Cancer Chemother Pharmacol (1983) 11: 167–171

Cancer Chemotherapy and Pharmacology

Effect of a single high dose and repeated small doses of dianhydrogalactitol (DAG; NSC-132313) on rat intestinal mucosa

Judit Kralovánszky[1], Noémi Prajda[1], Sándor Kerpel-Fronius[1], Ferenc Gál[2], and Zoltán Szentirmay[2]

[1] National Institute of Oncology, 1525 Budapest, Ráth Gy U 7
[2] Research Institute of Oncopathology, 1525 Budapest, Hungary

Summary. *The influence of the treatment schedule of dianhydrogalactitol on its effect on the activity of mucosal enzymes in rat intestine was studied. The effect of a single high dose (10 mg/kg) was compared with that of repeated small doses (4 × 2.5 mg/kg) given at daily intervals. At 48 h after a single high dose the activities of thymidine kinase, which is a marker of dividing crypt cells, and of alkaline phosphatase, sucrase, maltase, xanthine oxidase, which are markers of mature enterocytes, were strongly depressed. Even 96 h after the treatment low enzyme activities could be observed. Repeated small doses caused milder enzyme inhibition and almost total recovery had occurred by 96 h after administration of the last dose. The results indicate that fractionation of drug administration can reduce the toxic side-effects on the intestinal mucosa and might be partly responsible for the higher therapeutic index of such schedules in experimental tumor models.*

Introduction

Dianhydrogalactitol is an alkylating hexitol derivative with favorable antitumor activity. The drug suppresses proliferation of the tumor, the bone marrow, and the rapidly dividing small intestinal epithelial cells. In experimental animals that died following the LD_{50} of DAG gastrointestinal symptoms, e.g., ulceration and inflammation were dominant [19]. In clinical studies DAG was administered IV in small daily doses (20–30 mg/m^2) for 5 days [7], or in high doses (50–160 mg/m^2) given weekly or at longer intervals [10, 25]. The dose-limiting toxicity was myelosuppression [12]. Diarrhea occurred only rarely and was mild [3]. Probably the damage to the gut epithelium remains below the level necessary to elicit severe clinical symptoms. With the aim of developing appropriate clinical pharmacologic methods to study the functional consequences of intestinal drug effects, biochemical studies were undertaken in animals.

Preliminary studies [22] showed that a single high dose of DAG decreased the activity of TK, the characteristic enzyme of dividing cells and of AP, MAL, SUC, XO, which are markers of functional cells of intestinal mucosa. The effect was time- and dose-dependent and correlated well with cytomorphological changes.

Reprint requests should be addressed to: Judit Kralovánszky

Abbreviations used in this paper: DAG, dianhydrogalactitol; LD_{50}, dose of drug causing 50% lethality; TK, thymidine kinase EC 2.7.1.21; AP, alkaline phosphatase EC 3.1.3.1; SUC, sucrase EC 3.2.1.26; MAL, maltase EC 3.2.1.20; XO, xanthine oxidase EC 1.2.3.2

In our present work the effects of a single high dose and repeated small doses of DAG on the intestinal mucosal enzymes were compared, to check whether the change of dose schedule could reduce the damage to the small intestinal epithelium.

Materials and methods

H-Riop. Wistar outbred male rats (44) weighing 180–200 g were used in the experiments. DAG, dissolved in physiological saline, was given IV in three different dosages (Table 1).

Animals were killed 24, 48, or 96 h after the last dose of DAG together with the untreated controls. For 24 h prior to sacrifice the animals were deprived of food but had free access to water.

After sacrifice the entire small intestine was removed below the ligament of Treitz and washed with 154 m*M* NaCl containing 1 m*M* mercaptoethanol. Intestinal epithelial cells were isolated according to the method of Weiser [27] as modified by Kralovánszky et al. [16]. The method makes it possible to isolate the villus and crypt cells separately but in the present experiments, because of the great number of the samples, we collected the isolated cells in one mixed cell fraction. This cell isolation method offers a more homogenous experimental material free from underlying tissues, and the results obtained refer to the total excised gut length, so that proximal-distal biochemical changes are avoided.

