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Cytostatic activity and potential mechanism of action of 4-amino-N-(2-aminophenyl)-benzamide (Dinaline) on adriamycin sensitive (FL) and resistant (ARN) Friend leukemia cells

Dinaline, a N-phenylbenzamide derivative with cytostatic activity, was equally effective against adriamycin-sensitive and -resistant Friend erythroleukemia cells when continuously present during cell culture. Dinaline acted in two phases : an initial 24 h cytostatic phase was followed by a cytotoxic phase with rapidly progressing cell death within 120 h of exposure. During the cytostatic phase the following metabolic changes were observed at the single cell level by flow cytometric measurements : an accumulation of the cells in G_0/G_1-phase of the cell cycle by blocking cell entrance into S-phase, decreases of cell volume, carboxylesterase activity, intracellular pH and mitochondrial rhodamine 123 uptake, but an increase of the transmembrane potential in surviving FL and ARN cells. The cytotoxic effect of dinaline occurred irrespective of the cell cycle phase as demonstrated by simultaneous staining of the DNA in living and dead cells with Hoechst 33342 and propidium iodide. Dinaline may also be of interest for in vitro and in vivo synchronization of proliferating cells for experimental studies and therapy.

Key words : Dinaline — Accumulation — S-phase

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Introduction

Dinaline (Goe 1734 ; NSC 328786), a N-phenylbenzamide derivative, revealed a marked cytostatic and immunosuppressive capacity in various animal models [1]. Recent studies in the Brown Norway acute myelocytic leukemia (BNML) rat system confirmed dinaline's potential usefulness as an antitumor agent. A 8 log tumor cell kill compared with a 1 log kill of normal hematopoietic stem cells upon daily oral administration was obtained [2]. From the presence of an acetylated metabolite in rat serum it was assumed that dinaline had to be activated in vivo to become effective.

Dinaline belongs to none of the classical groups of cytostatic agents and its mechanism of action is still unknown. Dinaline was found to exhibit a low mutagenicity in the Ames test [3] and only few DNA single strand breaks and interstand crosslinks could be detected in liver cells of treated rats [3]. Regarding the effects of dinaline on cell metabolism, a 60 % decrease in cAMP pools after 2 h of incubation of L1210 cells with the drug has been reported [4]. Other benzamide derivatives were found to inhibit poly-ADP-ribosylation [5], glucose metabolism and DNA synthesis and to affect cell viability [6].

During the last years a series of functional flow cytometric assays has been established which allow the rapid and sensitive monitoring of the effectiveness of cytostatic drugs on cell

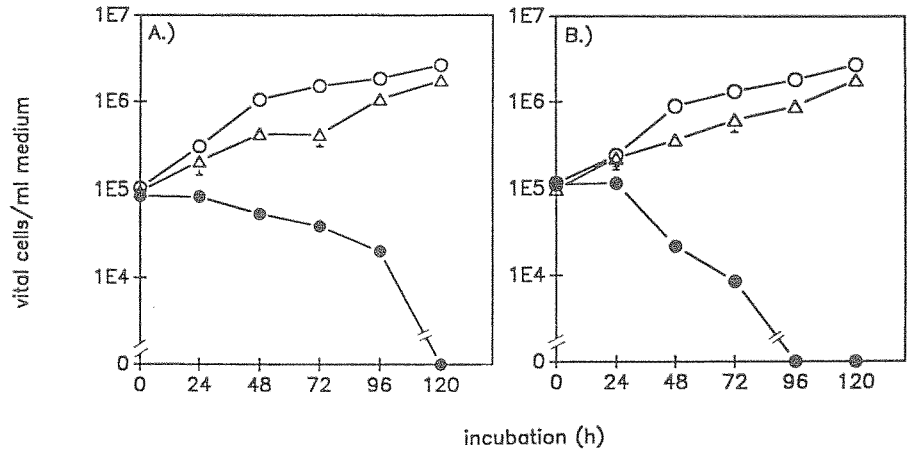

Fig. 1. Effect of dinaline on growth of FL *(A)* and ARN *(B)* cells. The cells were grown in the absence (o) or in the continuous presence of 20 μg/ml (•), 2 μg/ml (△ dinaline. The concentration of vital cells was determined in a hemocytometer by exclusion of trypan blue. Each point represents the mean ± SEM of 5 different cell culture experiments

metabolism and cell cycle [7, 8, 9]. Two cell lines, Friend erythroleukemia cells (FL cells) and an adriamycin resistant FL cell variant (ARN cells) [10] under continuous dinaline treatment were screened for changes in intracellular pH, carboxylesterase (E.C. 3.1.1.1) activity, transmembrane and transmitochondrial potential and DNA cell cycle distribution. These experiments were performed in order to study the mechanism of dinaline's influence on tumor cell growth and to clarify its possible interference with DNA synthesis.

