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PROTEIN SYNTHESIS, CELL DIVISION AND CELL DEATH

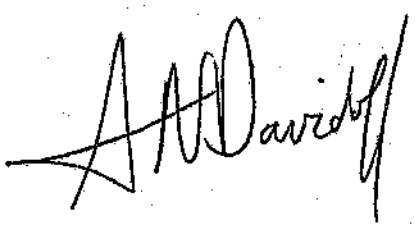
Avri Nava Davidoff

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa, in fulfilment of the requirements for the Degree of Doctor of Philosophy. This thesis is presented as a series of manuscripts.

1993

DECLARATION

I declare that this thesis is my own unaided work. It is being submitted to the University of the Witwatersrand, Johannesburg in fulfilment of the requirements for the degree of Doctor of Philosophy. It has not been submitted before for any degree or examination in any other University.



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MBBCh BSc(Hons) MSc(Biotechnology)

DEDICATION

This thesis is dedicated to those I love for their unfailing support and commitment.

PUBLICATIONS AND CONFERENCE PROCEEDINGS

Chapters 1, 3, 4 and 5 have already been published. Chapter 6 is currently in press. Chapter 2 has been presented as a poster at the XIIth International Society of Haematology Meeting in Vienna, August 28 - September 3, 1993. Chapters 2, 7 and 8 have been submitted for consideration for publication.

Publications

Davidoff AN and Mendelow BV (1993).
Substituted purines elicit differential cytokinetic, molecular and phenotypic effects in HL-60 cells.
Experimental Hematology 21, 456-460.

Davidoff AN and Mendelow BV (1993).
Cell-cycle disruptions and apoptosis induced by the cyclophosphamide derivative mafosfamide.
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Davidoff AN and Mendelow BV (1993).
c-myc Expression is down-regulated in mafosfamide-treated HL-60 cells undergoing apoptosis.
Anticancer Research (In Press).

Davidoff AN and Mendelow BV (1992).

Unexpected cytokinetic effects induced by puromycin include a G2-arrest, a metaphase-mitotic-arrest, and apoptosis.
Leukemia Research 16(11), 1077-1085.

Davidoff AN and Mendelow BV (1992).
Puromycin is a potent and specific inhibitor of tyrosine kinase activity in HL-60 cells.
Anticancer Research 12, 1761-1766.

Davidoff AN and Mendelow BV (1993).
G2 synchronous apoptosis is elicited in HL-60 cells by the protein synthesis inhibitor puromycin.
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Davidoff AN and Mendelow BV (1993).
Puromycin-elicited c-myc mRNA superinduction precedes apoptosis in HL-60 leukemic cells.
Manuscript submitted.

Davidoff AN and Mendelow BV (1993).
Protein synthesis inhibition is not a requisite for Puromycin- and Cycloheximide-elicited c-myc mRNA superinduction.
Manuscript submitted.

Conference Proceedings

XIIth Meeting of the International Society of Haematology, Vienna, August 28 - September 3, 1993.

Davidoff AN and Mendelow BV

G2-Synchronous apoptosis is elicited by protein synthesis inhibition in HL-60 cells.

24th Congress of the International Society for Haematology, London, 23 - 27 August, 1992.

Davidoff AN and Mendelow BV

Puromycin metabolites are potent and specific inhibitors of endogenous tyrosine kinase activity in HL-60 leukaemic cells.

Davidoff AN and Mendelow BV

Closely-related substituted purine nucleoside analogs elicit differential cytokinetic, molecular, and phenotypic effects in HL-60 cells.

34th Annual Meeting of the American Society of Hematology, Anaheim, December 4 - 8,1992

Davidoff AN and Mendelow BV

Cell-cycle disruptions, apoptosis, and down-regulation of c-myc mRNA levels induced in HL-60 cells by Mafosfamide.

33rd Annual Congress of the Federation of South African Societies of Pathology, Bophuthatswana, 4 - 6 August, 1993.

Davidoff AN and Mendelow BV

G2-Synchronous apoptosis is elicited by protein synthesis inhibition in HL-60 cells.

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Davidoff AN and Mendelow BV

Cell-cycle disruptions and apoptosis induced by the cyclophosphamide derivative Mafosfamide.

Davidoff AN and Mendelow BV

Up-regulation of c-myc mRNA levels is not a requisite for DNA repair mechanisms in Mafosfamide-treated HL-60 cells.

31st Annual Congress of the Federation of South African Societies of Pathology, Warmbaths, June 30 - July 3, 1992.

Davidoff AN and Mendelow BV

Low dose Puromycin-exposure and its postulated effects on tyrosine and serine/threonine-kinase activity.

10th Congress of the South African Biochemical Society, Pietermaritzburg, 27-30 January, 1991.

Davidoff AN, Port MZ, Tzitzivacos D and Mendelow BV

G2-Phase arrest in protein synthesis inhibited Hl-60 cells: a possible mechanism of cell death?

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Note: As the manuscripts presented in Chapters 1-8 had to comply with the

requirements of the different scientific journals to which they were submitted, editorial inconsistencies are evident. The most obvious of these concern the format of the reference lists at the end of each chapter.

ABSTRACT

In this study the morphologic, cytokinetic, biochemical, and molecular consequences of low-dose continuous Puromycin-exposure were examined in HL-60 cells, and in a variety of malignant and non-malignant human and murine cell types. Puromycin (PM) is a composite of the aminonucleoside dimethyladenosine and tyrosine-o-methylether. Functionally it is an analogue of the terminal aminoacyl-adenosine portion of aminoacyl-tRNA, more specifically of tyrosyl-tRNA. At high concentrations 5-50μg/ml (10-100μM) PM has been found to block protein synthesis completely by causing the premature release from the ribosomes of truncated peptide chains which are bound to PM through their carboxyl termini. The nascent PM-peptide complexes (PMPs) are rapidly degraded through a ubiquitin-dependent pathway and are of interest because of (i) their potential to compete for degradation with the natural substrates of the ubiquitin-dependent pathway, including cyclin B, and (ii) because their structure predicts an inhibitory effect on tyrosine kinase activity. In the current study then, special consideration was given to the effect of PM on the cell cycle, on apoptosis (programmed cell death), and on tyrosine kinase activity. As a means of comparison, certain of these effects were also examined with respect to another translation inhibitor Cycloheximide (CHX), to two other substituted purines Puromycin aminonucleoside (PAN) and 6-Dimethylaminopurine (6-DMAP), as well as to the cyclophosphamide derivative Mafosfamide (ASTA Z 7557).

The potent effects of low doses of PM on the cell cycle have to date been obscured by the conventional usage of this drug at high concentrations (5-50μg/ml) to inhibit protein synthesis. In unsynchronized cells, low doses of PM (0.1-0.5μg/ml) were found to disrupt significant phase-to-phase cell cycle transitions, causing a G2-Phase-arrest, a metaphase-mitotic-arrest, and apoptosis. These observations were elicited by PM concentrations as low as 0.1μg/ml, and were more pronounced at slightly higher PM concentrations, including that (0.5μg/ml) which inhibited [^{14}C]Leucine incorporation by approximately 20% after one hour, and by approximately 50% after 24 hours. A concentration of CHX (0.25μg/ml) which was equivalent to 0.5μg/ml of PM, both in terms of molarity (0.9uM) and degree of inhibition of [^{14}C]Leucine incorporation, failed to induce similar changes to those induced by PM. This suggests that at these particular concentrations the PM-induced changes were likely to have been related to the different mechanisms of protein synthesis inhibition exerted by these two "classical" translation inhibitors. PM but not CHX generates nascent peptidyl-PM complexes (PMPs), and it is therefore feasible to propose that the subsequent intracellular effects exerted by the PMPs may account, in part, for the differential cytokinetic effects elicited by these drugs. Furthermore the observation that the closely related substituted purines PAN and 6-DMAP did not exert detectable morphological and cytokinetic effects may also be attributable to their failure to generate nascent PMPs. Since the metaphase-to-anaphase transition is mediated by precipitous ubiquitin-dependent cyclin B degradation, and since PMPs are degraded by the same pathway, it is possible that in PM-treated cells this pathway was

preferentially diverted into PMP proteolysis. One or more of the ubiquitin pathway components might have become limiting for cyclin B degradation, resulting in the observed metaphase-mitotic-arrest. As far as the mechanism of the G2-Phase-arrest is concerned, this may be related to PM-mediated attenuated tyrosine phosphorylation of the proteins that mediate the G2-Phase/metaphase transition (see below).

In cells that were synchronised at the G0G1/S boundary and subsequently exposed to 2μg/ml PM, S-Phase progression was found to be retarded. A G2-Phase-arrest was evident at 8 hours and was found to precede acute, explosive apoptosis at 10 hours. Apoptosis was confirmed morphologically and biochemically. [C^{14}]Leucine incorporation studies revealed that in the 8th hour of PM-exposure, protein synthesis was inhibited by ±94%. Thus in synchronized, PM-treated cells, this study showed that the activation of the apoptotic cascade was cell cycle-dependent i.e. consequent on a G2-Phase-arrest, and that both the G2/apoptosis transition and the process of apoptosis itself were virtually protein synthesis-independent. The mechanisms by which the protein synthesis inhibitor PM retarded the passage of synchronised cells through S-Phase, disrupted the G2/metaphase transition, and activated the apoptotic cascade may be related to PM's protein synthesis inhibiting activity, or alternatively to its ability to disrupt cellular tyrosine phosphorylation of the regulatory proteins involved in mediating the G2-Phase/metaphase transition.

With regard to the latter suggestion, since PM-exposure is known to generate

PMPs whose structure predicts an inhibitory effect on tyrosine kinase activity, and since the search for specific tyrosine kinase inhibitors constitutes a novel approach to the development of anticancer agents, the effect of a low dose of PM (0.9 μM//0.5μg/ml) on tyrosine kinase activity was investigated. Experiments were specifically controlled to exclude inhibitory effects consequent on protein synthesis blockade. Soluble enzyme extracts derived from PM-treated cells showed attentuated phosphorylation of two independent synthetic tyrosine kinase-specific substrates, the polymer Poly(Glu:Tyr;4:1) and the peptide substrate Raytide. In contrast, Protein Kinase C-specific activity was unaffected by PM-exposure. It is likely that inhibition was due to metabolite/s of PM, perhaps PMPs, rather than to PM itself.

Low-dose PM could thus provide a potentially useful tool for dissecting out the role of tyrosine phosphorylation in the regulation and dysregulation of cell growth, and could conceivably prove beneficial in the treatment of tumours in which tyrosine kinase overactivity is linked to oncogenesis. PM could also prove useful as a low-dose component of a multidrug chemotherapeutic regimen in which its cell-cycle-specific effects could potentially be synergistic with other anticancer agents that elicit similar cell cycle arrests, as well as with chemotherapeutic drugs that activate the apoptotic cascade e.g. the Cyclophosphamide derivative Mafosfamide.

Cyclophosphamide is a widely employed alkylating chemotherapeutic agent whose cytotoxicity is mediated by the introduction into DNA of cross-links and

single-strand breaks. Mafosfamide (ASTA Z 7557), a cyclophosphamide derivative which rapidly generates 4-Hydroxycyclophosphamide after aqueous dissolution, was employed in vitro at doses ranging from 0.1 to 10 ug/ml. In unsynchronised cells Mafosfamide-exposure was associated with an S-Phase accumulation, a G2-Phase arrest, and morphological, ultrastructural, and biochemical evidence of apoptosis. In synchronised cells, S-Phase progression was considerably delayed in the presence of Mafosfamide. The apoptosis which was evident in Mafosfamide-treated cells 12 hours after release from the thymidine block was found to occur in the presence of S-Phase- and G2-Phase-cell-cycle-arrests. Taken together the current data suggest that Mafosfamide may well have potential synergism with PM in that both of these drugs elicit a G2-Phase-arrest as well as activate the apoptotic cascade.

Since the expression of the proto-oncogene c-myc has been strongly but variously implicated in the regulation of the apoptotic pathway, a study was undertaken to examine the levels of c-myc mRNA transcripts in PM- and Mafosfamide-treated cells in which the apoptotic cascade was activated. Mafosfamide was employed in vitro at doses ranging from 0.1 to 10μg/ml. No changes in c-myc mRNA levels were evident after 1 hour of exposure to Mafosfamide. After 6 and 24 hours of Mafosfamide-exposure however, there was a dose- and time-dependent decrease in c-myc transcripts. c-myc mRNA expression was reduced to a greater extent than either the beta-actin or GAPDH expression was. In the context of Mafosfamide-treated HL-60 cells therefore, upregulation of c-myc mRNA transcription was not fundamental for

the activation of the apoptotic cascade. As far as PM was concerned, for this part of the study doses of 0.9μM and 2μMPM were employed in vitro. Dose-dependent c-myc mRNA up-regulation was present at 1 hour of PM-exposure, and was associated with subsequent activation of the apoptotic cascade at 24 hours of exposure. Although high concentrations of protein synthesis inhibitors have been found by others to augment and stabilize mRNA transcript levels e.g. 36-180μM of PM has been found to elicit c-myc mRNA upregulation/superinduction, [C^{14}]Leucine incorporation studies at 1 hour demonstrated minimal protein synthesis inhibition at the doses of PM employed, suggesting that c-myc mRNA up-regulation in this context may have been the result of mechanisms which were independent of new protein synthesis.

In a further study undertaken to address this suggestion, the protein synthesis-inhibitory effects of PM and CHX were dissociated from their c-myc mRNA superinducing effects. When employed at concentrations which were sub-inhibitory with respect to protein synthesis (0.2μM), PM and CHX nevertheless elicited the phenomenon of superinduction, possibly through mechanisms related to interruptions in signal transduction. It is feasible that the tyrosine kinase inhibition demonstrated in PM-treated cells, and the Protein Kinase C inhibition evident in CHX-treated cells, may have contributed to the protein synthesis-independent c-myc mRNA upregulation elicited by low doses of these drugs.

The findings of this study are of interest because the use of PM previously in 1955 as a single antineoplastic agent proved to be ineffective in a range of clinical settings. Although significant protein synthesis inhibiting concentrations of PM are unattainable in vivo without serious toxicity, this limitation does not necessarily apply to the more potent effects elicited by low-dose PM. The data suggests that PM may yet prove to be a useful chemotherapeutic agent, not singly but as a component of a multidrug regimen in which its potent cell-cycle-specific and apoptotic effects could well be synergistic with other agents, and in which its potent and specific tyrosine kinase-inhibiting effects could be maximally exploited. This could be accomplished by first accumulating cells in specific non-G0G1-Phase compartments and then subsequently delivering appropriate cycle-active chemotherapy. Thus the metaphase-mitotic-arrest caused by low-dose PM-exposure could be synergistic with the metaphase-specific agent Vincristine, while the shunting of G2-Phase-arrested cells into apoptosis could act synergistically with Etoposide, Cisplatin, or Mafosfamide. Phase I/II veterinary clinical trials incorporating PM into such multidrug regimens for the treatment of spontaneous canine lymphomas are currently under way in our laboratories.

INTRODUCTION

Brief overview

The role of protein synthesis in the cell cycle and in the process of apoptosis is contentious, as is the relationship between cell cycle phase and apoptosis activation. Since each of these subjects have been reviewed extensively elsewhere, this introduction merely serves to highlight the relationships that are reported to exist between these variables, with special reference to the "classical" translation inhibitors, Puromycin (PM) and Cycloheximide (CHX), and also to the substituted purine nucleoside analogs Puromycin aminonucleoside (PAN), and 6-Dimethylaminopurine (6-DMAP). Also, because PM is known to generate peptides whose structure has been suggested to predict an inhibitory effect on tyrosine kinase activity, and because the search for specific tyrosine kinase inhibitors constitutes a novel and exciting approach to the development of anticancer agents, a discussion on the pivotal role that tyrosine phosphorylation plays in the regulation of normal cell growth and proliferation and in neoplastic transformation has been included.

To further address some of the above issues from a potentially practical point of view, the apoptosis-cell cycle relationship has been examined in the context of the chemotherapeutic agent Mafosfamide. Mafosfamide is the in vitro form of cyclophosphamide, one of the most versatile of the chemotherapeutic agents known, and one which is also prototypical of the alkylating agents.

Finally the issue of c-myc expression has been addressed in the specific contexts of apoptosis, protein synthesis inhibition, and chemotherapeutic agents.

PM, CHX, PAN, and 6-DMAP

PM, an antibiotic derived from the fermentation of the yeast Streptomyces alboniger (Baker et al, 1953), is well known for its inhibitory effect on protein synthesis (Yarmolinsky and De La Haba, 1959; Moazed and Noller, 1989), and indeed is considered to be one of the "classical" translation inhibitors. Structurally it is a composite of the aminonucleoside dimethyladenosine and tyrosine-o-methylether i.e. it is composed of 3 structural units : a purine, an amino sugar, and an amino acid (Yarmolinsky and De La Haba, 1959). Functionally it is an analogue of peptidyl-transferRNA, more specifically of tyrosyl-transferRNA. At concentrations of 5-50μg/ml (10-100μM), PM blocks protein synthesis completely by causing the premature release from the ribosomes of truncated peptide chains. These are bound to PM through their carboxyl termini, and are thought to contribute to PM's cytotoxic effects (Longnecker, 1972). Since the nascent PM-peptide complexes (PMPs) are rapidly degraded through a ubiquitin-dependent pathway (Ciechanover et al, 1984), their metabolic effects are poorly understood. The PMPs are of interest however, because of their potential to compete for degradation with the natural substitutes of the ubiquitin-dependent pathway (including cyclin, p53, c-myc, and c-fos (Glotzer et al, 1991; Reichsteiner, 1991)), and because their structure predicts an inhibitory effect on tyrosine kinase activity (Gordon

et al, 1986). In the latter regard it is noteworthy that the carboxyterminal tyrosine residues of PMPs are reminiscent of synthetic random polymers of tyrosine-containing aminoacids, some of which have been found to act as competitive inhibitors of endogenous tyrosine kinases (Braun et al, 1984).

Cycloheximide (CHX), like PM, is another "classical" translation inhibitor, which prevents chain elongation by inhibiting the peptidyl transferase activity of the 60S ribosomal subunit (Stryer, 1988), but which unlike PM, does not generate nascent polypeptides (Allen and Zamecnik, 1961).

The substituted purines, of which Puromycin (PM), Puromcyin aminonucleoside (PAN), and 6-Dimethylaminopurine (6-DMAP) are prototypes, have been found to exert a variety of biochemical and cytological effects when used at high concentrations ranging from 10-300μM. For example, 6-DMAP, the purine component of both PM and PAN, reversibly arrests cells in the G2-Phase of the cell cycle by inhibiting phosphorylation (Rime et al, 1990). The nucleoside analog PAN, which is a composite of 6-DMAP and 3-Aminoribose, has been found to inhibit HIV-1 replication in vitro (Vander Heyden, 1989), and PM, itself a composite of PAN and tyrosine-o-methylether, is as discussed above, well know for its inhibitory effect on protein synthesis (Yarmolinsky and De La Haba, 1959).

Protein synthesis, the cell cycle, and apoptosis

The role of protein synthesis in the cell cycle and in the process of apoptosis is

contentious, as is the relationship between cell cycle phase and apoptosis activation. It would appear that much of the confusion surrounding the interrelationships between the cell cycle, apoptosis, and protein synthesis reflects diverse contextual experimental scenarios in which the three variables have not been independently controlled.

As far as apoptosis and protein synthesis is concerned, not only is there conflicting opinion as to whether protein synthesis is (Nieto et al., 1992) or is not (Kruman et al., 1992) required for the process to occur, but in certain settings protein synthesis inhibitors may actually induce it (Martin et al., 1990) or augment the apoptosis induced by other agents (Bazar and Deeg, 1992).

With respect to the cell cycle and protein synthesis, and depending on cell synchronicity and on contextual diversity, protein synthesis inhibitors have been found to halt the entry of cells into S-Phase (Doida and Okada, 1972), retard the S/G2M transition (Nagata et al., 1992), inhibit the passage of cells through the G2-Phase (Kishimoto and Lieberman, 1964; Epifanova et al., 1969), induce cells to accumulate in the G2M-Phase (Akamatsu et al., 1991), completely block mitosis initiation (Epifanova et al., 1969), and prolong metaphase in those cells that are already in early mitosis when the drug is applied (Epifanova et al., 1969). There does appear to be an absolute requirement for protein synthesis for (i) progression through the G1b-subPhase of the cell cycle (Pardee, 1989), and for (ii) progression from the end of DNA synthesis to nuclear division and cytokinesis (Burke and Church, 1991).

Cell cycle phase and the activation of the apoptotic cascade have been variously linked. Apoptosis may be G0-Phase (Bruno et al., 1992) or S-Phase specific (Del Bino and Darzynkiewicz, 1991), it may occur subsequent to a G1-Phase (Chen and Rosenberg, 1992) or to a G2-Phase arrest (Eastman, 1990), it may occur early in M-Phase (Ishida et al., 1992), or it may occur simultaneously during all phases of the cell cycle (Glynn et al. 1992). Caution must be exercised, however, in interpreting data from unsynchronised cultures, because cell population fluxes may be occurring between different phases of the cell cycle, concomitantly with the occurrence of apoptosis. This problem is compounded if the agents used to induce apoptosis also impact on these population fluxes, either by causing transition blocks, or by activating cells to enter cycling status from the G0-Phase.

Mafosfamide and the cell cycle

Cyclophosphamide is a widely employed alkylating cancer chemotherapeutic agent (Ahmed and Hombal, 1984; Kessinger et al, 1991) whose cytotoxicity is mediated by the introduction into DNA of cross-links and single-strand breaks (Hilton, 1984; Crook et al, 1986). Cyclophosphamide derivatives are also frequently used in vitro to purge leukemic cells from bone marrow (Crook et al, 1986; Nara et al, 1988; Douay, 1985). One such derivative is the prodrug Mafosfamide (ASTA Z 7557), a cyclohexylamine salt that rapidly generates 4-Hydroxycyclophosphamide after aqueous dissolution. 4-Hydroxycyclophosphamide degrades irreversibly via aldophosphamide to phosphoramide mustard and acrolein (Niemeyer et al, 1989; Domeyer and

Daldek, 1980; Le Blanc and Waxman, 1990), the therapeutically significant metabolites of cyclophosphamide (Sharkis et al, 1980).

A number of anticancer agents are able to induce apoptosis or programmed cell death, e.g. etoposide, cisplatin, melphalan, methotrexate, camptothecin, teniposide, amsacrine, and 5-fluorouracil (Eastman, 1990; Eastman, 1992; Fernberg et al, 1991; Del Bino and Darzynkiewicz, 1991; Lock and Ross, 1990; Sorenson et al, 1990). In the cases of etoposide (Lock and Ross, 1990) and cisplatin (Sorenson et al, 1990), a preceding G2-Phase cell-cycle-arrest has been associated with the subsequent activation of the apoptotic cascade, whereas camptothecin, teniposide, and amsacrine have been found to induce S-Phase-specific internucleosomal DNA degradation (Del Bino and Darzynkiewicz, 1991).

Apoptosis and c-myc expression

The proto-oncogene c-myc has been strongly implicated in the apoptotic pathway. In certain contexts the process of apoptosis has been found to be contingent on c-myc expression (Bertrand et al, 1991; Askew et al, 1991; Grassilli et al,1992; Sen, 1992; Shi et al, 1992; Evan et al, 1992; Kyprianou et al, 1991), whereas in other scenarios down-regulation of c-myc mRNA levels have accompanied the activation of the apoptotic cascade (Oritani et al, 1992; Alnemri et al, 1992; Gunji et al, 1992). For example down-regulation of the c-myc gene has been found to precede IL-6- induced apoptosis of Y6 cells (Oritani et al, 1992), steroid-induced apoptosis of 697 pre-B lymphocytes

(Alnemri et al, 1992), and phorbol ester-induced apoptotis of U-937 cells (Gunji et al, 1992). On the other hand, elevated c-myc mRNA levels are seen in rat thymocytes undergoing apoptosis following dexamethasone treatment (Grassilli, 1992), and c-myc has been found to be a potent inducer of apoptosis in rat fibroblasts (Evan, 1992). c-myc expression is also a requirement for activation-induced apoptosis of T-cell hybridomas (Shi et al, 1992), for apoptotic regression of estrogen-treated MCF-7 human mammary cancers (Kyprianou et al, 1991), and for apoptosis elicited in c-myc-amplified cell lines (including HL-60) by the DNA topoisomerase II inhibitor VM-26 (Bertrand et al, 1991).

Chemotherapeutic agents and c-myc expression

Certain anticancer agents have been found to reduce c-myc mRNA levels (Arbiser et al, 1991; Futscher and Erickson, 1990; Pieper et al, 1989; Miller et al, 1987; Snyder et al, 1991), whereas others, including the cyclophosphamide derivative 4-Hydroperoxycyclophosphamide, have been found to elevate c-myc mRNA transcripts and/or c-myc oncoprotein levels (Futscher and Erickson, 1990; Sullivan and Willis, 1989). In Colo320HSR cells treated with the cyclophosphamide derivative (4-hydroperoxycyclophosphamide), c-myc mRNA levels were found to increase after 1 hour of exposure but to decrease subsequently, raising the possibility that alkylating agents may suppress the expression of specific transforming genes (Futscher and Erickson, 1990). Further evidence for this comes from in vitro experimentation which demonstrated that alkylating agents including nitrogen mustard and melphelan,

but not 4-S-(propionic acid)-sulfidocyclophosphamide, terminated c-myc transcription in a sequence-specific, drug-specific manner (Pieper et al, 1989). In addition mithramycin, which binds to G-C-rich sequences in the DNA minor groove, has been found to inhibit c-myc transcription in HeLa cells (and other genes which have G-C rich promoter sequences) by preventing the formation of the transcription initiation complex (Miller et al, 1987). However, increased c-myc protein has been observed to accumulate in a lymphoblastoid cell line in response to certain alkylating agents (Dimethylsulphonate, Ethylmethane sulphonate and Mitomycin C) (Sullivan and Willis, 1989), and elevated c-myc mRNA levels have been reported in SV40 transformed human keratinocytes in response to ultraviolet irradiation (Ronai et al, 1988) and in rat embryonic cells exposed to ionizing irradiation (Higo et al, 1989).

Protein synthesis and c-myc expression

The overall regulation of eukaryotic gene expression is inextricably linked, inter alia, to factors governing mRNA stability and degradation (Carter and Malter, 1991). The steady-state levels of a specific mRNA species can be controlled at any one of the multiple steps involved in its metabolism i.e transcription initiation, transcription elongation, post-transcriptional RNA processing (addition of the 5' cap, the 3' poly(A) tail, and splicing), mRNA transport from the nucleus to the cytoplasm, translation, and cytoplasmic mRNA accumulation and degradation (Carter and Malter, 1991; Atwater et al, 1990).

The term "superinduction" has traditionally been applied to the process by which complete protein synthesis inhibition results in the augmentation and stabilization of inducible mRNA transcripts (Mahadevan and Edwards, 1991). For example, high doses i.e. 36-180μM of the "classical" translation inhibitors PM and CHX have been used to elicit c-myc mRNA superinduction (Laird-Offringa et al, 1990). Within the context of protein synthesis inhibition, the phenomenon of superinduction has been attributed to a number of different processes including mechanisms relating to the inhibition of translation per se (Wisdom and Lee, 1991), the inhibition of translation-dependent poly(A) tail shortening (Laird-Offringa et al, 1990), and enhanced mRNA stability due to the loss of labile transcriptional repressors or RNAses (Greenberg and Ziff, 1984; Dani et al, 1984).

Recently it has been recognised that superinduction may also be elicited through mechanisms which involve signal transduction pathways, and which are distinct from protein synthesis inhibition (Mahadevan and Edwards, 1991; Frick et al, 1988; Hauguel-de Mouzon and Kahn, 1991). For example, sub-inhibitory concentrations of the protein synthesis inhibitor anisomycin has been found to superinduce c-fos and c-jun mRNA, specifically through the phosphorylation of two chromatin-associated proteins pp33 and pp15 (Mahadevan and Edwards, 1991).

Tyrosine kinase inhibitors as anticancer agents

Phosphorylation of protein tyrosine plays a pivotal role in the regulation of

normal cell growth and proliferation, and in neoplastic transformation. In the cell cycle, the G2- to M-Phase transition is controlled by phosphorylation/ dephosphorylation reactions involving the Tyrosine-15 residue of the $p34^{cdc2}$ kinase (Krek and Nigg, 1991). In addition a number of transmembrane growth factor receptors (including those of Epidermal Growth Factor, Platelet-derived Growth Factor, and Insulin-like Growth Factor), as well as a variety of cytosolic non-receptor-associated oncogene products (such as pp60src, p59fyn, p62yes, etc), exhibit tyrosine kinase activity (Bishop, 1983; Hollywood, 1991; Ullrich and Schlessinger, 1990; Cantley et al,1991; Aaronson, 1991).

The search for specific tyrosine kinase inhibitors represents a novel approach to the development of anticancer agents (Grunicke, 1991; Wong and Goldberg, 1981; Akiyama et al, 1987), in that it is based on the modification of transmembrane and intracytoplasmic tyrosine kinase signal transduction, rather than on the killing of tumour cells by inhibition of transcription, translation, and DNA synthesis (Powis et al, 1990; Newman and Dogramatzis, 1991). Quercetin (Glossman et al, 1981; Graziani et al, 1983), genistein (Akiyama et al, 1987), erbstatin (Gazit et al, 1989), amiloride (Davis and Czech, 1985), and herbimycin A (Uehara et al, 1989), have all proved effective against tyrosine kinases. Problems of toxicity, specificity, and drug delivery to tumours have limited their clinical applications however. For example, although genistein is a much more specific inhibitor of tyrosine kinases than is quercetin, its toxicity has precluded its in vivo use in patients to date. More recently, derivatives of tyrphostin other than erbstatin have been found to inhibit EGF-stimulated

growth of squamous carcinoma cells, in vitro, and in nude mice inoculated with such cells (Yoneda et al. 1991).

CHAPTER 1

Unexpected cytokinetic effects induced by Puromycin include a G2-arrest, a metaphase-mitotic-arrest, and apoptosis.

ABSTRACT

The potent effects of low doses of PM on the cell cycle have to date been obscured by the conventional usage of this drug at high concentrations (5-50μg/ml) to inhibit protein synthesis. In this in vitro study undertaken in a variety of malignant and non-malignant human and murine cell types, we found that low doses of PM (0.1-0.5μg/ml) disrupted significant phase-to-phase cell cycle transitions, causing a G2-arrest, a metaphase-mitotic-arrest, and apoptosis. In HL-60 cells these observations were elicited by PM concentrations starting at 0.1μg/ml, and were more pronounced at slightly higher PM concentrations, including that (0.5μg/ml) which inhibited [^{14}C]Leucine incorporation by approximately 20% after one hour, and by approximately 50% after 24 hours. A concentration of CHX (0.25μg/ml) which was equivalent to 0.5μg/ml of PM, both in terms of molarity (0.9uM) and degree of inhibition of [^{14}C]Leucine incorporation, failed to induce similar changes to those induced by PM. This suggests that at these particular concentrations the PM-induced changes were likely to have been related to the different mechanisms of protein synthesis inhibition exerted by these two "classical" translation inhibitors. PM but not CHX generates nascent peptidyl-PM complexes (PMPs), and we therefore propose that the subsequent intracellular effects exerted by the PMPs may account, in part, for the differential cytokinetic effects elicited by these drugs. The role of PM is currently being evaluated in vivo as a low-dose component of a multidrug chemotherapeutic regimen in which its cell-cycle-specific effects could

potentially be synergistic with other agents.

INTRODUCTION

The antibiotic Puromycin (PM), is a composite of aminonucleoside dimethyladenosine and tyrosine-o-methylether (1). It is a functional analogue of tyrosyl-transferRNA, and is well know for its inhibitory effect on protein synthesis (2). PM concentrations of 5-50μg/ml block protein synthesis completely by causing the premature release of truncated peptide chains which are bound through their carboxyl termini to PM. Since the nascent PM-peptide complexes (PMPs) are rapidly degraded through an ubiquitin-dependent pathway (3), their metabolic effects are poorly understood. The PMPs are of interest however because of their potential to compete for degradation with the natural substrates of the ubiquitin-dependent pathway (including cyclin, p53, c-myc, and c-fos) (4), and because their structure predicts an inhibitory effect on tyrosine kinase activity (5). In the latter regard it is noteworthy that the carboxy terminal tyrosine residues of the PMPs are reminiscent of certain synthetic random polymers of tyrosine-containing aminoacids, some of which have been found to act as competitive inhibitors of endogenous tyrosine kinases (6).

The regulation of cell cycle transitions has recently been elucidated and extensively reviewed (7,8,9). The G2- to M-Phase transition is regulated by a co-ordinated cascade of kinase- and phosphatase-mediated reactions (10), while the metaphase to anaphase transition is mediated by rapid, ubiquitin-dependent cyclin proteolysis (11). Consistent with what is now known to be

an absolute requirement for protein synthesis for progression through the G1b-subphase of the cell cycle (12), PM (5-20μg/ml) halts the entry of cells into S-Phase (13), inhibits the passage of cells through the G2-Phase (14,15), completely blocks mitosis initiation (15), and prolongs metaphase in those cells that are already in early mitosis when PM is applied (15).

Cell cycle events and the process of apoptosis (programmed cell death) have been linked through the recognition of an association existing between a G2-arrest and subsequent activation of the apoptotic cascade (16). As far as apoptosis is concerned, protein synthesis has a varied and sometimes controversial role to play in this process. Certain cells enter apoptosis by a process dependent on de novo protein synthesis (17,18), whereas in other cells complete protein synthesis inhibition does not prevent apoptosis (19). Furthermore, inhibition of protein synthesis may augment spontaneous apoptosis (20), and in certain circumstances inhibition of protein synthesis per se has been enough to trigger apoptosis (20,21,22,23). Specifically PM employed at 50μg/ml induced apoptosis in HL-60 cells (23).

The concentrations of PM (5-50μg/ml) achieved in vitro (13,14,15), and which significantly inhibit protein synthesis by greater than 95%, are unattainable in vivo without serious toxicity. Therefore to explore the in vitro effects of PM on the cell cycle further, specifically as a prelude to re-evaluating its potential in vivo application in a chemotherapeutic context, we employed very low concentrations of PM (<1μg/ml) which inhibited protein

synthesis to a limited extent. We also compared PM's cytokinetic effects to those elicited by an equivalent protein synthesis inhibiting concentration of another protein synthesis inhibitor Cycloheximide (CHX). We found that at these concentrations PM but not CHX induced apoptosis, and potently blocked late cell cycle transitions, disrupting both the G2-to M-Phase, and the metaphase to anaphase transitions. Since CHX prevents chain elongation by inhibiting peptidyl transferase activity of the 60S ribosomal subunit (24), but does not generate nascent polypeptides (25), we suggest that the intracellular effects exerted subsequently by the PM-generated PMPs may account for the differential cytokinetic effects elicited by doses of these drugs that inhibited protein synthesis to an equivalent extent.

MATERIALS AND METHODS

Cell culture

HL-60 human promyelocytic leukemic cells, THP-1 human monocytic leukemic cells, and SP-2 murine myeloma cells (ATCC Rockville, MD, USA) were maintained in logarithmic growth phase at 37°C in a humidified atmosphere of air and 5% CO_2. Four week old female (C3H/HeJ, endotoxin-resistant) mice were thymectomized and thymocyte suspensions prepared (26). All cells were seeded at $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% (v/v) of heat-inactivated fetal calf serum, as well as L-glutamine (2mM), and Penicillin (50U/ml) and Streptomycin (50μg/ml) antibiotics (all from GIBCO, Madison, WI, USA). Puromycin and Cycloheximide were obtained from Sigma. At completion of incubations, culture viability was assessed using the trypan blue dye exclusion technique. Cells were fixed and stained with May-Grünwald-Giemsa, and examined, using light microscopy, for mitotic figures and apoptotic cells. Previously defined criteria were used to identify apoptotic cells (27). The number of mitotic and apoptotic cells per 1000 cells counted was determined, and then expressed as the mitotic index and as the apoptotic index respectively.

Protein synthesis inhibition

0.5μCi[^{14}C]Leucine (specific activty 50Ci/mmol; Amersham) was added to cultures which were then further incubated at 37°C for one hour (28). At the conclusion of the incubation period, cells were washed three time in

phosphate-buffered saline, 1 ml of 10% trichloroacetic acid was added and the tubes chilled on ice for 30 minutes. The precipitate so formed was filtered on GF/C filter papers (Whatmann), and washed three times with 5% trichloroacetic acid. The filters were dried and counted after adding aqueous counting scintillant. [^{14}C]Leucine incorporation was determined in triplicate.

Flow cytometric analysis

Cells were prepared for analysis as described (29). The centrifuged cell pellet was resuspended in a hypotonic nuclear isolation medium containing Propidium Iodide (Sigma), incubated on ice, and then in RNase at 37°C. Fluorescence emitted from the Propidium Iodide-DNA complex was quantitated after laser excitation of the fluorescent dye by the Coulter EPICS-Profile-II flow cytometer (Coulter Electronics, Inc, Fla). Cell debris and doublets were excluded (30). The coefficient of variations of the peaks of the control G0G1- and G2M-Phases were less than 3%. Limits of cell cycle phases as determined in the control DNA distributions were retained in the subsequent analysis of the drug-treated populations.

DNA fragmentation analysis

DNA isolation, extraction and electrophoresis was carried out according to the following method. After 24 hours cells were sedimented and lysed, and DNA extracted prior to incubation with RNAse. Total DNA from $1.5x10^6$ cells was loaded onto a 1.5% agarose gel and electrophoresed for 12 hours at 1.5 V/cm. Both the gel and the electrophoresis buffer contained ethidium

bromide(1μg/ml).

RESULTS

Viability

The viability at 24 hours of PM- and CHX-treated cultures was unaffected by concentrations up to 0.5μg/ml (Figure 1a) . At higher concentrations ≥1μg/ml, the viability of both PM- and CHX-treated was compromised, with the viability of PM-treated cultures being affected to a greater extent.

Protein synthesis inhibition

The dose-response curves expressing the inhibitory activity of PM on protein synthesis (as measured by [^{14}C]Leucine incorporation), are presented in Figure 1b. At 1 hour and at 24 hours, at a concentration of 0.9μM (0.25μg/ml), CHX was found to inhibit [^{14}C]Leucine incorporation to a similar extent as a concentration of 0.9μM (0.5μg/ml) of PM did i.e. approximately 20-30% at 1 hour and approximately 50% at 24 hours (Figure 1c).

PM effects

Effects on the DNA histogram of HL-60 cells following continuous exposure to increasing PM concentrations confirmed a time- and a dose-related increase in the G2- and M-Phase populations, an almost reciprocal reduction in the S-Phase population, and an apparently unperturbed G0G1-Phase population (Figure 2, Figure 3a and b). Morphological studies showed a metaphase-mitotic-arrest and features of apoptosis, the apoptotic index being 12.4 in PM-treated cells as compared to 1.02 in control cells, and to 1.4 in CHX-treated

(0.25μg/ml) cells (Figure 5). Apoptosis was confirmed ultrastructurally (not shown), and by DNA electrophoresis which showed the pattern of double-stranded DNA fragments that results from the activation of an endogenous endonuclease (Figure 6).

CHX effects

After 24 hours of CHX-exposure (0.25μg/ml) there was a G0G1-arrest with concomitant limitation of the S-, the G2-, and the M-Phases (Figure 2, Figure 4a and b). The morphological sequelae of CHX-exposure were unremarkable except for a reduced number of mitotic figures (Figure 4b). Apoptosis above control levels was not evident at a CHX concentration of 0.25μg/ml (Figure 5c), nor were DNA fragments produced at this concentration (Figure 6). DNA fragmentation was evident when CHX was employed at a much higher concentration of 2μg/ml (Figure 6).

The morphological, cytokinetic and electrophoresis data obtained in 0.5μg/ml PM-treated HL-60 cells were representative of similar data obtained (but not shown) in 0.5μg/ml PM-treated THP-1 and SP-2 cell lines, as well as for 0.5μg/ml PM-treated mouse thymocytes.

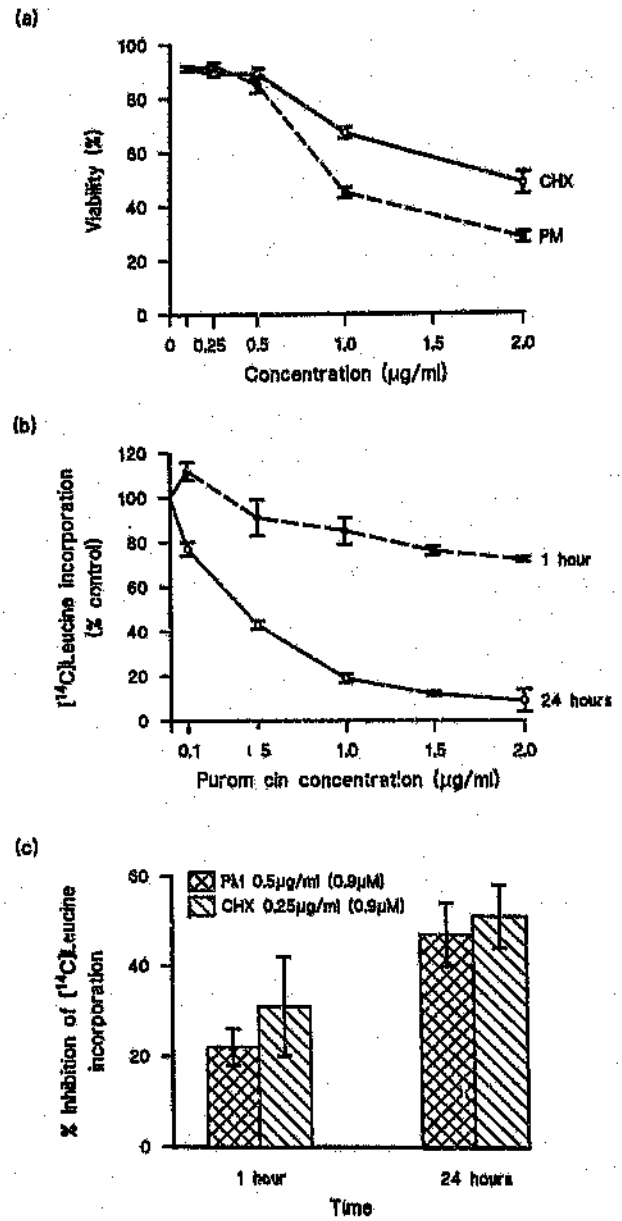

Figure 1

a) Viability of cultures (as determined by the trypan blue dye exclusion technique) after 24 hours of exposure to the indicated concentrations of PM and CHX (µg/ml). Results represent the mean ± SD of three separate experiments each done in duplicate.

b) The effect of increasing doses of PM (µg/ml) on [^{14}C]Leucine incorporation at one hour and at twenty-four hours in HL-60 cells. Results were expressed as the percentage of control values and represent the mean ± SD of three separate experiments each done in triplicate.

c) Effect of equivalent molar concentrations (0.9µM) of PM (0.5µg/ml) and CHX (0.25µg/ml) on inhibition of [^{14}C]Leucine incorporation. Results represent the mean ± SD of three separate experiments each done in triplicate.