After centrifugation for 15 min at 1,500 *g*, the isolated cells were homogenized in a Potter-Elvehjem homogenizer in isotonic KCl at +5° C.

For the determination of AP, SUC, and MAL activities the whole homogenate, and for TK and XO assays the

Table 1. Design of DAG treatments and enzyme determinations

	Days of DAG treatment				Days of enzyme determinations		
	0	1	2	3	4	5	7
Single high dose	–	–	–	4×	24[a]	48	96
Repeated small doses	×	×	×	×	24	48	96
Single small dose	–	–	–	×	–	48	96
Control	–	–	–	–	24	48	96

×, 2.5 mg/kg DAG; 4× = 10.0 mg/kg DAG
[a] Time after the last dose of DAG (h)

100,000 *g* supernatant fluid were used. AP activity was determined with *p*-nitrophenyl phosphate as substrate and the release of *p*-nitrophenol was determined spectrophotometrically at 405 nm [2]. SUC and MAL activities were estimated by measuring the rate of hydrolysis of sucrose or maltose by the use of glucose oxidase to measure the formation of glucose [5]. TK was measured by determining the conversion of [2-^{14}C]Tdr to [2-^{14}C]dTMP by the DEAE cellulose disk method [15]. XO activity was assayed by the formation of uric acid from xanthine, using the increase in absorbance at 293 nm [21]. Protein was determined by the method of Lowry et al. [17]. Enzyme activities were calculated as μmoles per hour and per centimeter of intestine. Statistical analyses were performed by using *t*-statistics for two means.

Two animals from each group were subjected to histological examination. Tissue sections were stained with hematoxylin and eosin.

Results

A single high dose (10 mg/kg) of DAG depressed the activities of all enzymes studied (Fig. 1). By 24 h after its administration the TK level had already fallen significantly (to 30%–50% of the control value) and levels of the brush border enzymes (AP, SUC, MAL) were subsequently also found to have fallen at 48 h after the treatment. This indicates that DAG acts primarily on the immature, dividing crypt cells and the damage to villus cells is only secondary. If 10 mg/kg was given in four equal parts, i.e., 2.5 mg/kg of DAG on each of 4 consecutive days, the enzmye-reducing effect decreased and was significant only in the case of TK and ALP (Table 2).

The time needed for regeneration was also different. With a single high dose, all enzyme activities were still depressed 96 h after DAG injection, while after repeated smaller doses the normal enzyme levels had already been recovered by this time, with the exception of TK. Nevertheless, the process of regeneration was not followed further.

The morphologic alterations correlate well with the results of the enzyme determinations.

At the maximum of the functional changes, which occurred on day 2 after drug administration, most of the villi became denuded after the injection of a single high dose. An intact epithelial cell covering is retained irregularly on some villi. The crypt cells seem to be morphologically intact (Fig. 2a).

After repeated small doses the structure of the epithelial layer is mostly undisturbed; impaired regions are only occasionally encountered (Fig. 2b).

The effects of three dose schedules of DAG are compared in Fig. 3.

It appears that neither the rate of enzyme inhibition after 48 h nor the regeneration after 96 h was significantly influenced by administration of the total dose in four equal parts 4 × 2.5 mg/kg, whereas the same total dose given in one single

