Urol Res (1996) 24:93–99

ORIGINAL PAPER

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In vivo, synergestic inhibition of MAT-LyLu rat prostatic adenocarcinoma growth by polyamine deprivation and low-dose cyclophosphamide

Received: 23 May 1995 / Accepted: 8 September 1995

Abstract Polyamine deprivation in vivo produces significant tumor growth inhibition of the hormone-resistant, metastatic Dunning Mat-LyLu murine prostatic carcinoma. In order to produce a cytotoxic effect in addition to the cytostatic effect of polyamine deprivation, various chemotherapy regimens, combined with drug-containing polyamine-deficient chow (DC-PDC), were assessed. Triple chemotherapy combining methotrexate, cyclophosphamide and vindesine; and monochemotherapy with high-dose cyclophosphamide (90 mg·kg^{-1}) and low-dose cyclophosphamide (20 mg·kg^{-1}) were studied alone and in combination with DC-PDC. A variant of DC-PDC excluding the polyamine oxidase inhibitor MDL 72527 was also studied in combination with low-dose cyclophosphamide. The triple-chemotherapy regimen alone or in combination with polyamine deprivation was effective on tumor growth inhibition but was also toxic. High-dose cyclophosphamide alone produced significant tumor growth inhibition and an increase in life span. High-dose cyclophosphamide in combination with DC-PDC was also effective on tumor growth but was also toxic. Low-dose cyclophosphamide alone was moderately effective on tumor growth inhibition with a marginal increase in life span. When combined with polyamine deprivation, results with low-dose cyclophosphamide compared favourably with those of high-dose cyclophosphamide alone and prevented the formation of lung metastases. The polyamine oxidase inhibitor does not appear to be mandatory to achieve this effect if DC-PDC is combined with low-dose cyclophosphamide. Polyamine deprivation appears to be an important tool in anticancer therapy, allowing the use of reduced doses of cytotoxic agents with the same antitumoral efficacy.

Key words Polyamines · Prostate cancer · Chemotherapy

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The naturally occurring polyamines putrescine, spermidine and spermine are involved in cellular proliferation and differentiation processes in most living organisms [16]. Inhibition of polyamine biosynthesis is currently considered a potential goal in cancer chemotherapy [16, 17]. One of the major reasons for the modest clinical success of this therapeutic approach up to now [23] is the fact that the mammalian organism has access to polyamines from several sources. Rapidly growing tissues are able to use circulating polyamines for growth. These may be released physiologically into the blood from normal cells, pathophysiologically from dying cells, or polyamines may be taken up from the gastrointestinal tract. Gastrointestinal tract polyamines originate from bacterial synthesis and dietary intake [1, 2].

Prevention of polyamine interconversion [24] by inhibition of polyamine oxidase (PAO) in addition to the inhibition of the intracellular de novo putrescine synthesis somewhat improved the antitumoral effect of 2-(difluoromethyl)ornithine (DFMO, Eflornithine) [9]. DFMO is a selective inhibitor of ornithine decarboxylase (ODC) [2]. An almost complete prevention of Lewis lung carcinoma (3LL) (grafted in mice) [25] and of human U-251 glioblastoma (xenografted in nude mice) growth [14] was achieved by (partial) decontamination of the gastrointestinal tract, using neomycin and

metronidazole, and feeding a polyamine-free diet, in addition to inhibition of ODC and PAO.

The activities of polyamine-synthesizing enzymes are considerably higher in the prostate than in other tissues of the male vertebrate organism [10, 22, 27]. An increased urinary excretion of spermidine in 70% of patients with prostatic adenocarcinoma has been shown [10–22]. Moreover, we found recently that red blood cell (RBC) polyamines (which correspond to more than 95% of the circulating spermidine and spermine) [12, 13, 20] are an index of malignant cell proliferation in patients suffering from prostatic cancer [6, 7]. Furthermore, patients with metastatic disease and high spermine erythrocyte levels at diagnosis are poor responders to androgen ablation and present significantly shorter progression-free and specific survivals than metastatic patients with normal spermine erythrocyte levels [8].

