Alterations in the cellular DNA and protein content determined by flow cytometry as indicators for chemically induced structural and numerical chromosome aberrations

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Cellular DNA and protein content were determined simultaneously in freshly isolated fibroblast-like rat cells by flow cytometry. After exposure to doxorubicin, nitrofurantoin, propranolol and practolol at a low, tissue like oxygen concentration (5% O_2), drug-induced alterations in cell cycle kinetics, in the distribution of DNA and in the protein content of G_1-phase cells (nucleus/cytoplasm ratio) were analysed. Optimal exposure time (5 or 24 h) and recovery interval (24 or 48 h) were determined. Variation in the exposure time and recovery period can affect cell cycle kinetics both qualitatively and quantitatively, whereas the distribution of DNA and protein content are affected quantitatively only. A 24-h exposure combined with a 24-h recovery period proved to be the most efficient approach. Each of the tested chemicals induced a specific, dose-dependent pattern of altered cellular DNA and protein content. Comparison with results obtained in other genotoxicity tests, and with data reported earlier, showed that this two-parameter protocol can be used to recognize and to characterize chemicals as clastogens, or as compounds with a combined cytostatic/clastogenic activity, or as spindle-poison-like compounds.

Introduction

In a given cell population, flow cytometry provides an efficient tool to analyse specific constituents of individual cells. By simultaneous analysis of the DNA and protein content of intact cells by two-parameter flow cytometry (Maier and Schawalder, 1986) relationships between the cytotoxic/cytostatic activities and the genotoxic activity following exposure to mutagens can be defined. Indicators of such activities are blockage at a specific cell cycle phase, alterations in the dispersion of cellular DNA and protein content in G_1-phase cells and an imbalance between the two parameters (nucleus/cytoplasm ratio). Using this protocol, surviving intact cells are analysed and drug-induced dead cells, with lysed or fragmented nuclei, can readily be discriminated.

During metaphase analysis, only those cells which terminate DNA synthesis and G_2 phase during the relative short arrest interval are analysed. In contrast, using flow cytometry, cells in all cycle phases can be analysed. Analysis of G_1-phase cells allows chemicals with an S-phase dependent activity to be recovered when a sufficient number of damaged cells enter the G_1 phase following treatment. This post-mitotic analysis might be advantageous, particularly for the detection of spindle poisons which induce numerical chromosome aberrations.

With the extension of the recovery period, the accumulation of aneuploid, often slowly cycling cells in the G_1 phase, might be increased. Alternatively, selection against these abnormal cells occurs as a function of the proliferative activity of the normal cells (cell density).

Therefore, the objective of the present study was (i) to evaluate further the suitability of the two-parameter flow cytometry protocol established in this laboratory (Maier and Schawalder, 1986) by varying exposure and recovery periods and (ii) to use this system to analyse two 'problematic' chemicals: nitrofurantoin and propranolol.

The anthracycline antibiotic doxorubicin (DX) was used as a positive control. This chemical is a powerful clastogen in vitro (Vig, 1977), in vivo in mice (Maier and Schmid, 1976; Au and Hsu, 1980) and man (Hacker-Klom et al., 1986). The drug is highly fluorescent (Krishan and Ganapathi, 1980), and when excited with a 350-nm argon laser beam, the emission spectrum (Krishan et al., 1985) is in the same range as the DNA fluorochrome (DAPI) used in the present investigation. This property provided an opportunity to investigate whether the drug could interfere with analysis of DNA.

Nitrofurantoin (NF) is an antibacterial agent used in human and veterinary medicine for the treatment of systemic infections specifically of the urinary tract. It is highly mutagenic in bacterial test systems (Cohen et al., 1973; Yahagi et al., 1976) and induces mitotic gene conversion in yeast (Siebert et al., 1979). However, in mammalian cells, the drug causes only a low incidence of sister chromatid exchanges (Shirai and Wang, 1980). No tumorigenic activity has so far been reported in rodents (Morris et al., 1969; Yahagi et al., 1974). This discrepancy between the mutagenic and carcinogenic activity and the pathways involved in drug activation in mammalian tissues is still a matter for debate (McCalla, 1983). In particular, it is not clear whether the reactivity of the chemical is influenced by the oxygen tension present in tissues.

The third chemical tested was propranolol, a β-adrenergic blocking agent with widespread use in the treatment of hypertension in man. This compound was reported positive in the micronucleus test (Okine et al., 1983; Aruna and Krishnamurthy, 1986) but its genotoxicity has not been confirmed in other assays. Practolol is also a β-blocking agent which has been withdrawn from the market because of its induction of a rare oculomucocutaneous syndrome (Wright, 1975) in humans. It was used as a negative control.

Primary cultures of fibroblast-like rat cells were used in the experiments. These euploid cells maintain a highly stable DNA content over several passages and their metabolism parallels that found in the corresponding tissue in vivo.

Materials and methods

Chemicals

The source of the chemicals was as follows: DX (Farmitalia, Carlo Erba, Milano, Italy), NF (1-[(5-nitrofurfurylidene) amino]-hydantoin) (Sigma Chemical Company, St Louis, MO, USA), practolol [N-4-(2-hydroxy)-3(1-metyl)-aminopropoxyphenyl-acetamine] and propranolol [1-(isopropylamino)-3(1-naphthyl-oxy)-2-propranolol]
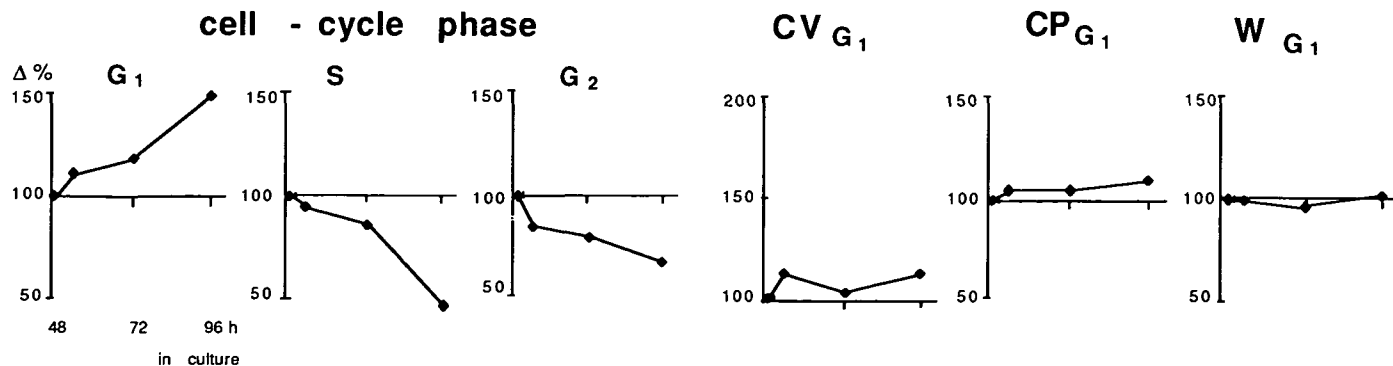
Fig. 1. Influence of exposure/recovery schedule on DNA/protein parameters in untreated cells. Data are pooled from control values of all chemicals tested. The culture period corresponds to the following exposure/recovery periods: 48 h = 24/0 h; 53 h = 5/24 h; 72 h = 24/24 h; 96 h = 24/48 h.

