Pergamon

*Biochemical Pharmacology*, Vol. 48, No. 1, pp. 75–82, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0128-8

# THE ACTION OF THE DNA INTERCALATING AGENTS 4′-(9-ACRIDINYLAMINO) METHANESULPHON-*M*-ANISIDIDE AND 1,4-BIS(BUTYLAMINO) BENZO[*G*]PHTHALAZINE IN U-937 HUMAN PROMONOCYTIC CELLS: RELATIONSHIP BETWEEN CELL CYCLE AND DIFFERENTIATION

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(*Received* 8 *June* 1993; *accepted* 9 *March* 1994)

**Abstract**—The action of two structurally related DNA intercalating agents has been studied and compared, namely 4′-(9-acridinylamino) methanesulphon-*m*-anisidide (amsacrine, *m*AMSA) and 1,4-bis(butylamino)benzo[*g*]phthalazine (ABP) on the cell cycle and differentiation of U-937 human promonocytic leukemia cells. *m*AMSA (0.1 μM) and ABP (4 μM) reduced the proliferation activity to a similar extent and caused little cell mortality. At these subcytotoxic concentrations *m*AMSA induced the cells to accumulate at the $G_2$ phase of the cycle, while cycle inhibition provoked by ABP was not phase specific. In addition, *m*AMSA caused an increase in the cell mass while ABP provoked cell shrinkage. This was consistent with the fact that ABP considerably inhibited protein synthesis, while *m*AMSA did not significantly affect this activity. SDS/$K^+$DNA precipitation assays indicated that *m*AMSA, but not ABP, stimulated protein–DNA covalent complex formation. Finally, it was found that *m*AMSA, but not ABP, elicited the expression of differentiation markers, namely nitroblue tetrazolium reduction, activation of vimentin and leukocyte integrin (CD11b/CD18 and CD11c/CD18) expression, and downregulation of c-*myc* expression. The DNA intercalators doxorubicin and mitoxantrone, which like *m*AMSA induced the cells to accumulate at the $G_2$ phase and increased the cell mass, induced the expression of differentiation markers. In contrast, the intercalators aclarubicin and caffeine and the non-intercalator novobiocin, which produced minor alterations on cell-cycle distribution and caused cell shrinkage, did not significantly elicit differentiation. These results support the conclusion that differentiation of myeloid leukemia cells by cytostatic drugs depends on the perturbations of the cell cycle, leading to disproportionate increases in cell mass.

*Key words:* DNA intercalation; amsacrine; 1,4-bis(butylamino)benzo[*g*]phthalazine; cell cycle; differentiation

DNA intercalating agents often present therapeutical interest as antibacterial, antiparasitic and anti-neoplastic drugs [1]. Intercalators contain a planar chromophore of fused aromatic rings able to be inserted into the DNA helix pair bases, as well as cationic groups which allow hydrogen bonding with the deoxyribophosphate backbone [2]. The 9-aminoacridine derivative amsacrine, *m*AMSA‖ is a DNA intercalator with strong antitumour activity [3, 4]. This property seems to be related to the capacity of the drug to affect topoisomerase II (DNA topoisomerase ATP-hydrolysing, EC 5.99.1.3) activity by stabilizing the cleavable enzyme–DNA covalent complexes in eukaryotic cells, with the consequence of DNA breakage [5, 6]. Although the DNA damage is rapidly repaired upon drug removal, the deleterous effect on the cells persists, eventually leading to cell death. Recently, a series of new DNA intercalators derived from the benzo[*g*]phthalazine molecule, with structural similarities to mitoxantrone and *m*AMSA, have been synthesized in these laboratories [7]. Preliminary results indicate that these compounds have strong cytostatic and antiparasitic activities [8], but other effects at the cellular and molecular level have not yet been determined.

A property of some cytostatic drugs is that they are able to induce differentiation when applied at low, subcytotoxic concentrations [9]. However, the mechanisms responsible for differentiation are unknown. In the present report the effects of *m*AMSA and ABP [10] on the growth and differentiation of U-937 human promonocytic leukemia cells [11] are analysed comparatively. The results indicated that subcytotoxic concentrations of *m*AMSA, but not of ABP, efficiently induced the expression of differentiation

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‖ Abbreviations: amsacrine, *m*AMSA, 4′-(9-acridinylamino) methanesulphon-*m*-anisidide; ABP, 1,4-bis(butylamino)benzo[*g*]phthalazine; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; PI, propidium iodide; NBT, nitroblue tetrazolium; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; ECL, enhanced chemiluminiscence; mAb, monoclonal antibody.

markers. Differentiation seemed to be conditioned by the drug effects on the cell cycle, defined as the coordination between the DNA division cycle and the cycle of growth in the cell mass [12].