Results

The cytostatic effect and the toxicity of dinaline were determined by counting the number of vital cells over a period of 5 days (Fig. 1). Dinaline significantly reduced cell proliferation of exponentially growing FL cells and ARN cells at a concentration of 2 μg/ml medium. Cell proliferation was blocked immediately and completely at 20 μg/ml dinaline in exponentially growing cell cultures (Fig. 1) but cells were still vital. After 24 h between 6.9 and 7.3 % dead cells were

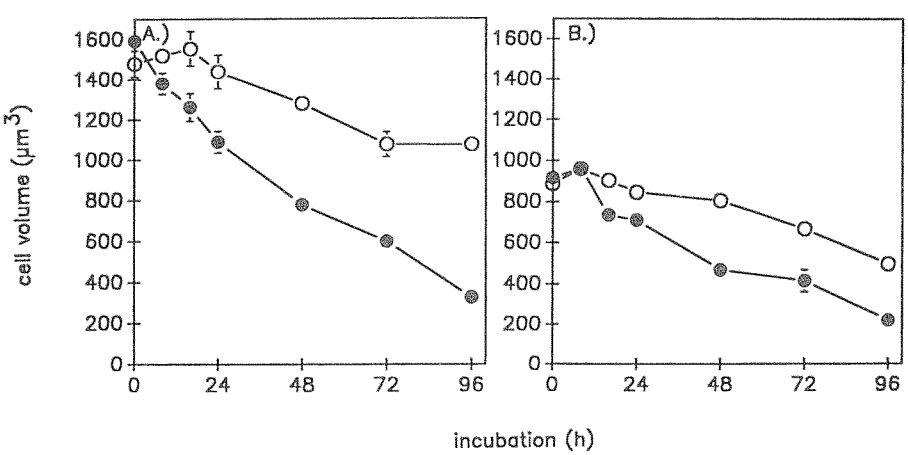

Fig. 2. Effect of dinaline on the mean cell volume of FL *(A)* and ARN *(B)* cells. The cells were grown in the absence (o) or continuous presence (•) of 20 μg/ml dinaline. Each point represents the mean ± SEM of 3 different cell cultures. Each cell volume value was determined 2 times

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observed in the 2 μg/ml and 20 μg/ml dinaline treated cell cultures as compared to dead cell numbers between 6.4 and 6.7 % in untreated control assays (n = 5). Later on the number of vital cells decreased (Fig. 1) and the amount of dead cells increased at 48 h to betwen 34.6 % and 35.5 % in dinaline-treated FL and ARN cells as compared to levels between 9.6 and 11.5 % in control assays.

The dinaline-induced cytostasis during the first 24 h was completely reversible, when dinaline was removed from the cells (data not shown). The velocity of the cell kill caused by dinaline was higher in ARN as compared to FL cells (Fig. 1). No viable cells were detectable in the FL cell cultures after 120 h and in the ARN cell cultures after 96 h. After a 48 h treatment dinaline had a similar cytotoxic effect in stationary cultures (25.5 % dead cells) and in exponentially growing cultures (23.7 % dead cells) which had been arrested to more than 80 % in the G_0/G_1-phase by isoleucin deprivation in the medium.

To study the influence of dinaline on the metabolism of FL and ARN cells on the single cell level several functional parameters were analyzed by flow cytometry. The initial cell volume of the vital cells was significantly lower in the ARN cells as compared to FL cells (Fig. 2). The cell volume of vital FL and ARN cells in the control assays diminished to 73 % and 61 % respectively while it decreased substantially more under dinaline treatment to 21 % and 22 % after 96 h (Fig. 2).

The intracellular pH (Fig. 3A, B) remained stable in FL and ARN control cells but decreased significantly by 0.20-0.22 units during the first 24 h of dinaline treatment. The carboxylesterase activity (Fig. 3C, D) decreased slightly in the controls but more pronounced in the dinaline treated assays.

