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Prophylaxis and Therapy of Mouse Mammary Carcinomas with Doxorubicin and Vincristine Encapsulated in Sterically Stabilised Liposomes

J. Vaage, D. Donovan, T. Loftus, P. Uster and P. Working

This study tested the prophylactic efficacies of doxorubicin hydrochloride and vincristine sulphate, encapsulated in sterically stabilised long circulating liposomes, against the spontaneous development of mammary carcinomas in C3H/He mice. Monthly prophylactic intravenous (i.v.) injections of 6 mg/kg doses of liposome-encapsulated doxorubicin (DOX-SL) or 1 mg/kg doses of liposome-encapsulated vincristine (VIN-SL) were begun when retired breeding mice were 26 weeks old. Mice that developed a mammary carcinoma while on the monthly prophylactic protocols were then given weekly i.v. injections of 6 mg/kg DOX-SL or 1 mg/kg VIN-SL to test the therapeutic efficacies of the drugs, and to determine whether the tumours were susceptible or resistant to therapy. The monthly prophylactic injections reduced the incidence of first mammary carcinomas from 87/88 (99%) in untreated mice to 24/42 (57%) in DOX-SL-treated mice and to 26/32 (81%) in VIN-SL-treated mice. Of the mice that developed a mammary tumour while on the prophylactic protocols, 12 of 30 mice were cured by the weekly therapeutic use of DOX-SL, and the growth of 18 tumours was inhibited. The weekly therapeutic use of VIN-SL cured 3 of 8 mice, and inhibited the growth of five tumours. Weekly DOX-SL therapy cured 7 of 22 previously untreated mice. The mean survival of tumour-bearing mice was extended from 24 days in untreated mice to 87 days in DOX-SL-treated mice, which had not received prophylactic treatment. Metastases were found in 29 of 54 untreated mice, and in 3 of 72 mice treated with DOX-SL and VIN-SL. Toxic side effects were limited to a transient weight loss during the weekly treatments. Drug resistance as a result of treatments was not observed.

Key words: liposomes, prophylaxis, mammary carcinoma, metastasis, therapy
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INTRODUCTION

BECAUSE BREEDING C3H/He female mice have a predictable incidence of mammary carcinoma development [1], these animals can be used as a model to test chemopreventive programmes against mammary cancer. During the first pregnancy, which normally starts at the age of 8 weeks, the 10 mammary glands begin to develop premalignant hyperplastic alveolar nodules (HAN). The HAN persist when the normal mammary parenchyma involutes following lactation, and increase in number with repeated pregnancies and advancing age [2]. The HAN are thought to give rise to most, if not all, mammary carcinomas in C3H/He mice [3], a situation with a possible parallel in human breast cancer aetiology [4]. Most breeding C3H/He mice will develop one or more mammary carcinomas before they are 1 year old [5]. The median age for first tumour development in breeding C3H/He mice is 268 days, but a first tumour has appeared as early as 173 days and as late as 530 days of age [1]. Between the development of the first HAN and the first tumour, there may, therefore, be an intervening premalignant phase of a few weeks or of several months. Genetic nutritional and hormonal factors, and reproductive status are known or strongly indicated aetiological factors in both human and murine mammary carcinoma development [3, 4]. These points of similarity make mice with primary mammary carcinomas one of the most relevant animal models in cancer research.

A number of studies in animal tumour models found that the therapeutic effects of anticancer drugs could be enhanced and prolonged, and toxic side effects reduced, when the drugs were encapsulated in liposomes [6–9]. The increased therapeutic effect was thought to be due to the slow release of drugs from liposomes. However, the effectiveness of drugs in conventional liposomes was limited by their rapid uptake by the cells of the reticulo-endothelial system (RES) [10, 11], reducing the amount of drug that reached the tumour [10]. By inserting lipids conjugated with polyethylene glycol into the lipid bilayers, small liposomes with greater membrane rigidity and a negative net surface charge (sterically stabilised liposomes) were produced [12, 13]. Such liposomes have a reduced uptake by the cells of the RES and, consequently, have a longer circulation time. The pharmacokinetic characteristics of these liposomes enable more of the drug to reach a tumour, improving its systemic therapeutic efficacy [14, 15]. Studies in C3H/He mice have found that the therapeutic efficacies of doxorubicin and vincristine against mammary carcinomas are increased when the drugs are encapsulated in sterically stabilised liposomes [16]. Empty liposomes were found to have no therapeutic effect on mouse mammary tumour growth [17].