## MATERIALS AND METHODS

*Cell culture and drug treatments.* U-937 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum and 0.2% (w/v) sodium bicarbonate, in a humidified 5% $CO_2$ atmosphere at 37°. *m*AMSA, as a lactate derivative (LAMASINE®, and mitoxantrone, as a hydrochloride derivative, were generous gifts from Bristol Myers S.A.E. (Madrid, Spain) and American Cyanamid (Pearl River, NY, U.S.A.), respectively. Doxorubicin, aclarubicin and novobiocin were purchased from Sigma Química (Madrid, Spain), and caffeine from Merck (Madrid, Spain). ABP was prepared in these laboratories. *m*AMSA, mitoxantrone, doxorubicin and aclarubicin were dissolved in distilled water at 1 mM and kept at −20°. Novobiocin and caffeine were dissolved in RPMI 1640 at 10 and 100 mM, respectively, just before application. ABP was dissolved at 1 mM in dimethyl sulphoxide and kept at −20°. At the final concentrations used cell growth and viability were not significantly affected by the solvent. Cells were seeded for experiments at the concentration of $2 \times 10^5$ cells/mL in a mixture of old medium and fresh medium (approximately 1:3, v/v). To prevent long-term cultures from reaching plateau densities or nutrient exhaustion, on the second day of treatment they were supplemented with an equal volume of fresh medium containing the corresponding drug. Both cell growth and viability were checked daily by using an hemocytometer and Trypan blue exclusion, respectively.

*Flow cytometry determinations.* Simultaneous determinations of DNA and protein content were carried out essentially as described by Crissman and Steinkamp [13]: cells were first incubated for 30 min at room temperature in PBS containing 20 μg/mL FITC, then extensively washed and incubated for 5 min at room temperature in PBS containing 0.05% (w/v) Nonidet P-40 and 50 μg/mL PI. The cells were analysed using an EPICS-CS flow cytometer (Coulter Cientifica, Mostoles, Spain) with an argon laser operated at 200 mV and an excitation wavelength at 488 nm. Appropriate colour filters were used to determine the PI-derived red–orange fluorescence (emission peak 590 nm) which measured DNA content and the FITC-derived green–yellow fluorescence (emission peak 530 nm) which measured protein content.

To detect the expression of the CD11b/CD18 (CR3) and CD11c/CD18 (p150,95) cell surface integrins, indirect immunofluorescence determinations were carried out using the monoclonal antibodies Bear 1 (anti-CD11b) [14] and HCl/1 (anti-CD11c) [15]. Cells were labelled with the monoclonal antibody for 30 min at 4°. After two washes with RPMI 1640 medium, FITC-labelled sheep anti-mouse IgG (Amersham, U.K.) was added and the incubation was continued for an additional period of 30 min at 4°. The background of non-specific fluorescence was measured by incubating cells with X63 hybridome supernatant instead of Bear 1 and HCl/1 mAbs. After the cells had been washed twice with RPMI 1640 medium their fluorescence was estimated by flow cytometry as above.

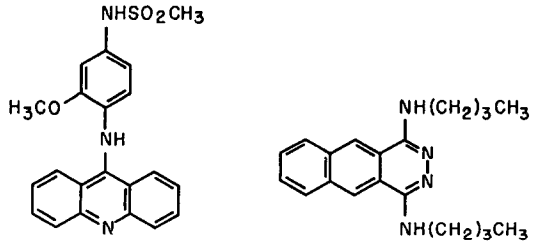
*Cytochemical assays.* The ability of cells to reduce NBT was determined by incubating them for 20 min at 37° in PBS containing 0.2% NBT (Sigma Madrid, Spain) and 0.15 μM 12-*O*-tetradecanoyl-phorbol-13-acetate (Sigma, Madrid, Spain) and then examining them for formazan precipitates with a microscope.

*Measurement of uridine and valine incorporation.* To measure RNA or protein synthesis activities cells were pulse labelled for 1 hr with either 4 μCi/mL of [5,6-$^3$H]uridine (46 Ci/mmol) or 3 μCi/mL of [3,4(*n*)-$^3$H]valine (33 Ci/mmol) (Amersham, U.K.), respectively. The cells were collected by centrifugation, washed twice with cold PBS and suspended for 1 hr in 10% (w/v) cold trichloroacetic acid. After centrifugation the pellets were processed to estimate the acid-precipitable counts [16].