When the mitochondrial Rh123 uptake of the two cell lines was moni-

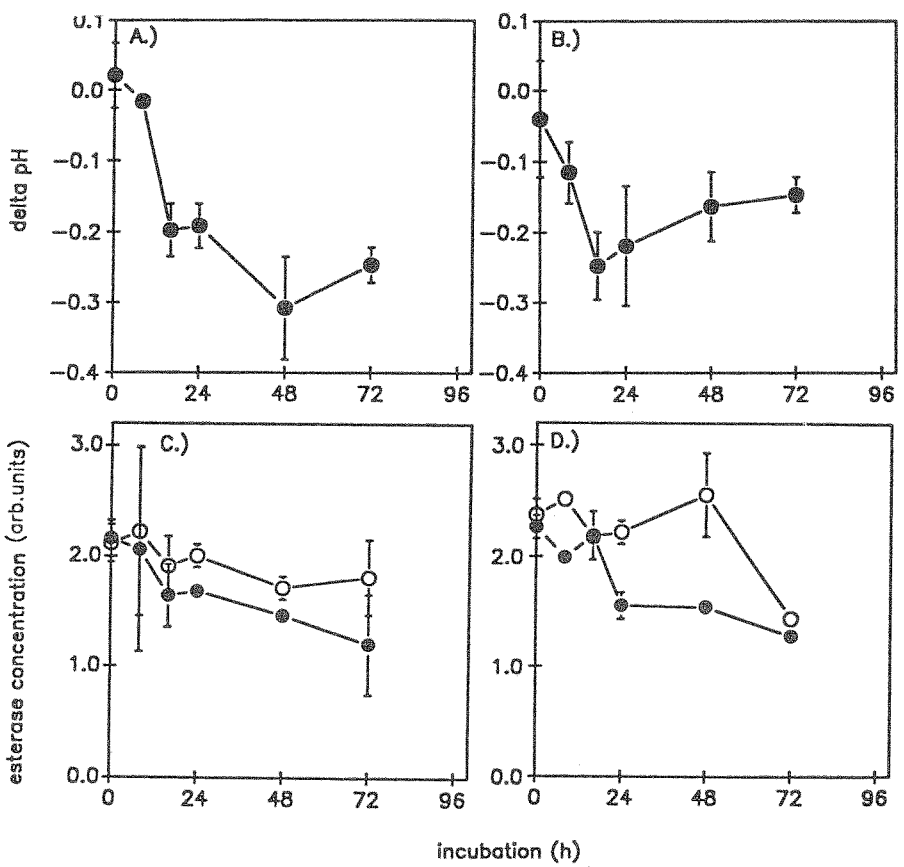

Fig. 3. Effect of dinaline on the intracellular pH *(A, B)* and the carboxylesterase activity *(C, D)* of vital FL *(A, C)* and ARN *(B, D)* cells in the absence (o) or continuous presence (•) of 20 μg/ml dinaline. Each point represents the mean ± SEM from 3 to 5 different experiments

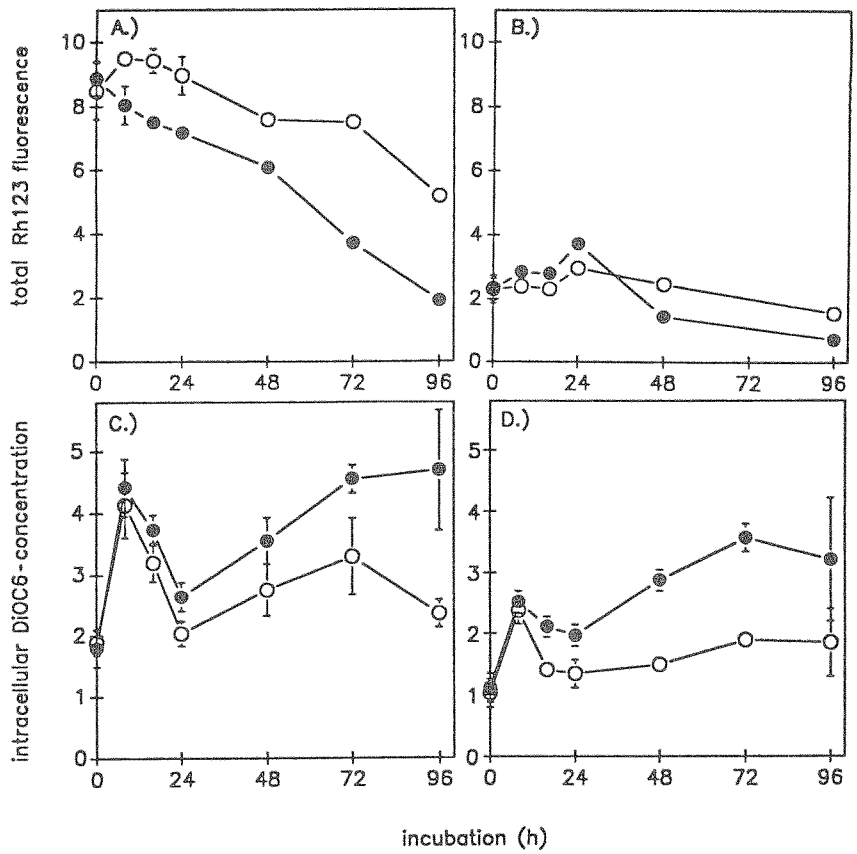

Fig. 4. Effect of dinaline on the mitochondrial Rh123 uptake *(A, B)* and on the transmembrane potential *(C, D)* of FL *(A, C)* and ARN *(B, D)* cells in the absence (o) or continuous presence (•) of 20 μg/ml dinaline. Each point represents the mean ± SEM from 6 different experiments

tored, significantly lower Rh123 levels were obtained in ARN cells (Fig. 4B) than in FL cells (Fig. 4A). After 8 h of dinaline treatment the FL cells began to loose Rh123 at a higher speed than untreated FL cells. In the ARN cell population dinaline caused an initial increase in Rh123 fluorescence. After more than 24 h incubation with dinaline ARN cells lost Rh123 faster than FL cells (Fig. 4B).

