all other treatment groups (Figure 4).

Measurement of percentage of fluorescent cells using flow cytometer demonstrated that, in the treatment combinations with the 48-h interval, the number of Doxo positive cells was significantly lower than in the treatment with Doxo alone or with both drugs injected simultaneously (VLB + Doxo). The lower percentage of fluorescent cells observed after the treatments can be ascribed to a significant cell kill induced by these treatments (Figure 5). This is in agreement with the results observed when the animals were treated with increasing doses of Doxo alone.

In the group treated with Doxo and VLB applied with the 48-h interval between the two drug administrations, the DNA distribution measurements of the samples showed an increased G_2M compartment. The same phenomenon was observed when the animals were treated with Doxo alone or when Doxo and VLB were applied simultaneously. The DNA histograms of the samples from VLB treated animals or when VLB preceded Doxo showed less distinctive peaks and cells with very high DNA values (Figure 6). Morphological changes corresponded to the measured DNA content in the cells (Figure 7). In the experiments when VLB preceded Doxo, the effect of VLB was dominant (multinucleated cells), while in the case when Doxo preceded VLB, the dominant effect on the cell morphology was due to Doxo (enlarged cells). However, in both cases, extremely enlarged cells with potocytosis were also observed. When the drugs were given simultaneously, a certain percentage of unaffected tumour cells were observed, the rest of them displayed morphological changes that could be ascribed predominantly to Doxo action. In other treatment groups (VLB 48 h and Doxo 48 h alone), the cells displayed typical changes pertinent to the action of the drug. The cells treated with VLB alone were enlarged. In addition, irregular mitoses and multinucleated cells with potocytosis were also observed. The cells treated with Doxo alone were also enlarged having enlarged nuclei and nucleoli.

In cytological smears of all treatment groups, a high percentage of inflammatory cells was observed, which was also shown in DNA histograms, as a diploid peak.

Effect of different VLB and Doxo treatment combinations on animal survival

Survival of animals was also determined after various schedules of Doxo and VLB treatments delivered with 24-h and 48-h intervals between the injections of the drugs. Despite the significant difference in reduction of EAT tumour cell number in the ascites after the treatment with Doxo and VLB delivered with the 48-h interval between the injections, this reduction did not translate into statistically significantly prolonged animal survival. Regardless of the schedule of the treatment, median survival times did not significantly differ between the treatment groups either delivered with the 24-h interval (data not shown) or 48-h interval between the drug administration (Figure 8). However, there was a trend to prolonged survival in the group treated with Doxo and 48h later with VLB.

Discussion

The results of our study show that the combination of VLB and Doxo is schedule-dependent. The time interval between the

drug administrations, but not the sequence, seems to be crucial for the obtained effects. The interval of 48 h between the drug administrations resulted in more pronounced antitumour effectiveness compared to the 24-h interval.

In planning multidrug (combined) chemotherapy in clinical setting, attention is focused mainly on defining an optimal dosage of the drugs in a protocol with acceptable toxicity. However, for the effect of a protocol, beside dosage and toxicity, an interaction of drugs with consequent schedule-dependency could be decisive. A combination of drugs can result in a synergistic, additive or antagonistic effect. In the literature on multidrug schedules in clinical chemotherapy, there are usually only the data on the dosage of drugs and their distribution according to days in a particular cycle of chemotherapy. There are mostly no data on the sequence and exact timing of drugs.[18-22] In the reports in which authors define the sequence of drugs, there are usually no data on their exact timing and intervals between drugs [1,4,6], *i.e.* factors which could have an important influence on the effect of chemotherapy. In clinical studies comparing multidrug chemotherapy to single agent chemotherapy, a clear advantage of multidrug chemotherapy over single agents was not always shown. In a meta analysis of randomized trials in metastatic breast cancer, Fossati[23] found an advantage of multidrug chemotherapy in the response rate, but only a very modest benefit in survival of patients. Similarly, Ejlertsen[19] described a higher response rate with no benefit in survival of patients, whereas Norris and Joensuu found no advantage of combined schedules in metastatic breast cancer.[22,24] Among other factors for these conflicting results, schedule-dependency could also play a role.

Recently, there have been many reports on schedule-dependency between antracyclins and microtubule active drugs.[7-11,25] In our work, we explored the schedule-dependency of VLB combined with Doxo in Ehrlich ascites tumour cells in mice. We found that the combination was schedule-dependent. The most cytotoxic combinations were VLB injected 48 h before Doxo and the reverse order of the drug injections, Doxo injected 48 h before VLB. Both orders of sequence were equally effective and showed a statistically significant reduction of tumour cell survival compared to all other combinations (Figure 4). The combination of VLB and Doxo was not better than Doxo alone when given simultaneously or with the 24-h interval between the drugs. In contrast to our results, Zeng [10] found an antagonistic effect of Doxo and a mitotic poison Docetaxel if the two drugs were applied simultaneously or in the sequence Doxo-Docetaxel. The reverse sequence Docetaxel-Doxo delivered with an interval of 12 h between the drugs resulted in an enhanced cytotoxicity. The explanation for the antagonism of the sequence Doxo-Docetaxel backed up by flow cytometry was that Doxo produced a block in G_2 phase of the cell cycle and thus prevented the mitotic arrest by Docetaxel.[10] Similarly, the best inhibition of the tumour by the schedule Docetaxel interval 12 h Doxo was reported by To *et. al.* in Ehrlich ascites cell tumours bearing mice.[9] In contrast to these results, Zoli found a synergistic and not antagonistic effect of the sequence Doxo and another microtubule stabilizing agent Paclitaxel.[11] The results of schedule-dependency studies are influenced by many factors, such as the dosage, time of exposure to the drugs and the intervals between the injections of drugs. In addition, the results could depend on the tumour model as different cell lines can respond with different cell kinetic changes to the same drug.[26]

