Cancer Chemother. Pharmacol. 4, 89–93 (1980)

Cancer Chemotherapy and Pharmacology

Therapy of Solid Walker Carcinosarcoma 256 with Bleomycin after Synchronization with Hydroxyurea

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Summary. *Rats with the solid Walker carcinosarcoma 256 were synchronized with hydroxyurea (6 × 50 mg/kg or 1 × 300 mg/kg body weight) and treated with bleomycin (32 mg/kg body weight) at different time points thereafter. Bleomycin clearly affects Walker carcinosarcoma cells at the G1/S boundary or in S-phase. The improvement in the results of bleomycin therapy after pretreatment with hydroxyurea can mainly be accounted for by the synchronization of the tumour cells.*

Introduction

The therapeutic effect of many cytostatic agents on tumour cells differs according to the cell cycle phase in which they are applied [4, 13, 21]. For this reason, attempts have been made to improve the results of chemotherapy by increasing the number of cells in the phase that is most sensitive to a particular cytostatic agent. This is normally achieved by means of substances that reversibly block cell proliferation. Examples of substances that can induce partial synchronization are bleomycin [2], cytosine arabinoside [8], 5-fluorouracil [7], hydroxyurea [27, 30], and vincristine [18]. When the concentration of these substances in the tissue decreases below a certain level the blockage is released and the cells synchronously traverse the next cell-cycle phase.

Various authors have reported favourable results of cancer chemotherapy after synchronization [9, 11, 12, 16, 19, 25, 28]. Nevertheless, doubts have repeatedly been raised as to whether the success of therapy in these cases is due to the synchronization of the tumour cells or simply to a particularly appropriate combination of the substances used [17, 24, 26, 39].

The present investigation was designed to test whether the effect of bleomycin therapy on the Walker carcinosarcoma of the rat can be improved by prior treatment with hydroxyurea (HU) and whether the improvement can be explained by synchronization of the tumour cells.

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Materials and Methods

1. Animals and Tumours

Sprague-Dawley rats (male, 200 g body weight, Mus-Rattus, D-8011 Brunnthal) were maintained under standard conditions: 21° C room temperature, 60% humidity, macrolon cages, water and "Altromin" standard diet (Altrogge, D-4937 Lage) ad libitum. The solid tumours of the Walker carcinosarcoma were induced by SC injection of ascites tumour cells under the back skin of the rats. As soon as the tumours had reached a measurable size (on Day 4 after tumour transplantation) the tumour-bearing animals were randomly distributed into the different experimental groups. Tumour size was determined three times daily by means of a caliper gauge (two diameters). The cell kinetic data for the Walker carcinosarcoma have previously been reported [37]: labelling index 32%; generation time 9 h; growth fraction 58%.

2. Synchronization Therapy

The rats were synchronized according to two different procedures: (a) 6 × 50 mg HU/kg body weight (IP at intervals of 1.5 h; interval between first and second injections was 1 h; the first injection took place at 23.30 h) [29, 37]. (b) 1 × 300 mg HU/kg body weight (IP; injection took place at 7.00 h) [22].

Bleomycin (Heinrich Mack Nachf., D-7918 Illertissen) at a dose of 32 mg/kg body weight was injected IP at different time points after synchronization.

Results

The rapidly growing Walker carcinosarcoma provides a very suitable model system for experimental chemotherapy; the tumours grow uniformly (Fig. 1) and the experimental results are very reproducible. Figure 1 also shows the protocol observed in therapy experiments: treatment

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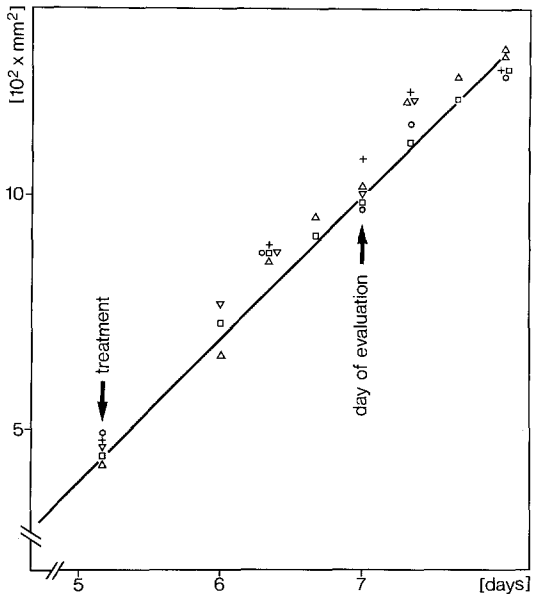

