Intracavitary Chemotherapy with Activated Cyclophosphamides and Simultaneous Systemic Detoxification with Protector Thiols in Sarcoma 180 Ascites Tumor

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ABSTRACT

Activated cyclophosphamides such as 4-sulfoethylthiocyclophosphamide (mafosfamide) are suitable for a local intracavitary chemotherapy, whereas cyclophosphamide requires a metabolic activation. Mafosfamide administered i.p. in mice was less toxic (50% lethal dose, 640 mg/kg) than its i.v. application (50% lethal dose, 480 mg/kg). A further remarkable reduction of toxicity with an increase of the 50% lethal dose of mafosfamide to 1500 mg/kg was obtained by the simultaneous i.v. application of the protector thiol cysteine (mafosfamide:cysteine ratio, 1:5 on molar weight basis). In comparison with the i.v. injection of mafosfamide, the local i.p. application resulted in a 20 times higher concentration versus time product of peritoneal drug levels. The molar ratio of sulfhydryl groups to activated cyclophosphamide (resorbed from the peritoneal cavity) remained high in blood. Therapy studies on Sarcoma 180 ascites tumor of mice revealed that the coadministration of cysteine i.v. in mafosfamide i.p. treatment is superior to mafosfamide i.p. application alone. On the contrary, the simultaneous i.p. application of cysteine is accompanied by a loss of antitumor efficacy. The regime of local i.p. chemotherapy with activated cyclophosphamide and simultaneous systemic detoxification by an appropriate thiol allows (a) the reduction of the systemic toxicity of treatment without influence on the cancerotoxic activity at the site of local injection and (b) the exposing of the intraperitoneal tumor to a much higher concentration of the cytostatic agent.

INTRODUCTION

Metastasizing cancers such as breast cancer and ovarian carcinoma are frequently accompanied by pleural and peritoneal carcinosis (1, 2). The metastatic spreading of the tumor may remain restricted to these body cavities for a long time. Local forms of therapy were therefore instituted at a very early stage at the beginning of the chemotherapy era (3, 4). The results were relatively unconvincing (4, 5), so that intracavitary applications of cytostatics were largely abandoned (1), and exclusively systemic chemotherapy was used. As observed by Jones et al. (6) among others, a series of pharmacokinetic conditions was disregarded in the local therapy applied so far. Furthermore, almost all local applications of cytostatics carried out were monotherapies, so that these results may only be compared with those of systemic monotherapy.

In 1978, Dedrick and DeVita (7) developed a concept of local chemotherapy for peritoneal carcinosis based on a pharmacokinetic rationale. This concept has also become known under the name "belly bath" or high volume i.p. chemotherapy. In the meantime, the first experience with this form of therapy is meantime, the first experience with this form of therapy is
volume i.p. chemotherapy is based on the pharmacokinetic rationale developed by Jones et al. (6) and Dedrick et al. (7).

Assay for "Activated Cyclophosphamide." The name activated cyclophosphamide as used in this paper denotes the sum of all cyclophosphamide derivatives (4-hydroxycyclophosphamide, its hemiaminal aldehyde, and stable activated 4-S,R-sulfidocyclophosphamides) that give rise to liberation of acrolein and can be determined by the fluorometric assay. A 10-μl blood sample was deproteinized with 0.6 ml of ice-cold 5% (w/v) trichloroacetic acid and centrifuged. One-half ml of the supernatant was placed in small conical tubes together with 0.6 ml of a solution containing 3-aminophenol (5 mg/ml) and hydroxylaminoniumchloride (6 mg/ml) dissolved in HCl (1 mol/liter). The further procedure was identical with the original fluorometric assay for activated cyclophosphamide as described previously (21, 22).

Assay for Sulfhydryl Groups. SH groups were measured with 4,4'-dithiobispyridine by the method of Grasset and Murray (23). Prior to cysteine administration, blood or ascites samples were taken as a blank to determine the endogenous SH levels.