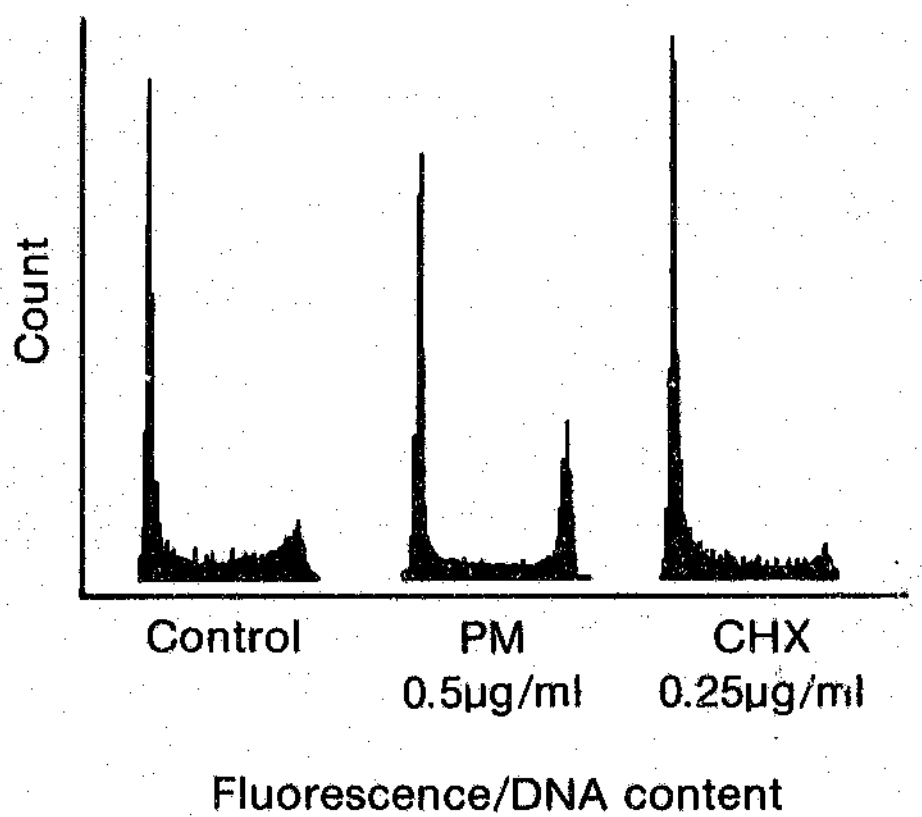

Figure 2
Representative flow cytometric analysis at 24 hours of Count versus Fluorescence/DNA content of control, PM-treated, and CHX-treated HL-60 cells. Twenty-five thousand cells were counted for each analysis. Histogram scaling was the same for all samples.

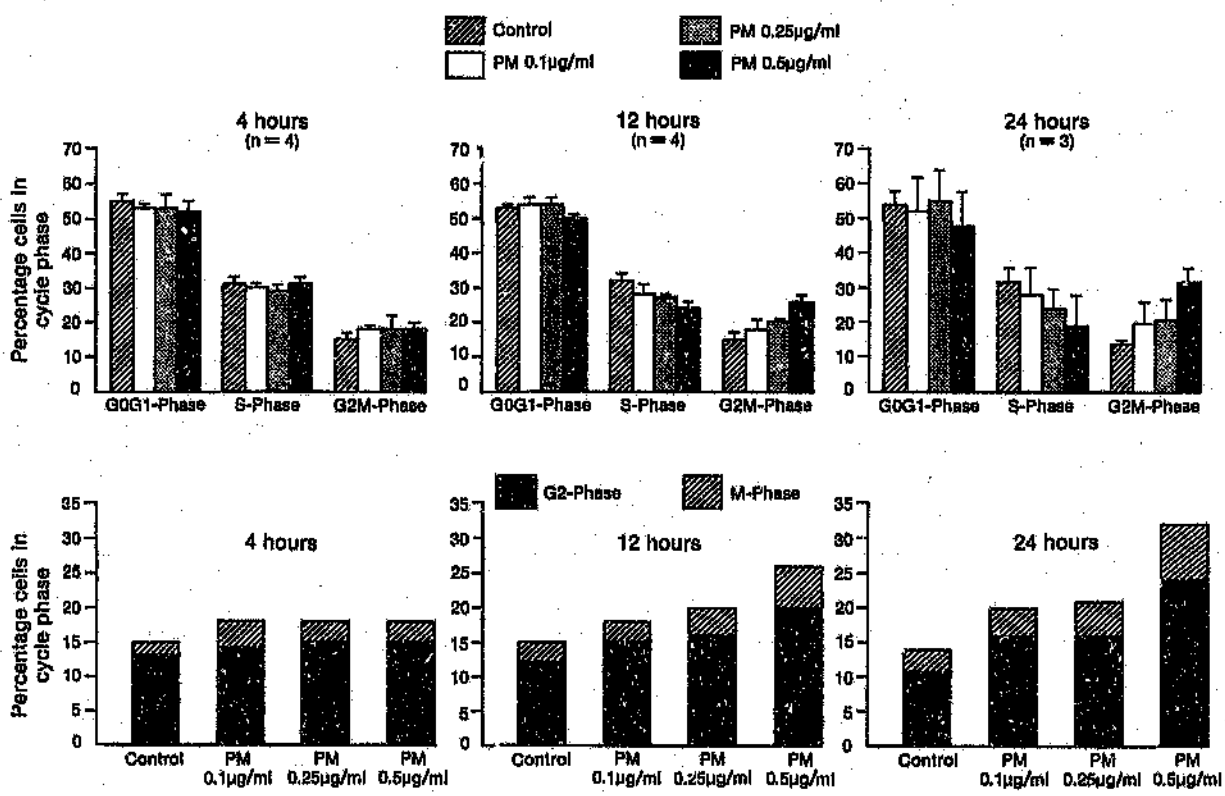

Figure 3

a) Effects on the DNA histogram of HL-60 cells following 4, 12, and 24 hours of exposure to increasing PM concentrations (µg/ml). Results are expressed as mean ± SD. (n=3/4)

b) Stacked-bar graphs illustrating the relative percentages of HL-60 cells in the G2- and M-Phases of the cell cycle consequent on 4, 12 and 24 hours of exposure to increasing PM concentrations (µg/ml). The M-Phase at each concentration point is representative of the mean mitotic index (number of mitotic figures per 100 cells) of two experiments done on separate occasions. The G2-Phase was calculated by subtracting the M-Phase value from the mean G2M-Phase value (n=3/4) obtained from Figure 2a.

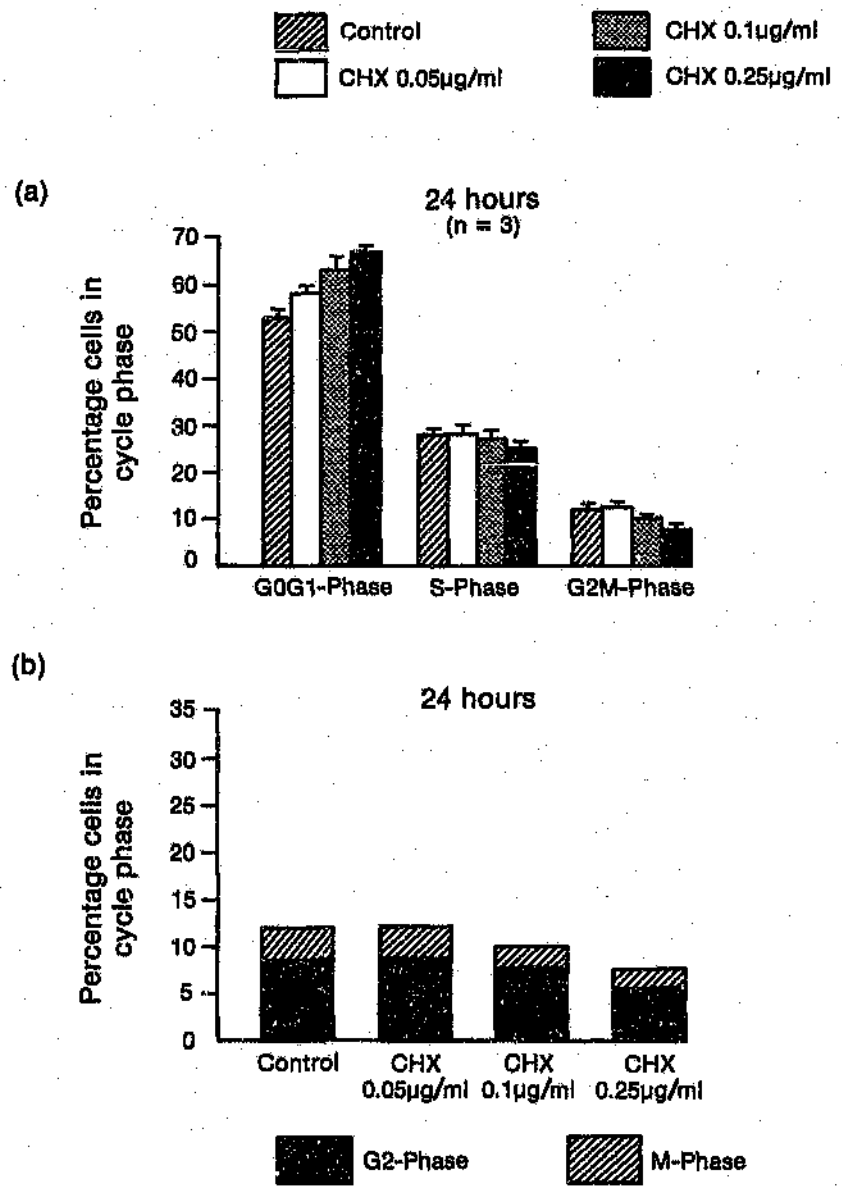

Figure 4

a) Effects on the DNA histogram of HL-60 cells following 24 hours of exposure to increasing CHX (µg/ml) concentrations. Results are expressed as mean ± SD (n=3).

b) Stacked-bar graphs illustrating the relative percentage of HL-60 cells in the G2- and the M-Phases of the cell cycle consequent on 24 hours of exposure to increasing CHX (µg/ml) concentrations. The percentage of cells in the G2- and in the M-Phases were calculated as described in the legend of Figure 3b.

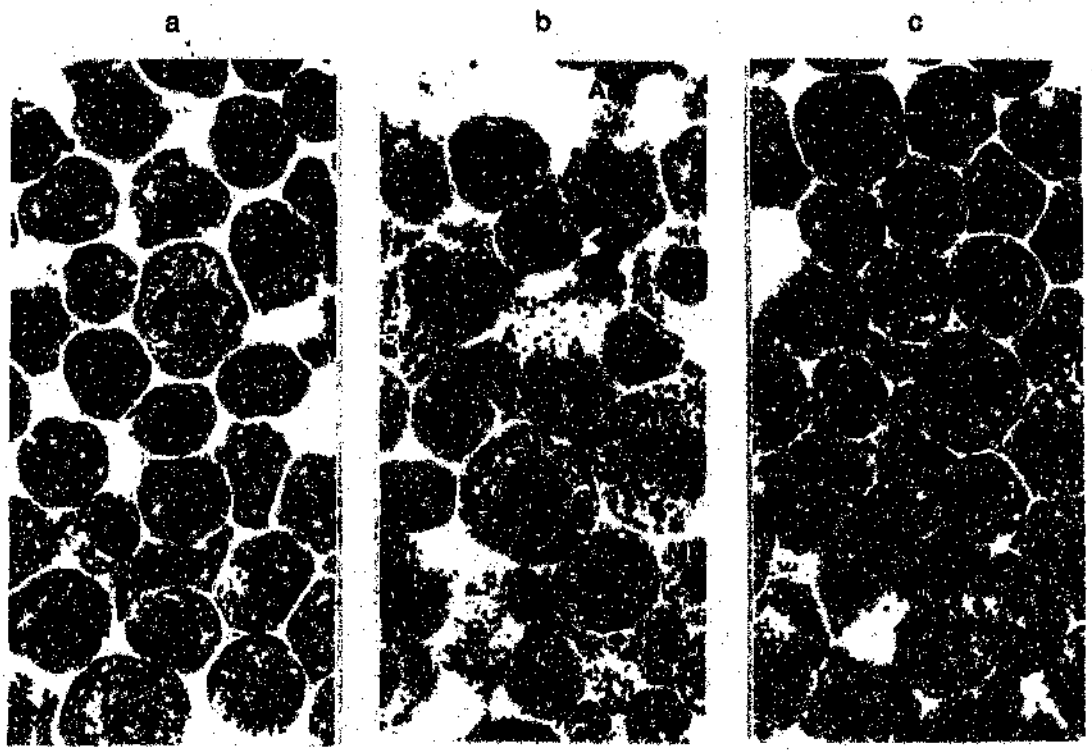

Figure 5

Giemsa-stained cytospin preparations (magnification x 1000) of 24 hour control and drug-treated HL-60 cells.

a) Control HL-60 cells

b) PM-treated HL-60 cells (0.5μg/ml) showed a marked metaphase mitotic accumulation "M" by 24 hours. Certain PM-treated cells bore histological evidence of apoptosis "A" (condensation of the nuclear chromatin and cytoplasm, nuclear fragmentation, and the formation of membrane-bound apoptotic bodies).

c) CHX-treated HL-60 cells (0.25μg/ml) were indistinguishable from control cells except for a reduced mitotic activity.

The apoptotic index in each case represents the mean ± SD of three separate experiments each done in duplicate.

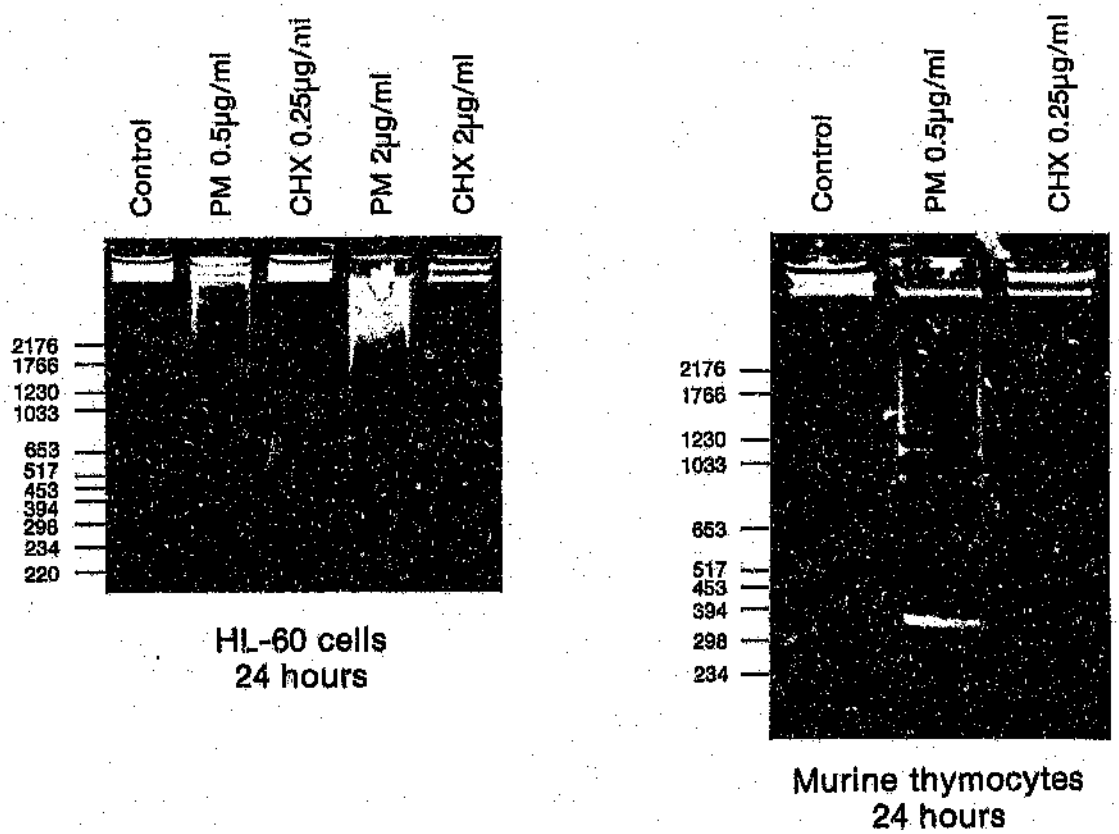

Figure 6
Agarose gel electrophoresis of total DNA extracted at 24 hours from 1.5 x 10^6 HL-60 cells, and from 2x10^6 murine thymocytes, treated as indicated. The size (in bases) of separated DNA molecular weight markers (BglI/HinfI cleaved pBR328) are shown.

DISCUSSION

In the current study we have shown that PM exerted a number of potent effects on the cell cycle when used at concentrations far below those used traditionally to completely block protein synthesis (5-50μg/ml). These effects included a G2-arrest, a metaphase-mitotic-arrest, and apoptosis, and were found in a variety of malignant and non-malignant cell types of both human and murine origin. In HL-60 cells these observations were elicited by PM concentrations starting at 0.1μg/ml, which not only did not inhibit [^{14}C]Leucine incorporation, but actually enhanced it, (at least for the first hour of exposure). The effects were more pronounced at slightly higher PM concentrations, including that (0.5μg/ml) which inhibited [^{14}C]Leucine incorporation by approximately 20% after one hour, and by approximately 50% after 24 hours. A concentration of CHX (0.25μg/ml) which was equivalent to 0.5μg/ml of PM, both in terms of molarity and degree of inhibition of [^{14}C]Leucine incorporation, failed to induce similar changes to those induced by PM. This suggests that at these particular concentrations the PM-induced changes were likely to have been related to the different mechanisms of protein synthesis inhibition exerted by these two "classical" translation inhibitors. PM (25), but not CHX (24), generates nascent peptidyl-PM complexes (PMPs), and we propose that the subsequent intracellular effects exerted by the PMPs may account, in part, for the differential cytokinetic effects elicited by these drugs.

The cytokinetic effects observed with PM-exposure included a time- and dose-

related increase in both the G2- and the metaphase populations, an almost reciprocal reduction in the S-Phase population, and an evidently unperturbed G0G1-Phase population. These observations suggest an undelayed passage through and egress from S-Phase of those cells that were beyond the G1b-subphase when PM was applied, but with subsequent partial hold-ups at the G2/metaphase and the metaphase/anaphase boundaries. Any predicted but not observed decreases in the G0G1-Phase population (on the basis of the metaphase-mitotic- arrest) are likely to have been offset by an accumulation of cells in the G1b-subphase consequent on PM's inhibitory effect on the mandatory protein synthesis requirement of this subphase (12). CHX-exposure induced a G0G1-arrest with concomitant limitations of the S-, the G2-, and the M-Phases, indicating that CHX blocked the entry of new cells into division consequent on the protein synthesis requirement of the G1b-subphase (12,31), but permitted those cells that were beyond this subphase at the time of CHX application, to complete mitosis (31). We did not observe the G2-delay that has been reported to occur with high-dose CHX-treatment (31).

The G2- to M-Phase transition is controlled by phosphorylation - dephosphorylation reactions involving the Tyrosine-15 residue of the $p34^{cdc2}$ kinase unit of Mitosis Promoting Factor (7,8,10,32). Since we have recently found PM metabolites to specifically inhibit tyrosine kinase activity (submitted data), we are investigating the possibility that such metabolites, by attenuating Tyrosine-15 phosphorylation/dephosphorylation reactions of $p34^{cdc2}$ kinase, may

have been responsible for the G2-arrest observed in PM-treated cells. Such an arrest has also been reported when PM was employed at much higher concentrations of 5-20μg/ml (14,15).

It is possible that there is a connection between the G2-arrest and the apoptosis observed in PM-treated HL-60 cells (0.5μg/ml), since a preceding G2-arrest has been found to be linked to subsequent activation of the apoptotic cascade in cells exposed to the anticancer agents Etoposide (33), Cisplatin (34),and Mafosfamide (submitted data).

The role of protein synthesis in the process of apoptosis is varied and controversial (17-23). Although 50μg/ml of PM was found previously to trigger apoptosis in HL-60 cells (23), we were able to elicit apoptosis at one hundredth of that PM concentration i.e. at 0.5μg/ml. CHX used in HL-60 cells at a dose that inhibited protein synthesis to a similar extent as 0.5μg/ml of PM did, failed to induce apoptosis. Although we cannot adequately explain the differential effects exerted on apoptosis by these concentrations of PM and CHX, we suggest that it might relate to the mechanism, rather to the presence, of protein synthesis inhibition produced by these two drugs. In the future we plan to evaluate the specific role, if any, of PMPs in apoptosis activation. It is worth noting that a higher concentration of CHX (2μg/ml) induced apoptosis in HL-60 cells, consistent with the apoptosis that has been induced in such cells by 50μg/ml of CHX (22,23), and consistent with the augmentation of spontaneous apoptosis in B-chronic lymphocytic leukemia cells elicited by

1μg/ml of CHX (20).

The metaphase to anaphase transition is mediated by precipitous ubiquitin-dependent cyclin degradation (11). Since PMPs are known to be degraded by the same pathway (3), it is possible that in PM-treated cells this pathway was preferentially diverted into PMP proteolysis. One or more of the ubiquitin pathway components might have become limiting for cyclin degradation, resulting in the observed metaphase-mitotic-arrest. This hypothesis is currently under investigation in our laboratory. In addition we are also evaluating the effect of low-dose PM on cyclin and on ubiquitin synthesis, and on the synthesis of the specific protease enzymes responsible for ubiquitin-dependent proteolysis, since it is necessary to exclude decreased production of these proteins as being implicated in the generation of the PM-induced metaphase-mitotic-arrest. In this regard however it is worthwhile noting that 0.25μg/ml CHX, which inhibited protein synthesis to the same extent as 0.5μg/ml of PM did, did not induce a metaphase-mitotic-arrest, indicating that this level of protein synthesis inhibition appears not to have compromised cyclin, ubiquitin, or protease synthesis. It is of interest that when employed at a high dose of 20μg/ml, PM completely blocks the ability of cells to initiate mitosis, and prolongs metaphase in those cells that are already in mitosis when PM is applied (15).

The findings of this study are of interest because the use of PM previously as a single antineoplastic agent proved to be ineffective in a range of clinical

settings (35). Although significant protein synthesis inhibiting concentrations of PM are unattainable in vivo without serious toxicity, this limitation does not necessarily apply to the more potent effects elicited by low-dose PM. Our data suggests that PM may yet proved to be a useful chemotherapeutic agent, not singly but as a component of a multidrug regimen in which its potent cell-cycle-specific effects could well be synergistic with other agents. This could be accomplished by first accumulating cells in specific non-G0G1-Phase compartments and then subsequently delivering appropriate cycle-active chemotherapy. Thus, the metaphase-arrest caused by low-dose PM could be synergistic with the metaphase-specific agent Vincristine, while the shunting of G2-arrested cells into apoptosis could act synergistically with Etoposide, Cisplatin, or Mafosfamide. Phase I/II veterinary clinical trials incorporating PM into such multidrug regimens for the treatment of spontanouus canine lymphomas are currently under way in our laboratories.

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CHAPTER 2

G2 synchronous apoptosis is elicited in HL-60 cells by the protein synthesis inhibitor Puromycin.

ABSTRACT

HL-60 human leukemic cells were synchronised at the G0G1/S boundary using the double thymidine block method. Upon release from the block, serial flow cytometric analyses revealed that cells exposed to the protein synthesis inhibitor Puromycin (2μg/ml) progressed more slowly through S-Phase than did untreated control cells. In Puromycin-treated cells, a G2-Phase arrest was evident at 8 hours which was found to precede acute, explosive apoptosis at 10 hours. Apoptosis was confirmed morphologically and biochemically. [C^{14}]Leucine incorporation studies revealed that in the 8th hour of PM-exposure, protein synthesis was inhibited by ±94%. Our study in this context showed that the activation of the apoptotic cascade was cell cycle-dependent, and that the G2/apoptosis transition and the process of apoptosis itself were virtually protein synthesis-independent. The mechanisms by which the protein synthesis inhibitor PM retarded the passage of synchronised cells through S-Phase, disrupted the G2/metaphase transition, and activated the apoptotic cascade may be related to PM's protein synthesis inhibiting activity, or alternatively to its ability to disrupt cellular tyrosine phosphorylation of the regulatory proteins involved in mediating the G2/metaphase transition. The data are helpful in reconciling some of the apparently conflicting opinions concerning the relationship between protein synthesis, apoptosis and the cell cycle.

INTRODUCTION

The role of protein synthesis in the cell cycle and in the process of apoptosis is contentious, as is the relationship between cell cycle phase and apoptosis activation (Figure 1).

As far as apoptosis and protein synthesis is concerned, not only is there conflicting opinion as to whether protein synthesis is [1] or is not [2] required for the process to occur, but in certain settings protein synthesis inhibitors may actually induce it [3] or augment the apoptosis induced by other agents [4].

With respect to the cell cycle and protein synthesis, and depending on cell synchronicity and on contextual diversity, protein synthesis inhibitors have been found to halt the entry of cells into S-Phase [5], retarded the S/G2M transition [6], inhibit the passage of cells through the G2-Phase [7,8], induce cells to accumulate in the G2M-Phase [9], completely block mitosis initiation [8], and prolong metaphase in those cells that are already in early mitosis when the drug is applied [8]. There does appear to be an absolute requirement for protein synthesis for (i) progression through the G1b-subPhase of the cell cycle in which the proteins required for DNA replication are produced [10], and for (ii) progression from the end of DNA synthesis to nuclear division and cytokinesis [11].

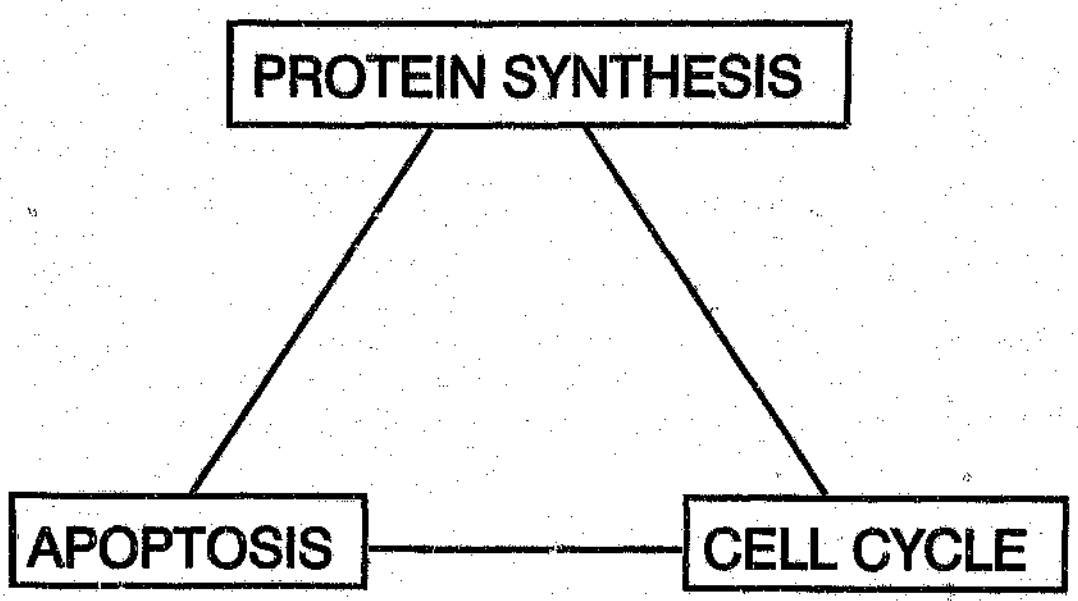

Figure 1
Diagrammatic representation of the relationships existing between cell cycle phase, apoptosis, and protein synthesis.

Cell cycle phase and the activation of the apoptotic cascade have been variously linked. Apoptosis may be G0-Phase [12] or S-Phase specific [13], It may occur subsequent to a G1-Phase [14] or to a G2-Phase arrest [15], it may occur early in M-Phase [16], or it may occur during all phases of the cell cycle [17]. Caution must be exercised, however, in interpreting data from unsynchronised cultures, because cell population fluxes may be occurring between different phases of the cell cycle, conincidentally with the occurrence of apoptosis. This problem is compounded if the agents used to induce apoptosis also impact on these population fluxes, either by causing transition blocking or by activating cells to enter cycling status from the GO-Phase.

It would appear that much of the confusion surrounding the interrelationship between the cell cycle, apoptosis and protein synthesis reflects diverse contextual experimental scenarios in which the three variables have not been independently controlled. In the present study, we have, investigated the apoptotic process in cells synchronised with respect to the cell cycle, while maintaining control over protein synthesis. The data suggest a working model which may help to reconcile at least some of the current apparently conflicting opinions on the issue.

MATERIALS AND METHODS

Cell culture and Puromycin-exposure

HL-60 cells (ATCC passage 17, Rockville, MD, USA) were seeded at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2mM L-glutamine, Penicillin (50U/ml), and Streptomycin (50μg/ml). Cells were maintained unagitated in logarithmic growth phase at 37°C in a humidified atmosphere of air and 5% CO_2. For synchronization experiments the double thymidine block method was employed [18]. Cells seeded at a concentration of 10^6/ml were incubated for two 24 hour periods in the presence of 2mM thymidine. The two 24 hour periods were separated by a 12 hour period in which cells were incubated in the absence of thymidine i.e. they were released from the first thymidine block. Thymidine was removed after each 24 hour-exposure by washing the cells three times in medium that had been prewarmed to 37°C. After release from the second thymidine block, cells were seeded at a final density of 10^6/ml, and aliquots of cell suspensions were collected after various incubation intervals and subjected to flow cytometric analysis and DNA agarose gel electrophoresis. Culture viability was assessed by the trypan blue dye exclusion technique, and after cells were fixed and stained with May-Grünwald-Giemsa, cytospin preparations were examined for mitotic figures and apoptotic cells.

Flow cytometric analysis

Cells were prepared for analysis as described elsewhere [19]. At the conclusion of the designated incubation periods, the pellet obtained by centrifugation from 10^6 cells was resuspended in 1ml of a hypotonic nuclear isolation medium (50mg/l Propidium Iodide; 1g/l Triton-X 100; 1g/l Sodium Citrate), incubated on ice for 30 minutes, and then in DNAase free RNAase at 37°C. Fluorescence emitted from the Propidium Iodide-DNA complex was quantitated after laser excitation of the fluorescent dye by the Coulter EPICS Profile II flow cytometer (Coulter Electronics, Inc, Fla.). Cell debris and doublets were excluded [20], and the coefficient of variations of the unsynchronised control G0G1-Phase and G2M-Phase peaks were less than 3%. 25 000 cells were counted for each analysis. Histogram scaling was the same for all samples. Limits of cell cycle phases as determined in the unsynchronised control DNA distribution, were retained in the subsequent analysis of the synchronised populations.

DNA fragmentation analysis

After appropriate incubation periods, cells which had initially been seeded at 10^6/ml were pelleted at 4°C and prepared for subsequent fragmentation analysis [21]. The pellet was resuspended in 70µl of lysis buffer (10mM EDTA; 50mM Tris-HCl, pH=8.0; 0.5% sodium lauryl sarkosinate; 0.5mg/ml proteinase K) and incubated for 1 hour at 50°C. A 10µl aliquot of 1mg/ml ribonuclease A was added to each sample prior to a further 1 hour incubation at 50°C. Samples were then heated to 70°C after which the reaction was

terminated by the addition of 20μl of loading buffer (10mM EDTA, pH=8.0; 0.25% bromophenol blue; 40% sucrose). Extracts were loaded into the wells of a 2% agarose gel and electrophoresed in a phosphate buffer (2mM EDTA; 800mM Tris-phosphate; pH=7.8). Both the gel and the buffer contained 1μg/ml of ethidium bromide. BglI/HinfI cleaved pBR328 served as molecular weig' t markers.

Protein synthesis inhibition studies

0.5μCi [^{14}C]Leucine (specific activity 50Ci/mmol; Amersham) was added to control and PM-treated cultures which were then further incubated at 37°C for one hour [22]. At the conclusion of the relevant incubation periods, cells were washed three times in phosphate-buffered saline, 1ml of 10% trichloroacetic acid was added, and the tubes were then chilled on ice for 30 minutes. The precipitate so formed was filtered on GF/C filter papers (Whatmann), and washed three times with 5% trichloroacetic acid. The filters were dried and counted after adding aqueous counting scintillant. [^{14}C]Leucine incorporation was determined in triplicate on three separate occasions.

Materials

All materials for cell culture were obtained from GIBCO, Madison, WI, USA. Propidium Iodide and Puromycin were from Sigma. All other reagents were of analytical grade and were from standard commercial sources.

RESULTS

Flow cytometric analysis

At 0 hours, HL-60 cells were synchronised at the G0G1/S boundary (Figure 2). Subsequent to their release from the double thymidine block, S-Phase progression was retarded in PM-treated (2μg/ml) cells as compared to control cells. By 8 hours the majority of control cells were in the G2M-Phase of the cell cycle, although a percentage had already completed cytokinesis and their daughter cells had moved into the G0G1-Phase. There was a G2M-Phase accumulation at 8 hours in PM-treated cells, an increase which reflected an absolute
G2-Phase arrest (Del Bino and Darzynkiewicz, 1991), since mitotic figures were not evident on cytospin preparations at this time-point (Figure 3).

Morphology

Synchronisation of HL-60 cells at 0 hours was confirmed morphologically by the absence of mitotic figures on cytospin preparations (Figure 3). By 6 hours mitotic figures had become prominent in the control population, but were absent in PM-treated cells. An occasional apoptotic cell was seen in the PM-treated population by 8 hours. Between 8 and 10 hours dramatic and virtually generalised apoptosis had occurred in this entire cell population, as evidenced at 10 hours by the presence of chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies.

DNA agarose gel electrophoresis

The striking morphological apoptosis seen at 10 hours in PM-treated cells was confirmed by DNA electrophoresis which showed the pattern of double-stranded DNA fragments that result from the activation of an endogenous endonuclease (Figure 4).

[C^{14}]Leucine incorporation studies

[C^{14}]Leucine incorporation was measured for the duration of both the 1st and the 8th hour post-release from the double thymidine block (Figure 5). With respect to control, [C^{14}]Leucine incorporation studies showed that PM inhibited protein synthesis by ±57% during the 1st hour of drug exposure, and by ±94% during the 8th hour post-release from the double thymidine block. Thus at the beginning of the 8th hour protein synthesis was effectively inhibited by ±94% in PM-treated cells.

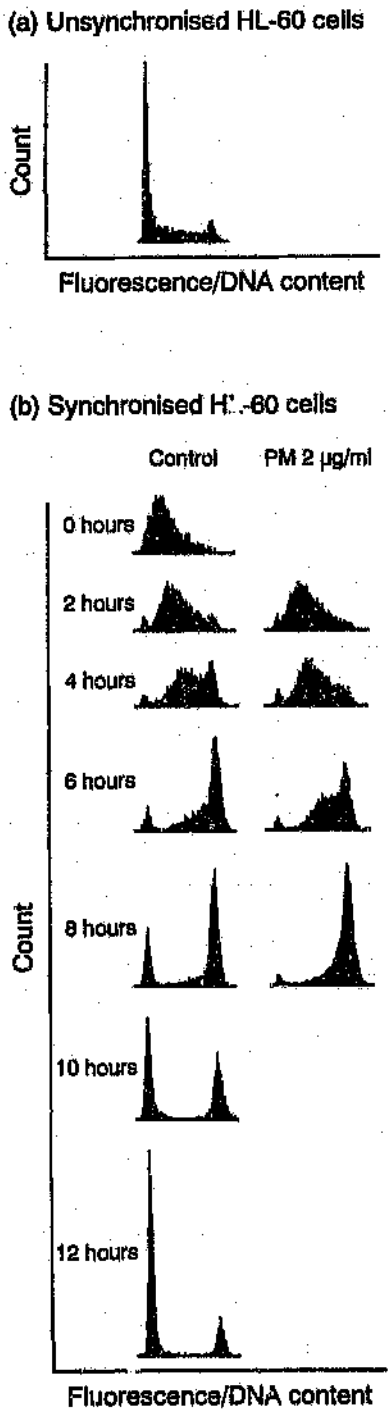

Figure 2
Effects of PM-exposure (2ug/ml) on cell cycle progression of HL-60 cells released from the double thymidine block. Note the delay in S-Phase progression in PM-treated cells, as well as the G2M-Phase accumulation of cells that was evident in both populations at 8 hours after release from the double thymidine block.

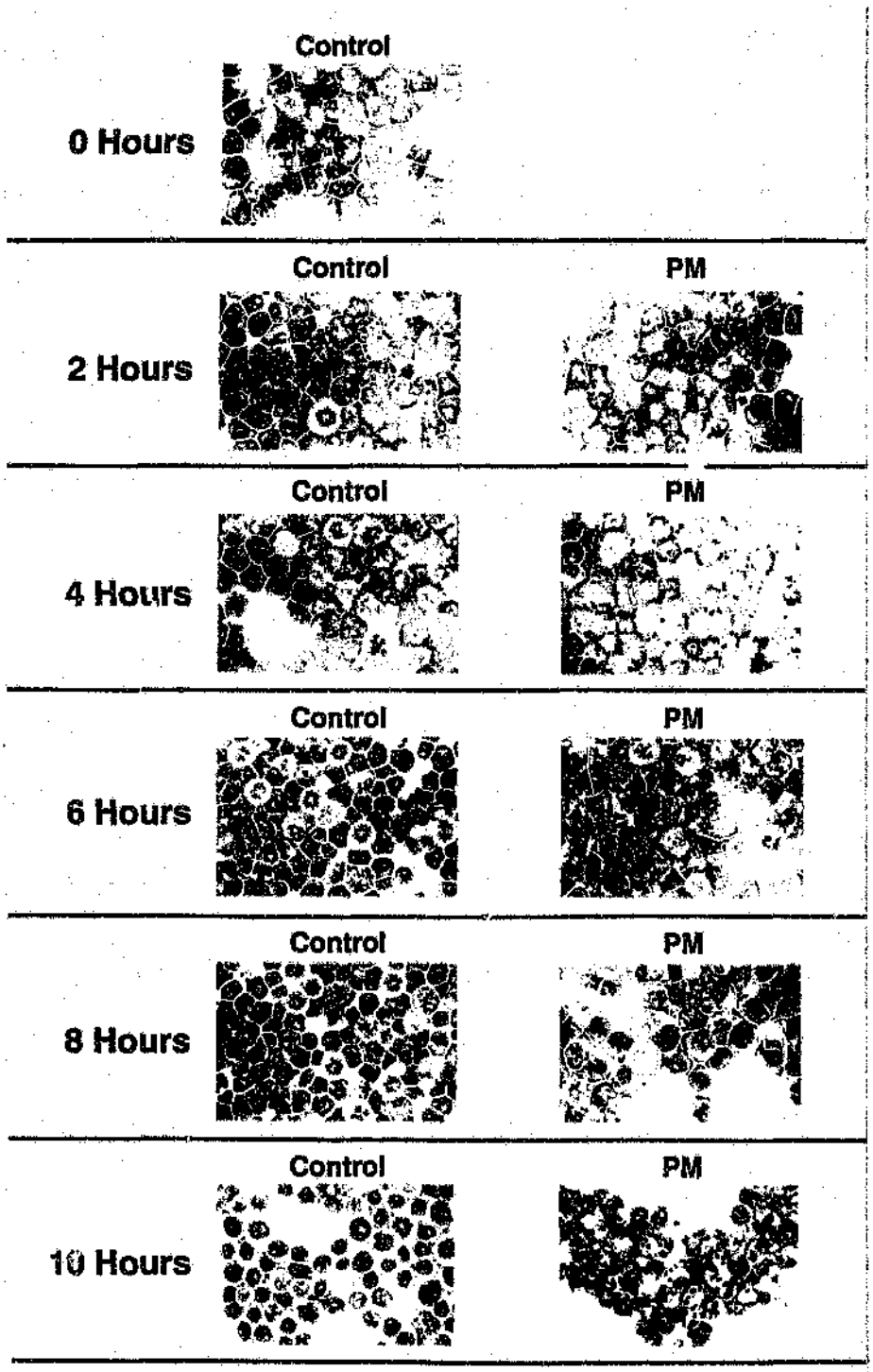

Figure 3
Giemsa-stained cytospin preparations (magnification X1000) of synchronised HL-60 cells at various time intervals after release from the double thymidine block. Note the preponderance of mitotic figures in control cells from 4 to 10 hours after release from the block. Mitotic figures were absent in PM-treated (2μg/ml) cells for up to 8 hours of drug-exposure. By 10 hours there was dramatic morphological evidence of mitosis in PM-treated cells in the form of chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies.

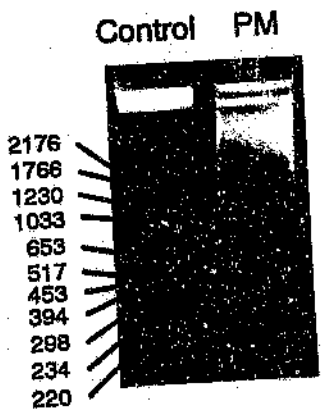

Figure 4
Agarose gel electrophoresis of total DNA extracted from control and PM-treated (2ug/ml) HL-60 cells at 10 hours after release from the double thymidine block (starting cell number at 0 hours was 10^6 cells). The sizes (in bases) of molecular weight markers are shown. Note the extensive DNA fragmentation at 10 hours of PM-exposure, coinciding with the dramatic morphological features of apoptosis at this timepoint (Figure 2).

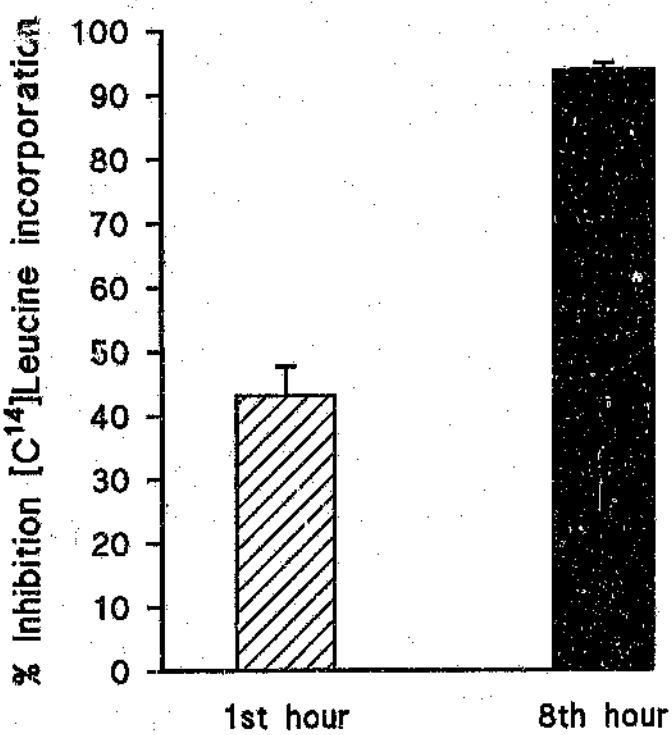

Figure 5
[C^{14}]Leucine incorporation studies in PM-treated HL-60 cells for the duration of the 1st and the 8th hour post-release from the double thymidine block. Results are expressed as a percentage of control [C^{14}]Leucine incorporation, and represent the mean±SD of three separate experiments, each done in triplicate.

DISCUSSION

The role of protein synthesis in the cell cycle and in the process of apoptosis is contentious, as is the relationship between cell cycle phase and apoptosis activation. It is likely however, that inconsistencies reported in the literature represent contextual variations (diversity in cell type, cell synchronicity, type and dose of protein synthesis inhibitors employed, extent of protein synthesis inhibition) rather than major controversies. In the present study undertaken in synchronized, PM-treated HL-60 cells, we have demonstrated that (i) the activation of the apoptotic cascade was cell cycle-dependent, and that (ii) the G2/apoptosis transition and (iii) the process of apoptosis itself were virtually protein synthesis-independent.

