CHAPTER 3

INFLUENCE OF GASTROINTESTINAL POLYAMINES ON THE CYTOSTATIC EFFECTS OF DFMO.


Janine

3.1 Microbial flora in the gastrointestinal tract abolishes cytostatic effects of α-difluoromethylornithine *in vivo*

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Summary

Although treatment with the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO) leads to depletion of intracellular polyamines and to related growth inhibition in vitro, its cytostatic effects in vivo are disappointing. This may be due to abolition of the DFMO induced growth inhibition by polyamines released during normal body cell turnover, to dietary polyamines, or to putrescine synthesized by the microbial flora in the GI tract. We studied selectively (aerobic) and totally (aerobic + anaerobic) GI-tract decontaminated L1210-bearing mice fed 3 three types of diet differing in their polyamine and carbohydrate residue contents and treated with combinations of intraperitoneal DFMO and oral deuterium-labelled putrescine. Our data show that, irrespective of diet type, total decontamination markedly potentiates the moderate tumor growth inhibition that is caused by DFMO alone. During total decontamination, growth-inhibited L1210 cells accumulate in the G_0/G_1 phase of the cell cycle. Although orally administered deuterium-labelled putrescine gave rise to deuterium labelling of L1210 putrescine, spermidine and spermine, the polyamine levels in our diets played only a minor role.

Introduction

Several studies of eukaryotic cells in culture indicate an essential role for the polyamines putrescine (Pu), spermidine (Sd) and spermine (Sp) in cellular growth.[1,2] This makes polyamine metabolism a potential target for cancer chemotherapy.

DL-*α*-difluoromethylornithine (DFMO) is a specific irreversible inhibitor of ornithine decarboxylase (ODC), which catalysis the first step in polyamine biosynthesis.[3] In a number of cultured cell types, treatment with DFMO leads to depletion of Pu and Sd and a connected growth inhibition.[4,5] The DFMO-induced growth inhibition *in vitro* prompted a number of investigators to study its effect in tumor-bearing animal models[1,5,6] and cancer patients.[7,8] However, although some cytostatic effects of DFMO were observed *in vivo*, overall results have been quite disappointing.

The reasons for the lack of *in vivo* activity of DFMO are unclear at present time. It is usually attributed to poor cellular uptake, rapid renal clearance[9] or compensatory increase of both ODC and the other key enzyme in polyamine metabolism, S-adenosylmethionine decarboxylase.[10]

All these hypotheses assume insufficient intracellular DFMO concentrations, and thus incomplete ODC inhibition. It is also possible, however, that, despite complete ODC inhibition, the cytostatic effect of DFMO is abolished by the availability of exogenous polyamines. The importance of exogenous polyamines is supported by the observations that polyamine uptake is increased during cell proliferation[11] and polyamine depletion[12,13], and that mutant L1210 cells deficient in polyamine uptake are highly sensitive to DFMO.[14]

No studies have been reported on possible origins of exogenous polyamines, and thus on the growth-inhibitory effects of DFMO if their sources are eliminated. Some possible sources are: polyamines liberated by normal body cell turnover, dietary polyamines and Pu produced in the GI tract by extracellular aminoacid decarboxylases from microbial origin.[15,16] The GI tract is a potential source of Pu and cadaverine (Cad), as previously demonstrated by the inhibition of diamine oxidase with methylglyoxal bis(guanylhydrazone) or aminoguanidine, leading to

reversal of DFMO-induced polyamine depletion and appearance of Cad in tumor cells[17,18] and increased excretion of Cad in the urine.[19] However, it is generally believed that, under conditions of uninhibited diamine oxidase activity, polyamines from the GI tract are not utilized. We have investigated the effect of GI tract decontamination of healthy persons on the excretion of polyamines in their urine. The results showed a dramatic decrease in total Cad, indicating that under normal conditions it is not only produced by the microbial flora, but that it is subsequently absorbed and transported through the body.[20]

The objective of our present study was to investigate the contributions of dietary and microbially synthesized polyamines in the presumed abolition of the cytostatic effect of DFMO *in vivo*. We made use of selectively and totally decontaminated L1210-bearing mice fed with 3 diets differing in their polyamine contents and treatment with combinations of intraperitoneal DFMO and oral deuterium labelled Pu. A preliminary report of this work has recently been presented.[21]

Animals, materials and methods

Animals

Specified-pathogen-free, 3 months-old female DBA/2 mice, initial body weight 18-22 g (Centrum für Versuchstiere, Hannover, FRG), were used throughout the study. Except in experiments including total intestinal tract decontamination, animals were housed under standard laboratory conditions in plastic cages with free access to standard "RMH-B" or "purified" diet and acidified water (pH 2.5-3.0). In the case of total decontamination mice were kept under isolation conditions in sterile cages with sterile sawdust bedding. They had access autoclaved standard "SRM-A" or "purified" diet (both pelleted) and sterile water supplemented with antibiotics.

Experimental tumor model

L1210 leukemia was maintained *in vivo* by weekly i.p. inoculation of 10^5 tumor cells into DBA/2 mice, for a maximum of 10 passages. The mice used for the experiments were inoculated i.p. with 10^5 cells (harvested from the donor at day 7) per animal in 0.2 ml sterile phosphate-buffered saline (PBS; 8.0 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4 and 0.2 g/l KH_2PO_4; pH 7.4). The day of inoculation was designated as day 0. Mice used for the experiments were killed between days 3 and 7 by cervical dislocation, 2 to 3 hr after the last DFMO injection.

Sample collection

L1210 cells were collected by peritoneal lavage with 2 x 10 ml ice-cold PBS. The resulting cell suspension was adjusted to 40 ml and cell density determined by counting in a cell Counter (type Dn, Coulter Electronics, UK).

Twenty-four-hour mouse faecal samples were collected from the sawdust bedding and stored at -20 °C until analysis.

Drug regimen and diets

DFMO (kindly donated by the Merrell Dow Research Institute, Cincinnati, OH) was administered at a dosage of 500 mg/kg every 12 hr by i.p. injection, starting 6 hr after tumor-cell inoculation.

Selective decontamination[22] was performed by oral administration of a 2 g/l aqueous solution of arginine-free aztreonam (kindly donated by Squibb, Rijswijk, The Netherlands) as the sole drinking fluid, beginning 7 days before tumor cell inoculation.

Total decontamination was carried out by oral administration of a combination of a nonabsorbable cephalosporin and an aminoglycoside[23]; *i.e.* cefamandol (Eli Lilly, Amsterdam, The Netherlands) and kanamycin (Gist-Brocades, Delft, The Netherlands). Each antibiotic was given at a concentration of 2 g/l in the drinking water from 7 days before tumor-cell inoculation. In addition 1 g/l of amphotericin-B (Squibb) was added to suppress potential growth of yeasts and fungi.

Putrescine-d4 [Pu-d4; $H_2NCD_2CH_2CH_2CD_2NH_2$] was prepared by catalytic reduction of succinonitrile with deuterium gas.[24] Pu-d4 was administered orally *via* drinking water, beginning immediately after tumor inoculation. The Pu-d4 concentration was 2.6 mmol/l in experiment IB and both 0.5 and 2.6 mmol/l in experiments IIA and IIB.

Mice were normally fed *ad libitum* with RMH-B standard diet. The SRM-A and purified diets were supplied at least 2 weeks before tumor inoculation. The nutritional composition of the purified diet was: 20 % casein, 68 % carbohydrates and 5 % soybean oil. The diet was supplemented with minerals, dl-methionine, choline chloride, standard vitamin mix and trace element mix. All diets were similar with respect to their macro-nutrient proportion, but different to the source. The polyamine and carbohydrate compositions of the diets are given in Table I. All diets were purchased from Hope Farms (Woerden, The Netherlands).

Analytical methods

Bacteriological cultures

As a check on the effect of antibiotic treatment, fresh faecal samples were collected twice a week from 2 mice in each cage. The samples were suspended in 10 ml brain-heart infusion broth (Difco, Detroit, MI) and incubated under aerobic conditions at 37 °C. Cultures were monitored by visual inspection for at least 3 days before being recorded as sterile.

Table I. Polyamine content and carbohydrate composition of diets.

Diet	Polyamine levels[1] (nmol/g)				Carbohydrate composition[2] (g/100g)		
	Pu	Cad	Sd	Sp	Starch	Cellulose	Mono sacharides
SRM-A	1,150	1,540	180	70	34	21	4
RMH-B	180	50	80	30	38	25	4
Purified	6	_	5	5	10	5	53

[1]Polyamine levels were determined by gas chromatography with nitrogen-phosphorus detection after acid hydrolysis. [2]As stated by the manufacturers.