*Quantification of covalent protein–DNA complex formation.* Drug-induced changes in covalent protein–DNA complex formation were determined essentially as described by Schneider *et al.* [17]. Cells were labelled for 24 hr with 0.2 μCi/mL of [methyl-$^3$H]thymidine (2 Ci/mmol) (Amersham, U.K.) either in the absence or presence of the drugs after which they were collected by centrifugation and washed at room temperature with PBS. The cells were lysed for 10 min at 65° in pre-warmed solution consisting of 1.25% (w/v) SDS, 5 mM EDTA, pH 8, containing 0.4 mg/mL calf thymus DNA. An aliquot of 50 μL of the lysate was taken to estimate the total acid-precipitable radioactivity. Pre-warmed (37°) 325 mM KCl solution (250 μL) was added to the rest of the lysate, followed by vigorous vortexing for 10 sec and cooling on ice for 30 min. After centrifugation in a microfuge for 10 min at 4° the pellets were resuspended in 1 mL of ice-cold wash solution consisting of 10 mM Tris–HCl, pH 7.5, 100 mM KCl and 2 mM EDTA, held for 10 min at 65° with occasional mixing and cooled on ice for 10 min. The suspensions were collected on glass fibre filters and the filters washed extensively with wash solution (see above) and dried prior to radioactive counting.

*RNA blot assays.* Total cytoplasmic RNA was prepared as described before [18]. RNA samples (15 μg) were denatured, electrophoresed in 1.1% agarose–formaldehyde gels and blotted onto nylon membranes. The RNA blots were hybridized with the 1.5 kilobase *Cla*I–*Eco*RI fragment of pMA413rc plasmid which contained the third exon of human c-*myc* [19]. The fragment was labelled to approximately $10^9$ cpm/μg of DNA with [α-$^{32}$P]dCTP (3000 Ci/mmol) (Amersham, U.K.) by random hexanucleotide priming [20].

*Immunoblot assays.* Cells were washed once with PBS and lysed in 62.5 mM Tris–HCl, pH 6.8, containing 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 10% (v/v) glycerol. After boiling for 2 min at 98° aliquots corresponding to 25 μg of total protein were separated in SDS–polyacrylamide (10%, w/v) mini-slab gels according to the Laemmli procedure [21]. Electrophoretic blotting onto nitrocellulose and immunological detection of




Amsacrine (mAMSA)

1,4-bis (butylamino) benzo[g] phthalazine (ABP)

Fig. 1. Structure of *m*AMSA and ABP.

proteins were carried out essentially as described by Towbin *et al.* [22], using a mouse anti-vimentin (Amersham, U.K.) as the first antibody and horseradish peroxidase–conjugated rabbit anti-mouse IgG (Dakopatts, Denmark) as the second antibody. The filters were developed with an ECL Western blotting detection kit (Amersham, U.K.) following the procedure described by the manufacturer.

## RESULTS

Figure 1 shows the structures of *m*AMSA and ABP. Both drugs have a tricyclic heteroaromatic ring which binds tightly to double-stranded DNA by intercalation, as shown by helix-unwinding studies with closed, circular DNA and by nuclear magnetic resonance [4, 7, 23]. Besides, the side chains of both *m*AMSA and ABP make the heterocyclic group easily protonable at physiological pH [7, 24, 25].

Figure 2 shows the effect of different concentrations of *m*AMSA and ABP on the proliferation activity of U-937 cells. *m*AMSA (0.1 μM) and ABP (4 μM) inhibited proliferation to a similar extent and caused little cell mortality (less than 20% at the third day of treatment). At higher doses proliferation was more efficiently inhibited but the drugs then caused excessive cell death. Hence, 0.1 μM *m*AMSA and 4 μM ABP were selected for further determinations.