Blood Cell Counts. Total WBC were determined manually using a Neubauer counting chamber. For WBC counting, erythrocytes were hemolyzed with 1% acetic acid solution.

Acute Toxicity of Mafosfamide. The lethal toxicity was estimated at least at 4 doses for each schedule, and each dose was evaluated in 10 to 20 animals. The observation period was 30 days. LD₅₀ and LD₁₀ were evaluated by probit analysis (24).

Antitumor Activity. Two lines of the Sarcoma 180 ascites sarcoma (Tumorbank, Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany), which are quite different in their response to treatment with oxazaphosphorine cytostatics, were used in all experiments. The ascites tumor was passaged weekly by the transfer of 5 × 10⁴ ascites cells suspended in tissue culture medium (Sera, Heidelberg, Federal Republic of Germany). The percentage of damaged cells, which were detected by the trypsin blue dye test, was usually below 5%. Therapy experiments were carried out according to exact body weight 48 h after implant. Control and treated mice were observed for 90 days. Survival was recorded in days as median or mean survival time, and the ILS was calculated by comparing these with the survival of the control group. Comparisons of antitumor activity were made on dose level of 50% of the LD₁₀. Other details are described in the legends.

Pharmacokinetic Analysis. The trapezoidal rule was used to calculate the AUC in blood and ascites. A regression analysis was performed to determine drug plasma half-life times. Drug clearance (Cl) from the peritoneal space and blood is calculated as: Cl = dose/AUC.

RESULTS

Pharmacokinetics. In a local therapy with activated cyclophosphamide (mafosfamide), the systemic bioavailability determines the extent of general toxicity, above all the limiting bone marrow toxicity. The question as to whether the systemic bioavailability of the cytostatic is restricted after i.p. administration of mafosfamide as compared with i.v. application was therefore investigated on the basis of AUC of the plasma levels. The plasma levels of activated cyclophosphamide were measured after i.p. and i.v. application of the same dose of mafosfamide. As demonstrated in the blood level curves shown in Fig. 2, the AUC of activated cyclophosphamide applied i.p. amounts to 75% of the values following i.v. application. Only 56% are reached after high-volume i.p. injection (Table 1). According to Voelcker et al. (20), this is attributable to a first pass effect with detoxification of the activated cyclophosphamide in the liver to carboxycyclophosphamide and 4-keto cyclophosphamide. This is enhanced by the delayed absorption with a large injection volume (Table 1).

In further investigations, the activity level of activated cyclophosphamide in the abdominal cavity was determined after i.p. and i.v. injection of mafosfamide (Fig. 3). The initial levels in the peritoneal cavity are naturally much higher after i.p. administration than the peak levels of activated cyclophosphamide obtained in the peritoneal fluid after i.v. injection of mafosfamide. However, the 20-fold higher concentration x time product in the peritoneal cavity is not only a manifestation of the automatically higher initial levels in i.p. injection and also reflects a very much lower peritoneal clearance for activated cyclophosphamide compared to systemic clearance. Thus, a peritoneal clearance of 4.2 μl × min⁻¹ × g⁻¹ is calculated from the pharmacokinetics in Figs. 2 and 3 as compared to a clearance of 33 μl × min⁻¹ × g⁻¹ calculated from the blood concentration curve.

The influence of sulfhydryl compounds on the general and cancerotoxic action of activated cyclophosphamide depends on the molar ratio between cytostatic and protector thiol (20). In order to investigate whether the antitumor effect at the site of local i.p. cytostatic injection can be inhibited by a systemic i.v. application of cysteine, the permeation of i.v. cysteine into the peritoneal fluid was measured (Fig. 4). An equilibrium between the blood and peritoneal levels of SH groups is seen after 30 min, which corresponds to a period of roughly 2 plasma half-lives. If the ratio of the blood levels of cysteine and activated cyclophosphamide after i.p. injection of 400 mg of mafosfamide per kg and a 5-fold molar excess of cysteine in relation to mafosfamide are calculated, a large excess of SH groups remains in the blood for more than 1 h. In the abdominal cavity, an equimolar ratio of SH groups and activated cyclophosphamide is likewise observed only after 1 h (Fig. 5). For the desired effect, namely systemic protection from the cytostatic effect without an influence on the local antitumor action, there is thus a favorable ratio between protector thiol and cytostatic in the compartments investigated.