Changes of the cellular transmembrane potential were measured by the uptake of the dye $DiOC_6(3)$. The influence of cell size was eliminated from the measurement by dividing the fluorescence of each cell by its volume. ARN cells had a lower transmembrane potential (Fig. 4D) than FL cells (Fig. 4C). An initial hyperpolarization was observed 8 h after the begin of the experiment, which was followed by a depolarization to the starting levels at 24 h in FL and ARN cells (Fig. 4C, D). In the presence of dinaline the membrane potential showed a similar time course in both cell types but hyperpolarization was more pronounced in the presence of dinaline.

The discrimination between the cell cycle distribution of vital and dead cells (Fig. 5) was achieved by simultaneous staining of the cells with Hoechst 33342 and PI [13]. FL cells after 24 h preincubation with dinaline consist of 3 populations (Fig. 5A) : The vital cells (78.6 %) emit strong Hoechst blue fluorescence, whereas the dead cells (17.5 %) show only PI red fluorescence. A third population of dying cells (3.8 %) are mostly in the G_0/G_1-phase and are simultaneously stained by Hoechst and PI.

The vital cells show a narrow DNA-distribution with a CV of the G_0/G_1-peak of 6.4 % (Fig. 5A), whereas the dead cells have a broader distribution with a CV of 10.2 % (Fig. 5B). 20 μg/ml dinaline caused a considerable accumulation of vital FL cells in G_0/G_1-phase already after 8 h of incubation as compared to the untreated control (Fig. 6A, B). The

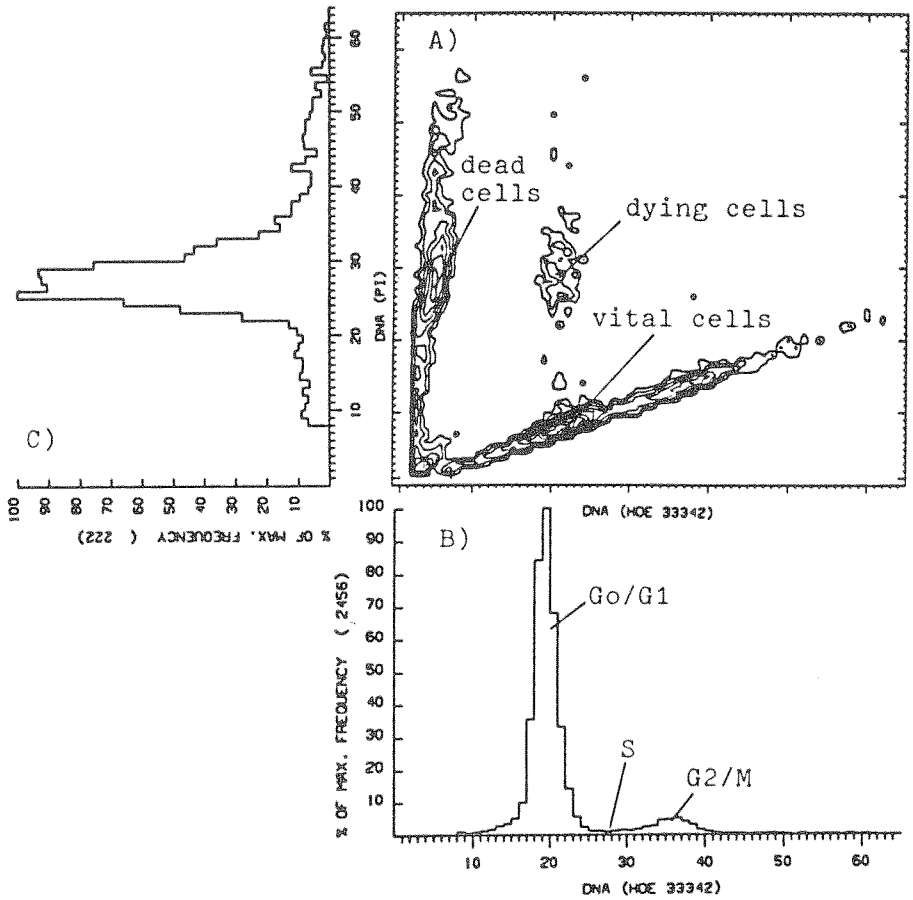

Fig. 5. Effect of dinaline on DNA distribution. Two dimensional contour plot (Fig. 5*A*) of FL cells after simultaneous staining with Hoechst 33342 and PI. The cells were preincubated with 20 μg/ml dinaline for 24 h, stained and analyzed as described in Materials and methods. 78,6 % of the cells were vital (blue fluorescence) 17.5 % dead (red fluorescence) and 3.8 % were at the live-dead boundary (dying cells). Vital and dead cells were separately projected as single DNA histograms onto the respective axis (Fig. 5*B* and Fig. 5*C*). The CV of the G_0/G_1-peak was 6.4 % for the vital cells (Fig. 5*B*) and 10.2 % for the dead cells (Fig. 5*C*)

percentage of G_0/G_1-cells increased up to 84.21 % (Fig. 6D, F) with a corresponding decrease in the percentage of S- and G_2M-phase cells until the end of the cytostatic phase, i.e. after 24 h. In the control assays only minor changes in cell cycle distribution were observed (Fig. 6A, C, E). Dinaline caused a similar decrease of S- and G_2M-phases (Table 1) in ARN cells with 71.7 % of the cells in G_0/G_1-phase after 24 h (Table 1). It is noteworthy that the ARN cells took up only 25-35 % as much Hoechst 33342 dye as the FL cells (data not shown).