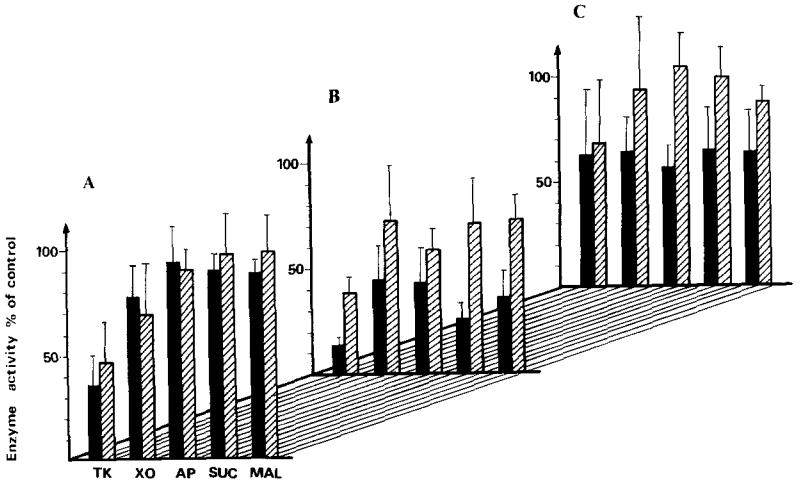

Fig. 1. Effect of DAG on the mucosal enzymes of rat intestine. Rats received DAG IV as a single high dose of 10 mg/kg (■) or four doses of 2.5 mg/kg (▨). Animals were killed 24 h (A), 48 h (B), or 96 h (C) after the last dose of DAG. Enzyme activities are expressed as percentages of control values. In each group mean values ± SD of four animals are given

Table 2. Statistical evaluation of enzyme activity changes after DAG treatment

Time after DAG treatment	Comparison	Enzyme				
		TK	XO	AP	SUC	MAL
24 h	Control vs 1 × 10 mg/kg	$P < 0.001$	NS	NS	NS	NS
	Control vs 4 × 2.5 mg/kg	$P < 0.001$	NS	NS	NS	NS
	1 × 10 vs 4 × 2.5 mg/kg	NS	NS	NS	NS	NS
48 h	Control vs 1 × 10 mg/kg	$P < 0.001$	$P < 0.05$	$P < 0.001$	$P < 0.05$	$P < 0.05$
	Control vs 4 × 2.5 mg/kg	$P < 0.001$	NS	$P < 0.01$	NS	NS
	1 × 10 vs 4 × 2.5 mg/kg	$P < 0.001$	NS	NS	$P < 0.001$	$P < 0.05$
96 h	Control vs 1 × 10 mg/kg	NS	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$
	Control vs 4 × 2.5 mg/kg	NS	NS	NS	NS	NS
	1 × 10 vs 4 × 2.5 mg/kg	NS	NS	$P < 0.01$	$P < 0.05$	$P < 0.05$