Toxicity. The acute toxicity of mafosfamide in the mouse and its modification by different ways of application and administration of protector thiol are shown in Table 2. In accordance...

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\[ \text{The abbreviations used are: SH, sulfhydryl; ILS, increase of life span; LD}_{50}, \text{50\% lethal dose; LD}_{10}, \text{10\% lethal dose; AUC, area under the concentration \times time curve; CD}_{50}, \text{50\% curative dose.} \]
Table 1  Comparative pharmacokinetic parameters of activated cyclophosphamide in NMRI mice after injection of mafosfamide (450 mg/kg) by different routes and injection volumes

<table>
<thead>
<tr>
<th>Application route</th>
<th>Injection volume (ml/kg)</th>
<th>$K_a$ (min⁻¹)</th>
<th>$K_m$ (min⁻¹)</th>
<th>$t_{1/2}$ (min)</th>
<th>AUC$_{blood}$ (µmol.min.ml⁻¹)</th>
<th>AUC i.p. injection (µmol.min.ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>20</td>
<td>0.051</td>
<td>13.5</td>
<td>26.8 ± 3.7</td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td>i.p.</td>
<td>10</td>
<td>0.042</td>
<td>16.5</td>
<td>20.2 ± 4.5</td>
<td></td>
<td>0.56</td>
</tr>
<tr>
<td>i.p.</td>
<td>150</td>
<td>0.014</td>
<td>49.5</td>
<td>15.0 ± 4.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Peritoneal concentration of activated cyclophosphamide in NMRI mice following i.v. (O) or i.p. (150 ml/kg) (•) injection of mafosfamide (450 mg/kg). Samples of approximately 50-µl volume were obtained at all points by i.p. puncture. Before i.v. application of mafosfamide, 150 ml of Ringer’s solution per kg, corresponding to the high-volume i.p. treatment, were injected i.p. to facilitate the permeation of activated cyclophosphamide into the peritoneal space and to allow measurements of i.p. levels. Dilution effect of the i.p. fluid depot on drug concentrations has not been corrected. Points, mean value obtained in 6 animals, bars, SE.

Fig. 4. Blood (x) and peritoneal (•) concentration of cysteine following i.v. injection of cysteine (1500 mg/kg) in 6 NMRI mice. For further details, see Fig. 3 and “Materials and Methods.”

with the restricted systemic bioavailability of mafosfamide applied i.p. in higher volume, this form of application was far less toxic than i.p. administration in a small volume or i.v. injection. As recently as has been shown by Voelcker et al. for 4-(S-ethanol)sulfidocyclophosphamide (20), a further lowering of the toxicity, above all local toxicity, is possible by i.p. administration of cysteine as protector thiol. As shown by the WBC curve in Fig. 6, in i.p. treatment with mafosfamide, the bone marrow toxicity becomes the dose-limiting factor. The development of severe leukopenia can be partially abolished by i.v. administration of cysteine and leads to a remarkable increase of the LD$_{50}$ value of mafosfamide (Table 2). Overall, the therapy concept of high-volume i.p. treatment with fractionated dosage over 2 days and systemic detoxification with i.v. cysteine allows an increase of dosage of mafosfamide by the factor 5–6 when compared with conventional i.p. injection.