Relative caecal weights (RCW)

After killing of the mice and aspiration of the peritoneal tumor cells, the caecum and its contents were collected and weighed. The relative caecal weight is defined as a percentage of the total body weight, and used as a measure of total decontamination.[25]

Volatile fatty acids

Aliquots of faeces (200-400 mg) were homogenized and the volatile fatty acids (acetic, propionic and butyric acids) were isolated by vacuum distillation.[26] The volatile fatty acids were determined by fused megabore column gas chromatography with flame ionization detection.[27]

Polyamines

The polyamines 1,3-diaminopropane, Pu, Cad, Sd and Sp in L1210 cells and faeces were determined by capillary gas chromatography with nitrogen-phosphorus detection.[28] For the measurement of polyamines in cells, L1210 suspensions were deproteinized with sulphosalicylic acid. Polyamines in the supernatant were isolated by adsorption onto silica and converted into their heptafluorobutyryl (HFB) derivatives. For measurement of faecal polyamines 50-100 mg aliquots of faeces were hydrolyzed in 6 M HCl, as previously described for urine.[29] Isolation and derivatization were carried out in the same way as for L1210 cells.

Polyamine deuterium-enrichment

After oral administration of Pu-d4 the enrichment of deuterium polyamines in L1210 cells was determined by gas chromatography-mass spectrometry (GC/MS). GC/MS was performed with a Hewlett Packard 5890 gas chromatograph directly coupled to a Trio-II mass spectrometer (VG Instruments, Manchester, UK), operated under the following conditions: injector temperature 250 °C; oven temperature program 110 °C, 10 °C/min to 300 °C; ion source temperature 150 °C; ionization energy 70 eV. The samples were

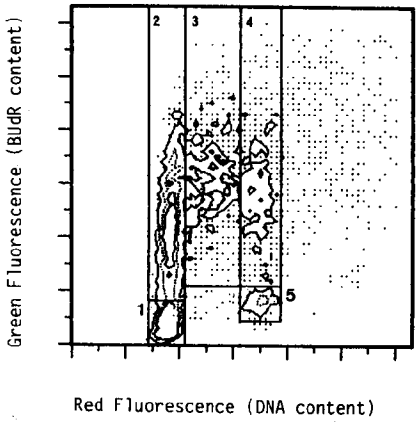
monitored in the electron impact mode on the positive ions m/z 226 and 228, corresponding with the $[HFB\text{-}NH{=}CH_2]^+$ fragment of naturally occurring Pu and the $[HFB\text{-}NH{=}CD_2]^+$ fragment of deuterated Pu. In the case of Sd and Sp, the ions at 536/540 and 576/580, corresponding with the $[M \text{ minus } HFB]^+$ fragments and the $[M \text{ minus } HFB, \text{ minus } NH_2\text{-}HFB]^+$ fragments, respectively, were monitored. The peak area ratios 228/226, 540/536 and 580/576 at the retention times corresponding to Pu, Sd and Sp, respectively, were calculated using the VG 11-250 data system, and used as a measure of deuterium enrichment.

Cell cycle analysis

in vivo BUdR labelling. Thirty minutes before cervical dislocation, mice were injected i.p. with 1.25 mg 5-bromo-2'-deoxyuridine (BUdR; Janssen, Beerse, Belgium) per mouse in 0.2 ml PBS. Approximately 5 x 10^6 L1210 cells were washed once with PBS and fixed with 70 % ice-cold ethanol.)

Cytochemistry. Cells were pelleted from ethanol and their DNA was partially denaturated by resuspension in 2 ml 2.7 M HCl (containing 0.5 % Triton X-100) at 20 °C for 20 min. The staining procedure for BUdR incorporation and DNA content was essentially the same as that described by Dolbeare *et al.*[30] Briefly, the procedure involves staining of BUdR, incorporated by S-phase cells, by labelling it with an indirect immunofluorescent technique, using a monoclonal anti-BUdR antibody (Becton-Dickinson, Montain View, CA, USA) and a fluorescein-isothiocyanate-conjugated (FITC) goat antimouse IgG antibody (Sigma, St Louis, MO, USA). Double stranded DNA was stained with propidium iodide (Sigma)

Flow cytometry. Cell cycle analyses were performed with a Becton-Dickinson FACSstar flow cytometer. A detailed description of techniques of cell cycle analysis have recently been reviewed by Gray and Darzynkiewicz.[31] Routinely, 10,000 cells were analyzed at a flow rate of about 200 cells/sec. Cells were excited at 488 nm (300 mW). Red fluorescence



Series of 5 boxes on each bivariate distribution was set, representing: 1, G_0/G_1-phase cells; 2, early S-phase cells; 3, mid S-phase cells; 4, late S-phase cells; 5, G_2/M-phase cells.

Figure 1. *BUdR/DNA contour-plot distribution of L1210 cells labelled in vivo with BUdR for 30 min.*

from propidium iodide was collected through a 630/30 nm band pass filter and its intensity was used as a measure for total DNA. Green fluorescence from FITC was collected through a 530/30 nm band pass filter and used as a measure of the amount of incorporated BUdR. Both signals were collected using linear amplification. The resulting bivariate DNA/BUdR distributions were displayed in contour plots (Fig. 1).

Data processing. Using a computer program (Consort 30, Becton and Dickinson) we set the following series of five boxes (Fig. 1) in each bivariate distribution, essentially as described by Pallavicini *et al.*[32]: (1) G_0/G_1-phase cells: a region containing all cells with 2n DNA content and no BUdR incorporation; (2) early S-phase cells: a region containing BUdR labelled cells with G_0/G_1 DNA content; (3) mid S-phase cells: a region containing BUdR labelled cells with an intermediate DNA content; (4) late S-phase cells: a region containing BUdR labelled cells and 4n DNA content; (5) G_2/M-phase cells: a region containing unlabelled cells with 4n DNA content.

Experimental protocol

Three separate experiments were carried out (numbered: I, II, III). In each experiment 60 mice were divided into 4 equal groups, 2 of which formed the controls (A), while the other 2 were treated with DFMO (B). From days 3 to 7 3 mice from each group were killed daily. The experimental protocol is depicted in table II. In addition, 8 mice were included in groups IB, IIA and IIB for treatment with different concentrations Pu-d4 in drinking water (see "drug regimen and diets").

Results

Parameters for selective and total decontamination

Figure 2 shows the faecal concentrations of polyamines and volatile fatty acids for groups of mice fed different diets and treated by different decontaminating regimens. Feeding with the SRM-A or RMH-B diets led to similar excretion levels of volatile fatty acids (bars 1 and 3), whereas levels for mice fed the purified diet were considerably lower (bars 5). Selective decontamination notably reduced the levels of propionic and butyric acids (bars 4 *vs.* 3 and bars 6 *vs.* 5). The lowest faecal levels of all 3 volatile fatty acids were achieved during total decontamination (bars 2 and 7), regardless of the type of diet.

Mice fed the purified diet had a lower RCW than those fed the SRM-A diet (Table III). Total decontamination consistently led to an increase in the RCW of mice fed the SRM-A diet, and to a lesser extent, to an increase in RCW in the case of the purified diet (Table III).

Table II. Schematic representation of treatments used in experiments I, II and III.

Experiment	Group	DFMO[1]	Diets[2]			Decontamination[3]	
			RMH-B	Purified	SRM-A	Selective	Total
I	A1		+				
	A2		+			+	
	B1	+	+				
	B2	+	+			+	
II	A1			+			
	A2			+		+	
	B1	+		+			
	B2	+		+		+	
III	A1				+		+
	A2			+			+
	B1	+			+		+
	B2	+		+			+

[1]DFMO, 500 mg/kg i.p. twice daily; [2]Mean daily consumption of the diets: RMH-B (3.7 g), purified (2.6 g), SRM-A (3.5 g). Mean daily fluid intakes were: RMH-B (3.9 ml), purified (2.7 ml), SRM-A (3.8 ml). [3]Selective decontamination was performed by 2 g/l aztreonam in the drinking water. Total decontamination was performed by 2 g/l cefamandole, 2g/l kanamycin and 1 g/l amphotericin-B in the drinking water.

Culture experiments did not reveal the presence of aerobic bacteria in the faeces of selectively and totally decontaminated mice.

L1210 tumor growth curve, cell cycle distribution and intracellular polyamine concentration studied under various conditions.

(a) Influence of DFMO and selective decontamination on L1210 in mice fed the RMH-B diet

Selective decontamination of mice fed the RMH-B diet did not show striking differences in L1210 growth characteristics (Fig. 3, IA), nor in their polyamine content (Fig. 4, IA).

Treatment with DFMO led to moderate L1210 growth inhibition and a moderate decrease (about 9 %) of the total number S-phase cells at days

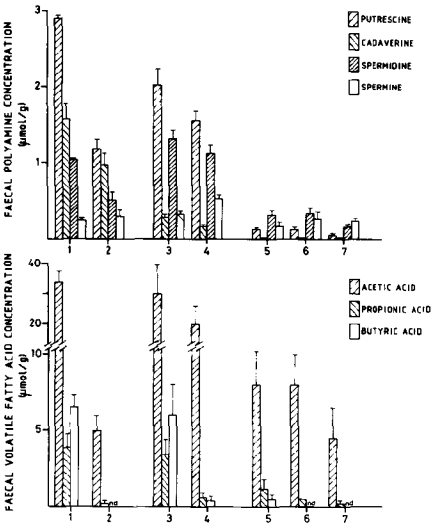


Figure 2. *Faecal polyamine (top) and volatile fatty acid (bottom) concentrations of mice fed different diets and treated by different decontaminating regimens.*
1, fed SRM diet; 2, fed SRM diet and totally decontaminated; 3, fed RMH-B diet; 4, fed RMH-B diet and selectively decontaminated; 5, fed purified diet; 6, fed purified diet and selectively decontaminated; 7, fed purified diet and totally decontaminated.

