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Fundamental and Molecular Mechanisms of Mutagenesis

DNA synthesis inhibition as an indirect mechanism of chromosome aberrations: comparison of DNA-reactive and non-DNA-reactive clastogens

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Abstract

Positive results in the in vitro assay for chromosome aberrations sometimes occur with test chemicals that apparently do not react with DNA, being negative in tests for mutation in bacteria, for DNA strand breaks, and for covalent binding to DNA. These chromosome aberrations typically occur over a narrow concentration range at toxic doses, and with mitotic inhibition. Indirect mechanisms, including oxidative damage, cytotoxicity and inhibition of DNA synthesis induced by chemical exposure, may be involved. Understanding when such mechanisms are operating is important in evaluating potential mutagenic hazards, since the effects may occur only above a certain threshold dose. Here, we used two-parameter flow cytometry to assess DNA synthesis inhibition (uptake of bromodeoxyuridine [BrdUrd]) associated with the induction of aberrations in CHO cells by DNA-reactive and non-reactive chemicals, and to follow cell cycle progression. Aphidicolin (APC), a DNA polymerase inhibitor, induces aberrations without reacting with DNA; 50 μM APC suppressed BrdUrd uptake during a 3-h treatment to $< 10\%$ of control levels. Several new drug candidates induced aberrations concomitant with marked reductions in cell counts at 20 h (to 50–60% of controls) and suppression of BrdUrd uptake ($< 15\%$ of control). Several non-mutagenic chemicals and a metabolic poison, which induce DNA double strand breaks and chromosome aberrations at toxic dose levels, also suppressed DNA synthesis. In contrast, the alkylating agents 4-nitroquinoline-1-oxide, mitomycin C, methylnitrosourea, ethylnitrosourea, methylmethane sulfonate and ethylmethane sulfonate, and a topoisomerase II inhibitor, etoposide, produced many aberrations at concentrations that were less toxic (cell counts $\geq 73\%$ of controls) and gave little inhibition of DNA synthesis during treatment (BrdUrd uptake $\geq 85\%$ of controls), although cell cycle delay was seen following the 3-h treatment. Thus, inhibition of DNA synthesis at the time of treatment is supporting evidence for an indirect mechanism of aberrations, when there is no direct DNA reactivity.

Keywords: Genotoxicity associated with cytotoxicity

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1. Introduction

For decades, the analysis of chromosome aberrations has provided an enormous amount of useful information about mechanisms of chromosomal mutations induced by physical and chemical DNA damaging agents, and has served as an indicator of human exposure to mutagens. Induction of chromosome aberrations in cultured cells has also been widely used as a method for screening agents for potential mutagenic hazard. As experience with hundreds of chemicals has accumulated, it has become clear that chemicals or their metabolites that do not directly damage DNA can induce chromosome aberrations by secondary mechanisms. It has been known since the work of Kihlman and others that chromosome aberrations can result from inhibition of DNA synthesis, through interference with metabolism of DNA or its precursors [1,2]. Inhibition of DNA polymerases by aphidicolin [3], for example, is an effective way to induce aberrations [4]. We now have evidence that inhibition of DNA synthesis is a possible mechanism of aberration induction by a variety of chemicals that are not obviously involved in controlling DNA precursor pools or the activity of polymerases.

We describe here our experience with some chemicals that are apparently not DNA-reactive, but induce chromosome aberrations in vitro concomitant with suppression of DNA synthesis. First, we investigated a compound that was a candidate for use as a drug. It was not mutagenic in the Ames test, but was positive in the in vitro assay for chromosome aberrations in CHO cells. The increases in aberrations showed a steep dose relation, and were associated with substantial reductions in cell counts, and perturbations of the cell cycle seen as mitotic suppression followed by recovery. Using a series of structurally related compounds, we assessed evidence for direct DNA reactivity (Ames test, measurements of single and double-stranded DNA breakage, and DNA adducts) and indirect (toxicity, inhibition of DNA synthesis) mechanisms of aberrations. The evidence suggested an indirect mechanism of aberration induction through perturbation of DNA synthesis. Some of the observations were presented previously in abstract form [5].

We also found inhibition of DNA synthesis here with some non-mutagenic chemicals known to cause DNA strand breaks as a result of cytotoxicity, and chromosome aberrations associated with cytotoxicity [6]. In contrast to the inhibition of DNA synthesis seen during 3-h treatments with these non-DNA-reactive chemicals, the classical alkylating clastogens and the topoisomerase inhibitor etoposide caused dramatic increases in aberrations without markedly suppressing DNA synthesis at the time of treatment.

Since indirect mechanisms of aberrations may operate only above a critical concentration of the test compound, and not at lower, non-cytotoxic doses, recognition of these mechanisms is vital in the safety assessment of chemicals.

2. Materials and methods

2.1. Test chemicals and solvents

Chemicals were from the following sources: bisphenol A (CAS No. 80-05-7), methyl methanesulfonate (MMS; CAS No. 66-27-3), ethyl methanesulfonate (EMS; CAS No. 65-50-0), menthol (CAS No. 15356-70-4), sodium iodoacetate (CAS No. 305-53-3), 2,4-dichlorophenol (DCP; CAS No. 120-83-2) and diethyldithiocarbamic acid (dithiocarb; CAS No. 20624-25-3): Aldrich, Milwaukee, WI; ethyl nitrosourea (ENU; CAS No. 759-73-9): Pfaltz and Bauer; aphidicolin (APC; CAS No. 38966-21-1), methyl nitrosourea (MNU; CAS No. 684-93-5), phenformin HCl (CAS No. 834-28-6), tacrine (CAS No. 1684-40-8), cytosine arabinoside (ara C; CAS no. 69-74-9), phenylephrine HCl (CAS No. 61-76-7), ephedrine sulfate (CAS N0. 134-72-5), etoposide (CAS No. 33419-42-0), mitomycin C (MMC; CAS NO. 50-07-7), 4-nitroquinoline oxide (4-NQO; CAS No. 56-57-5) and dimethylsulfoxide (DMSO): Sigma; distilled water (dH_2O): Gibco BRL, Grand Island, NY. Novel compounds A1-7, B and C were prepared at Merck Research Laboratories.

Test chemicals were prepared fresh as 20–100 × concentrations to give a final concentration of 1% DMSO (A1-7, B, C; APC, bisphenol A, dithiocarb, MNU, MMS, ENU, EMS, 4-NQO, menthol, 2,4-DCP, tacrine, etoposide), or dH_2O (Na iodoacetate, MMC, ephedrine sulfate, phenylephrine HCl) or 5% dH_2O (phenformin HCl) in cultures.

2.2. Cell cultures

CHO cells, clone WBL, were cultured in McCoy's 5A medium (Gibco BRL) with 10% fetal bovine serum (Biowhittaker), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium, all Gibco BRL) and were used for no more than 15 passages since cloning. Cells were maintained in sub-confluent, exponential growth at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.3. Cell treatment and harvest

About 24 h before treatment, cells were seeded at 1.2×10^6 cells/10 ml in 75-cm^2 flasks. Parallel sets of cultures were incubated with test compounds for 3 h. Incubation with S-9 was in serum-free medium containing 15 μl/ml S9 (from phenobarbital/β-naphthoflavone induced male Sprague–Dawley rats), 0.8 mg/ml NADP, and 1.5 mg/ml trisodium isocitrate. Incubation without S-9 was in medium with 10% fetal calf serum. After 3 h, cultures were washed twice and re-fed with complete medium. One set of cultures was incubated for an additional 17 h and harvested for cytogenetic analysis (see below). The remaining three sets of cultures, designated for flow cytometric analysis, each contained duplicate cultures for each time point, and received 0.1 μM 5-bromodeoxyuridine (BrdUrd; Sigma) for the last 30 min of incubation with drug. After removal of drug, one set of cultures was immediately harvested ('0 Hour'). The last two sets were incubated in medium supplemented with 40 μM thymidine (Sigma) for 5 or 17 h prior to harvest with trypsin.