Discussion

The knowledge about the biochemical effects of dinaline on tumor cells is very limited. In our experiments dinaline had antiproliferative and cytotoxic effects on FL and ARN cells (Fig. 1), which demonstrates that there is no cross-resistance of adriamycin resistant cells to dinaline.

Flow cytometric measurements under continuous exposure to dinaline revealed that this drug induces a cytostatic phase during the first 24 h of treatment (Fig. 1), which is followed by a cytotoxic phase between 24 and 72 h.

During the cytostatic phase several dinaline-induced biophysical, functional and metabolic changes occurred, such as cell shrinkage, carboxylesterase activity decrease, pH decrease, cell membrane hyperpolarization, loss of the Rh123 retaining ability and inhibition of cell cycle progression.

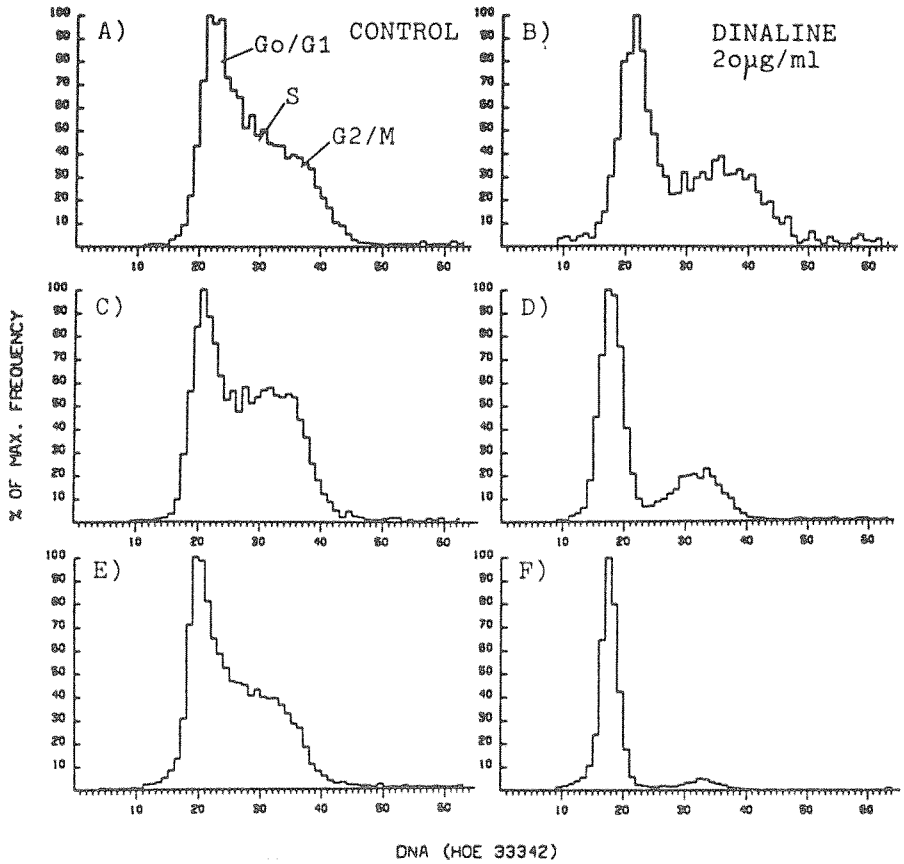

Fig. 6. Effect of dinaline on the cell cycle progression of dinaline-treated *(right column)* and untreated *(left column)* FL cells. The cells were incubated in the absence or continuous presence of 20 µg/ml dinaline. They were stained and analyzed as described in Materials and methods after 8 h (Fig. 6*A, B*), 16 h (Fig. 6*C, D*) and 24 h (Fig. 6*A, F*)

The fact, that dinaline treatment leads to cellular shrinking (Fig. 2) is surprising, because many chemotherapeutic agents, especially those which act via cell cycle arrest, on the contrary induce an « unbalanced growth » of the cell body with a 3- to 4-fold increase of the cell volume [14]. Cell shrinking is usually considered to be an early indicator for apoptosis, i.e. for the onset of programmed cell death [15]. Interestingly, apoptosis is promoted by substances which are benzamide analogues as dinaline [16, 17]. Various benzamide analogues inhibit poly-ADP-ribosylation [5], which is a prerequisite for DNA repair [18]. Dinaline however does not cause a significant intravital DNA degradation which is observed after treatment of vital cells with other benzamide derivatives [16, 17] since the amount of cellular DNA remains constant during dinaline treatment as indicated by DNA staining with Hoechst 33342 (Fig. 6).