In order to study the influence of polyamine deprivation on the growth of prostatic adenocarcinoma, the MAT-LyLu subline of the Dunning adenocarcinoma was chosen as model [26]. This tumor is androgen insensitive and possesses one of the shortest doubling times among the Dunning sublines [26]. Copenhagen rats bearing this murine adenocarcinoma were treated, once the tumor was palpable, with neomycin, metronidazole, DFMO and the PAO inhibitor N^1,N^4-bis-(2,3-butadienyl)-1,4-butanediamine (MDL 72527), in combination with a polyamine-free diet. Almost complete inhibition of tumor growth was observed during the treatment, but tumor growth was reversible after stopping the treatment [15]. In an attempt to amplify the antitumor effect, we assessed different chemotherapy modalities combined with polyamine deprivation. Because of encouraging results obtained with 3LL Lewis lung carcinoma tumor-bearing mice [18], we started our study testing a triple-chemotherapy combination with polyamine deprivation. We gradually decreased concentrations of the dose of the cytotoxic agents and finally used low-dose cyclophosphamide. As the PAO inhibitor MDL 72527 is not available for human trials, we assessed the efficacy of polyamine deprivation without the PAO inhibitor and in combination with low-dose cyclophosphamide.

Materials and methods

Chemicals and drugs

Reagents were from Sigma, St. Louis, (Mo., USA). N^1,N^4-bis-(2,3-Butadienyl)-1,4-butanediamine (MDL 72527) [21] and 2-(difluoromethyl)ornithine (MDL 71782; DFMO; Eflornithine) [22] were a gift from Marion Merrell Dow (Strasbourg, France). Metronidazole was from Rhône-Poulenc (Paris, France), neomycin from Usiphar (Compiègne, France), cyclophosphamide from Asta France (Mérignac, France), methotrexate from Roger Bellon (Neuilly sur Seine, France) and vindesine from Lilly France (Saint Cloud, France).

Tumor graft

Animals used in this study were Fisher-Copenhagen F1 male rats locally obtained, weighing 180–200 g. Cells of P83F1/PV2 MAT-LyLu, kindly provided by J. A. Schalken and F. M. J. Debruyne, St. Radboud University Hospital, Nijmegen, The Netherlands, were maintained in vivo in male Copenhagen rats. When the tumor volume was 3–4 cm^3, malignant tissue was surgically removed from the flank of the rat bearing the P83F1/PV2 MAT-LyLu rat adenocarcinoma. The tumor was mechanically dissociated in RPMI 1640. After counting the viable cells by trypan blue exclusion, $2 \cdot 10^6$ tumor cells were injected subcutaneously in the right flank of the animals. Tumor volume and body weight were determined 3 times/week until death. Tumor volume was measured as follows:

$$V\,(cm^3) = 4/3\pi p \cdot (d/2)^2 \cdot (D/2)\ (d = \text{small diameter},\ D = \text{large diameter}).$$

Drug regimen

Drug-containing polyamine-deficient chow. Polyamine-deficient chow (PDC) was prepared as previously described [21]. Drugs [DFMO (3%), MDL 72527 (0.05%), neomycin (0.2%) and metronidazole (0.0034%)] were included in the PDC. This drug-containing polyamine-deficient chow was designated as "DC-PDC + ". Polyamine-deficient chow in which the PAO inhibitor was not added was designated as "DC-PDC – ". To obtain clinical conditions, all animals received a synthetic rodent chow (SRC) until the tumor had a volume of 0.5 cm^3, treatment being started afterwards (approximately 8 days after the tumor graft). The SRC corresponded to the drug-free PDC, to which was added putrescine · 2HCl (21.0 $mg \cdot kg^{-1}$), spermidine · 3HCl (153.0 $mg \cdot kg^{-1}$) and spermine 4HCl (48.7 $mg \cdot kg^{-1}$). The quantity of the polyamines in SRC corresponded to the concentration of putrescine, spermidine and spermine found in standard commercial rodent chow (Charles River, Villemoisson-sur-Orge, France). DC-PDC+ and DC-PDC– were given 5 days/week (from Monday to Friday). During the remaining days (Saturday and Sunday) all animals received SRC. In all experiments the mean survival time was determined.