In a recent review [23], Martin has drawn attention to the concept of an apoptosis pathway comprising a number of signalling entry points, distinct from the process of apoptosis itself. As some entry points occur proximal to a requirement for new protein synthesis, and others occur distal to this requirement, protein synthesis may or may not be required for apoptosis to occur. In the present study, we extend this concept by suggesting that the various entry points are dictated by the cell cycle. Thus our data show that synchronised cells in which protein synthesis is inhibited proceed more slowly through S-Phase, and subsequently undergo synchronous apoptosis at the G2/M transition point. The effect on synchronized cells is dramatic, virtually all cells entering apoptosis at precisely the same time. The data also show

that under these particular experimental circumstances the process of apoptos is to all intents and purposes independent of new protein synthesis, since protein synthetic activity immediately preceeding apoptosis was found to be reduced by 94%. Because there is an absolute requirement for protein synthesis during the G1b subphase of the cells cycle in order to produce the proteins required for subsequent DNA synthesis [10], blockade of new protein synthesis at this stage would preclude cells from reaching the G2/M transition point at which stage apoptosis occurs. Thus, under such conditions, protein synthesis inhibition would not only not cause apoptosis, but would in fact prevent it. The latter scenario clearly is applicable to cells in the G0-Phase stimulated to enter the cell cycle but also subjected to protein synthesis inhibition. This model is outlined in Figure 6 according to which apoptosis is viewed as a cell cycle checkpoint, concomitant with the G2/M transition. Clearly it does not preclude the existence of other checkpoints elsewhere in the cell cycle, some of which may also be mediated by apoptosis [24].

In addition, there is evidence for additional checkpoints mediated by other mechanisms. We have previously shown that PM-treatement of unsynchronized cells results in both a G2-Phase-arrest and a metaphase-mitotic-arrest [25]. The data from that study are consistent with a further block at the

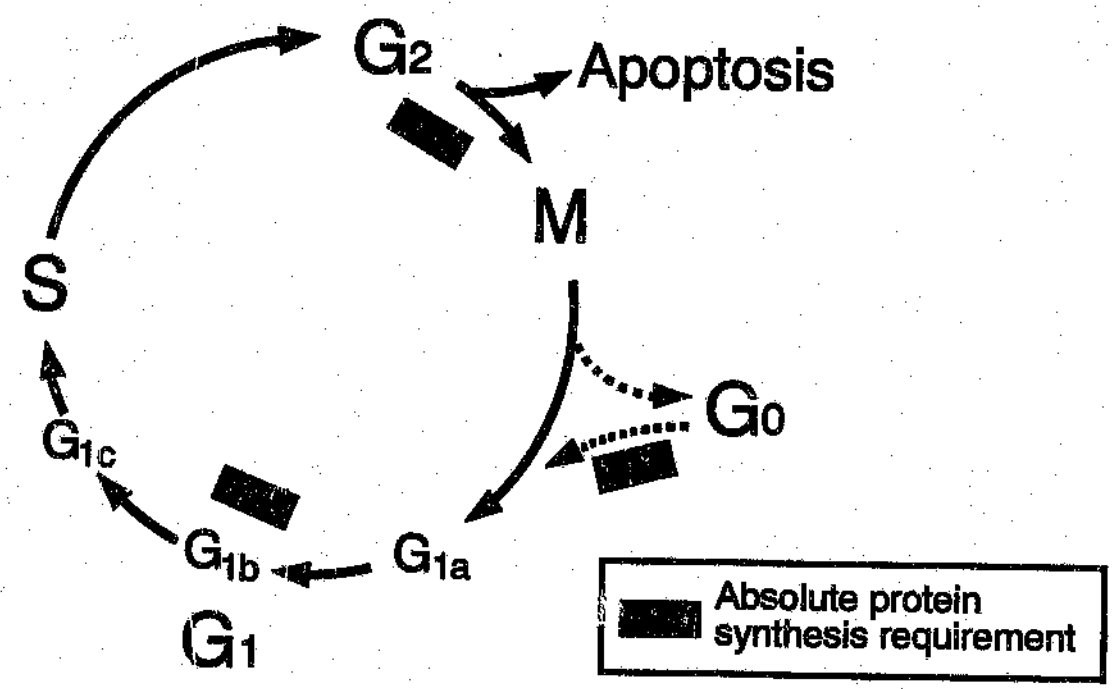

Figure 6
Cell cycle indicating the protein synthesis-dependent phases and checkpoints. Note that the G2-Phase to Apoptosis transition is protein synthesis - independent, whereas the G2-Phase to Mitosis transition has an absolute protein synthesis requirement. Similarly the G0-Phase to G1-Phase transition and the G1b-subPhase of the cell cycle are protein synthesis-dependent.

metaphase-to-anaphase transition, applicable to unsynchronized cells which had already entered metaphase by the time protein synthesis was disrupted. In the previous and in the present study, the proportion of cells in G0/G1-Phase was reduced, concomitant with the appearance of apoptosis. Clearly, in view of the data presented here, it would be inappropriate to attribute the reduction in G0-Phase cells to apoptosis occurring in the G0-Phase, as has been interpreted by others on the basis of similar data [12]. Our data and conclusions are, however, consistent with the findings of Colombel et al, 1992 [26]. In the present study we have not addressed the question of the existence of checkpoints at the G1/S transition, apoptosis mediated or not, as has been described previously [24].

As far as the mechanisms of the PM-induced G2-Phase arrest is concerned, it may have been consequent on protein synthesis inhibition per se, leading to inadequate constitution of the catalytic ($p34^{cdc2}$kinase) and/or the regulatory (cyclin B) subunits of Mitosis Promoting Factor (MPF), the activation of which is responsible for mediating the G2/M transition [27,28]. Secondly PM may in some way have disrupted the tyrosine phosphorylation status of the regulatory amino acid/protein involved in activating MPF, including the Tyr-15 residue of $p34^{cdc2}$kinase and the tyrosine phosphatase cdc25 [29]. In this regard, we have previously shown PM to inhibit tyrosine kinase activity globally in HL-60 cells [30].

In conclusion, our data show that the process of apoptosis itself occured

convincingly despite there being no appreciable evidence of new protein synthesis. By default then it would appear that any protein changes that occured were postranslational in nature. In this regard it is tempting to suggest that postranslational modificaton such as phosphorylation status, were instrumental. Candidate proteins could include hypothetical or actual endonucleases [31], and perhaps histone proteins themselves, since these are known to undergo changes class in both tyrosine and non-tyrosine phosphorylation status at the time of the G2/M transition [32]. Such postranslational modifications could result either in altered access of endonucleases, to the DNA or in mechanical stresses at the nucleosomal level, both of which could result in DNA fragmentation patterns characteristic of apoptosis.

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CHAPTER 3

Puromycin is a potent and specific inhibitor of tyrosine kinase activity in HL-60 cells.

ABSTRACT

The search for specific tyrosine kinase inhibitors constitutes a novel approach to the development of anticancer agents. Puromycin (PM) inhibits protein synthesis by causing the premature release of truncated PM-peptide complexes (PMPs), the structure of which predicts an inhibitory effect on tyrosine kinase activity (21). The present study was undertaken to investigate the effects of a low dose of PM (0.9 μM) on tyrosine kinase activity in HL-60 leukaemic cells. Experiments were specifically controlled to exclude inhibitory effects consequent on protein synthesis blockade. Soluble enzyme extracts derived from PM-treated cells showed attentuated phosphorylation of two independent synthetic tyrosine kinase-specific substrates, the polymer Poly(Glu:Tyr;4:1) and the peptide substrate Raytide. In contrast, Protein Kinase C-specific activity was unaffected by PM-exposure. It is possible that inhibition was due to metabolite/s of PM, perhaps PMPs, rather than to PM itself. Whether the inhibition of tyrosine kinase activity observed with the substrates used in this study is equally applicable to all tyrosine kinases, is not yet clear. Nevertheless, low-dose PM could provide a useful tool for dissecting out the role of tyrosine phosphorylation in the regulation and dysregulation of cell growth, and could conceivably prove beneficial in the treatment of tumours in which tyrosine kinase overactivity is linked to oncogenesis.

INTRODUCTION

Phosphorylation of protein tyrosine plays a pivotal role in the regulation of normal cell growth and proliferation, and in neoplastic transformation. In the cell cycle, the G2- to M-Phase transition is controlled by phosphorylation/ dephosphorylation reactions involving the Tyrosine-15 residue of the $\gamma 4^{cdc2}$ kinase (1). In addition a number of transmembrane growth factor receptors (including those of Epidermal Growth Factor, Platelet-derived Growth Factor, and Insulin-like Growth Factor), as well as a variety of cytosolic non-receptor-associated oncogene products (such as pp60src, p59fyn, p62yes, etc), exhibit tyrosine kinase activity (2,3,4,5,6).

The search for specific tyrosine kinase inhibitors represents a novel approach to the development of anticancer agents (7,8,9), in that it is based on the modification of transmembrane and intracytoplasmic tyrosine kinase signal transduction, rather than on the killing of tumour cells by inhibition of transcription, translation, and DNA synthesis (10,11). Quercetin (12,13), genistein (9), erbstatin (14), amiloride (15), and herbimycin A (16), have all proved effective against tyrosine kinases. Problems of toxicity, specificity, and drug delivery to tumours have limited their clinical applications however. For example, although genistein is a much more specific inhibitor of tyrosine kinases than is quercetin, its toxicity has precluded its in vivo use in patients to date. More recently, derivatives of tyrphostin other than erbstatin have been found to inhibit EGF-stimulated growth of squamous carcinoma cells, in vitro,

and in nude mice inoculated with such cells (17).

The antibiotic Puromycin (PM) is well known for its inhibitory effect on protein synthesis (18). Structurally it is a composite of the aminonucleoside dimethyladenosine, and tyrosine-o-methylether (19). Functionally it is an analogue of peptidyl-transferRNA, more specifically of tyrosyl-transferRNA. At concentrations of 10-100μM PM blocks protein synthesis completely by causing the premature release of truncated peptide chains which are bound to PM through their carboxyl termini. Since the nascent PM-peptide complexes (PMPs) are rapidly degraded through a ubiquitin-dependent pathway (20), their metabolic effects are poorly understood. The PMPs are of interest however because their structure predicts an inhibitory effect on tyrosine kinase activity (21). In this regard it is noteworthy that the carboxyterminal tyrosine residues of PMPs are reminiscent of synthetic random polymers of tyrosine-containing aminoacids, some of which have been found to act as competitive inhibitors of endogenous tyrosine kinase (22).

The present study was undertaken to investigate the effects of PM and its metabolites on cellular tyrosine kinase activity. The experiments were specifically controlled to exclude inhibitory effects on tyrosine kinase activity consequent to protein synthesis blockade.

METHODS AND MATERIALS

Cell Culture

HL-60 human promyelocytic leukaemic cells (Passage 17, ATCC, Rockville, MD, USA), were maintained in logarithmic growth phase at 37°C in a humidified atmosphere of air and 5% CO_2. Cells were seeded at $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, as well as 2mM L-glutamine, 50U/ml Penicillin, and 50ug/ml Streptomycin (all from GIBCO, Madison, WI, USA). Puromycin (PM) and Cycloheximide (CHX) were obtained from Sigma, and were used as described. Culture viability was assessed using the trypan blue dye exclusion technique. The viability at 24 hours of PM- and CHX-treated cultures was essentially unaffected by concentrations up to 0.5ug/ml (Figure 1a). At higher concentrations $\geq$1ug/ml, the viability of both PM- and CHX-treated cultures was compromised, with the viability of PM-treated cultures being affected to a greater extent.

Protein synthesis inhibition

0.5μCi [^{14}C]Leucine (specific activity 50 Ci/mmol; Amersham) was added to cultures which were then further incubated at 37°C for one hour. At the conclusion of incubation [^{14}C]Leucine incorporation was determined in triplicate as described (23). The dose-response curves expressing the inhibitory activity of PM on protein synthesis, as measured by [^{14}C]Leucine incorporation, are presented in Figure 1b. At 1 hour and at 24 hours, at a

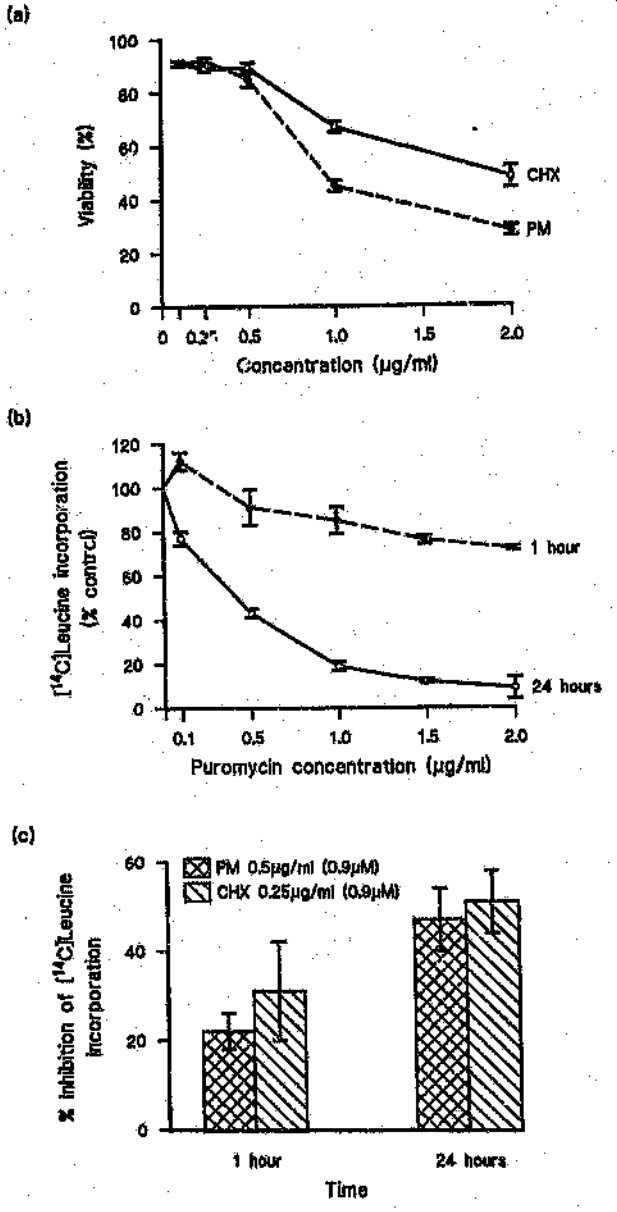

Figure 1

a) Viability of cultures (as determined by the trypan blue dye exclusion technique) after 24 hours of exposure to the indicated concentrations of PM and CHX. Results represent the mean ± SD of three separate experiments each done in duplicate.

b) The effect of increasing doses of PM (µg/ml) on [^{14}C]Leucine incorporation in HL-60 cells after one hour and 24 hours of incubation. Results were expressed as the percentage of control values and represent the mean ± SD of three separate experiments each done in triplicate.

c) Effect of equivalent molar concentrations (0.9µM) of PM (0.5µg/ml) and CHX (0.25µg/ml) on inhibition of [^{14}C]Leucine incorporation. Results represent the mean ± SD of three separate experiments each done in triplicate.found to inhibit [^{14}C]Leucine incorporation to a similar extent as did PM used at a concentration of 0.9µM (0.5µg/ml) i.e. approximately 20-30% at 1 hour and approximately 50% at 24 hours (Figure 1c).

concentration of 0.9μM (0.25μg/ml), CHX was found to inhibit [^{14C}]Leucine incorporation to a similar extent as did PM used at a concentration of 0.9uM (0.5ug/ml) i.e. approximately 20-30% at 1 hour and approximately 50% at 24 hours (Figure 1c).

Preparation of solubilized enzyme extracts

Cellular extracts representing soluble tyrosine kinases were prepared as described (24). Briefly, at the conclusion of incubation, cells from 10ml of suspension culture were collected by low-speed centrifugation (500g) and washed three times in PBS. Culture viability (as assessed by the trypan blue dye exclusion technique) was consistently $\geq$85%. The final cell pellet was resuspended in four volumes of extraction buffer (50mM HEPES/NaOH, pH=7.5; 2mM EGTA; 10mM DTT; 1mM PMSF; 5μg/ml aprotinin; 0.1%(w/v) Triton X-100). The cell suspension so formed was sonicated for 10 seconds at 4°C, and then centrifuged at 15 000g for 30 minutes at 4°C. The supernatant was collected and 10%(v/v) of glycerol was added. Protein content of an aliquot of the supernatant was determined (25) using bovine serum albumin as control.

Determination of tyrosine kinase activity using Poly(Glu:Tyr;4:1) as substrate

Tyrosine kinase activity was assessed using a substrate assay described previously (26), involving the incorporation of [^{32}P] from [γ-^{32}P]ATP into the exogenous synthetic polymer substrate Poly(Glu:Tyr;4:1). The final reaction volume of 50μl contained 50mM Tris-HCl (pH=7.5), 30mM $MgCl_2$, 100μM

Sodium-ortho-vanadate, 150μM ATP, 30μCi of [γ-^{32}P]ATP (6000 Ci/mmol; 10mCi/ml), 2mg/ml of exogenous tyrosine kinase-specific substrate Poly(Glu:Tyr;4:1), and suitably diluted enzyme preparation (10μg protein). The reaction was initiated by the addition of ATP at 30°C. Termination of the reaction after 15 minutes was by applying 25μl of the reaction mixture to Whatman 3MM paper squares (3cm x 3cm). These were washed four times for 15 minutes each in a 10% TCA solution containing 10 mM sodium pyrophosphate. Two final washes for a further 15 minutes each were first in ethanol and then in acetone. The papers were dried and then [^{32}P] incorporation of reaction triplicates was determined by liquid scintillation spectrometry. Appropriate reaction mixtures with or without substrate, and with or without extract served as controls. The activity of purified pp60$^{c\text{-}src}$ tyrosine kinase was also measured using Poly(Glu:Tyr;4:1) as substrate.

Tyrosine Kinase activity using Raytide as substrate.

Tyrosine kinase activity was determined with Raytide as substrate (27) in a final reaction volume of 50μl containing 50mM HEPES (pH=7.5), 0.1mM EDTA, 0.015% Brij 35, 150μM ATP, 30mM $MgCl_2$, 30μCi of [γ-^{32}P]ATP (6000 Ci/mmol; 10 mCi/ml), 0.2 mg/ml of the exogenous tyrosine kinase-specific substrate Raytide, and 10 μg suitably diluted enzyme preparation. The reaction was terminated by adding 120μl of 10% phosphoric acid. The sample was vortexed, and 120μl applied onto 3cm x 3cm Whatman P81 ion exchange chromatography paper. The papers were washed extensively in 0.5% phosphoric acid before a further wash with acetone. The papers were dried

and [^{32}P] incorporation into Raytide of reaction triplicates was determined by liquid scintillation counting. Appropriate controls were used (as described above).

Analysis of Tyrosine Kinase Activity

Tyrosine kinase activity was determined in solubilized enzyme extracts derived from 1 hour and 24 hour control, PM-treated (0.9μM), and CHX-treated (0.9μM) HL-60 cells. Appropriate reaction mixtures with or without substrate, and with or without extract served as controls. Extract incorporation of [^{32}P] in the absence of substrate represented endogenous phosphorylation. Net specific tyrosine phosphorylation was represented by the difference between extract incorporation of [^{32}P] in the presence and in the absence of substrate, and corrected for substrate background. Net tyrosine kinase-specific activity of extracts of PM- and CHX-treated cells were expressed as a percentage of the corresponding tyrosine kinase-specific activity of extracts of untreated cells.

Endogenous phosphorylation of extracts of PM- and CHX-treated cells were expressed as a percentage of the endogenous phosphorylation present in the extracts derived from untreated cells. In order to try and ascertain whether PM itself, or whether its metabolites were affecting tyrosine kinase activity, a series of experiments were done in which PM (0.9μM) and CHX (0.9μM) were added directly to reaction mixtures containing either extracts of untreated cells, or to purified pp60$^{c\text{-}src}$.

Protein Kinase C activity

Protein Kinase C activity was determined using a commercially available Protein Kinase C enzyme assay system from Amersham. The incorporation of [^{32}P] from [γ-^{32}P]ATP into a Protein Kinase C specific peptide substrate was determined. The final reaction volume of 75μl contained the recommended kit components as well as 20μg of suitably diluted enzyme preparation and 30μCi of [γ-^{32}P]ATP (6000 Ci/mmol; 10mCi/ml). Reaction samples were equilibrated at 25°C for 5 minutes. Each reaction was initiated by the addition of ATP at 25°C. After 15 minutes the reaction was terminated by adding 100μl of dilute acid. One hundred and twenty-five microlitres of the reaction mixture was applied onto 3cm x 3cm Whatman P81 ion exchange chromatography paper. The papers were washed twice, for 10 minutes each time, in 75mM ortho Phosphoric acid. After washing, the papers were dried before [^{32}P] incorporation of reaction triplicates was determined by liquid scintillation spectrometry. Relevant controls were used as described previously. Analysis of Protein Kinase C activity followed the same method as that used for analysis of tyrosine kinase activity.

Flow cytometric analysis of phosphotyrosine content

Simultaneous flow cytometric analysis of phosphotyrosine and DNA content was carried out by a modification of a method described by others (28). $2x10^6$ HL-60 cells were fixed in 2 ml of ice cold 70% ethanol for 30 minutes at 4°C. Cells were washed twice in PBS, and then resuspended in 380μl of PBS containing 1% bovine serum albumin. Twenty microlitres of fluorescein

FITC-conjugated anti-phosphotyrosine monoclonal antibody (Boehringer Mannheim; Batch 12244820-01) was added to the suspension which was then incubated for 45 minutes in the dark at room temperature. Following two further washes with PBS, the cells were resuspended in 1ml of PBS containing RNAse (10μg/ml) at 37°C for 10 minutes. The cells were washed again and then resuspended in 1ml of propidium iodide (10μg/ml) in PBS for 30 minutes. After laser excitation (488 nm) of the fluorescent complexes by the Coulter-EPICS-Profile II flow cytometer, red fluorescence from propidium iodide was recorded on a linear scale as a measure of total DNA content. Green fluorescence from FITC was logarithmically amplified to represent phosphotyrosine content. Twenty-five thousand cells were counted for each sample. According to the manufacturers, in this context, anti-phosphotyrosine fluorescence staining is proportional to the amount of tyrosine phosphorylated proteins as determined by phosphoaminoacid analysis, since the antibody shows no cross-reactivity with any other phosphorylated amino acids (Boehringer Mannheim). Since the FITC-anti-phosphotyrosine monoclonal antibody was of the IgG1 subtype, FITC-conjugated control IgG1 antibody was used to exclude the presence of non-specific binding.

RESULTS

Tyrosine kinase activity of extracts of PM-treated cells

Using Poly (Glu:Tyr; 4:1) as substrate we showed inhibition of tyrosine kinase activity in extracts of PM-treated cells (0.9μM) - approximately 15% inhibition after 1 hour, and approximately 25% inhibition after 24 hours (Figure 2a). In contrast no inhibition of tyrosine kinase activity was seen in extracts of CHX-treated cells after these time intervals. At the dose used (0.9μM), CHX inhibited [^{14}C]Leucine incorporation into HL-60 cells to a similar extent as did 0.9μM of PM, i.e. approximately 20-30% after 1 hour, and approximately 50% after 24 hours (Figure 1c). There was no inhibition of tyrosine kinase activity when the same concentration of PM was added directly to reaction mixtures containing 10μg control enzyme extracts (Figure 2a), suggesting that some metabolite/s of PM, rather than the drug PM itself, could have been responsible for the observed tyrosine kinase inhibition. To explore this phenomenon further, we used purified pp60$^{c\text{-}src}$ as a source of specific tyrosine activity, and poly(Glu:Tyr;4:1) as a tyrosine kinase-specific substrate (Figure 3). When PM drug (0.9μM) was added directly to this system, specific tyrosine kinase activity of pp60$^{c\text{-}src}$ was not inhibited.

Inhibition of tyrosine kinase activity in extracts of PM-treated cells was confirmed using another tyrosine kinase-specific substrate Raytide (Figure 4a). In fact inhibition was more dramatic (approximately 80%) with Raytide substrate than with Poly(Glu:Tyr;4:1) substrate.

Endogenous kinase activity of extracts of PM-treated cells

Endogenous kinase activity of extracts of PM-treated cells was indistinguishable from control extract activity (Figure 2b and 4b). This confirmed that the reduced ability of extracts of PM-treated cells to phosphorylate tyrosine kinase-specific substrates was due to specific inhibition of tyrosine kinase activity, rather than to the overall reduction in endogenous kinase activity.

Phosphotyrosine content of PM-treated HL-60 cells

After 24 hours PM-treated HL-60 cells had a reduced phosphotyrosine content as reflected by the downwards shift on the Y-axis of log green fluorescence (Figure 5). This was consistent with the inhibition of tyrosine kinase activity observed in extracts of PM-treated cells when Raytide and Poly(Glu:Tyr;4:1) were used as the tyrosine kinase-specific substrates (Figures 2a and 4a). Whether the reduced phosphotyrosine content was a result of decreased synthesis or increased degradation of phosphotyrosine-containing proteins, or due to reduced phosphorylation of tyrosine residues by tyrosine-specific kinases, remains to be determined.

Protein Kinase C activity of PM-treated extracts

There was no inhibition of Protein Kinase C activity in extracts of PM-treated (0.9μM) cells after 1 hour and after 24 hours of exposure (Figure 6). Protein Kinase C activity was somewhat inhibited (± 10%) in extracts of CHX-treated (0.9μM) cells at 24 hours but not at 1 hour.

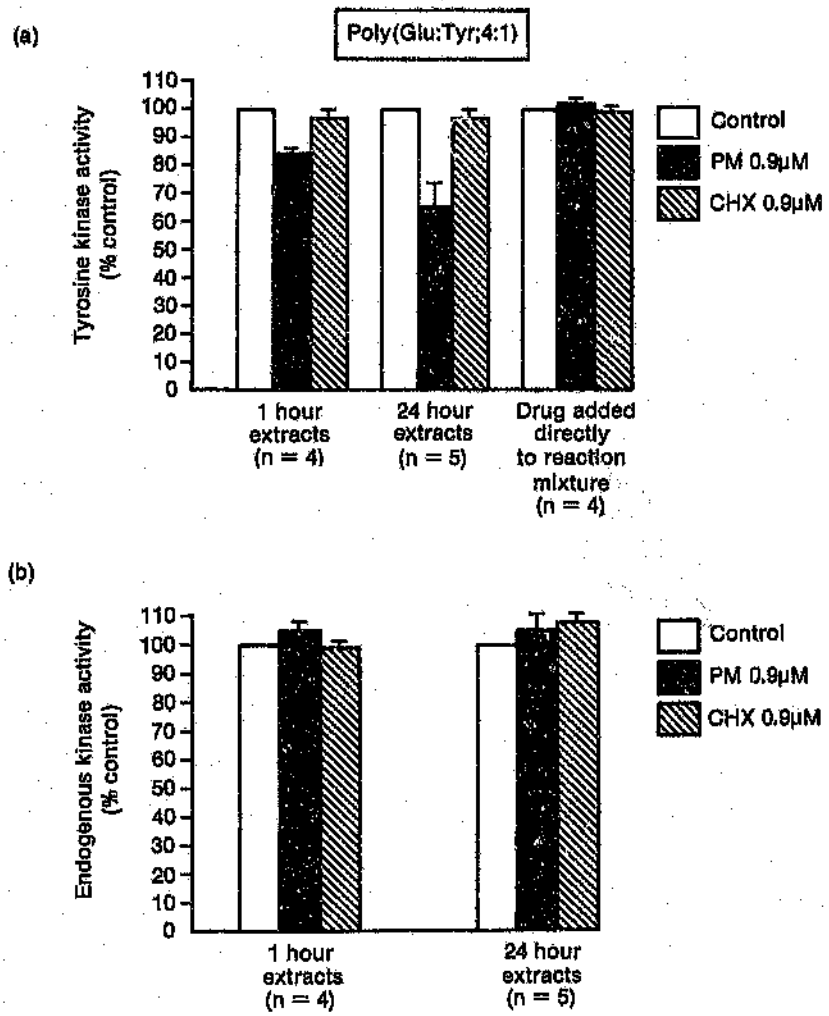

Figure 2

a) Soluble tyrosine kinase activity using Poly(Glu:Tyr;4:1) as substrate in cellular extracts derived from untreated HL-60 cells and from cells pre-treated for 1 and 24 hours with 0.9μM PM, or 0.9μM CHX. Shown also are the results of four experiments in which each of the drugs were added directly to reaction mixtures containing 10 μg of soluble enzyme protein extract derived from untreated cells.

b) Endogenous kinase activity in the cellular extracts described in (a). Results are expressed as (a) a percentage of tyrosine kinase activity in untreated extracts, or as (b) a percentage of endogenous kinase activity in untreated extracts, and are represented as mean ± SEM derived from separate experiments (n=4/5) each done in triplicate.

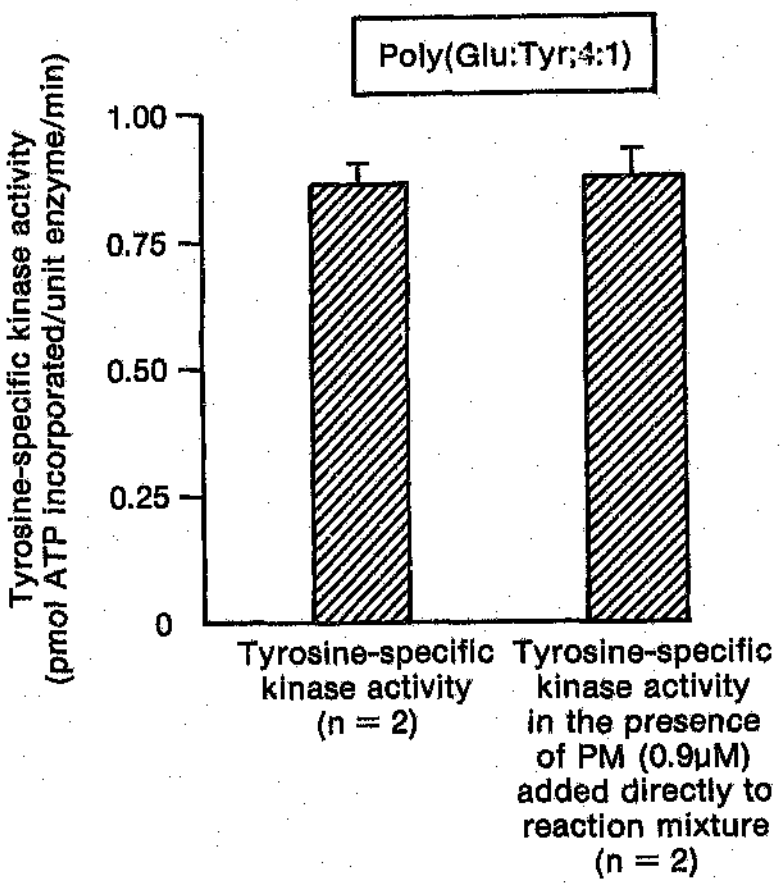

Figure 3
pp60$^{c\text{-}src}$ Tyrosine kinase activity determined with the tyrosine kinase-specific substrate Poly(Glu:Tyr;4:1) in the presence or absence of 0.9μM PM. Results are expressed as pmol ATP incorporated per unit of enzyme per 15 min assay, and are represented as mean ± SD derived from separate experiments (n=2) each done in triplicate.

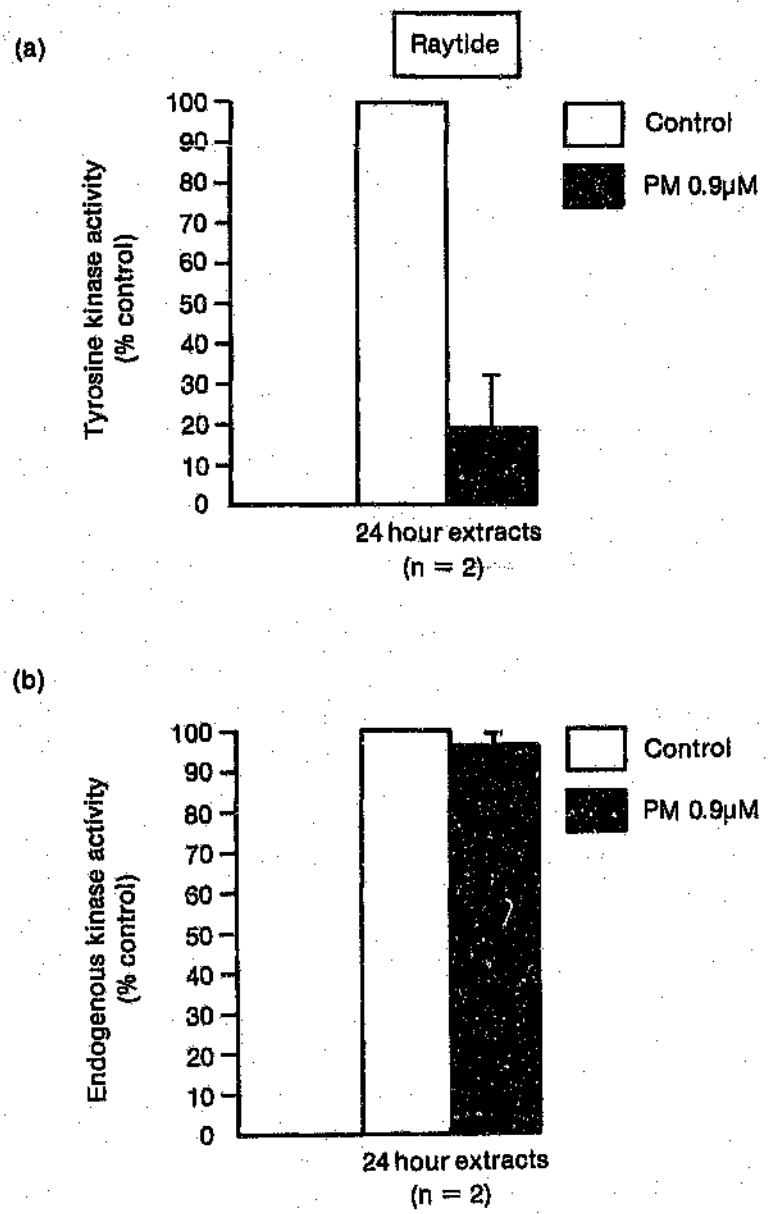

Figure 4

a) Soluble tyrosine kinase activity (using Raytide as substrate) in extracts from untreated and from PM-treated (0.9μM) HL-60 cells after 24 hours of exposure.

b) Endogenous kinase activity in the cellular extracts described in (a). Results are given as the mean ± SD derived from separate experiments (n=2) each done in triplicate.

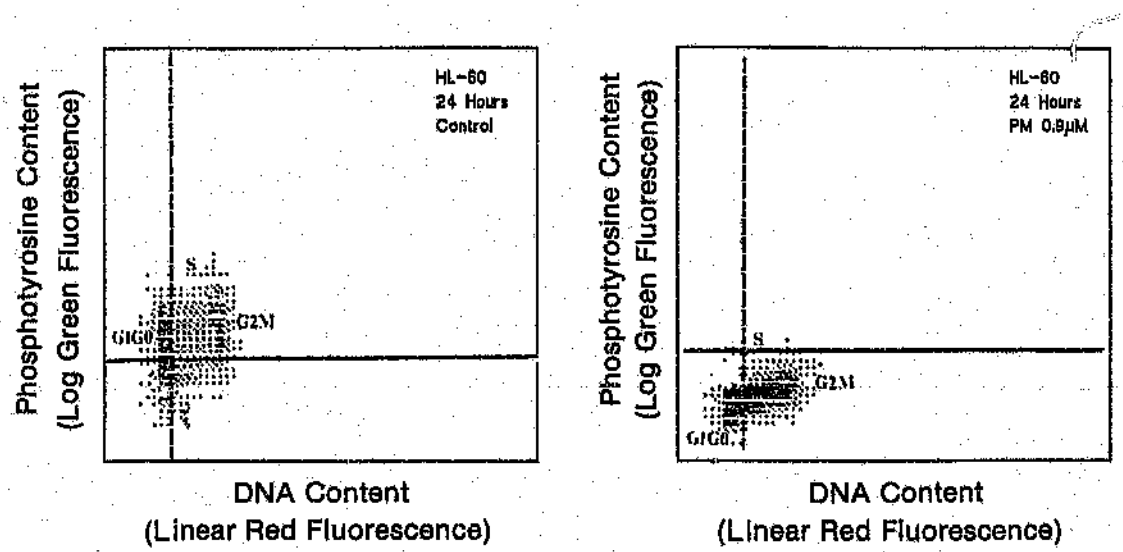

Figure 5
Dual parameter flow cytometric histograms of 24 hour control and PM-treated HL-60 cells, showing phosphotyrosine content (log-scale, Y-axis) and DNA content (linear-scale, X-axis).

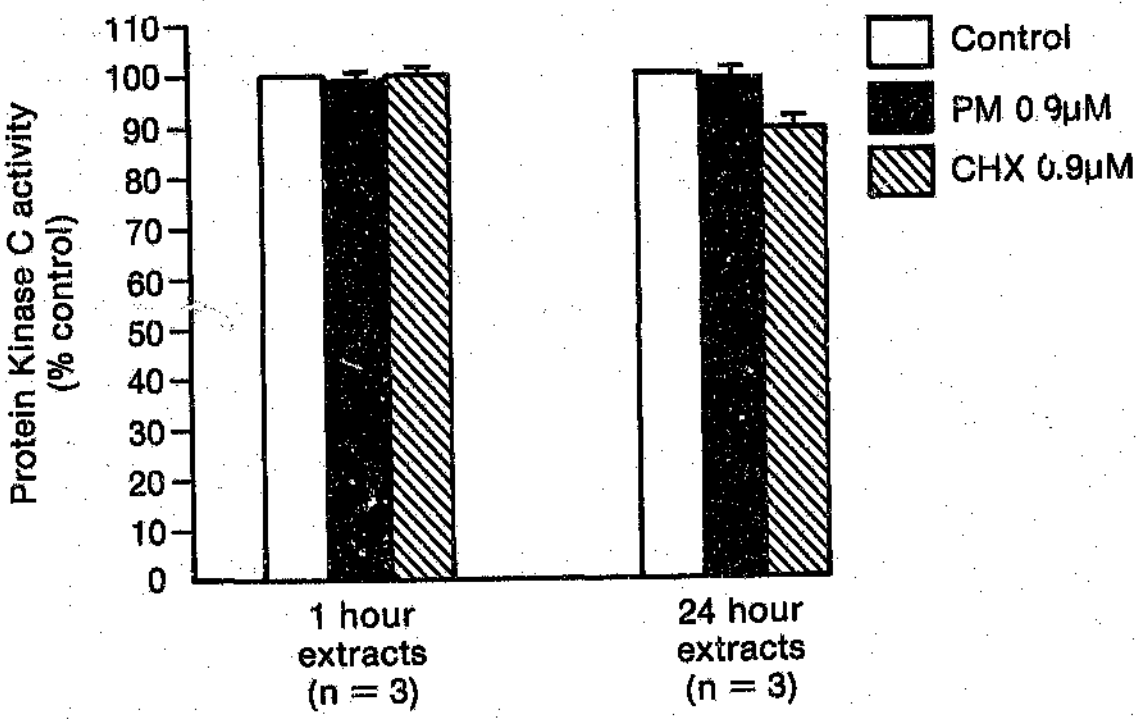

Figure 6
Protein Kinase C activity (using a specific Protein Kinase C peptide substrate) in cellular extracts derived from untreated HL-60 cells and from cells pre-treatd for 1 hour and 24 hours with 0.9μM PM, or 0.9μM CHX.

DISCUSSION

We have shown that low-dose PM-exposure resulted in potent and specific inhibition of tyrosine kinase activity as determined in soluble enzyme extracts prepared from 1 hour and 24 hour PM-treated HL-60 cells. That this inhibition was tyrosine kinase-specific was supported by several observations. Firstly, soluble enzyme protein derived from PM-treated cells showed attenuated phosphorylation of two independent synthetic tyrosine kinase-specific substrates, i.e. the polymer Poly(Glu:Tyr;4:1), and the peptide substrate Raytide. We do not know why the extent of inhibition observed with Raytide as the substrate was greater than that demonstrated with Poly(Glu:Tyr;4:1), although we suggest that it might relate to the different structures of these substrates (peptide as opposed to polymer). Secondly, PM-exposure did not result in the inhibition of endogenous phosphorylation - endogenous phosphorylation effectively represents serine/threonine kinase activity since the relative abundance of phospho-amino-tyrosine in both normal and transformed cells is less than 0.5% (29). Thirdly, and in corroboration of the previous observation, Protein Kinase C activity (representing substrate-specific serine/threonine kinase activity) was not affected by PM-exposure.

Soluble enzyme extracts derived from HL-60 cells treated with a dose of CHX (0.9μM) that was equimolar to the dose of PM employed (0.9μM), and that also inhibited [^{14}C]Leucine incorporation to a similar extent as PM did, failed to inhibit phosphorylation of the tyrosine kinase-specific substrate

Poly(Glu:Tyr;4:1). This indicates that the tyrosine kinase-specific inhibition induced by PM was not likely to have been the consequence of protein synthesis blockade per se, but may relate to the different mechanisms of action of these two "classical" protein synthesis inhibitors. Unlike PM (30), CHX does not generate drug-linked nascent polypeptides, instead it prevents peptide chain elongation by inhibiting the peptidyl transferase activity of the 60S ribosomal subunit (31).

It is possible that inhibition of tyrosine kinase-specific activity could have been due to some metabolite/s of PM, rather than to PM itself, since PM drug (0.9μM) added directly to reaction mixtures containing either extracts of untreated HL-60 cells, or to reaction mixtures containing purified $pp60^{c\text{-}src}$ tyrosine kinase enzyme, failed to inhibit the capacity of either of these to phosphorylate the tyrosine kinase specific substrate Poly(Glu:Tyr;4:1). In this regard the PMPs are of interest because their structure predicts an inhibitory effect on tyrosine kinase activity (21). In fact the concept of a "pseudosubstrate" sequence interacting with protein kinase catalytic domains and thereby turning off the protein kinase is not a new one (32,33), and indeed certain synthetic tyrosine polymers with ordered sequences, e.g. (Tyr-Glu-Ala-Gly)n and (Ala-Tyr-Gly)n have been found to serve as potent inhibitors of tyrosine-specific protein kinases (22). It is therefore possible that PMPs with their carboxyterminal tyrosine residues reminiscent of synthetic random polymers of tyrosine-containing aminoacids might have exerted an inhibitory effect by occupying the protein substrate site of the catalytic domain of

tyrosine-specific protein kinases.

Since PM is a structural analogue of ATP, it may have been competitive with the ATP subsite of the enzyme, as has been reported with amiloride, an ATP analogue which is a potent inhibitor of protein tyrosine kinase activity (15). As an alternative to this scenario, it is feasible that PM could have interfered with emerging tyrosine kinase molecules at their nascent stage.

We found that in HL-60 leukemic cells, low-dose PM-exposure specifically inhibited tyrosine kinase activity, an effect which may be related to some metabolite/s of PM (perhaps PMPs), rather than to PM itself. These phenomena warrant further investigation, since as a specific inhibitor of tyrosine kinase activity, PM could provide a useful tool for dissecting out the physiological role of tyrosine phosphorylation. Finally PM may in the future have some benefit in the treatment of tumours in which tyrosine kinase overactivity is linked to oncogenesis - its effect is specific (at least in HL-60 cells), the drug is easily taken up in vitro and in vivo (34, own unpublished results), and toxicity is unlikely to limit its utility if it is used in vivo at concentrations which in vitro elicit these biochemical sequelae (34). In a poorly controlled study in 1955, intermittent dose PM used as a single antineoplastic agent proved to be ineffective in the treatment of a variety of cancers (35). However the drug scheduling in that study would not have resulted in cellular exposure to a constant level of PM sufficient to accumulate PM metabolites to any degree. Clinical trials using appropriate PM scheduling as a component of a multidrug chemotherapeutic regimen are

currently under investigation in our centre in dogs presenting with spontaneous lymphoproliferative disorders.

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CHAPTER 4

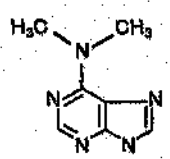
Substituted purines elicit differential cytokinetic, molecular, and phenotypic effects in HL-60 cells.