Fig. 1. Growth curve of the Walker carcinosarcoma. *Ordinate*, tumour size; *abscissa*, days after tumour transplantation. The values shown are the averages from 7–10 tumours. The symbols (○, □, ▽, △, +) represent different experiments. $n = 44$ rats

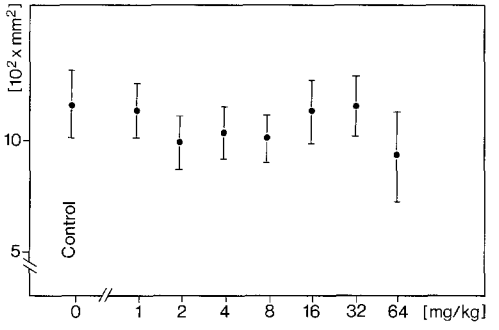

Fig. 2. Effect of bleomycin at different dose levels on tumour size of Walker carcinosarcomas. *Ordinate*, tumour size; *abscissa*, bleomycin concentration. Administration (IP) took place on Day 5 and measurement of tumour size on Day 7 after tumour transplantation. Average values ± SD from seven tumours; $n = 56$ rats

was carried out on Day 5 after tumour transplantation and the effect of therapy was evaluated 7 days later (cf. Fig. 5).

Figure 2 shows the effect of bleomycin at different dose levels on tumour size 7 days after tumour transplantation. Only at the highest dose level applied (64 mg/kg body weight, corresponding to about one-quarter of the LD_{50} [6]) did bleomycin show a small effect on tumour size. Variance analysis of the data, however, showed that this effect was not statistically significant ($\alpha = 0.05$).

Synchronization with HU was then carried out to increase the effect of bleomycin therapy on the tumours.

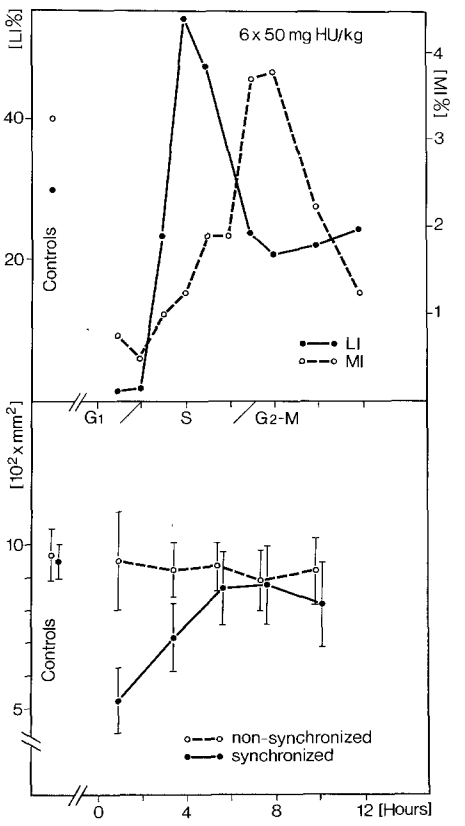

Fig. 3. Effect of bleomycin on the growth of Walker carcinosarcomas after six injections of 50 mg HU/kg body weight. *Top:* labelling index (LI) and mitotic index (MI) (from [37]). Controls, untreated rats; $n = 70$ rats. *Bottom:* tumour size (*ordinate*) of solid Walker carcinosarcomas after treatment with 32 mg bleomycin/kg body weight or with no treatment (controls). Tumour size was measured on Day 7 after tumour transplantation (second day after therapy). *Abscissa*, hours after last HU injection. Average values ± SD from ten tumours; $n = 120$ rats. Statistical analysis: groups $P \leq 0.001$; time $P \leq 0.001$; interaction $P \leq 0.001$ (double-variance analysis)

Six applications of HU (Fig. 3) result in an inhibition of DNA synthesis (labelling index) in the tumour cells for a period of about 9 h. Two hours after the last HU treatment a sharp increase in DNA synthesis is observed, which reaches a maximum 11–12 h after synchronization is started (4–5 h after the last HU treatment). The mitotic index reaches a maximum 14–15 h after synchronization is started (7–8 h after the last HU treatment). A single treatment with 300 mg HU/kg body weight inhibits DNA synthesis for 4 h (Fig. 4). The maximum DNA synthesis is subsequently reached 6–8 h after treatment, with the peak in mitoses following after 10–11 h. Both single and repeated injections of HU have the effect of inducing synchronous passage of Walker carcinosarcoma cells through the cell cycle.