(Imperial Chemical Industries, Macclesfield, UK). DX, practolol and propranolol were dissolved in culture media (Dulbecco's modified Eagle's medium, DMEM) and NF in dimethylsulphoxide (DMSO) to a maximal final concentration of 0.3% at the highest exposure level. Appropriate controls were carried out with the highest DMSO concentration. The fluorochromes used were sulphorhodamine 101 (SR 101, Sigma) for protein 4,6-diamidino-2-phenyl indole dihydrochloride (DAPI, Serva, Munich, FRG) for DNA.

Cells and treatment

Fibroblast-like cells were isolated from subcutaneous granulation tissue of nearly adult male rats (220 ± 15 g, n = 15, Sprague−Dawley, SIV 50, Ivanovas, Kisslegg, FRG). Growth of this tissue was initiated on the inside wall of an air pouch at the scapular region of young adult male rats (Maier, 1984). Four days later, cells were isolated according to standardized procedures. Fibroblast-like cells were then cultured for 3 days in DMEM supplemented with 10% fetal calf serum and 5 μl/ml gentamycin under the following conditions: 12% CO_2, 5% O_2, 36.5°C, pH 7.4, RH 95%. Exposure of cells occurred at physiologic tissue pO_2 which corresponds to an O_2 concentration in the incubator of 5% (Maier et al., 1987). In a series of experiments with NF, hyperoxic conditions (31% O_2) were chosen.

On day 3, cells were trypsinized and the cells seeded per dish were adapted according to the exposure/recovery period in order to prevent contact inhibition of growth. After 24 h in culture, the test compounds were added for 5 or 24 h to these asynchronous, rapidly proliferating cell populations. The short exposure time (5 h) was assumed to approximate to the exposure time which would be expected after an *in vivo* treatment. The 24-h exposure interval covers more than the whole cell cycle of proliferating cells.

After replacement of the medium, treated cells were subjected to a recovery period of 24 or 48 h. The extended recovery period was designed to increase further the accumulation of aneuploid cells in G_1 phase. Subsequently, cultures were washed with phosphate-buffered saline (PBS), trypsinized (trypsin/EDTA) and the dispersed cells fixed in cold 70% ethanol added to the culture dishes. After centrifugation cells were fixed in 90% ethanol for 30 min to 2 h.

Staining

The staining solution was a mixture of 10 ml SR 101 solution (3 mg sulphorhodamine 101 dissolved in 100 ml Tris−HCl, 180 mM, pH 7.4, 200 mM NaCl) and 0.3 ml DAPI solution (10 mg DAPI in Tris−HCl) as described earlier (Stöhr et al., 1978; Maier and Schawalder, 1986). This cold staining solution was added to the cell pellet. Cells were suspended and stained for 15 min on a Coulter Mixer in the dark. Subsequently, they were stored on ice (1−2 h) and filtered through a 14-μm nylon mesh immediately before flow analysis.

Flow measurements

Stained samples were analysed in the staining solution on a PAS II flow cytometer (Partec, Arlesheim, Switzerland) with the following conditions: excitation—100-W mercury lamp, UG1 (290−410 nm, 3 mm), chromatic beam splitter (460 nm); emission—beam splitter (610 nm), 610-nm barrier filter for SR 101 analysis and a GG 455 filter for DAPI signals. Flow rate: 200−400 cells/s; objective: 40× Nikon UV-F for epifluorescence (numerical aperture 1.3) with glycerine immersion and Köhler illumination. As an internal standard for DNA we took freshly isolated and fixed rat thymocytes. Their coefficients of variation (CV) in the DNA content of G_1 cells before measurements were always <2.5%. Standards for protein were cryopreserved fibroblast-like human cells. Protein measurements were triggered by the DNA fluorescence signals.

From each treated cell population, 5 × 10⁴ cells were analysed. Each chemical was tested in one experiment over the whole dose range including the control Measurements were repeated in 3−5 separate experiments (Table III). The controls

were rerun after each individual series. Only when the same modal DNA values were obtained as at the beginning were data further analysed.

Data acquisition was carried out with a FDAS II computer (Ahrens et al., 1980). From each sample the single-parameter frequency distribution histograms of DNA and protein, the two-parameter contour display, the three-dimensional perspective display and the protein single-parameter histogram of gated G_1 cells were recorded and stored on floppy disks.

Data analysis

DNA and protein single-parameter histograms were analysed with fitting programs (FIT 4.24) provided by O.Ahrens (Bargteheide, FRG). The DNA dispersion of the G_1 peak was described by the coefficient of variation of the G_1 cell population (CV_{G1}). The relative contribution of cells in the G_1/G_0, S and G_2/M phases was calculated by means of a modified least-square fitting method (Gray and Dean, 1980) (Figure 2b).

The values of the cellular protein content in gated G_1 cells were best fitted by a X-log-normal distribution (Figure 2c). The channel position of the modus (CP_{G1}) and the width of the distribution $(W_{G1}$ value) were used to describe the drug-induced effects. An excellent correlation existed between the position of the modus of the protein distribution and the protein content of these cells as determined by the method of Bradford (1976) (data not shown).