6 and 7 (Fig. 3, IB). The latter could be attributed to a fall in the number of mid S phase cells (Fig. 5). Intracellular polyamine contents of the DFMO treated groups showed the well known partial depletion of Pu and Sd, and an increase in Sp (Fig. 4, IB). Selective decontamination did not potentiate the moderate tumor-growth-inhibitory effect of DFMO, nor did it change the percentage S-phase cells (Fig. 3, IB), or the intracellular polyamine contents (Fig. 4, IB).

(b) Influence of DFMO and selective decontamination on L1210 in mice fed purified diet

Selective decontamination of mice fed purified diet did not alter L1210 growth characteristics (Fig. 3, IIA), or their polyamine content (Fig. 4, IIA).

Table III. Relative caecal weights (RCW) of L1210 bearing mice fed SRM or purified diets, with and without total decontamination[1].

Diet	Decontamination	n	RCW (%)	SD (%)
SRM-A	-	4	1.8[2,4]	0.3
SRM-A	Total	12	6.3[2]	1.1
Purified	-	4	1.3[3,4]	0.2
Purified	Total	12	2.2[3]	0.3

[1]Purified diet and antibiotics for total decontamination (see "Material and Methods") were administered from 2 weeks and 1 week before tumor inoculation, respectively. RCW, defined as the caecum weight with content expressed as a percentage of the body weight, was determined from days 4 to 7 after tumor inoculation and was independent of tumor growth. n, number of mice; S.D. standard deviation. [2]$p < 0.01$; [3]$p < 0.01$; [4]$p < 0.05$ (unpaired Student's-t-test with a 2-sided probability).

Treatment with DFMO showed similar changes in L1210 growth and L1210 polyamine content (Figs 3 and 4, IIA *vs.* IIB) as previously noted for mice fed the RMH-B diet (Figs 3 and 4, IA *vs.* IB). However, at day 7, the number of S-phase cells in the DFMO-treated group was significantly increased from 46 to 82 % compared with those in the DFMO non-treated group. The observed rise of the number of S-phase cells at day 7 could totally be attributed to an increase in early S-phase cells (Fig. 5).

There were no striking differences between growth parameters and intracellular polyamine contents of L1210 in mice treated with the combination of selective decontamination and DFMO and those treated with DFMO alone (Figs 3 and 4, IIB).

(c) Influence of the purified diet, SRM-A diet and DFMO on L1210 in totally decontaminated mice

Total decontamination, regardless of diet, did not induce striking alterations in L1210 growth characteristics (Fig. 3, IIIA *vs.* IA) or L1210 polyamine contents (Fig. 4, IIIA *vs.* IA).

Regardless of the diet type, treatment of totally decontaminated mice with DFMO strongly inhibited L1210 growth and strongly reduced the number of S-phase cells (Fig. 3, panel IIIB *vs.* IIIA). When compared with the DFMO non-treated controls, the decrease in total number of S-phase

cells could for the greater part be attributed to a fall in the percentage mid S-phase cells from 35 % to 17 % at days 5 and 6 (Fig. 5), and to an increase in G_0/G_1 phase cells from 36 % to 62 %. The intracellular Sd concentration dropped below values previously noted for the other DFMO-treated groups (Fig. 4, IIIB *vs.* IIB and IB). There was no compensatory increase in intracellular Sp as a consequence of DFMO treatment (Fig. 4, IIIB *vs.* IIIA).

Effect of oral Pu-d4 administration on L1210 tumor growth, DFMO-induced growth inhibition, and deuterated polyamine enrichment in L1210 cells

With Pu concentrations in the SRM-A, RMH-B and purified diets of 1150, 180 and 6 nmol/g (Table 1) and a measured daily food intake of about 3.5, 3.7 and 2.6 g/mouse of the respective diets, we estimated an average daily Pu intake of 4.0, 0.6 and 0.02 μmol/mouse via these diets, respectively. With Pu-d4 concentrations of 2.6 (IB), 2.6 (IIA/IIB) and 0.5 (IIA/IIB) mmol/l in the drinking water and an average daily intake of about 3.9 , 2.7 and 2.7 ml of the Pu-d4 solutions, we estimated a daily Pu-d4

Explanatory notes to figures 3 and 4.
Panels A: Animals not treated with DFMO, panels B: animals treated i.p. with DFMO twice daily (500 mg/kg). Panel I: mice fed RMH-B diet (——); ●, no additional treatment; ○, selectively decontaminated with 2 g/l aztreonam in drinking water from 7 days before tumor inoculation; ▲, 10 μmol/mouse/day Pu-d_4 administered via drinking water from day 0. Panel II: mice fed purified diet from 2 weeks before tumor inoculation (---); ●, no additional treatment; ○, selectively decontaminated as described above; ▲, 6,5 μmol/mouse/day Pu-d4 (panels IIA and IIB) administered via drinking water from day 0. Panel III: totally decontaminated mice fed purified diet (---) or SRM diet (——); □. Total decontamination was performed with 2 g/l kanamycin, 2 g/l cefamandole and 1 g/l amphotericin B in drinking water from 7 days before tumor inoculation. Each point represents the mean of 3 determinations on separate animals; vertical bars represent standard deviations.