2.4. Cytogenetic analysis

Cells were collected 20 h after the beginning of treatment [7]. Colcemid (0.1 μg/ml, Gibco BRL) was added 2–3 h before cells were harvested by trypsin-EDTA treatment, and fixed. Giemsa-stained slides were scored under code. Two hundred metaphase cells containing 19–23 chromosomes were scored for each treatment unless aberration frequencies were high, or few cells were available for analysis due to mitotic suppression. We noted but did not include in aberration totals the following: gaps (achromatic regions less than or equal to the width of a chromatid, or larger lesions with visible connecting material across the gap), polyploid and endoreduplicated cells, and pulverized and double-minute chromosomes. Cells with 10 or more aberrations were classified as severely damaged (SD) cells, and scored as one aberrant cell but as 10 aberrations when calculating total frequency.

2.5. Flow cytometry

2.5.1. Anti-BrdUrd staining procedure

At 0, 5 or 17 h after treatment, cell pellets were washed once with Dulbecco's PBS without Ca^{2+} and Mg^{2+} (PBS), fixed with ice-cold 70% ethanol, and stored refrigerated for up to 3 days before they were stained by the method of Dolbeare et al. [8]. Up to 5×10^6 fixed cells per culture were stained for BrdUrd detection (FITC-labeled secondary antibody to mouse anti-BrdUrd) and total DNA (propidium iodide; PI). All staining was done at room temperature unless noted; reagents were from Sigma. The cells were removed from ethanol and resuspended in 1 ml of PBS containing 0.5 mg/ml RNAse for 20 min. After centrifugation, the cells were resuspended in 1 ml ice-cold 0.1 N HCl containing 0.5% Triton X-100, and placed on ice for 10 min. After one wash with 5 ml dH_2O, the cells were resuspended in 1.5 ml dH_2O and placed in a 90°C water bath for 10 min. The samples were placed in an ice-water bath for 5 min, then washed with 5 ml PBS containing 0.5% Tween (PBT). The IU-4 anti-BrdUrd monoclonal antibody (kindly provided by Dr Frank Dolbeare, Lawrence Livermore National Laboratory) was prepared by diluting it 1:1000 in PBT with 0.5% bovine serum albumin. Pellets were resuspended in 0.2 ml of antibody solution for 20 min, washed with 5 ml PBT, gently pelleted to avoid clumping, and incubated for 20 min with 100 μl of FITC-labeled goat anti-mouse IgG that was diluted 1:20 in PBT. Nuclei were then washed (volume to 5 ml with PBT), gently pelleted as above and counterstained with 0.5 ml 10 μg/ml PI in PBS. The stained suspensions could be stored at 4°C for up to a month before flow cytometric analysis.

2.5.2. Flow cytometric analysis

Analysis of these dual stained CHO cells was performed in EPICS flow cytometers (Coulter Elec-

tronics, Hialeah, FL), equipped with two Innova 90-6 argon-ion lasers (Coherent, Palo Alto, CA). Compounds A1-7, B and C were analyzed in the EPICS 753, and the remaining compounds in the EPICS Elite ESP instrument. A single laser tuned to 488 nm was used to excite the two fluorochromes (FITC and PI). Bivariate BrdUrd-FITC/DNA-PI histograms were constructed using a linear scale on the *X*-axis to display the DNA content (PI-red integrated fluorescence) and a logarithmic scale on the *Y*-axis to display BrdUrd incorporation (FITC-green integrated fluorescence). Information from 10^4 cells was collected for each sample.

The relative quantity of BrdUrd incorporated per S phase cell was calculated from the difference in the mean fluorescent intensity (channel number) between the BrdUrd (+) cell population (S phase cells) and the BrdUrd (−) population (G_1, G_2 and M cells).

Analysis of the proportion of cells in each stage of the cell cycle was refined by dividing the standard two-parameter histogram into 10 compartments, called 'integration areas', and classifying cells according to green and red fluorescence, and into G_1, G_2 + M, or various stages from early through late S phase (Fig. 1). All 10 integration areas from treated samples were individually compared with respective control samples, after normalization against controls, where necessary, to take account of any change in the total number of cells labeled with BrdUrd caused by accumulation or deletion of specific compartments, or by a generalized change in numbers of BrdUrd incorporating cells. Data were then analyzed by ANOVA and Student's *t*-test (with Dunnett's and Duncan's adjustment for multiple comparisons) to locate regions containing abnormal numbers of cells.

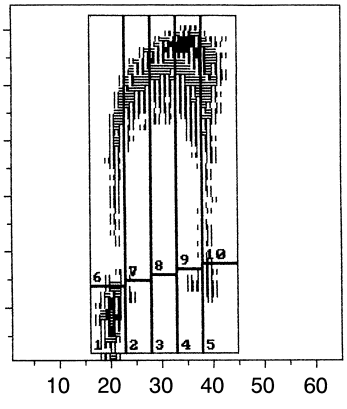

Fig. 1. Flow histogram (EPICS 753) of green fluorescence (BrdUrd uptake into DNA) on the vertical axis (a 3 decade log scale) and red fluorescence (total DNA content) on the horizontal axis (arithmetic scale), showing cell cycle compartments. Numbers on the *x* axis are channel numbers.

2.5.3. Comparison of two cytometers

To show that the data generated by the new instrument were consistent with those produced previously, we re-analyzed in the new cytometer (EPICS Elite) some samples that had been analyzed in the EPICS 753. The calculated BrdUrd uptake in S phase cells as a percentage of controls was very similar in the two instruments; for example, aphidicolin at 50 μM had 9.9% and 10.6% of control BrdUrd uptake in the old and new cytometers, respectively, and Mitomycin C at 1.5 μM had 95.9% and 91.4% of control. The control data for percentages of cells in different compartments of the cell cycle also remain similar. In DMSO controls without S-9, at the end of the 30 min pulse with BrdUrd the percentages of cells were typically 33% in G_1, 61% in S and 6% in G_2 + M.

3. Results

3.1. Novel compounds

Drug candidates with two different pharmacological targets, A2 and B were not mutagenic in *Salmonella* (Ames test) and *E. Coli*, but were positive in the in vitro assay for chromosome aberrations in CHO cells. First, to investigate drug A2, a series of structurally related compounds was tested in the genetic toxicology battery (the molecular structures had quite substantial differences). Results are described here for 7 group A compounds, six parent molecules and a metabolite of one: A5 is a cleavage product of A6. All were negative in the microbial mutation assay, and in the alkaline elution assay for DNA single-strand breaks when cytotoxicity was taken into account (see Section 4). Four (A1, A4, A5 and A6) were tested for DNA adducts and were negative. Six of the seven were positive in the

chromosome aberration assay. The increases in aberrations tended to show a steep dose relation (Figs. 2 and 3), and to occur at doses that reduced cell counts substantially at the time of harvest. Cell cycle perturbation was apparent since there was mitotic suppression 7 h from the beginning of the 3 h treatments, but mitotic indices recovered by 20–24 h, and in some cases surpassed the control levels (see A1, 4 and 6 in Figs. 2 and 3), indicating that some degree of synchrony had resulted from the 3-h drug treatments.