To test whether the effect of bleomycin therapy is increased by synchronization with HU, the cytostatic was injected to tumour-bearing rats at different time points

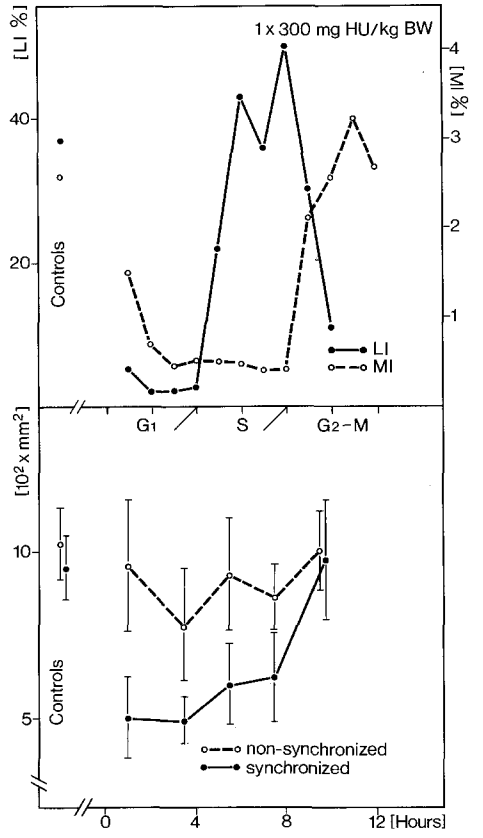

Fig. 4. Effect of bleomycin on the growth of Walker carcinosarcomas after a single injection of 300 mg HU/kg body weight. *Top:* labelling index (LI) and mitotic index (MI) (from [22]). Controls, untreated rats; $n = 52$ rats. *Bottom:* tumour size (*ordinate*) of solid Walker carcinosarcomas after treatment with 32 mg bleomycin/kg body weight or with no treatment (controls). Size was measured on Day 7 after tumour transplantation (second day after therapy); *abscissa*, hours after last HU injection. Average values ± SD from ten animals; $n = 120$ rats. Statistical analysis: groups $P \leq 0.001$; time $P \leq 0.001$; interaction $P \leq 0.001$ (double-variance analysis)

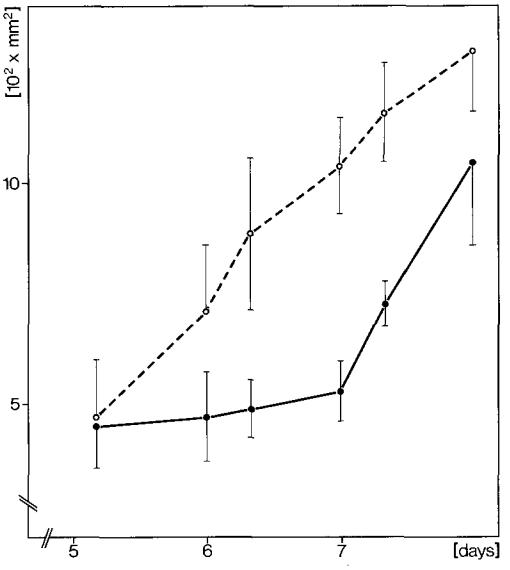

Fig. 5. Tumour size of Walker carcinosarcomas at different days after transplantation. *Ordinate*, tumour size; *abszissa*, days after tumour transplantation. ●——●, tumours synchronized with 1 × 300 mg HU/kg body weight and treated with 32 mg bleomycin/kg body weight 3.5 h after the HU application; O - - - O, nonsynchronized and untreated controls. The values shown are the averages from ten tumours ± SD

Table 1. Tumour size of Walker carcinosarcomas at different time points after treatment with bleomycin during HU blockage

	Tumour size[a]		
	Controls	I[c]	II[d]
Without HU application	977 ± 232	1,016 ± 185	999 ± 224
During HU application[b]	1,043 ± 84	1,025 ± 215	1,090 ± 160