ABSTRACT

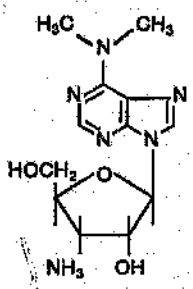
This study investigated and compared the cytokinetic, phenotypic, and molecular effects elicited in HL-60 human leukemic cells by low doses (0.6μM) of three closely related, substituted purines (Puromycin (PM), Puromycin aminonucleoside (PAN), and 6-Dimethylaminopurine (6-DMAP)). PM, but not 6-DMAP or PAN, inhibited [^{14}C]Leucine incorporation, induced a time-related cytotoxic effect, a G2-arrest, a metaphase-mitotic-arrest, apoptosis, and c-myc mRNA superinduction. PAN and 6-DMAP exerted no detectable morphological or cytokinetic effects, although exposure to these drugs resulted in down-regulation of c-myc mRNA levels. We suggest that the specific effects exerted by PM relate to the generation of nascent peptidyl-PM complexes by PM, but not by 6-DMAP or PAN.

INTRODUCTION

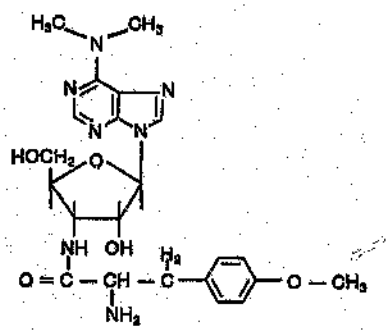
The substituted purines, of which Puromycin (PM), Puromcyin aminonucleoside (PAN), and 6-Dimethylaminopurine (6-DMAP) are prototypes (Figure 1), have been found to exert a variety of biochemical and cytological effects, when used at high concentrations ranging from 10-300μM. For example, 6-DMAP, the purine component of both PM and PAN, reversibly arrests cells in the G2-Phase of the cell cycle by inhibiting phosphorylation [1]. The nucleoside analog PAN, which is a composite of 6-DMAP and 3-Aminoribose, has been found to inhibit HIV-1 replication in vitro [2], and PM, itself a composite of PAN and tyrosine-o-methylether, is a functional analog of tyrosyl-transfer RNA and is well know for its inhibitory effect on protein synthesis [3]. Since low-dose PM was unexpectedly found to cause a metaphase-mitotic-arrest (submitted data), and because of its close structural relationship to PAN and 6-DMAP, we undertook to investigate and compare the cytokinetic, phenotypic, and molecular effects which these drugs elicited in HL-60 leukemic cells when employed specifically at low concentrations (0.6μM).



6-Dimethylaminopurine



Puromycin aminonucleoside



Puromycin

Figure 1
Structures of 6-DMAP, PAN and PM.

MATERIALS AND METHODS

Cell culture

HL-60 human promyelocytic cells (passage 17, ATCC, USA) were maintained in RPMI 1640 medium supplemented with fetal calf serum, L-glutamine and Penicillin/Streptomycin antibiotics (GIBCO, USA). Cell cycle time was approximately 22 hours. PM, PAN, and 6-DMAP (Sigma) were used at a final concentration of 0.6μM. Culture viability was assessed using the trypan blue dye exclusion technique. Cells were fixed and stained with May-Grünewald-Giemsa, and then examined for mitotic figures and apoptotic cells.

Protein synthesis inhibition

0.5μCi [^{14}C]Leucine (specific activity 50Ci/mmol; Amersham) was added to cultures which were then further incubated at 37°C for one hour [4]. At the conclusion of the incubation period, cells were washed three times in phosphate-buffered saline, 1ml of 10% trichloroacetic acid was added, and the tubes were then chilled on ice for 30 minutes. The precipitate so formed was filtered on GF/C filter papers (Whatmann), and washed three times with 5% trichloroacetic acid. The filters were dried and counted after adding aqueous counting scintillant. [^{14}C]Leucine incorporation was determined in triplicate.

DNA fragmentation analysis

At the conclusion of the incubation period the cells were pelleted at 4°C and

prepared for subsequent fragmentation analysis [5]. The pellet was resuspended in 70ul of lysis buffer (10mM EDTA; 50mM Tris-HCl, pH=8.0; 0.5% sodium lauryl sarkosinate; 0.5mg/ml proteinase K) and incubated for 1 hour at 50°C. A 10ul aliquot of 1mg/ml ribonuclease A was added to each sample prior to a further 1 hour incubation at 50°C. Samples were then heated to 70°C after which the reaction was terminated by the addition of 20ul of loading buffer (10mM EDTA, pH=8.0; 0.25% bromophenol blue; 40% sucrose). Extracts were loaded into the wells of a 2% agarose gel and electrophoresed in a phosphate buffer (2mM EDTA; 800mM Tris-phosphate, pH 7.8). Both the gel and the buffer contained 1ug/ml of ethidium bromide. BglI/HinfI cleaved pBR328 served as molecular weight markers.

Flow cytometric analysis

Cells were prepared for flow cytometric analysis as described [6]. The centrifuged cell pellet was resuspended in a hypotonic nuclear isolation medium (50mg/l Propidium Iodide; 1g/l Triton-X 100; 1g/l Sodium Citrate), incubated on ice for 30 minutes ,and then at 37°C in 100μg/ml RNAse A. Fluorescence emitted from the Propidium Iodide-DNA complex was quantitated after laser excitation of the fluorescent dye by the a Coulter EPICS-Profile-II flow cytometer. Cell debris and doublets were excluded [7]. The coefficient of variations of the peaks of the control G0G1- and the G2M-Phases were less than 3%. Limits of cell cycle phases as determined in the control distributions were retained in the subsequent analysis of the drug-treated populations.

c-myc mRNA analysis

c-myc mRNA expression was investigated using standard techniques [8]. 10μg of total extracted RNA was size-fractionated by 1.2% agarose formaldehyde gel electrophoresis, and then transferred and ultraviolet cross-linked to nylon membrane. The membrane was prehybridized at 42°C for 4 hours. Sequential hybridization at 42°C was to the [α-^{32}P]dCTP (Amersham) random-primer labelled 1.6kB SstI fragment from the 5' end of the c-myc gene (ATCC,USA), and to the [γ-^{32}P]ATP (Amersham) end-labelled 40mer oligonucleotide antisense GAPDH probe (Oncogene Science, USA). GAPDH served as an internal control for constitutive gene expression. Autoradiograms were scanned densitometrically.

RESULTS

Effects on protein synthesis

At 1 hour of incubation 0.6μM PM had inhibited [^{14}C]Leucine incorporation by ± 20% (Figure 2). In contrast 6-DMAP (0.6μM) and PAN (0.6μM) exerted no detectable effect on [^{14}C]Leucine incorporation.

Phenotypic, apoptotic, and cytotoxic effects

After 12 hours (Figure 3), HL-60 cells treated with equimolar concentrations of 6-DMAP and PAN were morphologically indistinguishable from control cells. In contrast PM-treated cells showed marked metaphase mitotic accumulation and some evidence of apoptosis (chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies). Apoptosis in PM-treated cells was confirmed ultrastructurally (not shown), and by DNA electrophoresis at 24 hours which showed the pattern of fragmentation that results from endogenous endonuclease activation (Figure 4). PM, but not PAN or 6-DMAP, exerted a time-related toxic effect on HL-60 cells as demonstrated by the reduction in viable cell number in PM-treated cells (Figure 5).

Cytokinetic effects

PM induced a G2-arrest as well as a marked M-Phase increase, with an associated reduction in the S-Phase of the cell cycle (Figure 6a, 6b, 6c). These effects were time-related in that they were present at 12 hours, and

were more marked at 24 hours. The increase in the M-Phase reflected a metaphase-arrest (Figures 3 and 6c). PAN and 6-DMAP exerted no obvious cytokinetic effects at 12 and at 24 hours (Figure 6).

Effect on c-myc mRNA levels

After 12 hours of exposure, PM was found to superinduce c-myc mRNA levels, whereas PAN- and 6-DMAP-exposure resulted in a decrease in the level of c-myc transcripts (Figure 7a). It is of interest that PM-exposure also appeared to superinduce GAPDH to ± 122% of control levels (Figure 7b), although it superinduced c-myc to a much greater extent (± 200% of control levels).

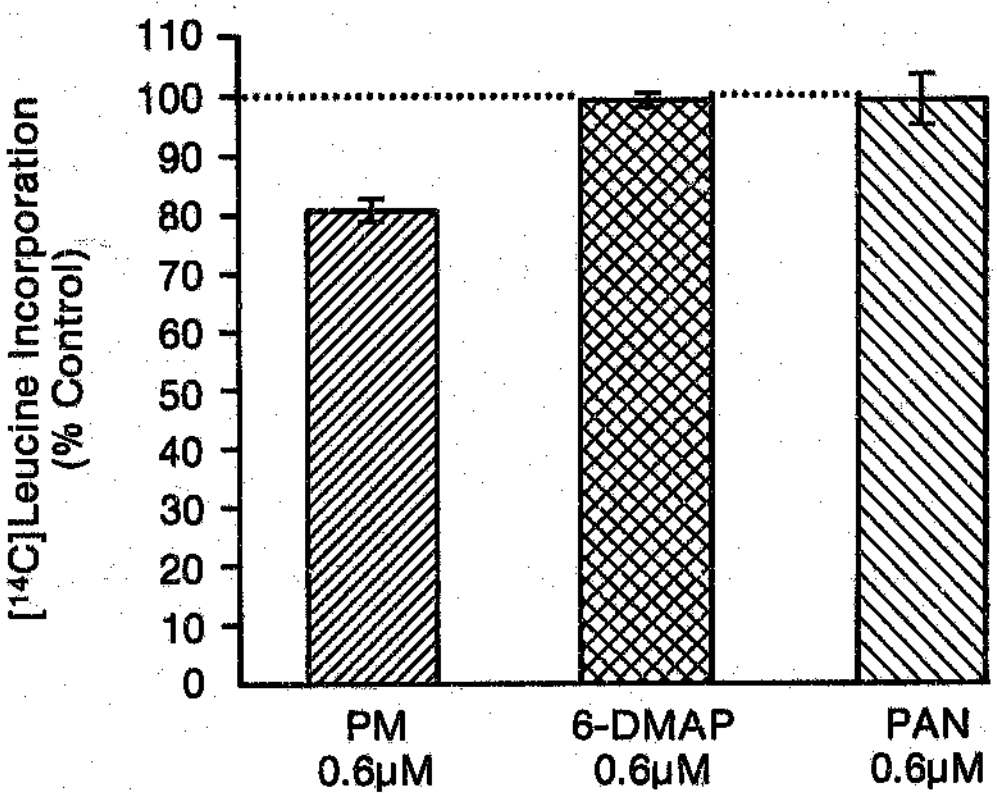


Figure 2
The effect of PM, 6-DMAP, and PAN, on [^{14}C]Leucine incorporation in HL-60 cells at one hour of incubation. Results were expressed as the percentage of control values, and represent the mean ± SD of three separate experiments each done in triplicate.

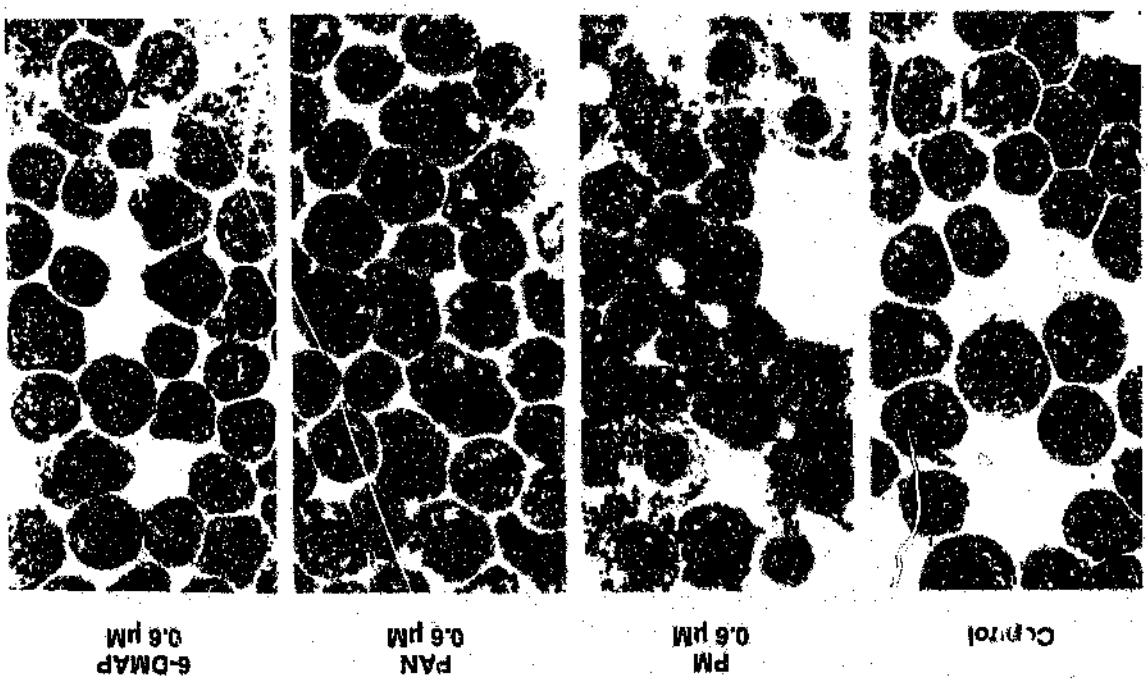


Figure 3
Giemsa-stained cytospin preparations (magnification x 1000) of 12 hour control and drug-treated HL-60 cells.

a) Control HL-60 cells.
b) PM-treated HL-60 cells showed a marked metaphase mitotic accumulation "M" by 12 hours. Certain PM-treated cells showed evidence of apoptosis "A".
c) 6-DMAP-treated HL-60 cells.
d) PAN-treated HL-60 cells.

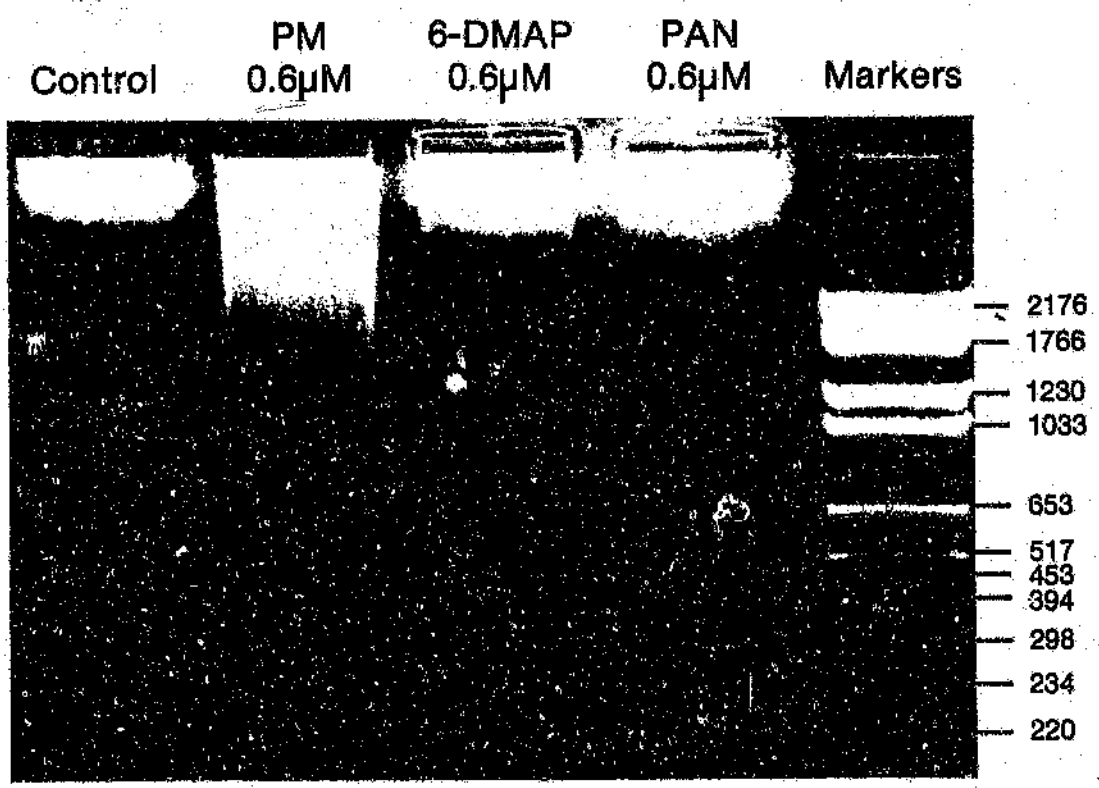


Figure 4
Agarose gel electrophoresis of total DNA extracted at 24 hours from $1x10^6$ HL-60 cells treated as indicated. The size (in bases) of separated DNA molecular weight markers are shown.

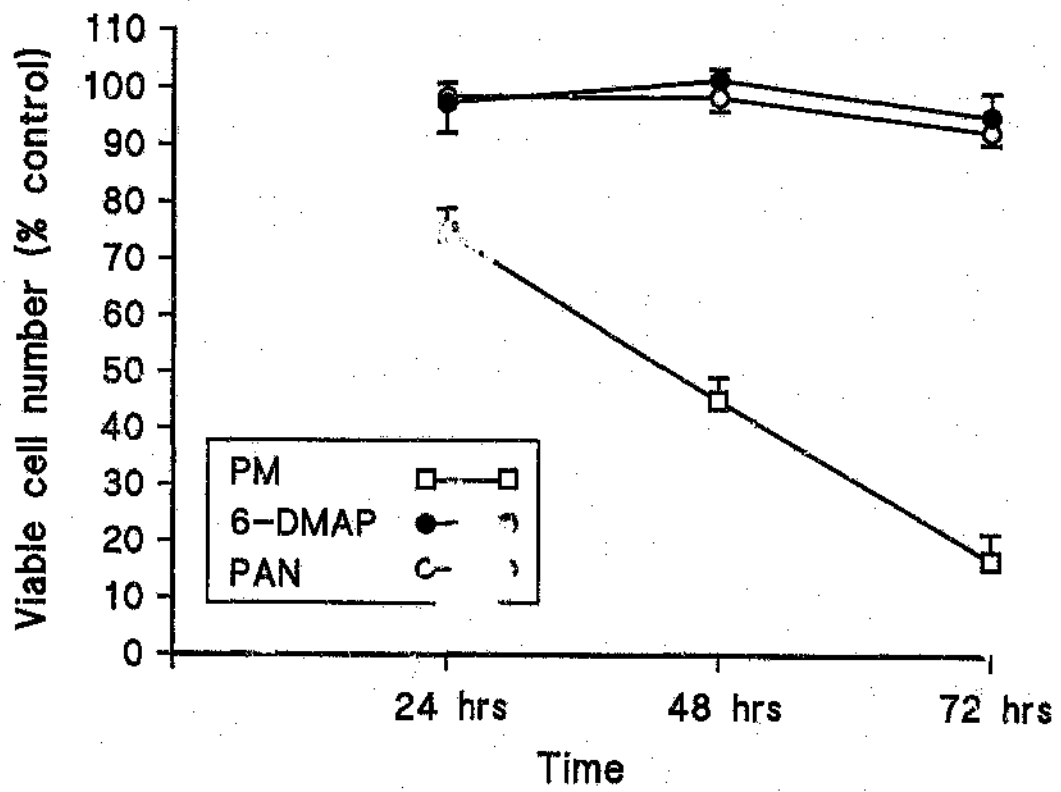


Figure 5
Viable cell number in PM-, 6-DMAP-, and PAN-treated HL-60 cells after 24, 48, and 72 hours. Results are expressed as a percentage of control values and represent the mean ± SD of three separate experiments each done in duplicate. Viable cell number was not affected in relation to control by drug-treatment at 12 hours.

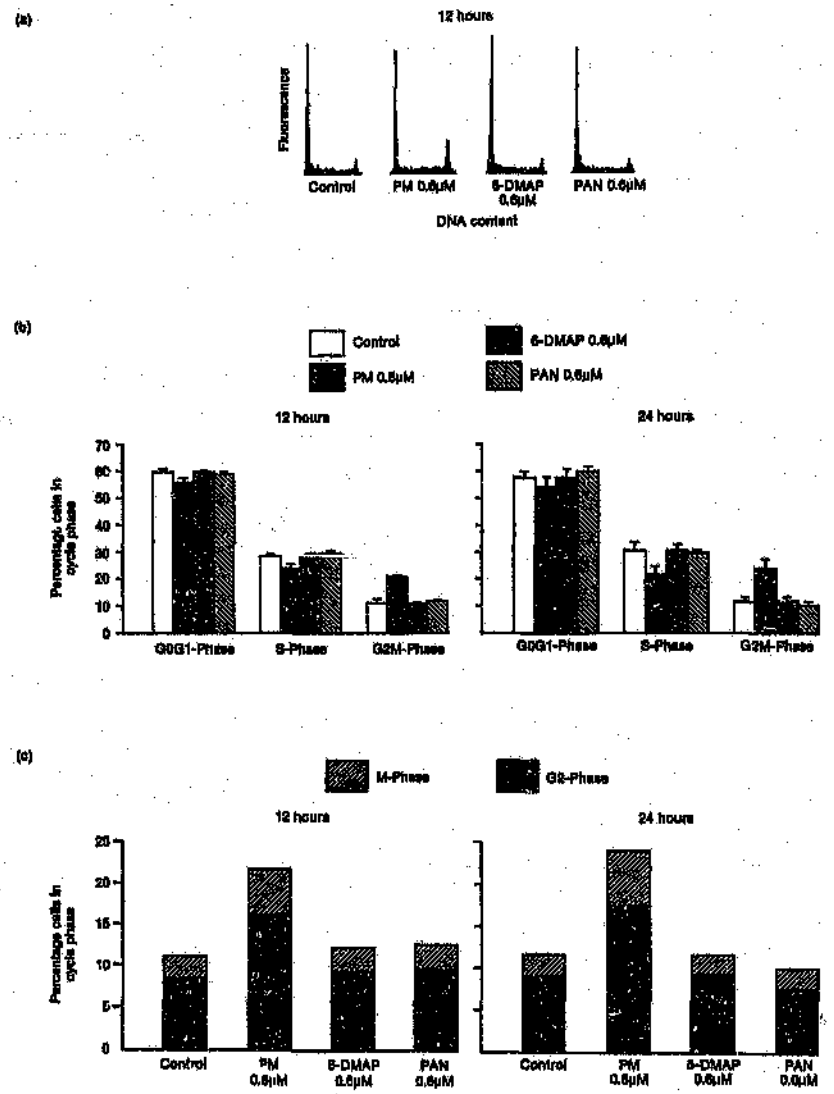


Figure 6

a) Representative flow cytometric analysis at 12 hours of count versus Fluorescence/DNA content of control, PM-treated, 6-DMAP-treated, and PAN-treated HL-60 cells. Twenty-five thousand cells were counted for each analysis. Histogram scaling was the same for all samples.

b) Effects on the DNA histogram of HL-60 cells following 12 hours of exposure to PM-6-DMAP, and PAN. Results are expressed as mean ± SD (n=3).

c) Stacked-bar graphs illustrating the relative percentages of HL-60 cells in the G2- and M-Phases of the cell cycle consequent on 12 hours of exposure to PM, PAN, and 6-DMAP. The M-Phase at each concentration point is representative of the mean mitotic index (number of mitotic figures per 100 cells) of two experiments done on separate occasions. The G2-Phase was calculated by subtracting the M-Phase value from the mean G2M-Phase value (n=3) obtained from Figure 2b.

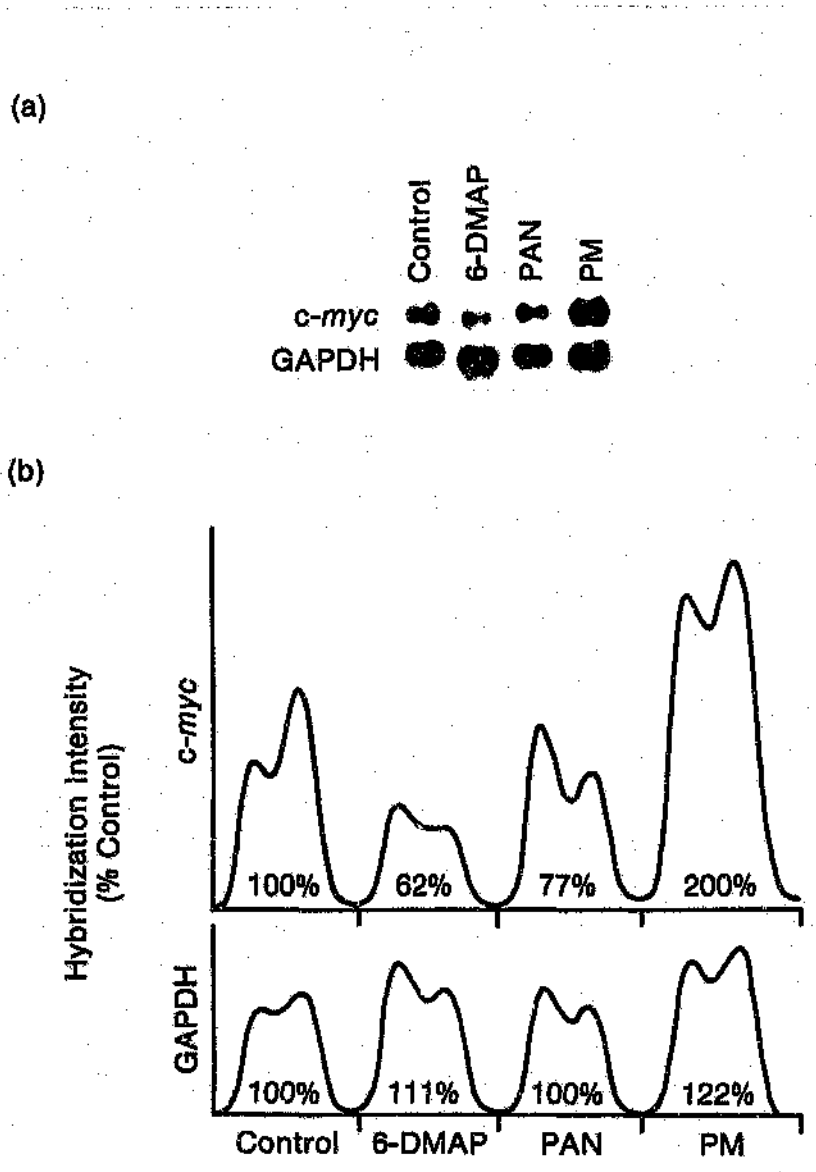


Figure 7

a) c-myc and (for reference) GAPDH mRNA expression levels at 12 hours in HL-60 cells treated with 0.6μM PM, 6-DMAP, and PAN, as indicated. 10μg total RNA was loaded per lane.

b) Hybridization intensities were determined by densitometric scanning of the autoradiogram. The hybridization intensities of treated cells are shown relative to those of untreated control cells which were normalized to 100%. Similar results were elicited on one further occasion.

DISCUSSION

We found that low-dose equimolar concentrations of the structurally related purines PM, PAN, and 6-DMAP, elicited strikingly different morphological, cytokinetic, and molecular effects in HL-60 human leukemic cells. PM, but not 6-DMAP or PAN, inhibited [^{14}C]Leucine incorporation, induced a time-related cytotoxic effect, a G2-arrest, a metaphase-mitotic-arrest, morphological and biochemical evidence of apoptosis, and c-myc mRNA superinduction. PAN and 6-DMAP exerted no detectable morphological or cytokinetic effects on HL-60 cells, although exposure to these drugs results in down-regulation of c-myc mRNA levels.

The effects seen with PM-exposure are interesting since they were observed at a dose (0.6μM) which was orders of magnitude lower than the doses of PM traditionally employed to inhibit protein synthesis (10-200μM). This dose of PM (0.6μM) nevertheless inhibited [^{14}C]Leucine incorporation by $\pm$ 20% after 1 hour. PM-treatment is known to cause the premature release of truncated peptide chains which are bound through their carboxyl termini to PM [3], and which are rapidly degraded through an ubiquitin-dependent pathway [9]. Since the metaphase-to-anaphase transition is also mediated by precipitous ubiquitin-dependent cyclin degradation [10], it is likely that this pathway was preferentially diverted into PM-peptide proteolysis in PM-treated cells. One or more of the ubiquitin pathway components might have become limiting for cyclin degradation, resulting in the observed metaphase-mitotic-arrest.

The G2-arrest observed in PM-treated HL-60 cells might have been linked to subsequent apoptosis activation, since such an association has been recognised to exist in cells exposed to the anticancer agents etoposide [11], cisplatin [12], and mafosfamide (own unpublished data). There are also precedents for the principle that cells deprived of signalling input enter the apoptotic pathway [13], and although the actual mechanism of the PM-induced G2-arrest remains unresolved at this stage, we suggest that it may relate to the attenuated tyrosine kinase signal transduction that we have observed in PM-treated cells (submitted data). It is of interest that low-dose PM has recently been found to induce apoptosis in Erythropoietin-dependent murine erythroblasts [14].

As far as the PM-induced c-myc mRNA superinduction is concerned, our finding is consistent with that of others [15], and can be attributed to a number of different processes related to protein synthesis inhibition. These include the inhibition of translation per se [16], the inhibition of translation-dependent poly(A) tail shortening [15] and enhanced mRNA stability due to the loss of labile transcriptional repressors or RNAses [17,18]. Again it is of interest that we were able to elicit the phenomenon of c-myc mRNA superinduction at a much lower dose than used previously - 0.6μM compared to 180μM [15].

In contrast to the c-myc mRNA superinduction observed with PM-exposure, both 6-DMAP and PAN down-regulated c-myc transcripts in HL-60 cells. As far as 6-DMAP is concerned, this may have been related to the known kinase inhibiting properties of 6-DMAP [1,19], since another substituted purine, 2-

Aminopurine, an inhibitor of double-stranded RNA-dependent protein kinase, has been found to down-regulate c-myc mRNA levels [20,21,22].

Although 6-DMAP arrests Xenopus oocytes in the G2-phase of the cell cycle when employed at a concentration of 300μM [1], no detectable cytokinetic effects were elicited in HL-60 cells when 6-DMAP (and also PAN) were used at a much lower dose (0.6μM).

Despite their close structural relationships (6-DMAP is the purine component of both PM and PAN, PAN is a composite of 6- DMAP and 3-Aminoribose, and PM is a composite of PAN and tyrosine-o-methylether), PM, 6-DMAP, and PAN varied greatly in their capacity to elicit morphological, cytokinetic and molecular effects when employed at low, equimolar concentrations in HL-60 cells. This is likely to relate to the ability of PM, but not 6-DMAP and PAN, to generate the nascent polypeptide chains likely to be responsible for the effects elicited by PM, phenomena which are currently being investigated further in our laboratory. Of particular note are the unexpected effects exerted by PM, and in this context it is likely that the traditional usage of PM at high doses to inhibit protein synthesis has obscured its potent cytokinetic effects. In this regard, low-dose PM should be a useful adjunctive investigative tool in studies pertaining to the cell cycle and to apoptosis. Furthermore, although the use of PM as a single antineoplastic agent was not fruitful [23], this drug may nevertheless have role to play, at low doses, in rational combination cancer chemotherapy. At such doses PM could

differentially accumulate cells in metaphase to possibly increase their susceptibility to administration of a metaphase-specific agent such as vincristine, and in synergism with etoposide, cisplatin and mafosfamide, PM could potentially promote tumour regression consequent on apoptotic cascade activation. Clinical trials using low-dose PM in combination with some of these agents are currently under investigation in our laboratory in dogs presenting with spontaneous lymphoproliferative disorders.

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CHAPTER 5

Cell cycle disruptions and apoptosis induced by the Cyclophosphamide derivative Mafosfamide.

ABSTRACT

The effect of the cyclophosphamide derivative Mafosfamide (ASTA Z 7557) was investigated in vitro in HL-60 leukaemic cells. Mafosfamide, which rapidly generates 4-hydroxycyclophosphamide after aqueous dissolution, was employed at doses ranging from 0.1 to 10 ug/ml. In unsynchronised cells Mafosfamide-exposure was associated with an S-Phase accumulation, a suggestion of a G2-Phase arrest, and morphological and biochemical evidence of apoptosis. In cells that had been synchronised by the double thymidine block method, S-Phase progression was considerably delayed in the presence of Mafosfamide. The apoptosis which was evident in Mafosfamide-treated cells 12 hours after release from the thymidine block was found to occur in the presence of S-Phase- and G2-Phase-cell-cycle-arrests. Taken together the current data suggest that Mafosfamide may have potential synergism with other anticancer agents that elicit similar cell-cycle-arrests, as well as with chemotherapeutic drugs that activate the apoptotic cascade.

INTRODUCTION

Cyclophosphamide is a widely employed alkylating cancer chemotherapeutic agent [1,2] whose cytotoxicity is mediated by the introduction into DNA of cross-links and single-strand breaks [3,4]. Cyclophosphamide derivatives are also frequently used in vitro to purge leukemic cells from bone marrow [4,5,6]. One such derivative is the prodrug Mafosfamide (ASTA Z 7557), a cyclohexylamine salt that rapidly generates 4-Hydroxycyclophosphamide after aqueous dissolution. 4-Hydroxycyclophosphamide degrades irreversibly via aldophosphamide to phosphoramide mustard and acrolein [7-9], the therapeutically significant metabolites of cyclophosphamide [10].

A number of anticancer agents are able to induce apoptosis or programmed cell death, e.g. etoposide, cisplatin, melphalan, methotrexate, camptothecin, teniposide, amsacrine, and 5-fluorouracil [11-16]. In the cases of etoposide [15] and cisplatin [16], a preceding G2-Phase cell-cycle-arrest has been associated with the subsequent activation of the apoptotic cascade, whereas camptothecin, teniposide, and amsacrine have been found to induce S-Phase-specific internucleosomal DNA degradation [14]. In the current study undertaken in vitro in human HL-60 leukemic cells, we have investigated the cytokinetic consequences of the cyclophosphamide derivative Mafosfamide, and have evaluated its capacity to induce double-stranded DNA breaks and apoptosis.

MATERIALS AND METHODS

Cell culture and Mafosfamide-exposure

HL-60 cells (ATCC passage 17, Rockville, MD, USA) were seeded at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2mM L-glutamine, Penicillin (50U/ml), and Streptomycin (50μg/ml). Cells were maintained unagitated in logarithmic growth phase at 37°C in a humidified atmosphere of air and 5% CO_2. For synchronization experiments the double thymidine block method was employed [17]. Cells seeded at a concentration of 10^6/ml were incubated for two 24 hour periods in the presence of 2mM thymidine. The two 24 hour periods were separated by a 12 hour period in which cells were incubated in the absence of thymidine i.e. they were released from the first thymidine block. Thymidine was removed after each 24 hour-exposure by washing the cells three times in medium that had been pre-warmed to 37°C. After release from the second thymidine block, cells were seeded at a final density of 10^6/ml, and aliquots of cell suspensions were collected subsequent to various incubation intervals. Culture viability was assessed by the trypan blue dye exclusion technique, and after cells were fixed and stained with May-Grünwald-Giemsa, they were examined for mitotic figures and apoptotic cells. Aliquots of cells were subjected to flow cytometric analysis and DNA agarose gel electrophoresis. Mafosfamide crystalline powder (MW=500.5) was received as a kind gift from Dr J. Pohl, ASTA PHARMA Pr Brock, Asta-Werke, Bielefeld, West Germany. Fresh Mafosfamide solution was prepared in RPMI medium immediately prior

to use at final concentrations ranging from 0.1 ug/ml to 10 ug/ml.

Flow cytometric analysis

Cells were prepared for analysis as described elsewhere [18]. At the conclusion of the designated incubation periods, the pellet obtained by centrifugation from 10^6 cells was resuspended in 1ml of a hypotonic nuclear isolation medium (50mg/l Propidium Iodide; 1g/l Triton-X 100; 1g/l Sodium Citrate), incubated on ice for 30 minutes, and then in DNAase-free RNAase at 37°C. Fluorescence emitted from the Propidium Iodide-DNA complex was quantitated after laser excitation of the fluorescent dye by the Coulter EPICS Profile II flow cytometer (Coulter Electronics, Inc, Fla.). Cell debris and doublets were excluded [19], and the coefficient of variations of the control G0G1-Phase and G2M-Phase peaks were less than 3%. 25 000 cells were counted for each analysis. Histogram scaling was the same for all samples. Limits of cell cycle phases as determined in the control DNA distributions were retained in the subsequent analysis of the Mafosfamide-treated populations.

DNA fragmentation analysis

After appropriate incubation periods, cells which had initially been seeded at 10^6/ml were pelleted at 4°C and prepared for subsequent fragmentation analysis [20]. The pellet was resuspended in 70µl of lysis buffer (10mM EDTA; 50mM Tris-HCl, pH=8.0; 0.5% sodium lauryl sarkosinate; 0.5mg/ml proteinase K) and incubated for 1 hour at 50°C. A 10µl aliquot of 1mg/ml

ribonuclease A was added to each sample prior to a further 1 hour incubation at 50°C. Samples were then heated to 70°C after which the reaction was terminated by the addition of 20μl of loading buffer (10mM EDTA, pH=8.0; 0.25% bromophenol blue; 40% sucrose). Extracts were loaded into the wells of a 2% agarose gel and electrophoresed in a phosphate buffer (2mM EDTA; 800mM Tris-phosphate; pH=7.8). Both the gel and the buffer contained 1μg/ml of ethidium bromide. BglI/HinfI cleaved pBR328 served as molecular weight markers.

Materials

All materials for cell culture were obtained from GIBCO, Madison, WI, USA. Propidium Iodide was from Sigma. All other reagents were of analytical grade and were from standard commercial sources.

RESULTS

Cell cycle arrests

Asynchronous cells

Flow cytometric changes in asynchronous cells were unremarkable after 6 hours of Mafosfamide-exposure (data not shown). By 24 hours however, an S-Phase accumulation was evident which occurred progressively earlier in S-Phase with the higher doses of Mafosfamide (Figure 1a and b). At 24 hours there was also a dose-dependent increase in the G2M-Phase (Figure 1a and b). This increase reflects an absolute increase in the G2-Phase [14], since the M-Phase decreased with increasing Mafosfamide concentrations at this time-point (Figure 1c). Further evidence for hold-ups at the S-Phase and G2-Phase boundaries at 24 hours is the progressive decrease in the G0G1-Phase (Figure 1a and b) with increasing Mafosfamide concentrations, suggesting continuing egress out of G0G1-Phase into the S-Phase, but limited if any influx into the G0G1-Phase from the G2M-Phase.

Synchronized cells

S-Phase progression was retarded in synchronized Mafosfamide-treated HL-60 cells released from the double thymidine block (Figure 2). By 8 hours $\pm$60% of synchronized control cells had reached the G2M-Phase compared with only $\pm$15% of the Mafosfamide-treated cells. By 12 hours when $\pm$73% of control cells had completed the previous G2M-Phase and were back in the G0G1-Phase, the majority ($\pm$70%) of the

Mafosfamide-treated cells were still held up in the previous S- and G2-Phases.

Morphological and biochemical evidence of apoptosis

Unsynchronised cells

Apoptosis was not seen in unsynchronized Mafosfamide-treated cells after 6 hours of exposure (data not shown). At 24 hours however, there was morphological evidence of apoptosis (chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies) in Mafosfamide-treated cells (Figure 3). (An apoptotic body is a fragment of a larger apoptotic cell that may or may not contain nuclear components in the form of cleaved DNA). This was confirmed ultrastructurally (data not shown), and by DNA electrophoresis which showed the pattern of double-stranded DNA fragments that result from the activation of an endogenous endonuclease (Figure 4). Apoptosis was also observed in Mafosfamide-treated THP-1 and K562 cells (data not shown).

Synchronized cells

Apoptosis was not evident in synchronized Mafosfamide-treated cells for up to 10 hours after release from the double thymidine block. At 12 hours morphological apoptosis was present in these cells - this was confirmed by DNA electrophoresis (Figure 5). The presence of apoptosis in these cells was coincident with both a late S-Phase, and a suggestion of a G2-Phase accumulation (Figure 2). (The G2M-Phase

accumulation observed in Mafosfamide-exposed cells at 12 hours in Figure 2 reflects an absolute increase in the G2-Phase [14] since mitotic figures were not evident at this time-point in these cells (data not shown)).

Cytotoxic effects

By 24 hours a dose-dependent cytotoxicity was evident in unsynchronized Mafosfamide-treated cells (Figure 6).

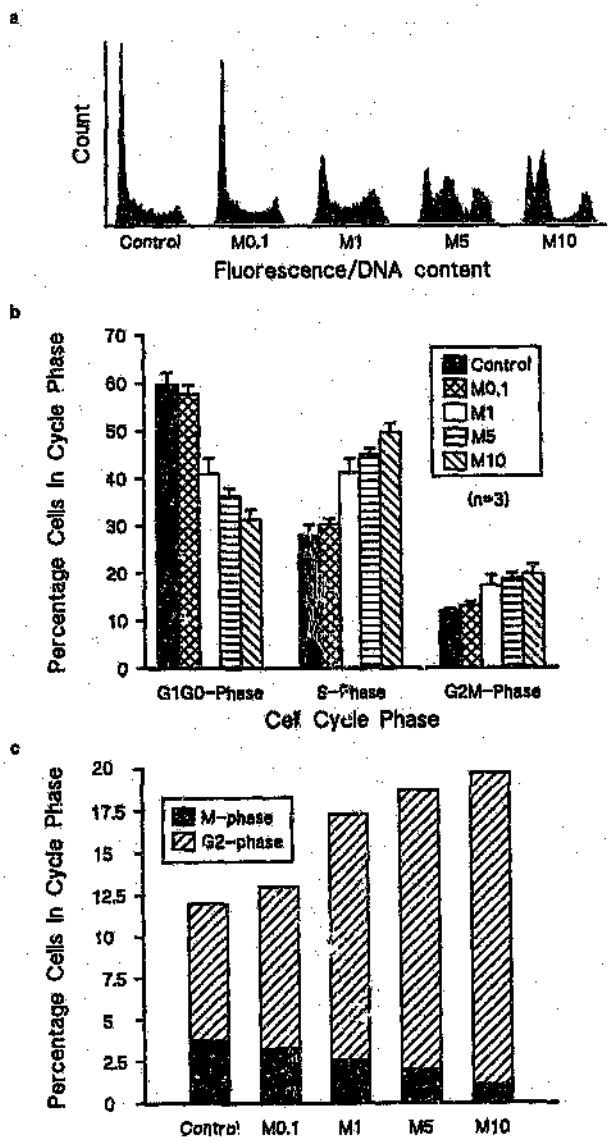


Figure 1

a) Representative flow cytometric analysis at 24 hours of of unsynchronised control and Mafosfamide-treated HL-60 cells.

b) Effects on the DNA histogram of unsynchronised HL-60 cells following 24 hours of exposure to increasing Mafosfamide concentrations. Results are expressed as mean ± SD (n=3).

c) Stacked-bar graphs illustrating the relative percentages of HL-60 cells in the G2- and M-Phases of the cell cycle consequent on 24 hours of Mafosfamide-exposure. The M-Phase at each concentration point is representative of the mean mitotic index of two experiments done on separate occasions. The G2-Phase was calculated by subtracting the M-Phase value from the mean G2M-Phase value (n=3) obtained from Figure 2b [14].

All Mafosfamide concentrations (M) are in ug/ml.