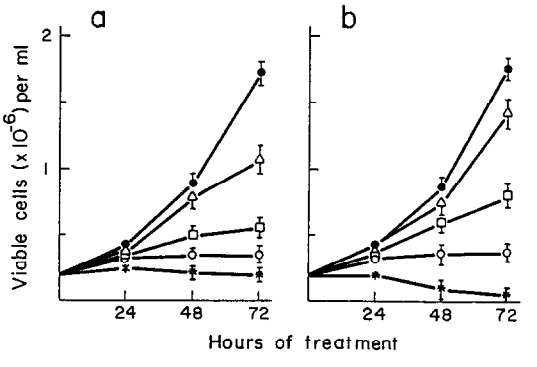



Fig. 2. Effect of *m*AMSA and ABP on U-937 cell proliferation; (a) *m*AMSA at 0.02 (△), 0.05 (□), 0.1 (○) and 0.2 (*) μM; (b) ABP at 1 (△), 2 (□), 4 (○) and 8 (*) μM. The symbol (●) represents drug-untreated (control) cells. Results are the mean ± SD of three determinations.

*Cell cycle*

The cell-cycle distribution and the relative mass of the cells were simultaneously determined by means of flow cytometry after double staining with PI, which measured DNA content, and FITC, which measured total protein content. It was found that *m*AMSA preferentially accumulated cells at the post-replicative stage, as indicated by the increased number of them with $G_2$/M DNA content. In contrast, ABP did not significantly alter cell-cycle distribution (Fig. 3a). Proliferation inhibition by *m*AMSA was accompanied by an increase in cell mass, as measured by protein content, which affected both the $G_1$ and $G_2$ subpopulations. In contrast, ABP caused a reduction in cell mass (Fig. 3b).

*RNA and protein synthesis*

The action of *m*AMSA and ABP on macromolecule synthesis was determined by measuring the incorporation of [$^3$H]uridine and [$^3$H]valine, respectively. The results are summarized in Fig. 4. Both drugs inhibited RNA synthesis, but the inhibition was more prominent in the case of ABP. In addition, ABP significantly inhibited protein synthesis while this activity was little affected by *m*AMSA. This result was consistent with the different actions of *m*AMSA and ABP on cell mass (as indicated above).

*Protein–DNA covalent complex formation*

Since it is known that DNA intercalating agents often inhibit topoisomerase activities by provoking topoisomerase–DNA cleavable complex stabilization, it was of interest to comparatively analyse the capacity of *m*AMSA and ABP to stimulate protein–DNA covalent associations. SDS/ClK precipitation assays indicated that *m*AMSA significantly increased the frequency of covalent associations even at the subcytotoxic concentration of 0.1 μM. In contrast, ABP was without significant effect at both subcytotoxic (4 μM) and highly toxic (40 μM) concentrations (Table 1).

*Cell differentiation*

Since cytostatic drugs often induce myeloid cell maturation, it was decided to study and compare the effect of *m*AMSA and ABP on the expression of differentiation markers in U-937 cells. This was carried out by measuring NBT reduction, surface expression of the CD11b/CD18 and CD11c/CD18 leukocyte integrins, accumulation of the intermediate filament protein vimentin and expression of the c-*myc* oncogene. The results are expressed in Table 2 and Fig. 5. It was found that *m*AMSA greatly stimulated NBT reduction as well as integrin and vimentin expression, as observed earlier using several differentiation inducers [26–29]. In contrast, none of these markers was induced by ABP. In addition, *m*AMSA greatly reduced c-*myc* mRNA levels, a result which was consistent with earlier reports indicating that myeloid cell differentiation is