[a] mm^2, measured at Day 7 after transplantation, average values from 10 tumour, ± standard deviations
[b] 6 × 50 mg HU/kg body weight
[c] Bleomycin treatment (32 mg/kg body weight) after the second HU application
[d] Bleomycin treatment (32 mg/kg body weight) after the fourth HU application

after HU treatment. The time points for bleomycin treatment were chosen in such a way that the tumour cells were exposed to the substance during different cell cycle phases. The dose of bleomycin used was not sufficient by itself to influence the growth rate of the tumours (32 mg/kg body weight; cf. Fig. 2). Similarly, the doses of HU given did not lead to any diminution of tumour growth. However, when the effect of bleomycin was measured 1 or 3.5 h after the last HU treatment (6 × 50 mg HU/kg body weight), a significant inhibition of tumour growth was observed (lower part of Fig. 3).This means that the effect of bleomycin is increased when tumour cells synchronously enter and pass through S-phase. On the other hand, if bleomycin is administered at a later time point, when the blockage in DNA synthesis has been released and the cells are predominantly in the G2- or M-phases, no improvement is observed in the effect of therapy.

When synchronization was carried out with a single dose of HU (300 mg/kg body weight), bleomycin had significantly reduced tumour growth when its effect was measured 1, 3.5, 5.5, and 7.5 h later (lower part of Fig. 4). In this case the growth of the tumour cells was also inhibited, mainly at the G1/S boundary or during S-phase. Administration of bleomycin at a later time point (G2-, M-phase) again did not lead to any detectable improvement in the results of therapy.

Discussion

Previous investigations in this laboratory have demonstrated that only rapidly growing tumours with a large enough growth fraction are suitable for 'synchronization therapy' [37, 38]. Whereas no improvement in the results of therapy after HU synchronization was observed for the slowly growing neurosarcoma [36], the therapeutic effects of cytosine arabinoside, hydroxyurea and vincristine on the rapidly growing Walker carcinosarcoma were increased by prior synchronization [22, 37]. Since all three substances exert cytotoxic effects primarily on cells synthesizing DNA, the improvement in the results of therapy was only observed when the tumour cells were predominantly in the S-phase. On the other hand, when cytostatic agents that are active during different cell cycle phases and have a relatively long biological half-life (e.g., cyclophosphamide) were used, no improved results of therapy were obtained after synchronization [37].

Various authors have demonstrated that HU is suitable for the synchronization of solid tumours [21, 27, 29, 35–38]. HU is known to prevent the passage of cells from the G1- to the S-phase and to block the DNA synthesis, and in higher doses it also kills most of the cells that are in S-phase. In this way HU causes a build-up of cells at the G1/S boundary [30].

The aim of the present investigation was to determine whether the effect of bleomycin on the growth of the Walker carcinosarcoma can be increased by synchronization with HU. Bleomycin, which is isolated from *Streptomyces vertillus* [34], is used mainly in the treatment of squamous epithelial carcinomas [5, 14, 34]. The substance causes DNA – strand breaks and reduces DNA synthesis by the inhibition of DNA-dependent DNA polymerase [23, 31, 32]. Before bleomycin is used in synchronization therapy, accurate information should be available on its activity during different cell-cycle phases. The literature data on this are, however, contradictory. According to Barranco and Humphrey [1], Iversen et al. [15], and Watanabe et al. [40], cells are particularly sensitive to bleomycin in late the G1- and the S-phase. Increased sensitivity has also been reported for cells in G2- and M-phases [1, 3, 15, 33].

The tumour size was measured on different days after application of cytostatic agents, and the reduction in tumour size 2 days after treatment (7 days after tumour transplantation) was taken as a measure of the success of therapy (Fig. 5). In our test system, bleomycin has the strongest effect on Walker carcinosarcoma cells that are just entering S-phase from G1 or are already in S-phase. However, when bleomycin was applied to Walker carcinosarcoma cells that are in the G2- or the M-phase, no improvement in the results of therapy was detected. The reason for this may be related to a variation in the duration of different cell-cycle phases in the tumour cells and to a partial desynchronization of the cells. In addition, the G2- and M-phases are extremely short, and it can be expected that only a small number of cells would be simultaneously passing through these phases.

It is often difficult to decide whether successful treatment by synchronization therapy is simply due to the synchronization of the tumour cells or is a result of the combination of drugs used [10, 26]. Neither bleomycin (32 mg/kg body weight) nor HU (6×50 mg/kg or 1×300 mg/kg body weight) had any effect on tumour growth if administered alone (cf. Figs. 2–4). Bleomycin also failed to affect tumour growth if applied during the HU blockage. Table 1 demonstrates a lack of correlation between bleomycin sensitivity and HU concentration in the tumour. Thus the improved results observed in our investigations after synchronization with HU are obviously due to the synchronization.

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Received May 10, 1979/Accepted November 15, 1979