DC-PDC+ and polychemotherapy. Twenty MAT-LyLu-bearing Copenhagen rats were randomized into four groups of five animals. The first group received SRC (controls); the second group received DC-PDC+ 5 days/week; the third group was given SRC and rats were injected i.p. once weekly with methotrexate (1.4 $mg \cdot kg^{-1}$) on Mondays, cyclophosphamide (75 $mg \cdot kg^{-1}$) on Tuesdays and vindesine (0.2 $mg \cdot kg^{-1}$) on Wednesdays; the fourth group received DC-PDC+ 5 days/week and the triple chemotherapy as described above. The triple chemotherapy was designated as M-C-V.

DC-PDC+ and cyclophosphamide 90 $mg \cdot kg^{-1}$. Twenty-four MAT-LyLu-bearing Copenhagen rats were randomized into four groups of six animals. The first group received SRC (controls); the second group received DC-PDC+ 5 days/week; the third group was given SRC and rats were injected with 90 $mg \cdot kg^{-1}$ (i.p.) cyclophosphamide weekly; the fourth group received DC-PDC+ 5 days/week and cyclophosphamide weekly with SRC as described above.

DC-PDC+ and cyclophosphamide 20 $mg \cdot kg^{-1}$. Twenty-four MAT-LyLu-bearing Copenhagen rats were randomized into four groups of six animals. The first group received SRC (controls); the second group received DC-PDC+ 5 days/week; the third group was given SRC and rats were injected with 20 $mg \cdot kg^{-1}$ (i.p.) cyclophosphamide weekly; the fourth group received DC-PDC+ 5 days/week and cyclophosphamide weekly with SRC as described above.

DC-PDC− and cyclophosphamide 20 mg·kg^{-1}. Twenty-four MAT-LyLu-bearing Copenhagen rats were randomized into four groups of six animals. The first group received SRC (controls); the second group received DC-PDC − 5 days/week; the third group was given SRC and rats were injected with 20 mg·kg^{-1} (i.p.) cyclophosphamide weekly; the fourth group received DC-PDC − 5 days/week and cyclophosphamide weekly with SRC as described above.

DC-PDC − and cyclophosphamide 20 mg·kg^{-1}, effect on lung metastasis. In order to evaluate the effect of this combination regimen on the development of lung metastases, a fifth experiment was performed under the same conditions with only three animals in each group, as described above, but the animals were killed 14 days after grafting because mortality occurs in the control groups after 15 days. Lung metastases were recorded as the percentage of the pulmonary surface invaded by metastases, which is the best way of accounting for number and volume of metastasis.

Statistical analysis

Results of tumor growth are expressed as mean ± SD. For all calculations non-parametric tests were used (Mann and Whitney U-test for unpaired values). Kaplan-Meier survival curves were calculated for each experiment; differences were assessed using the Mantel Cox test.

Results

DC-PDC+ and polychemotherapy M-C-V

Tumor growth. As shown in Fig. 1, 25 days after tumor graft, mean tumor volumes (± SD) were 121 ± 9, 45 ± 8, 3.8 ± 1.1 and 2.6 ± 1.9 cm^3 in control animals and animals treated by DC-PDC +, M-C-V, and DC-PDC+ with M-C-V respectively. Tumor growth inhibition was thus 63% ($P < 0.02$), 97% ($P < 0.014$) and 99% ($P < 0.02$), respectively, compared to controls.

Influence of treatment on life span. M-C-V and DC-PDC+ with M-C-V treated animals had shorter life spans [mean survival time (MST) = 22 and 32 days, respectively] than controls and DC-PDC+ treated animals (MST = 35 and 36 days, respectively).

DC-PDC+ and cyclophosphamide 90 mg·kg^{-1}

Tumor growth. As shown in Fig. 2A, 25 days after tumor graft, mean tumor volumes (±SD) were 79 ± 34, 66 ± 29, 2 ± 1.5 and 0.8 ± 0.6 cm^3 in control animals and animals treated by DC-PDC +, 90 mg·kg^{-1} cyclophosphamide and DC-PDC+ with 90 mg·kg^{-1} cyclophosphamide, respectively. Tumor growth inhibition thus obtained by cyclophosphamide and DC-PDC+ with 90 mg·kg^{-1} cyclophosphamide was 97% and 99%, respectively ($P < 0.006$) compared to controls.