DNA and protein data are summarized in Tables I, II and III. Values represent the mean from individual experiments, including standard deviations.

For illustration, the dose−response effect of DX is shown in representative single-parameter DNA and protein histograms with the corresponding contour plots (Figure 2). Histograms selected best fit the mean value at this exposure levels as indicated in Table III. They are representative for the analysis of the other chemicals.

Results

From untreated cell populations, the mean of the CV of the DNA content in G_1 cells ranged from 2.7 to 4.9% between individual independent experiments. This variability may be due to differences in cell preparations (fixation), in the staining solutions and most importantly on the condition of the high-pressure mercury arc lamp or in the adjustments of the optical system of the flow cytometer. CV values respond most sensitively to these parameters. Therefore, only when experiments are performed over the entire dose range of a chemical, in the cell population from the same rat, analysed on the same day and with reference samples always used after each abnormal histogram, will drug-induced CV alterations be discriminated from artefacts.

Alterations in the cell cycle kinetics of untreated cells might arise from handling of the cells during culture. This was demonstrated by the analysis of six untreated cell populations cultured in parallel from two animals (Table I). Clearly, growth behaviour from one culture dish to the other from the same animal does not significantly contribute to the observed variations. The variability derives from the use of cell populations from different rats.

Alterations in cell cycle phases of untreated cells were also found with increasing culture time in the protocols chosen (Table
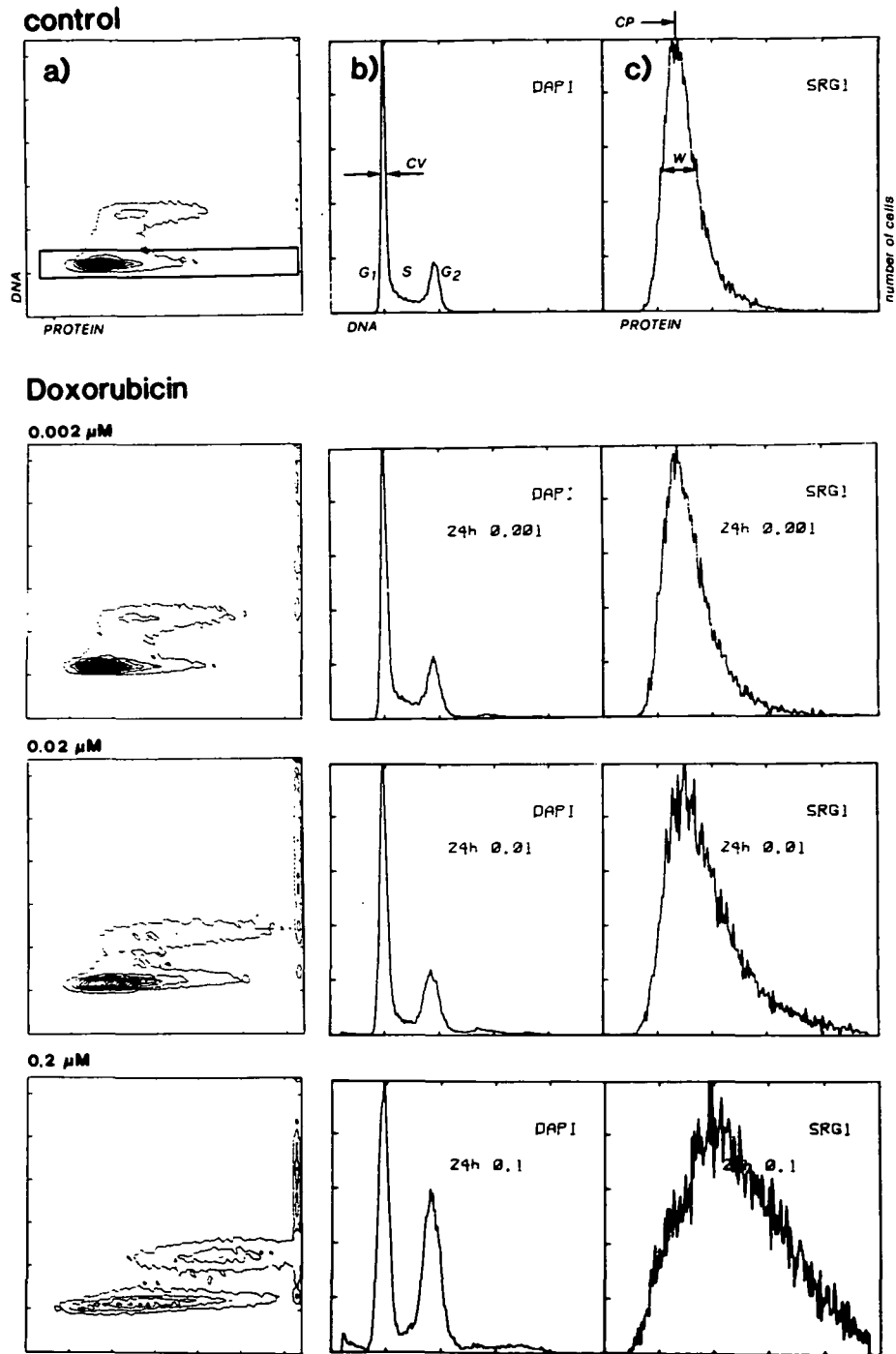
Fig. 2. Analysis of cellular DNA and protein content. (a) Gate for protein analysis in G_1 cells defined in the contour plots. (b) Analysis of DNA parameters [coefficient of variation of G_1-phase cells (CV_{G1}) and G_1-, S- and G_2-phase calculations] from the DNA histogram. (c) Analysis of protein histograms of G_1 cells: channel position (CP_{G1}) and W value (W_{G1}) and drug induced effects by doxorubicin.

III). This was due to the decrease of the trypsin-induced synchronization after subcloning and, despite the reduction of cell number in the 24/24- and 24/48-h schedules, to a density-dependent inhibition of growth. The altered growth rate alone does not affect CV, CP and W values as shown in Figure 1. Accordingly a chemical which affects growth behaviour alone will not affect these parameters. At the time of treatment, however (24 h after seeding), these differences between the protocols do not exist.