The present investigation had two purposes. Firstly, to determine the chemopreventive effects of intravenous (i.v.) injections of liposome-encapsulated doxorubicin (DOX-SL) or liposome-encapsulated vincristine (VIN-SL) at low doses (6 mg/kg and 1 mg/kg, respectively) during the latent phase of mammary carcinoma development. The chemopreventive treatments, at easily tolerated monthly treatment schedules, were begun at an age when HAN would have developed in all of the mice and when the probability of imminent tumour development was high; secondly, to determine whether tumours that developed in mice on the chemopreventive protocols were resistant or susceptible to drug therapy.

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MATERIALS AND METHODS

Liposome components

The liposome components were: cholesterol (Croda, Fullerton, California), hydrogenated soy phosphatidylcholine (HSPC) (Lipoid K.G., Ludwigshafen, Germany), and distearoyl-phosphatidyl-ethanolamine (DSPE) (Genzyme Corp., Cambridge, Massachussetts) conjugated at its amino position with a 1900 molecular weight segment of methoxypolyethylene glycol carbamate of distearoyl phosphatidylethanolamine (MPEG-1900-DSPE) [12]. Proton NMR spectra of MPEG-1900-DSPE showed the appropriate polyoxyethylene band at 3.64 ppm and integration of this signal showed the degree of polymerisation with oxyethylene groups to be $n = 49$.

Test material

DOX-SL™ and VIN-SL™ were provided by Liposome Technology, Inc., Menlo Park, California. Doxorubicin concentration was 2.0 mg/ml, drug encapsulation efficiency was >90%, and the mean particle size was 96 nm. Vincristine concentration was 1.0 mg/ml, drug encapsulation efficiency was >95%, and the mean particle size was 90 nm. Non-liposomal doxorubicin hydrochloride (Adriamycin RDF™,2.0 mg/ml) was from Farmitalia Carlo Erba, Milan, Italy. Non-liposomal vincristine sulphate (Oncovin™; 1.0 mg/ml) was from Eli Lilly, Indianapolis, Indiana. The final ratios of drug to total lipid were 1 to 8 (DOX-SL), and 1 to 17 (VIN-SL). Control mice received saline.

Mice

The animals used were pedigree breeding mice of the C3H/He strain, raised and maintained in a filtered-air environment. All had been bred from 8 weeks of age and had weaned their third litters by age 22–24 weeks.

Statistical analysis

The mice were randomly assigned to prophylaxis and to control groups when they reached 26 weeks of age. The mice that developed a tumour were than randomly assigned to treatment and control groups. The incidence and the size of tumours in treated mice and in control mice were recorded weekly. Tumour volumes were calculated by the formula $0.4(ab^2)$ where "a" is the larger and "b" the smaller diameter.

Differences in mean tumour volume, mean survival and mean weight, were evaluated by the Student's t-test. Differences in the incidence of primary tumours and differences in the incidence of metastases between groups of mice were evaluated by the χ^2 test. Differences were considered significant when the P value of comparison was less than 0.05 ($\chi^2 > 3.8$, and $t > 1.7$). A mouse was considered cured if its tumour remained unpalpable for at least 6 weeks.

Treatment schedules

All injections were given via the tail vein in volumes ranging from 0.06 to 0.11 ml as determined by the animal's weight, which was recorded weekly. Prophylactic injections of 6 mg/kg DOX-SL or 1 mg/kg VIN-SL were given on a monthly schedule starting when the mice were 26 weeks old. These dose levels had been found in earlier studies to be therapeutically effective and well tolerated [16, 17]. The injections were repeated every 28th day until a tumour developed, or until the mice on the DOX-SL

protocol were from 455 to 703 (mean 581 ± 22) days old, and the mice on the VIN-SL protocol were from 553 to 735 (mean 645 ± 30) days old, at which ages the treatments were halted and the mice were observed for tumour development until advanced age with failing health necessitated euthanasia.