Therapy of Sarcoma 180 Ascites Tumor. The therapeutic effect of mafosfamide on a sensitive and a resistant line of Sarcoma 180 ascites tumor of the mouse was investigated. The CD$_{50}$ and the therapeutic index LD$_{50}$/CD$_{50}$ was determined in the sensitive line of Sarcoma 180 ascites tumor. In the relatively resistant line of the tumor, only the number of survivals could be recorded, and a percentage comparison on the basis of ILS at a dose of 50% of the LD$_{50}$ could be made, since the CD$_{50}$ already overlapped with the lethal toxic range. The results of therapy shown in Table 3 reveal that local i.p. therapy is far superior to systemic cytostatic application in this ascites tumor. Thus, i.v. therapy with mafosfamide remained largely ineffectiv in the relatively chemoresistant line on Sarcoma 180 ascites tumor. In the chemosensitive line, the i.p. therapy was roughly 60 times as effective as the i.v. administration measured in terms of the therapeutic index.

Fig. 7 shows the influence of the protector thiol cysteine on the cytostatic efficacy of mafosfamide. Whereas i.p. cysteine in a 5-fold molar ratio to activated cyclophosphamide largely abolished its cancerotoxic action, the local antitumor effect is
The number of 60-day survivors also reveals a clear advantage of the combination therapy with protector thiol applied i.v.

**DISCUSSION**

Advantages are only to be expected from a topical cytostatic therapy when the tumor is exposed locally to a far higher concentration versus time product ($c \times t$, AUC) than in systemic administration, and the general toxicity is simultaneously reduced compared to systemic therapy (6, 7). These requirements are fulfilled using the activated cyclophosphamide compound mafosfamide according to our animal experimental data. In i.p. application of this compound, the systemic bioavailability of the cytostatic was found to be reduced to about 56% compared to an equal i.v. dose due to a first-pass effect (20). As was to be expected on the basis of these pharmacokinetic data, the acute toxicity of mafosfamide in the NMRI mouse was also lower after high-volume i.p. administration than after i.v. injection. Due to the comparatively low peritoneal clearance for activated cyclophosphamide, a 20-fold higher concentration versus time product could be measured in the peritoneal cavity of the mouse after i.p. injection than after i.v. application of the same dose. Therefore, it was concluded that the local intracavitary chemotherapy with mafosfamide is superior to systemic cytostatic administration in a tumor with disseminated growth restricted to the abdominal cavity. This was confirmed in the Sarcoma 180 ascites tumor of the mouse.

Despite the advantages of local i.p. therapy, the doses of mafosfamide used also give rise to a substantial general toxicity. The curative and lethal toxic dose ranges overlap in the resistant line of Sarcoma 180 ascites tumor investigated. This result is comparable with the clinical situation in ovarian carcinoma (25). The question was therefore examined as to whether the...
therapeutic range of activated cyclophosphamide can be enlarged by additional administration of protector thiols.

A protective action of sulphydryl compounds against alkylating cytostatics was known as early as 1951 (26). The SH groups serve as nucleophilic partners which are preferentially alkylated (27). By the irreversible loss of the alkylation capacity, not only the general toxicity of the alkylating agents is reduced, but the antitumor action is also substantially restricted. As a rule, a therapeutic advantage is thus not to be expected from these detoxification procedures with protector thiols (27). The toxicity of oxazaphosphorine cytostatics (e.g., cyclophosphamide and ifosfamide) can also be influenced by protector thiols. However, the reaction with the SH groups takes place preferentially on a second functional group at the 4-hydroxy position (20) bearing to 4-sulfidocyclophosphamides. From these thioglycosides, the activated metabolite 4-hydroxycyclophosphamide can be released once more by hydrolysis depending upon the molar ratio of the reaction partners (cf. Fig. 1). The alkylating capacity of this metabolite is thus not abolished by the reaction with sulphydryl groups.