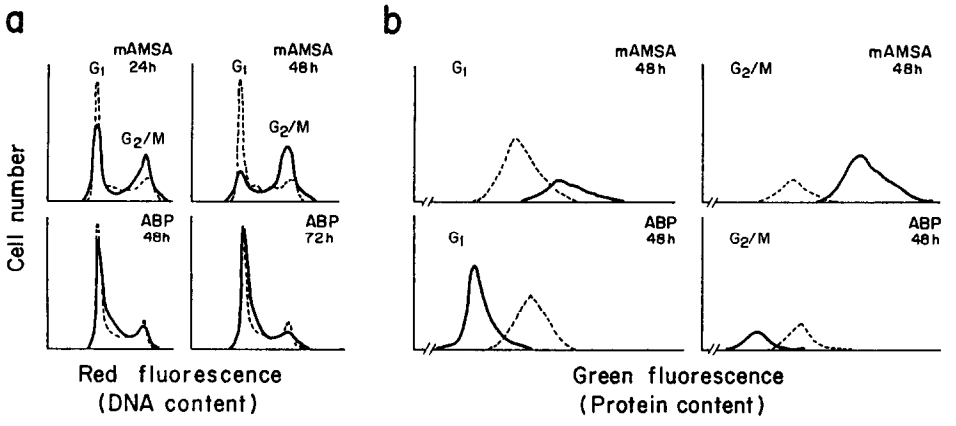



Fig. 3. Effect of 0.1 μM *m*AMSA and 4 μM ABP on U-937 cell cycle and cell mass. (a) Cell-cycle distribution in untreated cultures (dotted lines) and cultures incubated with the drugs for the indicated times (continuous lines). The DNA histograms were generated by flow cytometry analyses of PI-stained cells. (b) Relative cell mass in the $G_1$ and $G_2/M$ phase subpopulations of untreated cultures (dotted lines) and cultures treated for 48 hr with the indicated drugs (continuous lines), as measured by protein content. The histograms were generated by flow cytometry analyses of FITC/PI-stained cells. Results are representative of one of two experiments, with similar results.

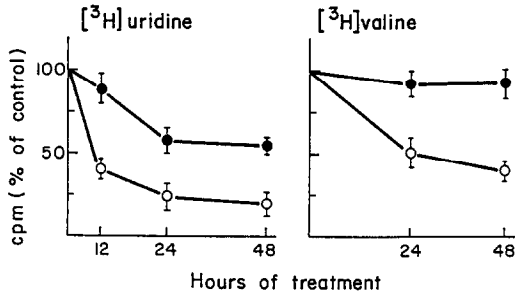



Fig. 4. Effect of *m*AMSA and ABP on RNA and protein synthesis activities. U-937 cells were incubated with the drugs for the indicated times with either 0.1 μM *m*AMSA (●) or 4 μM ABP (○), and pulse-treated with the indicated labelled precursors during the last hour of incubation. Acid precipitable radioactivities were calculated and expressed as percentages of those in drug-untreated (control) cultures. Values represent the means ± SD of four determinations. The mean absolute values in controls were 45,160 and 325,426 cpm per $10^6$ cells for [$^3$H]uridine and [$^3$H]valine labelling, respectively.

Table 1. Formation of protein–DNA covalent associations, as determined by SDS/ClK DNA precipitation assays

| Drug | Precipitated [$^3$H]DNA (%) |
|---|---|
| None | 2.90 ± 0.42 |
| *m*AMSA | |
| 0.1 μM, 12 hr | 11.65 ± 2.42 |
| 0.1 μM, 24 hr | 13.02 ± 2.52 |
| 1 μM, 1 hr | 43.25 ± 4.31 |
| ABP | |
| 4 μM, 12 hr | 3.12 ± 0.42 |
| 4 μM, 24 hr | 3.32 ± 0.25 |
| 40 μM, 1 hr | 3.62 ± 0.64 |

The cells were labelled for 24 hr with [$^3$H]thymidine. For treatments, the drugs were applied during the last hour, the last 12 hr or the whole labelling period. Results are the means ± SD of at least three determinations. The mean absolute radioactivity values in drug-untreated cells were 472, 735 and 13,709 cpm per $10^6$ cells, for total and precipitated radioactivity, respectively.

associated with the downregulation of c-*myc* expression [29, 30]. In contrast, ABP only slightly decreased c-*myc* mRNA levels, in spite of the fact that this agent inhibited the overall RNA synthesis more efficiently than *m*AMSA. Taken together, these results indicated that *m*AMSA induced U-937 cell differentiation, while ABP did not.

*Effects of other cytostatic agents*

To further investigate whether differentiation was conditioned by the drug effects on the cell cycle, the action of the DNA intercalators doxorubicin (15 nM), mitoxantrone (2.5 nM), aclarubicin (70 nM) and caffeine (3 mM), and of the non-intercalator novobiocin (0.15 mM) on U-937 cell-cycle distribution, cell mass and expression of differentiation markers was analysed. Doxorubicin and mitoxantrone exhibit structural similarities with *m*AMSA and ABP [7] and, like *m*AMSA, provoke topoisomerase II–DNA cleavable complex stabilization and DNA breakage [5]. Caffeine has been reported to produce *in vivo* protein-linked DNA breaks, in a similar manner to typical topoisomerase II inhibitors [31]. Aclarubicin and novobiocin inhibit topoisomerase II activity by mechanisms which do not involve cleavable complex stabilization nor

Table 2. Effect of *m*AMSA and ABP on the expression of differentiation markers

| Drug | NBT | CD11b/CD18 | CD11c/CD18 |
|---|---|---|---|
| None | 5.2 ± 2.1 | 4.3 ± 1.4 | 2.3 ± 1.1 |
| *m*AMSA (0.1 μM) | 31.5 ± 4.2 | 54.9 ± 6.2 | 33.3 ± 4.2 |
| ABP (4 μM) | 6.1 ± 1.8 | 3.2 ± 1.6 | 2.9 ± 0.9 |

The data represent the percentage of cells which reduced NBT and expressed the indicated surface integrins in untreated cultures and in cultures treated for 72 hr with the indicated drugs. Results are the means ± SD of three determinations. Five hundred cells were scored in each determination.

