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Original Paper

Efficacy of Dinaline and its Methyl and Acetyl Derivatives Against Colorectal Cancer *In Vivo* and *In Vitro*

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Dinaline [4-amino-*N*-(2′-aminophenyl)-benzamide, Din], p-*N*-methyldinaline (Me-Din) and p-*N*-acetyldinaline (Ac-Din) were evaluated for their antineoplastic efficacy in acetoxymethylmethylnitrosamine-induced colorectal carcinomas in Sprague–Dawley rats and in two human colon cancer cell lines. Din was very effective at all dosages (10, 7.7 and 5.9 mg/kg) as indicated by the ratio of median tumour volume of treated and control groups (T/C%) values of 0.4, 16 and 10.6, respectively, but also caused a corresponding mortality of 87, 47 and 13%, respectively, as opposed to 15% in the control group. Me-Din also showed significant tumour growth inhibition at all dosages (13.8, 10.6, 8.2 and 6.2 mg/kg), as evidenced by T/C% values of 2, 5.7, 8.4 and 25, respectively. The corresponding mortality was 47, 20, 27 and 30%, respectively. Ac-Din showed the lowest mortality with 20, 13 and 20% at dosages of 9.1, 7.0 and 5.3 mg/kg, respectively, whereas application of 11.9 mg/kg resulted in 100% mortality. T/C values of 18.3, 11.1 and 21.6%, respectively, demonstrated again high anticancer efficacy. Compared to the combination therapy with 5-FU and leucovorin (25 mg/kg each), p-*N*-acetyldinaline (7.0 mg/kg) was 4-fold more effective as indicated by T/C% values of 81.4 versus 21.9 at similar toxicity. *In vitro*, all three compounds were similarly active with IC_{50} concentrations between 1 and 2.2 μg/ml after 48 h of exposure and 0.6 to 1.6 μg/ml after 72 h of incubation. The MTT dye conversion assay correlated well with cell counts obtained by cell counting except for low dosages after short incubation periods when it stimulated cell proliferation. These results suggest that dinaline and its derivatives have clinical potential. Copyright © 1996 Published by Elsevier Science Ltd

Key words: acetoxymethylmethylnitrosamine-induced colorectal carcinoma, dinaline, MTT-test

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INTRODUCTION

COLORECTAL CARCINOMAS belong to the most frequent malignant neoplasms in the western world, where they are the second most common cancer in both men and women [1]. The current standard treatment of colorectal carcinoma with curative intention is radical surgical resection following standardised operative schemes. The same applies to solitary liver or lung metastases. Nevertheless, almost 50% of surgically treated patients die because of local tumour recurrence or metastatic growth [2]. For this reason, adjuvant chemotherapy has been recommended after extirpation of Dukes' B and C carcinomas (NIH consensus conference 1990). In patients suffering from advanced unresectable colorectal carcinoma, chemotherapy remains the only possibility of obtaining a retardation of tumour progression.

So far, multiple chemotherapeutic regimens have been evaluated, but none have exerted more than marginal therapeutic effect, and the corresponding increase in survival time remains minimal [3]. Currently, only two drug combinations have been established in clinical use: 5-fluorouracil (5-FU) in combination with leucovorin [4] or with levamisole [5].

To overcome this generally disappointing situation, several attempts have been made to select other, more effective

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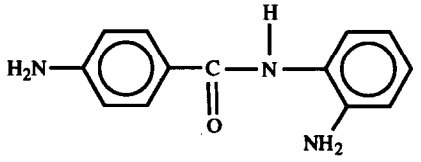
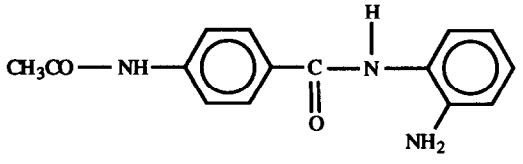
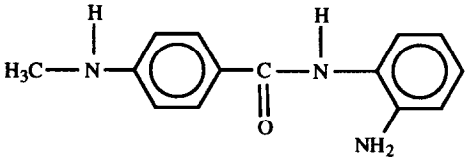
drugs. Our group has recently concentrated on ruthenium- and platinum-derived metal complexes that show antineoplastic activity against chemically induced autochthonous colorectal carcinoma in rats [6, 7]. This tumour model provides an interesting possibility of evaluating antineoplastic compounds, because tumour histology, route of metastasis, original tumour/host interaction, slow growth kinetics, and high resistance against conventionally used chemotherapeutic agents seem to mimic the human situation relatively closely [8]. The model also permits prediction of general side-effects and organ-related toxicity and is used as an instrument to evaluate antineoplastic agents against colorectal carcinoma in an advanced screening system. The information obtained is complementary to data gained *in vitro* which allow an initial characterisation of new compounds and is instrumental in assessing the mechanism of action. In this study, we concentrated on a new group of anticancer compounds, which are derived from a simple ortho-phenylene-diamine structure. The first agent of this group, dinaline (4-amino-*N*-(2′-aminophenyl)-benzamide, Din), has recently been found to exhibit high antineoplastic activity in a series of slowly growing tumours such as chemically induced rat mammary and colorectal carcinomas, osteosarcoma C22LR and Brown Norway myeloid leukaemia. The drug is inactive against many of the typically hypersensitive signal tumours, i.e. mouse leukaemias P388 and L1210, sarcoma 180 and Yoshida sarcoma [9–11]. Subsequent investigations focused on structure–activity relationships of dinaline derivatives. Acetyldinaline has been found to be similarly effective as dinaline in the Brown Norway rat leukaemia [12]. Here, we present the results of *in vivo* and *in vitro* experiments that were performed to compare the therapeutic efficacy and toxicity of dinaline with its methyl- and acetyl-derivatives in colorectal carcinoma.