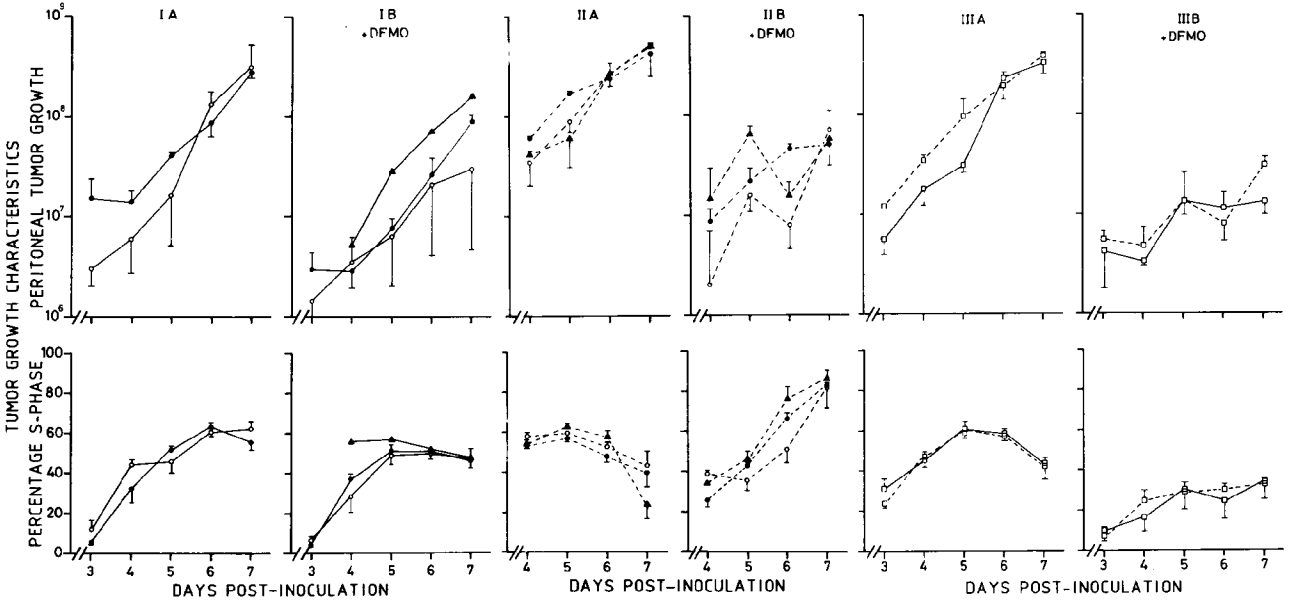


Figure 3. *Peritoneal L1210 growth and percentage S-phase cells in DBA/2 mice.*

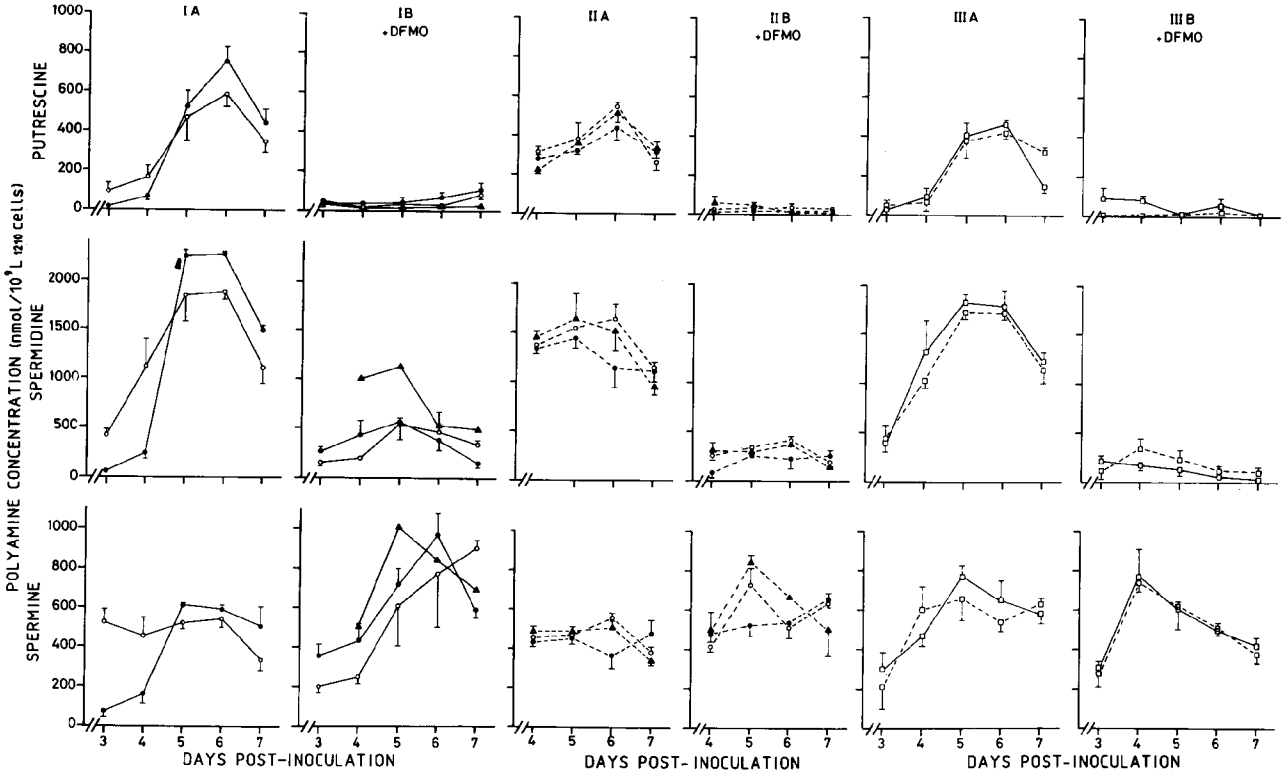


Figure 4. Intracellular putrescine, spermidine and spermine concentrations in L1210 cells.

intake of 10.0, 6.5 and 1.3 μmol/mouse, respectively.

Daily oral administration of 1.3 μmol Pu-d4/mouse to L1210-bearing mice fed the purified diet did not lead to detectable deuterium labelling of L1210 polyamines, whereas the additional treatment with DFMO did (Table IV).

The same experiments conducted with 6.5 μmol Pu-d4/day led to considerably higher deuterium labelling in both L1210 and faeces, but did not affect L1210 growth characteristics (Fig. 3, IIA and IIB), or intracellular polyamine concentrations (Fig. 4, IIA and IIB). In this instance, the highest deuterium enrichment of L1210 and faecal polyamines were achieved during treatment with DFMO.

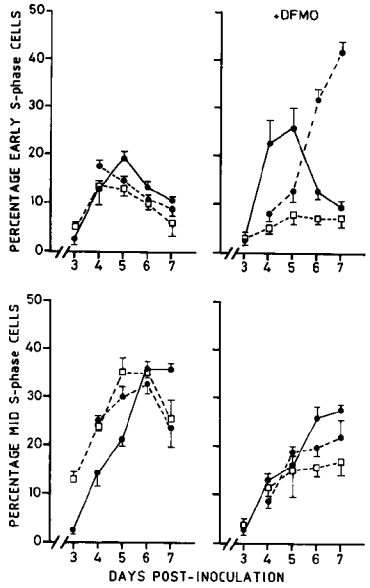


Figure 5. *Distribution of percentages early- and mid S-phase L1210 cells during growth in DBA/2 mice.*

Left panel: mice not treated with DFMO; right panel: mice treated with DFMO. Animals were fed RMH-B (●——●), purified diet (●---●) or purified diet in combination with total decontamination (□---□).

L1210 bearing mice fed the RMH-B diet and treated with DFMO together with an oral dose of 10 μmol Pu-d4/mouse daily, equivalent to

Table IV. Measurement for deuterium enrichment of polyamines in L1210 cells and faeces after oral administration of tetradeuterated putrescine (Pu-d_4) to L1210 bearing mice.

Experimental conditions		Daily Pu-d_4 intake[2]	Deuterium enrichment of polyamines[3]			
			In L1210 cells			In faeces
Diet	DFMO[1]	μmol/mouse	Pu-d_4/Pu-d_0	Sd-d_4/Sd-d_0	Sp-d_4/Sp-d_0	Pu-d_4/Pu-d_0
Purified	-	1.3	ND	ND	ND	NM
Purified	+	1.3	0.04 ± 0.02	0.06 ± 0.02	0.08 ± 0.01	0.18 ± 0.04
Purified	-	6.5	0.04 ± 0.02	0.08 ± 0.01	0.08 ± 0.03	0.75 ± 0.02
Purified	+	6.5	0.22 ± 0.07	0.25 ± 0.04	0.27 ± 0.02	1.22 ± 0.05
RMH-B	+	10	0.18 ± 0.02	0.37 ± 0.05	0.32 ± 0.09	0.03 ± 0.02

[1]DFMO was injected i.p. twice daily at a dose of 500 mg/kg. [2]Estimated average daily Pu-d_4 intake from days 0 to 7. Pu-d_4 was administered via the drinking water, starting immediately after tumor inoculation. [3]The Polyamine-d_4/polyamine-d_0 ratios (mean ± S.D., n=8) were measured from days 3 to 7 and were independent of the time after tumor inoculation. N.M: not measured, N.D.: not detectable.

2.5 times the daily Pu intake via SRM-A diet, showed relatively high deuterium labelling of Sd and Sp in L1210 in particular (Table IV) and a partial reversal of DFMO- induced growth inhibition (Fig. 3, IB).

Polyamines in faeces

Faecal polyamine excretion proved highly dependent on the type of diet (Fig. 2, bars 1, 3 and 5), and seemed related to its polyamine content (Table I).

Both selective (bars 4 *vs.* 3) and total decontamination (bars 2 *vs.* 1) reduced the faecal excretion of Pu, Cad and Sd, but not of Sp, in the case of mice fed the RMH-B and SRM-A diets, respectively.

The very low faecal polyamine levels of mice fed the purified diet were not further reduced by selective decontamination (bars 6 *vs.* 5), whereas total decontamination led to a fall in faecal Pu and Cad to nearly undetectable levels (bars 7 *vs.* 5). Sp levels were not systematically affected by any of the experimental conditions.

Discussion

Parameters for selective and total decontamination

Bacterial fermentation of residual carbohydrates in the colon leads to the production of volatile fatty acids. Although more than 90% of the produced volatile fatty acids are subsequently absorbed, considerable quantities eventually reach the faeces.[33] The decrease of faecal volatile fatty acids during selective (aerobic) decontamination and the sharp drop of faecal volatile fatty acids during total (aerobic and anaerobic) decontamination (Fig. 2) may therefore be expected to correspond to a successful partial and total removal of the intestinal flora, respectively. The much lower faecal volatile fatty acid levels in mice fed purified diet are most probably due to the high absorbability of its carbohydrates and consequently to the low amount of carbohydrate residues (incompletely digested starch and cellulose; Table I), which eventually enter the colon. This results in a low supply of fermentation substrates to the bacterial flora.

The increase in RCW to 6.3 % (Table III) during total decontamination of mice fed the SRM-A diet is in good agreement with the RCW of 6.5 % for germ-free mice fed the same diet[25], and therefore confirms successful decontamination. The smaller initial RCW for mice fed purified diet has been described by Koopman et al.[34] The moderate increase of the RCW of this group during total decontamination confirms the high absorbability of the carbohydrates in this type of diet.

Effect of DFMO under various conditions

The impressive increase in the number of early S phase cells at day 7 during DFMO treatment of mice fed purified diet, but not with RMH-B diet, reflects a seriously retarded progression through this cell-cycle phase. The outcome of this effect is, however, poorly reflected in the corresponding L1210 growth curves. The origin of such delayed S-phase progression should be sought in differences between the compositions of the purified and the RMH-B diets. Table I shows 2 striking differences, *i.e.* a considerably higher polyamine content and a potentially higher residual carbohydrate content of the RMH-B diet. Theoretically, both dietary polyamines (directly) and residual carbohydrates (indirectly) may abolish DFMO-induced growth inhibition.