To examine DNA synthesis and cell cycle effects further, we measured DNA synthesis by incorporation of BrdUrd into DNA during the last 30 min of the 3-h incubation with test compounds. As a positive control, we measured DNA synthesis inhibition and cell cycle perturbation by aphidicolin and ara C. As expected, aphidicolin markedly inhibited BrdUrd incorporation (Fig. 4; note that the vertical axis is a log scale, so the amount of DNA synthesis at 25 μM APC is only 17% of control) and induced chromosome aberrations (Fig. 5). There were also cell cycle delays, with accumulation of cells in G_1 and in early and mid S phase. Ara C, which inhibits DNA synthesis [9] and induces aberrations [1,10] also gave the expected suppression of BrdUrd incorporation, to 8% of controls at 30 μM (data not shown). Dramatic suppression of BrdUrd incorporation was seen with all six of the group A compounds that induced chromosome aberrations in vitro but were negative in the other genotoxicity studies (A1-3 and A5-7). Representative histograms of data from the flow

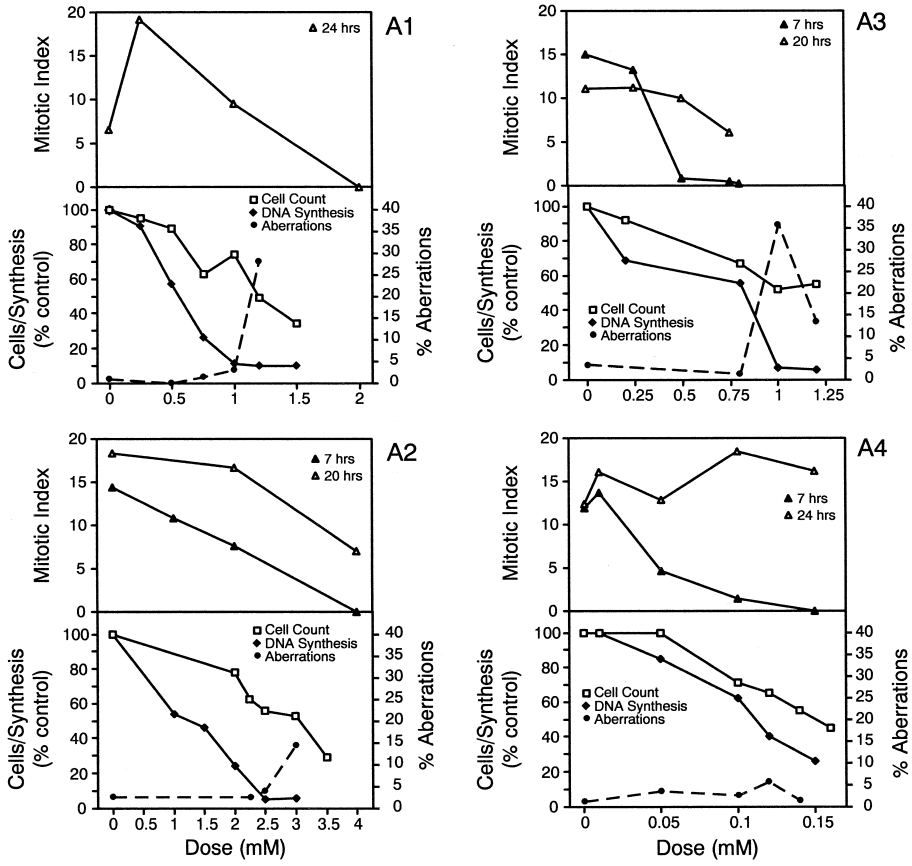

Fig. 2. Percentages of cells with aberrations compared with the degree of suppression of DNA synthesis and reductions in cell counts and mitotic indices. Since results were comparable with and without S-9 for A 1–4, the data for only one set of conditions are shown. Data for A1 are from experiments without S-9, and the remainder are from experiments with S-9 metabolic activation.

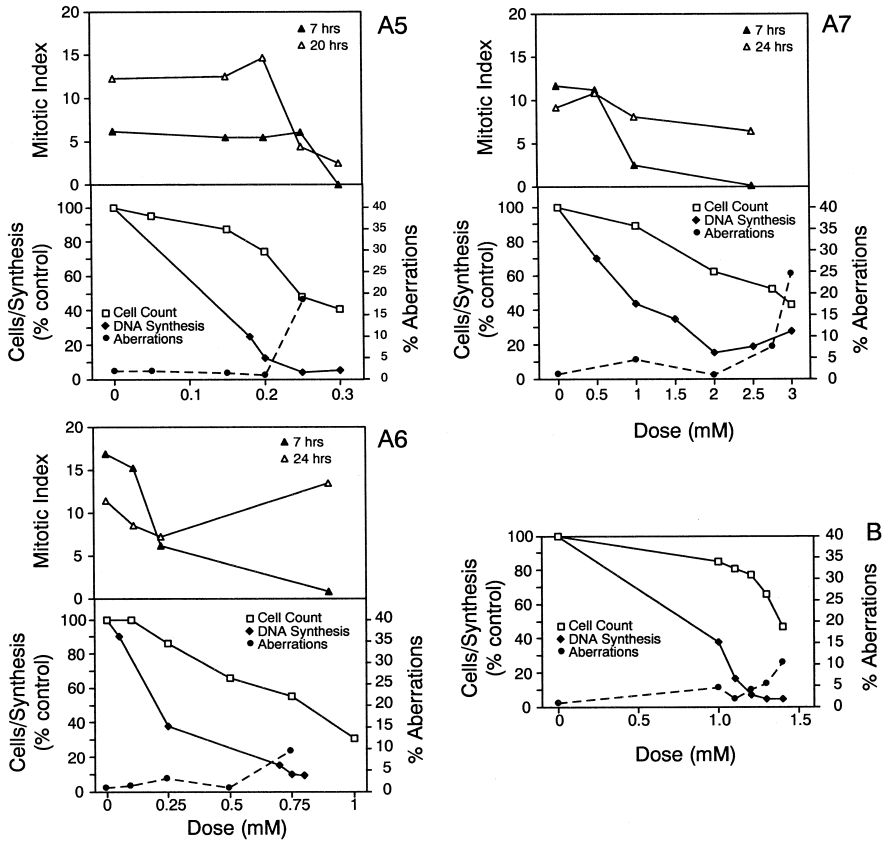

Fig. 3. Percentages of cells with aberrations compared with the degree of suppression of DNA synthesis and reductions in cell counts and mitotic indices. Since results were comparable with and without S-9 for A6 and B, the data for only one set of conditions are shown. Data for A5 and B are from experiments without S-9, and the remainder are from experiments with S-9 metabolic activation.

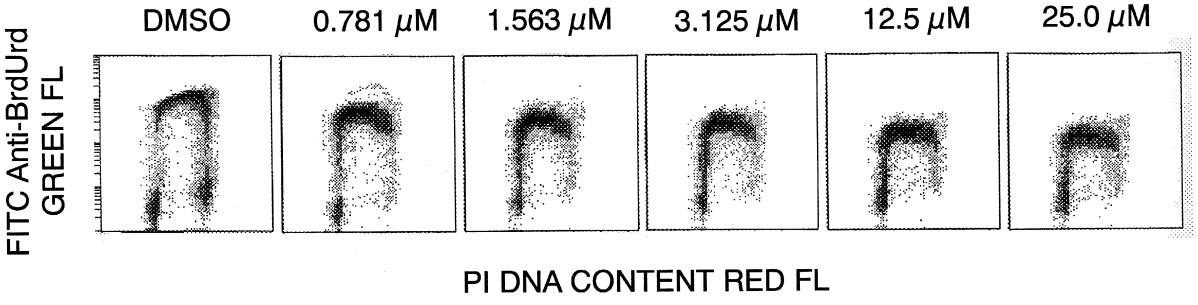

Fig. 4. Aphidicolin: Flow histogram (EPICS Elite ESP) of green fluorescence (BrdUrd uptake into DNA) on the vertical axis (a 4-decade log scale) and red fluorescence (total DNA content) on the horizontal axis (arithmetic scale), showing suppression of DNA synthesis and accumulation of cells in G_1 and in early S.