Dinaline (GOE 1734)



p-*N*-Acetyldinaline (GOE 5549)



p-*N*-Methyldinaline (GOE 4902)

Figure 1. Chemical structures of dinaline [4-amino-*N*-(2′-aminophenyl)-benzamide, Din], p-*N*-methyldinaline (Me-Din) and p-*N*-acetyldinaline (Ac-Din).

MATERIALS AND METHODS

Substances

Acetoxymethylmethylnitrosamine (AMMN) was synthesised [13] and kindly provided by Professor Wiessler, German Cancer Research Centre. 5-Fluorouracil (5-FU) was provided by Medac GmbH (Hamburg, Germany) and leucovorin (LV) by Lederle Company (Wolfratshausen). Dinaline [4-amino-N-(2′-aminophenyl) benzamide, Din], p-*N*-methyldinaline (Me-Din) and p-*N*-acetyl-dinaline (Ac-Din) were supplied by Goedecke AG (Freiburg, Germany) in a purity required for clinical use (Figure 1). The latter three compounds were suspended in 0.8% Methocel (Nordmann-Rasmann, Hamburg, Germany) immediately before application.

Animals and tumour induction

Two hundred and seventy male Sprague–Dawley rats (Charles River Breeding, Sulzfeld, Germany) were purchased at a weight of 140–160 g and thereafter kept under conventional conditions, i.e. two rats per Macrolon III cage, tap water and Altromin pellets *ad libitum*, dark–light cycle of 12 h. Colorectal carcinomas were induced using fresh 0.2% solutions of AMMN in physiological saline: 2 mg/kg was administered intrarectally at weekly intervals for 10 weeks using a rectal tube, the tip of which was inserted up to the colonic flexure.

Diagnosis of tumours and their evaluation

At the beginning of week 5 after completion of the 10-week induction period, the animals were anaesthetised using chloral hydrate (320 mg/kg i.p., diluted in physiological saline). Endoscopic examination of the colon was carefully performed using a paediatric bronchoscope (Olympus 3F, Type 4C2, Olympus Optical, Tokyo; [14, 15]), and animals with evident tumours were randomly allocated to treatment and control groups. Endoscopic examination was repeated at intervals of 2 weeks as long as no macroscopically visible tumour could be detected; this procedure was applied up to four times. Treatment started immediately after diagnosis of tumour occurrence and was continued for 10 weeks. Dinaline and its derivatives were administered orally 5 days per week using a stomach tube whereas 5-FU and LV were injected i.p. and s.c. twice weekly (for experimental design see Table 1). The i.p. injection of 5-FU was chosen because this mode of administration is easier to perform and is as effective as oral application [16].

The animals were sacrificed after 10 weeks of treatment except for animals found in a moribund state which were killed prematurely. Dead animals were dissected and the last 20 cm of the gut removed, opened and weighed. The volume of each tumour was estimated by measuring three diameters according to the formula $a \times b \times c/2$. Results were analysed with the Kruskal–Wallis test [17, 18]. Animals which were treated for less than 8 weeks were excluded from evaluation of therapeutic efficacy, but included for evaluation of toxicity. For all animals, the colorectum and specimens of liver, lung, spleen, kidney, suprarenal gland, testicle, epididymis and bone were fixed in 7% formalin solution and processed for histological examination (Prof. Dr

Table 1. Experimental design

Experiment number	Group number	Animals (*n*)	Compound	Administration route	Single dose (mg/kg)	Single dose (μmol/kg)	Total dose (mg/kg)*
1	1	15	–	–	–	–	–
	2	20	–	–	–	–	–
	3	15	Din	p.o.	10	44	500
	4	15	Din	p.o.	7.7	34	385
	5	15	Din	p.o.	5.9	26	295
	6	5	Ac-Din	p.o.	11.9	44	595
	7	15	Ac-Din	p.o.	9.1	34	455
	8	15	Ac-Din	p.o.	7	26	350
	9	15	Ac-Din	p.o.	5.3	19	265
	10	15	Me-Din	p.o.	13.8	57	690
	11	15	Me-Din	p.o.	10.6	44	530
	12	15	Me-Din	p.o.	8.2	34	410
	13	10	Me-Din	p.o.	6.2	26	310
2	14	15	–	–	–	–	–
	15	20	–	–	–	–	–
	16	15	Ac-Din	p.o.	7	26	350
	17	15	5-FU/Leucovorin	i.p./s.c.	25/25	216/49	500/500†

* Administration fives times weekly for 10 weeks. † Administration twice weekly for 10 weeks.
p.o., orally; Din, dinaline; Ac-Din, p-*N*-acetyldinaline; Me-Din, p-*N*-methyldinaline.

Komitowski, Institute of Experimental Pathology, German Cancer Research Centre).