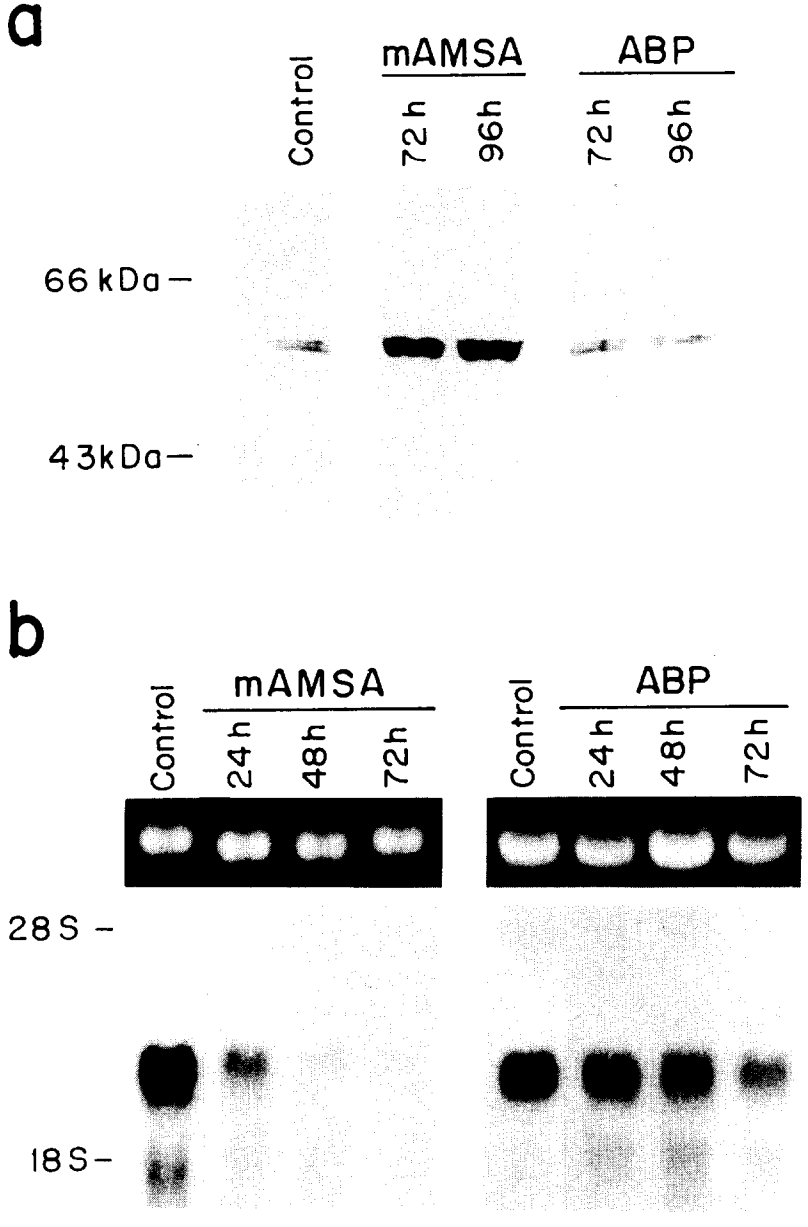



Fig. 5. Effect of 0.1 μM *m*AMSA and 4 μM ABP on vimentin and c-*myc* expression. (a) Whole lysates were obtained from untreated (Cont) and drug-treated cells. Samples of 25 μg of total protein per lane were electrophoresed, transferred to nitrocellulose and assayed for reactivity with an anti-vimentin antibody. (b) Total cytoplasmic RNA was extracted from untreated (Cont) and drug-treated cells. Samples of 15 μg per lane were electrophoresed, transferred to nylon membrane and hybridized with a $^{32}$P-labelled c-*myc* cDNA probe. Ethidium bromide staining of 28S rRNA is shown at the top of the panel as control of sample loading into the gels. All panels are representative of one of two different determinations which gave similar results.