In untreated cell populations, the positions of the mode of the protein histograms (CP_{G1}) in relation to the cellular standard (cryopreserved fibroblast-like human cells) do not vary more than $\pm 5\%$. The W values as an indicator for the heterogeneity in the protein histograms varied between 1.32 and 1.47.

Exposure levels were selected so that no signs of acute (detachment of cells) or delayed toxicity (protein leakage) within the 24-h or the 48-h recovery period were observed. Extension of the exposure period enhanced the dose-dependent increase of the dispersion of DNA content (CV_{G1} values) by DX and NF. This is probably due to the fact that more cells were dividing in the

Table I. DNA and protein parameters determined from histograms of untreated cells cultured in parallel

	Culture no.	DNA (contribution in %)				Protein	
		G_1	S	G_2	CV_{G1}	CP_{G1}	W_{G1}
Rat 1	1	48.3	30.4	21.2	4.7	67	1.46
	2	46.6	31.0	22.5	5.6	80	1.42
	3	43.4	32.5	24.1	4.6	75	1.40
	4	46.0	30.0	24.0	4.5	77	1.40
	5	44.8	33.0	22.3	4.8	77	1.40
	6	45.3	31.8	22.9	5.1	71	1.42
Mean ± SD		45.7 ± 1.7	31.5 ± 1.2	22.8 ± 1.1	4.9 ± 0.4	75 ± 5	1.41 ± 0.03
Rat 2	1	52.7	25.8	21.6	3.1	79	1.35
	2	53.5	24.7	21.8	4.8	68	1.37
	3	52.2	25.3	22.6	4.2	83	1.40
	4	48.4	25.0	26.2	4.5	82	1.40
	5	52.1	26.1	21.8	4.0	70	1.38
	6	48.4	27.3	24.3	4.9	70	1.40
Mean ± SD		51.3 ± 2.1	25.7 ± 0.9	23.1 ± 1.8	4.3 ± 0.7	75 ± 7	1.38 ± 0.02

Data are obtained with cells from two rats. Six dishes were cultured in parallel and analysed individually 48 h after seeding.

Table II. Influence on DNA and protein parameters as a function of cell density and conformational changes of G_1-phase cells to G_0-phase cells

Culture	DNA (contribution in %)					Protein	
	G_1	S	G_2	CV_{G1}	CV_{G2}	CP_{G1}	W_{G1}
48-h	48.4 ± 1.1	27.0 ± 0.7	24.6 ± 0.4	3.9 ± 1.4	4.3 ± 1.6	68 ± 1	1.42 ± 0.06
72-h	63.3 ± 7.7	20.2 ± 5.7	16.6 ± 2.0	3.3 ± 1.0	3.6 ± 1.3	70 ± 3	1.37 ± 0.03
96-h	81.7 ± 4.5	6.7 ± 1.2	11.7 ± 3.3	3.2 ± 0.4	3.5 ± 0.4	69 ± 15	1.45 ± 0.16

Values are from four independent continuous cultures analysed 48, 72 and 96 h after seeding of 3×10^5 cells into 15-cm dishes. Values = mean ± SD.

Table III. DNA and protein parameters calculated from single-parameter histograms of cells exposed to test compounds

Exposure	Treatment interval (h)	Recovery period (h)	n	DNA (contribution in %)				Protein	
				G_1	S	G_2	CV_{G1}	CP_{G1}	W_{G1}
Doxorubicin									
Control	5	24	3	48.7 ± 3.2	28.9 ± 1.5	22.4 ± 2.1	4.2 ± 1.1	66 ± 4	1.44 ± 0.05
	24	24	3	59.6 ± 8.3	22.1 ± 5.2	18.3 ± 3.3	3.5 ± 0.8	70 ± 2	1.36 ± 0.3
	24	48	3	70.8 ± 14.0	11.3 ± 8.0	17.6 ± 6.7	4.9 ± 1.4	62 ± 4	1.47 ± 0.08
0.002 μM	5	24	3	45.4 ± 3.2	28.0 ± 5.9	26.6 ± 2.8	4.0 ± 1.0	76 ± 9*	1.42 ± 0.01
	24	24	3	54.0 ± 3.0	24.6 ± 0.4	21.4 ± 2.8*	5.0 ± 0.2	65 ± 14	1.42 ± 0.04
	24	48	3	70.7 ± 11.5	11.6 ± 8.5	17.8 ± 3.9	5.4 ± 1.0	59 ± 10	1.45 ± 0.05
0.01 μM	5	24	3	48.0 ± 3.7	28.3 ± 2.7	23.7 ± 1.3	4.9 ± 0.3	71 ± 15	1.40 ± 0.08
	24	24	3	56.4 ± 1.4	22.5 ± 0.3	21.1 ± 1.7*	5.3 ± 1.1*	71 ± 5	1.46 ± 0.06
	24	48	3	69.0 ± 10.6	12.3 ± 5.7	18.8 ± 4.9	6.8 ± 2.1	75 ± 8*	1.53 ± 0.09
0.02 μM	5	24	3	47.2 ± 1.7	26.7 ± 1.2*	26.1 ± 0.5	6.1 ± 1.3	68 ± 15	1.45 ± 0.04
	24	24	3	59.0 ± 4.4	18.3 ± 1.7	22.7 ± 4.0*	5.1 ± 2.0	78 ± 8	1.56 ± 0.05*
	24	48	3	68.6 ± 10.6	11.8 ± 5.3	19.6 ± 5.6	6.1 ± 0.9	68 ± 12	1.57 ± 0.15
0.1 μM	5	24	3	54.9 ± 2.2*	20.5 ± 3.5*	24.5 ± 2.5	5.9 ± 1.6	82 ± 23	1.54 ± 0.09*
	24	24	3	54.8 ± 4.4	10.8 ± 2.0*	34.4 ± 2.4*	7.7 ± 1.8*	107 ± 14*	1.88 ± 0.13*
	24 ·	48	3	60.7 ± 2.5	8.2 ± 1.8	31.1 ± 1.0*	8.1 ± 1.8*	98 ± 7*	1.93 ± 0.15*
0.2 μM	5	24	3	48.0 ± 5.2	19.7 ± 2.3*	32.3 ± 7.2	7.6 ± 1.4	84 ± 23	1.59 ± 0.12*
	24	24	3	48.9 ± 4.6	11.9 ± 2.9	39.1 ± 2.1*	8.3 ± 1.2*	116 ± 30*	1.93 ± 0.04*
	24	48	3	51.6 ± 0.5	11.5 ± 3.2	36.9 ± 2.8*	9.4 ± 1.8*	112 ± 13*	2.08 ± 0.38*
Nitrofurantoin									
Control									
5% O_2	5	24	2	70.8 ± 22.2	17.3 ± 16.1	12.0 ± 6.2	3.4 ± 0.8	70 ± 1	1.32 ± 0.01
	24	0	2	49.3 ± 1.5	32.1 ± 3.2	18.8 ± 1.6	2.6 ± 0.5	65 ± 2	1.41 ± 0.03
	24	24	4	58.7 ± 2.6	25.5 ± 1.9	15.8 ± 1.2	3.3 ± 1.3	67 ± 14	1.36 ± 0.06
	24	48	4	72.8 ± 9.6	15.3 ± 5.5	11.9 ± 4.3	2.7 ± 0.5	78 ± 9	1.39 ± 0.08