Influence of dietary polyamines

The relatively low deuterium enrichment of polyamines in L1210 cells and faeces following oral administration of Pu-d4 (Table IV) shows that a small proportion of the dietary Pu is absorbed in the GI tract and made available to L1210 cells. Additional treatment with DFMO leads to higher deuterium enrichment of Pu, Sd and Sp in L1210 cells. This may be ascribed to a DFMO-induced enhancement of polyamine uptake from exogenous sources[12] and a DFMO-induced depletion of intracellular, naturally occurring, polyamines, but not a DFMO induced downregulation of intestinal diamine oxidase activity.[17]

Attempts to abolish DFMO-induced growth inhibition by oral administration of Pu-d4 showed that a daily dosage of 10 μmol, but not 6.5 or 1.3 μmol, was able to accomplish this goal. However, the dosage of 10 μmol proved to be well beyond the estimated daily consumption of naturally occurring Pu from either of the diets in our study. The minor influence of dietary Pu in the abolition of a DFMO-induced growth inhibition was confirmed in the experiment on totally decontaminated mice (Fig. 3, IIIB), which shows equally strong L1210 growth inhibition, irrespective of whether mice were fed SRM-A diet (4 μmol Pu daily) or the purified diet (0.02 μmol Pu daily). We therefore conclude that the polyamines in the diets were not major contributors to the relatively poor L1210 growth-inhibitory effects of DFMO in experiments I and II.

Influence of dietary residual carbohydrates

The substantially reduced excretion of faecal volatile fatty acids by mice fed purified diet (Fig. 2), and the concomitant reduction of faecal polyamine excretion, suggest a reduction in the number of intestinal bacteria and/or decreased fermentation activity. It is conceivable that the low carbohydrate residue of this diet that may be expected to reach the colon deprives the bacterial flora of a carbohydrate source, resulting in decreased volatile fatty acid production and excretion. As a consequence of this markedly reduced volatile fatty acid production, a moderate drop in pH may take place. Therefore there does not seem to be any need for the bacterial flora to adjust their environmental pH by the induction of biodegradative arginine, ornithine and lysine decarboxylases, which finally

produce Pu and Cad.[15,16] The suggested close association between faecal volatile fatty acid and polyamine levels is illustrated in Fig. 2. An additional source of faecal polyamines is certainly the diet itself: (1) orally administered Pu-d4 is recovered from faeces (Table IV), (2) total decontamination of mice fed SRM-A diet (high in polyamines) leads to faecal polyamine levels higher than those of mice fed purified diet (low in polyamines) (Fig. 2).

Totally decontaminated L1210 bearing mice treated with DFMO (Fig. 3, IIIB) showed that abolition of DFMO-induced growth inhibition is related to the presence of bacteria in the intestine. As recently shown by us[20] in humans, Cad produced by intestinal bacteria is absorbed and excreted in urine. It is therefore conceivable that also Pu, especially that produced by intestinal bacteria to counteract a drop in environmental pH due to fermentation of residual carbohydrates, is also able to enter the body and to subsequently abolish DFMO-induced growth inhibition. This hypothesis is supported by the following observations (1) DFMO has a curative effect on mutant L1210 cells that are deficient in polyamine uptake[34] and (2) Sarhan *et al.*[35] have observed a prevention of Lewis lung carcinoma growth and a marked prolongation of the average life span of L1210 bearing mice on combined treatment with GI tract decontamination, ODC inhibition (DFMO) and polyamine oxidase inhibition (MDL 72527).

Since total decontamination of cancer patients is not feasible, other approaches for eliminating GI microbial production of polyamines (notably Pu), absorption of polyamines in the GI tract (notably the colon), or uptake of polyamines by tumor cells may need to be investigated. Theoretical possibilities include total parenteral nutrition, oral feeding with diets essentially free of carbohydrate residues, oral administration of non-absorbable inhibitors of bacterial arginine and ornithine decarboxylases, and oral administration of absorbable or non-absorbable polyamine transport inhibitors.

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3.2 Growth inhibition of two solid tumors in mice caused by polyamine depletion is not attended by alterations in cell cycle phase distribution.

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Summary

We studied the effect of polyamine depletion on growth and cell cycle characteristics of subcutaneously grown Lewis lung carcinoma (LLC) and fibrosarcoma (FIO 26) in mice. Polyamine depletion was achieved by inhibition of ornithine decarboxylase using 2-(difluoromethyl)ornithine, limitation of exogenous polyamines by administration of polyamine poor diet, decontamination of the gastrointestinal tract, and inhibition of endogenous polyamine reutilization by N,N'-bis-(2,3-butadienyl)putrescine (MDL 72527). Determination of S-phase cells was performed in tumor cell suspensions by flowcytometry and in tumor tissue sections by microscopy, following in vivo labelling with 5-bromo-2'-deoxyuridine (BUdR). DNA synthesis rate was estimated from the incorporation of in vivo injected [^{3}H]-thymidine ([^{3}H]-TdR). Both solid tumors almost completely stopped growing after access to polyamines was blocked. Growth inhibition was, however, not attended by changes in cell cycle phase distribution. Paradoxically, we measured increased in vivo [^{3}H]-TdR incorporation rates and unaltered BUdR-linked staining intensity in treated tumors. Injection of putrescine into treated LLC bearing mice resulted in an increase of intracellular putrescine and spermidine concentrations, a slight increase in the number of S-phase cells and a marked drop in DNA synthesis rate within the following 9 hours.

Introduction

The naturally occurring polyamines putrescine, spermidine and spermine are ubiquitous in eukaryotic cells. Several studies indicated that polyamines are required for optimal rates of DNA synthesis and therefore play a role in cell proliferation.[1,2] The oscillations of polyamines during the cell cycle, especially the peak in G_1, suggest that one of their roles might be the traverse of cells through G_1 into S phase.[3-5]

Treatment with DL-2-(difluoromethyl)ornithine (DFMO), a potent and specific inactivator of ornithine decarboxylase (ODC)[6], inhibits growth of cultured tumor cells as a consequence of the resulting polyamine depletion.[7-9] Some tumor cells were shown to accumulate in G_1[3,10,11], while others accumulated in S and/or G_2 phase.[9,12,13] In some instances of ODC inhibition prolongation of the cell cycle time was observed, without changes in cell cycle distribution.[14,15] It was concluded that the effect of DFMO on cell cycle traverse depends on the cell line studied.[9]

Attempts to inhibit tumor cell growth in tumor bearing animals by treatment with DFMO gave frequently disappointing results.[9,16,17] However, we demonstrated recently that limitation of exogenous polyamine sources by feeding polyamine deficient chow and treatment with antibiotics for gastrointestinal tract decontamination, strongly potentiates the cytostatic effect of DFMO in both L1210[18] and Lewis lung carcinoma (LLC) bearing mice.[19,20] This growth inhibition was attended by accumulation of L1210 cells in G_1 phase of the cell cycle.[18] Inhibition of polyamine interconversion[21] by additional treatment with a polyamine oxidase inhibitor N,N'-bis(2,3-butadienyl)putrescine (MDL 72527)[22] resulted in an even more pronounced growth inhibition of LLC tumors.[19,23]

The objective of our study is to investigate the mechanism of growth inhibition following polyamine deprivation in LLC and fibrosarcoma (FIO 26) tumors in mice in relation to changes in cell cycle characteristics. During growth inhibition and its subsequent abolition by putrescine administration, we measured DNA synthesis rates by determining [^{3}H]-TdR incorporation and S-phase cells by 5-bromo-2'-deoxyuridine (BUdR) incorporation using flowcytometry and microscopy.

Animals, materials and methods

Animals, diets and treatment

Female, 3 months-old C57BL mice, weighing 19 ± 2 g, were used throughout the study. Mice used for experiment I were from Charles River (St. Aubin-les Elbeuf, France) and for experiments II from Centrum für Versuchstiere (Hannover, Germany). The animals were housed under standard laboratory conditions with 12 h-light, 12 h-dark cycle and had free access to water and standard rodent chow, unless otherwise stated.

Polyamine-deficient chow used for experiment I was prepared as previously described.[20] For decontamination of the gastrointestinal (GI) tract it contained 200 mg neomycin and 3.4 mg metronidazole (both from Sigma Chemical Co., St. Louis, USA). This diet is henceforward denoted "PDC". Part of this diet also contained 3 g DFMO[24] and 50 mg MDL 72527[22] per 100 g diet as ODC and polyamine oxidase inhibitors, respectively (PDC I). For experiment II polyamine-deficient chow was obtained from Hope Farms (Woerden, The Netherlands). The composition of the diet has previously been described.[18] For GI tract decontamination it was supplemented with 200 mg kanamycin and 200 mg cephamandol per 100 g diet (PDC). Part of this diet was supplemented with 3 % DFMO as ODC inhibitor (PDC II). In some cases DFMO was administered via drinking water (3 % solution) and MDL 72527 by i.p. injection (25 mg/kg per day).

Tumor models

LLC spontaneously originated as a carcinoma of the lung in a C57BL/6 mouse[25] and was subsequently propagated *in vivo* by serial passages in the same strain. The s.c. grown LLC is characterized by formation of lung metastases and by necrosis formation after the tumor has reached a certain size.