NS = not significant

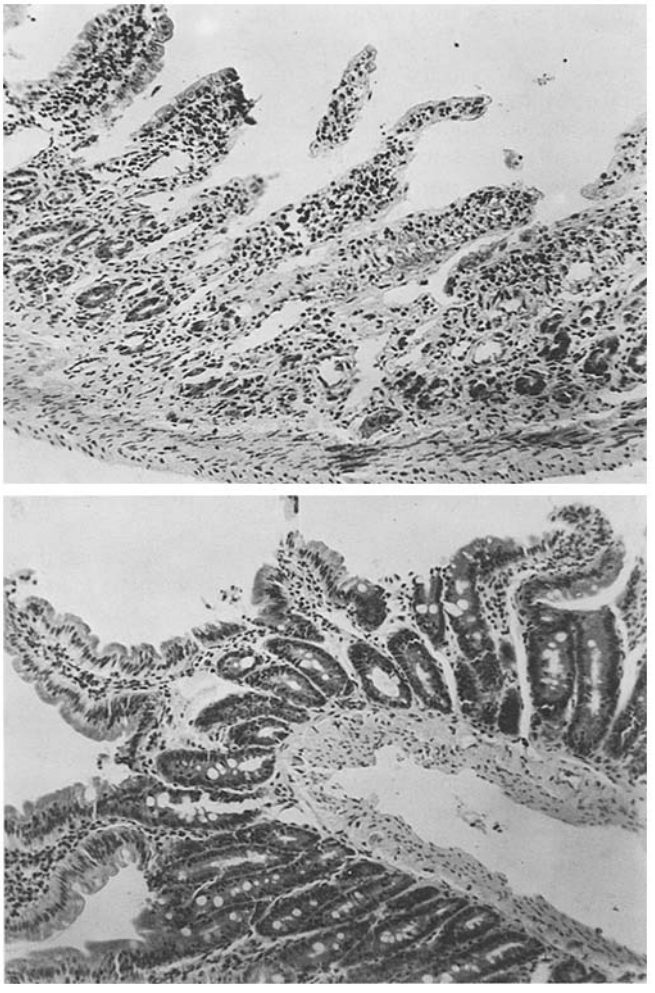

Fig. 2. a Intestinal epithelium 48 h after administration of 10 mg DAG/kg. The villi are denuded; intact epithelial cell covering is retained at some islands HE × 150. **b** Intestinal epithelium 48 h after administration of 4 × 2.5 mg DAG/kg. The functional organization is well preserved. Loss of epithelial cells is visible only at some sites. HE × 170

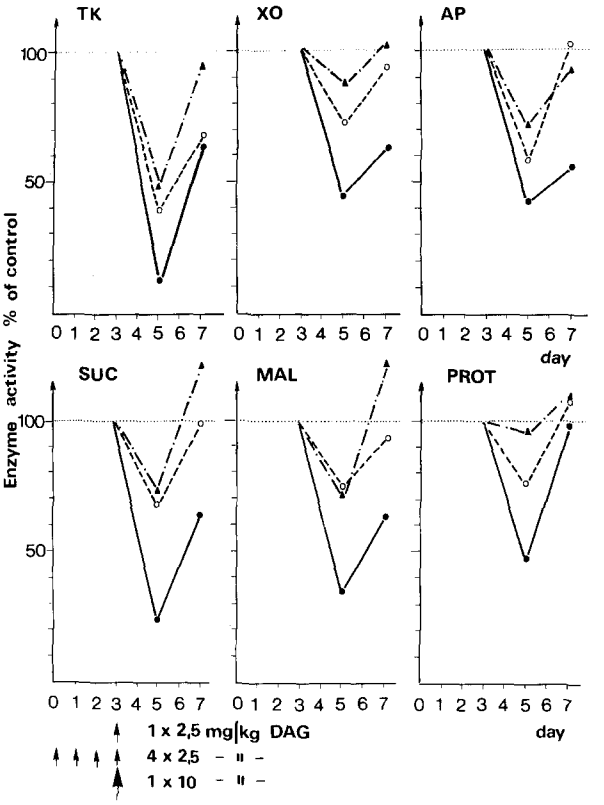

Fig. 3. Enzyme activity changes in rat intestinal mucosa cells caused by different dose schedules of DAG: (●——●) 1 × 10 mg/kg DAG on day 3; (▲–·–·–▲) 1 × 2.5 mg/kg DAG on the day 3; (○– – –○), 4 × 2.5 mg/kg DAG on days 0–3. Animals were killed on the days 5 and 7, i.e., 48 and 96 h after the last dose of DAG (mean values of four animals are shown)