Exposure	Treatment interval (h)	Recovery period (h)	n	DNA (contribution in %)				Protein	
				G_1	S	G_2	CV_{G1}	CP_{G1}	W_{G1}
31% O_2	24	0	2	67.0 ± 9.6	20.2 ± 1.8	12.9 ± 7.8	3.3 ± 0.1	72 ± 6	1.47 ± 0.01
	24	24	4	54.6 ± 3.5	29.6 ± 1.7	15.8 ± 2.3	3.6 ± 0.6	74 ± 21	1.39 ± 0.09
	24	48	3	56.7 ± 8.3	25.4 ± 3.2	17.9 ± 5.0	3.1 ± 0.2	83 ± 16	1.38 ± 0.03
20 μM									
5% O_2	5	24	1	56.8	29.6	13.6	4.5	73	1.33
	24	0	2	53.0 ± 4.0	31.4 ± 1.4	15.6 ± 5.5	3.2 ± 0.3	72 ± 6	1.37 ± 0.07
	24	24	4	59.5 ± 4.3	25.8 ± 1.1	14.9 ± 3.3	3.8 ± 1.5	72 ± 17*	1.40 ± 0.08
	24	48	2	60.6 ± 5.6	20.8 ± 0.1	18.7 ± 5.7	3.7 ± 1.2	64 ± 12	1.42 ± 0.11
31% O_2	24	0	2	71.4 ± 14.8	12.2 ± 0.4	16.4 ± 15.1	3.6 ± 0.1*	75 ± 6	1.50 ± 0.10
	24	24	4	54.0 ± 8.7	30.3 ± 4.1	15.7 ± 4.7	3.5 ± 0.7	74 ± 24	1.42 ± 0.08*
	24	48	2	49.1 ± 6.3	26.4 ± 2.9*	24.6 ± 3.4	3.4 ± 0.1	80 ± 9	1.44 ± 0.01
40 μM									
5% O_2	5	24	2	68.9 ± 18.0	18.3 ± 12.7	12.2 ± 6.5	4.0 ± 1.4	68 ± 8	1.32 ± 0.03
	24	0	2	53.3 ± 2.3	32.7 ± 2.4	14.0 ± 4.7	3.1 ± 0.4	76 ± 2*	1.44 ± 0.06
	24	24	4	57.5 ± 4.9	27.0 ± 2.3	15.5 ± 2.7	3.9 ± 1.4*	76 ± 23	1.40 ± 0.10
	24	48	4	67.6 ± 10.3*	17.8 ± 7.1	14.7 ± 3.4	2.9 ± 0.3	77 ± 5	1.38 ± 0.09
31% O_2	24	0	2	65.3 ± 20.5	19.8 ± 6.8	14.9 ± 13.7	3.9 ± 0.3	78 ± 20	1.51 ± 0.01
	24	24	4	57.6 ± 9.4	31.8 ± 4.1	10.7 ± 6.4	4.4 ± 0.5*	86 ± 21	1.57 ± 0.14*
	24	48	3	53.0 ± 14.6	25.4 ± 5.0	21.7 ± 9.6	3.8 ± 0.2	92 ± 21	1.46 ± 0.05*
100 μM									
5% O_2	24	0	2	59.8 ± 23.9	23.8 ± 7.8	16.5 ± 16.0	4.1 ± 1.6	81 ± 6	1.44 ± 0.10
	24	24	4	62.9 ± 9.4	23.2 ± 1.8	14.0 ± 8.2	4.8 ± 1.1	89 ± 24*	1.47 ± 0.07*
	24	48	4	58.3 ± 8.6*	22.3 ± 1.8*	19.5 ± 7.4	3.5 ± 0.4	91 ± 12*	1.53 ± 0.16
31% *$_2$	24	0	2	69.5 ± 17.1	17.8 ± 0.9	12.6 ± 16.2	4.0 ± 0.9	71 ± 13	1.53 ± 0.02
	24	24	5	55.7 ± 10.9	26.7 ± 6.0	17.5 ± 15.2	4.6 ± 0.7	88 ± 16	1.50 ± 0.05
	24	48	3	46.6 ± 8.4*	28.3 ± 1.1	25.1 ± 7.3*	3.8 ± 0.6	88 ± 19	1.47 ± 0.09
200 μM·									
5% O_2	5	24	2	70.1 ± 9.4	18.6 ± 9.4	11.4 ± 0	4.5 ± 1.2	72 ± 13	1.33 ± 0.01
	24	0	2	55.3 ± 20.9	30.4 ± 11.6	14.3 ± 9.3	3.8 ± 0.6	84 ± 4.7*	1.42 ± 0.03
	24	24	4	61.0 ± 14.9	25.5 ± 6.4	13.6 ± 9.0	5.2 ± 0.6	92 ± 14*	1.51 ± 0.09
	24	48	4	53.3 ± 15.7*	26.1 ± 4.8*	20.6 ± 12.1	3.7 ± 0.2	101 ± 15*	1.54 ± 0.16