A fibrosarcoma was originally induced in C57BL mice by skin painting with methylcholanthrene.[26] A cell line with fibroblast-like morphology was derived from this solid tumor and designated as FIO 26. In contrast to LLC, FIO 26 is a non-metastasizing tumor that becomes much less necrotic at the same tumor volume. A specimen of this tumor, conserved in liquid nitrogen, was obtained from the Department of Radiopathology of the University of Groningen, and maintained by s.c. passage in C57BL mice.[27] For both tumors growth was monitored by measuring their cross sections using vernier callipers.[28]

Preparation of tumor cell suspension for transplantation

Cell suspensions of LLC and FIO 26 tumors were prepared by mechanical dispersion of tumor tissue as follows. Tissue was cut with scissors into small pieces, which were gently dissociated using a layer of nylon gauze, supported by a metal gauze, and at the same time rinsed with 20 ml ice cold Hanks' solution (7.40 g NaCl, 0.20 g KCl, 0.05 g KCl, 0.18 g $MgCl_2.6H2O$, 0.75 g $Na_2HPO_4.2H_2O$, 0.05 g KH_2PO_4 and 1.00 g glucose in 1 l distilled water; pH adjusted to 7.35). After centrifugation at 4 °C for 10 min. at 800 g the cell pellet was resuspended in ice cold Hanks' solution. Viable cells were counted using trypan

blue exclusion. A total of 4 x 10^6 viable cells in 0.1 ml Hanks' solution were injected s.c. in the right thigh of each mouse. The day of inoculation was designated as day 0.

Animal experiments

Experiment I: cell cycle kinetics of LLC during polyamine depletion and subsequent putrescine administration

Experimental design. A group of 42 C57BL mice received PDC (not containing DFMO or MDL 72527) from 7 days before tumor-cell inoculation in order to accustom them to the diet. A control group, consisting of 12 mice, received standard diet throughout. All mice were injected s.c. with 4 x 10^6 viable LLC cells in the right thigh. At day 4, when tumors became palpable, the treatment group received PDC I. At day 8 all mice of the treatment group were injected i.p. with putrescine (30 mg/kg in 0.2 ml PBS). Time of injection was designated as "t = 0 h". To prevent its catabolism, aminoguanidine (20 mg/kg in 0.2 ml PBS; i.p.) (Sigma) was administered one hour before putrescine injection. Mice of the treatment group were killed at t = 0, 1, 2, 3, 4, 6 and 9 hr, and mice of the control group at t = 0 and 9 hr. At each time tumors were collected from six mice, of which 3 mice had received [^{3}H]-TdR (70 Ci/mmol; 20 μCi/mouse in 0.1 ml PBS; s.c.) (NEN, Dreieich, Germany) and 3 mice BUdR (2.50 mg/mouse in 0.1 ml PBS; i.p.) (Janssen, Beerse, Belgium) 1 hr prior to death.

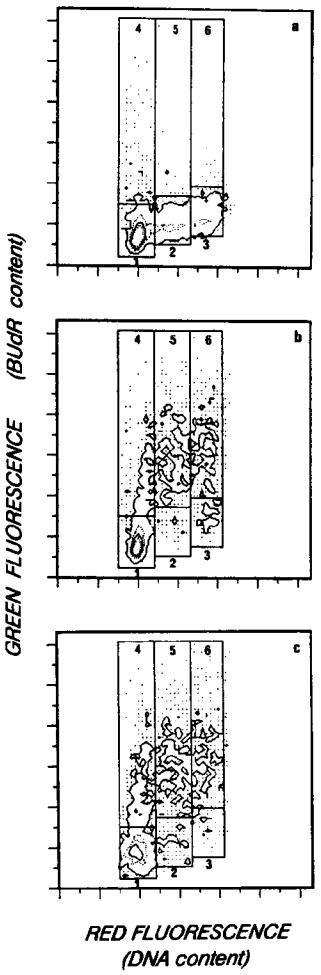
Analytical methods. The tumors labelled with [^{3}H]-TdR were homogenized in 10 vol ice cold 0.2 M $HClO_4$. After centrifugation and 1:10 dilution of the perchloric acid extracts, polyamines were determined by a reversed-phase ion-pair high performance liquid chromatographic method.[29] DNA was separated from RNA and proteins by a version of the method of Schmidt-Thannhauser.[30] DNA was determined based on the deoxyribose method of Burton.[31] Thymidine content of perchloric acid extracts was determined with a high performance liquid chromatographic method with UV detection (254 nm).[32] A pre-purification step was introduced by application of 1 ml neutralized perchloric acid extract on a small Dowex 50 WX8 column (0.5 ml) and elution with 1 ml 0.2 M HClO4. Radioactivity levels of the perchloric acid extracts were determined with a liquid scintillation counter. Cell cycle analyses of BUdR-labelled LLC tumors were performed as described for L1210 cells.[18] Briefly, single-cell suspensions from the tumors were washed once with Hanks' solution and fixed in 5 ml ice cold 70 % ethanol. About 5 x 10^6 cells were used for analysis of cell cycle distribution. The pretreatment procedure included partial denaturation of DNA with HCl and staining of the BUdR, incorporated into S-phase cells, by labelling with an indirect immuno-fluorescent technique. For this, we used an anti-BUdR monoclonal antibody (MAb) (Becton-Dickinson, Mountain-View, CA) and a fluorescein-isothiocyanate-conjugated (FITC) goat anti-mouse IgG antibody (Sigma). Double stranded DNA was stained with propidium iodide (Sigma). The staining procedure was essentially the same as described by Dolbeare *et al.*[33] Measurements were performed with a Becton Dickinson FACSstar flow cytometer. Cells were excited at 488 nm (300mW). The intensity of the red fluorescence from propidium iodide, collected through a 630/30 nm band pass filter, was used as a measure of total DNA. That of green fluorescence from FITC was collected through a 530/30 nm band pass filter and used as a measure of BUdR incorporation. The resulting bivariate

DNA/BUdR distributions were displayed in contour plots (Fig. 1). In these plots we set series of 6 boxes using a computer program (Consort 30, Becton Dickinson). Boxes 1-3 are regions without BUdR incorporation: [1] G_0/G_1-phase cells (2n DNA content); [2] unlabelled S-phase cells (intermediate DNA content); [3] G_2/M-phase cells (4n DNA content). Boxes 4-6 are regions with BUdR incorporation and therefore designated as S-phase cells: [4] early S-phase cells (2n DNA content); [5] mid S-phase cells (intermediate DNA content); [6] G_2/M-phase cells (4n DNA content). To ascertain that the green fluorescence represents specific labelling of incorporated BUdR, cells were also treated according to standard staining procedure without addition of anti-BUdR antibody (Fig. 1a)

Experiment II: cell-cycle kinetics and tumor growth of FIO 26 during polyamine depletion

Experimental design. A group of 19 C57BL mice were fed PDC without DFMO 7 days before FIO 26 tumor cell inoculation. From 5 days after inoculation, when tumors were palpable, they were fed with PDC II. Four mice of this group were, in addition, injected daily with 25 mg/kg MDL 72527 in 0.2 ml PBS (i.p.) from days 8 to 12. Eight tumor-bearing mice received standard diet. Four mice of this group received no treatment (control group) and 4 were treated by administration of a 3 % solution of DFMO as the sole drinking fluid from day 5 on. At day 12, 4 mice of each group were killed in order to measure polyamine content and percentage S-phase cells with both flowcytometry in single-cell suspensions and by microscopy in tissue sections. For the measurement of polyamine content and S-phase cells the mice were injected i.p. with 2.5 mg BUdR/mouse in 0.1 ml PBS, one hour prior to decapitation. Tumor growth was monitored by measuring tumor cross section at various days. From day 12 on tumor growth of the 11 remaining mice of the PDC II group was monitored until their average tumor cross-section was comparable with those of the control group at day 12.

Analytical methods. To quantitate and localize BUdR incorporation in whole tissue, BUdR staining was performed on 4 μm frozen sections of the FIO 26 tumor, according to the method of Harms *et al.*[34] BUdR was visualized with peroxidase-conjugated rabbit anti-mouse antibody (Dakopatts, Copenhagen, Denmark) against anti-BUdR mouse MAb, using 3-amino-9ethyl-carbazole (AEC, EGA-Chemie, Steinheim/Albuch, FRG) as a chromogen. The nuclei were counterstained with celestin blue 66 (Gurr, BDH Chemicals Ltd, Poole, Dorset) and Mayer's haematoxylin (Sigma). The number of S-phase cells (labelling-index) was determined by microscopy as the number of labelled nuclei per thousand counted nuclei x 100 (%). Another part of the tumor tissue was mechanically dispersed, the cells fixed in ice cold 70 % ethanol and used for cell cycle analysis with flowcytometry as described above. The remaining part of the tumor was homogenized in 6 % sulphosalicylic acid together with 1,6-diaminohexane, monoacetyl-1,6-diaminohexane, N-(3-aminopropyl)1,5-diaminopentane and N,N'-bis(3-aminopropyl)-1,5-diaminopentane as internal standards (25 nmol each per 50 mg tissue) and used for polyamine measurements. Polyamine content was determined by gas chromatography with nitrogen/phosphorus detection.[35]



Cells were labelled *in vivo* with BUdR for 60 min. DNA content was measured with propidiumiodide (red fluorescence) and BUdR content is measured with anti-BUdR antibody/FITC (green fluorescence). *(a)* LLC cells (from untreated C57BL mice) stained without anti-BUdR antibody; *(b)* Cells from the same sample stained with anti-BUdR antibody; *(c)* LLC cells (from mice treated for 4 days with PDC I) stained with anti-BUdR antibody. Series of 6 boxes were set in each plot, representing: 1, G_0/G_1-phase cells; 2, unlabelled S-phase cells; 3, G_2/M-phase cells; 4, early S-phase cells; 5, mid S-phase cells; 6, late S-phase cells.