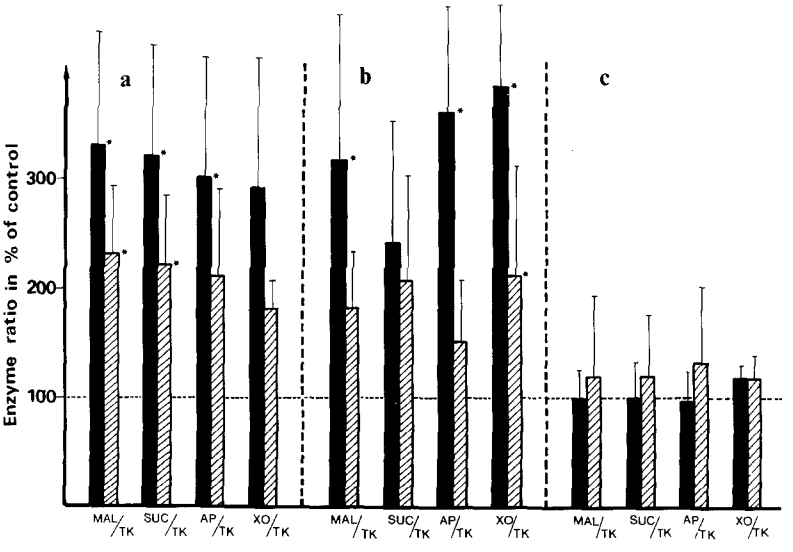

Fig. 4. Changes in the enzyme ratios 24 h (**a**), 48 h (**b**), and 96 h (**c**) after DAG treatment. They were calculated as follows:
$$\frac{\text{enzyme ratio of the treated animal}}{\text{enzyme ratio of the control animal}} \times 100 .$$
Mean values ± SD of four animals are expressed. × = significantly different from normal control ($P < 0.05$)

injection caused more serious enzyme inhibition and reduced regeneration.

DAG influenced the various intestinal enzymes to different extents; the level of TK, for example, declined much more markedly than that of ALP. Therefore, not only the activities of the different enzymes but also the enzyme ratios changed during the treatment. Biochemical regeneration of the mucosa means normalization of both the enzyme activities and enzyme ratios.

All enzyme ratios differed from the control ratios as soon as 24 h after the last dose of DAG in animals treated with a single high dose or repeated small doses. By 48 h after treatment the difference between enzyme ratios of animals treated according to different dose schedules of DAG became more pronounced. Enzyme quotients approached the normal level 96 h after treatment despite the decreased enzyme values found in the group treated with a single high dose of DAG (Fig. 4).

Discussion

The influence of various anticancer drugs, in particular antimitotics and antimetabolites, on the small intestinal mucosal enzymes has been studied extensively [1, 4, 11, 13, 18]; however, only sporadic results are available on the effects of alkylating agents. The morphological and functional disturbances caused by cyclophosphamide have been presented by several authors [8, 20, 23, 26]. The early signs of crypt cell damage, i.e., reduction in the number of cells in crypt columns and slow fall in mitotic index, appear 12 h after the administration of 100 mg/kg cyclophosphamide. At this time no changes are seen in the activity of different brush border enzymes and the in vivo absorption of galactose [8]. These changes cannot be expected until 24–48 h after the treatment at the earliest, as up to then the differentiated epithelium can maintain its function.

In a preliminary study from our laboratory the time course of the intestinal damage and regeneration was determined after the administration of 5 mg/kg DAG. The nadir of the inhibition of TK was found at 24 h but the activity remained low even at 48 h. The maximum inhibition of the functional enzymes was not observed until 48 h after DAG injection. The recovery of all enzymes was completed at 96 h. The morphologic changes closely followed the alteration of enzyme values. However, histologic regeneration was not yet complete by the time normalization of the enzyme activities was achieved [22].

In the present work a 4-day course with daily doses of 2.5 mg/kg DAG was selected, which caused marked tumor regression in various animal tumor models. The equivalent total dose of 10 mg/kg, if given on a single occasion, proved toxic or lethal to the animals [19].

The time of maximal inhibition was not influenced either by the dose or by the schedule of administration. The extent of enzyme inhibition and morphologic damage is clearly dependent on the daily and not on the cumulative dose (Figs. 1–3). On the other hand, the rate of regeneration is related to the dose applied. After 2.5 or 5 mg/kg DAG it is completed in 96 h, while after 10 mg/kg more days are needed. A single large dose of DAG damages crypt cells much more than repeated small doses, and consequently the compensatory increase of DNA synthesis, as judged from the TK activity, is much larger after high-dose treatment. Nevertheless, the replacement of the large number of lost enterocytes takes longer than after repeated doses. It is thus very likely that during prolonged treatment the production and maturation of villus cells are not completely inhibited but proceed at a slower rate.