31% O_2	24	0	2	60.8 ± 15.1	17.6 ± 4.4	21.6 ± 10.6	4.0 ± 0.5	71 ± 4	1.49 ± 0.13
	24	24	4	63.4 ± 1.43	16.7 ± 6.1*	24.4 ± 15.6	4.6 ± 0.6	79 ± 24	1.51 ± 0.05
	24	48	2	43.3 ± 10.8	24.3 ± 1.5*	32.5 ± 9.3	3.7 ± 0.6	90 ± 11	1.63 ± 0.15
Propranolol									
Control	5	24	3	49.7 ± 7.5	30.3 ± 2.6	20.1 ± 5.8	3.7 ± 1.0	68 ± 7	1.39 ± 0.04
	24	24	4	56.9 ± 10.6	26.2 ± 4.1	16.9 ± 6.5	3.1 ± 0.4	69 ± 14	1.34 ± 0.05
1 μM	5	24	3	48.5 ± 6.7	33.2 ± 1.9	18.2 ± 4.7	4.2 ± 1.2*	70 ± 14	1.39 ± 0.03
	24	24	4	55.9 ± 9.1	26.3 ± 4.5	17.9 ± 5.0	3.7 ± 0.4*	76 ± 11	1.35 ± 0.04
10 μM	5	24	3	49.5 ± 9.1	32.4 ± 3.8	18.0 ± 5.9	3.9 ± 0.4	71 ± 9	1.34 ± 0.08
	24	24	4	55.4 ± 6.5	27.4 ± 2.2	17.1 ± 4.6	4.2 ± 1.0*	74 ± 11	1.34 ± 0.05
32 μM	5	24	2	46.0 ± 1.3	30.2 ± 0.7	23.8 ± 0.6	3.7 ± 0.1	66 ± 10	1.35 ± 0.01
	24	24	2	51.3 ± 7.5	27.4 ± 2.0	21.3 ± 5.2	4.0 ± 0.2	67 ± 1	1.35 ± 0.03
100 μM	5	24	3	46.6 ± 3.0	32.4 ± 1.8	20.9 ± 1.8	3.6 ± 0.5	72 ± 10	1.36 ± 0.02
	24	24	4	53.5 ± 6.6	27.4 ± 1.7	19.0 ± 5.1	4.3 ± 0.4*	75 ± 13	1.32 ± 0.06
KP 7-cells									
Control	24	24	2	70.0 ± 1.3	15.4 ± 1.4	14.6 ± 0.1	2.8 ± 0.2	78 ± 20	1.38 ± 0.08
1 μM	24	24	2	70.0 ± 3.3	15.0 ± 0.8	15.0 ± 1.5	2.9 ± 0.6	81 ± 18	1.36 ± 0.01
3.2 μM	24	24	2	70.1 ± 1.9	15.3 ± 0.5	14.7 ± 1.3	3.3 ± 0.1	73 ± 27	1.37 ± 0.08
10 μM	24	24	2	69.6 ± 1.8	14.9 ± 0.7	15.4 ± 01.1	3.1 ± 0.4	68 ± 25	1.35 ± 0.01
32 μM	24	24	2	68.5 ± 2.0	14.9 ± 0.7	16.6 ± 0.8	3.2 ± 0.8	83 ± 40	1.33 ± 0.07
100 μM	24	24	2	57.0 ± 0.1	17.0 ± 0.4	26.0 ± 0.4	3.5 ± 1.3	76 ± 23	1.37 ± 0.02
Practolol									
Control	5	24	3	50.4 ± 9.1	30.9 ± 2.8	18.7 ± 6.3	3.6 ± 1.1	71 ± 4	1.40 ± 0.05
	24	24	3	54.0 ± 10.8	27.4 ± 4.0	18.5 ± 6.8	3.1 ± 0.5	66 ± 6	1.35 ± 0.06
1 μM	5	24	3	50.5 ± 9.1	31.5 ± 3.1	18.0 ± 6.1	3.6 ± 0.8	77 ± 23	1.34 ± 0.03
	24	24	3	55.9 ± 3.0	27.3 ± 0.7	16.8 ± 2.6	2.7 ± 0.3	68 ± 8	1.34 ± 0.05
10 μM	5	24	3	46.9 ± 9.6	32.2 ± 4.0	20.9 ± 7.2	4.0 ± 0.6	74 ± 22	1.36 ± 0.04
	24	24	2	57.5 ± 5.9	27.5 ± 2.8	15.0 ± 3.2	2.8 ± 0.1	73 ± 9	1.36 ± 0.08
100 μM	5	24	3	50.0 ± 11.0	31.7 ± 5.0	18.6 ± 6.4	3.8 ± 0.8	68 ± 14	1.34 ± 0.02
	24	24	3	55.3 ± 7.5	26.6 ± 2.8	18.1 ± 4.9	3.5 ± 0.4	76 ± 13	1.36 ± 0.04
320 μM	5	24	2	41.3 ± 0.1	36.6 ± 2.6	22.0 ± 2.0	3.5 ± 1.1	84 ± 15	1.34 ± 0.04
	24	24	2	49.8 ± 1.8	30.1 ± 0.5	20.1 ± 1.4	3.0 ± 0.4	63 ± 5	1.39 ± 0.01