Dinaline induced cell shrinkage may be caused by K^+ loss or by interference of dinaline with the Na^+/H^+-exchange pump. Shrinkage of vital cells is usually accompanied by initial intracellular acidification followed by regulatory alkalinization of the cytoplasm through the activity of the Na^+/H^+-pump with consecutive normalization of the cell volume [19]. The dinaline induced intracellular acidification (Fig. 3) is compatible with an effect of dinaline on the Na^+/H^+-exchange. The observed cell membrane hyperpolarization (Fig. 4) is not contradictory, because the Na^+/H^+-ion exchange is electroneutral. Changes in the intracellular Rh123 accumulation (Fig. 4) may reflect changes of the transmitochondrial potential [20], of the p-glycoprotein mediated rhodamine 123 efflux [21] or the mitochondrial mass.

The Hoechst dye uptake was also diminished in ARN cells confirming a previous report, which demonstrated

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Table 1. Cell cycle distribution of FL- and ARN-cells under dinaline

treatment	time (h)	FL-cells G_0/G_1* (%)	FL-cells S* (%)	FL-cells G_2/M* (%)	ARN-cells G_0/G_1* (%)	ARN-cells S* (%)	ARN-cells G_2/M* (%)
Dinaline 20 μg/ml	0	26.9 ± 4.1	26.1 ± 1.8	47.0 ± 4.4	38.3 ± 1.9	28.1 ± 0.1	33.6 ± 1.8
	8	45.4 ± 8.1	19.1 ± 1.8	35.5 ± 8.2	44.4 ± 1.8	23.4 ± 0.8	32.2 ± 2.4
	16	60.6 ± 2.3	10.8 ± 0.4	28.6 ± 2.0	52.3 ± 2.5	20.0 ± 0.6	27.8 ± 2.6
	24	84.2 ± 2.6	3.6 ± 0.5	12.1 ± 2.4	71.7 ± 1.0	9.2 ± 0.2	19.1 ± 0.8
Controls	0	26.1 ± 3.2	28.8 ± 1.1	45.2 ± 4.3	41.2 ± 1.2	28.4 ± 0.2	30.4 ± 1.4
	8	28.5 ± 1.5	33.0 ± 0.8	38.5 ± 0.8	45.8 ± 1.3	27.8 ± 0.5	26.5 ± 1.8
	16	32.3 ± 0.3	28.0 ± 0.2	39.7 ± 0.5	41.3 ± 1.9	29.6 ± 1.0	29.2 ± 2.9
	24	29.4 ± 6.1	34.1 ± 2.5	36.5 ± 3.6	44.8 ± 1.2	26.2 ± 0.1	29.0 ± 1.4

* Mean ± SEM

that Hoechst 33342 was actively effluxed by adriamycin resistant P388-cells [22].

Intracellular $DiOC_6(3)$ concentration is a measure for the transmembrane potential [23]. Dinaline induced a hyperpolarization in the FL and in the ARN cells (Fig. 4). The effect on membrane potential may explain why dinaline, which was originally developed as an anticonvulsive agent, effectively disrupts « petit mal » as well as « grand mal » epileptic seizures in mice [24]. The reason for the triphasic time course of membrane potential which occurred in both cell types after splitting of the cells in culture with and without dinaline (Fig. 4C, D), in unclear. More detailed studies are necessary to understand the underlying mechanisms for the observed changes of the functional cell parameters.

The influence of dinaline on cell cycle distribution, in contrast can be better explained. The cell cycle progression of the exponentially growing FL and ARN cells was almost totally blocked after 24 h of incubation with 20 μg/ml dinaline (Fig. 6). The cells accumulated in G_0/G_1-phase, i.e. a block of S-phase entrance had occurred. The Hoechst/PI staining technique does not permit to distinguish whether the block is at the G_0/G_1-stage or at the G_1/S-stage of the cell cycle. The DNA-histogram of the dead cells is similar to that of the living cells (Fig. 5B, C). From this one can conclude, that dinaline does not induce cycle-specific cell death in the S- and G_2M-phase, which alternatively could explain the disappearance of S- and G_2/M-phase cells from the vital cell fraction of the dinaline-treated cultures.

Several authors described the marked efficacy of dinaline preferentially in slowly growing tumors (acetoxymethyl-methylnitrosamine-induced rat colorectal adenocardinoma [3, 26], methylnitrosourea-induced rat mammary carcinoma [3, 27], mouse osteosarcoma C22LR [28], BNML [2], human ovarian, colon and lung tumors [28]). These tumors are characterized by a high degree of quiescent cells in G_0-phase. Quiescent cells, i.e. cells in G_0-phase, are usually less sensitive to the cytotoxic effect of antitumor agents than proliferating cells [25]. This is not so with dinaline because FL and ARN cells, which were synchronized in G_0/G_1-stage by isoleucin deprivation, were as effectively killed by dinaline as exponentially growing cells.