The changes in the ratio of brush border enzymes to TK indicate the functional site of action of DAG within the intestinal epithelium. At first only the function of crypt cells is interfered with, as judged by the low TK and almost intact brush border enzyme activities. The latter are not affected until 48 h after treatment, while the inhibition of TK deepens. As a result, the enzyme ratios show a progressive increase up to 48 h and a very quick normalization due to the rapid increase of TK activity and the sluggish brush border enzyme regeneration. This dynamics indicates that DAG interferes with the replacement of specialized cells by blocking the division of crypt cells. Since 48 h are needed for the production of mature enterocytes [9], the time course of the damage of the brush border enzymes provides further support for the indirect nature of the drug action. Most of the cytostatic agents and low-dose X-ray treatment have a similar effect on the small intestine [6].

Alkylating agents are preferably given in a high-dose intermittent schedule, which is less toxic to the bone marrow. Comparing high-dose intermittent and 5-day schedules of DAG treatment, Hernadi et al. [14] found that the number of CFU_c (colony forming units in culture) is much lower in the bone marrow 24 h after a large dose than following repeated administration. Regeneration is complete in a week after either treatment but the number of CFU_c oscillates below the control value for a long time after repeated doses. On the other hand, treatment with small repeated doses is of significant advantage in the gut, as it does not lead to pronounced disruption of the specific cellular organization and enzyme pattern. Consequently functional damage is probably also lower. Both schedules were found to produce similar antitumor effects in Walker ascites carcinoma [24], but the therapeutic index was higher after repeated doses because of the rare loss of animals due to gastrointestinal toxicity. In fact the LD_{50} of DAG can be doubled if the dose is divided into four equal parts. In summary, we can conclude that the fractionated dosage of DAG can reduce toxic side-effects on the intestinal mucosa with no loss of antitumor effect.

Acknowledgements. We wish to thank Miss Márta Kovács and Mrs Judit Kutvölgyi for their excellent technical assistance, Mrs Mária Olasz for treatment and care of animals, and Mrs Judit Bodi for typing the manuscript.

References

1. Achord JL (1969) The effect of methotrexate on rabbit intestinal mucosal enzymes and flora. Am J Dig Dis 14: 315
2. Bessey OA, Lowry OH, Brock MI (1965) Determination in serum with *p*-nitrophenylphosphate. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie, Weinheim/Academic Press, New York London, p 783
3. Chiuten DF, Rozencweig M, von Hoff DD, Muggia FM (1981) Clinical trials with hexitol derivatives in the US. Cancer 47: 447
4. Cohen MI, McNamara H (1970) The effect of colchicine on guinea pig intestinal enzyme activity. Am J Dig Dis 15: 247
5. Dahlquist A (1968) Assay of intestinal disaccharidases. Anal Biochem 22: 99
6. De Both NJ, Plaisier H (1974) The influence of changing cell kinetics on functional differentiation in the small intestine of the rat. J Histochem Cytochem 22: 352