Cell lines

For *in vitro* experiments, the two human colon cancer cell lines SW707 and SW948 were used. They were derived from patients with colorectal cancer and provided by the tumour bank of the Institute of Experimental Pathology, German Cancer Research Centre (Heidelberg, Germany). Their characteristics were as follows: SW707 and SW948 were derived from well-differentiated adenocarcinomas of the rectum and colon, respectively, with modal chromosome numbers of 47 for SW707 and 76 for SW948. Microscopically, SW707 cells showed no microvesicular bodies in contrast to SW948 cells. The latter cell line synthesised considerably more carcinoembryonic antigen (CEA) and produced more mucus than SW707 cells [19]. Both cell lines were routinely checked for their mucus production to exclude the possibility that fibroblasts had overgrown the carcinoma cells. Moreover, they were tested for mycoplasma contamination and proved to be negative. The cell lines were grown as monolayer cultures in MEM medium (Gibco) supplemented with 10% heat inactivated (57°C; 40 min) fetal calf serum, streptomycin (100 μg/ml),

Table 2. Anticancer activity of dinaline and its derivatives

Experiment number	Group number	Animals (*n*)	Treatment mode (dose in mg/kg)	Median tumour volume* (mm^3)	T/C%†	Median number of tumours (95% CI)
1	1‡	15	–	50 (17–97)‖	10.1	3 (2–4)
	2§	20	–	492 (281–984)	100	9 (5–14)
	3	15	Din (10)	1.7 (0–3.5)¶	0.4	0.5 (0–1)¶
	4	15	Din (7.7)	79 (28–220)¶	16	2.5 (1–4)¶
	5	15	Din (5.9)	52 (22–148)¶	10.6	4 (2–6)¶
	6	5	Ac-Din (11.9)	25.5 (16–35)¶	5.2	1 (1–1)
	7	15	Ac-Din (9.1)	90 (6–181)¶	18.3	3 (3–4)¶
	8	15	Ac-Din (7)	54 (21–171)¶	11.1	3 (3–7)¶
	9	15	Ac-Din (5.3)	106 (35–348)¶	21.6	6 (3–9)¶
	10	15	Me-Din (13.8)	10 (0–54)¶	2	2 (0–5)¶
	11	15	Me-Din (10.6)	28 (11–106)¶	5.7	4 (1–7)¶
	12	15	Me-Din (8.2)	41 (9–284)¶	8.4	6 (2–7)¶
	13	10	Me-Din (6.2)	125 (9–411)¶	25	4 (2–10)¶
2	14‡	15	–	67 (44–92.5)	12.8	4 (3–8)
	15§	20	–	521 (303–973)	100	8 (5–14)
	16	15	Ac-Din (7)	114 (63–229)¶	21.9	7 (3–11)
	17	15	5-FU/Leucovorin (25/25)	424 (234–584)	81.4	8 (3–10)

* Calculated using the equation $a \times b \times c/2$. † Ratio of median tumour volume of treated and control × 100. ‡ Control at the beginning of treatment. § Control after termination of therapy. ‖ CI, confidence interval. ¶ Difference significant relative to control ($P < 0.05$).

penicillin (100 IU/ml) and L-glutamine (2 μmol/l) all from Serva, Heidelberg, Germany. The cells were maintained in a humidified atmosphere (5% CO_2/95% air) at 37°C.

MTT test

Monolayer cell cultures were trypsinised and single cell suspensions were obtained by repeated pipetting. The percentage of viability was determined by the Trypan Blue exclusion test. A final concentration of 1×10^4 cells/ml medium was prepared, and then 0.5 ml was added to each well of a 96-well culture plate. One day after plating the cells, the double concentrated test compounds were dissolved in 500 μl medium and added to eight wells per concentration, respectively. The MTT assay was performed on days 2–5 after start of incubation. Concomitantly, cell counts were determined in triplicate with a Coulter Counter in parallel treated cells (see Results for the drug concentrations used).

According to a previously described procedure [20], 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Munich, Germany) was dissolved in phosphate-buffered saline (Oxoid Ltd, Basingstoke, U.K.) at 5 mg/ml and filtered through a 0.22 μm filter (Millipore, Molsheim, Frankreich); 200 μl of this MTT solution was added to each well. After incubation of the plates with MTT for 2 h at 37°C, the medium was discarded and 200 μl isopropanol (0.04 N HCl in isopropanol) was added to each well to stop the enzyme reaction. Within 1 h of this addition, the level of the coloured formazan derivative dissolved in acid-isopropanol was determined on a Flow Multiscan MC plate reader at a wavelength of 540 nm (reference wavelength 690 nm).