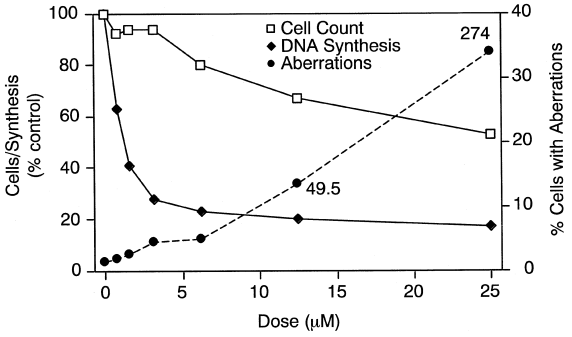

Fig. 5. Aphidicolin; Percentages of cells with aberrations compared with the degree of suppression of DNA synthesis and reductions in cell counts. Numbers beside two points are the frequencies of aberrations per 100 cells, since many cells had multiple aberrations at 12.4 and 25.0 μM.

cytometer are shown in Fig. 6, and the degree of inhibition of BrdUrd uptake for each compound is plotted in Figs. 2 and 3 for comparison with the cell count and chromosome aberration data. The data are summarized in Table 1. Metabolic activation was not required for the responses, except with A7, which was positive only with S-9 (Table 1); A5 was positive only without S-9. There were profound decreases in BrdUrd uptake, which seemed to reach a low plateau at the high doses (Fig. 6). In examining the histograms, note that the decrease in green fluorescence represents a reduction in the average uptake of BrdUrd per cell. Even when cell numbers are decreased by cytotoxicity, the information collected

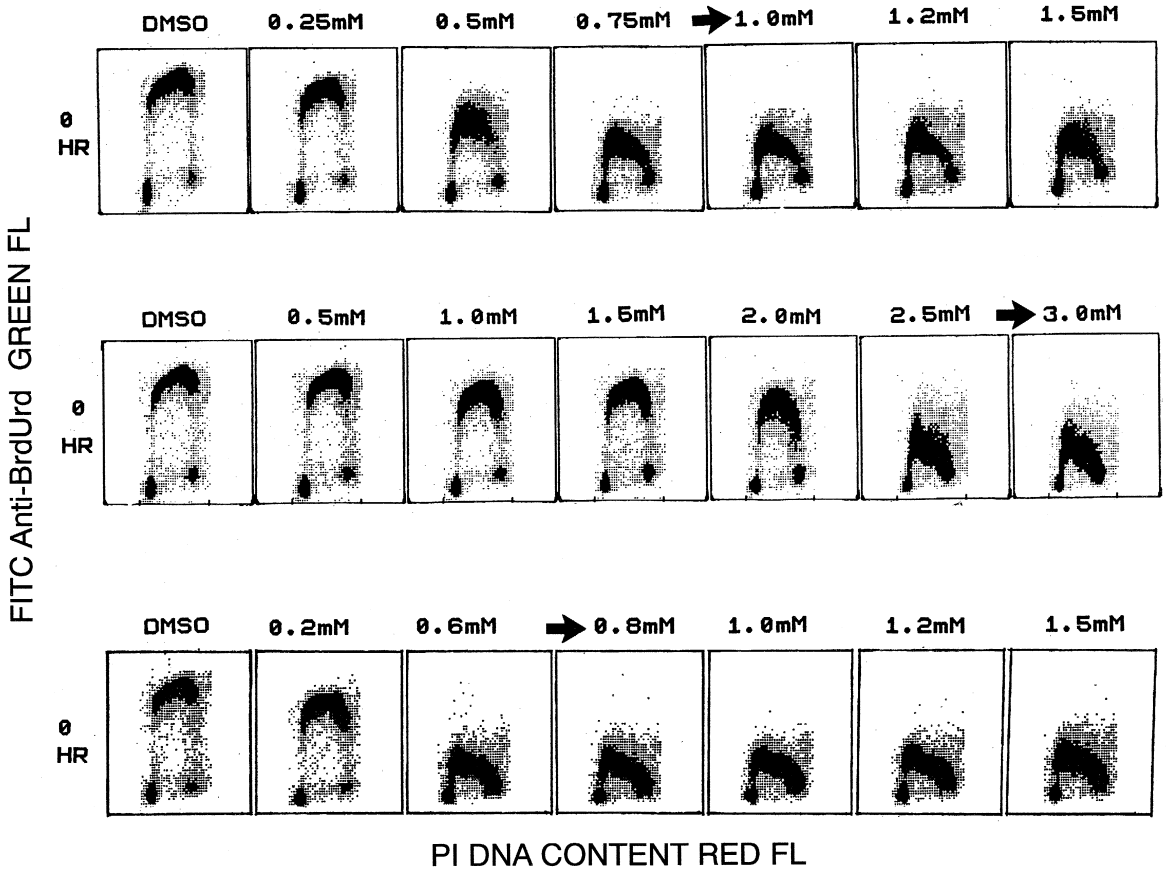

Fig. 6. Compounds A1 (top), A2 and A3. Flow histograms (EPICS 753) of green fluorescence (BrdUrd uptake into DNA) on the vertical axis (a 3 decade log scale) and red fluorescence (total DNA content) on the horizontal axis (arithmetic scale), showing suppression of DNA synthesis. The arrows show the dose levels at which chromosome aberration induction was apparent.

Table 1
% DNA synthesis inhibition at the lowest dose to increase chromosome aberrations, or highest tested dose for non-clastogens

Drug	S-9	Dose (mM)	20 h		3 h[a]
			Cell count (% control)	% Cells with aberrations	BrdUrd uptake of S phase cells (% control)
A1	+	1.0	49	49	6.1
	−	1.2	49	28	9.9
A2	+	3.0	53	14.5	8.2
	−	3.0	51	10.0	5.6
A3	+	1.0	52	36	6.7
	−	1.2	49	14.5	5.0
A4	+	0.12[b]	65	5.75 sig	39.5
		0.10[b]	86	5.00 ns	
		0.16[b]	57	2.00 ns	
	−	0.24	49	3.50 neg	42.3
A5	+	0.1	82	4.5	not done
		0.25	60	4.0 neg	
	−	0.25	48	18.5	3.8
A6	+	0.75	55	9.5	9.8
	−	0.8	55	4.0	10.8
		0.9	49	10.75	
A7	+	2.0	62	9.0	14.6
	−	2.0[b]	50	2.5	22.5
B	+	1.2	64	7.5	5.1
	−	1.3	66	5.5	4.7
		1.4	47	10.5	4.4

[a] Pulse treatment for the last 30 min of 3 h incubation with test compound.
[b] Three separate experiments.
Flow cytometry was not done concurrently with aberration experiments, except for compound B.
The mean control values for these experiments based on 44 cultures was 1.75% cells with aberrations (range 0.75 to 5.0%).
ns: not significant; sig: statistically significant.

is always from the same number of cells, 10,000 per sample.

While there were strong associations among cell cycle perturbation (mitotic index suppression followed by recovery), inhibition of DNA synthesis and increases in chromosome aberrations, the correlation was not complete; A4 showed these indicators of cell cycle perturbation and reduced cell counts to 55% and 49% of controls (+ or −S-9 respectively) but was considered negative overall in the aberration assay. A borderline but statistically significant increase in aberrations was seen at one dose level of A4 with S-9 (5.75% at 0.12 mM, cf. 1.25% in control), but was not reproduced in two other studies. While A4 depressed DNA synthesis, the suppression was not as profound as that seen at the dose levels of the related compounds that induced aberrations. Uptake of BrdUrd was reduced to about 40% of controls (Table 1, Fig. 7), compared with 4–15% with the compounds that induced aberrations (Table 1). Similarly, A7 induced aberrations reproducibly with S-9, but not without S-9, yet DNA synthesis was inhibited both with and without S-9, the lowest level of BrdUrd uptake being 14.5% of control with S-9 and 22.5% without S-9 (Table 1).