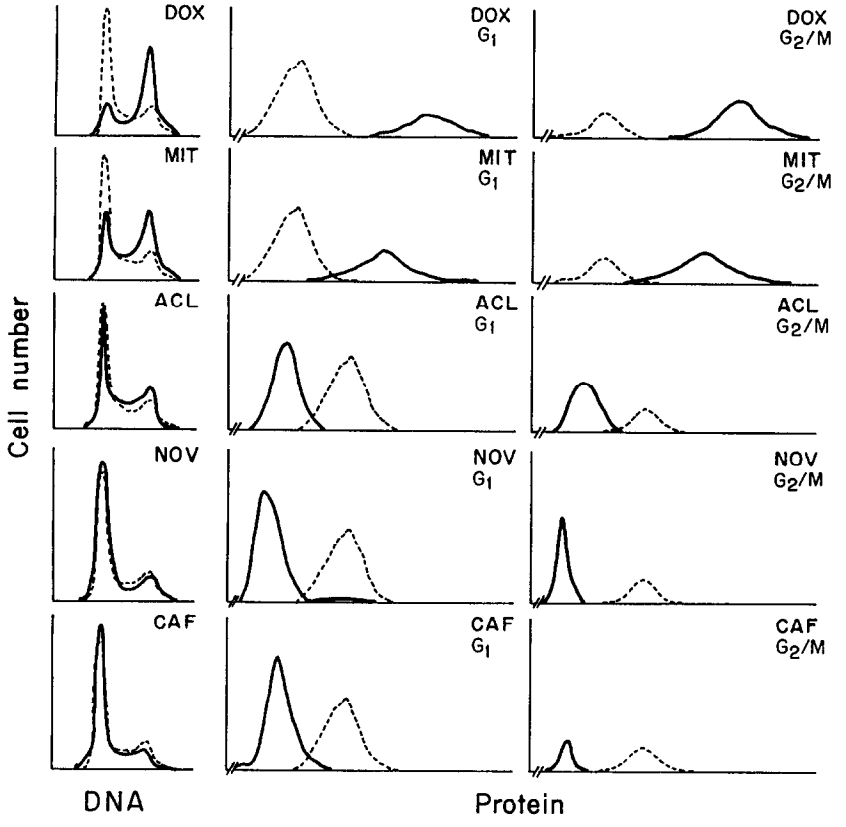



Fig. 6. Effect of 15 nM doxorubicin (DOX), 2.5 nM mitoxantrone (MIT), 70 nM aclarubicin (ACL), 0.15 mM novobiocin (NOV) and 3 mM caffeine (CAF) on cell cycle and cell mass. Conditions were the same as in Fig. 3, except that all determinations were carried out at 72 hr of treatment. Results are representative of one of two experiments, with similar results.

Table 3. Effect of doxorubicin, mitoxantrone, aclarubicin, novobiocin and caffeine on the expression of differentiation markers

| Drug | NBT | CD11b/ CD18 | CD11c/ CD18 |
|---|---|---|---|
| None | 6.8 ± 1.9 | 5.9 ± 1.8 | 3.8 ± 1.3 |
| DOX (15 nM) | 41.2 ± 3.9 | 65.4 ± 7.1 | 48.3 ± 5.4 |
| MIT (2.5 nM) | 22.8 ± 2.7 | 42.4 ± 4.3 | 48.5 ± 5.2 |
| ACL (70 nM) | 8.1 ± 2.1 | 7.8 ± 1.6 | 5.1 ± 1.2 |
| NOV (0.15 mM) | 8.2 ± 2.8 | 11.0 ± 3.1 | 9.4 ± 1.7 |
| CAF (3 mM) | 7.4 ± 0.9 | 3.4 ± 0.6 | 3.3 ± 1.1 |

For experimental conditions see Table 2 and Fig. 6.

DNA breakage [32, 33]. In addition, aclarubicin, novobiocin and caffeine inhibit other activities, such as RNA synthesis [34, 35, our unpublished observations]. At the assayed concentrations all drugs decreased cell proliferation to a similar extent as 0.1 μM *m*AMSA and 4 μM ABP, and caused little cell mortality (results not shown). The results in Fig. 6 and Table 3 indicate that mitoxantrone and doxorubicin induced cell accumulation at the $G_2$ phase, caused an increase in cell mass and stimulated NBT reduction and CD11b/CD18 and CD11c/CD18 surface integrin expression, in the same manner as *m*AMSA. In contrast, aclarubicin, caffeine and novobiocin had little effect on cell-cycle distribution. They induced a slight increase in the proportion of cells at either late S and $G_2$ phases in the case of aclarubicin, or at $G_1$ phase in the case of novobiocin and caffeine. The three agents decreased cell mass and had little effect on the expression of differentiation markers, as was the case with ABP.