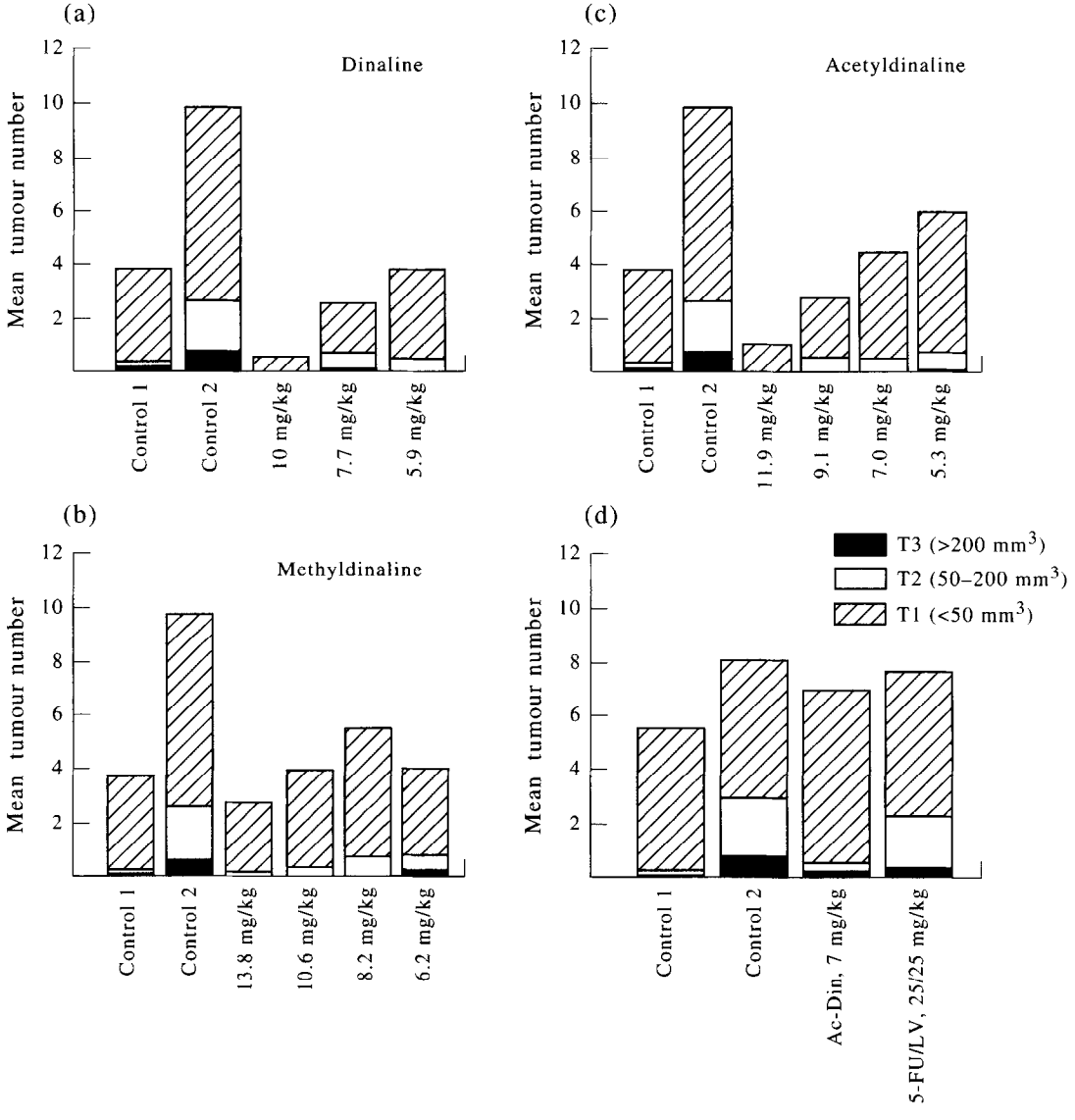


Figure 2. Mean numbers of tumours per animal classified, according to size, are given for treatment with dinaline (a), methyldinaline (b), acetyldinaline (c, all experiment 1) and the comparison of acetyldinaline with 5-fluorouracil/leucovorin (d, experiment 2). Controls 1 and 2 refer to groups 1 and 2 (experiment 1) and to groups 14 and 15 (experiment 2; Tables 1–3).

RESULTS

Tumour induction

262 of 270 animals induced with AMMN developed one or several endoscopically detectable colorectal tumours. All tumours were classified as adenocarcinomas; metastases were seen in 5 animals only (liver, lung).

In vivo *experiment 1*

The median tumour volume, the ratio of tumour volume in treated over control groups, and the median tumour number of all experimental groups are given in Table 2 as parameters of therapeutic efficacy. The median tumour volume of untreated controls increased almost 10-fold from 50 to 492 mm^3 within the 10-week period of observation and the median tumour number increased 3-fold from 3 to 9 (Table 2, groups 1, 2).

All three substances showed a significant reduction in tumour volume compared to control 2 (group 2, Table 2), but only treatment with Me-Din induced a strictly dose-related effect on tumour growth. Compared to the initial tumour volume (control 1, group 1, Table 2), the therapeutic effect can be characterised as tumour regression (Table 2, groups 3, 6, 10, 11), as 'no change' (Table 2, groups 5, 8, 12) or as tumour progression (Table 2, groups 4, 7, 9, 13). Me-Din was the only compound that achieved dose-dependently complete remissions in up to 26% of the animals (13.8 mg/kg, group 10, Table 2). Regression of tumours diagnosed at the beginning of therapy, was also caused by Din and Ac-Din, but to a lesser degree. Figure 2 compares tumour size at autopsy of all treatment arms. The mean tumour numbers did not or just barely increased during the 10-week treatment period and the appearance of large tumours was effectively inhibited compared to controls.

The spontaneous mortality as a consequence of tumour growth was 15% within the control group; these animals showed a slightly positive body weight gain of 3% during the period of observation (group 2, Table 3).

Treatment with Din resulted in a dose-related mortality of 87, 47 and 13%, respectively. The toxicity was also reflected by decreases in body weight of 24, 23 and 12%, respectively (Table 3, groups 3, 4, 5), indicating that only the low dose group had a benefit from the treatment without concomitant toxicity.

The therapy with Ac-Din was ceased at the highest dosage used (11.9 mg/kg), since the majority of animals died before completion of treatment. In the next two higher dose groups, a dose-related reduction in body weight of 15 and 8% was seen, whereas the body weight remained constant at the lowest dosage. The corresponding rates of mortality were 20, 13 and 20%, respectively (Table 3, groups 6–9).

Administration of Me-Din resulted in a less pronounced diminution of the median body weight (−12, −9, −2, −8%), and the respective rates of mortality were generally lower than for the other two compounds (47, 20, 27 and 30%; Table 3, groups 10–13).

In vivo *experiment 2*

In this experiment, only one dosage (7.0 mg/kg) of Ac-Din, that had been found to be slightly toxic, was tested in comparison with the established combination therapy 5-FU and LV. The median tumour volume of the untreated controls increased 7.7-fold from 67 to 521 mm^3, the median tumour number doubled from 4 to 8.