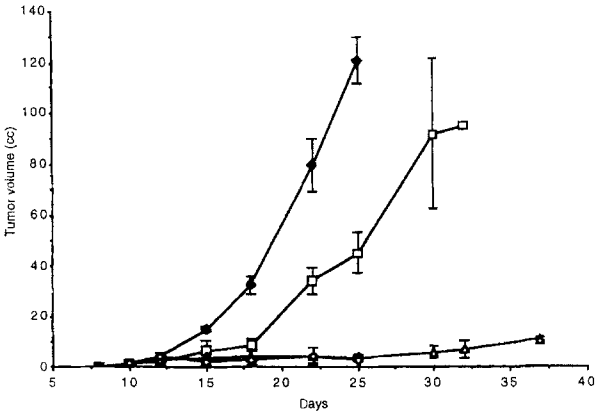

Fig. 1 Tumor volume measured in control animals and animals treated with DC-PDC+, the triple-chemotherapy combination methotrexate (1.4 mg·kg^{-1}), cyclophosphamide (75 mg·kg^{-1}) and vindesine (0.2 mg·kg^{-1}) i.p. weekly designated M-C-V, DC-PDC+ combined with M-C-V (mean values ± SD). Each treatment group constituted five animals, with treatment starting 8 days after tumor graft. ◆ Controls; □ DC-PDC+; △ M-C-V; ◇ DC-PDC+ and M-C-V

Influence of treatment on life span. As shown in Fig. 2B, animals treated by 90 mg·kg^{-1} cyclophosphamide alone had an increased survival (MST = 48 days) compared to controls (MST = 24 days) ($P < 0.0001$), whereas the combination regimen was more toxic than cyclophosphamide alone, as evidenced by the reduced survival time (MST = 27 days).

DC-PDC+ or DC-PDC− with cyclophosphamide 20 mg·kg^{-1}

Tumor growth. The evolution of individual tumor volumes of the four groups is reported in Fig. 3A for animals fed with DC-PDC+ and Fig. 4A for animals fed with DC-PDC−. Control animals in each experiment, 25 days after tumor graft, had a mean tumor volume (±SD) of 43 ± 20 and 75 ± 12 cm^3, respectively. Maximum inhibition obtained by low-dose cyclophosphamide alone was <54%, and mean tumor volumes (± SD) were 20 ± 15 and 59 ± 13 cm^3, respectively. Rats treated with DC-PDC+ and DC-PDC− showed 79% (9 ± 3 cm^3) ($P < 0.01$) and 67% (25 ± 3 cm^3) ($P < 0.01$) tumor growth inhibition, respectively, whereas rats treated with the combination treatment showed 95% (1.4 ± 1 cm^3) ($P < 0.002$ with DC-PDC +) and 81% (14 ± 3 cm^3) ($P < 0.006$ with DC-PDC−) tumor growth inhibition. Although less important, tumor gowth inhibition was maintained until death of the animals.

Influence of treatments on life span. Survival curves are shown in Figs. 3B and 4B. The combination-treated animals had longer survivals than controls or monotherapy-treated animals ($P < 0.0003$ and $P < 0.001$).