Figure 1. *Contour plots of bivariate DNA/BUdR distribution measured for LLC cells.*

TABLE I. Cell cycle phase distribution of LLC cells from tumors of untreated mice, PDC I treated mice and PDC I treated mice following putrescine administration.

	LLC cell-cycle phase distribution (%)					
treatment	G_0/G_1	early-S	Mid-S	Late-S	G_2/M	Unlabelled-S
Control	31 ± 2	17 ± 1	24 ± 3	18 ± 3	3.8 ± 0.6	5.7 ± 0.9
PDC I	27 ± 2	18 ± 1	24 ± 2	20 ± 1	5.7 ± 0.4	5.3 ± 1.0
1 hr^1	23 ± 3	23 ± 1	24 ± 3	21 ± 1	4.2 ± 0.6	4.7 ± 0.4
2 hr^1	25 ± 2	21 ± 2	24 ± 2	19 ± 3	4.9 ± 0.2	6.8 ± 2.0
3 hr^1	24 ± 1	19 ± 2	22 ± 1	25 ± 2	5.5 ± 0.4	5.6 ± 1.4
4 hr^1	23 ± 2	22 ± 3	25 ± 1	21 ± 2	4.0 ± 0.1	5.4 ± 1.8
6 hr^1	26 ± 3	24 ± 4	23 ± 4	18 ± 2	4.5 ± 1.0	5.3 ± 1.9
9 hr^1	32 ± 1	16 ± 3	19 ± 2	20 ± 1	6.7 ± 1.5	5.9 ± 0.3

Data represent means ± S.D. for 3 (treatment groups) or 6 (control group) animals. Treatment groups and their drug schedules are specified in the legend of Fig 2. Cell cycle phase distributions were determined by flowcytometry, using measurement of cellular DNA content (propidium iodide) and DNA incorporated BUdR content, as described in Materials and Methods (see also Fig. 1). ^{1}; time after putrescine administration (30 mg/kg; i.p.) to PDC I treated mice.

Results

Experiment I: cell-cycle kinetics of LLC during polyamine depletion and subsequent putrescine administration.

Four days after inoculation, the LLC tumor cross-section amounted to 27 ± 9 mm^2 and 20 ± 3 mm^2 for the control (n = 12) and the treatment (n = 42) groups, respectively. Although these differences are statistically significant (P = 0.015; Mann-Whitney U-test) further tumor growth was not affected by polyamine deficient chow containing antibiotics.[19] At day 7, after 3 days of treatment with PDC I, the tumor cross-sections were 59 ± 20 mm^2 and 22 ± 5 mm^2, respectively (p = 0.001). As tumor polyamine content and cell cycle characteristics for the control group at t = 0 and 9 hr showed no differences, these data were taken together and designated as control values (Table I and Fig. 2).

Figure 1 shows representative plots of bivariate DNA/BUdR distributions of LLC cells. To determine the height of the boxes for unlabelled cells, ethanol-fixed LLC cells were treated according to the standard staining procedure without addition of anti-BUdR antibody (Fig. 1a). In practice the boxes were set in such a manner that unlabelled cells (boxes 1-3) were optimally separated from cells exhibiting specific labelling against BUdR (S phase cells; boxes 4-6). The position of the boxes were kept constant within one series. The data show that LLC cells from both untreated tumors (Fig 1b) and PDC I treated tumors (Fig 1c) contain considerable numbers of S-phase cells. Almost all S-phase cells incorporated BUdR. The fraction of unlabelled S-phase cells amounted to 5-7 % (Table I). Table I shows that there were no striking differences in LLC cell-cycle-phase distributions between control and PDC I treated tumors. Administration of putrescine (30 mg/kg) resulted in a small decrease of G_0/G_1-phase cells and a concomitant increase of early and late S-phase cells within 1 hr. Nine hours afterwards cell-cycle distribution parameters returned to near control values.

Treatment with PDC I resulted in a 3-fold increase in relative [^{3}H]-TdR labelling. There were no marked differences in the percentage of total number of S-phase cells, when compared with control values; 62 ± 2 (n=3) and 59 ± 2 (n=6), respectively (Fig. 2). Administration of putrescine resulted in an increase of putrescine content in tumor tissue within 1 hr after injection and a concomitant increase of spermidine at 4 hr. However, it did not restore spermidine levels. One hour after putrescine administration the percentage of total S-phase cells increased from 62 ± 2 to 68 ± 3 and returned below pretreatment values after 9 hours (55 ± 2). [^{3}H]-TdR labelling gradually decreased as a function of time after putrescine injection (Fig. 2)

There was no difference in the radioactivity of the acid soluble [^{3}H]-TdR nucleoside/nucleotide pool between the control and the treatment groups; 5539 ± 529 (n=6) and 5463 ± 319 DPM/ml extract (n=21), respectively. These radioactivity levels were independent from the time after putrescine administration. Moreover, there was no difference in the radioactivity of the acid soluble fraction, expressed in terms of pmol thymidine: 16.1 ± 3.2 (control) and 19.1 ± 4.1 DPM/pmol thymidine

(treatment group), respectively. The DNA content of the LLC tumors, expressed in nmol deoxyribose per g wet tissue, at day 8 was 201 ± 42 (n = 6) for the control group and 166 ± 58 (n = 21) for the treatment group. This was not a statistically significant difference (Mann-Whitney U-test).

Experiment II: cell-cycle kinetics and tumor growth of FIO 26 during polyamine depletion.

Treatment of FIO 26-bearing mice with PDC II from day 5 on resulted in an impressive growth inhibition, whereas feeding with standard diet and treatment with DFMO alone had no growth-inhibitory effect (Fig. 3). Cross-section of tumors from the PDC II treated group at day 32 was comparable with that of the control group at day 12. However, mice of the former group were in poor health at this time. Additional treatment with MDL 72527 from day 8 on resulted in further polyamine depletion (Table II) and growth inhibition (Fig. 3). On day 12 the percentage S-phase cells in this group was not changed, and the number of S-phase cells measured by flowcytometry (DNA/BUdR distributions) did not differ from that measured by microscopy (BUdR staining) (Table II).

TABLE II. FIO 26 tumor cross section, polyamine content and cell cycle characteristics after polyamine depletion by various treatments.

Treatment	Cross-section (mm^2)	Polyamine content (nmol/g tissue)			S-phase cells (%)	
		Pu	Sd	Sp	Flow Cytometry	Microscopy
None	117 ± 13	439 ± 109	1360 ± 191	552 ± 79	36.1 ± 2.6	39.2 ± 6.2
PDC II	29 ± 21	16 ± 2	160 ± 16	777 ± 60	NM	NM
PDC II + MDL 72527	16 ± 4	5 ± 1	52 ± 7	426 ± 93	38.7 ± 3.5	41.9 ± 4.6

Data represent means ± s.d. of 4 animals, sacrificed at day 12 after FIO 26 tumor cell inoculation. Treatment groups and their drug schedules are specified in the legend of Fig. 2. NM: not measured.

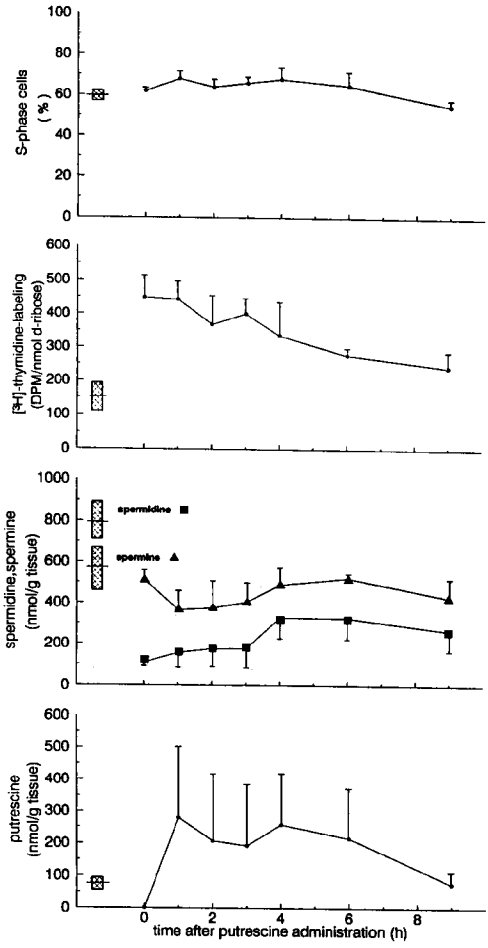


Figure 2. *Percentage S-phase, [^{3}H]-TdR labelling and polyamine content of growth inhibited LLC tumors following putrescine administration.*

LLC bearing mice (n = 42), fed polyamine deficient chow and antibiotics, were treated with DFMO and MDL 72527 from 4 days after tumor inoculation. After 4 days of treatment, when tumor growth was nearly completely inhibited, mice were injected with putrescine (30 mg/kg, i.p.) and killed at t = 0, 1, 2, 3, 4, 6 and 9 h. Each time-point represents the values (mean ± s.d.) for 3 mice. Shaded blocks represent the values (mean ± s.d.) for 6 untreated LLC bearing mice sacrificed at t = 0 and 9 h. Percentage S-phase cells is the sum of early, mid and late S-phase cells given in Table 1.