Therapy with Ac-Din resulted in a tumour growth inhibition of approximately 78% compared to control 2 (group 15, Table 2), whereas the combination therapy with 5-FU/LV was ineffective as evidenced by a tumour growth inhibition of only approximately 18%. No differences were found with respect to the increase in new tumours as shown by the median tumour numbers. However, the distribution of tumour sizes at autopsy (Figure 2c) revealed a clear inhi-

Table 3. Parameters of toxicity following therapy with dinaline and its derivatives

Experiment number	Group number	Treatment mode (dose in mg/kg)	Median body weight (g)		Change inmedian body weight (%)*	Mortality	
			Therapy week 1	Therapy week 10		*n*	%
1	1†	–	470 (450–505)‡	–	–	0	0
	2§	–	520 (490–545)	535 (505–575)	+ 3	3	15
	3	Din (10)	485 (460–530)	370 (310–430)	− 24	13	87
	4	Din (7.7)	500 (470–550)	380 (320–460)	− 23	7	47
	5	Din (5.9)	500 (460–535)	440 (375–470)	− 12	2	13
	6	Ac-Din (11.9)	465 (450–545)	Toxic	N/A	4	80
	7	Ac-Din (9.1)	505 (480–515)	430 (320–460)	− 15	3	20
	8	Ac-Din (7)	480 (455–520)	440 (420–480)	− 8	2	13
	9	Ac-Din (5.3)	500 (490–525)	505 (485–550)	+ 1	3	20
	10	Me-Din (13.8)	520 (500–540)	455 (410–475)	− 12	7	47
	11	Me-Din (10.6)	480 (465–500)	435 (375–495)	− 9	3	20
	12	Me-Din (8.2)	480 (460–530)	470 (385–505)	− 2	4	27
	13	Me-Din (6.2)	505 (495–525)	465 (280–595)	− 8	3	30
2	14†	–	575 (520–590)	–	–	–	–
	15§	–	550 (515–575)	570 (535–610)	+ 4	1	5
	16	Ac-Din (7)	540 (510–570)	505 (460–550)	− 6	7	47
	17	5-FU/LV (25/25)	550 (510–560)	530 (500–620)	− 4	6	40

* Weight difference at the end of treatment in per cent of initial body weight. † Control at the beginning of therapy. ‡ Values in parentheses represent 95% confidence intervals. § Control after termination of therapy. N/A, not available; 5-FU, 5-fluorouracil; LV, leucovorin.

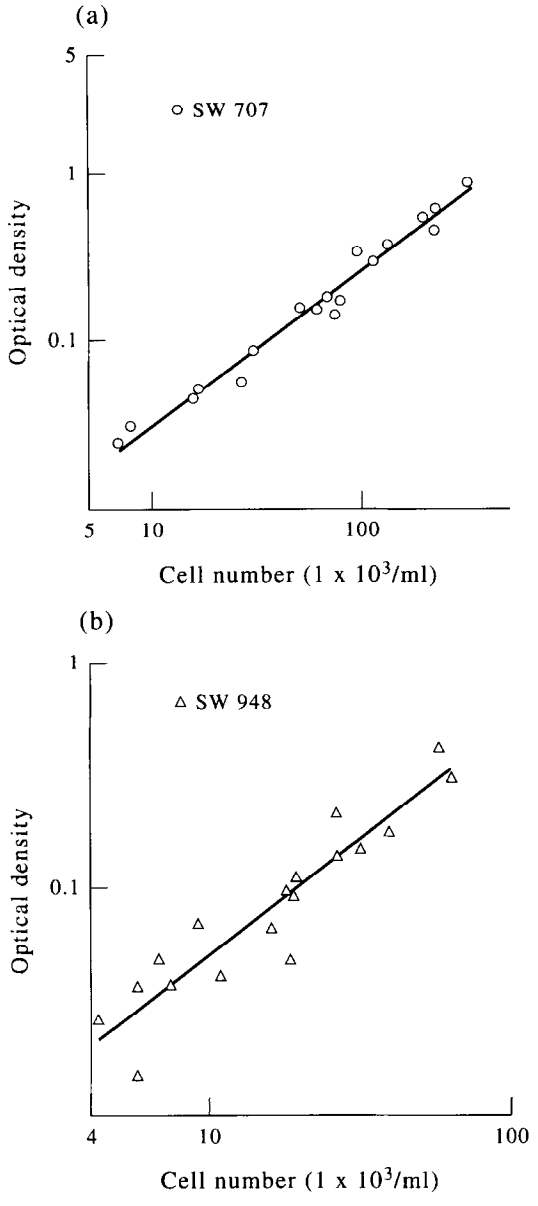


Figure 3. Correlation of optical density with cell number as estimated by MTT assay and cell counts (Coulter Counter) in cell lines SW707 ($r = 0.99$) and SW948 ($r = 0.93$).

bition of appearance of large sized tumours (>50 mm^3) by treatment with acetyldinaline, but not for the combination of 5-FU/LV.

In terms of toxicity, both treatment arms were equally toxic as indicated by a mortality rate of 47% and 40% and a loss of body weight of 6% and 4%, respectively.

Histopathologically, the toxicity of Din and its congeners was characterised by depressed haematopoiesis and atrophy of bone marrow. The spleen, thymus and lymph nodes showed high-grade depletion of their lymphatic portions. On this basis, opportunistic infections developed, e.g. bronchopneumonia, that were ultimate causes of death.