Flow cytometric study showed two distinctive patterns of DNA distribution after

the treatment with VLB or Doxo. The latter drug produced a block of cells in the G_2M compartment as shown previously[27,28], whereas after VLB, a wide scatter of DNA values with less distinctive peaks in comparison with Doxo were observed. When both drugs were given simultaneously, the effect of Doxo prevailed, whereas in combinations delivered with an interval of 24 or 48 h between the drugs, the pattern of the first drug prevailed (Figure 6). Similarly, cytomorphologic studies showed two distinctive patterns of changes after VLB or Doxo. After Doxo, the tumour cells were enlarged with enlarged both nuclei and nucleoli. Mitoses in the smears were very rare, which means that the signals appearing in the DNA histograms in the G_2M compartment represent nuclei blocked in the G_2 phase of the cell cycle. In contrast, after VLB, cells with irregular mitoses as well as multinucleated cells as a consequence of unaccomplished cell division were observed. In the DNA histograms, scattered, very high DNA values depassing the G_2M peak of the tumour were found, correspondingly. VLB exerts its action at least in part by binding to tubulin and disturbs the function of microtubules, necessary for the formation of mitotic spindle. After discovery of VLB, it was believed that its primary action is depolymerization of microtubules. Only recently it was discovered that, at low doses, VLB stabilizes microtubule dynamics and blocks or slows down the mitosis by acting on microtubule dynamics and not by depolymerization as at high doses.[29,30] The consequence of impaired function of mitotic spindle is the mitotic arrest and inhibition of cell proliferation. However, as antiproliferative activity of VLB does not correlate well with binding to tubulin, there must be other targets for VLB such as RNA, DNA and lipid biosynthesis.[29]

Better results of the combinations of VLB and Doxo applied with the 48-h intervals over the drugs applied simultaneously can only partly be explained by DNA measurements and cell morphology studies. The block in G_2 could prevent cells entering mitosis, which is the part of the cell cycle where VLB can exert its maximal effect. We could speculate that the sequence of Doxo and VLB could therefore be self-limiting. Such an explanation could be valid for the simultaneous application of both drugs or when the time interval was 24 h where the effect was not better than that of Doxo alone. On the other hand, such an assumption does not explain the best results obtained after administering the drugs in 48-h intervals between them irrespective of their sequence. Our DNA measurements in this experiment showed that the block of the cells in G_2 was still present 48 h and even 72 h after the application of Doxo, yet the tumour cell toxicity was enhanced with the combination of Doxo and VLB provided the drugs were delivered with the 48-h interval between the drug administrations. Moreover, not the sequence of drugs, but the time interval between them was crucial for obtaining better effect. According to our results, the explanation could be that the action of either of the drugs, Doxo or VLB, needs 48 h to make the tumour cells either prone to the action of the other drug or to trigger a cell death pathway.

Our second objective was to explore whether pretreatment with VLB will increase the accumulation of Doxo in tumour cells. In our previous preclinical study, we showed that VLB increased cell membrane fluidity.[16] Pretreatment with VLB increased the uptake of cisplatin into the tumour cells, which led to an increased antitumour effectiveness of cisplatin.[14] An increase antitumour effectiveness was also demonstrated for bleomycin applied after VLB.[15] In the present study, the pretreatment with VLB did not result either in an increased accumulation of Doxo or in the increased

antitumour effectiveness compared to the treatment with reverse order of the drugs. There are reports that the effect of Doxo is not dependent solely on entering the cell and binding to DNA, but also on binding to the cell membrane, which is very important for inducing the cell death.[31-33] Therefore, we assumed that the cytotoxic effect of the combination of Doxo and VLB applied with the 48-h interval between the drug administration might be the result of the effects on DNA, cell membrane events and transmembrane signalling.[31-34] Another explanation for better results of the schedule with 48-h interval could be a repopulation of tumour cells which might occur after that interval and the elimination of the repopulated cells by the second drug.

The fact that no drug combination resulted in a prolonged survival of animals (Figure 9) is not surprising. We deliberately used low doses of VLB and Doxo with the aim to demonstrate a possible interaction of the combinations. Even after the best combinations with 48-h interval between the drug injections, there were still some unaffected tumour cells in the specimens. In view of rapid repopulation of tumour cells in this fast growing tumour model, the difference in animal survival could not be expected after only one cycle of chemotherapy.

In conclusion, the combination of VLB and Doxo is schedule dependent. It seems that for the effect of treatment the time interval between the drug administrations, but not the sequence of drugs, is crucial. Pretreatment with VLB does not increase the accumulation of Doxo in EAT tumour cells. When translating the results of preclinical studies to clinical setting, we need to be cautious, since different tumour models used can yield controversial results. Nevertheless, the data from preclinical studies should be taken into consideration when planning combined treatment schedules in clinical situation.

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