mean ± SD.

n number of experiments.

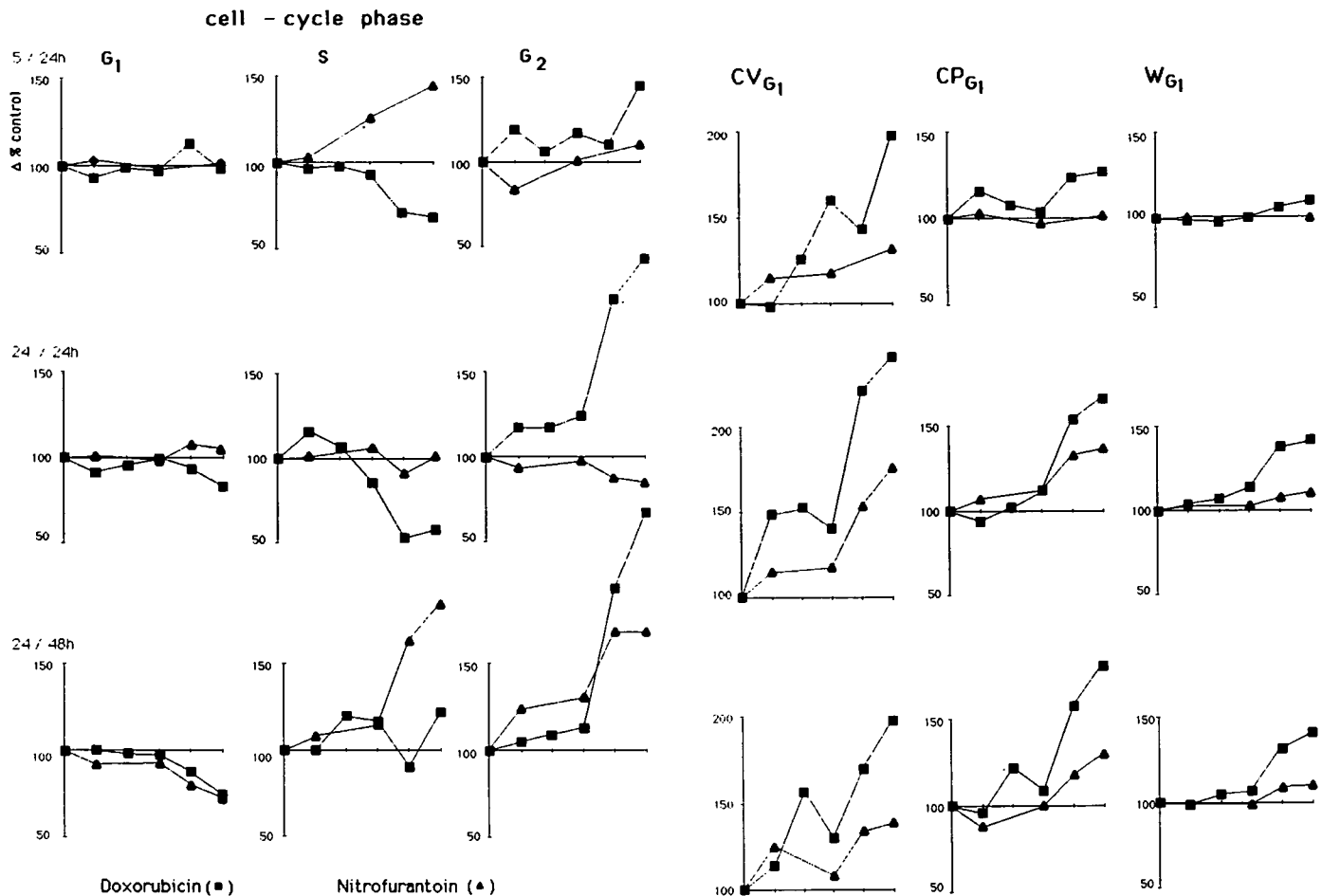* Significantly different from the control culture paired t-test ($P > 0.05$).

Fig. 3. Drug-induced exposure-response effect of the DNA/protein parameter after treatment with doxorubicin and nitrofurantoin. Control values are set at 100%.

presence of the drug and providing sensitive targets for the potential clastogenic activity of the two test agents. With both drugs, the extension in the recovery period from 24 to 48 h resulted in a slight decrease in the CV and protein values. Most likely, after 24 h few additional damaged cells enter the cell cycle to reach the G_1 phase and give rise to a viable aneuploid cells. After 48 h the fraction of unaffected cells selectively increases. With DX no abnormal position of the G_1 DNA peak was detectable which could be attributed to the fluorescence of the test compound itself.

Alterations in the cell cycle phases change dramatically depending on the exposure/recovery intervals chosen and the drug used (Figure 3). In all experimental protocols, DX accumulated cells at G_2/M phase with a feedback control to S phase. The increase of S- and G_2/M-phase cells induced by NF at the 5/24-h and 24/48-h schedule is probably caused by a release of the cells from the chemically induced cytostatic blockage in the G_1 phase. More cells then begin to divide and enter the S and G_2/M phases with a corresponding decrease in G_1 phase. Clearly the effect of DX is longer lasting than that of NF.

With both chemicals the increase in cellular protein content (CP_{G1}) and in heterogeneity (W_{G1} values) is most efficiently induced after a 24-h exposure/24-h recovery interval; qualitatively, a 5/24-h schedule gives similar results. Exposure of cells to nitrofurantoin under hyperoxic conditions (31% O_2) or the shortening of the recovery period did not result in increased activity of the chemical (Table III).

With propranolol, no statistically significant dose-dependent alterations in the cell cycle phase were detected, but an increase in the CV_{G1} value at the 24/24-h schedule was noted. These trends were more clearly apparent in experiments with human fibroblasts. Here the drug reduced the contribution of G_1 phase and increased the CV_{G1} in a dose-dependent way: neither the CP_{G1} values nor the W values (Table III) were affected. With practolol, no dose-dependent effect was detectable on any parameter over the entire dose range tested.

Discussion

Binding of fluorochrome to DNA can be influenced by conformational changes in the heterochromatin−DNA complex and this effect can be observed between different cell types in the same animal. Reversible changes are also observed in normal dividing cells in different phases of the cell cycle, e.g. between G_0- and G_1-phase cells (Moser et al., 1981). These conformational changes can be induced chemically, possibly by causing a shift of cells from G_0 to G_1 phase (Moser and Maier, 1987). However, in continuous cultures in which cell-density-dependent inhibition is achieved 96 h after seeding (Table II), G_0-phase cells accumulated but CV_{G1} did not increase significantly (Table II). Even slightly decreased values derive from the absence of cells just entering the S phase (CV_{G1}) and from the absence of cells in metaphase (CV_{G2}). So far, no evidence exists to indicate that with our protocols conformational changes in the

Table IV. Pattern of changes in the DNA and protein histograms obtained by two-parameter flow cytometry 24 h after treatment