7. Eagan RT, Moertel CG, Hahn RG, Schutt AJ (1976) Phase I study of a five-day intermittent schedule for 1,2:5,6-dianhydrogalactitol (NSC-132313). J Natl Cancer Inst 56: 179
8. Ecknauer R (1976) Morphology and enzyme activity in rat small intestinal epithelium 6 and 12 h after an alkylating agent. Acta Hepatogastroenterol (Stuttg) 23: 365
9. Ecknauer R, Rommel K (1978) Zytostatika und Dünndarm. Klin Wochenschr 56: 579
10. Espana P, Wiernik PH, Walker MD (1978) Phase II study of dianhydrogalactitol in malignant glioma. Cancer Treat Rep 62: 1199
11. Grand RJ, Chong OA, Isselbacher KJ (1971) Intracellular processing of disaccharidases. The effect of actinomycin D. Biochim Biophys Acta 261: 341
12. Haas CD, Stephens RL, Hollister M, Hoogstraten B (1976) Phase I evaluation of dianhydrogalactitol (NSC-132313). Cancer Treat Rep 60: 611
13. Hartwich G (1974) Side-effects of a cytostatic treatment on the gastrointestinal tract. Acta Hepatogastroenterol (Stuttg) 21: 89
14. Hernádi F, Kovács P, Ujj G, Kerpel-Fronius S, Somfai-Relle S (1980) Effect of repeated doses of dianhydrogalactitol on murine bone marrow colony-forming units in culture. In: Nelson JD, Grassi C (eds) Current chemotherapy and infectious diseases, vol 2. American Society for Microbiology, Washington, p 1608
15. Klemperer C, Haynes J (1968) Thymidine kinase in rat liver during development. Biochem J 108: 541
16. Kralovánszky J, Prajda N, Kerpel-Fronius S, Szentirmay Z (1981) Biological model system for investigating gastrointestinal side-effects caused by cytostatic agents. In: Mózsik Gy, Hänninen O, Jávor T (eds) Gastrointestinal defence mechanisms. Pergamon Press, London/Akadémiai Kiadó, Budapest, p 327
17. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurment with the Folin phenol reagent. J Biol Chem 193: 265
18. Marano R, Pastore G, Manghisi O, Schiraldi O (1969) Effect of aminopterin and antibiotics on disaccharidase activity of the rat small bowel. Experientia 25: 1284
19. Németh L, Institóris L, Somfai S, Gál F, Pályi I, Sugár J, Csuka O, Szentirmay Z, Kellner B (1972) Pharmacologic and antitumor effect of 1,2:5,6-dianhydrogalactitol (NSC 132313). Cancer Chemother Rep 56: 593
20. Palme G, Liss E, Wiebel F (1964) Autoradiographische Untersuchungen über das Verhalten einzelner Interphasenabschnitte des Dünndarmepithels der Maus unter der Einwirkung von Cyclophosphamid. Naturwissenschaften 51: 197
21. Prajda N, Weber G (1975) Malignant transformation-linked imbalance: Decreased xanthine oxidase activity in hepatomas. FEBS Lett 59: 245
22. Prajda N, Kralovánszky J, Kerpel-Fronius S, Gál F (1981) Effect of dianhydrogalactitol on biochemical and morphological characteristics of the rat intestinal mucosa. In: Mózsik Gy, Hänninen O, Jávor T (eds) Gastrointestinal defence mechanisms. Pergamon Press, London/Akadémiai Kiadó, Budapest, p 339
23. Rommel K, Naupert Ch, Grimmel K (1969) Wirkung von Cyclophosphamid (Endoxan) auf die Disaccharidasenaktivität der Dünndarmschleimhaut der Ratte. Z Gastroenterol 7: 474
24. Somfai-Relle S, Németh L (1982) Cumulation of toxic effects of DBD. In: Eckhardt S (ed) Dibromodulcitol. Medicina Press, Budapest, p 38
25. Vogel CL, Winton EF, Moore MR, Sohner S (1976) Phase I trial of dianhydrogalactitol administered i.v. in a weekly schedule. Cancer Treat Rep 60: 895
26. Waldeck HH (1972) Die Dünndarmschleimhaut nach hohen Dosen Cyclophosphamid. Morphologische und histochemische Untersuchungen beim Goldhamster. Z Gastroenterol 10: 543
27. Weiser MM (1973) Intestinal epithelial cell surface membrane glycoprotein synthesis. J Biol Chem 248: 2536

Received March 20, 1983/Accepted August 3, 1983