Cell cycle perturbation was seen by flow cytometry with all the compounds. Compounds A 1-7 all had accumulation of cells in early S at 0 h, (typically a 2-fold increase in number of cells in early S) and in $G_2 + M$, e.g., A4 and A5 had more than a 3-fold increase in the number of cells in this compartment. Cell cycle progression was assessed by sampling

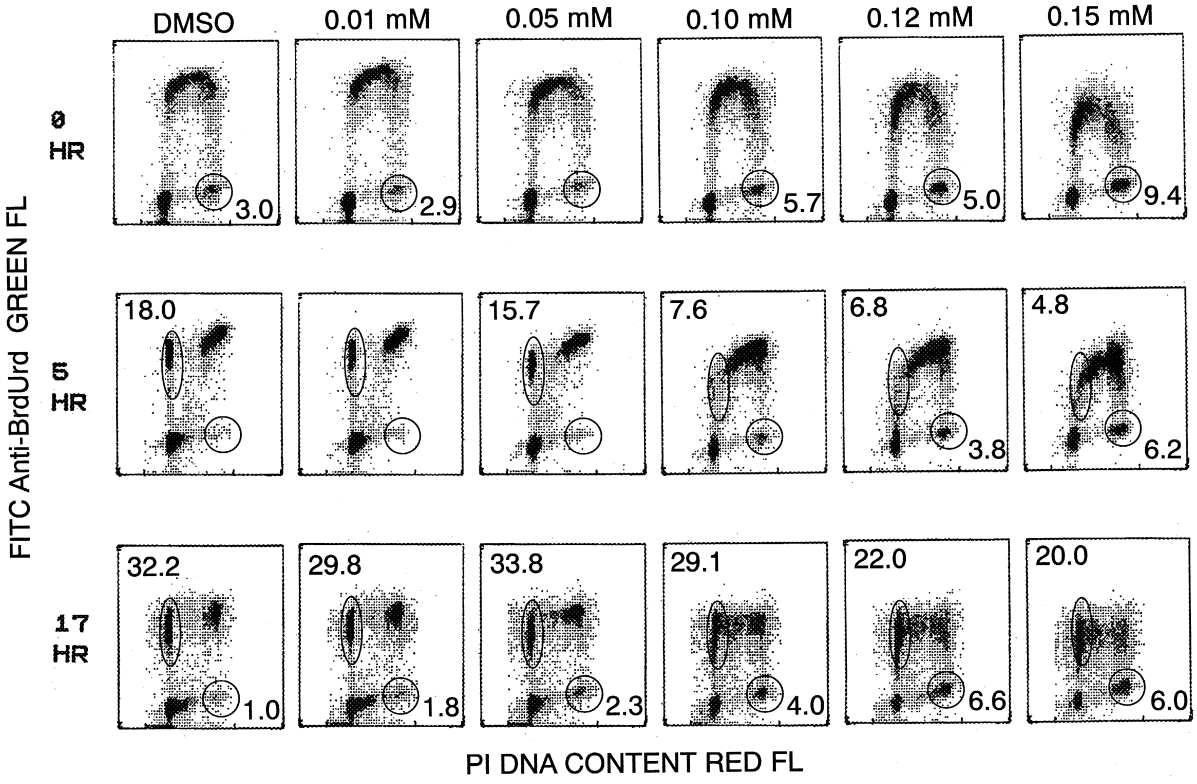

Fig. 7. Compound A4. Flow histograms (EPICS 753) of green fluorescence (BrdUrd uptake into DNA) on the vertical axis (a 3 decade log scale) and red fluorescence (total DNA content) on the horizontal axis (arithmetic scale), showing suppression of DNA synthesis and accumulating percentages of cells in early S and in $G_2 + M$.

cells 5 and 17 h after the end of treatment, as illustrated for A4 in Fig. 7. At time zero, accumulation of cells in S and in $G_2 + M$ is seen. By 5 h, control cultures contain BrdUrd labeled cells that have divided and reached G_1 of a new cell cycle; the $G_2 + M$ compartment has emptied, and labeled cells have progressed well through S phase. In contrast, treated cultures at 5 h have fewer or no cells that have reached the next G_1 phase, cells are blocked in $G_2 + M$, and other labeled cells are lagging in S phase. By 17 h, cells at the higher doses have now divided and reached G_1, but there are cells still lagging in the previous S phase, and in $G_2 + M$. Thus, the blocks seen immediately after treatment appear to be reversible, but some delay persists.

These compounds did not cause rapid cell killing on a large scale, with cell counts at the end of the 3 h treatment generally around 70% or higher. This early cell loss explains some cases of depletion of the numbers of cells in G_1 at 0 h that was too great to be accounted for by the G_2/M block.

The characteristics of A 1-7 are not restricted to a single class of drugs, but have been seen with several diverse types of molecules. One example is compound B, shown in Fig. 3 and in Table 1. Once again, this drug, which has a different molecular target, induced chromosome aberrations in vitro, but was negative in the Ames and alkaline elution assays, and did not produce DNA adducts detectable by ^{32}P post-labeling in calf thymus DNA (data not shown). It produced DNA synthesis inhibition (Fig. 3), and cell cycle perturbation, with accumulations in early S and $G_2 + M$.

3.2. DNA-reactive clastogens and a topoisomerase inhibitor

For comparison, some ‘positive control’ clastogens were tested for their effects on DNA synthesis

and cell cycle progression. The data are summarized in Table 2, and include the data for a single dose level of MMC analyzed at 0 h only. Since such clastogens generally do not cause much cell killing within 20 h at doses that induce many aberrations, very high doses of these mutagens were tested in order to reduce cell counts towards 50% of control, similar to the growth reductions seen with A 1-7 and B. There was very little inhibition of DNA synthesis at doses that gave a large increase in aberrations (Table 2), although there was cell cycle perturbation. For example, 4-NQO (Fig. 8) shows accumulation of cells in early S, corresponding depletion in mid S, and accumulation in $G_2 + M$. Cells were still delayed by 17 h (data not shown).

3.3. Toxicity-associated clastogens

We have previously demonstrated induction of chromosome aberrations in CHO cells by a series of non-mutagenic chemicals that are not rodent carcinogens, and by metabolic poisons [6]. These had been shown to induce double-stranded DNA breakage in cultured hepatocytes at toxic doses, and the aberra-

Table 2
DNA synthesis inhibition and chromosome aberrations in CHO cells treated with alkylating agents or etoposide

Treatment	20 h		3 h[a]
	Cell counts (% control)	% Cells with aberrations[b]	BrdUrd uptake of S-phase cells (% control)
DMSO 1%		1.7[c]	
Mitomycin C 50 μM	77	52.0	91
Ethylnitrosourea			
1.25 mM	85	13.5	89
2.50 mM	67	80[d]	79
5.00 mM	54	100[d]	56
Ethylmethane sulfonate			
7.50 mM	81	16.5	85
15.0 mM	55	84[d]	61
30.0 mM	49	100[d]	26
Methylnitrosourea			
0.63 mM	94	22.5	93
1.25 mM	80	47[e]	93
2.50 mM	62	88[d]	85
Methylmethane sulfonate			
0.3 mM	84	20.5	99
0.6 mM	53	90[d]	26
1.2 mM	47	100[f]	6
4-Nitroquinoline-1-oxide			
1.25 μM	93	4.0	98
2.5 μM	73	31.0[e]	92
5.0 μM	47	52.0[e]	56
Etoposide			
5 μM	92	37.0[ce]	88
10 μM	81	72.0[d]	88
20 μM	61	96.0[d]	90

[a] Pulse treatment for the last 30 min of 3 h incubation with test compound.
Flow cytometry was done concurrently with aberration analysis.
[b] 200 cells scored unless noted; [e] 100, [d] 50 or [f] 25 cells scored.
[c] Combined mean from 3 experiments.