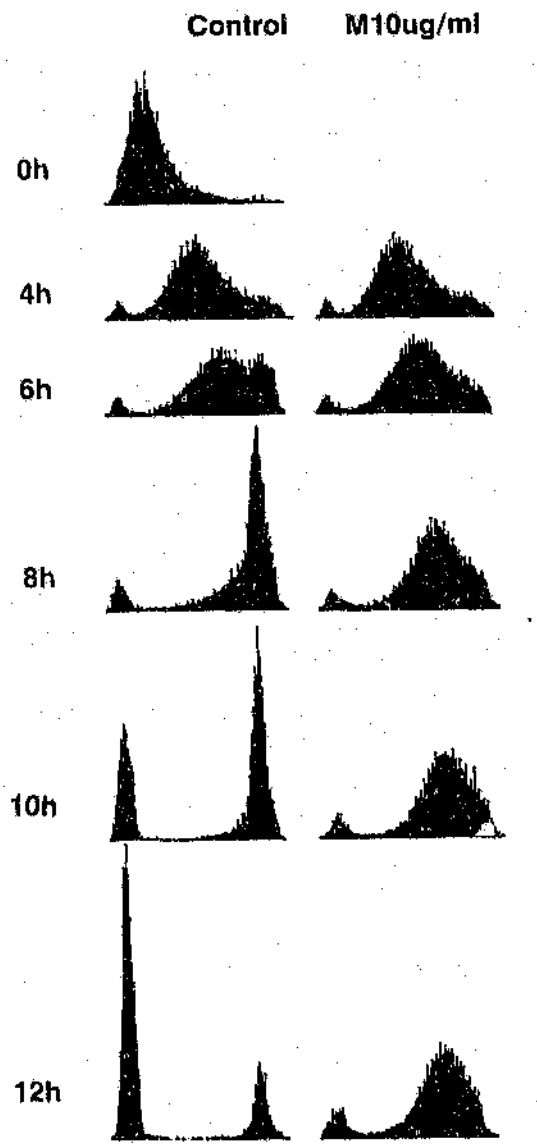
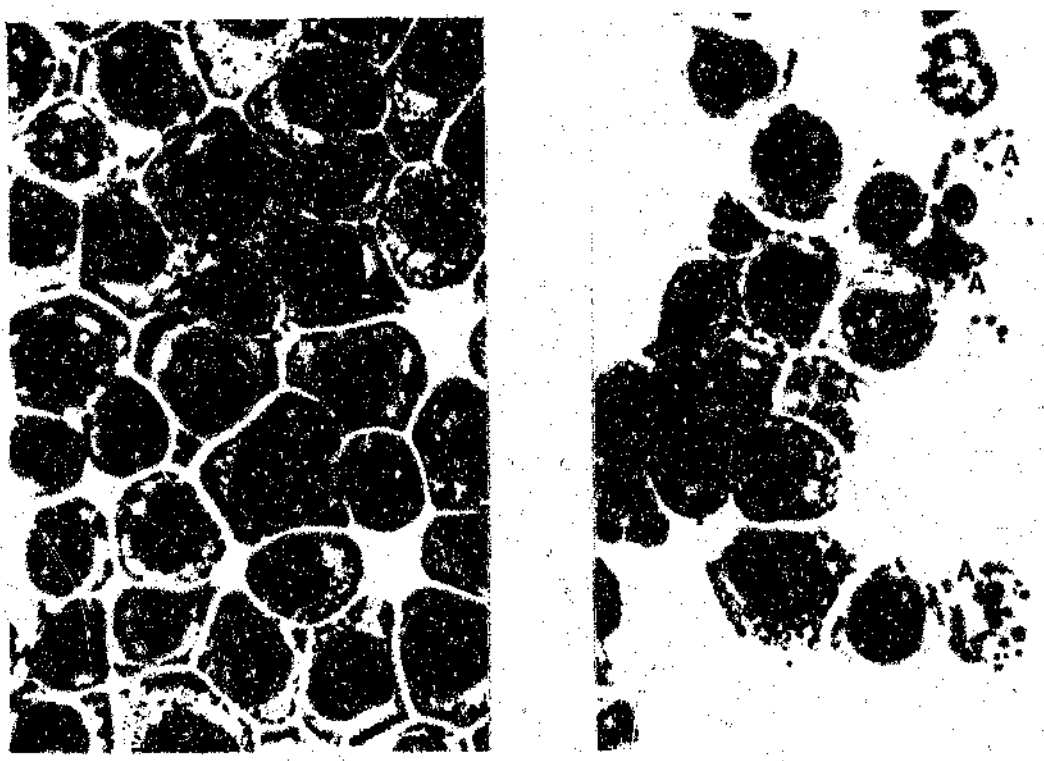


Figure 2
Effects of Mafosfamide-exposure (10ug/ml) on cell cycle progression of HL-60 cells released from the double thymidine block. Note the delay in S-Phase progression in Mafosfamide-treated cells, as well as the late S-Phase accumulation and G2M-Phase arrest that was evident 12 hours after release from the thymidine block.



Control

Mafosfamide 5µg/ml

Figure 3
Giemsa-stained cytospin preparations (magnification X1000) of unsynchronised HL-60 cells.

a) Control HL-60 cells at 24 hours.

b) After 24 hours of Mafosfamide-exposure (5ug/ml) there was morphological evidence of apoptosis "A" in the form of chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies.

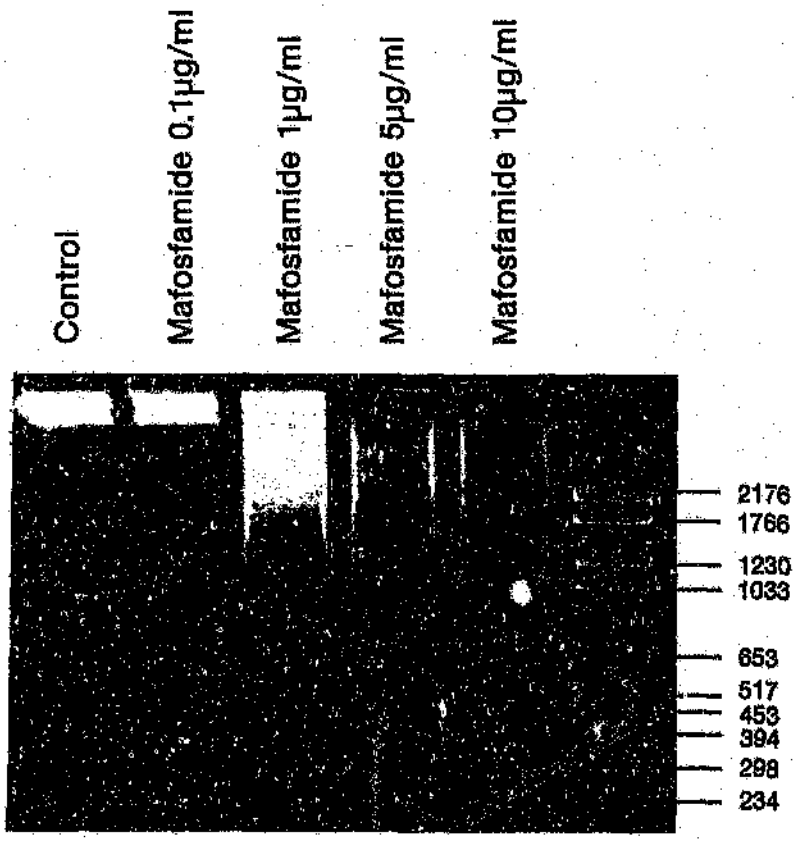


Figure 4
Agarose gel electrophoresis of total DNA extracted at 24 hours from $1x10^6$ unsynchronised control and Mafosfamide-treated HL-60 cells (starting cell number at 0 hours was 10^6 cells). The sizes (in bases) of molecular weight markers are shown.
Note the DNA fragmentation in cells treated with Mafosfamise at concentrations $\geq$10ug/ml.

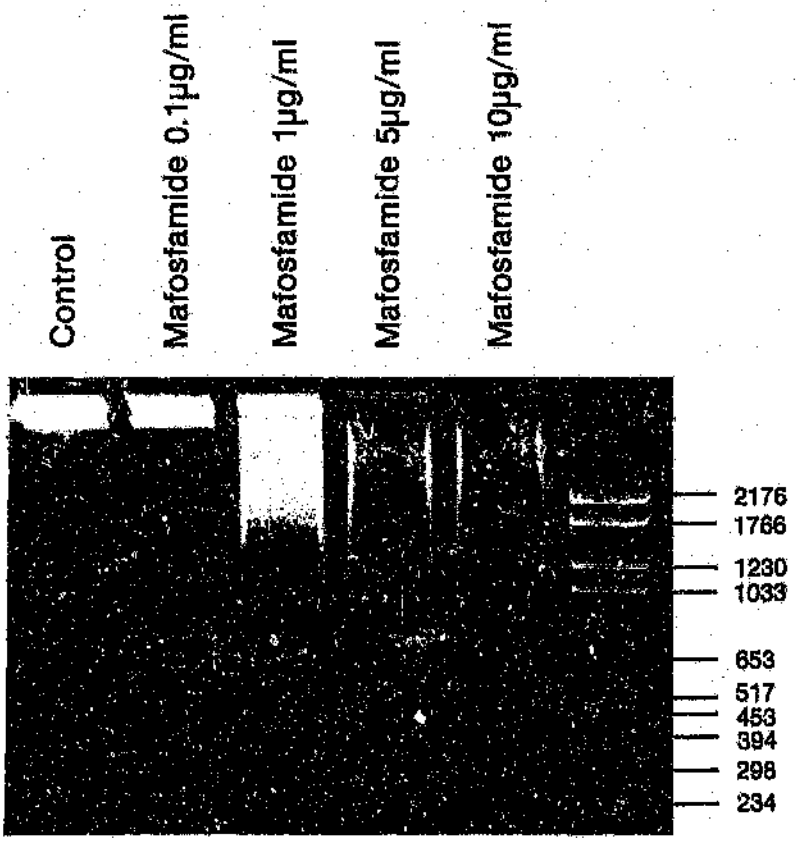


Figure 5
Agarose gel electrophoresis of total DNA extracted from control and "M" Mafosfamide-treated (10ug/ml) HL-60 cells at progressive time intervals after release from the double thymidine block (starting cell number at 0 hours was 10^6 cells). The sizes (in bases) of molecular weight markers are shown. Note the sudden onset of DNA fragmentation between 10 and 12 hours, corresponding to the late S-Phase- and G2-Phase-cell-cycle-arrests (see Figure 2).

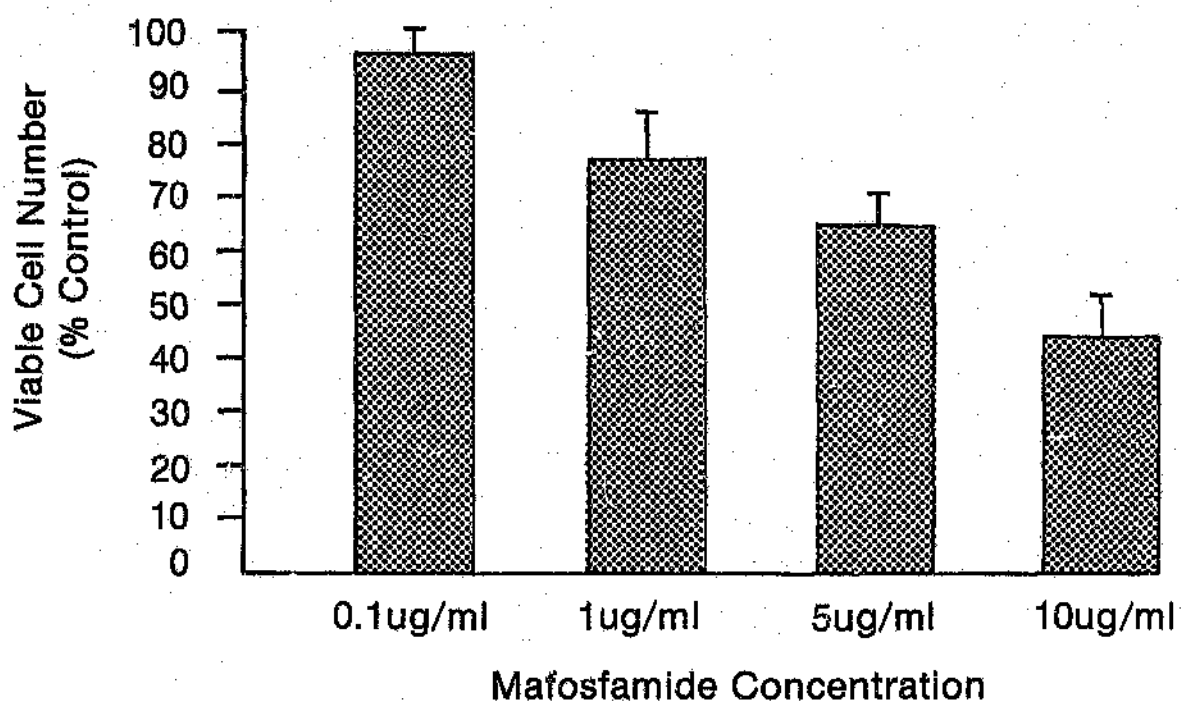


Figure 6
Effect of Mafosfamide on the viable cell number of unsynchronised HL-60 cells after 24 hours of exposure.
Results are expressed as a percentage of control values and reflect the mean ± SD of two separate experiments, each done in duplicate.

DISCUSSION

In this study we investigated the in vitro effects of the cyclophosphamide derivative Mafosfamide. Mafosfamide, which rapidly generates 4-hydroxycyclophosphamide after aqueous dissolution, was employed at doses ranging from 0.1 to 10 ug/ml in HL-60 human leukemic cells. We found that Mafosfamide induced morphological and biochemical evidence of apoptosis in both asynchronous and in synchronysed HL-60 cells. This is similar to the findings of Marks and Fox [22], and also to the apoptosis that has been observed in response to numerous other chemotherapeutic agents including etoposide, cisplatin, melphalan, methotrexate, camptothecin, teniposide, amsacrine, and 5-fluorouracil [11-16].

Consistent with the known alkylating effects of cyclophosphamide [9], and also with its capacity to introduce cross-links and single-strand breaks into DNA [4,8], we found that Mafosfamide caused an S-Phase accumulation in both synchronised and unsynchronized HL-60 cells. Since the progression of DNA synthesis during S-Phase is dependent on the sequential activation of banks of replicons so that activation of later replicons is contingent on the successful replication of previously activated replicons [21], it is likely that alkylating damage to DNA by Mafosfamide precluded successful sequential replicon activation, disrupted S-Phase progression, and was responsible for the S-Phase accumulation. Because apoptosis was found to occur simultaneously with the S-Phase-arrest in Mafosfamide-treated cells, it is possible that apoptosis may have been activated, at least in part, subsequent to the arrest, as has been

reported with the cancer chemotherapeutic agents camptothecin, teniposide, and amsacrine, all of which induce S-Phase-specific internucleosomal cleavage [14].

In addition to the apoptosis and the S-Phase accumulation induced by Mafosfamide, ther was a suggestion of a G2-Phase arrest in both synchronized and in unsynchronised HL-60 cells, similar to the G2-Phase arrest elicited by cisplatin and etoposide [15,16]. We suggest that the apoptosis in Mafosfamide-treated cells, in addition to occurring in S-Phase, may also have occurred in the G2-Phase, as has been shown to occur with both etoposide and cisplatin [15,16], where a preceding G2-Phase-cell-cycle-arrest is associated with subsequent activation of the apoptotic cascade.

In conclusion we have shown that the cyclophosphamide derivative Mafosfamide induced apoptosis in HL-60 cells, and that the apoptosis was associated with intercurrent S-Phase and G2-Phase cell-cycle-arrests. As such we propose that Mafosfamide may have potential synergism with other anticancer agents that elicit similar cell-cycle-arrests e.g. cisplatin and etoposide, and with cancer chemotherapeutic drugs that induce apoptosis e.g. melphalan, methotrexate, camptothecin, teniposide, amsacrine, 5-fluorouracil, etoposide, cisplatin.

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CHAPTER 6

c-myc Expression is down-regulated in Mafosfamide-treated HL-60 cells undergoing apoptosis.

ABSTRACT

The expression of the proto-oncogene c-myc has been strongly implicated in the regulation of apoptosis. Since we have previously shown apoptosis to occur in HL-60 leukemic cells exposed to the prodrug Mafosfamide (ASTA Z 7557), the present study was undertaken to examine the levels of c-myc mRNA transcripts in such cells. Mafosfamide, which is a cyclophosphamide derivative that rapidly generates 4-Hydroxycyclophosphamide after aqueous dissolution, was employed in doses ranging from 0.1 to 20ug/ml. No changes in c-myc mRNA levels were evident after 1 hour of exposure to Mafosfamide. After 6 and 24 hours of Mafosfamide-exposure however, there was a dose- and time-dependent decrease in c-myc transcripts. c-myc mRNA expression was reduced to a greater extent than either the beta-actin or GAPDH expression was. Morphological, biochemical, and ultrastructural evidence of apoptosis accompanied the Mafosfamide-induced c-myc mRNA down-regulation at 24 hours. We conclude that in the context of Mafosfamide-treated HL-60 cells, upregulation of c-myc mRNA transcription was not fundamental for the activation of the apoptotic cascade.

INTRODUCTION

The proto-oncogene c-myc has been strongly implicated in the apoptotic pathway. In certain contexts the process of apoptosis has been found to be contingent on c-myc expression (1-7), whereas in other scenarios down-regulation of c-myc mRNA levels have accompanied the activation of the apoptotic cascade (8-10).

Cyclophosphamide is a widely employed alkylating cancer chemotherapeutic agent (11-12). the derivatives of which are frequently used in vitro to purge leukemic cells from bone marrow (13-20). One such derivative is the prodrug Mafosfamide (ASTA Z 7557), a cyclohexylamine salt that rapidly generates 4-Hydroxycyclophosphamide after aqueous dissolution. 4-Hydroxycyclophosphamide degrades irreversibly via aldophosphamide to phosphoramide mustard and acrolein (21-23), the therapeutically significant metabolites of cyclophosphamide (24). We have recently found that Mafosfamide-exposure induces apoptosis in HL-60 leukemic cells (25). Since certain anticancer agents have been found to reduce c-myc mRNA levels (26-29), whereas others, including the cyclophosphamide derivative 4-Hydroperoxycyclophosphamide, have been found to elevate c-myc mRNA transcripts and/or c-myc oncoprotein levels (27, 30-31), the present study was undertaken to examine the levels of c-myc mRNA transcripts in Mafosfamide-treated HL-60 cells undergoing apoptosis.

METHODS AND MATERIALS

Cell culture and Mafosfamide-exposure

HL-60 human promyelocytic leukemic cells (ATCC passage 17, Rockville, MD, USA) were seeded in RPMI 1640 medium supplemented with fetal calf serum, L-glutamine and Penicillin/Streptomycin antibiotics (GIBCO, Madison, WI, USA). Cells were maintained at 37°C in a humidified atmosphere of air and 5% CO_2. Mafosfamide crystalline powder (MW=500.5) was a kind gift from Dr J. Pohl, ASTA PHARMA, Pr Brock, Asta-Werke, Bielefeld, West Germany. Fresh Mafosfamide solution was prepared in RPMI medium immediately prior to use at final concentrations ranging from 0.1 μg/ml to 20μg/ml.

DNA fragmentation analysis

At the conclusion of the incubation period the cells were pelleted at 4°C and prepared for subsequent fragmentation analysis (32). The pellet was resuspended in 70μl of lysis buffer (10mM EDTA; 50mM Tris-HCl, pH=8.0; 0.5% sodium lauryl sarkosinate; 0.5mg/ml proteinase K) and incubated for 1 hour at 50°C. A 10μl aliquot of 1mg/ml ribonuclease A was added to each sample prior to a further 1 hour incubation at 50°C. Samples were then heated to 70°C after which the reaction was terminated by the addition of 20μl of loading buffer (10mM EDTA, pH=8.0; 0.25% bromophenol blue; 40% sucrose). Extracts were loaded into the wells of a 2% agarose gel and electrophoresed in a phosphate buffer (2mM EDTA; 800mM Tris-phosphate;

pH=7.8). Both the gel and the buffer contained 1μg/ml of ethidium bromide. BglI/HinfI cleaved pBR328 served as molecular weight markers.

c-myc mRNA expression

c-myc mRNA expression was investigated using standard techniques (33). Extracted total RNA (10ug) was size fractionated by 1.2% agarose formaldehyde gel electrophoresis, and then transferred and UV-crosslinked to nylon membrane. Hybridization at 42°C for 16 hours was to the [gamma-^{32}P]dCTP (Amersham, England) random primer-labelled 1.6Kb SstI fragment from the 5' end of the human c-myc gene (ATCC, USA). Following stringency washes at 42°C and at room temperature, the membrane was exposed at -70°C to film using image-intensifying screens. Finally the membrane was stripped before rehybridization either to the [α^{32}P]dCTP random primer-labelled 1.9Kb BamHI fragment of ß-actin (ATCC, USA), or to the [γ-^{32}P]ATP (Amersham, England) end-labelled 40mer oligonucleotide antisense GAPDH (Oncogene Science, USA). ß-actin and GAPDH served as internal controls for constitutive gene expression.

All other reagents were of analytical grade and were from standard commercial sources.

RESULTS

DNA fragmentation analysis

Apoptosis was not seen in Mafosfamide-treated cells after 1 and 6 hours of exposure (data not shown). At 24 hours however, there was morphological evidence of apoptosis (chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies) in Mafosfamide-treated cells (data not shown). This was confirmed ultrastructurally (data not shown), and by DNA electrophoresis which showed the pattern of double-stranded DNA fragments that result from the activation of an endogenous endonuclease (Figure 1).

c-myc mRNA expression

There was no change in c-myc, ß-actin and GAPDH mRNA expression after 1 hour of Mafosfamide-exposure in HL-60 cells (data not shown). After 6 hours and 24 hours however, there was a dose-dependent decrease in the expression of these genes in Mafosfamide-treated cells (Figure 2). c-myc mRNA expression was reduced more than either the ß-actin or GAPDH expression was (Figure 2 and Figure 3).

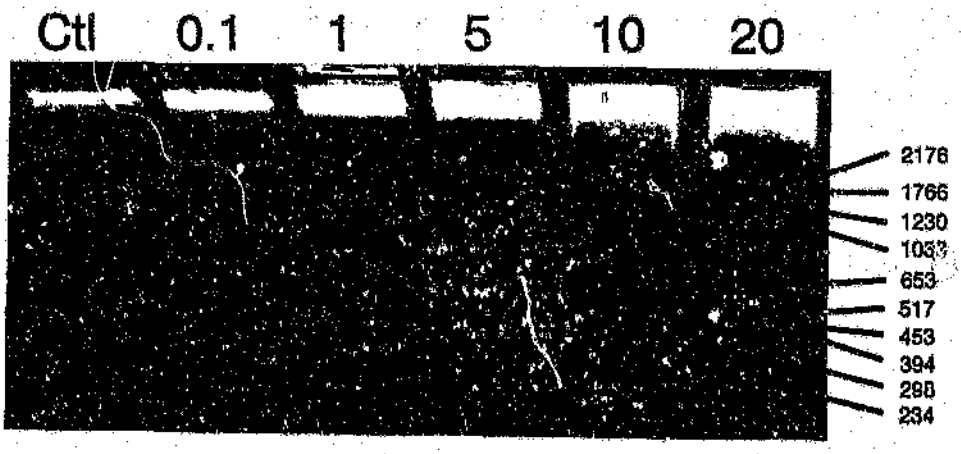

Figure 1
Agarose gel electrophoresis of total DNA extracted from control and Mafosfamide-treated (ug/ml) HL-60 cells at 6 hours and 24 hours after drug exposure. The sizes in bases of molecular weight markers are shown.

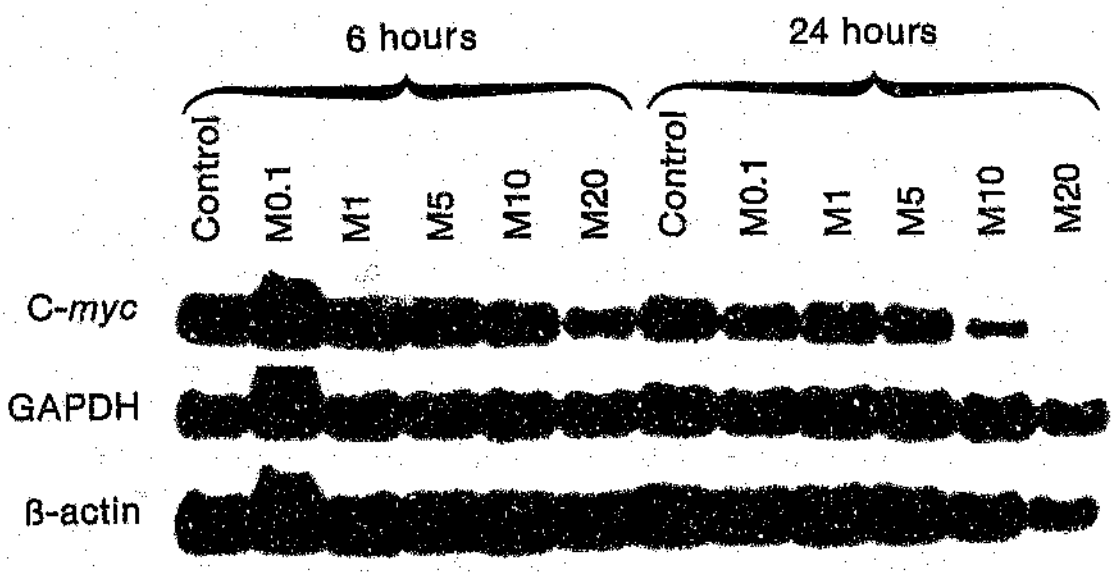

Figure 2
c-myc, ß-actin, and GAPDH mRNA expression at 6 hours and at 24 hours in control and in Mafosfamide-treated (μg/ml) HL-60 cells (10μg total RNA loaded per lane). The results were reproduced on one further occasion.

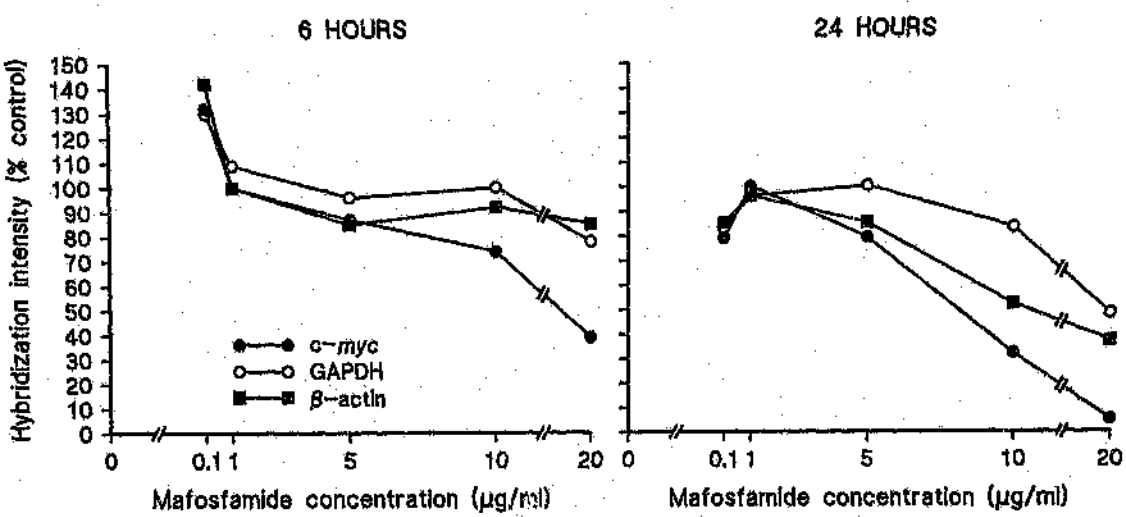

Figure 3
Hybridization intensities at 6 hours and at 24 hours in Mafosfamide-treated HL-60 cells are shown relative to that in untreated control cells (normalised to 100%). The results were quantitated by densitometric scanning of the autoradiogram.

DISCUSSION

We recently found that the apoptotic pathway was activated in HL-60 cells following exposure to the prodrug Mafosfamide, a derivative of the alkylating cancer chemotherapeutic agent cyclophosphamide (25). Since the expresson of the proto-oncogene c-myc has been strongly implicated in the regulation of the apoptotic cascade (1-10), we undertook to examine the effect of Mafosfamide on c-myc mRNA levels in HL-60 cells. In the current study we found no change in c-myc mRNA levels after 1 hour of Mafosfamide-exposure, and a dose-dependent decrease in c-myc mRNA transcripts after 6 hours and 24 hours in Mafosfamide-treated cells.

These findings suggest that in the context of Mafosfamide-treated HL-60 cells, upregulation of c-myc mRNA transcription was not fundamental for apoptosis induction. This is consistent with the down-regulation of the c-myc gene that has been found to precede IL-6 induced apoptosis of Y6 cells (8), steroid-induced apoptosis of 697 pre-B lymphocytes (9), and phorbol ester-induced apoptotis of U-937 cells (10). Our findings are in contrast with the elevated c-myc mRNA levels seen in rat thymocytes undergoing apoptosis following dexamethasone treatment (3), with the evidence that c-myc is a potent inducer of apoptosis in rat fibroblasts (6), and with the observations that c-myc expression is a requirement for activation-induced apoptosis of T-cell hybridomas (5), for apoptotic regression of estrogen-treated MCF-7 human mammary cancers (7), and for apoptosis elicited in c-myc-amplified cell lines

(including HL-60) by the DNA topoisomerase II inhibitor VM-26 (1).

In the context of anticancer agents, it is of interest that in Colo320HSR cells treated with another cyclophosphamide derivative (4-hydroperoxy-cyclophosphamide), c-myc mRNA levels increased after 1 hour of exposure but decreased subsequently, raising the possibility that alkylating agents may suppress the expression of specific transforming genes (27). Further evidence for this comes from in vitro experimentation which demonstrated that alkylating agents including nitrogen mustard and melphelan, but not 4-S-(propionic acid)-sulfidocyclophosphamide, terminated c-myc transcription in a sequence-specific, drug-specific manner (28). In addition mithramycin, which binds to G-C-rich sequences in the DNA minor groove, has been found to inhibit c-myc transcription in HeLa cells (and other genes which have G-C rich promoter sequences) by preventing the formation of the transcription initiation complex (29). However, increased c-myc protein has been observed to accumulate in a lymphoblastoid cell line in response to certain alkylating agents (Dimethylsulphonate, Ethylmethane sulphonate and Mitomycin C) (30), and elevated c-myc mRNA levels have been reported in SV40 transformed human keratinocytes in response to ultraviolet irradiation (20) and in rat embryonic cells exposed to ionizing irradiation (21).

It is clear that although c-myc expression has been strongly implicated in both apoptosis and the cellular response to DNA-damaging agents, the precise nature of its regulation of and/or participation in such processes still requires

elucidation. To address the issue more comprehensively in the future, c-myc mRNA expression should be evaluated not only at the levels of RNA and of protein, but also at the level of DNA, since c-myc gene-specific DNA repair may itself be differentially affected according to the eliciting insult (36). Finally it would be worthwhile to examine the transcription of mammalian genes termed gadd (growth arrest and DNA damage inducible), that specifically code for transcripts which are increased by DNA damage (37), and to examine their role, if any, in the apoptotic response to cancer chemotherapeutic drugs, particularly alkylating agents such as Mafosfamide.

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CHAPTER 7

Puromycin-elicited c-myc mRNA superinduction precedes apoptosis in HL-60 leukaemic cells.

ABSTRACT

The phenomenon of superinduction refers to the process by which high concentrations of protein synthesis inhibitors augment and stabilize mRNA transcript levels e.g. 36-180μM of Puromycin (PM) has been found to elicit c-myc mRNA superinduction. The expression of the proto-oncogene c-myc has been strongly but variously implicated in the regulation of the apoptotic cascade. Since we recently found that a low dose of the protein synthesis inhibitor PM activated the apoptotic cascade in HL-60 leukemic cells, the current study was undertaken to examine c-myc mRNA transcript levels in such cells, and to assess the relationship, if any, between PM-elicited c-myc mRNA superinduction and subsequent activation of the apoptotic cascade. PM was employed in vitro at doses of 0.9μM and 2μM. Dose-dependent c-myc mRNA superinduction was present at 1 hour of PM-exposure, and was associated with subsequent activation of the apoptotic cascade at 24 hours of exposure. Apoptosis was confirmed morphologically as evidenced by chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies, and by DNA agarose gel electrophoresis which showed showed the pattern of double-stranded DNA fragments that result from the activation of an endogenous endonuclease. [C^{14}]Leucine incorporation studies at 1 hour demonstrated minimal protein synthesis inhibition at doses used, suggesting that c-myc mRNA superinduction in this context may have been the result of mechanisms which were independent of the inhibition of synthesis of new proteins, such as interruptions in signal transduction.

INTRODUCTION

The steady-state levels of a specific mRNA can be controlled at any one of the multiple steps involved in its metabolism (1,2). The term "superinduction" has traditionally been applied to the process by which protein synthesis inhibition results in the augmentation and stabilization of mRNA transcripts (3) through a variety of mechanisms which include the inhibition of translation per se (4,5), the inhibition of translation-dependent poly(A) tail shortening (5), and the loss of labile transcriptional repressors or RNases (6,7). For example high doses i.e. 36-180μM of the "classical" protein synthesis inhibitor Puromycin (PM) have been found to elicit c-myc mRNA superinduction (5).

The proto-oncogene c-myc has recently been strongly but variously implicated in the apoptotic pathway. In certain contexts the process of apoptosis has been found to be contingent on c-myc expression (8-12), whereas in other scenarios down-regulation of c-myc mRNA levels have accompanied the activation of the apoptotic cascade (13-16).

The role of protein synthesis in the process of apoptosis is contentious since there is evidence that depending on the context, apoptosis may (17) or may not (18) require de novo protein synthesis, and that apoptosis may be induced by protein synthesis inhibitors themselves (19).

We recently found that a low dose (0.9μM) of the protein synthesis inhibitor

Puromycin (PM) activated the apoptotic cascade in HL-60 leukaemic cells (20). In the current study we therefore undertook to examine c-myc mRNA transcript levels in such cells and to assess the relationship, if any, between PM-elicited c-myc mRNA superinduction and subsequent activation of the apoptotic cascade.

METHODS AND MATERIALS

Cell culture

HL-60 human promyelocytic leukemic cells (ATCC passage 17, Rockville, MD, USA) were seeded in RPMI 1640 medium supplemented with 10%(v/v) heat-inactivated fetal calf serum, 2mM L-glutamine, 50U/ml Penicillin, and 50μg/ml Streptomycin (GIBCO, USA). Cells were maintained at 37°C in a humidified atmosphere of air and 5% CO_2. PM (Sigma) was used as described.

Protein synthesis inhibition

0.5μCi [^{14}C]Leucine (specific activity 50Ci/mmol; Amersham) was added to cultures which were then further incubated at 37°C for one hour (21). At the conclusion of the incubation period, cells were washed three times in phosphate-buffered saline, 1ml of 10% trichloroacetic acid was added, and the tubes were then chilled on ice for 30 minutes. The precipitate so formed was filtered on GF/C filter papers (Whatmann), and washed three times with 5% trichloroacetic acid. The filters were dried and counted after adding aqueous counting scintillant. [^{14}C]Leucine incorporation was determined in triplicate. Replicates differed from each other by less than 10%.

c-myc mRNA expression

c-myc mRNA expression was investigated using standard techniques (22). 10μg of total extracted RNA was size-fractionated by 1.2% agarose

formaldehyde gel electrophoresis, and then transferred to and subsequently ultraviolet cross-linked to nylon membrane. The membrane was prehybridized at 42°C for 4 hours. Hybridization was to the [α-^{32}P]dCTP (Amersham) random-primer labelled 1.6kB SstI fragment from the 5' end of the c-myc gene (ATCC,USA). Following stringency washes at 42°C and at room temperature, the membrane was exposed at -70°C to film using image-intensifying screens. Finally the membrane was stripped before re-hybridization to the [γ-^{32}P]ATP (Amersham) end-labelled 40mer oligonucleotide antisense GAPDH probe (Oncogene Science, USA). GAPDH served as an internal control for constitutive gene expression. Autoradiograms were scanned densitometrically.

DNA fragmentation analysis

After appropriate incubation periods, cells which had initially been seeded at 10^6/ml were pelleted at 4°C and prepared for subsequent fragmentation analysis (23). The pellet was resuspended in 70μl of lysis buffer (10mM EDTA; 50mM Tris-HCl, pH=8.0; 0.5% sodium lauryl sarkosinate; 0.5mg/ml proteinase K) and incubated for 1 hour at 50°C. A 10μl aliquot of 1mg/ml ribonuclease A was added to each sample prior to a further 1 hour incubation at 50°C. Samples were then heated to 70°C after which the reaction was terminated by the addition of 20μl of loading buffer (10mM EDTA, pH=8.0; 0.25% bromophenol blue; 40% sucrose). Extracts were loaded into the wells of a 2% agarose gel and electrophoresed in a phosphate buffer (2mM EDTA; 800mM Tris-phosphate; pH=7.8). Both the gel and the buffer contained

1μg/ml of ethidium bromide. BglI/HinfI cleaved pBR328 served as molecular weight markers.

Materials

All other reagents were of analytical grade and were from standard commercial sources.

RESULTS

[C^{14}]Leucine incorporation studies

After both 1 hour and 24 hours, PM employed at 0.9μM and 2μM was seen to inhibit [C^{14}]Leucine incorporation (Figure 1). The extent of [C^{14}]Leucine incorporation inhibition at 24 hours (±43% and ±80%) was greater than at 1 hour (±9% and ±15%).

c-myc mRNA Expression

After 1 hour, PM superinduced c-myc mRNA levels above those of the control (Figure 2). At 2μM, PM superinduced GAPDH as well as c-myc, although c-myc was superinduced to a greater extent than GAPDH was.

DNA fragmentation analysis

After 1 hour of PM-exposure, and coincident with c-myc mRNA superinduction, there was no evidence of DNA fragmentation on agarose gel electrophoresis (Figure 3). At 24 hours however, 0.9μM and 2μM of PM were both seen to induce DNA fragmentation.

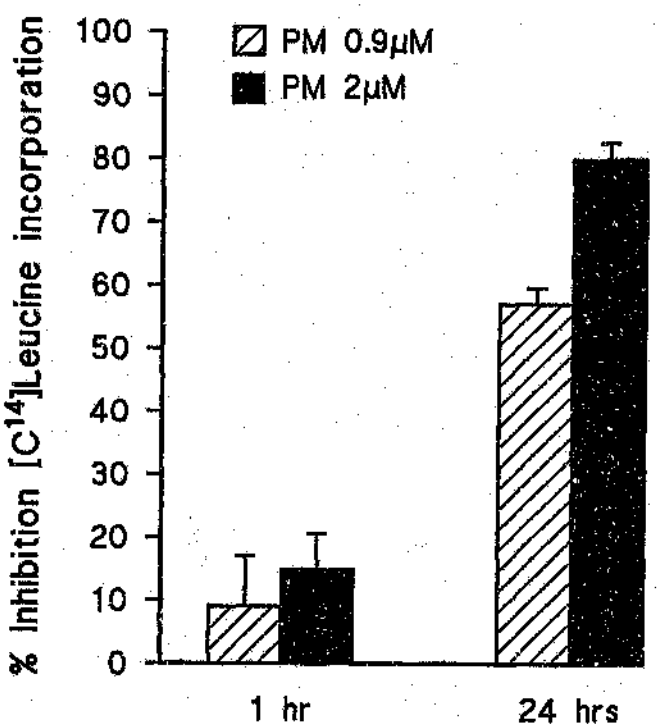

Figure 1
[C14]Leucine incorporation studies in HL-60 cells at 1 hour and at 24 hours of PM-exposure. Results are expressed as the percentage inhibition of [C14]Leucine incorporation, and represent the mean ± SD of three separate experiments each done in triplicate.

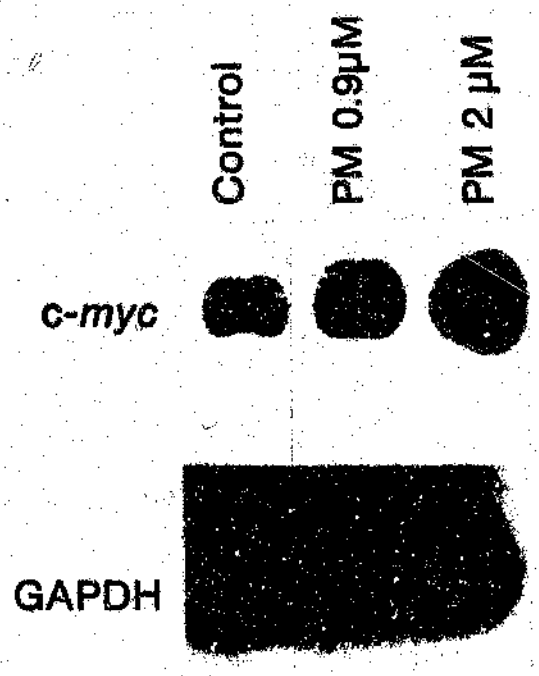

Figure 2
Dose-response representation of c-myc and (for reference) GAPDH mRNA levels after 1 hour in control and PM-treated HL-60 cells (10μg total RNA loaded per lane).

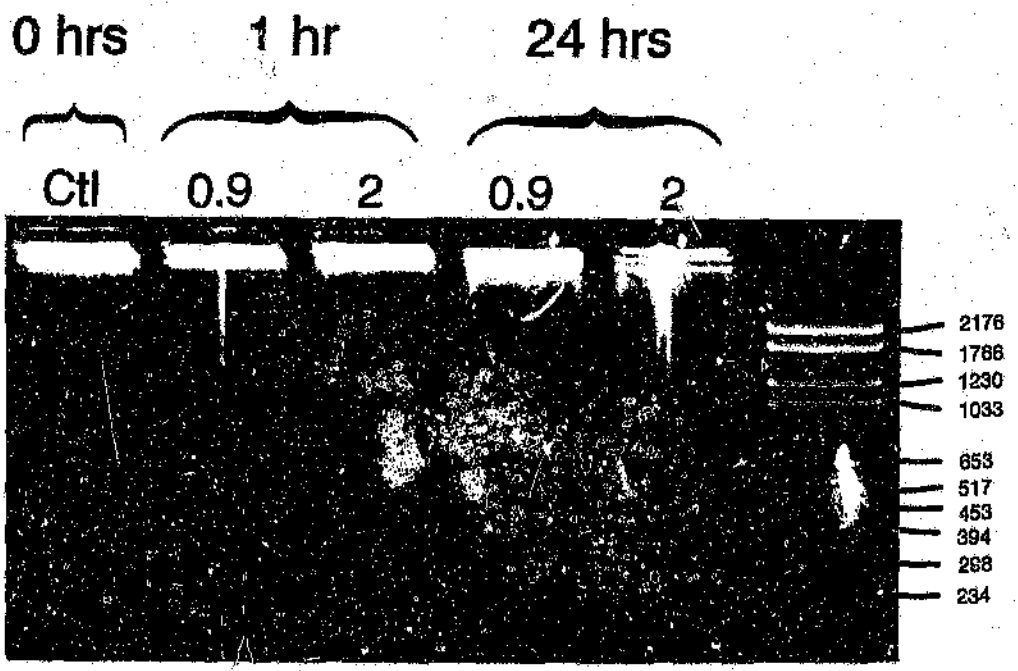

Figure 3
Agarose gel electrophoresis of total DNA extracted from HL-60 cells at 1 hour and at 24 hours of PM-exposure. The sizes (in bases) of molecular weight markers are shown.

DISCUSSION

In the current study undertaken in HL-60 leukaemic cells, we have investigated the in vitro effects of low doses of the protein synthesis inhibitor Puromycin (PM), specifically with regard to its effects on c-myc mRNA levels and on apoptosis induction. We have shown that PM-elicited c-myc mRNA superinduction at 1 hour was associated with subsequent activation of the apoptotic cascade at 24 hours.