Authors such as Kline (19), Gurtoo et al. (18), and Berrigan et al. (17) were able to show a reduction in toxicity in oxazaphosphorine cytostatics by thiols without a loss of cancerotoxic activity. According to Voelker et al. (20), the application of cysteine as protector thiol in the new class of activated cyclophosphamide leads to a raised therapeutic range when a molar ratio of protector thiol to activated cyclophosphamide of 5:1 is not exceeded. In contrast to these results, our present investigations on the relatively resistant line of Sarcoma 180 ascites sarcoma of the mouse have shown that the general lowering of toxicity is accompanied by an equally large loss of antitumor activity in the therapeutic dose range in simultaneous i.p. administration of activated cyclophosphamide and protector thiol. Accordingly, as with the simple alkylating agents, a therapeutic advantage thus does not result generally even with the oxazaphosphorine cytostatics despite a different mechanism of cytostatic inactivation by protector thiols. A predictive test which allows an appraisal of whether a loss of cancerotoxic activity is to be expected from administration of protector thiols in treatment of a cancer with oxazaphosphorine cytostatics does not exist. Furthermore, in contrast to leucovorin protection in high-dose methotrexate therapy (28), there is no molecular biological basis, which could explain a different effect of protector thiols on normal host cells and on tumor cells in cyclophosphamide therapy. A simultaneous systemic or local administration of cyclophosphamide or activated cyclophosphamide and protector thiol at the same site of injection therefore cannot be recommended clinically at present.

On the other hand, a separate administration of cytostatic and protector thiol with i.p. administration of the activated cyclophosphamide and systemic detoxification by cysteine applied i.v. appears to provide therapeutic advantages. According to the data we obtained, the molar concentration ratio of protector thiol to activated cyclophosphamide in the blood remains high over several half-lives, whereas in the peritoneal cavity, an excess of activated cyclophosphamide in relation to the detoxifying agent is present. Accordingly, the systemic detoxification by protector thiols should not appreciably influence the cancerotoxic action at the site of local application, as was confirmed by therapeutic studies on the Sarcoma 180 ascites tumor of the mouse. If the protector thiol cysteine is administrated additionally i.v. with a dose of 50% of the LD10 of mafosfamide, the therapeutic efficacy is maintained, as measured by the increase of life span. Since the systemic toxicity is appreciably lowered at the same time, a greater therapeutic range can be attained. Thus, higher doses of mafosfamide can be applied under the protection of cysteine, resulting in a marked rise of the rate of healing and an increase of the life span.

A successful clinical example of a protector thiol application in oxazaphosphorine cytostatics is the regional detoxification of the urinary tract with the uroprotector 2-mercaptoethanesulfonate (mesna) (29). An influence on the antitumor effect is not observed here (29), since mesna is rapidly oxidized systematically to the inactive disulfide, and free SH groups at high concentration are only released again by reduction in the renal tubules. Our investigations on animal models allow the conclusion that the opposite approach, namely, effective systemic protection by thiols in regional cytostatic therapy, is possible without loss of cancerotoxic activity. However, some possible limitations in the clinical application of this therapeutic concept have to be considered. It is a general problem of i.p. chemotherapy in human cancer (e.g., ovarian carcinoma) that we meet not only a diffuse carcinosis with free floating ascites cells but also solid i.p. masses. With the increase of the amount of diameter of solid tumor masses, the value of i.p. therapy may decrease. Under these circumstances, the optimum type of i.p. therapy would be with an agent, which results in high i.p. levels and at the same time leads to blood levels within the therapeutic range to provide an additional cytotoxic effect delivered via capillary circulation. According to our animal model, this is true for activated cyclophosphamide. As shown in Fig. 2, despite the reduced bioavailability of i.p.- applied mafosfamide, systemic drug levels are still in a range which is observed at the end of the distribution phase following i.v. injection of the drug. However, it cannot be excluded that the systemic cytostatic action is partially abolished by the protector thiol cysteine. This possible shortcoming of our therapeutic model also applies to a similar approach of i.p. cis-platinum treatment concomitant with systemic sodium thiosulfate protection according to Howell et al. (9). Although until now some successful clinical studies were performed with cis-platinum and sodium thiosulfate (9, 30), the question above is not answered and requires further clinical evaluation.

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REFERENCES

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