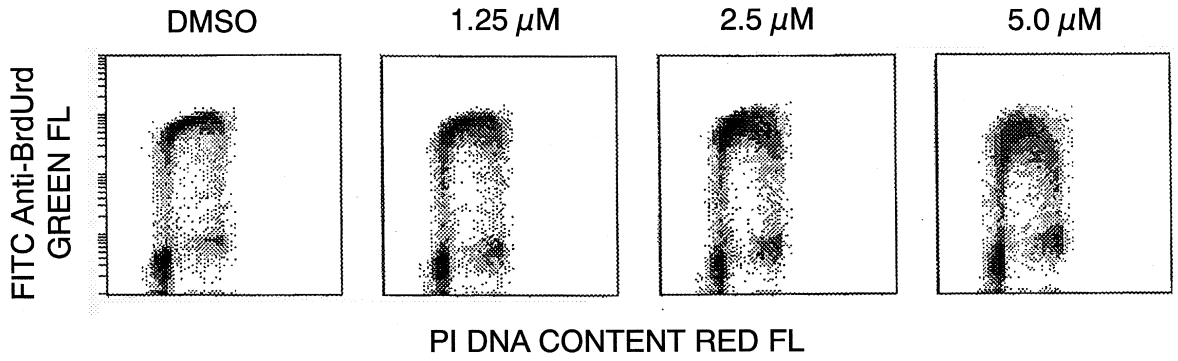

Fig. 8. 4-Nitroquinoline-1-oxide. (4-NQO). Flow histograms (EPICS Elite ESP) of green fluorescence (BrdUrd uptake into DNA) on the vertical axis (a 4-decade log scale) and red fluorescence (total DNA content) on the horizontal axis (arithmetic scale), showing only slight suppression of DNA synthesis and accumulation of cells in early S and in $G_2 + M$.

tions were also considered to be secondary to the cytotoxicity. Here, we found that these chemicals also suppressed DNA synthesis (Tables 3 and 4; Fig. 9). There was a wide variety of responses: dithiocarb caused very little inhibition of DNA synthesis, and may have mechanism(s) for inducing chromosome aberrations other than toxicity, since it is less toxic at clastogenic doses than other chemicals in this set. At the opposite extreme, phenformin is a good example of a chemical that dramatically suppressed BrdUrd uptake, to 8% of controls, yet did not induce aberrations (Table 4). Because of the cell cycle delay caused by phenformin, we sampled cells at 32 h and 48 h in addition to the routine 20 h, to see if damaged cells might reach mitosis later, but no increases in aberrations were found. Tacrine is mutagenic in *Salmonella* strain TA97 [11] and was not one of the toxic, non-mutagenic clastogens in the

Table 3
% DNA synthesis inhibition at the lowest dose to increase chromosome aberrations, or highest tested dose for non-clastogens

Chemical	Dose (mM)	20 h		3 h[a]
		Cell count (% control)	% Cells with aberrations	BrdUrd uptake of S phase cells (% control)
Clastogens				
Menthol (A)	1.5	45	7	41
2,4-dichlorophenol (B)	1.2	67	4	18
	1.4	45	9	33
Dithiocarb (C)	0.01	60	9.5	91
Na iodoacetate (D)	0.5	76	6	46
Bisphenol A (E)	0.4	45	7	26
	0.45	29	23	51
Non-clastogens				
Tacrine	0.45	40	3.0	20
Phenformin	10	49	1.0	8
Ephedrine	10	66	1.0	100
Phenylephrine	10	87	3.0	133

Letters in parentheses under chemical names refer to the figures in Fig. 9.
Flow cytometry was done concurrently with aberration experiments.
[a] Pulse treatment for the last 30 min of 3 h incubation with test compound.
The mean control value for these experiments (22 cultures) was 1.49% cells with aberrations (range 0.5 to 3.0).

Table 4
DNA synthesis inhibition by two toxic non-clastogens

Chemical	Dose (mM)	20 h		3 h[c]
		Cell count (% control)	% Cells with aberrations	BrdUrd uptake of S phase cells (% control)
Phenformin HCl[a]	1.0	70	4.0	102
	2.5	61	1.5	85
	5.0	54	1.0	64
	7.5	50	3.0	15
	10.0	49	1.0	8
Tacrine[b] expt 1	0.25	78		96
	0.30	73	6.0	77
	0.35	60	1.0	59
	0.40	40	2.0	37
	0.45	40	3.0	20
Tacrine expt 2	0	100	1.25	not done
	0.30	88	2.5	
	0.35	52	3.0	
	0.40	47	2.5	
	0.45	51	2.0	

Flow cytometry was done concurrently with aberration experiments.
[a]A non-mutagen that caused DNA double-strand breaks at toxic concentrations in hepatocytes.
[b]*Salmonella* mutagen; strand breaks not investigated.
[c]Pulse treatment for the last 30 min of 3 h incubation with test compound.

study of Hilliard et al. [6]; it was included here because it induced cytotoxicity but not aberrations (Table 4). An unexpected dose response was seen with bisphenol A and 2,4-dichlorophenol (Table 3 and Fig. 9B,E) in which BrdUrd uptake decreased with increasing dose, but then increased again at the highest doses. The experiment with 2,4-DCP was repeated, and gave the same result (Fig. 10). Three possible explanations for this can be discounted. First, it seemed possible that if there were a mixture of S phase cells some of which had taken up much less BrdUrd than others because they were more severely damaged, then as the dose increased, these cells might be killed and removed from the population, resulting in a higher average BrdUrd uptake. However, the histograms do not show evidence for this, since the height of the 'top of the horseshoe', i.e., the amount of BrdUrd incorporated by the S phase cells, actually increased at the highest two doses (Fig. 10).

Second, although we examined cultures carefully and saw no evidence of precipitate of test compounds even by dark field microscopy, a potential explanation for less effect at high doses would be if some of the compound was coming out of solution, but was invisible.[1] To rule out this possibility, bisphenol A and 2,4-dichlorophenol were added to medium to give the concentrations tested by flow cytometry. After incubation at 37° for 15 min, 1 h or 3 h, samples were ultracentrifuged at 37°C for 20 min at $100{,}000 \times g$, a procedure that has revealed previously non-visible microprecipitates for other chemicals. However, there was no evidence for precipitate with either chemical.

Finally, to make sure that fluorescence by the chemicals themselves was not giving a misleading signal in the flow cytometer, we checked for excitation at 488 nm, but found no evidence for this by spectrophotometry, either of DMSO stock solutions or of 1 in 100 dilutions into saline.

Of the chemicals that neither induced aberrations nor suppressed DNA synthesis, phenylephrine had an increase in mean BrdUrd content of the S phase cells

[1] Since cell counts continued to decrease with increasing dose, any putative precipitate would still have to be inhibiting cell growth, but not BrdUrd uptake.