In vitro experiment

Control cells showed a doubling time of 24 (SW707) and 33 (SW948) h. The correlation between cell numbers, as determined by Coulter Counter and MTT due conversion, was found to be 0.99 and 0.93 for SW707 and SW948 cells, respectively (Figure 3). Equally, very similar IC_{50} values were obtained following incubation of 48 h or more with dinaline, acetyldinaline or methyldinaline (Table 4). They ranged between 1 and 2.2 μg/ml after 48 h and from 0.6 to 1.6 μg/ml after 72 h of incubation.

Representative concentration-dependent curves of inhibition of cell proliferation versus time following exposure to methyldinaline are given in Figures 4 and 5 for SW707 and SW948 cells. As can be seen, concentrations exceeding 1.9 μg/ml were cytotoxic and decreased the initial cell number, whereas lower concentrations were cytostatic or ineffective.

Interestingly, incubation for short periods (24 h) with low concentrations of the test compounds caused an increase in dye conversion as determined by the optical density at 540 nm which was not correlated to an increase in cell number (Figure 6). This difference disappeared following incubation periods of more than 24 h.

DISCUSSION

Chemically, Din and its p-*N*-methyl and p-*N*-acetyl derivatives represent a group of pharmacologically active lipophilic substances with a relatively simple structure derived from *N*-acyl-*o*-phenylenediamine. Din was developed originally as an anticonvulsive agent, that showed significant inhibitory effects on peripheral blood cells and spermatogenesis when administered continuously [10]. It significantly reduced the frequency of spontaneously developing tumours in male and female Sprague–Dawley rats following long-

Table 4. IC_{50} concentrations following continuous exposure to dinaline, acetyldinaline and methyldinaline*

Incubation period†	Cell line	Dinaline		Acetyldinaline		Methyldinaline	
		CC‡	MTT	CC	MTT	CC	MTT
48 hours (day 3)	SW707	1.0§ (0.5–1.6)‖	2.2 (1.8–2.6)	1.2 (0.5–1.8)	1.7 (1.2–2.1)	1.3 (0.9–1.6)	2.0 (1.6–2.4)
	SW948	1.5 (0.9–2.1)	1.0 (0.2–1.8)	1.2 (?–2.6)	1.7 (0.4–3.4)	1.8 (0.3–3.3)	1.2 (0.2–2.1)
72 hours (day 4)	SW707	1.3 (0.1–2.4)	1.2 (0.8–1.5)	0.7 (0.02–1.3)	1.1 (0.8–1.4)	1.6 (1.2–2.1)	1.1 (0.4–1.8)
	SW948	0.8 (?–1.9)	0.8 (?–1.8)	1.1 (?–2.4)	0.9 (?–1.9)	0.6 (?–1.6)	0.7 (?–1.6)

* IC_{50} concentrations were obtained from linear regression applied to log/log plots of concentration/effect curves. All correlations were significant ($P < 0.05$) with correlation coefficients ≤ -0.9. † Concentrations used for continuous incubation (μg/ml): 0.47, 0.95, 1.9, 3.8, 7.5, 15. ‡ Cell count determined by Coulter Counter. § In μg/ml. ‖ 95% confidence limits.

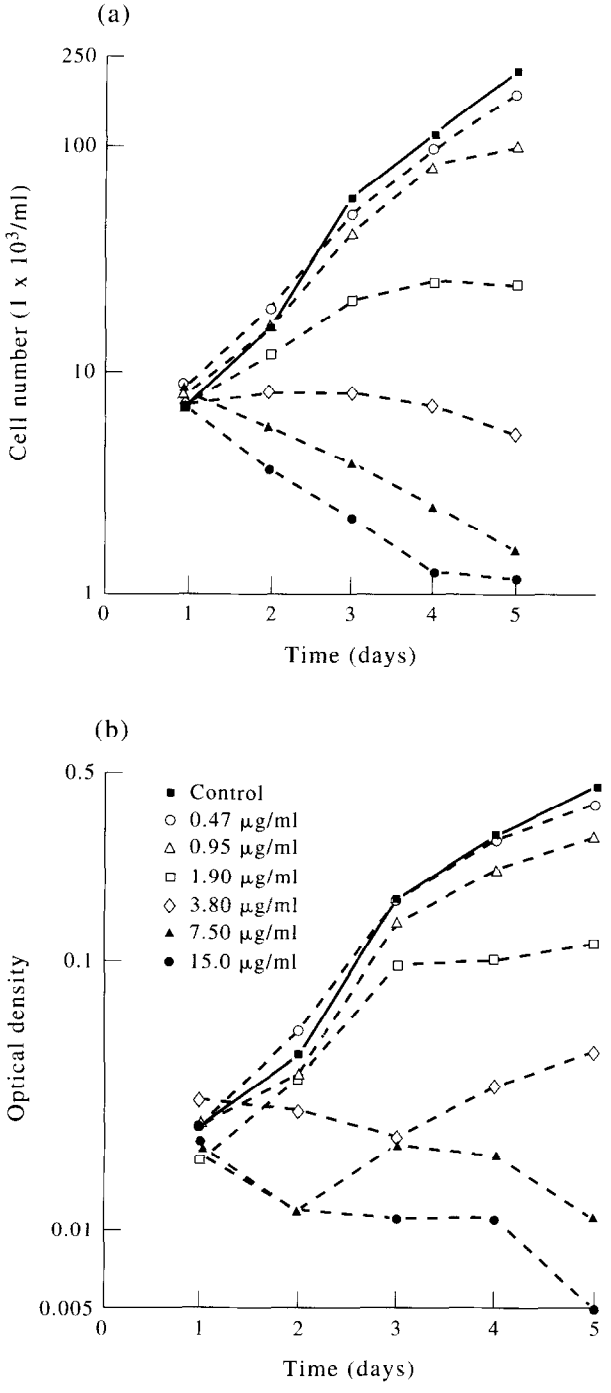


Figure 4. Efficacy of methyldinaline on SW707 cells during 5 days of continuous incubation as shown by cell count (a) and optical density (b). Standard deviation bars were omitted for clarity; standard deviation was below 15%.