The expression of the c-myc proto-oncogene has been strongly but variously implicated in the regulation of the apoptotic cascade (8-16). Our finding that the up-regulation of c-myc mRNA transcription was found to precede subsequent apoptosis induction is consistent with the elevated c-myc mRNA levels seen in rat thymocytes undergoing apoptosis following dexamethasone treatment (8), and with the evidence that c-myc is a potent inducer of apoptosis in rat fibroblasts (9). Our data are also consistent with the observations that c-myc expression is a requirement for activation-induced apoptosis of T-cell hybridomas (10), for apoptotic regression of oestrogen-treated MCF-7 human mammary cancers (11), and for apoptosis elicited in c-myc amplified cell lines by the DNA topoisomerase II inhibitor VM-26 (12). Our findings are in contrast with our recent observation that c-myc mRNA levels are down-regulated in Mafosfamide treated cells undergoing apoptosis (13), with the down-regulation of the c-myc gene that has been found to precede IL-6-induced apoptosis of Y6 cells (14), steroid-induced apoptosis of

697 pre-B lymphocytes (15), and phorbol ester-induced apoptosis of U-937 cells (16).

The concentrations of PM that were employed to elicit the c-myc mRNA superinduction i.e. 0.9μM and 2μM, were much lower than those used previously (36-180μM) by others to generate this effect (5). Although some protein synthesis inhibition was noted at these low doses of PM, the magnitude of the superinduction (±180% at 2μM) seems out of proportion to the degree of protein synthesis inhibition which occurred (±15% at 2μM for 1 hour). In fact we have recently shown that c-myc mRNA superinduction was found to occur following exposure to PM at 0.2μM, a concentration which actually enhanced [C^{14}]Leucine incorporation (data not shown). These observations suggest that the c-myc mRNA superinduction may have been mediated through mechanisms which were distinct from protein synthesis inhibition (3,23,24), and which have been found by others to include interactions with signal transduction (3,23,24). For example, sub-inhibitory concentrations of the protein synthesis inhibitor anisomycin have been found to superinduce c-fos and c-jun mRNA through the phosphorylation of two chromatin-associated proteins pp53 and pp15 (3). Furthermore we have recently found low doses of PM to inhibit endogenous tyrosine kinase activity in HL-60 cells (25).

As far as the role of protein synthesis in the process of apoptosis is concerned, in the current context the apoptotic cascade was found to be activated despite protein synthesis inhibition which at 24 hours ranged from ±57% at 0.9μM to

±80% at 2μM, indicating that in this particular scenario the process of apoptosis was probably not dependent on de novo protein synthesis (18). Alternatively it may have been that apoptosis was dependent on de novo protein synthesis as has been reported by others (17). If so, it would follow that any proteins required for the apoptotic process were among those proteins whose synthesis was not inhibited. Finally it is possible that PM may itself have directly induced apoptosis, a phenomenon that has been reported to occur with other protein synthesis inhibitors (19).

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CHAPTER 8

Protein synthesis inhibition is not a requisite for Puromycin- and Cycloheximide-induced c-myc mRNA superinduction.

ABSTRACT

The phenomenon of superinduction refers to the process by which high doses (30-180μM) of the protein synthesis inhibitors Puromycin (PM) or Cycloheximide (CHX) augment and stabilize mRNA transcript levels through mechanisms which are dependent on the complete inhibition of protein synthesis. The current study undertaken in HL-60 leukaemic cells has dissociated the protein synthesis-inhibitory effects of PM and CHX from their c-myc mRNA superinducing effects. When employed at concentrations which were sub-inhibitory with respect to protein synthesis (0.2μM), PM and CHX nevertheless elicited the phenomenon of superinduction, possibly through mechanisms related to intracellular signalling.

INTRODUCTION

The overall regulation of eukaryotic gene expression is inextricably linked, inter alia, to factors governing mRNA stability and degradation, as evidenced by the strong implication of dysregulated mRNA turnover in the pathogenesis of a number of diseases including thalassemia and certain haematological malignancies (1). The steady-state levels of a specific mRNA species can be controlled at any one of the multiple steps involved in its metabolism i.e transcription initiation, transcription elongation, post-transcriptional RNA processing (addition of the 5' cap, the 3' poly(A) tail, and splicing), mRNA transport from the nucleus to the cytoplasm, translation, and cytoplasmic mRNA accumulation and degradation (1,2).

The term "superinduction" has traditionally been applied to the process by which complete protein synthesis inhibition results in the augmentation and stabilization of inducible mRNA transcripts (3). For example, high doses i.e. 36-180μM of the "classical" translation inhibitors Puromycin (PM) and Cycloheximide (CHX) have been used to elicit c-myc mRNA superinduction (4). Within the context of protein synthesis inhibition, the phenomenon of superinduction has been attributed to a number of different processes including mechanisms relating to the inhibition of translation per se (5), the inhibition of translation-dependent poly(A) tail shortening (4), and enhanced mRNA stability due to the loss of labile transcriptional repressors or RNAses (6,7).

Recently it has been recognised that superinduction may also be elicited through mechanisms which involve signal transduction pathways, and which are distinct from protein synthesis inhibition (3,8,9). For example, sub-inhibitory concentrations of the protein synthesis inhibitor anisomycin has been found to superinduce c-fos and c-jun mRNA, specifically through the phosphorylation of two chromatin-associated proteins pp33 and pp15 (3).

In the light of these findings we undertook to investigate the effects of the protein synthesis inhibitors PM and CHX on c-myc mRNA levels in HL-60 leukemic cells. We specifically employed concentrations which were sub-inhibitory with respect to protein synthesis in order to ascertain whether the phenomenon of superinduction persisted under these circumstances.

METHODS AND MATERIALS

Cell culture

HL-60 human promyelocytic leukemic cells (ATCC passage 17, Rockville, MD, USA) were seeded in RPMI 1640 medium supplemented with 10%(v/v) heat-inactivated fetal calf serum, 2mM L-glutamine, 50U/ml Penicillin, and 50μg/ml Streptomycin (GIBCO, USA). Cells were maintained at 37°C in a humidified atmosphere of air and 5% CO_2. PM and CHX (Sigma) were used as described.

Protein synthesis inhibition

0.5μCi [^{14}C]Leucine (specific activity 50Ci/mmol; Amersham) was added to cultures which were then further incubated at 37°C for one hour (10). At the conclusion of the incubation period, cells were washed three times in phosphate-buffered saline, 1ml of 10% trichloroacetic acid was added, and the tubes were then chilled on ice for 30 minutes. The precipitate so formed was filtered on GF/C filter papers (Whatmann), and washed three times with 5% trichloroacetic acid. The filters were dried and counted after adding aqueous counting scintillant. [^{14}C]Leucine incorporation was determined in triplicate. Replicates differed from each other by less than 10%.

c-myc mRNA expression

c-myc mRNA expression was investigated using standard techniques (11). 10μg of total extracted RNA was size-fractionated by 1.2% agarose

formaldehyde gel electrophoresis, and then transferred to and ultraviolet cross-linked to nylon membrane. The membrane was prehybridized at 42°C for 4 hours. Hybridization was to the [α-^{32}P]dCTP (Amersham) random-primer labelled 1.6kB SstI fragment from the 5' end of the c-myc gene (ATCC,USA). Following stringency washes at 42°C and at room temperature, the membrane was exposed at -70°C to film using image-intensifying screens. Finally the membrane was stripped before re-hybridization to the [γ-^{32}P]ATP (Amersham) end-labelled 40mer oligonucleotide antisense GAPDH probe (Oncogene Science, USA). GAPDH served as an internal control for constitutive gene expression. Autoradiograms were scanned densitometrically.

Materials

All other reagents were of analytical grade and were from standard commercial sources.

RESULTS

[C^{14}]Leucine incorporation studies

[^{14}C]Leucine incorporation studies showed that at a dose of 0.2μM, both PM and CHX were sub-inhibitory on protein synthesis (Figure 1). In fact at this concentration PM was actually found to boost protein synthesis.

c-myc mRNA Expression

Despite being employed at sub-inhibitory concentrations with respect to protein synthesis i.e. 0.2μM, both PM and CHX superinduced c-myc mRNA levels above that of the control at 1 hour (Figure 2). PM-elicited c-myc mRNA superinduction was not maximal at this concentration however, since higher concentrations of PM (0.9μM and 2μM) that inhibited [^{14}C]Leucine incorporation by $\geq$ 20%, were able to elicit greater degrees of superinduction (data not shown).

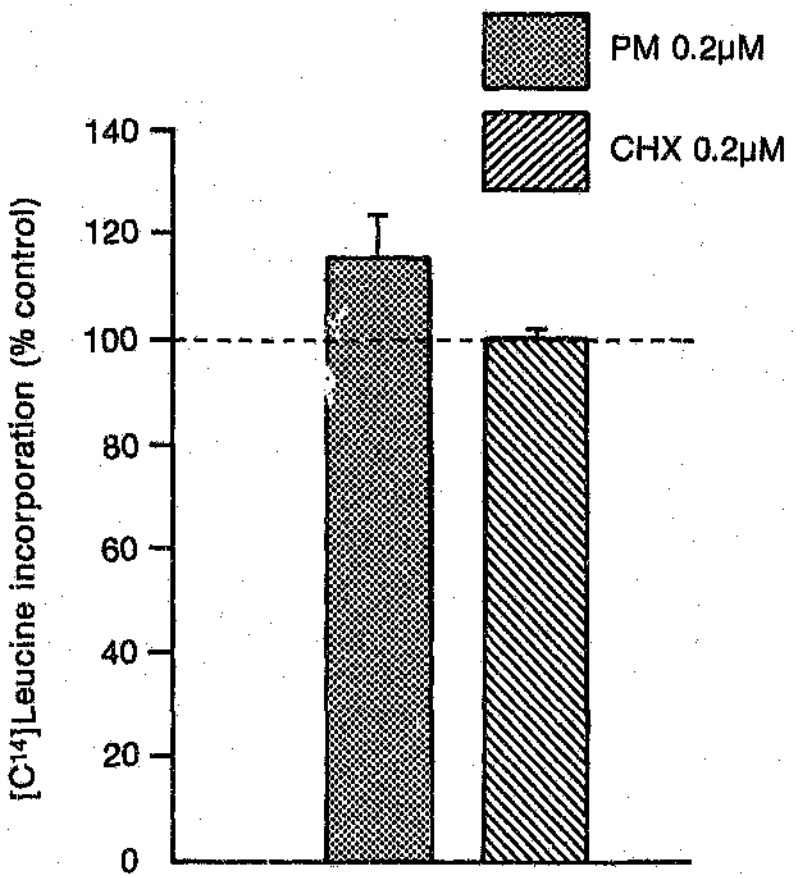

Figure 1
The effect of equivalent concentrations (0.2μM) of PM (0.1μg.ml) and CHX (0.05μg/ml) on [^{14}C]Leucine incorporation after one hour in HL-60 cells. Results are expressed as a percentage of the mean control value, and represent the mean ± SEM of triplicates.

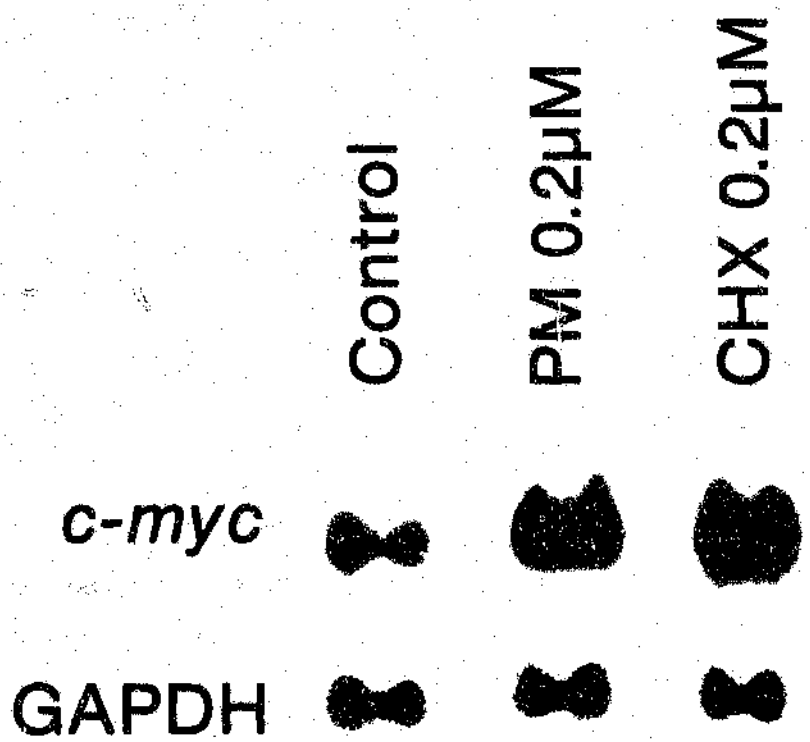

Figure 2
c-myc and (for reference) GAPDH mRNA levels after one hour in control, and in PM- and CHX-treated HL-60 cells (10μg total RNA loaded per lane). Similar results were obtained in three further experiments.

DISCUSSION

In this study we have been able to dissociate the protein synthesis-inhibitory effects of two "classical" protein synthesis inhibitors, PM and CHX, from their c-myc mRNA superinducing effects. When employed at sub-inhibitory concentrations (0.2μM) in HL-60 leukemic cells, PM and CHX nevertheless elicited superinduction, a phenomenon that has been evoked previously by high concentrations of these drugs that completely abolish protein synthesis i.e. 30-200μM (4,12,13,14).

Our findings are consistent with the recent recognition that superinduction may also occur through mechanisms which are distinct from protein synthesis inhibition (3,8,9). Such mechanisms have been found to include interactions with signal transduction pathways. For example, sub-inhibitory concentrations of the protein synthesis inhibitor anisomycin have been found to superinduce c-fos and c-jun mRNA, specifically through the phosphorylation of two chromatin-associated proteins pp33 and pp15 (3). EGTA has been found to superinduce PDGF- and Ca^{+2}-modulated c-myc mRNA expression (8), and Insulin-like-growth factor I has been found to up-regulate c-fos expression through the stimulation of tyrosine kinase activity (9). Since we have recently found low doses of PM to inhibit endogenous tyrosine kinase activity in HL-60 cells (15), we suggest that PM's superinducing effect in the current study may have been mediated through disruption of tyrosine kinase signal transduction pathways.

This report supports the notion that superinduction can be attributed to different mechanisms depending on the concentration of the protein synthesis inhibitor employed to elicit this phenomenon. Thus the superinduction demonstrated at sub-inhibitory concentrations of both PM and CHX clearly cannot be attributed to any of those mechanisms relying on protein synthesis inhibition (4,5,6,7). At higher, protein synthesis inhibitory concentrations, enhancement of c-myc mRNA transcript has been found by others (4,12) and ourselves (data not shown) in both PM- and CHX-treated cells (4,12), consistent with the interpretation of superinduction as being the consequence of impaired protein synthesis (4,5,6,7). The phenotypic and functional consequences of c-myc mRNA superinduction are likely to be very different depending on whether or not there is a concomitant inhibition of protein synthesis. Thus, at low concentrations of PM and CHX, the enhanced c-myc mRNA may be associated with equivalent enhancement of c-myc oncoprotein, whereas high concentrations of PM and CHX which inhibit protein synthesis are likely to attenuate c-myc oncoprotein levels. These issues, as well as the impact on signal transduction pathways of sub-inhibitory concentrations of PM and CHX, are currently being addressed in our laboratory.

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CONCLUSIONS

1. PM exerted a number of potent effects on the cell cycle when used at concentrations far below those used traditionally to completely block protein synthesis. These effects included a G2-arrest, a metaphase-mitotic-arrest, and apoptosis, and were found in a variety of malignant and non-malignant cell types of both human and murine origin. Since CHX failed to elicit similar effects, the data suggests that the PM-induced changes were likely to have been related to the different mechanisms of protein synthesis inhibition exerted by these two "classical" translation inhibitors Puromycin (PM) and Cycloheximide (CHX). PM, but not CHX, generates nascent peptidyl-PM complexes (PMPs), whose subsequent intracellular effects, may account, at least in part, for the differential cytokinetic effects and the tyrosine kinase inhibition (see point 3) elicited by these drugs. Furthermore the observation that the closely related substituted purines Puromycin aminonucleoside (PAN) and 6-Dimethylaminopurine (6-DMAP) did not exert detectable morphological and cytokinetic effects may also be attributable to their failure to generate nascent PMPs.

2. Although the role of protein synthesis in the cell cycle and in the process of apoptosis has been contentious, as has the relationship between cell cycle phase and apoptosis activation, it would appear that

much of the confusion surrounding the interrelationship between these variables reflects diverse contextual experimental scenarios in which they have not been independently controlled. This study therefore investigated the apoptotic process in cells synchronized with respect to the cell cycle, while maintaining tight control over protein synthesis. The data showed that in synchronized, PM-treated HL-60 cells, (i) the activation of the apoptotic cascade was cell cycle-dependent, and that (ii) the G2/apoptosis transition and (iii) the process of apoptosis itself were virtually protein synthesis-independent. The data suggest a working model which may help to reconcile at least some of the current apparently conflicting opinions on the issue, and also confirms the likelihood that inconsistencies reported in the literature represent contextual experimental variations (diversity in cell type, cell synchronicity, type and dose of protein synthesis inhibitors employed, extent of protein synthesis inhibition) rather than major controversies.

3. In addition low-dose PM-exposure resulted in potent and specific inhibition of tyrosine kinase activity as determined in soluble enzyme extracts prepared from 1 hour and 24 hour PM-treated HL-60 cells. Enzyme extracts derived from HL-60 cells treated with a dose of CHX that was equimolar to the dose of PM employed and that also inhibited protein synthesis to a similar extent as PM did, failed to inhibit phosphorylation of tyrosine kinase-specific substrate. This indicates that the tyrosine kinase-specific inhibition induced by PM cannot be

attributable to protein synthesis blockade per se, but may relate to the different mechanisms of action of PM and CHX.

4. When the in vitro effects of the cyclophosphamide derivative Mafosfamide were investigated in HL-60 cells, it was found that Mafosfamide induced apoptosis and that the apoptosis was associated with intercurrent S-Phase and G2-Phase cell-cycle-arrests in both synchronized and unsynchronized cells. Furthermore in the context of Mafosfamide-treated HL-60 cells, upregulation of c-myc mRNA transcription was found not to be fundamental for apoptosis induction since there was no change in c-myc mRNA levels after 1 hour of Mafosfamide-exposure, and a dose-dependent decrease in c-myc mRNA transcripts after 6 hours and 24 hours in Mafosfamide-treated cells. On the other hand PM-elicited c-myc mRNA superinduction evident at 1 hour was shown to be associated with subsequent activation of the apoptotic cascade at 24 hours.

5. In this study it has been possible to dissociate the protein synthesis-inhibitory effects of the two "classical" protein synthesis inhibitors, PM and CHX, from their c-myc mRNA superinducing effects. When employed at sub-inhibitory concentrations (0.2μM) with respect to protein synthesis in HL-60 cells, PM and CHX nevertheless elicited superinduction, a phenomenon that has been evoked previously onlyby high concentrations of these drugs that completely abolish protein

synthesis i.e. 30-200μM. This report supports the notion that superinduction can be attributed to different mechanisms depending on the concentration of the protein synthesis inhibitor employed to elicit this phenomenon. Thus the superinduction demonstrated at sub-inhibitory concentrations of both PM and CHX clearly cannot be attributed to any of those mechanisms relying on the inhibition of production of new proteins, but possibly may relate to interactions with signal transduction pathways.

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ABBREVIATIONS

PM	Puromycin
CHX	Cycloheximide
6-DMAP	6-Dimethylaminopurine
PAN	Puromycin aminonucleoside

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Cell-cycle disruptions and apoptosis induced by the cyclophosphamide derivative mafosfamide



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Abstract. The effect of the cyclophosphamide derivative mafosfamide (ASTA Z 7557) was investigated in vitro in HL60 leukemic cells. Mafosfamide, which rapidly generates 4-hydroxycyclophosphamide after aqueous dissolution, was employed at doses ranging from 0.1 to 10 μg/mL. In unsynchronized cells, mafosfamide exposure was associated with an S-phase accumulation, a suggestion of a G_2-phase arrest and morphological and biochemical evidence of apoptosis. In cells that had been synchronized by the double thymidine block method, S-phase progression was considerably delayed in the presence of mafosfamide. The apoptosis that was evident in mafosfamide-treated cells 12 hours after release from the thymidine block was found to occur in the presence of S-phase and G_2-phase cell-cycle arrests. Taken together, the current data suggest that mafosfamide may have potential synergism with other anticancer agents that elicit similar cell-cycle arrests as well as with chemotherapeutic drugs that activate the apoptotic cascade.

Key words: DNA fragments—Apoptosis—G_2-phase—Cell cycle—HL60—S-phase

Introduction. Cyclophosphamide is a widely employed alkylating cancer chemotherapeutic agent [1,2] whose cytotoxicity is mediated by the introduction into DNA of cross-links and single-strand breaks [3,4]. Cyclophosphamide derivatives are also frequently used in vitro to purge leukemic cells from bone marrow [4–6]. One such derivative is the prodrug mafosfamide, a cyclohexylamine salt that rapidly generates 4-hydroxycyclophosphamide after aqueous dissolution. 4-hydroxycyclophosphamide degrades irreversibly via aldophosphamide to phosphoramide mustard and acrolein [7-9], the therapeutically significant metabolites of cyclophosphamide [10].

A number of anticancer agents are able to induce apoptosis or programmed cell death, e.g., etoposide, cisplatin, melphalan, methotrexate, camptothecin, teniposide, amsacrine and 5-fluorouracil [11-16]. In the cases of etoposide [15] and cisplatin [16], a preceding G_2-phase cell-cycle arrest has been associated with the subsequent activation of the apoptotic cascade, whereas camptothecin, teniposide and amsacrine have been found to induce S-phase–specific internucleosomal DNA degradation [14]. In the current study, undertaken in vitro in human HL60 leukemic cells, we have investigated the cytokinetic consequences of the cyclophosphamide derivative mafosfamide and have evaluated its capacity to induce double-stranded DNA breaks and apoptosis.

Materials and methods

Cell culture and mafosfamide exposure. HL60 cells (ATCC passage 17, Rockville, MD) were seeded at 10^6 cells/mL in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin (50 U/mL) and streptomycin (50 μg/mL). Cells were maintained unagitated in logarithmic growth phase at 37°C in a humidified atmosphere of air and 5% CO_2. For synchronization experiments, the double thymidine block method was employed [17]. Cells seeded at a concentration of 10^6/mL were incubated for two 24-hour periods in the presence of 2 mM thymidine. The two 24-hour periods were separated by a 12-hour period in which cells were incubated in the absence of thymidine; i.e., they were released from the first thymidine block. Thymidine was removed after each 24-hour exposure by washing the cells 3 times in medium that had been prewarmed to 37°C. After release from the second thymidine block, cells were seeded at a final density of 10^6/mL and aliquots of cell suspensions were collected subsequent to various incubation intervals. Culture viability was assessed by the trypan blue dye exclusion technique, and after cells were fixed and stained with May-Grünwald-Giemsa, they were examined for mitotic figures and apoptotic cells. Aliquots of cells were subjected to flow cytometric analysis and DNA agarose gel electrophoresis. Mafosfamide crystalline powder (molecular weight 500.5) was a kind gift from Dr J. Pohl, ASTA PHARMA Pr Brock, Asta-Werke, Bielefeld, Germany. Fresh mafosfamide solution was prepared in RPMI medium immediately prior to use at final concentrations ranging from 0.1 to 10 μg/mL.

Flow cytometric analysis. Cells were prepared for analysis as described elsewhere [18]. At the conclusion of the designated incubation periods, the pellet obtained by centrifugation from 10^6 cells was resuspended in 1 mL of a hypotonic nuclear isolation medium (50 mg/L propidium iodide; 1 g/L Triton-X 100; 1 g/L sodium citrate), incubated on ice for 30 minutes, and then in DNAase-free RNAase at 37°C. Fluorescence emitted from the propidium iodide–DNA complex was quantitated after laser excitation of the fluorescent dye by the Coulter EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL). Cell debris and doublets were excluded [19], and the coefficient of variations of the control G_0G_1-phase and G_2M-phase peaks were less than 3%. Twenty-five thousand cells were counted for each analysis. Histogram scaling was the same for all samples. Limits of cell cycle phases as determined in the control DNA distributions were retained in the subsequent analysis of the mafosfamide-treated populations.

DNA fragmentation analysis. After appropriate incubation periods, cells that had initially been seeded at 10^6/mL were pelleted at 4°C and prepared for subsequent fragmentation analysis [20]. The pellet was resuspended in 70 µL of lysis buffer (10 mM EDTA; 50 mM Tris-HCl, pH=8.0; 0.5% sodium lauryl sarkosinate; 0.5 mg/mL proteinase K) and incubated for 1 hour at 50°C. A 10-µL aliquot of 1 mg/mL ribonuclease A was added to each sample prior to a further 1-hour incubation at 50°C. Samples were then heated to 70°C, after which the reaction was terminated by the addition of 20 µL of loading buffer (10 mM EDTA, pH=8.0; 0.25 bromophenol blue; 40% sucrose). Extracts were loaded into the wells of a 2% agarose gel and electrophoresed in a phosphate buffer (2 mM EDTA; 900 mM Tris-phosphate, pH=7.8). Both the gel and the buffer contained 1 µg/mL of ethidium bromide. *Bgl* I/*Hin*fI-cleaved pBR328 served as molecular weight markers.

Materials. All materials for cell culture were obtained from Gibco, Madison, WI. Propidium iodide was from Sigma. All other reagents were of analytical grade and were from standard commercial sources.

Results

Cell cycle arrests

Asynchronous cells. Flow cytometric changes in asynchronous cells were unremarkable after 6 hours of mafosfamide exposure (data not shown). By 24 hours, however, an S-phase accumulation was evident; it occurred progressively earlier in S-phase with higher doses of mafosfamide (Fig. 1A, B). At 24 hours, there was also a dose-dependent increase in the G_2M-phase (Fig. 1A, B). This increase reflects an absolute increase in the G_2-phase [14], since the M-phase decreased with increasing mafosfamide concentrations at this time-point (Fig. 1C). Further evidence for hold-ups at the S-phase and G_2-phase boundaries at 24 hours is the progressive decrease in the G_0G_1-phase (Fig. 1A, B) with increasing mafosfamide concentrations, suggesting continuing egress out of G_0G_1-phase into the S-phase, but limited, if any, influx into the G_0G_1-phase from the G_2M-phase.

Synchronized cells. S-phase progression was retarded in synchronized mafosfamide-treated HL60 cells released from the double thymidine block (Fig. 2). By 8 hours, more than 60% of synchronized control cells had reached the G_2M-phase compared with only ±15% of the mafosfamide-treated cells. By 12 hours, when ±73% of control cells had completed the previous G_2M-phase and were back in the G_0G_1-phase, the majority (>70%) of the mafosfamide-treated cells were still held up in the previous S- and G_2-phases.

Morphological and biochemical evidence of apoptosis

Unsynchronized cells. Apoptosis was not seen in unsynchronized mafosfamide-treated cells after 6 hours of exposure (data not shown). At 24 hours, however, there was morphological evidence of apoptosis (chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies) in mafosfamide-treated cells (Fig. 3). (An apoptotic body is a fragment of a larger apoptotic cell that may or may not contain nuclear components in the form of cleaved DNA.) This was confirmed ultrastructurally (data not shown) and by DNA electrophoresis, which showed the pattern of double-stranded DNA fragments that result from the activation of an endogenous endonuclease (Fig. 4). Apoptosis was also observed in mafosfamide-treated THP-1 and K562 cells (data not shown).

Synchronized cells. Apoptosis was not evident in synchronized mafosfamide-treated cells for up to 10 hours after release from the double thymidine block. At 12 hours, morphological apoptosis was present in these cells; this was confirmed by DNA electrophoresis (Fig. 5). The presence of apoptosis in these cells was coincident with both a late S-phase and a suggestion of a G_2-phase accumulation (Fig. 2). (The G_2M-phase accumulation observed in mafosfamide-exposed cells at 12 hours in Figure 2 reflects an absolute increase in the G_2-phase [14], since mitotic figures were not evident at this time-point in these cells (data not shown).

Cytotoxic effects

By 24 hours, a dose-dependent cytotoxicity was evident in unsynchronized mafosfamide-treated cells (Fig. 6).

Discussion

In this study, we investigated the in vitro effects of the cyclophosphamide derivative mafosfamide. Mafosfamide, which rapidly generates 4-hydroxycyclophosphamide after aqueous dissolution, was employed at doses ranging from 0.1 to 10 µg/mL in HL60 human leukemic cells. We found that mafosfamide induced morphological and biochemical evidence of apoptosis in both asynchronous and synchronized HL60 cells. This is similar to the findings of Marks and Fox [21] and to the apoptosis that has been observed in response to numerous other chemotherapeutic agents including etoposide, cisplatin, melphalan, methotrexate, camptothecin, teniposide, amsacrine and 5-fluorouracil [11-16]. Consistent with the known alkylating effects of cyclophosphamide [9], and with its capacity to introduce cross-links and single-strand breaks into DNA [4,8], we found that mafosfamide caused an S-phase accumulation in both synchronized and unsynchronized HL60 cells. Since the progression of DNA synthesis during S-phase is dependent on the sequential activation of banks of replicons so that activation of later replicons is contingent on the successful replication of previously activated replicons [22], it is likely that alkylating damage to DNA by mafosfamide precluded successful sequential replicon activation, disrupted S-phase progression and was responsible for the S-phase accumulation. Because apoptosis was found to occur simultaneously with the S-phase arrest in mafosfamide-treated cells, it is possible that apoptosis may have been activated, at least in part, subsequent to the arrest, as has been reported with the cancer chemotherapeutic agents camptothecin, teniposide and amsacrine, all of which induce S-phase-specific internucleosomal cleavage [14].

In addition to the apoptosis and the S-phase accumulation induced by mafosfamide, there was a suggestion of a G_2-phase arrest in both synchronized and unsynchronized HL60 cells, similar to the G_2-phase arrest elicited by cisplatin and etoposide [15,16]. We suggest that the apoptosis in mafosfamide-treated cells, in addition to occurring in S-phase, may have occurred in the G_2-phase, as has been shown to occur with both etoposide and cisplatin [15,16], where a preceding G_2-phase cell-cycle arrest is associated with subsequent activation of the apoptotic cascade.

In conclusion, we have shown that the cyclophosphamide derivative mafosfamide induced apoptosis in HL60 cells and that the apoptosis was associated with intercurrent S-phase and G_2-phase cell-cycle arrests. As such, we propose that mafosfamide may have potential synergism with other anticancer agents that elicit similar cell-cycle arrests, e.g., cisplatin and etoposide, and with cancer chemotherapeutic drugs that induce apoptosis, e.g., melphalan, methotrexate, camptothecin, teniposide, amsacrine, 5-fluorouracil, etoposide and cisplatin.

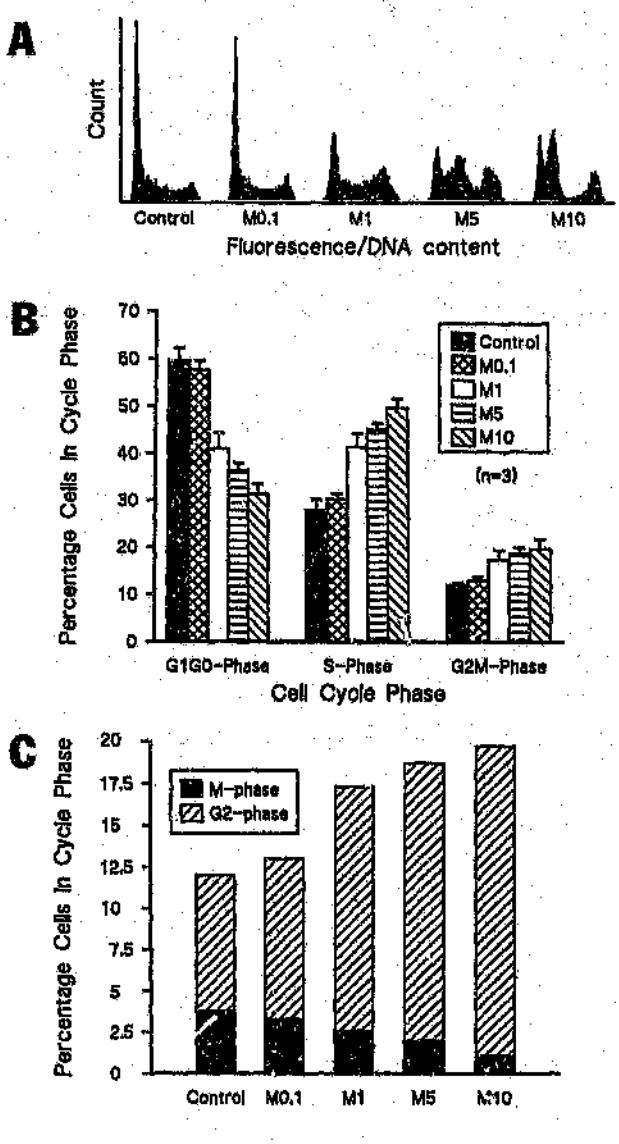


Fig. 1. A. Representative flow cytometric analysis at 24 hours of unsynchronized control and mafosfamide-treated HL60 cells. B. Effects on the DNA histogram of unsynchronized HL60 cells following 24 hours of exposure to increasing mafosfamide concentrations. Results are expressed as mean ± SD (n=3). C. Stacked-bar graphs illustrating the relative percentages of HL60 cells in the G_2- and M-phases of the cell cycle consequent on 24 hours of mafosfamide exposure. The M-phase at each concentration point is representative of the mean mitotic index of 2 experiments done on separate occasions. The G_2-phase was calculated by subtracting the M-phase value from the mean G_2M-phase value (n=3) obtained from Figure 2B [14]. All mafosfamide concentrations (M) are in µg/mL.

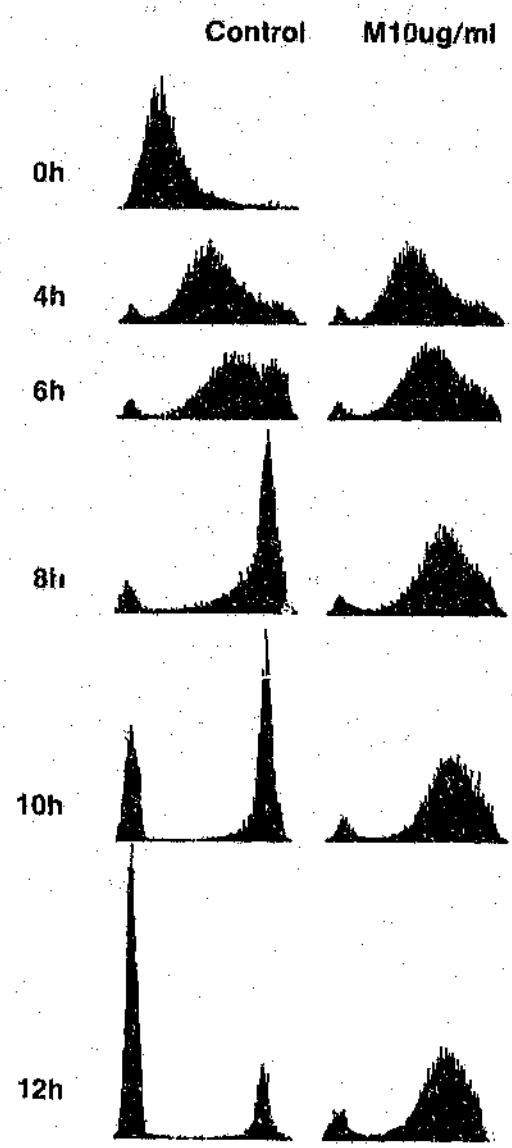


Fig. 2. Effects of mafosfamide exposure (10 µg/mL) on cell cycle progression of HL60 cells released from the double thymidine block. Note the delay in S-phase progression in mafosfamide-treated cells, as well as the late S-phase accumulation and G_2M-phase arrest that was evident 12 hours after release from the thymidine block.

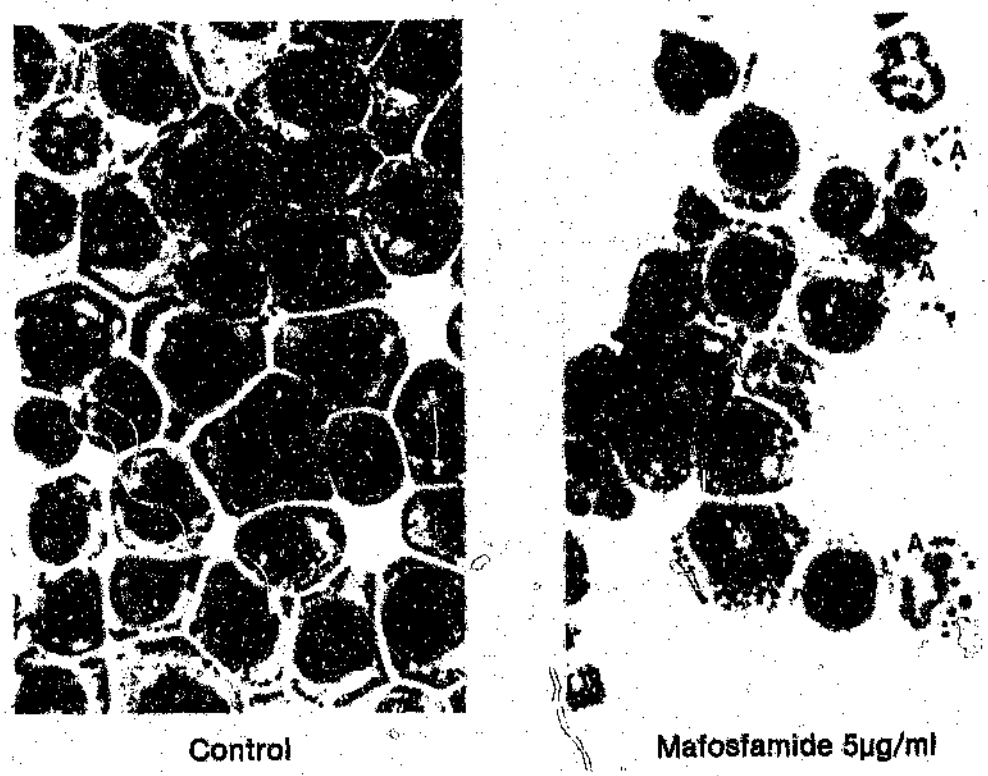
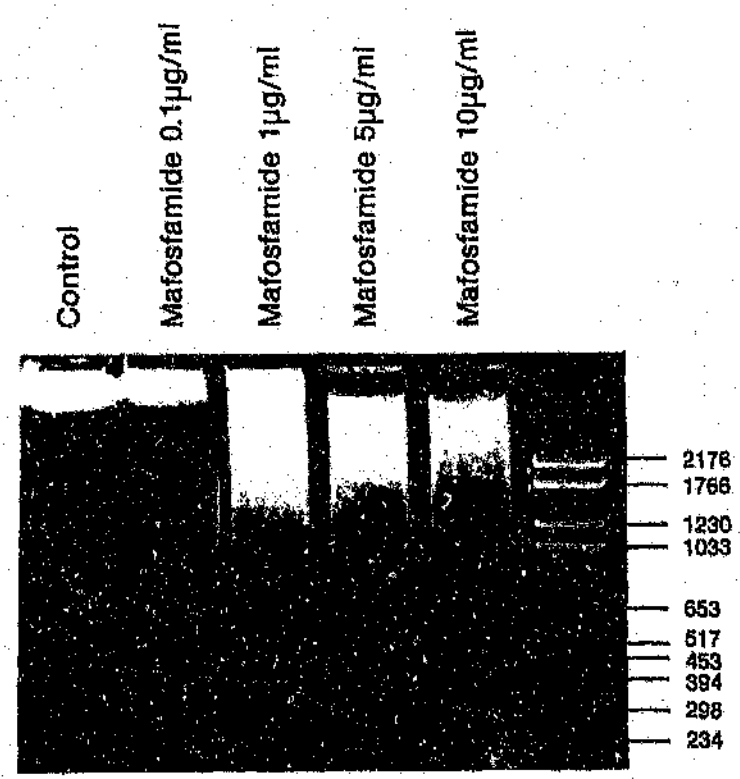


Fig. 3. Giemsa-stained cytospin preparations (magnification x1000) of unsynchronized HL60 cells. **A.** Control HL60 cells at 24 hours. **B.** After 24 hours of mafosfamide exposure (5 µg/mL), there was morphological evidence of apoptosis (A) in the form of chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies.

Fig. 4. Agarose gel electrophoresis of total DNA extracted at 24 hours from $1x10^6$ unsynchronized control and mafosfamide-treated HL60 cells (starting cell number at 0 hours was 10^6 cells). The sizes (in bases) of molecular weight markers are shown. Note the DNA fragmentation in cells treated with mafosfamide at concentrations >10 µg/mL.



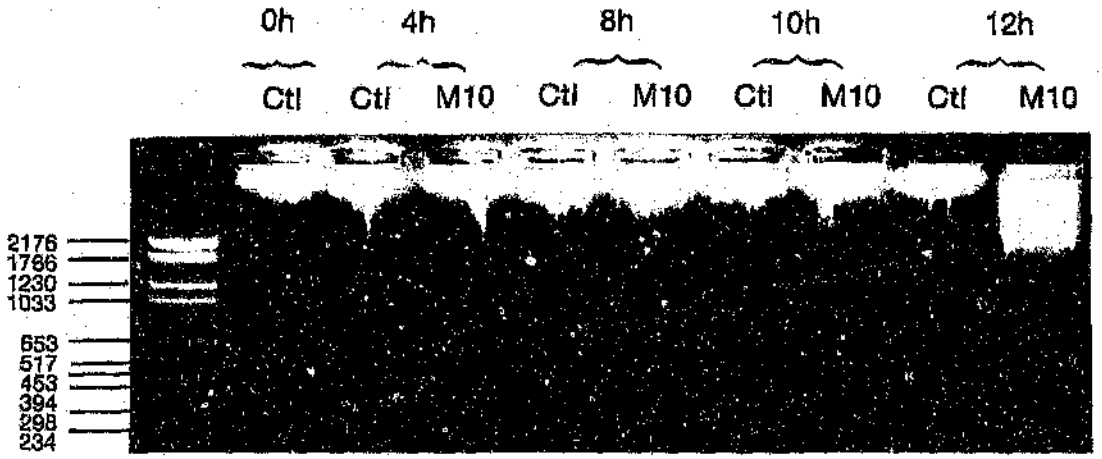


Fig. 5. Agarose gel electrophoresis of total DNA extracted from control and mafosfamide-treated (10 μg/mL) (M) HL60 cells at progressive time intervals after release from the double thymidine block (starting cell number at 0 hours was 10^6 cells). The sizes (in bases) of molecular weight markers are shown. Note the sudden onset of DNA fragmentation between 10 and 12 hours, corresponding to the late S-phase and G_2-phase cell-cycle arrests (see Fig. 2).

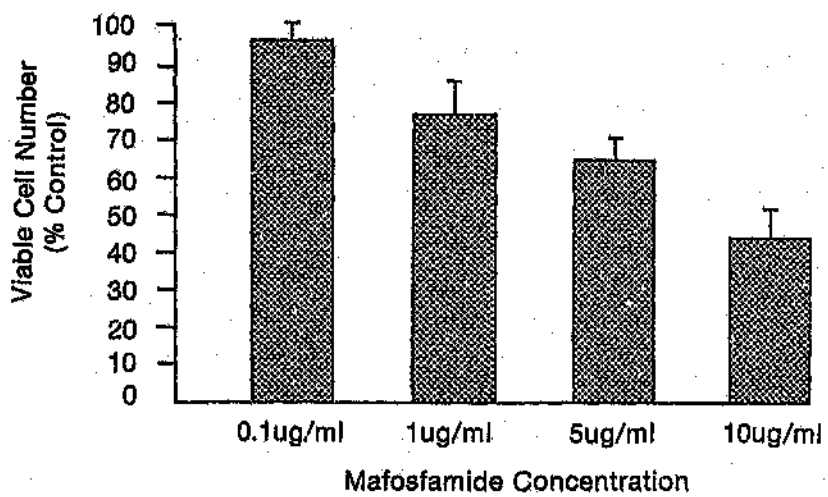


Fig. 6. Effect of mafosfamide on the viable cell number of unsynchronized HL60 cells after 24 hours of exposure. Results are expressed as a percentage of control values and reflect the mean ± SD of 2 separate experiments, each done in duplicate.