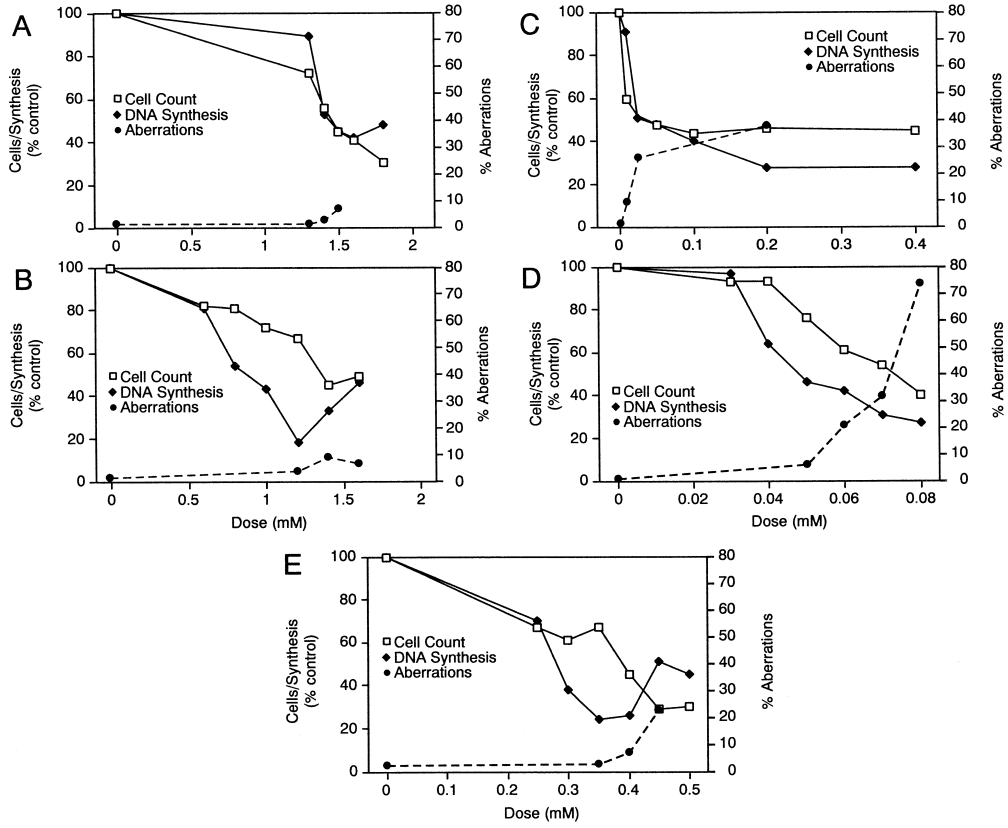

Fig. 9. 'Toxic clastogens'; Percentages of cells with aberrations compared with the degree of suppression of DNA synthesis and reductions in cell counts; A: menthol; B: 2,4-dichlorophenol; C. dithiocarb; D: Na iodoacetate; E: bisphenol A.

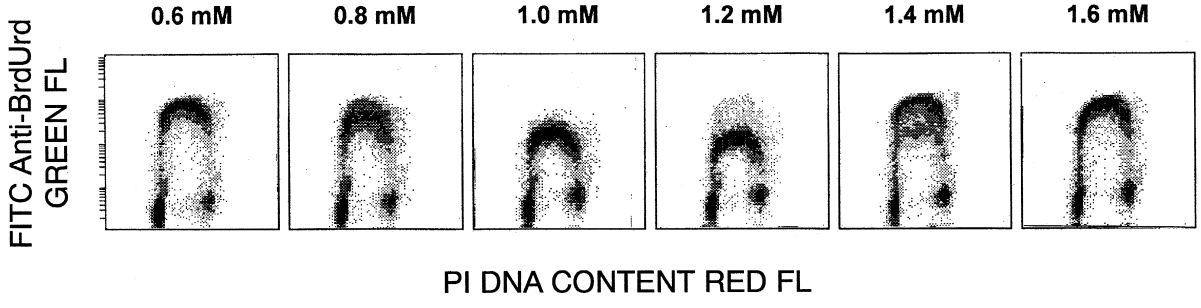

Fig. 10. 2,4-dichlorophenol. Flow histograms (EPICS Elite ESP) of green fluorescence (BrdUrd uptake into DNA) on the vertical axis (a 4-decade log scale) and red fluorescence (total DNA content) on the horizontal axis (arithmetic scale), showing unusual dose relation for suppression of DNA synthesis.

(Table 3). This is apparently the result of a depletion of cells in early S (area 6) and accumulation of cells in mid S (area 8), so that the average S phase cell has a higher BrdUrd content than controls.

3.4. Cell cycle blocks

Table 5 summarizes the areas of the histograms in which cells accumulated. The numbers in Table 5 show the fold increase in the percentage of cells in a given compartment over the control value. The prototype inhibitor of DNA synthesis, aphidicolin, caused cells to accumulate in G_1 (1.5 times the control number) and in very early to mid S (up to 3 times the control numbers.) The percentage of S phase cells was increased at the lowest dose, by about one-third, but the fraction of S phase cells decreased from that level at the higher doses as the accumulation in G_1 became more (see Section 1). Accumulation in $G_2 + M$ was a common feature (Table 5), as it was with the A group and B (see Section 1). There was no obvious characteristic that differentiated the non-DNA-reactive 'toxic' clastogens (Table 5c) from the positive control clastogens (Table 5b), except a tendency for the toxic compounds to give more pronounced blockage in early or mid S. Endoreduplication was frequently seen with the compounds tested here (up to about 8% of metaphases), but did not show any obvious correlation with particular blocks at 0 h, including $G_2 + M$, since endoreduplicated cells were seen with most of the chemicals, including the A group, B and etoposide, but not with the mutagens that cause DNA adducts. The only other compounds that did not induce endoreduplication were tacrine, phenformin and phenylephrine (data not shown).

Table 5
Cell cycle perturbation

Cell cycle compartment		Ratio of treated to control for % cells in each compartment*						
		G_1	S					$G_2 + M$
			Very early	Early	Mid	Late	Total % S	
a	Aphidicolin	1.5	3.0	2.0	1.5		1.3	
b	ENU						1.09	3.4
	MNU					1.6	↑, ns	1.9
	EMS			1.06			0.75	
	MMS				1.3		0.84	1.4
	4-NQO			1.08			1.11	2.4
	Etoposide			1.21			1.14	2.4
c	Na iodoacetate			1.3		1.4		4.1
	2,4-DCP					1.5	1.19	3.2
	Menthol		1.3	1.3				2.6
	Dithiocarb			1.2			1.16	3.0
	Bisphenol A			1.8	1.5		1.22	2.0
d	Tacrine				1.2		↑, ns	2.9
	Phenformin				1.2		0.81	1.8
e	Ephedrine				1.7		1.06	1.8
	Phenylephedrine				1.5		0.86	

* shown only when statistically significant.
ns: increase, but not significant.
a: DNA polymerase inhibitor.
b: Positive control clastogens.
c: 'toxic' clastogens.
d: 'toxic' non-clastogens with DNA synthesis inhibition.
e. Non-clastogens with no DNA synthesis inhibition.

4. Discussion

The evidence presented here indicates that chromosome aberration induction associated with inhibition of DNA synthesis may be more common than previously suspected, with chemicals that are not obvious candidates for interference with DNA replication. The rate of DNA synthesis may be reduced by slowing the rate of replication fork displacement, terminating progression of the growing points or preventing replicon initiation. Each of these mechanisms has been shown to occur with various types of DNA damage (reviewed in Ref. [12]). DNA strand gaps produced during synthesis might remain open when replication is perturbed, increasing the likelihood of conversion into double strand gaps and chromosome aberration formation. Resumption of replication following interruption might also be faulty, and perhaps abnormal tertiary structures in DNA might be targets for DNA repair, leading to opportunities for mis-repair and aberration formation.