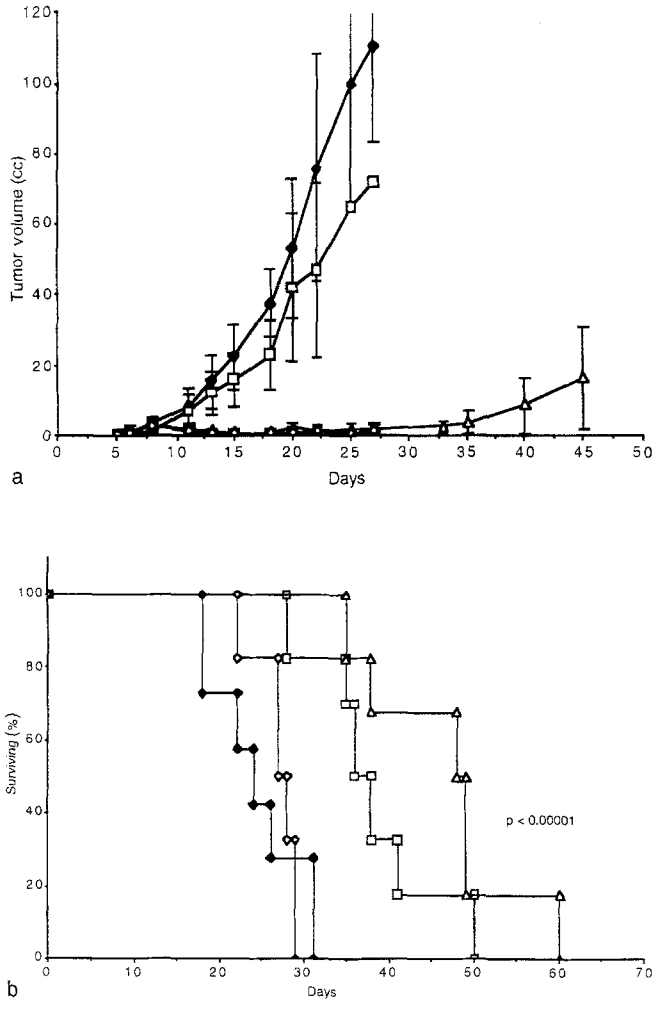

Fig. 2 **a** Tumor volume and **b** survival curves in control animals and animals treated with DC-PDC+, cyclophosphamide (90 mg·kg^{-1}) i.p. weekly and DC-PDC+ combined with cyclophosphamide (90 mg·kg^{-1}) i.p. weekly (mean values ± SD). Each treatment group constituted six animals, with treatment starting 8 days after tumor graft. ◆ Controls; □ DC-PDC+; △ cyclophosphamide 90 mg; ◇ DC-PDC + and cyclo, 90 mg. **b** ◆ Controls; □ DC-PDC; △ cyclophosphamide 90 mg; ◇ DC-PDC+ cycloph. 90 mg