Of the DOX-SL-treated mice that did develop a tumour, 12 were given 5–13 weekly therapeutic injections of 6 mg/kg DOX-SL, 8 mice were given 4–10 weekly therapeutic injections of 1 mg/kg VIN-SL, and 4 mice were not treated. Of the VIN-SL-treated mice that did develop a tumour, 18 were given 5–14 weekly injections of 6 mg/kg DOX-SL, and 8 mice were not treated. Twenty-two retired breeding mice that had not received prophylactic injections were given 5–17 weekly therapeutic injections of 6 mg/kg DOX-SL from the time they developed a mammary tumour. The number of therapeutic tail vein injections that could be given was limited by the development of epidermal necrosis, a consequence of drug extravasation at the sites of i.v. injections. Following the therapeutic injections, the mice were then observed for 1–20 more weeks. Mice were selected for euthanasia by CO_2 asphyxiation according to two criteria: (1) when its tumour had reached a size of 2 cm^3; or (2) if it appeared to be in poor health. All treated mice were necropsied, and the organs examined for metastases.

Tumour implantation

Tumour tissue was removed from one of the DOX-SL treated mice at the time of euthanasia and necropsy. Two 1-mm^3 pieces of tumour were implanted by sterile procedures into the right and left fourth mammary glands with an 18 gauge biopsy trocar through an incision in the skin of anaesthetised (Penthrane, Abbot, N. Chicago, Illinois) mice. The incisions were closed with wound clips.

Histology

The liver, the kidneys, the ovaries, the small and large intestine, the spleen, the heart, the lungs, and the brain, were removed at necropsy from all mice and examined for metastases and for evidence of toxic effects. The tissue specimens were formalin-fixed and paraffin-embedded. Four stepwise, 3 μm serial sections were taken of each of the specimens. The tissue sections were stained with haematoxylin–eosin.

RESULTS

Chemoprevention

Figure 1 shows the effects of monthly injections of 6 mg/kg DOX-SL or 1 mg/kg VIN-SL on the incidence of spontaneous tumours, compared with the tumour incidence in control mice that received no treatment. The results are summarised in Table 1. Of the 42 mice given monthly injections of DOX-SL, 24 (57%) developed a mammary carcinoma at a mean age of 416 ± 18 days (range = 318–679 days). Of the 32 mice given monthly injections of VIN-SL, 26 (81%) developed a mammary carcinoma at a mean age of 461 ± 30 days (range = 301–791 days). Of 88 untreated mice, 87 (99%) developed a mammary carcinoma at a mean age of 322 ± 10 days, range 182 to 528 days. One untreated mouse died tumour-free of intestinal obstruction at 413 days old. The differences in tumour incidence and mean age at first tumour between treated and untreated mice were highly significant.

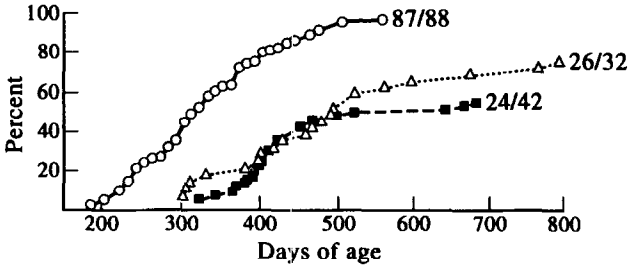

Figure 1. Effects of DOX-SL and VIN-SL chemoprevention. Cumulative curves for mammary tumour development in untreated mice (○), in mice treated with DOX-SL (■), and in mice treated with VIN-SL (△). The figures at the end of each curve are the number of mice that developed tumours out of the total number.

Chemotherapy

Figure 2 shows the effects of weekly treatments with DOX-SL or VIN-SL on the progressive growth of tumours that developed in normal mice and in mice on chemopreventive protocols. The results are summarised in Table 2. Of the 24 mice that developed a tumour while on prophylactic DOX-SL, 12 were given 5–13 (mean 8.0 ± 0.7) weekly injections of 6 mg/kg DOX-SL, 8 were given 4–10 (mean 7.0 ± 0.8) weekly injections of VIN-SL, and 4 mice were not treated. The latter grew to a size necessitating euthanasia in an average of 67 ± 9 days. The DOX-SL therapy cured 3 of 12 mice and prolonged survival to a mean of 76 ± 5 days. The VIN-SL therapy cured 3 of 8 mice and prolonged survival to a mean of 89 ± 10 days. The tumours in the cured mice remained unpalpable and were not found at necropsy, or following histological examination of the tumour sites.

Of the 26 mice that developed a tumour while on prophylactic VIN-SL protocol, 18 were given 5–14 (mean 9.0 ± 0.5) weekly injections of 6 mg/kg DOX-SL, and 8 mice were not treated. The latter grew to a size necessitating euthanasia in an average 47 ± 6 days. The DOX-SL therapy cured 9 of 18 mice, and prolonged survival to 94 ± 9 days. The tumours in the cured mice remained unpalpable until euthanasia. Areas of viable tumour cell were found in only one tumour at necropsy.