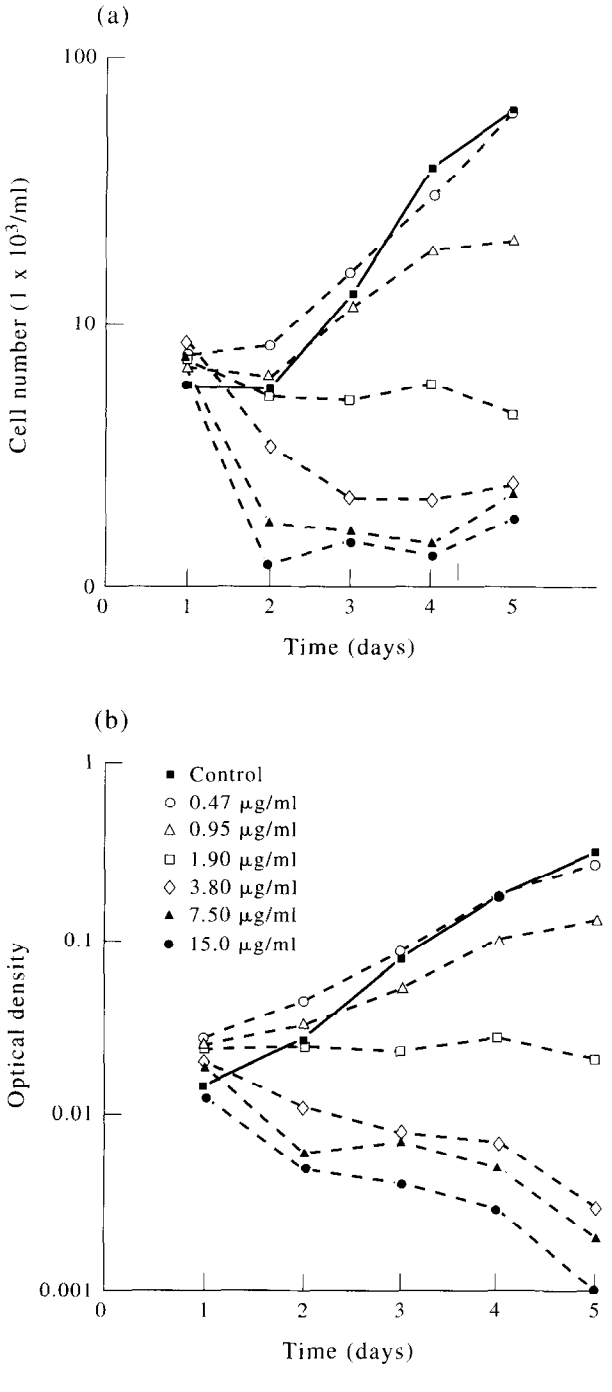


Figure 5. Efficacy of methyldinaline on SW948 cells during 5 days of continuous incubation as shown by cell count (a) and optical density (b). Standard deviation bars were omitted for clarity; standard deviation was below 15%.

term application [21]. Preclinical evaluation according to former NCI standard protocols found Din to be totally ineffective against mouse leukaemia P388. Further experiments demonstrated marginal activity against leukaemia L1210, moderate activity against Lewis lung carcinoma and high anticancer activity against C22LR osteosarcoma, Brown Norway myelocytic leukaemia [9] and methylnitrosourea (MNU)-induced autochthonous rat mammary carcinoma [10, 22].

Exposure of L1210-cells to Din *in vitro* resulted in a significant reduction of intracellular c-AMP pools [23]. The same effect was determined in tumours and livers of Din-treated rats bearing MNU-induced mammary carcinoma [22, 24].

The present experiments were designed to compare the therapeutic effect of dinaline with its N-acetyl and N-methyl derivatives in AMMN-induced autochthonous colorectal rat carcinoma and in two human colon cancer cell lines. The