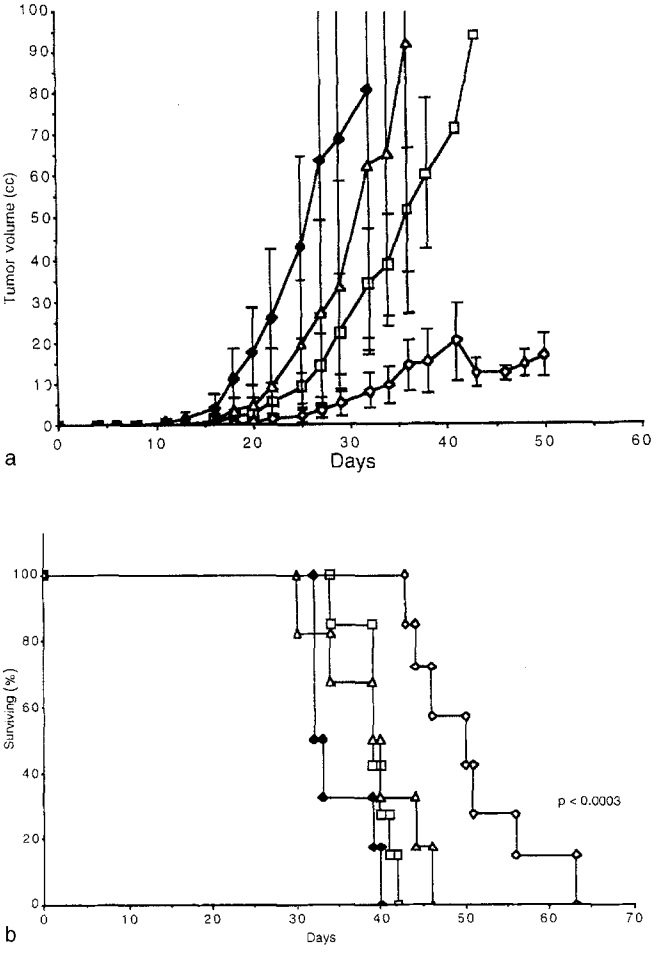

Fig. 3 **a** Tumor volume and **b** survival curves in control animals and animals treated with DC-PDC+, cyclophosphamide (20 mg·kg^{-1}) i.p. weekly and DC-PDC+ combined with cyclophosphamide (20 mg·kg^{-1}) i.p. weekly (mean values ± SD). Each treatment group constituted six animals, with treatment starting 8 days after tumor graft. **a** ◆ Controls; □ DC-PDC+; △ cyclophosphamide 20 mg; ◇ DC-PDC + and cyclo. 20 mg. **b** ◆ Controls; □ DC-PDC; △ cyclophosphamide 20 mg; ◇ DC-PDC + cyclo. 20 mg

The MST of animals treated by DC-PDC+ or DC-PDC− with cyclophosphamide 20 mg·kg^{-1} was 50 or 43 days compared to an MST of 32 or 28 days in control animals, respectively.

Influence of DC-PDC− and cyclophosphamide 20 mg·kg^{-1} on lung metastasis

As shown in Table 1, polyamine deprivation alone and cyclophosphamide 20 mg·kg^{-1} alone decreased the number of lung metatastasis, whereas no lung metastasis occurred, 14 days after tumor graft, after administration of the combination regimen.

Discussion

Owing to inhibition of de novo putrescine formation from ornithine, decontamination of the gastrointestinal tract (of which the bacteria are an important source of polyamines) and restriction of alimentary polyamines, DC-PDC is known to decrease tumor putrescine and spermidine concentrations whereas spermine concentrations are less affected [25]. The fact that a very significant inhibition of the growth of Lewis lung carcinoma [25], human glioblastoma xenografted in nude mice [14], but also of the MAT-LyLu adenocarcinoma grafted in Copenhagen rats [15] is possible by this treatment indicates that systematic polyamine deprivation is presumably a general approach to reduce tumor growth rate.