Acknowledgments

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Experimental Hematology

Substituted purines elicit differential cytokinetic, molecular and phenotypic effects in HL-60 cells

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Abstract. This study investigated and compared the cytokinetic, phenotypic and molecular effects elicited in HL-60 human leukemic cells by low doses (0.6 μM) of 3 closely related, substituted purines, puromycin (PM), puromycin aminonucleoside (PAN) and 6-dimethylaminopurine (6-DMAP). PM, but not 6-DMAP or PAN, inhibited [^{14}C]leucine incorporation, induced a time-related cytotoxic effect, a G_2-arrest, a metaphase-mitotic-arrest, apoptosis and c-*myc* mRNA superinduction. PAN and 6-DMAP exerted no detectable morphological or cytokinetic effects, although exposure to these drugs resulted in downregulation of c-*myc* mRNA levels. We suggest that the specific effects exerted by PM relate to the generation of nascent peptidyl-PM complexes by PM, but not by 6-DMAP or PAN.

Key words: HL-60—Puromycin—Puromycin aminonucleoside—6-dimethylaminopurine—c-*myc* mRNA

Introduction. The substituted purines, of which PM, PAN and 6-DMAP are prototypes (Fig. 1), have been found to exert a variety of biochemical and cytological effects when used at high concentrations ranging from 10 to 300 μM. For example, 6-DMAP, the purine component of both PM and PAN, reversibly arrests cells in the G_2 phase of the cell cycle by inhibiting phosphorylation [1]. The nucleoside analog PAN, which is a composite of 6-DMAP and 3-aminoribose, has been found to inhibit HIV-1 replication in vitro [2], and PM, itself a composite of PAN and tyrosine-o-methylether, is a functional analog of tyrosyl-transfer RNA and is well known for its inhibitory effect on protein synthesis [3]. Since low-dose PM was unexpectedly found to cause a metaphase-mitotic-arrest (submitted data), and because of its close structural relationship to PAN and 6-DMAP, we undertook to investigate and compare the cytokinetic, phenotypic and molecular effects these drugs elicited in HL-60 leukemic cells when employed specifically at low concentrations (0.6 μM).

Materials and methods

Cell culture. HL-60 human promyelocytic cells (passage 17, ATCC, Rockville, MD) were maintained in RPMI 1640 medium supplemented with fetal calf serum, L-glutamine and penicillin/streptomycin antibiotics (Gibco, Gaithersburg, MD). Cell cycle time was approximately 22 hours. PM, PAN and 6-DMAP (Sigma, St. Louis, MO) were used at a final concentration of 0.6 μM. Culture viability was assessed using the trypan blue dye exclusion technique. Cells were fixed and stained with May-Grünwald-Giemsa, and then examined for mitotic figures and apoptotic cells.

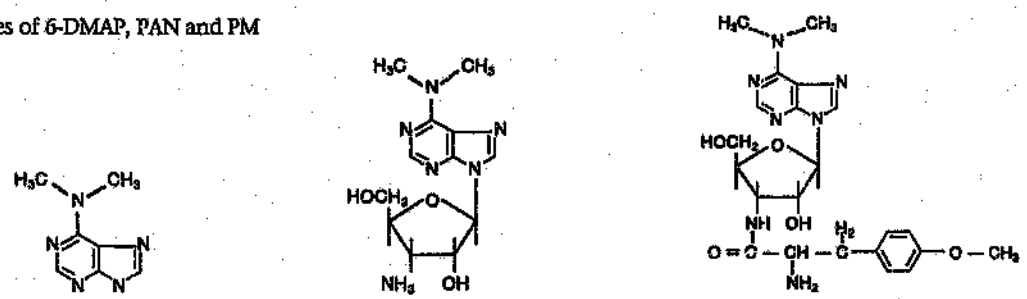
Protein synthesis inhibition. 0.5 μCi [^{14}C]leucine (specific activity 50 Ci/mM; Amersham, Aylesburg, Buckinghamshire, England) was added to cultures which were then further incubated at 37°C for 1 hour [4]. At the conclusion of the incubation period, cells were washed 3 times in phosphate buffered saline, 1 mL of 10% trichloroacetic acid was added, and the tubes were then chilled on ice for 30 minutes. The precipitate formed was filtered on GF/C filter papers (Whatmann Scientific Ltd., Maidstone, Kent, England), and washed 3 times with 5% trichloroacetic acid. The filters were dried and counted after adding aqueous counting scintillant. [^{14}C]leucine incorporation was determined in triplicate.

DNA fragmentation analysis. At the conclusion of the incubation period, the cells were pelleted at 4°C and prepared for subsequent fragmentation analysis [5]. The pellet was resuspended in 70 μL of lysis buffer (10 mM EDTA; 50 mM tris-HCl, pH 8.0; 0.5% sodium lauryl sarkosinate; 0.5 mg/mL proteinase K) and incubated for 1 hour at 50°C. A 10 μL aliquot of 1 mg/mL ribonuclease A was added to each sample prior to a further 1-hour incubation at 50°C. Samples were then heated to 70°C after which the reaction was terminated by the addition of 20 μL of loading buffer (10 mM EDTA, pH 8.0; 0.25% bromophenol blue; 40% sucrose). Extracts were loaded into the wells of a 2% agarose gel and electrophoresed in a phosphate buffer (2 mM EDTA; 800 mM tris-phosphate, pH 7.8). Both the gel and the buffer contained 1 μg/mL of ethidium bromide. *BglI/HinfI* cleaved pBR328 served as molecular weight markers.

Flow cytometric analysis. Cells were prepared for flow cytometric analysis as described [6]. The centrifuged cell pellet was resuspended in a hypotonic nuclear isolation medium (50 mg/L propidium iodide; 1 g/L Triton-X 100; 1 g/L sodium citrate), incubated on ice for 30 minutes and then at 37°C in 100 μg/mL RNAse A. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after laser excitation of the fluorescent dye by a Coulter EPICS-Profile-II flow cytometer (Coulter Electronics, Hialeah, FL). Cell debris and doublets were excluded [7]. The coefficients of variation of the peaks of the control G_0G_1 and the G_2M phases were less than 3%. Limits of cell cycle phases as determined in the control distributions were retained in the subsequent analysis of the drug-treated populations.

c-myc mRNA analysis. c-*myc* mRNA expression was investigated using standard techniques [8]. 10 μg of total extracted RNA

Fig. 1. Structures of 6-DMAP, PAN and PM



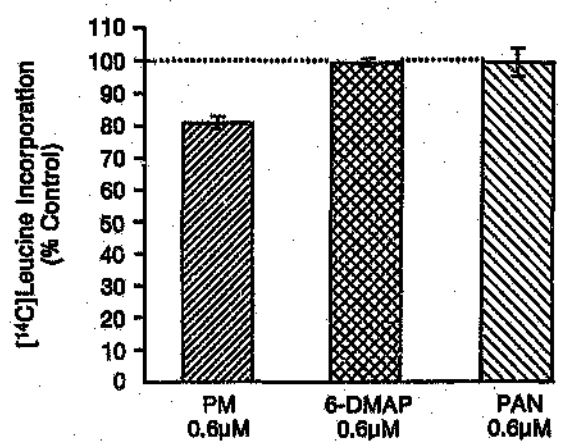


Fig. 2. The effect of PM, 6-DMAP and PAN on [^{14}C]leucine incorporation in HL-60 cells at 1 hour of incubation. Results were expressed as the percentage of control values, and represent the mean ± SD of 3 separate experiments, each done in triplicate.

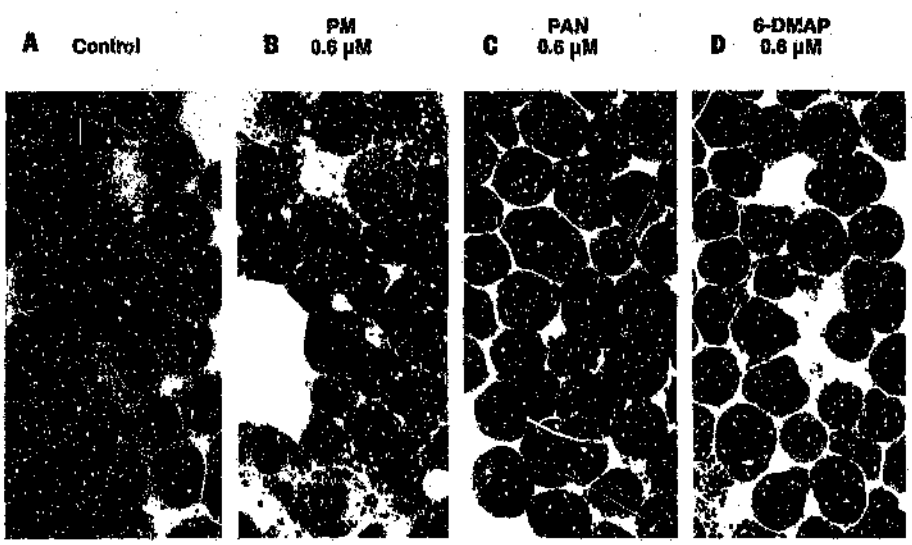


Fig. 3. Giemsa-stained cytospin preparations (magnification x1000) of 12-hour control and drug-treated HL-60 cells
A. Control HL-60 cells
B. PM-treated HL-60 cells showed a marked metaphase mitotic accumulation (M) by 12 hours. Certain PM-treated cells showed evidence of apoptosis (A).
C. 6-DMAP-treated HL-60 cells
D. PAN-treated HL-60 cells

was size-fractionated by 1.2% agarose formaldehyde gel electrophoresis, and then transferred and ultraviolet cross-linked to nylon membrane. The membrane was prehybridized at 42°C for 4 hours. Sequential hybridization at 42°C was to the [α-^{32}P]dCTP (Amersham) random-primer labeled 1.6kB *Sst*I fragment from the 5' end of the c-*myc* gene (ATCC), and to the [γ-^{32}P]ATP (Amersham) end-labeled 40mer oligonucleotide antisense glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Oncogene Science, Uniondale, NY). GAPDH served as an internal control for constitutive gene expression. Autoradiograms were scanned densitometrically.

Results

Effects on protein synthesis. At 1 hour of incubation, 0.6 μM PM had inhibited [^{14}C]leucine incorporation by >20% (Fig. 2). In contrast, 6-DMAP (0.6 μM) and PAN (0.6 μM) exerted no

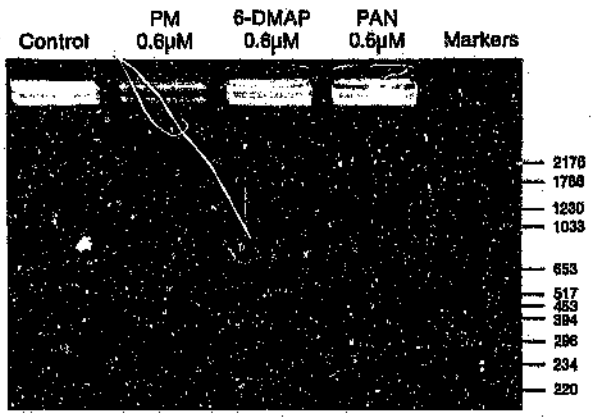


Fig. 4. Agarose gel electrophoresis of total DNA extracted at 24 hours from $1x10^6$ HL-60 cells treated as indicated. The size (in bases) of separated DNA molecular weight markers are shown.

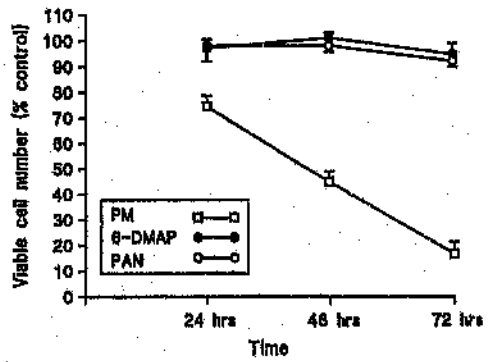


Fig. 5. Viable cell number in PM-, 6-DMAP- and PAN-treated HL-60 cells after 24, 48 and 72 hours. Results are expressed as a percentage of control values and represent the mean ± SD of 3 separate experiments, each done in duplicate. Viable cell number was not affected by drug treatment at 12 hours in relation to control.

detectable effect on [^{14}C]leucine incorporation.

Phenotypic, apoptotic and cytotoxic effects. After 12 hours (Fig. 3), HL-60 cells treated with equimolar concentrations of 6-DMAP and PAN were morphologically indistinguishable from control cells. In contrast, PM-treated cells showed marked metaphase mitotic accumulation and some evidence of apoptosis (chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies). Apoptosis in PM-treated cells was confirmed ultrastructurally (not shown), and by DNA electrophoresis at 24 hours which showed the pattern of fragmentation that results from endogenous endonuclease activation (Fig. 4). PM, but not PAN or 6-DMAP, exerted a time-related toxic effect on HL-60 cells as demonstrated by the reduction in viable cell number in PM-treated cells (Fig. 5).

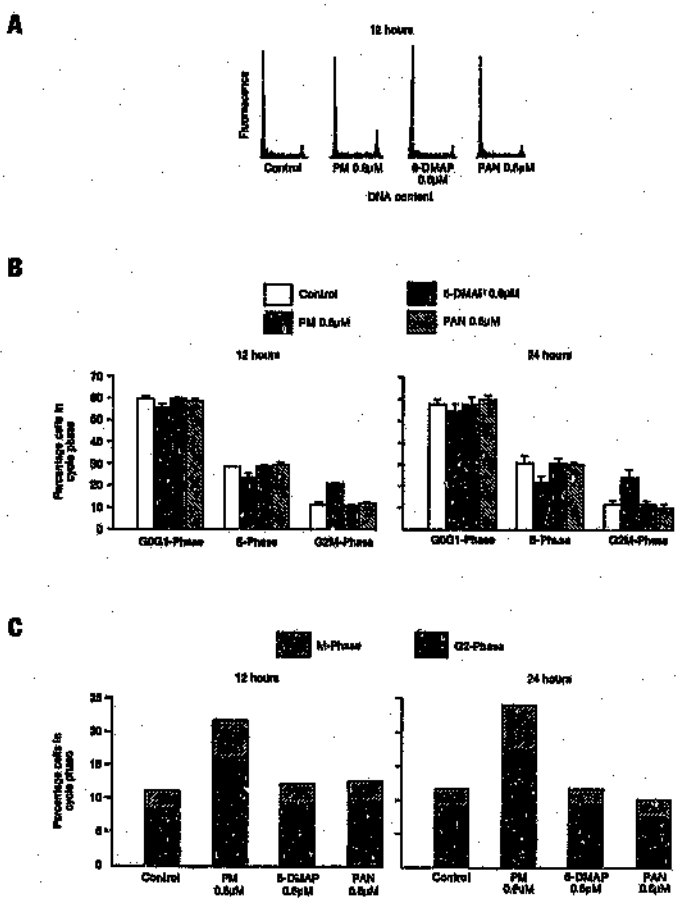


Fig. 6.
A. Representative flow cytometric analysis at 12 hours of count vs. fluorescence/DNA content of control, PM-, 6-DMAP- and PAN-treated HL-60 cells. 25,000 cells were counted for each analysis. Histogram scaling was the same for all samples.
B. Effects on the DNA histogram of HL-60 cells following 12 hours of exposure to PM, 6-DMAP and PAN. Results are expressed as mean ± SD (n=3).
C. Stacked-bar graphs illustrating the relative percentages of HL-60 cells in the G_2 and M phases of the cell cycle consequent on 12 hours of exposure to PM, PAN and 6-DMAP. The M phase at each concentration point is representative of the mean mitotic index (number of mitotic figures per 100 cells) of 2 experiments done on separate occasions. The G_2 phase was calculated by subtracting the M phase value from the mean G_2M phase value (n=3) obtained from Figure 2B.

Cytokinetic effects. PM induced a G_2 arrest as well as a marked M phase increase, with an associated reduction in the S phase of the cell cycle (Fig. 6A,B,C). These effects were time-related in that they were present at 12 hours, and were more marked at 24 hours. The increase in the M phase reflected a metaphase-arrest (Figs. 3,6C). PAN and 6-DMAP exerted no obvious cytokinetic effects at 12 and at 24 hours (Fig. 6).

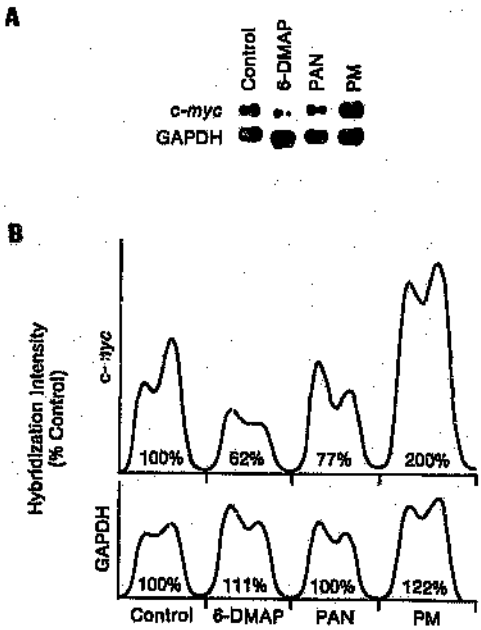


Fig. 7.
A. c-*myc* and (for reference) GAPDH mRNA expression levels at 12 hours in HL-60 cells treated with 0.6 μM PM, 6-DMAP and PAN, as indicated. 10 μg total RNA was loaded per lane.
B. Hybridization intensities were determined by densitometric scanning of the autoradiogram. The hybridization intensities of treated cells are shown relative to those of untreated control cells, which were normalized to 100%. Similar results were elicited on 1 further occasion.

Effect on c-myc mRNA levels. After 12 hours of exposure, PM was found to superinduce c-*myc* mRNA levels, whereas PAN and 6-DMAP exposure resulted in a decrease in the level of c-*myc* transcripts (Fig. 7A). It is of interest that PM exposure also appeared to superinduce GAPDH to ≥122% of control levels (Fig. 7B), although it superinduced c-*myc* to a much greater extent (≥200% of control levels).

Discussion

We found that low-dose equimolar concentrations of the structurally related purines PM, PAN and 6-DMAP elicited strikingly different morphological, cytokinetic and molecular effects in HL-60 human leukemic cells. PM, but not 6-DMAP or PAN, inhibited [^{14}C]leucine incorporation, induced a time-related cytotoxic effect, a G_2 arrest, a metaphase-mitotic arrest, morphological and biochemical evidence of apoptosis and c-*myc* mRNA superinduction. PAN and 6-DMAP exerted no detectable morphological or cytokinetic effects on HL-60 cells, although exposure to these drugs results in downregulation of c-*myc* mRNA levels.

The effects seen with PM-exposure are interesting since they were observed at a dose (0.6 μM) that is orders of magnitude lower than the doses of PM traditionally employed to inhibit protein synthesis (10 to 200 μM). This dose of PM (0.6 μM) nevertheless inhibited [^{14}C]leucine incorporation by >20% after 1 hour. PM-treatment is known to cause the premature release of truncated peptide chains which are bound through their carboxyl termini to PM [3], and which are rapidly degraded through an ubiquitin-dependent pathway [9]. Since the metaphase-to-anaphase transition is also mediated by precipitous ubiquitin-dependent cyclin degradation [10], it is likely that this pathway was preferentially diverted into PM-peptide proteolysis in PM-treated cells. One or more of the ubiquitin pathway components might have become limiting for cyclin degradation, resulting in the observed metaphase-mitotic arrest.

The G_2 arrest observed in PM-treated HL-60 cells might have been linked to subsequent apoptosis activation, since such an association has been recognized to exist in cells exposed to the anticancer agents etoposide [11], cisplatin [12] and mafosfamide (unpublished data). There are also precedents for the principle that cells deprived of signalling input enter the apoptotic pathway [13], and although the actual mechanism of the PM-induced G_2 arrest remains unresolved at this stage, we suggest that it may relate to the attenuated tyrosine kinase signal transduction that we have observed in PM-treated cells (submitted data). It is of interest that low-dose PM has recently been found to induce apoptosis in erythropoietin-dependent murine erythroblasts [14].

As far as the PM-induced c-*myc* mRNA superinduction is concerned, our finding is consistent with that of others [15], and can be attributed to a number of different processes related to protein synthesis inhibition. These include the inhibition of translation per se [16], the inhibition of translation-dependent poly(A) tail shortening [15] and enhanced mRNA stability due to the loss of labile transcriptional repressors or RNAses [17,18]. Again it is of interest that we were able to elicit the phenomenon of c-*myc* mRNA superinduction at a much lower dose than used previously—0.6 μM compared to 180 μM [15].

In contrast to the c-*myc* mRNA superinduction observed with PM-exposure, both 6-DMAP and PAN downregulated c-*myc* transcripts in HL-60 cells. As far as 6-DMAP is concerned, this may have been related to the known kinase-inhibiting properties of 6-DMAP [1,19], since another substituted purine, 2-aminopurine, an inhibitor of double-stranded RNA-dependent protein kinase, has been found to downregulate c-*myc* mRNA levels [20,21,22].

Although 6-DMAP arrests *Xenopus* oocytes in the G_2 phase of the cell cycle when employed at a concentration of 300 μM [1], no detectable cytokinetic effects were elicited in HL-60 cells when 6-DMAP (and also PAN) were used at a much lower dose (0.6 μM).

Despite their close structural relationships (6-DMAP is the purine component of both PM and PAN, PAN is a composite of 6-DMAP and 3-aminoribose, and PM is a composite of PAN and tyrosine-o-methylether), PM, 6-DMAP and PAN varied greatly in their capacity to elicit morphological, cytokinetic and molecular effects when employed at low, equimolar concentrations in HL-60 cells. This is likely to relate to the ability of PM, but not 6-DMAP and PAN, to generate the nascent polypeptide chains likely to be responsible for the effects elicited by PM, phenomena which are currently being investigated further in our laboratory. Of particular note are the unexpected effects exerted by PM, and in this context it is likely that the traditional usage of PM at high doses to inhibit protein synthesis has obscured its potent cytokinetic effects. In this regard, low-dose PM should be a useful adjunctive investigative tool in studies pertaining to the cell cycle and to apoptosis. Furthermore, although the use of PM as a single antineoplastic agent was not fruitful [23], this drug may nevertheless have a role to play, at low doses, in rational combi-

nation cancer chemotherapy. At such doses PM could differentially accumulate cells in metaphase to possibly increase their susceptibility to administration of a metaphase-specific agent such as vincristine, and in synergism with etoposide, cisplatin and mafosfamide. PM could potentially promote tumor regression consequent on apoptotic cascade activation. Clinical trials using low-dose PM in combination with some of these agents are currently under investigation in our laboratory in dogs presenting with spontaneous lymphoproliferative disorders.

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Puromycin is a Potent and Specific Inhibitor of Tyrosine Kinase Activity in HL-60 cells

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Abstract. *The search for specific tyrosine kinase inhibitors constitutes a novel approach to the development of anticancer agents. Puromycin (PM) inhibits protein synthesis by causing the premature release of truncated PM-peptide complexes (PMPs), the structure of which predicts an inhibitory effect on tyrosine kinase activity (21). The present study was undertaken to investigate the effects of a low dose of PM (0.9 μM) on tyrosine kinase activity in HL-60 leukaemic cells. Experiments were specifically controlled to exclude inhibitory effects consequent on protein synthesis blockade. Soluble enzyme extracts derived from PM-treated cells showed attentuated phosphorylation of two independent synthetic tyrosine kinase-specific substrates, the polymer Poly(Glu:Tyr; 4:1) and the peptide substrate Raytide. In contrast, Protein Kinase C-specific activity was unaffected by PM-exposure. It is possible that inhibition was due to metabolites of PM, perhaps PMPs, rather than to PM itself. Whether the inhibition of tyrosine kinase activity observed with the substrates used in this study is equally applicable to all tyrosine kinases, is not yet clear. Nevertheless, low-dose PM could provide a useful tool for dissecting out the role of tyrosine phosphorylation in the regulation and dysregulation of cell growth, and could conceivably prove beneficial in the treatment of tumours in which tyrosine kinase overactivity is linked to oncogenesis.*

Phosphorylation of protein tyrosine plays a pivotal role in the regulation of normal cell growth and proliferation, and in neoplastic transformation. In the cell cycle, the G2- to M-Phase transition is controlled by phosphorylation/ dephosphorylation reactions involving the Tyrosine-15 residue of the $p34^{cdc2}$ kinase (1). In addition a number of transmembrane growth factor receptors (including those of Epidermal Growth Factor, Platelet-derived Growth Factor, and Insulin-like Growth Factor), as well as a variety of cytosolic non-receptor-associated oncogene products (such as pp60*src*, p59*fyn*, p62*yes*, etc), exhibit tyrosine kinase activity (2, 3, 4, 5, 6).

The search for specific tyrosine kinase inhibitors represents a novel approach to the development of anticancer agents (7, 8, 9), in that it is based on the modification of transmembrane and intracytoplasmic tyrosine kinase signal transduction, rather than on the killing of tumour cells by inhibition of transcription, translation, and DNA synthesis (10, 11). Quercetin (12, 13), genistein (9), erbstatin (14), amiloride (15), and herbimycin A (16), have all proved effective against tyrosine kinases. Problems of toxicity, specificity, and drug delivery to tumours have limited their clinical applications however. For example, although genistein is a much more specific inhibitor of tyrosine kinases than is quercetin, its toxicity has precluded its *in vivo* use in patients to date. More recently, derivatives of tyrphostin other than erbstatin have been found to inhibit EGF-stimulated growth of squamous carcinoma cells, *in vitro*, and in nude mice inoculated with such cells (17).

The antibiotic Puromycin (PM) is well known for its inhibitory effect on protein synthesis (18). Structurally it is a composite of the aminonucleoside dimethyladenosine, and tyrosine-o-methylether (19). Functionally it is an analogue of peptidyl-transferRNA, more specifically of tyrosyl-transfer RNA. At concentrations of 10-100μm PM blocks protein synthesis completely by causing the premature release of truncated peptide chains which are bound to PM through their carboxyl termini. Since the nascent PM-peptide complexes (PMPs) are rapidly degraded through a ubiquitin-dependent pathway (20), their metabolic effects are poorly understood. The PMPs are of interest however because their structure predicts an inhibitory effect on tyrosine kinase activity (21). In this regard it is noteworthy that the carboxy-

Abbreviations: PM, Puromycin; CHX, Cycloheximide.

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terminal tyrosine residues of PMPs are reminiscent of synthetic random polymers of tyrosine-containing aminoacids, some of which have been found to act as competitive inhibitors of endogenous tyrosine kinase (22).

The present study was undertaken to investigate the effects of PM and its metabolites on cellular tyrosine kinase activity. The experiments were specifically controlled to exclude inhibitory effects on tyrosine kinase activity consequent to protein synthesis blockade.

Materials and Methods

Cell culture. HL-60 human promyelocytic leukaemic cells (Passage 17, ATCC, Rockville, MD, USA), were maintained in logarithmic growth phase at 37°C in a humidified atmosphere of air and 5% CO_2. Cells were seeded at $1X10^6$ cells/ml in RMPI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, as well as 2mM L-glutamine, 50U/ml Penicillin, and 50µg/ml Streptomycin (all from GIBCO, Madison, WI, USA). Puromycin (PM) and Cycloheximide (CHX) were obtained from Sigma, and were used as described. Culture viability was assessed using the trypan blue dye exclusion technique. The viability at 24 hours of PM- and CHX-treated cultures was essentially unaffected by concentrations up to 0.5µg/ml (Figure 1a). At higher concentrations ≥1µg/ml, the viability of both PM- and CHX-treated cultures was compromised, with the viability of PM-treated cultures being affected to a greater extent.

Protein synthesis inhibition. 0.5µCi [^{14}C]Leucine (specific activity 50 Ci/mmol; Amersham) was added to cultures which were then further incubated at 37°C for one hour. At the conclusion of incubation [^{14}C]Leucine incorporation was determined in triplicate as described (23). The dose-response curves expressing the inhibitory activity of PM on protein synthesis, as measured by [^{14}C]Leucine incorporation, are presented in Figure 1b. At 1 hour and at 24 hours, at a concentration of 0.9µM (0.25µg/ml), CHX was found to inhibit [^{14}C]Leucine incorporation to a similar extent as did PM used at a concentration of 0.9µM (0.5µg/ml) i.e. approximately 20-30% at 1 hour and approximately 50% at 24 hours (Figure 1c).

Preparation of solubilized enzyme extracts. Cellular extracts representing soluble tyrosine kinases were prepared as described (24). Briefly, at the conclusion of incubation, cells from 10ml of suspension culture were collected by low-speed centrifugation (500g) and washed three times in PBS. Culture viability (as assessed by the trypan blue dye exclusion technique) was consistently ≥85%. The final cell pellet was resuspended in four volumes of extraction buffer (50mM HEPES/NaOH, ph=7.5; 2mM EGTA; 10mM DTT; 1mM PMSF; 5µg/ml aprotinin; 0.1%(w/v) Triton X-100). The cell suspension so formed was sonicated for 10 seconds at 4°C, and then centrifuged at 15,000g for 30 minutes at 4°C. The supernatant was collected and 10%(v/v) of glycerol was added. Protein content of an aliquot of the supernatant was determined (25) using bovine serum albumin as control.

Determination of tyrosine kinase activity using Poly(Glu:Tyr;4:1) as substrate. Tyrosine kinase activity was assessed using a substrate assay described previously (26), involving the incorporation of [^{32}P] from [γ-^{32}P]ATP into the exogenous synthetic polymer substrate Poly (Glu:Tyr;4:1). The final reaction volume of 50µl contained 50mM Tris-HCL (pH=7.5), 30mM $MgCl_2$, 100µM Sodium-*ortho*-vanadate, 150µM ATP, 30µCi of [γ-^{32}P]ATP (6000 Ci/mmol; 10mCi/ml), 2mg/ml of exogenous tyrosine kinase-specific substrate Poly(Glu:Tyr;4:1), and suitably diluted enzyme preparation (10µg protein). The reaction was initiated by the addition of ATP at 30°C. Termination of the reaction after 15 minutes was by applying 25µl of the reaction mixture to Whatman 3MM paper squares (3cm X 3cm). These were washed four times for 15 minutes each in a 10% TCA solution containing 10 mM sodium pyrophosphate. Two final washes for a further 15 minutes each were first in ethanol and then in acetone. The papers were dried and then [^{32}P] incorporation of reaction triplicates was determined by liquid scintillation spectrometry. Appropriate reaction mixtures with or without substrate, and with or without extract served as controls. The activity of purified $pp60^{c\text{-}src}$ tyrosine kinase was also measured using Poly(Glu:Tyr;4:1) as substrate.

Tyrosine Kinase activity using Reytide as substrate. Tyrosine kinase

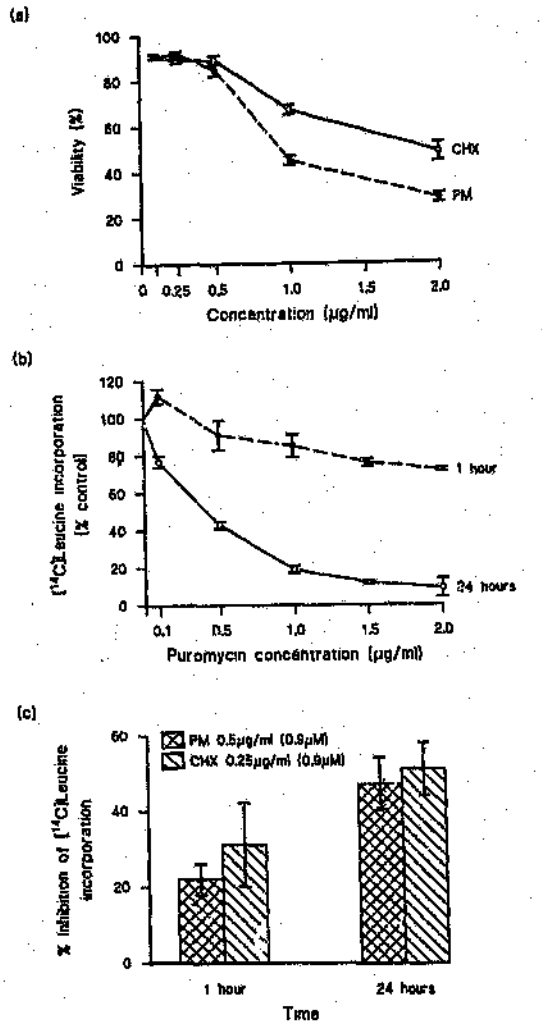

Figure 1 *a) Viability of cultures (as determined by the trypan blue dye exclusion technique) after 24 hours of exposure to the indicated concentrations of PM and CHX. Results represent the mean ± SD of three separate experiments each done in duplicate.*
b) The effect of increasing doses of PM (µg/ml) on [^{14}C]Leucine incorporation. Results were expressed as the percentage of control values and represent the mean ± SD of three separate experiments each done in triplicate.
c) Effect of equivalent molar concentrations (0.9µM) of PM (0.5µg/ml) and CHX (0.25µg/ml) on inhibition of [^{14}C]Leucine incorporation. Results represent the mean ± SD of three separate experiments each done in triplicate.

activity was determined with Raytide as substrate (27) in a final reaction volume of 50µl containing 50mM HEPES (pH=7.5), 0.1mM EDTA, 0.015% Brij 35, 150µM ATP, 30mM $MgCl_2$, 30µCi of [γ-^{32}P]ATP (6000 Ci/mmol; 10 mCi/ml) 0.2 mg/ml of the exogenous tyrosine kinase-specific substrate Raytide, and 10µg suitably diluted enzyme preparation. The reaction was terminated by adding 120µl of 10% phosphoric acid. The sample was vortexed, and 120µl applied onto 3cm X 3 cm Whatman P81 ion exchange chromatography paper. The papers were washed extensively in 0.5% phosphoric acid before a further wash with acetone. The papers were dried and [^{32}P] incorporation into Raytide of reaction triplicates was determined by liquid scintillation counting. Appropriate controls were used (as described above).

Analysis of tyrosine kinase activity. Tyrosine kinase activity was determined in solubilized enzyme extracts derived from 1 hour and 24 hour control, PM-treated (0.9µM), and CHX-treated (0.9µM) HL-60 cells. Appropriate reaction mixture with or without substrate, and with or without extract served as controls. Extract incorporation of [^{32}P] in the absence of substrate represented endogenous phosphorylation. Net specific tyrosine phosphorylation was represented by the difference between extract incorporation of [^{32}P] in the presence and in the absence of substrate, and corrected for substrate background. Net tyrosine kinase-specific activity of extracts of PM- and CHX-treated cells were expressed as a percentage of the corresponding tyrosine kinase-specific activity of extracts of untreated cells. Endogenous phosphorylation of extracts of PM- and CHX-treated cells were expressed as a percentage of the endogenous phosphorylation present in the extracts derived from untreated cells. In order to try and ascertain whether PM itself, or whether its metabolites were affecting tyrosine kinase activity, a series of experiments were done in which PM (0.9µM) and CHX (0.9µM) were added directly to reaction mixtures containing either extracts of untreated cells, or to purified $pp60^{c\text{-}src}$.

Protein kinase C activity. Protein kinase C activity was determined using a commercially available Protein Kinase C enzyme assay system from Amersham. The incorporation of [^{32}P] from [γ-^{32}P]ATP into a Protein Kinase C specific peptide substrate was determined. The final reaction volume of 75µl contained the recommended kit components as well as 20µg of suitably diluted enzyme preparation and 30µCi of [γ-^{32}P]ATP (6000 Ci/mmol; 10mCi/ml). Reaction samples were equilibrated at 25°C for 5 minutes. Each reaction was initiated by the addition of ATP at 25°C. After 15 minutes the reaction was terminated by adding 100µl of dilute acid. One hundred and twenty-five microlitres of the reaction mixture was applied onto 3cm X 3 cm Whatman P81 ion exchange chromatography paper. The papers were washed twice, for 10 minutes each time, in 75mM *ortho* Phosphoric acid. After washing, the papers were dried before [^{32}P] incorporation of reaction triplicates was determined by liquid scintillation spectrometry. Relevant controls were used as described previously. Analysis of Protein Kinase C activity followed the same method as that used for analysis of tyrosine kinase activity.

Flow cytometric analysis of phosphotyrosine content. Simultaneous flow cytometric analysis of phosphotyrosine and DNA content was carried out by a modification of a method described by others (28). $2X10^6$ HL-60 cells were fixed in 2ml of ice cold 70% ethanol for 30 minutes at 4°C. Cells were washed twice in PBS, and then resuspended in 380µl of PBS containing 1% bovine serum albumin. Twenty microlitres of fluorescein FITC-conjugated anti-phosphotyrosine monoclonal antibody (Boehringer Mannheim; Batch 12244820-01) was added to the suspension which was then incubated for 45 minutes in the dark at room temperature. Following two further washes with PBS, the cells were resuspended in 1ml of PBS containing RNAse (10µg/ml) at 37°C for 10 minutes. The cells were washed again and then resuspended in 1ml of propidium iodide (10µg/ml) in PBS for 30 minutes. After laser excitation (488 nm) of the fluorescent complexes by the Coulter-EPICS-Profile II flow cytometer, red fluorescence from propidium iodide was recorded on a linear scale as a measure of total DNA content. Green fluorescence from FITC was logarithmically amplified to represent phosphotyrosine content. Twenty-five thousand cells were counted for each sample. According to the manufacturers, in this context, anti-phosphotyrosine fluorescence staining is proportional to the amount of tyrosine phosphorylated proteins as determined by phosphoamino acid analysis, since the antibody shows no cross-reactivity with any other phosphorylated amino acids (Boehringer Mannheim). Since the FITC-anti-phosphotyrosine monoclonal antibody was of the IgG1 subtype, FITC-conjugated control IgG1 antibody was used to exclude the presence of non-specific binding.

Results

Tyrosine kinase activity of extracts of PM-treated cells. Using Poly (Glu:Tyr;4:1) as substrate we showed inhibition of tyrosine kinase activity in extracts of PM-treated cells (0.9µM) - approximately 15% inhibition after 1 hour, and approximately 25% inhibition after 24 hours (Figure 2a). In contrast no inhibition of tyrosine kinase activity was seen in extracts of CHX-treated cells after these time intervals. At the dose used (0.9µM), CHX inhibited [^{14}C]Leucine incorporation into HL-60 cells to a similar extent as did 0.9µM of PM, i.e. approximately 20-30% after 1 hour, and approximately 50% after 24 hours (Figure 1c). There was no inhibition of tyrosine kinase activity when the same concentration of PM was added directly to reaction mixtures containing 10µg control enzyme extracts (Figure 2a), suggesting that some metabolite/s of PM, rather than the drug PM itself, could have been responsible for the observed tyrosine kinase inhibition. To explore this phenomenon further, we used purified $pp60^{c\text{-}src}$ as a source of specific tyrosine activity, and poly(Glu:Tyr;4:1) as a tyrosine kinase-specific substrate (Figure 3). When PM drug (0.9µM) was added directly to this system, specific tyrosine kinase activity of $pp60^{c\text{-}src}$ was not inhibited.

Inhibition of tyrosine kinase activity in extracts of PM-treated cells was confirmed using another tyrosine kinase-specific substrate Raytide (Figure 4a). In fact inhibition was more dramatic (approximately 80%) with Raytide substrate than with Poly(Glu:Tyr;4:1) substrate.

Endogenous kinase activity of extracts of PM-treated cells. Endogenous kinase activity of extracts of PM-treated cells was indistinguishable from control extract activity (Figure 2b and 4b). This confirmed that the reduced ability of extracts of PM-treated cells to phosphorylate tyrosine kinase-specific substrates was due to specific inhibition of tyrosine kinase activity, rather than to the overall reduction in endogenous kinase activity.

Phosphotyrosine content of PM-treated HL-60 cells. After 24 hours PM-treated HL-60 cells had a reduced phosphotyrosine content as reflected by the downwards shift on the Y-axis of log green fluorescence (Figure 5). This was consistent with the inhibition of tyrosine kinase activity observed in extracts of PM-treated cells when Raytide and Poly(Glu:Tyr;4:1) were used as the tyrosine kinase-specific substrates (Figures 2a and 4a). Whether the reduced phosphotyrosine content

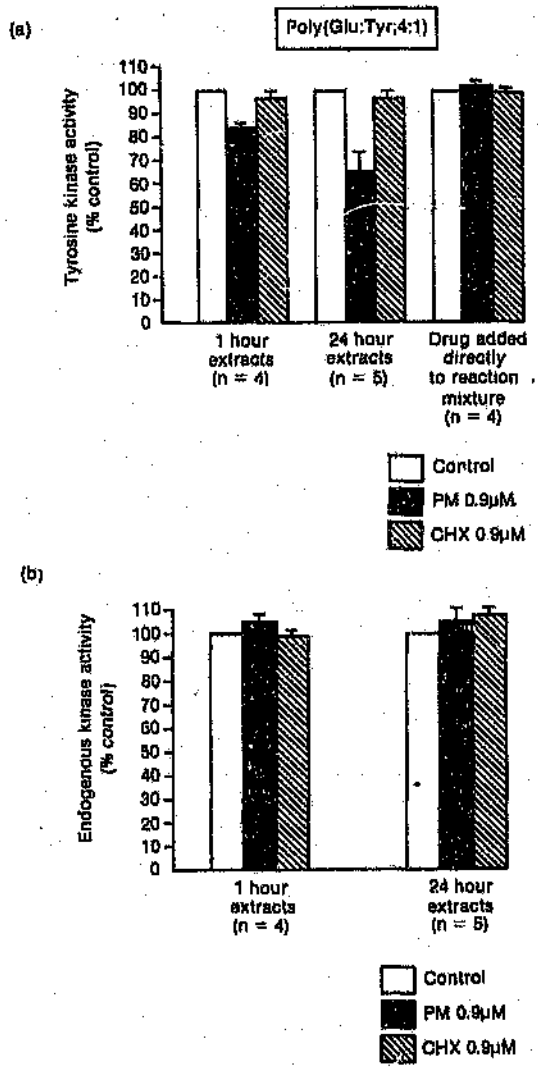

Figure 2 *a) Soluble tyrosine kinase activity using Poly(Glu:Tyr;4:1) as substrate in cellular extracts derived from untreated HL-60 cells and from cells pre-treated for 1 and 24 hours with 0.9μM PM, or 0.9μM CHX. Shown also are the results of four experiments in which each of the drugs were added directly to reaction mixtures containing 10 μg of soluble enzyme protein extract derived from untreated cells.*
b) Endogenous kinase activity in the cellular extracts described in (a).

Results are expressed as (a) a percentage of tyrosine kinase activity in untreated extracts, or as (b) a percentage of endogenous kinase activity in untreated extracts, and are represented as mean ± SEM derived from separate experiments (n=4/5) each done in triplicate.

was a result of decreased synthesis or increased degradation of phosphotyrosine-containing proteins, or due to reduced phosphorylation of tyrosine residues by tyrosine-specific kinases, remains to be determined.