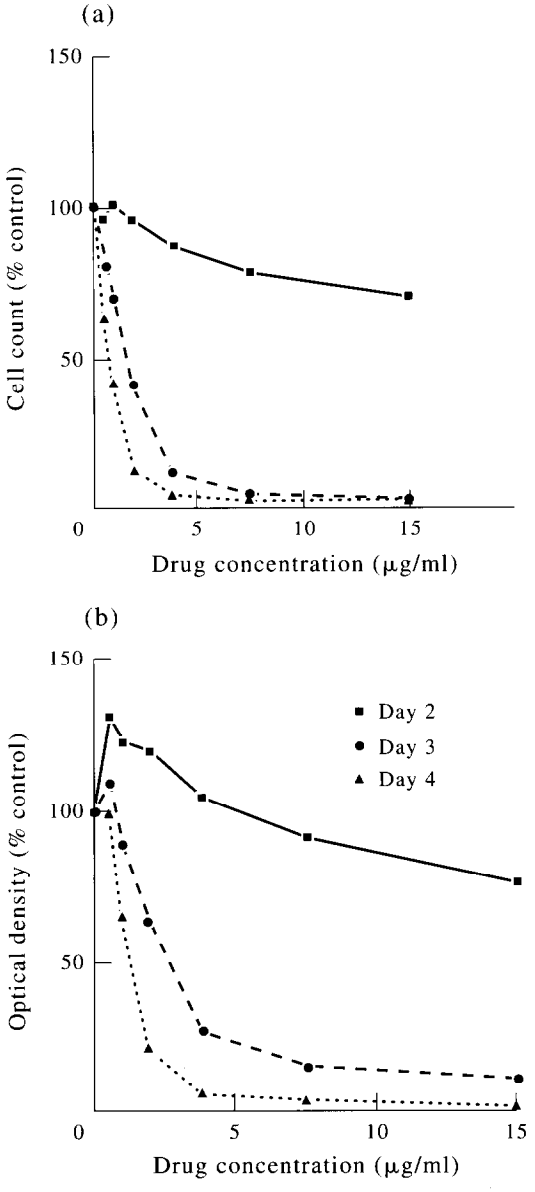


Figure 6. Concentration effect curves of acetyldinaline in SW948 cells; comparison of cell count and optical density as a percentage of the respective control. Standard deviation bars were omitted for clarity; standard deviation was below 15%.

animal model was chosen because its properties mimic the human situation more closely than transplanted tumours [8] in terms of intact tumour histology, route of metastasis, tumour–host interaction, and slow growth kinetics. The onset of tumour growth, as assessed by endoscopy, varied by 3 weeks between experiments 1 and 2 resulting in a difference of approximately 20% between mean initial tumour volumes and body weights. This variability did not influence the evaluation of anticancer activity since all comparisons were made only within the respective experiments. Clearly, Din and its derivatives proved to be highly effective in AMMN-induced autochthonous colorectal carcinoma. Their antineoplastic potency surpassed that of all other agents tested so far in this extremely chemoresistent model [8]. The slight variability in body weight explains the difference in toxicity of Ac-Din between the two experiments, which indicates that this derivative is characterised by a dose–response curve as steep as that of dinaline [10]. The antitumour efficacy of Ac-Din was clearly superior to that of the clinically used regimen 5-FU/LV. A comparison between the two treatment arms is difficult due to the high morality: 47% and 40% for Ac-Din and 5-FU/LV, respectively (Table 3). Nevertheless, at comparable toxicity, the activity of the experimental drug was 4-fold higher than that of the clinically used combination therapy.

For effective tumour growth inhibition, the compounds had to be administered without long treatment-free intervals since discontinuation of therapy was followed by immediate tumour proliferation. This was found in a preliminary experiment on animal survival following a treatment period of 10 weeks. After cessation of therapy, tumour growth resumed rapidly and thus only minimal increase in lifespan was observed (data not shown). Among several possible mechanistic explanations, this rapid regrowth of colorectal tumours could be caused by reversible inhibition of intracellular signal transduction or by inhibition of angiogenesis. Regardless of these speculations, the compounds need to be administered on a protracted basis to generate increased survival in the model used. This differs from the effect in certain transplanted tumours, in which administration for a short period of time led to highly increased survival [11, 26].

The daily oral schedule was another reason for a slightly increased mortality rate of treated rats as compared to what could have been expected on the basis of tumour growth suppression and overt drug toxicity. We assume that drug administration by gavage application imposed some additional risk that was not balanced by vehicle administration in controls.

The structural modifications at the amino group of dinaline are of importance since they influence the metabolism of this drug. Dinaline is known to be rapidly N-acetylated by microsomal liver enzymes to N-acetyldinaline, whereas metabolism of methyldinaline proceeds more slowly. Demethylation of the amino group in position 4 of the benzamide moiety to yield dinaline is followed by N-acetylation. The former reaction is rate limiting by its slower kinetics [25]. Accordingly, the half-life of dinaline is shorter than that of methyldinaline. Interestingly, the *in vivo* toxicity of the three test compounds can be ranked in the order of methyldinaline < dinaline < acetyldinaline (Table 3). *In vitro*, however, this ranking was not evident with similar ranges of IC_{50} values (Table 4). This could imply that the mechanism of antineoplastic action of the compounds is independent of metabolic alteration. So far, the definitive mechanism of action remains to be elucidated. A recent publication suggests a small phosphoprotein to be involved in the growth inhibition of C26 murine colon and HCT-8 human colon adenocarcinoma cells *in vitro* [26]. This and our *in vitro* studies show that human colon cancer cells are basically sensitive to dinaline and its derivatives. There was no significant difference in drug concentrations causing 50% growth inhibition between the three congeners. This is in line with experiments on HCT-8 human colon cancer cells which showed comparably high sensitivity to Ac-Din and Din *in vitro*, as well as transplanted to nude mice *in vivo* [27, 28]. Recent findings have also shown that Brown

Norway leukaemic cells are equally sensitive to Ac-Din and Din [12].

The difference in SW707 and SW948 cell proliferation rates did not influence their sensitivity to the test compounds. The similar efficacy of drugs *in vitro* reflects their corresponding antitumour activity *in vivo*, since a slight superiority of Me-Din *in vivo* was based mainly on differences in toxicity.

The results obtained certainly warrant a clinical investigation of these compounds. However, the following differences between the model used and the clinical situation should be kept in mind. This experiment showed that primary colorectal rat carcinomas are sensitive to treatment with Din and its derivatives as are human colon cancer cell lines *in vitro*. Metastases formation was too low in the rat model to be evaluated for treatment effects. Clinically however, chemotherapeutic agents are being used after primary resection for adjuvant or palliative treatment [5]. The question remains, therefore, whether metastases can also successfully be treated with the new group of agents. This will hopefully be answered in clinical trials which have recently started with Ac-Din.

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