We describe here our evaluation of the potential mutagenic hazard for a candidate for development as a drug, A2. In a series of structurally related compounds (A 1-7), six were positive in the chromosome aberration assay, yet none induced mutation in *Salmonella* or *E. Coli*. They were also negative in the alkaline elution assay for single strand breaks once toxicity was taken into account [13–15]. For example, with A4 (A2RA in Ref. [13]) mitochondrial destruction was seen in electron micrographs of treated cells, ATP levels were dramatically reduced, and neutral elution and pulsed field gel experiments showed that double strand DNA breaks were occurring in the dying cells, accounting for the strand breakage in the alkaline elution assay [13–15]. A representative compound was tested in the elution assay in vivo in rat liver, and no DNA strand breakage was found even up to doses that killed a quarter of the animals.

The lack of microbial mutagenicity or DNA breakage indicated that these chemicals did not damage DNA directly. As a stringent test for the capacity to bind to DNA, four of the compounds were tested for formation of DNA adducts in purified calf thymus DNA, by the ^{32}P post-labeling technique [16]. No evidence for DNA adducts was found using either nuclease P1 digestion or butanol extraction to look for adducts with different characteristics [16]. Also, there was no evidence for any capacity to induce strand breakage in lambda phage DNA, showing it was not likely that there were small adducts formed of types that were not detectable by the ^{32}P postlabeling technique [17]. In contrast, we demonstrated dramatic suppression of DNA synthesis with all seven chemicals tested, suggesting a secondary mechanism for induction of chromosome aberrations.

Taken together, the evidence shows lack of reactivity with DNA, steeply increasing dose relations for aberrations at doses associated with toxicity, and profound suppression of DNA synthesis during treatment. The elution data also demonstrated induction of DSB at toxic doses with this class, in hepatocytes. All this suggests an indirect mechanism for induction of aberrations, which might occur only above a certain threshold level. In vivo assays for chromosome aberrations were negative for the three compounds tested. Single oral treatments at three dose levels were followed by bone marrow sampling at 6, 24 and 48 h. For A2, the result was negative at doses up to 1500 mg/kg, at which 3/30 females and 3/30 males died. A3 was tested in males and was negative at doses up to 5000 mg/kg, without lethality, and A6 was tested in females and was negative up to 500 mg/kg, a dose at which 2 of 30 mice died.

Since the in vivo assays were negative at doses known to give bone marrow exposure (plasma levels), thousands of times higher than human clinical plasma levels, the drug candidate in this set, A2 was considered not to represent a risk of genotoxicity to humans.

For comparison with these non-DNA-reactive chemicals, some ‘positive control’ DNA-reactive clastogens were tested for their effects on DNA synthesis and cell cycle progression. We used methylating and ethylating agents, which produce different spectra of DNA adduction [18]; 4-NQO, which produces bulky adducts; and etoposide, which traps the DNA-topoisomerase II complex, leading to open DNA double strand breaks and chromosome aberrations [19]. Such clastogens are not immediately toxic at concentrations that induce aberrations, although they reduce colony-forming efficiency, largely due to the aberrations. In contrast to A1-3, A5-7 and B, which induced aberrations associated with severe

inhibition of DNA synthesis (Table 1), these strong DNA damaging agents caused little inhibition of DNA synthesis in the 3-h treatments at doses that induced many aberrations, (Table 2), and produced less marked cell cycle blockage in early S. A variety of other non-DNA-reactive clastogens had a range of severity of suppression of DNA synthesis (Table 3).

How can aberrations induced indirectly be differentiated from direct induction of aberrations by DNA-reactive chemicals? First, the remainder of the genotoxicity test profile should be negative, including tests for DNA binding. By itself, the observation of DNA synthesis inhibition associated with aberration induction does not allow us to dismiss the potential importance of the chromosome breakage. For example, we have a case of a compound (C), which induced both DNA synthesis inhibition in CHO cells and DNA adducts in calf thymus DNA, in the presence of S-9. A major metabolite of compound C proved positive in the Ames test. However, evidence for DNA reactivity does not necessarily explain chromosome aberration induction. It is possible that more than one mechanism is operating in the various tests of compound C. Although adducts were seen when purified DNA was exposed to drug with S-9, we do not know whether adducts were formed in the DNA of the CHO cells in which we saw chromosome aberrations. We have seen cases of compounds that formed adducts detectable in calf thymus DNA with S-9, or in cultured hepatocytes, but not in CHO cells with S-9, presumably because active species formed in the incubation mix do not penetrate to the nuclei of the CHO cells in detectable quantities. Thus, it may not be the adducts formed by compound C that induce chromosome aberrations. Careful evaluation of the compound must take into account metabolism, e.g., there was no DNA strand breakage in the alkaline elution assay; perhaps the hepatocytes can detoxify compound C.

Second, can the severity of DNA synthesis inhibition help ‘diagnose’ indirect aberration induction? Based on the data here, one might tentatively generalize that to cause aberrations, the inhibition of DNA synthesis must be quite profound, perhaps to less than about 20% of controls (Table 1). However, we must be very cautious about trying to calibrate the amount of DNA synthesis inhibition against the number of aberrations induced even with, for example, aphidicolin (Fig. 5), because we scored aberrations at only one time (20 h), and the yield of aberrations is highly dependent on sampling time. Also, we cannot conclude that there is a critical degree of suppression of DNA synthesis at which aberrations will begin to appear, because we have examples of compounds where BrdUrd uptake may be equally low at two or three successive dose levels, but aberrations are increased only at the highest doses (see, for example, A1 and A2, Fig. 2). We are currently examining the time course of recovery of DNA synthesis in our system; Painter and Howard [12] and Painter [20] has shown that, in many cases, the rate of recovery from inhibition can be used to distinguish DNA-reactive agents from non-DNA damaging agents that suppress DNA replication. Agents that react with DNA leave persistent lesions that continue to inhibit replication after the agent is removed, while more rapid recovery of the rate of DNA synthesis is typically seen following removal of agents that suppress DNA synthesis by metabolic or other non-DNA-reactive means.

The data on the set of ‘toxic clastogens’ illustrate the variety of response possible among chemicals (Table 3). The sequence of events in producing cytotoxicity and inhibition of DNA synthesis may differ among compounds, and may or may not involve activation of cell cycle checkpoints to cause inhibition of replication. Interference with synthesis of DNA precursors can result from many different types of insults, including disruption of ATP production, and the possible signals for checkpoint activation are many, including pool imbalance, the resulting defective DNA itself, or signals initiated by membrane damage, for example. Many less specific cytotoxic effects can be envisaged that will interfere with DNA synthesis, including changes in local ion concentration or pH, or non-specific inhibition of a variety of enzymes. We do not know what causes the cytotoxicity of drugs such as group A and B; they are receptor antagonists and an enzyme inhibitor whose targets are not likely to be expressed in CHO cells, and their high-dose effects are unlikely to be related to their pharmacological activity.

One cannot make the simple association ‘toxicity leads to aberrations’, because one may see cytotoxicity without aberrations. Similarly, a one-to-one correlation between DNA synthesis inhibition and aberra-

tions does not always exist; it will depend on the mechanism of toxicity/DNA synthesis inhibition. Since we saw suppression of DNA synthesis without aberration induction, it may be that some other key event is necessary to trigger aberrations. Further evidence that DNA synthesis inhibition is the mechanism for aberration induction here would be to demonstrate that aberrations are induced only in S phase.

The types of aberrations seen do indicate that replication is involved in their formation, since all the non-DNA-reactive clastogens here produced convincing increases in chromatid exchanges and isochromatid breaks in addition to simple chromatid deletions. [2]

These studies illustrate some complexities of the investigations of significance of aberrations induced at toxic doses of compounds. The data, however, support the concept that when there is good evidence that a compound and its metabolites do not react with DNA (or topoisomerases), aberrations at toxic doses that inhibit DNA synthesis may be induced indirectly, and may not constitute a risk of mutagenicity at low exposure levels associated with a good safety margin.

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