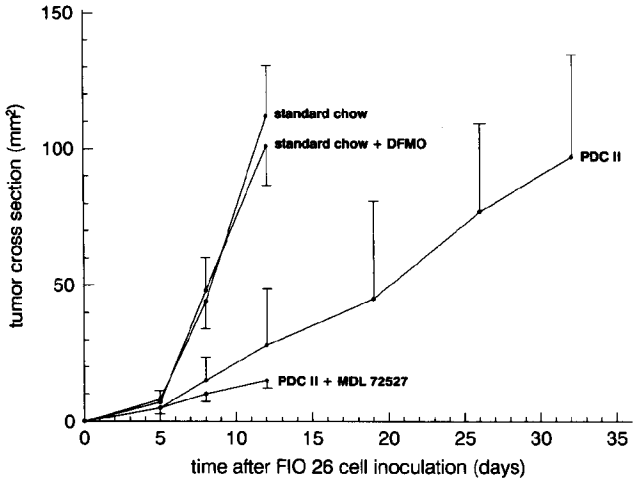


Figure 3. *Growth of fibrosarcoma (FIO 26) tumors in C57BL mice.*
Mice were fed standard chow (n=4); standard chow with DFMO (n=4); PDC with antibiotics and DFMO (PDC II) (n = 15; n = 11 from day 12 on); PDC II with MDL 72527 (n = 4). PDC II treatment was started from 5 days after tumor inoculation. For mice fed standard chow, DFMO was administered by offering a 3 % solution as the sole drinking fluid. MDL 72527 was injected i.p. (25 mg/kg) from days 8 to 12. At day 12, 4 mice of each group were killed for measurement of polyamine content and percentage S-phase (see Table II).

Discussion

From the present study it is obvious that polyamine deprivation applied to the FIO 26-bearing mice results in a similar growth-inhibitory effect to that described for the LLC-bearing mice.[20] However, after 32 days of treatment with PDC II the mice were in poor health, in agreement with previous observations after treatment with PDC I.[36] The reasons for the toxicity are at present unknown.

The DFMO-induced growth inhibition of L1210 cells is caused by their accumulation in the G_0/G_1-phase of the cell cycle.[18] In contrast with ascitic L1210 cells, none of the two solid tumors, LLC and FIO 26, showed any changes in the cell-cycle distribution (Table I), even though no net tumor growth was observed. The high number of S-phase cells, as measured with DNA/BUdR distributions by flowcytometry, cannot be

ascribed to selective destruction of G_1-phase cells during mechanical dispersion of the tumor, since similar results were obtained by BUdR-microscopy in tissue sections (Table II). There are several potential explanations for the apparently contradictory finding of unaltered cell-cycle distribution and effective growth inhibition.

(I): Polyamine depletion may cause excessive prolongation of the total cell- cycle time, with unchanged cell-cycle phase distribution.

Such a phenomenon has previously been described for Chinese hamster ovary cells during polyamine depletion *in vitro*.[15] A slow progression through the cell cycle is, however, accompanied by a decreased DNA replication rate. This is not consistent with the observed increase in [^{3}H]-TdR incorporation rate in PDC I-treated LLC tumors. The increase in [^{3}H]-TdR incorporation rate was reversed by administration of putrescine. This argues in favour of a relationship between this phenomenon and polyamine depletion.

Theoretically, it is possible that in non-treated LLC tumors the measured [^{3}H]-TdR incorporation is too low, as a consequence of tumor necrosis. Necrotic tissue is obviously not incorporating [^{3}H]-TdR, but still contains a considerable amount of DNA debris. This may be derived from the comparable levels of deoxyribose in treated and non-treated tumors, despite apparently less necrosis in the former. A decreased DNA replication rate should, however, be accompanied by a decrease in BUdR incorporation rate. BUdR linked fluorescence intensity is related with the BUdR content, although this is not a linear relationship for HCl-denaturated cells.[37] We found a similar, and in some instances even increased BUdR staining intensity in LLC cells from growth inhibited tumors employing both flowcytometry (Fig. 1) and microscopy (data not shown). We therefore conclude that a higher number of dead cells in non-treated tumors cannot explain relatively high [^{3}H]-TdR incorporation in treated tumors.

It should also be considered that the increased [^{3}H]-TdR incorporation in polyamine depleted cells is caused by a higher specific activity of the intracellular thymidine pool. Pitfalls in the use of [^{3}H]-TdR for measurements of cell proliferation kinetics are reviewed by Maurer.[38] The

pool of thymidine nucleotides for DNA synthesis is largely derived from endogenous synthesis via the thymidylate synthetase reaction. Exogenous [^{3}H]-TdR as well as BUdR are incorporated exclusively via the salvage pathway, which plays an inferior role in normal DNA biosynthesis. The rate limiting step for [^{3}H]-TdR and BUdR incorporation into DNA is thymidine kinase. Other requirements for incorporation are uptake in the cell via active transport and enzymatic polymerization into DNA. Furthermore, both nucleosides may be susceptible to degradation by catabolic enzymes. Seyfried and Morris[39] showed that polyamine depletion led to suppression of thymidine kinase activity in extracts of phytohemagglutinin-stimulated lymphocytes, probably accompanied by decrease in thymidine uptake. However, to explain the observed increase in [^{3}H]-TdR incorporation in our study as an artefact, the LLC cells would need increased [^{3}H]-TdR uptake and thymidine kinase activity, and/or decreased catabolism of the nucleosides following PDC I treatment. DFMO-induced changes in uptake or catabolism of [^{3}H]-TdR are not supported by our finding of comparable radioactivity levels in the acid soluble fraction (free [^{3}H]-TdR nucleoside/nucleotide pool) of control and treated tumors. From these observations, the possibility of excessively prolonged cell cycle time does not appear to be very likely, but it cannot be excluded with certainty.

(II): Polyamine depletion leads to increased cell-cycle traverse (corresponding with increased [^{3}H]-TdR incorporation), with no change in cell-cycle phase distribution.

As there is no net increase in tumor volume, this implies an equilibrium between cell growth and cell loss. It was previously shown that treatment of cultured SV40 virus-transformed 3T3 fibroblasts with methylglyoxal bis(guanylhydrazone), an inhibitor of S-adenosylmethionine decarboxylase[40], considerably slows the effective growth rate, while the cells progress through the cell cycle at an appreciable rate.[41] The authors suggested a balance between proliferation and cell death, since their cultures contained considerable numbers of floating dead cells.

In our case cell loss may either take place via metastasis formation, immunologically induced enhanced cell death, and spontaneous cell

death. Cell loss due to metastasis is unlikely, since the formation of lung metastases was nearly completely prevented by our treatment of LLC bearing mice.[19,20] Interleukin 2 (IL-2) production by murine lymphocytes is increased after their exposure to DFMO or MDL 72527. This effect was reversed by addition of polyamines.[42,43] Since we used both inhibitors, it seems likely that the immune response against tumor cells was enhanced by macrophage activation.

Indirect evidence for the enhancement of cell death by treatment with PDC I in normal, and especially in tumor bearing, mice comes from the accumulation of spermine in the circulation.[36] Spermine is considered to be an intracellular compound which is released only by non-viable cells. Furthermore, the hypothesis that completely growth inhibited tumors still contain a high number of rapidly proliferating cells is indirectly supported by the observation that the PDC I treated tumors started growing within 1 day after withholding the treatment, at the same rate as the control tumors.[19]

(IIII): The growth of solid tumors, in contrast with ascitic cells, is dependent on angiogenesis.

It seems possible that, following polyamine deprivation, impairment of blood vessel formation is a major reason for the observed differences in growth effects between tumor cells in culture and ascitic cells, on the one hand, and solid tumors on the other. Indeed, DFMO was shown to inhibit B16 melanoma induced angiogenesis in chick embryo chorioallantoic membrane and subsequently the growth of the tumor on this membrane.[44] It can, however, as yet not be excluded that the observed differences in cell kinetics between ascitic tumor cells and solid tumors, following polyamine deprivation, are mere reflections of the accomplished degree of polyamine depletion.

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