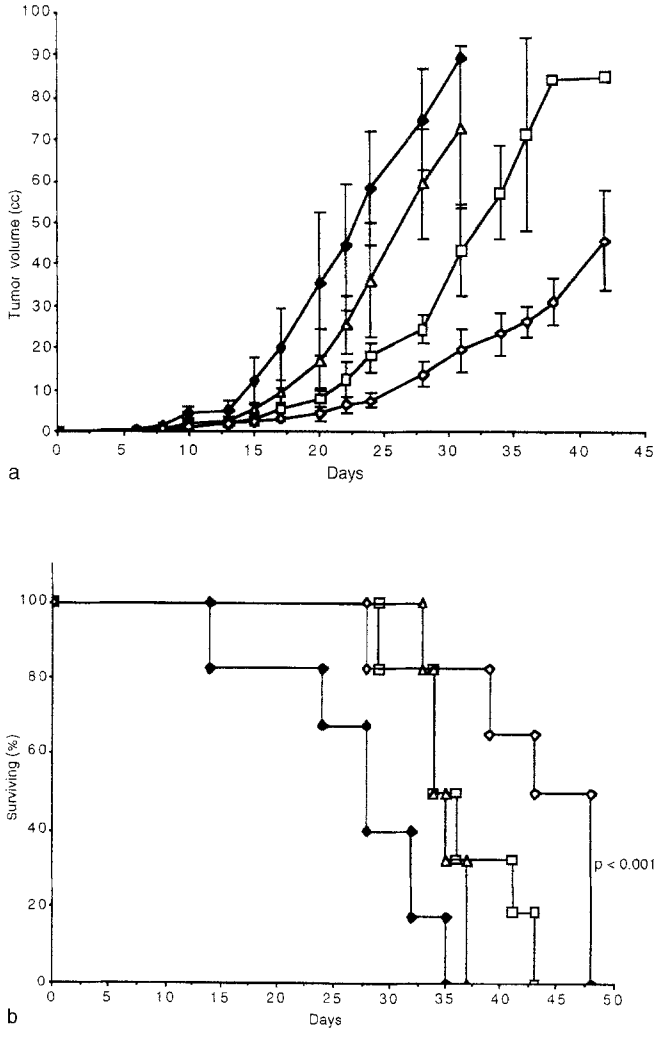

Fig. 4 **a** Tumor volume and **b** survival curves in control animals and animals treated with DC-PDC−, cyclophosphamide (20 mg·kg^{-1}) i.p. weekly and DC-PDC− combined with cyclophosphamide (20 mg·kg^{-1}) i.p. weekly (mean values ± SD). Each treatment group constituted six animals with treatment starting 8 days after tumor graft. **a** ◆ Controls; □ DC-PDC−; △ cyclophosphamide 20 mg; ◇ DC-PDC− and cyclo. 20 mg. **b** ◆ Controls; □ DC-PDC−; △ cyclophosphamide 20 mg; ◇ DC-PDC−cyclo. 20 mg

Table 1 Lung metastasis, 14 days after tumor graft, expressed as percentage metastasis per pulmonary surface in control animals, animals treated with DC-PDC−, cyclophosphamide (20 mg·kg^{-1}) i.p. weekly and DC-PDC− combined with cyclophosphamide (20 mg·kg^{-1}) i.p. weekly (mean values ± SD). Each treatment group constituted three animals

Treatment	Lung metastasis (% pulmonary surface)
Controls	65 ± 15
DC-PDC−	20 ± 5
Cyclophosphamide 20 mg·kg^{-1}	30 ± 30
DC-PDC− and cyclophosphamide 20 mg·kg^{-1}	0

The considerable variation of tumor growth and MST observed in the control animals in the different experiments may be explained by the fact that tumor cells injected in each group were harvested from surgically removed tumors and not from cultured Mat-LyLu cells. Cells thus collected are heterogeneous as the separated cells come from different "mother" tumors, their vitality and aggressivity may therefore differ considerably. It was therefore essential to relate the data obtained from the treatment groups to the respective control group. Our experimental conditions mimic as closely as possible clinical conditions in which not one tumor evolution is identical to another.

This work confirms that polyamine deprivation alone, under clinical conditions (treatment started only when tumors were palpable), produces 63–79% tumor growth inhibition, 25 days after tumor graft, of the highly aggressive prostatic Dunning Mat-LyLu rodent tumor model. But polyamine deprivation alone is only effective during the first 30 days of treatment; tumor growth is uncontrolled afterwards. Interruption of the treatment with DC-PDC is accompanied by a significant increase in tumor growth [15]. This demonstrates that tumor growth inhibition by polyamine deprivation is reversible. Two reasons may explain this observation. Firstly, we have noticed that, with time and increasing tumor burden, control and treated animals significantly decreased their food consumption proportionally with decrease in drug intake. Secondly, with growing tumor mass, the accessibility of the drugs by diffusion was interfered with.

Having obtained a significant inhibition of tumor growth without significant toxicity in 3LL Lewis lung carcinoma bearing mice treated by a triple-chemotherapy combination (methotrexate + cyclophosphamide + vindesine) and polyamine deprivation [18], we chose to use the same treatment protocol for Dunning Mat-LyLu tumor-bearing Copenhagen rats. Adriamycin had been used in a previous experiment with no positive effect [15]. The cytotoxic treatment alone or in combination with polyamine deprivation proved effective on tumor volume but none of the treated animals survived longer than the controls. The treated animals died with practically no tumor but with significant loss of body weight. We assumed that the toxicity of the treatment regimen was responsible for the death of treated animals and decided to use cyclophosphamide 90 mg·kg^{-1} i.p. alone to reduce toxicity. This also proved effective on tumor growth, with improvement of life span by 100% compared to controls. The combination of 90 mg·kg^{-1} cyclophosphamide i.p. weekly with polyamine deprivation was as effective on tumor growth as cyclophosphamide alone but the median survival time was the same as for controls. We speculated that polyamine deprivation potentiates cyclophosphamide toxicity, and therefore investigated the possibility of using low-dose cyclophosphamide. Polyamine deprivation combined with low-dose

cyclophosphamide apparently has synergistic effects. Inhibition of tumor growth and prevention of lung metastasis formation was highly significant and the MST was increased by 55%. The PAO inhibitor seemed not to contribute significantly to tumor growth inhibition when polyamine deprivation was combined with low-dose cyclophosphamide.