The fact that dinaline causes an accumulation of cells in G_0/G_1-phase followed by cell death is unusual. Drewinko who investigated 17 antitumor agents of various classes found that all of them caused a delay in G_2-traverse, leading to an accumulation of cells in G_2-phase [29]. A delay at the G_0/G_1-transition or in the early S-phase is characteristic for some antimetabolites and protein inhibitors such as methotrexate [30], hydroxyurea [31], 5-fluoruracil [32] and 1-α-D-arabinofuranosylcytosine [33]. The need for the prolonged and continuous presence of dinaline for an effective treatment suggests that dinaline may act as a competitive inhibitor in an important metabolic pathway. It also may explain why no

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effects on DNA$^-$, RNA$^-$, protein$^-$ or lipid-synthesis of L1210 cells and even no changes in cell cycle distribution of chinese hamster ovary cells were found after only 4 h of incubation with dinaline [4]. Similarly, no diminished virulence of L5222 leukemia cells in intracerebrally transplanted rats was found following 2 h incucation of the cells with dinaline [3].

The cell growth kinetics (Fig. 1) show that cytostasis occurs during the first 24 h of dinaline treatment because the cells do not divide any more. Dinaline-induced cell kill starts only after this time which corresponds approximately to 2 doubling times of the cells. Whith regard to the cell cycle distribution during this period (Fig. 6) it may be concluded that dinaline inhibits cell proliferation by a reversible block in the G_0/G_1-phase or in the G_1/S-traverse.

Under normal conditions the rate of DNA synthesis in cultivated cells is strongly correlated with their intracellular pH. In stimulated lymphocytes the lowering of the intracellular pH about 0.3 units reduces DNA synthesis by about 90 % [34]. Likewise the significant pH drop in FL and ARN cells, after 8 h of incubation with dinaline (Fig. 3A, B) may contribute to the observed block of DNA-synthesis in tumor cells.

Considering the decrease in cAMP-levels [4], the loss of mitochondrial potential (Fig. 4A, B), and the changes of transmembrane potential (Fig. 4C, D) in malignant cells following dinaline treatment, our results are compatible with the concept that dinaline deteriorates the cell energy metabolism. This could lead to the observed inability of the tumor cells to enter the S-phase and finally cause cell shrinkage and cell death.

Materials and methods

Materials

Multiple drug resistant ARN cells were derived from drug-sensitive Friend virus transformed murine leukemia cells (FL cells) [10]. Dulbecco's modified Eagle's medium (No. 041-02320H), MEM Select-Amin-Kit (No. 062-09050A) and fetal calf serum (No. 013-06290H) were obtained from Gibco (Eggenstein, FRG) and 50 ml tissue culture flasks from Falcon (Heidelberg, FRG, No. 3013). Dinaline was provided by Gödecke (Freiburg, FRG) as dry powder. It was solubilized at 4 mg/ml in dimethylformamide (DMF) (Merck, Darmstadt, FRG). 3,3'-dihexyloxacarbocyanine ($DiOC_6(3)$) was from Eastman Kodak (No. 138141), rhodamine 123 (Rh123) (No. R8004), propidium iodide (PI) (No. P5264) and Hoechst 33342 (No. B2261) from Sigma (Deisenhofen, FRG). 1,4-diacetoxy-2,3-dicyanobenzene (ADB) and 2,3 dicyano-hydroquinone (DCH) were obtained from Paesel (Frankfurt, FRG).

Cell culture

1×10^5 FL cells or ARN cells were seeded and cultured in 10 ml complete medium (DMEM, 10 % FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin) or in 10 ml isoleucin deprived DMEM in presence or absence of 2 or 20 µg/ml dinaline to a density of 2×10^6 cells/ml. 1 ml aliquots were transferred to 1.5 ml Eppendorf tubes after the indicated time intervals for flow cytometric analysis.

Estimation of cell growth and viability

The cells were counted daily during dinaline incubation in an improved Neubauer hemocytometer chamber (0.1 mm depth). Viability was checked by mixing an aliquot of cells 1:1 with a 0.5 % trypan blue solution in HEPES buffered saline (HBS) (0.15 M NaCl, 5 mM HEPES, pH 7.35) followed by 2 min incubation at room temperature, and microscopic scoring of the stained cells. The viability estimate was defined as the ratio of trypan blue stained cells to the total cells.

Staining procedures

The cells were twice washed by centrifugation (30 s, 3,000 g) with 1.5 ml ice-cold HBS, resuspended in 250 µl HBS and stained for 5 min at room temperature following the addition of 5 µl ADB/PI cocktail (1 mg/ml, 2 mg/ml in DMF) for the pH- and carboxylesterase-measurements. ADB is cleaved by intracellular carboxylesterases into DCH and acetate. DCH is fluorescent, accumulates in vital cells and indicates pH changes by a shift of fluorescence colour [7]. The fluorescence of DCH-stained or PI-stained cells was excited between 350 and 380 nm. Blue fluorescent light from DCH was collected between 410-440 nm. Green fluorescence of DCH and red fluorescence of the DNA of dead cells were collected between 500 and 750 nm (green and red fluorescence). The intracellular pH of each cell was calculated from the ratio of both fluorescences using a calibration table in the DIAGNO 1 computer program (see below).