Of the 87 mice not on a prophylactic protocol that developed a tumour, 54 were not treated and 22 mice were given 5–17 (mean 10.0 ± 0.6) weekly injections of 6 mg/kg DOX-SL. The untreated tumours grew to a size necessitating euthanasia in an average 24 ± 2 days. The DOX-SL therapy cured 7 of 22 mice, and prolonged survival to a mean of 87 ± 5 days. The tumours in the cured mice remained unpalpable and were not found at necropsy.

Metastases were found in 29 of 54 (54%) untreated mice, and in 3 of 72 (4%) mice on therapeutic protocols. Metastases were only found in the lungs.

Toxicity

The mice showed no notable toxic effects nor weight loss during the monthly prophylactic treatments. During the weekly treatments with VIN-SL, an average weight loss of 7% was recorded. The body weight was recovered after 3–6 injections. The weekly treatment with DOX-SL resulted in an average weight loss of 5%. The weight was recovered after 3–5 injections. None of the treated mice showed symptoms of systemic toxicity. Histological examinations revealed no pathological changes in internal organs of any of the mice examined. All the mice eventually developed some degree of dermal fibrosis and epidermal necrosis from drug extravasation at the sites of i.v. injections.

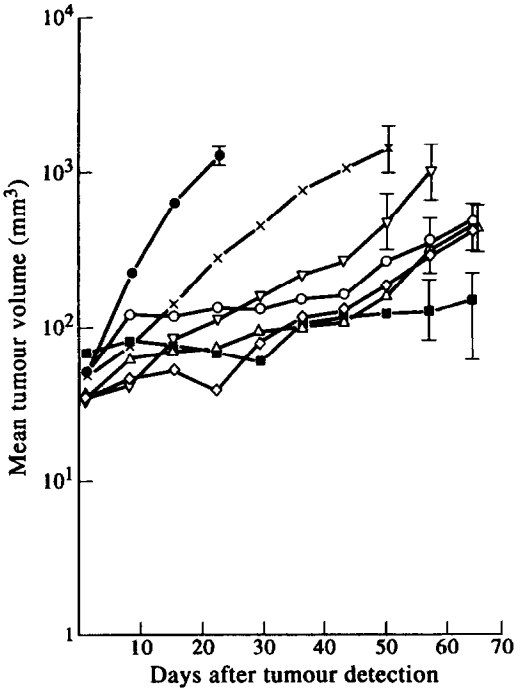

Figure 2. Effect of therapy on the growth of primary mammary tumours. The mice received weekly i.v. injections from the day after their tumour was detected. The mice were killed and necropsied when a tumour reached 1.0–1.4 cm³, or when a mouse seemed in poor health. The symbols for prophylaxis/therapy are the following: VIN/SL/none (X), DOX-SL/none (▽), none/DOX-SL (○), DOX-SL/DOX-SL (△), DOX-SL/VIN-SL (◇), VIN-SL/DOX-SL (■), untreated mice (●). Bars represent S.D.

Drug resistance

The 24 mice on DOX-SL prophylaxis (Table 1) that developed a primary tumour received 3–11 monthly prophylactic injections (median = 6) with 12 mice receiving less than the median, with a mean of 4.1 ± 0.3 injections, and 12 receiving more than the median with a mean of 7.6 ± 0.4 injections. A comparison of the susceptibility to DOX-SL therapy of the tumours between these two subsets found that the tumour volumes from day 1 to day 64 were not significantly different ($P = 0.1$, day 1; and $P = 0.3$, day 64). The mean number of weekly therapeutic injections given to the mice above and below the median number of prophylactic injections were almost indentical (9.0 ± 0.8 versus 9.0 ± 1.3, $P = 1.0$), and the survival times were not significantly different (83 ± 9 days versus 69 ± 5 days, $P = 0.2$).