The mechanisms of potentiation of the antitumor effect of our drug combination are unclear. One may hypothesize that polyamine-depleted cells are more sensitive to cytotoxic agents than cells with a physiologic polyamine pool. Furthermore, polyamine deprivation may potentiate the known immunomodulatory effect of cyclophosphamide. At 100 mg/kg, cyclophosphamide induces immunostimulatory reactions on host tumoral immunity in the Dunning Mat-LyLu model, whereas a low-dose regimen (30 mg/kg) has an immunosuppressive effect [4]. But the low-dose cyclophosphamide immunosuppressive effect is still debatable because an immunostimulatory effect has been observed in other tumor models [19]. Recently, our group has demonstrated that 3LL Lewis lung carcinoma bearing mice have decreased natural killer cytotoxic activities and that these activities are restored by polyamine deprivation [5]. Polyamine deprivation combined with low-dose cyclophosphamide may therefore enhance the immune response in tumor-bearing animals. This interesting possibility is presently under investigation.

Acknowledgements This work was supported by grants from the Association pour la Recherche sur les Tumeurs de la Prostate (1991), CNRS and the Association pour la Recherche sur le Cancer (ARC). The authors wish to thank Professor N. Seiler for revising this manuscript.

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Editorial comment

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In vivo, synergestic inhibition of MAT-LyLu rat prostatic adenocarcinoma growth by polyamine deprivation and low-dose cyclophosphamide

This study clearly demonstrates that polyamine deprivation, through a mechanism not yet fully understood, can enhance the efficacy of certain anticancer drugs such as cyclophosphamide. The results suggest that not only tumor growth, but also tumor metastasis is greatly inhibited by the combined regimen of polyamine depletion and cyclophosphamide administration. Our own studies with MAT-LyLu cells have indeed demonstrated that polyamine depletion results in reduced tumor cell motility, which is one of the factors implicated in metastatic behavior. In the present study, however, a distinction between effects on growth and on metastasis is difficult to make since the detection of metastases depends on their growth. It might therefore be interesting to determine the extent of metastatic spread also after a more prolonged exposure than the relatively short period of 14 days as was used in the present work. More specifically, the question might be addressed whether tumors developed in animals treated with DC-PDC plus cyclophosphamide give rise to the same number and amount of metastases as controls (at 14 days) with a comparable tumor burden.

A point of concern is the fact that the survival of the animals undergoing combination therapies seems to increase much less than would be expected on the basis of the observed antitumor effects. In fact, rats treated with DC-PDC/20 mg cyclophosphamide died within 60 days with a relatively low tumor load. One possibility is that these animals did develop metastatic disease, which can easily be checked by histological examination. Alternatively, the combination therapy itself might have a high toxicity. In the latter case the authors' statement that reduced drug doses can be applied to achieve similar antitumor activity has little practical value. The survival of rats treated with 90 mg cyclophosphamide is similar to those treated with 20 mg in combination with DC-PDC. The reasons for the relatively minor increase in survival should therefore be clarified before recommendations to apply this type of therapy in human trials can be made.

J. C. Romijn

Reply to the editorial comment

We greatly thank Dr. J. C. Romijn for his interesting comments. The work published above was performed using $2 \cdot 10^6$ cells harvested from a mother MAT-LyLu tumor and injected into rats. We have subsequently used $2 \cdot 10^6$ MAT-LyLu cultured cells injected into rats, thus changing the conditions of the study.

Under these new conditions, with more homogeneous tumors, we have confirmed that 20 mg cyclophosphamide associated with DC-PDC significantly inhibits metastasis formation 24 days after tumor graft and that at the time of death ($\simeq 52$ days) treated animals show no evidence of pulmonary metastases (unpublished data). The cause of death of these animals with a minimal tumor burden is of evident concern to us and is currently under investigation.

Finally, the fact that 20 mg cyclophosphamide with DC-PDC is as effective as 90 mg cyclophosphamide alone is of great interest to us. Understanding the reasons for this phenomenon remains a major challenge in the search for maximum efficacy of chemotherapy with minimal toxicity.

B. Cipolla