Changes of the transmembrane potential were determined with the dye $DiOC_6(3)$; measurement of the transmitochondrial potential was done with Rh123. Stock solutions contained 0.01 mg $DiOC_6(3)$/ml or 0.5 mg Rh123/ml both in DMF. 1 ml aliquots of the cells were centrifuged and resuspended in 250 µl HBS. 5 µl of the respective stock solution were added and the cells were incubated for 30 min at 37° C. The cells were then centrifuged for 60 s at 3,000 × g, the supernatant was removed, the cell pellet was washed once with 1.5 ml HBS by centrifugation and the cell sediment was resuspended in 250 µl ice-cold HBS. PI (5 µl of a stock solution 2 mg/ml in HBS) was added to the assay to counterstain the DNA of dead cells. The cells were placed on ice and analyzed at least 5 min, but not later than 15 min, after PI addition. The fluorescence of $DiOC_6(3)-$, Rh123 – and PI – stained cells was excited between 458 and 502 nm and collected between

510-555 nm (yellow fluorescence, F1) for $DiOC_6(3)$ and Rh123 and between 555-700 nm (red fluorescence, F2) for PI. Simultaneous Hoechst/PI staining was performed by adding 10 μl of a 2 mM solution of Hoechst 33342 dye in DMF to 1 ml aliquots of the cells in 1.5 ml Eppendorf tubes and 1 h incubation at 37° C. The stained cells were centrifuged and washed with 1.5 ml ice-cold HBS, resuspended in 1 ml HBS and 5 μl of a PI-stock solution 2 mg/ml in HBS were added. The cells were kept at 4° C and analyzed not later than 30 min after PI staining.

Flow cytometry

The flow cytometric measurements were performed with a Fluvo-Metricell flow cytometer (HEKA-Elektronik, Lambrecht/Pfalz), equipped with a HBO-100 high pressure mercury arc lamp and interfaced to an AT-compatible personal computer (80286/80287 processors, 640 KB memory, 4 Mbyte RAM disk, 70 Mbyte harddisk, 60 Mbyte streamer tape, 1.2 Mbyte floppy disk). The instrument was calibrated by day-to-day measurement of either 5 μm porous, NH_2-group bearing beads prestained with FITC or DCH or of 4.5 μm fluorescent and monodisperse microspheres (BB or YG, Polysciences, St Goar, FRG).

The electronic cell volume was measured in a cylindrical orifice of 80 μm diameter and 90 μm lenght with hydrodynamic focusing of the cells through the center of the orifice at an electrical current of 0.2-0.4 mA. The electrical cell volume and the fluorescence pulses were amplified by 3 decade logarithmic amplifiers with photomultiplier settings of 560 V for the F1-fluorescence channel (F1) and of 510 V for the F2-fluorescence channel (F2) of the flow cytometer when DCH, $DiOC_6(3)$ and Rh123 were measured. The fluorescence of Hoechst 33342/PI stained cells was linearly amplified at photomultiplier voltages of 470 V (F1) and 435 V (F2). The electronic volume signal and two fluorescences of each cell were measured simultaneously and the 3 measured values per cell were stored sequentially on the hard disk of the computer (list-mode operation).

10,000-20,000 cells were acquired per measurement in list mode operation. The list mode data were evaluated after the measurement by the DIAGNO 1 program system [11] (HEKA-Elektronik, Lambrecht/Pfalz) and displayed in the F1 versus F2 projection. The PI (red fluorescence) stained dead cells were well separated in the F1 versus F2 fluorescence histogram from the vital cells. Evaluation areas for vital and dead cell clusters in the histogram were established with the DIAGNO 1 software [12]. The cell volume versus F1 projection of the vital cells was then recalculated from the list mode data and displayed. The mean values of the cell volume and fluorescence intensity of the cells of a given cluster were calculated. For the analysis of the DCH-stained cells the cell volume was plotted in addition versus the intracellular pH. The coefficient of variation (CV) of the G_0/G_1-peak of the DNA distributions was calculated according to the equation :

$$CV = \frac{HW \times 100}{PC \times 2.35}$$

where PC is the peak channel and HW the total width of the peak in histogram channels at half of the peak height. The S− and G_2M-phases of the cell cycle were determined in the following way : The left side of the G_0/G_1-distribution was imaged along a vertical line through the G_0/G_1-peak to the right side. The symmetrical G_0/G_1-distribution thus obtained was subtracted from the whole DNA histogram. The remainder of the cells to the right of the G_0/G_1-peak were the S− and G_2M-phase cells.

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