## DISCUSSION

The results of this work indicate that subcytotoxic concentrations of the DNA intercalator *m*AMSA inhibited the proliferation of U-937 cells by preferentially affecting their transit throughout the $G_2$ phase of the cycle. At the same time, the drug produced positive unbalanced growth, which is defined as excess cell mass in relation to the degree of ploidy. This may be the consequence of the fact that inhibition of the cell-division cycle was not paralleled by protein synthesis inhibition. In contrast, the proliferation decrease caused by the DNA intercalator ABP, which exhibits great structural

similarity to *m*AMSA, was not phase specific and the drug significantly inhibited protein synthesis with the result of a reduction in cell mass. Finally, proliferation inhibition by *m*AMSA was accompanied by the induction of myeloid-specific differentiation markers, such as NBT reduction, expression of vimentin and leukocyte integrins, and great downregulation of c-*myc* oncogene expression. In contrast, none of these markers were induced by ABP.

A common property of many DNA intercalators, such as *m*AMSA, mitoxantrone, doxorubicin, 2-methyl-9-OH-ellipticinium, ditercalinium and actinomycin D, is that they inhibit eukaryotic DNA topoisomerase II activity [5, 6]. The drugs act by stabilizing the cleavable topoisomerase–DNA covalent complexes with the consequence of DNA breakage [5]. The present results indicated that subcytotoxic concentrations of *m*AMSA stimulated protein–DNA covalent associations significantly. Hence, the accumulation of cells at the $G_2$ phase upon treatment with *m*AMSA could be the consequence of the necessity for the cells to repair the drug-generated DNA damage before entering into mitosis, as occurs in general with clastogenic agents [36]. In addition, $G_2$ blockade could be the result of topoisomerase II inhibition itself, since it is known that this activity is required for cell transition from the $G_2$ phase into mitosis [37]. Actually, in these experiments aclarubicin, which is a non-clastogenic topoisomerase II inhibitor [32], slightly accumulated cells at the $G_2$ phase. According to these observations, the failure of ABP to promote protein–DNA covalent complex formation, as well as to accumulate cells at the $G_2$ phase suggested that under the conditions used the drug did not significantly affect topoisomerase activity.

The potential value of differentiation inducers as therapeutic agents resides in their ability to overcome the maturation blockade of leukemia cells, with the consequence of irreversibly stopping their uncontrolled proliferation. Hence, it is of great interest to understand the mechanisms by which certain cytostatic drugs cause cell maturation when used at subcytotoxic concentrations. It has been proposed that reduction of topoisomerase activities could be the actual trigger by which anti-topoisomerase drugs such as etoposide and camptothecin induce the differentiation of myeloid cells [38, 39]. This proposal must be considered with caution, since (1) as this group observed, the topoisomerase II inhibitors aclarubicin and novobiocin had little or no capacity to induce differentiation, and (2) differentiation could also be produced by cytostatic agents which were not topoisomerase inhibitors, as is the case of the DNA replication inhibitors cytarabine and hydroxyurea [29]. Interestingly, these compounds provoked an increase in cell mass [40, our unpublished observations]. In addition, Wangenheim and Howard [41] proposed that the positive unbalanced growth resulting from X-irradiation may have been a cause of cell differentiation. Our present results indicating that differentiation was produced by *m*AMSA, mitoxantrone and doxorubicin (which greatly affected cell transit throughout the $G_2$ phase and, as a consequence, increased cell mass) but not by ABP, aclarubicin, caffeine and novobiocin (which had slight or no effects on cell-cycle distribution and decreased cell mass) support such a conclusion. Hence, differentiation of myeloid leukemia cells by cytostatic drugs appears to be a response to specific uncoupling of the DNA-division cycle and the cell-growth cycle, leading to an increase in cell mass.

*Acknowledgements*—This work was supported by DGICYT (Spain) Grant PB91-0062 and FIS (Spain) Grant 94/0008-01. The authors wish to thank Drs C. Bernabeu and C. Cabañas for generous gifts of Bear 1 and HCl/1 mAbs, Dr C. de la Torre for critical reading of the manuscript, and Ms E. de Blas and Mr P. Lastres for excellent technical assistance.

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