When one of the tumours began to grow rapidly, 63 days after its appearance, resulting in euthanasia, the question was asked whether this tumour, which developed after 4 monthly DOX-SL injections, and had then been treated weekly with DOX-SL for 9 weeks, had become drug resistant. To study this, tumour tissue removed at the time of euthanasia was transplanted into the right and left number 4 mammary glands of two groups of 5 mice each. One group of mice received 4 i.v. injections of 6 mg/kg DOX-SL on days 1, 8, 15, and 22 after the implantation. The second group received four injections of saline. The mean tumour volumes are presented in Figure 3. The final incidence of tumour growth in the DOX-SL and saline-treated mice was 5/10 and 10/10 ($P < 0.05$), 50 days after implantation. By this time, the tumours had either regressed or had grown to a size beyond possible regression, necessitating euthanasia. These results show that the treatments with DOX-SL had not resulted in the emergence of a drug-resistant population of cancer cells.

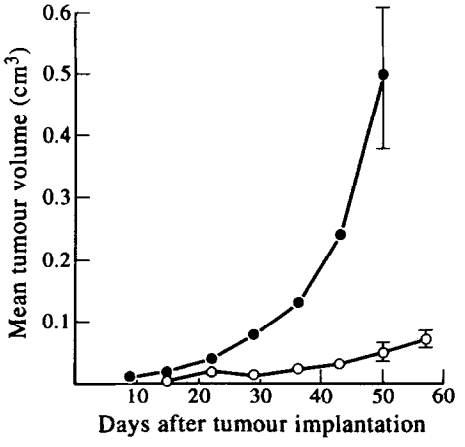

Figure 3. Drug sensitivity test of transplanted tissue from a tumour that grew rapidly in a mouse that had received 4 monthly and then 9 weekly DOX-SL injections. Each of 5 mice per group had two tumour pieces implanted into the right and left number 4 mammary glands on day 0. The mice were treated on days 1, 8, 15, and 22 after tumour implantation. DOX-SL (○), liposomal doxorubicin hydrochloride. Placebo (●), saline. Bars represent S.D.

Table 1. *Tumour incidence with monthly chemoprevention*

Treatment*	Tumour incidence	Mean no. of injections† (range)	Mean no. of injections‡ (range)	Mean age at first tumour (range)	Tumour-free survival (range)
DOX-SL	24/42§¶	5.9 ± 0.4 (3–11)	11 ± 0.6¶ (8–15)	416 ± 18 days§ (318–679)	581 ± 22 days¶ (455–708)
VIN-SL	26/32‖¶	6.2 ± 0.8 (2–12)	8 ± 1.7¶ (3–12)	461 ± 30 days§ (301–791)	645 ± 30 days§ (553–735)
None	87/88			322 ± 10 days (182–528)	413 days

*DOX-SL, liposomal doxorubicin hydrochloride, 6 mg/kg/month from 26 weeks of age; VIN-SL, liposomal vincristine sulphate, 1 mg/kg/month from 26 weeks of age. †Injections before the first tumour. ‡Total for tumour-free mice. Treated versus untreated: §$P < 0.0001$; ‖$P < 0.001$. DOX-SL versus VIN-SL: ¶$P = 0.1$.

DISCUSSION

Because of the predictable incidence of mammary carcinomas in C3H/He mice, these animals were used as a model to test chemopreventive programmes against latent cancers. The median age at first tumour is 38 weeks, but a tumour may develop as early as 26 weeks [1]. Accordingly, prophylactic treatments were started when the mice were 26 weeks old, when premalignant HAN were present and when the probability of occult malignant development was high. Preliminary studies have found that VIN-SL is well tolerated, and although less effective than DOX-SL when used as single agent therapy, VIN-SL is very effective when used with DOX-SL on an alternating schedule [16]. Therefore, VIN-SL prophylaxis followed by DOX-SL therapy, rather than followed by VIN-SL therapy, was tested as the more promising protocol. Because of the high mortality resulting from repeated i.v. injections of doxorubicin and vincristine in the free forms (data not shown), comparisons of the effects of DOX-SL and VIN-SL with the effects of the free drugs were not possible.

The results presented in Figure 1 and Table 1 show that the preventive treatments with DOX-SL or VIN-SL resulted in a significant reduction in the development of primary mammary carcinomas. The complete chemoprevention of mammary tumours in 24 of 74 treated mice is remarkable in view of the high probability of multiple tumour development in this strain.

In an earlier investigation of the influence of tumour size and frequency on the spontaneous development of metastases in C3H primary tumour hosts, the 36 mice in one group were surgically cured as soon as a tumour appeared, at a mean size of 5.0 ± 0.1 mm. The mean number of tumours removed was 17.3 ± 2.5 (range 8–37), during a mean life span of 622 ± 23 days (range 378–892) [18].