Compound	Type of interaction	Exposure level	DNA				Protein	
			G_1	S	G_2	CV_{G1}	CP	W
Model compounds (5/24-h schedule; from Maier and Schawalder, 1986)								
Bleomycin	Clastogenic	Low	0	−	0	+	+	+
		High	0	− −	+ +	+ + +	+ + +	+ + +
Amethopterin	DNA-synthesis inhibiting	Low	0	+	0	0	+	0
		High	0	+ +	− −	+ +	+ +	0
Vincristine	Spindle poison	Low	0	0	0	+ +	0	+
		High	−	+	+	+ + +	0	+ + +
Test chemicals (24/24-h schedule)								
Doxorubicin	Clastogenic	Low	0	−	+ +	+ +	0	+
		High	−	− − −	+ + +	+ + +	+ +	+ + +
Nitrofurantoin	Cytostatic/ clastogenic	Low	0	0	0	+ +	0	0
		High	0	0	− −	+ + +	+ +	0
Propranolol	Spindle-poison like	Low	0	0	+	+ +	0	0
		High	0	0	+ +	+ + +	0	0
		Human cells	−	(+)	+ +	+ + +	0	0
Practolol	None	Low	0	0	0	0	0	0
		High	0	0	0	0	(+)	0

Characterization of dose−response curves: Each + and − represents an exposure level with value (mean) above 115% (+) or below 85% (−) of that of untreated controls and this in the lower (low) or upper (high) exposure range tested.
( ) = trends to be observed.

DNA−chromatin complex contribute to enhanced CV values in the rat cell population.

An attempt was made to classify the four chemicals according to their effects upon the CV_{G1}, cell cycle phase, CP_{G1} and W_{G1} values (Table IV). Data from the previously tested model compounds bleomycin, amethopterin and vincristine (Maier and Schawalder, 1986) were used as standards. Classification was based on the data obtained after a 24-h exposure/24-h recovery period. Results obtained after a 48-h recovery period probably reflected non-specific recovery phenomena in the cell population rather than the drug-specific response of the cells to the initial insult.

The pattern observed with DX (Tables III and IV) is consistent with the known clastogenic activity of this chemical. G_2-phase blockage confirmed reports on the inhibitory activity of this drug in late S phase (Silvestrini et al., 1970) and in G_2 phase (Wheatley, 1972). The doubling of the CV_{G1} values is in agreement with the unusually high frequency of structural chromosome aberrations found in vitro (Vig, 1971). The concomitant increase in the protein content suggests that uncoupling between protein and DNA synthesis might be typical for chemicals which preferentially induce structural chromosome aberrations. This alteration in the nucleus/cytoplasma ratio in G_1-phase cells was observed previously with bleomycin (Maier and Schawalder, 1986). Protein synthesis proceeded independently of DNA synthesis and continued until the normal G_2 phase protein level was reached (Table II, Figure 3). The breakdown of this drug-induced effect was seen at the highest exposure level (0.1 μg/ml). Typically, the DNA parameters are affected (S and G_2 phase and CV_{G1}, Table II) without evidence of cytotoxicity (protein leakage).

From CV values obtained with NF, the drug appeared to be metabolized to genotoxic intermediates in the fibroblast-like cells of the granulation tissue. Activation of nitrofurans can occur via the prostaglandin-synthetase pathway (Zenser et al., 1984). This pathway is functional in the fibroblast-like cells used in the present

study (Lang et al., 1986). Prostaglandin synthesis is associated with cell proliferation (Cornwell and Morisaki, 1984) and initiation of DNA synthesis (Otto et al., 1982). It could well be that NF induces, concomitant with the metabolic inhibition of cell proliferation, chromosome aberrations in all cell cycle stages without further progression and accumulation in one particular cell cycle stage. From this synchronous arrest a rapid recovery of non-damaged cells from the cytostatic activity is possible, as suggested by data from cell cycle phases and CV analysis in the 5/24-h and 24/48-h schedules. We can thus postulate that the combined cytostatic and clastogenic activity (CV_{G1} values) of the chemical may prevent the progression of aneuploid cells. Such a failure of progression of particular cell types may explain the non-carcinogenic activity of NF (Morris et al., 1969, Yahagi et al., 1974).

The pO_2 does not seem to be critical for the metabolic conversion of NF to mutagenic metabolites. The relatively high effective exposure levels required indicate that in vivo this chemical will most likely exert its mutagenic activity after direct interaction with target tissues. In fact, when given intraperitoneally to rats, NF caused DNA strand breaks in liver (Russo et al., 1982) but had no effect on micronuclei formation in bone marrow cells (Goodman et al., 1977).

The pattern induced by propranolol in rat and human cells resembles qualitatively that of a spindle poison (Table IV). This could explain why no mutagenic activity was found in bacterial tests while an increased formation of micronuclei in mouse bone marrow was observed (Okine et al., 1983). At an exposure of 3/4 LD_{50} an increase of nearly 3-fold in the micronuclei frequency, without interference with the proliferative capacity of the bone marrow cells, was reported (Aruna and Krishnamurthy, 1986). The activity found in our investigation with human cells suggests that they metabolize propranolol more efficiently than rodent cells (Elliott et al., 1984).

Quantitatively, the aneuploidy-inducing activity of propranolol seems to be much weaker than with vinblastin. Differences in

the incidence of chromosomal loss in post-mitotic cells between different spindle poisons are well described. In the micronucleus test, colchicine induces a much lower incidence of micronuclei than the alkaloid vincristine (Maier and Schmid, 1976). This difference in micronucleus-inducing capability could be a consequence of the different binding sites of the two chemicals (Dustin, 1984). It is interesting that propranolol exhibits tumour-promoting activity for rat liver (Boyd and Martin, 1977) similar to that of phenobarbital (PB). Also, PB has been reported to interefere with tubulin assembly (Albertini *et al.*, 1985). However, further data are needed from chemicals with well-characterized interactions with individual steps in the chromosomal segregation process, in order to investigate the potential correlation between flow data and the mechanisms involved.

In conclusion, the concomitant analysis of cellular protein and DNA content in living G_1-phase cells by flow cytometry appears to allow the detection of chemicals which induce numerical and structural aberrations. In addition, the interaction with chromosomal material can be characterized. In a tier approach this information enables the efficient selection of the most suitable additional mutagenicity test for confirmation or risk evaluation.

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