The results presented in Figure 2 and Table 2 show that the tumours that developed during the prophylactic monthly treatments were susceptible to therapeutic protocols of 6 mg/kg/week of DOX-SL or 1 mg/kg/week of VIN-SL. Of the 52 tumour-carrying mice treated with weekly injections of DOX-SL, 19 mice were cured after 5–17 injections. Of the 8 tumour-carrying mice treated with VIN-SL, 3 were cured after 4–10 injections. The development of metastases was inhibited by the weekly chemotherapy even more effectively than was the growth of primary tumours. Metastases were found in only 3 of 72 treated mice compared with 29 of 54 untreated mice.

It is indicated by the results in Figure 1 that there must have been differences in drug sensitivity among individual HAN and/or mammary carcinomas because tumours continued to appear, although in reduced numbers, during the preventive monthly treatments. The montly preventive treatments during tumour latency did not affect the sensitivities of emerging tumours to weekly DOX-SL or VIN-SL therapies. When the mice that did develop a tumour were treated with weekly injections, a difference in the degree of drug sensitivity among individual tumours was observed. Of 60 tumours, 22 were destroyed and 38 remained stable or grew slowly during the period of weekly therapy. One tumour that started to grow rapidly after 9 weeks of DOX-SL therapy was found to be highly sensitive to DOX-SL therapy in syngeneic mice with intra-mammary implants of the tumour (Figure 3), demonstrating that this tumour had not developed drug resistance.

These studies focused on chemoprevention of mammary cancer in a model system where such cancers were expected to develop with high frequency. Prevention was attempted at a time when malignant cells were likely to be present, and premalignant cells were certainly present. The treatments could have exerted their antineoplastic effect either on the premalignant cells of HAN, or on cells already transformed, or both. A difference in the tumour incidences in the control group and in the treatment groups became apparent soon after the treatments were started (Figure 1). This suggests that occult carcinomas were already formed, and were affected by the preventive treatments. The tumours that emerged during chemoprevention were, nonetheless, susceptible to intensified therapy, and the development of metastases was significantly reduced in a comparison with untreated mice. Because monthly and weekly

Table 2. Tumour progression with weekly chemotherapy

Treatment* (prophylaxis/therapy)	No. of cures	Mean no. of injections (range)	Mean survival† (range)	Metastases found‡
DOX-SL/DOX-SL	3/12§	8 ± 0.7 (5–13)	76 ± 5 days (57 ± 113)	1/12
DOX-SL/VIN-SL	3/8§	7 ± 0.8 (4–10)	89 ± 10 days (50–169)	0/8
DOX-SL/None	0/4		67 ± 9 days (49–85)	0/4
VIN-SL/DOX-SL	9/18§‖	9 ± 0.5 (5–14)	94 ± 9 days (57–197)	0/18
VIN-SL/None	0/8‖		47 ± 6 days¶ (26–65)	1/8
None/DOX-SL	7/22§	10 ± 0.6 (5–17)	87 ± 5 days (56–157)	1/22
None/None	0/54		24 ± 2 days** (9–55)	29/54††

*See Table 1 for definitions. †From time of tumour detection. ‡By gross and histological examinations at necropsy. §Significantly greater than 0/54, $P < 0.001$. ‖9/18 versus 0/8, $P < 0.05$. ¶Significantly less than all therapy groups, $P < 0.01$. **Significantly less than all other groups, $P < 0.001$. ††3/72 treated versus 29/54 untreated, $P < 0.0001$.

injections of 6 mg/kg DOX-SL or 1 mg/kg VIN-SL are moderate protocols with only mild systemic toxic side-effects (temporary weight loss during weekly injections), the significant prophylactic and therapeutic benefits observed are encouraging.

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Opposite Effect of Miltefosine on the Antineoplastic Activity and Haematological Toxicity of Cyclophosphamide

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The effect of pretreatment with miltefosine (MIL) on the antineoplastic activity of cyclophosphamide (CPA) was evaluated in subcutaneous benzo(a)pyrene-induced sarcomas (BPS) of the rat. MIL alone had no antineoplastic effect on this autochthonous tumour, but enhanced the chemotherapeutic effect of CPA. Conversely, MIL counteracted the myelotoxicity of CPA in normal adult rats. Although the nadir of the leucocyte count remained unchanged, the recovery phase was considerably shortened, an effect which resembled the pharmacological action of GM-CSF.

Key words: miltefosine, cyclophosphamide, interactions

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