Protein Kinase C activity of PM-treated extracts. There was no inhibition of Protein Kinase C activity in extracts of PM-

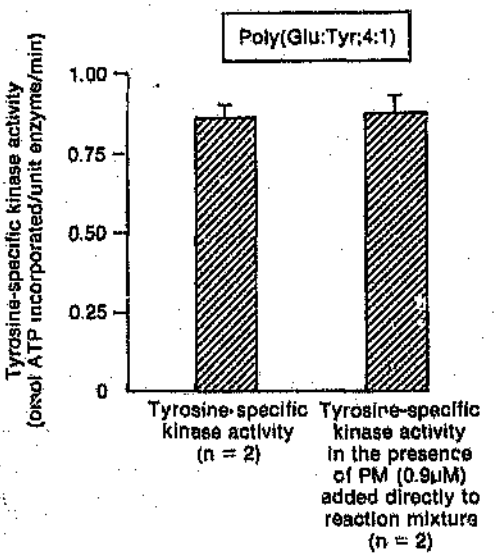

Figure 3 *pp60$^{c\text{-}src}$ Tyrosine kinase activity determined with the tyrosine kinase-specific substrate Poly(Glu:Tyr;4:1) in the presence or absence of 0.9μM PM. Results are expressed as pmol ATP incorporated per unit of enzyme per 15 min assay, and are represented as mean ± SD derived from separate experiments (n=2) each done in triplicate.*

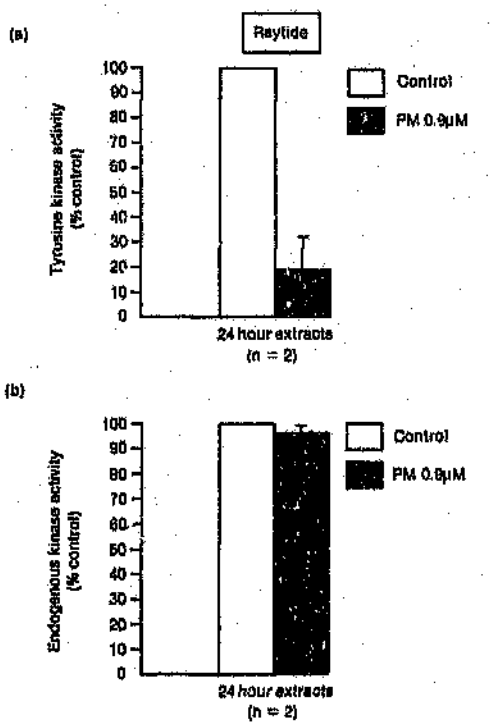

Figure 4 *a) Soluble tyrosine kinase activity (using Raytide as substrate) in extracts from untreated and from PM-treated (0.9μM) HL-60 cells after 24 hours of exposure.*
b) Endogenous kinase activity in the cellular extracts described in (a).

Results are given as the mean ± SD derived from separate experiments (n=2) each done in triplicate.

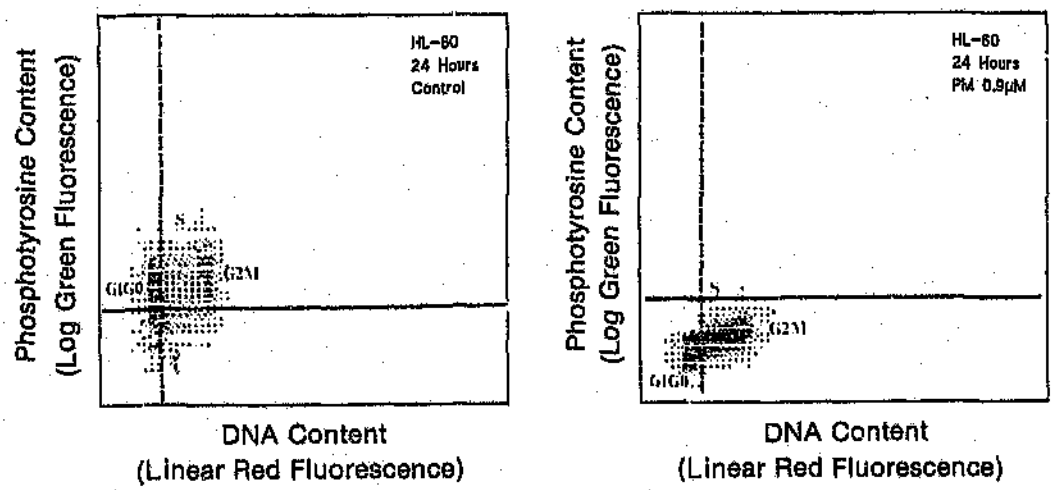

Figure 5 Dual parameter flow cytometric histograms of 24 hour control and PM-treated HL-60 cells, showing phosphotyrosine content (log-scale, Y-axis) and DNA content (linear-scale, X-axis).

treated (0.9μM) cells after 1 hour and after 24 hours of exposure (Figure 6). Protein Kinase C activity was somewhat inhibited (± 10%) in extracts of CHX-treated (0.9μM) cells at 24 hours but not at 1 hour.

Discussion

We have shown that low-dose PM-exposure resulted in potent and specific inhibition of tyrosine kinase activity as determined in soluble enzyme extracts prepared from 1 hour and 24 hour PM-treated HL-60 cells. That this inhibition was tyrosine kinase-specific was supported by several observations. Firstly, soluble enzyme protein derived from PM-treated cells showed attenuated phosphorylation of two independent synthetic tyrosine kinase-specific substrates, i.e. the polymer Poly(Glu:Tyr;4:1), and the peptide substrate Raytide. We do not know why the extent of inhibition observed with Raytide as the substrate was greater than that demonstrated with Poly(Glu:Tyr;4:1), although we suggest that it might relate to the different structures of these substrates (peptide as opposed to polymer). Secondly, PM-exposure did not result in the inhibition of endogenous phosphorylation - endogenous phosphorylation effectively represents serine/threonine kinase activity since the relative abundance of phospho-amino-tyrosine in both normal and transformed cells is less than 0.5% (29). Thirdly, and in corroboration of the previous observation, Protein Kinase C activity (representing substrate-specific serine/threonine kinase activity) was not affected by PM-exposure.

Soluble enzyme extracts derived from HL-60 cells treated with a dose of CHX (0.9μM) that was equimolar to the dose of PM employed (0.9μM), and that also inhibited [^{14}C]leucine incorporation to a similar extent as PM did, failed to inhibit phosphorylation of the tyrosine kinase-specific substrate Poly(Glu:Tyr;4:1). This indicates that the tyrosine kinase-specific inhibition induced by PM was not likely to have been the consequence of protein synthesis blockade per se, but may relate to the different mechanisms of action of these two "classical" protein synthesis inhibitors. Unlike PM (30), CHX does not generate drug-linked nascent polypeptides, instead it prevents peptide chain elongation by inhibiting the peptidyl transferase activity of the 60S ribosomal subunit (31).

It is possible that inhibition of tyrosine kinase-specific activity could have been due to some metabolite/s of PM, rather than to PM itself, since PM drug (0.9μM) added directly to reaction mixtures containing either extracts of untreated HL-60 cells, or to reaction mixtures containing purified pp60$^{c\text{-}src}$ tyrosine kinase enzyme, failed to inhibit the capacity of either of these to phosphorylate the tyrosine kinase specific substrate Poly(Glu:Tyr;4:1). In this regard the PMPs are of interest because their structure predicts an inhibitory effect on tyrosine kinase activity (21). In fact the concept of a "pseudosubstrate" sequence interacting with protein kinase catalytic domains and thereby turning off the protein kinase is not a new one (32, 33), and indeed certain synthetic tyrosine polymers with ordered sequences, e.g. (Tyr-Glu-Ala-Gly)n and (Ala-Tyr-Gly)n have been found to serve as potent inhibitors of tyrosine-specific protein kinases (22). It is therefore possible that PMPs with their carboxyterminal tyrosine residues reminiscent of synthetic random polymers of tyrosine-containing aminoacids might have exerted an inhibitory effect by occupying the protein substrate site of the catalytic domain of tyrosine-specific protein kinases. Since PM is a structural analogue of ATP, it may have been competitive with the ATP subsite of the enzyme, as has been reported with amiloride, an ATP analogue which is a potent inhibitor of protein tyrosine kinase activity (15). As an alternative to this scenario, it is feasible that PM could have interfered with emerging tyrosine kinase molecules at their nascent stage.

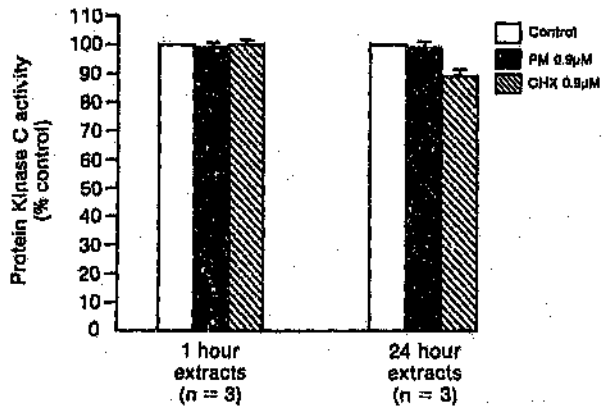

Figure 6 *Protein Kinase C activity (using a specific Protein Kinase C peptide substrate) in cellular extracts derived from untreated HL-60 cells and from cells pre-treated for 1 hour and 24 hours with 0.9μM PM, or 0.9μM CHX.*

We found that in HL-60 leukemic cells, low-dose PM-exposure specifically inhibited tyrosine kinase activity, an effect which may be related to some metabolite/s of PM (perhaps PMPs), rather than to PM itself. These phenomena warrant further investigation, since as a specific inhibitor of tyrosine kinase activity, PM could provide a useful tool for dissecting out the physiological role of tyrosine phosphorylation. Finally PM may in the future have some benefit in the treatment of tumours in which tyrosine kinase overactivity is linked to oncogenesis - its effect is specific (at least in HL-60 cells), the drug is easily taken up *in vitro* and *in vivo* (34, own unpublished results), and toxicity is unlikely to limit its utility if it is used *in vivo* at concentrations which *in vitro* elicit these biochemical sequelae (34). In a poorly controlled study in 1955, intermittent dose PM used as a single antineoplastic agent proved to be ineffective in the treatment of a variety of cancers (35). However the drug scheduling in that study would not have resulted in cellular exposure to a constant level of PM sufficient to accumulate PM metabolites to any degree. Clinical trials using appropriate PM scheduling as a component of a multidrug chemotherapeutic regimen are currently under investigation in our centre in dogs presenting with spontaneous lymphoproliferative disorders.

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UNEXPECTED CYTOKINETIC EFFECTS INDUCED BY PUROMYCIN INCLUDE A G_2-ARREST, A METAPHASE-MITOTIC-ARREST, AND APOPTOSIS

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Abstract—The potent effects of low doses of PM on the cell cycle have to date been obscured by the conventional usage of this drug at high concentrations (5–50 μg/ml) to inhibit protein synthesis. In this *in vitro* study undertaken in a variety of malignant and non-malignant human and murine cell types, we found that low doses of PM (0.1–0.5 μg/ml) disrupted significant phase-to-phase cell cycle transitions, causing a G_2-arrest, a metaphase-mitotic-arrest, and apoptosis. In HL-60 cells these observations were elicited by PM concentrations starting at 0.1 μg/ml, and were more pronounced at slightly higher PM concentrations, including that (0.5 μg/ml) which inhibited [^{14}C]leucine incorporation by approximately 20% after one hour, and by approximately 50% after 24 h. A concentration of CHX (0.25 μg/ml) which was equivalent to 0.5 μg/ml of PM, both in terms of molarity (0.9 μM) and degree of inhibition of [^{14}C]leucine incorporation, failed to induce similar changes to those induced by PM. This suggests that at these particular concentrations the PM-induced changes were likely to have been related to the different mechanisms of protein synthesis inhibition exerted by these two 'classical' translation inhibitors. PM but not CHX generates nascent peptidyl-PM complexes (PMPs), and we therefore propose that the subsequent intracellular effects exerted by the PMPs may account, in part, for the differential cytokinetic effects elicited by these drugs. The role of PM is currently being evaluated *in vivo* as a low-dose component of a multidrug chemotherapeutic regimen in which its cell cycle-specific effects could potentially be synergistic with other agents.

Key words: Puromycin, cycloheximide, cell cycle, DNA fragments, protein synthesis inhibition, apoptosis, G_2-phase-arrest, metaphase-mitotic-arrest.

INTRODUCTION

THE antibiotic puromycin (PM), is a composite of aminonucleoside dimethyladenosine and tyrosine-*o*-methylether [1]. It is a functional analogue of tyrosyl-transfer RNA, and is well known for its inhibitory effect on protein synthesis [2]. PM concentrations of 5–50 μg/ml block protein synthesis completely by causing the premature release of truncated peptide chains which are bound through their carboxyl termini to PM. Since the nascent PM-peptide complexes (PMPs) are rapidly degraded through an ubiquitin-dependent pathway [3], their metabolic effects are poorly understood. The PMPs are of interest however because of their potential to compete for degradation with the natural substrates of the ubiquitin-dependent pathway (including cyclin, *p53*, c-*myc*, and c-*fos*) [4], and because their structure predicts an inhibitory effect on tyrosine kinase activity [5]. In the latter regard it is noteworthy that the carboxy terminal tyrosine residues of the PMPs are reminiscent of certain synthetic random polymers of tyrosine-containing amino acids, some of which have been found to act as competitive inhibitors of endogenous tyrosine kinases [6].

The regulation of cell cycle transitions has recently been elucidated and extensively reviewed [7–9]. The G_2- to M-phase transition is regulated by a co-ordinated cascade of kinase- and phosphatase-mediated reactions [10], while the metaphase to anaphase transition is mediated by rapid, ubiquitin-dependent cyclin proteolysis [11]. Consistent with what is now known to be an absolute requirement for protein synthesis for progression through the G_1b-subphase of the cell cycle [12], PM (5–20 μg/ml) halts the entry of cells into S-phase [13], inhibits the passage of cells through the G_2-phase [14, 15], completely blocks mitosis initiation [15], and prolongs metaphase in

Abbreviations: PM, puromycin; *CHX*, cycloheximide; *PMPs*, puromycin peptides; *ATCC*, American type culture collection; [^{14}C], carbon 14-labeled.

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those cells that are already in early mitosis when PM is applied [15].

Cell cycle events and the process of apoptosis (programmed cell death) have been linked through the recognition of an association existing between a G_2-arrest and subsequent activation of the apoptotic cascade [16]. As far as apoptosis is concerned, protein synthesis has a varied and sometimes controversial role to play in this process. Certain cells enter apoptosis by a process dependent on *de novo* protein synthesis [17, 18], whereas in other cells complete protein synthesis inhibition does not prevent apoptosis [19]. Furthermore, inhibition of protein synthesis may augment spontaneous apoptosis [20], and in certain circumstances inhibition of protein synthesis *per se* has been enough to trigger apoptosis [20–23]. Specifically PM employed at 50 μg/ml induced apoptosis in HL-60 cells [23].

The concentrations of PM (5–50 μg/ml) achieved *in vitro* [13–15], and which significantly inhibit protein synthesis by greater than 95%, are unattainable *in vivo* without serious toxicity. Therefore to explore the *in vitro* effects of PM on the cell cycle further, specifically as a prelude to re-evaluating its potential *in vivo* application in a chemotherapeutic context, we employed very low concentrations of PM (<1 μg/ml) which inhibited protein synthesis to a limited extent. We also compared PM's cytokinetic effects to those elicited by an equivalent protein synthesis inhibiting concentration of another protein synthesis inhibitor cycloheximide (CHX). We found that at these concentrations PM but not CHX induced apoptosis, and potently blocked late cell cycle transitions, disrupting both the G_2- to M-phase, and the metaphase to anaphase transitions. Since CHX prevents chain elongation by inhibiting peptidyl transferase activity of the 60 S ribosomal subunit [24], but does not generate nascent polypeptides [25], we suggest that the intracellular effects exerted subsequently by the PM-generated PMPs may account for the differential cytokinetic effects elicited by doses of these drugs that inhibited protein synthesis to an equivalent extent.

MATERIALS AND METHODS

Cell culture

HL-60 human promyelocytic leukemic cells, THP-1 human monocytic leukemic cells, and SP-2 murine myeloma cells (ATCC Rockville, MD, U.S.A.) were maintained in logarithmic growth phase at 37°C in a humidified atmosphere of air and 5% CO_2. Four-week-old female (C3H/HeJ, endotoxin-resistant) mice were thymectomized and thymocyte suspensions prepared [26]. All cells were seeded at 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) of heat-inactivated fetal calf serum, as well as L-glutamine (2 mM), and penicillin (50 U/ml) and streptomycin (50 μg/ml) antibiotics (all from GIBCO, Madison, WI, U.S.A). Puromycin and cycloheximide were obtained from Sigma. At completion of incubations, culture viability was assessed using the trypan blue dye exclusion technique. Cells were fixed and stained with May–Grünwald–Giemsa, and examined, using light microscopy, for mitotic figures and apoptotic cells. Previously defined criteria were used to identify apoptotic cells [27]. The number of mitotic and apoptotic cells per 1000 cells counted was determined, and then expressed as the mitotic index and as the apoptotic index respectively.

Protein synthesis inhibition

The 0.5 μCi[^{14}C]leucine (specific activity 50 Ci/mmol; Amersham) was added to cultures which were then further incubated at 37°C for 1 h [28]. At the conclusion of the incubation period, cells were washed three times in phosphate-buffered saline, 1 ml of 10% trichloroacetic acid was added and the tubes chilled on ice for 30 min. The precipitate so formed was filtered on GF/C filter papers (Whatmann), and washed three times with 5% trichloroacetic acid. The filters were dried and counted after adding aqueous counting scintillant. [^{14}C]Leucine incorporation was determined in triplicate.

Flow cytometric analysis

Cells were prepared for analysis as described [29]. The centrifuged cell pellet was resuspended in a hypotonic nuclear isolation medium containing propidium iodide (Sigma), incubated on ice, and then in RNAse at 37°C. Fluorescence emitted from the propidium iodide–DNA complex was quantitated after laser excitation of the fluorescent dye by the Coulter EPICS-Profile-II flow cytometer (Coulter Electronics, FL). Cell debris and doublets were excluded [30]. The coefficient of variation of the peaks of the control G_0G_1- and G_2M-phases were less than 3%. Limits of cell cycle phases as determined in the control DNA distributions were retained in the subsequent analysis of the drug-treated populations.

DNA fragmentation analysis

DNA isolation, extraction and electrophoresis was carried out according to the following method. After 24 h cells were sedimented and lysed, and DNA extracted prior to incubation with RNAse. Total DNA from 1.5×10^6 cells was loaded onto a 1.5% agarose gel and electrophoresed for 12 h at 1.5 V/cm. Both the gel and the electrophoresis buffer contained ethidium bromide (1 μg/ml).

RESULTS

Viability

The viability at 24 h of PM- and CHX-treated cultures was unaffected by concentrations up to 0.5 μg/ml (Fig. 1a). At higher concentrations ≥1 μg/ml, the viability of both PM- and CHX-treated cultures was compromised, with the viability of PM-treated cultures being affected to a greater extent.

Protein synthesis inhibition

The dose–response curves expressing the inhibitory activity of PM on protein synthesis (as measured

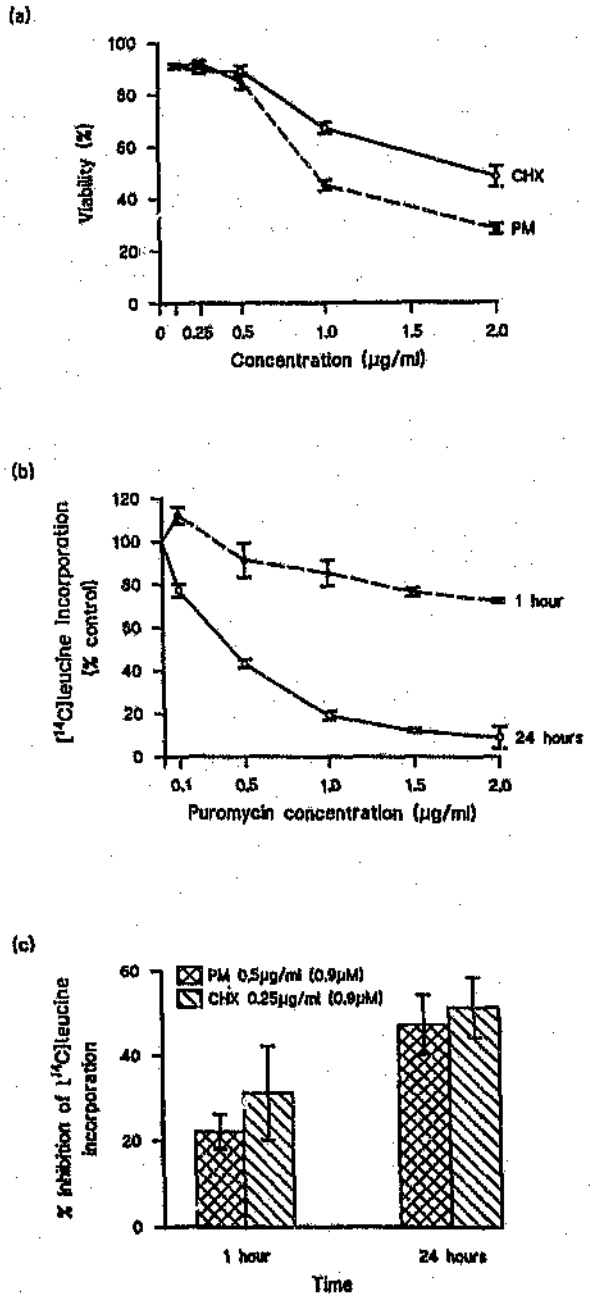

FIG. 1 (a). Viability of cultures (as determined by the trypan blue dye exclusion technique) after 24 h of exposure to the indicated concentrations of PM and CHX (μg/ml). Results represent the mean ± SD of three separate experiments each done in duplicate. (b) The effect of increasing doses of PM (μg/ml) on [^{14}C]leucine incorporation at 1 h and at 24 h in HL-60 cells. Results were expressed as the percentage of control values and represent the mean ± SD of three separate experiments each done in triplicate. (c) Effect of equivalent molar concentrations (0.9 μM) of PM (0.5 μg/ml) and CHX (0.25 μg/ml) on inhibition of [^{14}C]-leucine incorporation. Results represent the mean ± SD of three separate experiments each done in triplicate.

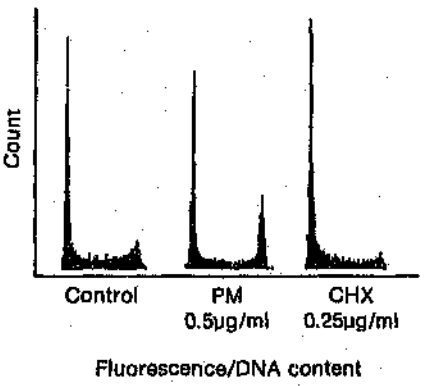

FIG 2. Representative flow cytometric analysis at 24 h of count vs fluorescence/DNA content of control, PM-treated, and CHX-treated HL-60 cells. Twenty-five thousand cells were counted for each analysis. Histogram scaling was the same for all samples.

by [^{14}C]leucine incorporation), are presented in Fig. 1b. At 1 h and at 24 h, at a concentration of 0.9 μM (0.25 μg/ml), CHX was found to inhibit [^{14}C]leucine incorporation to a similar extent as a concentration of 0.9 μM (0.5 μg/ml) of PM did, i.e. approximately 20–30% at 1 h and approximately 50% at 24 h (Fig. 1c).

PM effects

Effects on the DNA histogram of HL-60 cells following continuous exposure to increasing PM concentrations confirmed a time- and a dose-related increase in the G_2- and M-phase populations, an almost reciprocal reduction in the S-phase population, and an apparently unperturbed G_0G_1-phase population (Fig. 2, Fig. 3a, b). Morphological studies showed a metaphase-mitotic-arrest and features of apoptosis, the apoptotic index being 12.4 in PM-treated cells as compared to 1.02 in control cells, and to 1.4 in CHX-treated (0.25 μg/ml) cells (Fig. 5). Apoptosis was confirmed ultrastructurally (not shown), and by DNA electrophoresis which showed the pattern of double-stranded DNA fragments that result from the activation of an endogenous endonuclease (Fig. 6).

CHX effects

After 24 h of CHX-exposure (0.25 μg/ml) there was a G_0G_1-arrest with concomitant limitation of the S-, G_2-, and M-phases (Fig. 2, Fig. 4a, b). The morphological sequelae of CHX-exposure were unremarkable except for a reduced number of mitotic figures (Fig. 4b). Apoptosis above control levels was not evident at a CHX concentration of 0.25 μg/ml (Fig. 5c), nor were DNA fragments produced at this concentration (Fig. 6). DNA fragmentation was

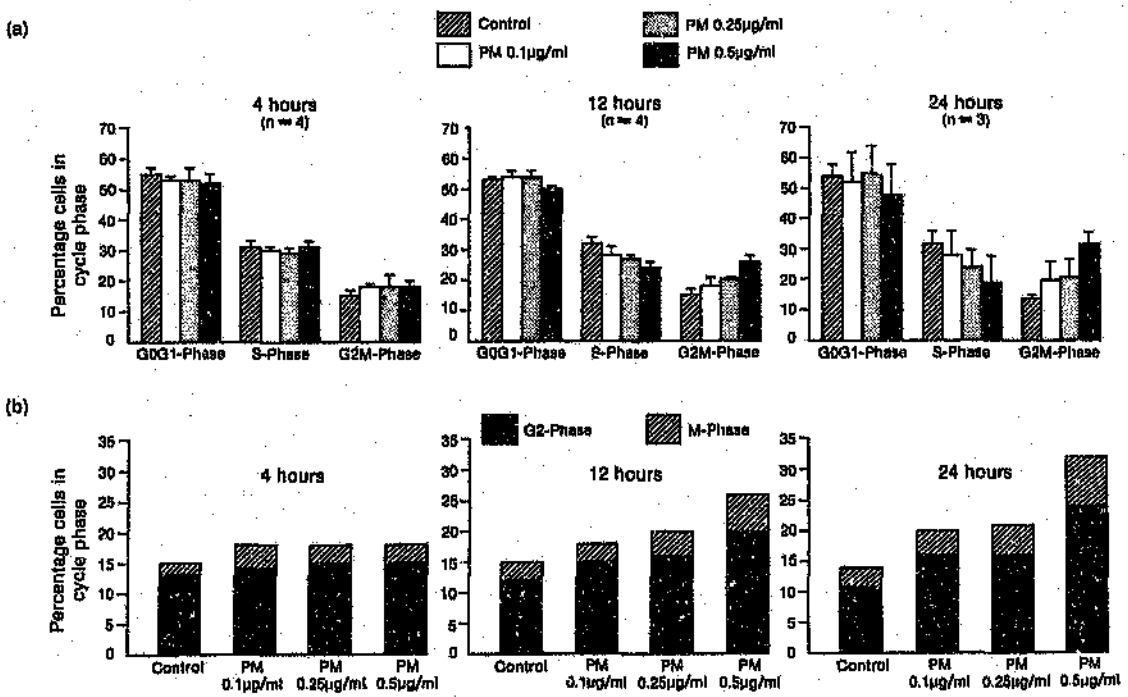

FIG. 3. (a) Effects on the DNA histogram of HL-60 cells following 4, 12, and 24 h of exposure to increasing PM concentrations (μg/ml). Results are expressed as mean ± SD ($n = 3/4$). (b) Stacked-bar graphs illustrating the relative percentages of HL-60 cells in the G_2- and M-phases of the cell cycle consequent on 4, 12, and 24 h of exposure to increasing PM concentrations (μg/ml). The M-phase at each concentration point is representative of the mean mitotic index (number of mitotic figures per 100 cells) of two experiments done on separate occasions. The G_2-phase was calculated by subtracting the M-phase value from the mean G_2M-phase value ($n = 3/4$) obtained from Fig. 2a.

evident when CHX was employed at a much higher concentration of 2 μg/ml (Fig. 6).

The morphological, cytokinetic and electrophoresis data obtained in 0.5 μg/ml PM-treated HL-60 cells were representative of similar data obtained (but not shown) in 0.5 μg/ml PM-treated THP-1 and SP-2 cell lines, as well as for 0.5 μg/ml PM-treated mouse thymocytes.

DISCUSSION

In the current study we have shown that PM exerted a number of potent effects on the cell cycle when used at concentrations far below those used traditionally to completely block protein synthesis (5–50 μg/ml). These effects included a G_2-arrest, ametaphase-mitotic-arrest, and apoptosis, and were found in a variety of malignant cell types of both human and murine origin. In HL-60 cells these observations were elicited by PM concentrations starting at 0.1 μg/ml, which not only did not inhibit [^{14}C]leucine incorporation, but actually enhanced it (at least for the first hour of exposure). The effects were more pronounced at slightly higher PM concentrations, including that (0.5 μg/ml) which inhibited [^{14}C]leucine incorporation by approximately 20% after 1 h, and by approximately 50% after 24 h. A concentration of CHX (0.25 μg/ml) which was equivalent to 0.5 μg/ml of PM, both in terms of molarity and degree of inhibition of [^{14}C]-leucine incorporation, failed to induce similar changes to those induced by PM. This suggests that at these particular concentrations the PM-induced changes were likely to have been related to the different mechanisms of protein synthesis inhibition exerted by these two 'classical' translation inhibitors.

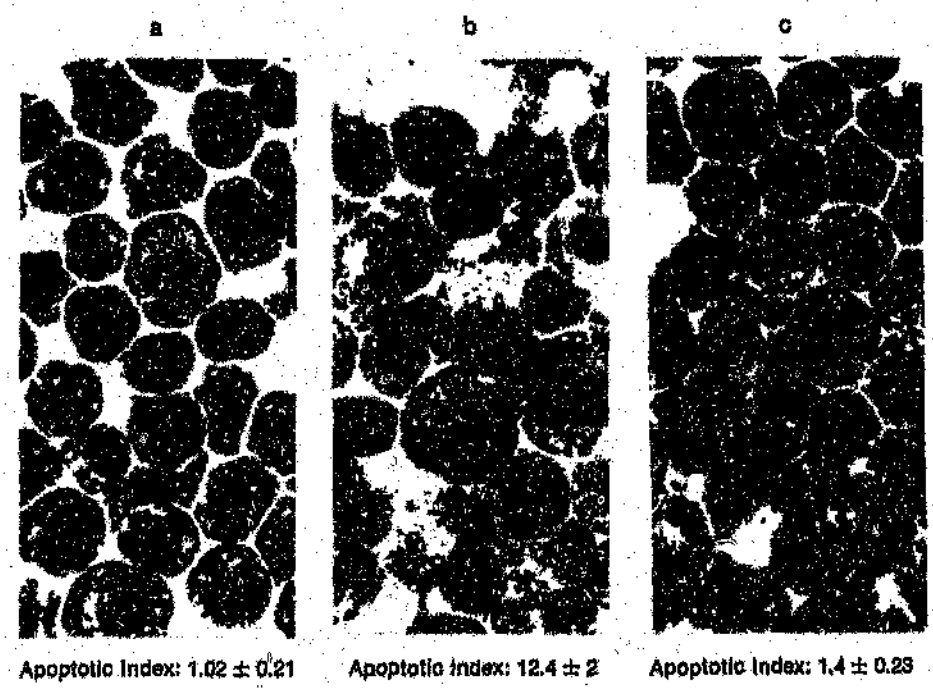

FIG. 5. Giemsa-stained cytospin preparations (magnification ×1000) of 24 h control and drug-treated HL-60 cells. (a) Control HL-60 cells. (b) PM-treated HL-60 cells (0.5 μg/ml) showed a marked metaphase mitotic accumulation 'M' by 24 h. Certain PM-treated cells bore histological evidence of apoptosis 'A' (condensation of the nuclear chromatin and cytoplasm, nuclear fragmentation, and the formation of membrane-bound apoptotic bodies). (c) CHX-treated HL-60 cells (0.25 μg/ml) were indistinguishable from control cells except for a reduced mitotic activity. The apoptotic index in each case represents the mean ± SD of three separate experiments each done in duplicate.

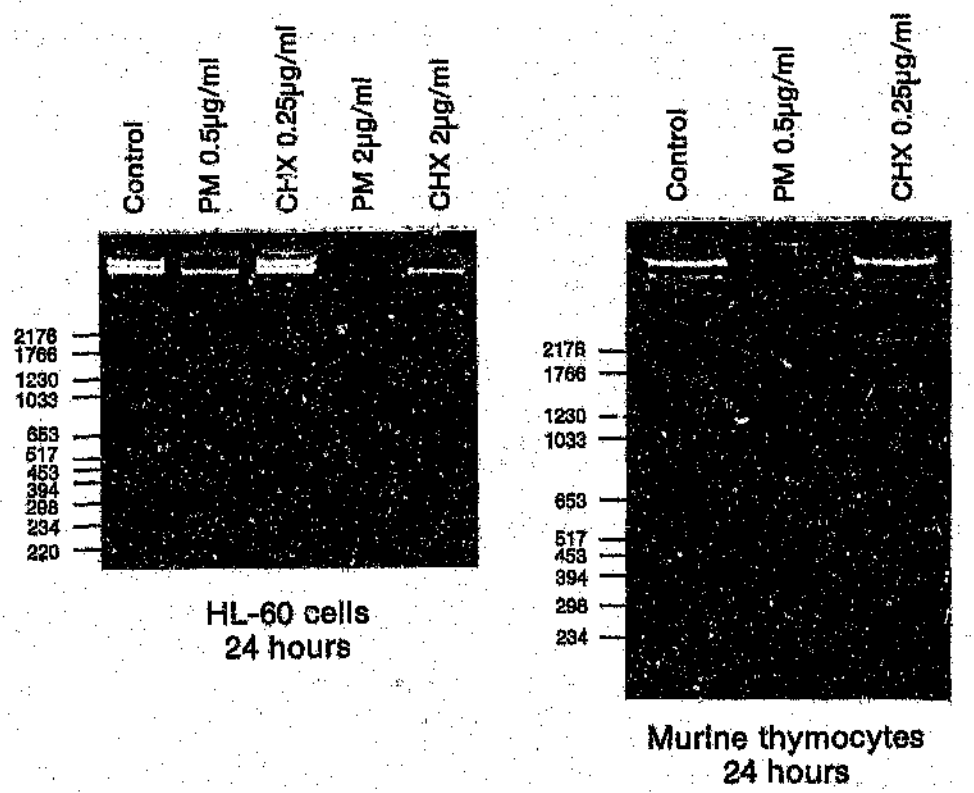

FIG. 6. Agarose gel electrophoresis of total DNA extracted at 24 h from 1.5×10^6 HL-60 cells, and from 2×10^6 murine thymocytes, treated as indicated. The size (in bases) of separated DNA molecular weight markers (BglI/HinfI cleaved pBR328) are shown.

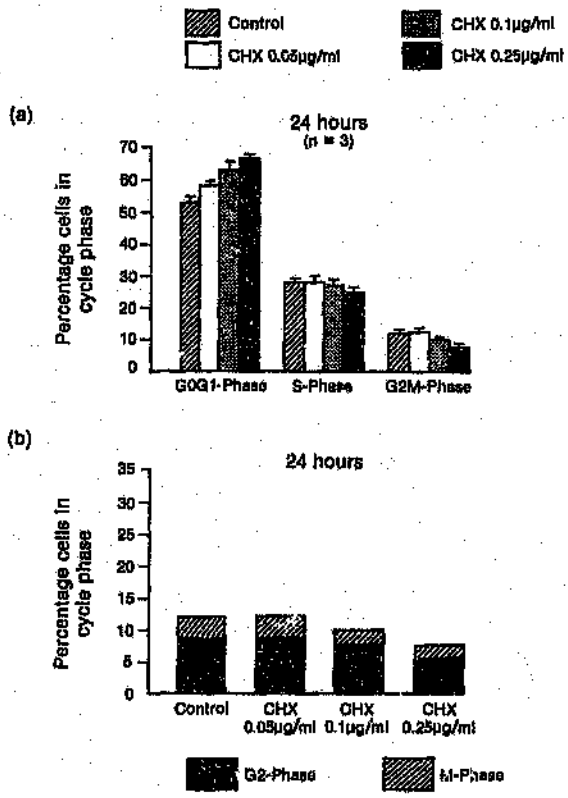

FIG. 4. (a) Effects on the DNA histogram of HL-60 cells following 24 h of exposure to increasing CHX (µg/ml) concentrations. Results are expressed as mean ± SD (n = 3). (b) Stacked-bar graphs illustrating the relative percentage of HL-60 cells in the G_2- and the M-phases of the cell cycle consequent on 24 h of exposure to increasing CHX (µg/ml) concentrations. The percentage of cells in the G_2- and in the M-phases were calculated as described in the legend of Fig. 3b.

PM [25], but not CHX [24], generates nascent peptidyl-PM complexes (PMPs), and we propose that the subsequent intracellular effects exerted by the PMPs may account, in part, for the differential cytokinetic effects elicited by these drugs.

The cytokinetic effects observed with PM-exposure included a time- and dose-related increase in both the G_2- and the metaphase populations, an almost reciprocal reduction in the S-phase population, and an evidently unperturbed G_0G_1-phase population. These observations suggest an undelayed passage through and egress from S-phase of those cells that were beyond the G_1b-subphase when PM was applied, but with subsequent partial hold-ups at the G_2/metaphase and the metaphase/anaphase boundaries. Any predicted but not observed decreases in the G_0G_1-phase population (on the basis of the metaphase-mitotic-arrest) are likely to have been offset by an accumulation of cells in the G_1b-subphase consequent on PM's inhibitory effect on the mandatory protein synthesis requirement of this subphase [12]. CHX-exposure induced a G_0G_1-arrest with concomitant limitations of the S-, G_2-, and M-phases, indicating that CHX blocked the entry of new cells into division consequent on the protein synthesis requirement of the G_1b-subphase [12, 31], but permitted those cells that were beyond this subphase at the time of CHX application, to complete mitosis [31]. We did not observe the G_2-delay that has been reported to occur with high-dose CHX-treatment [31].

The G_2- to M-phase transition is controlled by phosphorylation/dephosphorylation reactions involving the tyrosine-15 residue of the $p34^{cdc2}$ kinase unit of mitosis promoting factor [7, 8, 10, 32]. Since we have recently found PM metabolites to specifically inhibit tyrosine kinase activity (submitted data), we are investigating the possibility that such metabolites, by attenuating tyrosine-15 phosphorylation/dephosphorylation reactions of $p34^{cdc2}$ kinase, may have been responsible for the G_2-arrest observed in PM-treated cells. Such an arrest has also been reported when PM was employed at much higher concentrations of 5–20 µg/ml [14, 15].

It is possible that there is a connection between the G_2-arrest and the apoptosis observed in PM-treated HL-60 cells (0.5 µg/ml), since a preceding G_2-arrest has been found to be linked to subsequent activation of the apoptotic cascade in cells exposed to the anticancer agents etoposide [33], cisplatin [34], and mafosfamide (submitted data).

The role of protein synthesis in the process of apoptosis is varied and controversial [17–23]. Although 50 µg/ml of PM was found previously to trigger apoptosis in HL-60 cells [23], we were able to elicit apoptosis at 1/100 of that PM concentration, i.e. at 0.5 µg/ml. CHX used in HL-60 cells at a dose that inhibited protein synthesis to a similar extent as 0.5 µg/ml of PM did, failed to induce apoptosis. Although we cannot adequately explain the differential effects exerted on apoptosis by these concentrations of PM and CHX, we suggest that it might relate to the mechanism, rather than to the presence, of protein synthesis inhibition produced by these two drugs. In the future we plan to evaluate the specific role, if any, of PMPs in apoptosis activation. It is worth noting that a higher concentration of CHX (2 µg/ml) induced apoptosis in HL-60 cells, consistent with the apoptosis that has been induced in such cells by 50 µg/ml of CHX [22, 23], and consistent with the augmentation of spontaneous apoptosis in B chronic lymphocytic leukemia cells elicited by 1 µg/ml of CHX [20].

The metaphase to anaphase transition is mediated by precipitous ubiquitin-dependent cyclin degradation [11]. Since PMPs are known to be degraded by the same pathway [3], it is possible that in PM-treated cells this pathway was preferentially diverted into PMP proteolysis. One or more of the ubiquitin pathway components might have become limiting for cyclin degradation, resulting in the observed metaphase-mitotic-arrest. This hypothesis is currently under investigation in our laboratory. In addition we are also evaluating the effect of low-dose PM on cyclin and on ubiquitin synthesis, and on the synthesis of the specific protease enzymes responsible for ubiquitin-dependent proteolysis, since it is necessary to exclude decreased production of these proteins as being implicated in the generation of the PM-induced metaphase-mitotic-arrest. In this regard, however, it is worthwhile noting that 0.25 μg/ml CHX, which inhibited protein synthesis to the same extent as 0.5 μg/ml of PM did, did not induce a metaphase-mitotic-arrest, indicating that this level of protein synthesis inhibition appears not to have compromised cyclin, ubiquitin, or protease synthesis. It is of interest that when employed at a high dose of 20 μg/ml, PM completely blocks the ability of cells to initiate mitosis, and prolongs metaphase in those cells that are already in mitosis when PM is applied [15].

The findings of this study are of interest because the use of PM previously as a *single* antineoplastic agent proved to be ineffective in a range of clinical settings [35]. Although significant protein synthesis inhibiting concentrations of PM are unattainable *in vivo* without serious toxicity, this limitation does not necessarily apply to the more potent effects elicited by low-dose PM. Our data suggest that PM may yet prove to be a useful chemotherapeutic agent, not singly but as a component of a multidrug regimen in which its potent cell cycle-specific effect could well be synergistic with other agents. This could be accomplished by first accumulating cells in specific non-G_0G_1-phase compartments and then subsequently delivering appropriate cycle-active chemotherapy. Thus, the metaphase-arrest caused by low-dose PM could be synergistic with the metaphase-specific agent vincristine, while the shunting of G_2-arrested cells into apoptosis could act synergistically with etoposide, cisplatin, or mafosfamide. Phase I/II veterinary clinical trials incorporating PM into such multidrug regimens for the treatment of spontaneous canine lymphomas are currently under way in our laboratories.

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small c

c-*myc* Expression is Down-Regulated in Mafosfamide-Treated HL-60 Cells Undergoing Apoptosis

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4-Hydroperoxycyclophos-phamide

italics Abstract. *The expression of the proto-oncogene c-myc has been strongly implicated in the regulation of apoptosis. Since we have previously shown apoptosis to occur in HL-60 leukemic cells exposed to the prodrug Mafosfamide (ASTA Z 7557), the present study was undertaken to examine the levels* italics *of c-myc mRNA transcripts in such cells. Mafosfamide, which is a cyclophosphamide derivative that rapidly generates 4-Hydroxycyclophosphamide after aqueous dissolution, was employed in doses ranging from 0.1 to 20 μg/ml. No changes* italics *in c-myc mRNA levels were evident after 1 hour of exposure to Mafosfamide. After 6 and 24 hours of Mafosfamide-exposure, however, there was a dose - and time-dependent decrease in* italics *c-myc transcripts. c-myc mRNA expression was reduced to a greater extent than was either β-actin of GAPDH expression. Morphological, biochemical and ultrastructural evidence of* italics *apoptosis accompanied the Mafosfamide-induced c-myc mRNA down-regulation at 24 hours. We conclude that, in the context of Mafosfamide-treated HL-60 cells, upregulation of* italics *c-myc mRNA transcription was not fundamental for the activation of the apoptotic cascade.*

The proto-oncogene c-*myc* mRNA has been strongly implicated in the apoptotic pathway. In certain contexts the process of apoptosis has been found to be contingent on c-*myc* expression (1-7), whereas in other scenarious down-regulation of c-*myc* mRNA levels has accompanied the activation of the apoptotic cascade (8-10).

Cyclophosphamide is a widely employed alkylating cancer chemotherapeutic agent (11-12), the derivatives of which are frequently used *in vitro* to purge leukemic cells from bone marrow (13-20). One such derivative is the prodrug Mafosfamide (ASTA Z 7557), a cyclohexylamine salt that rapidly generates 4-Hydroxycyclophosphamide after aqueous dissolution. 4-Hydroxycyclophosphamide degrades irreversibly via aldophosphamide to phosphoramide mustard and acrolein (21-23), the therapeutically significant metabolites of cyclophosphamide (24). We have recently found that Mafosfamide-exposure induces apoptosis in HL-60 leukemic cells (25). Since certain anticancer agents have been found to reduce c-*myc* mRNA levels (26-29), whereas others (including the cyclophosphamide derivative 4-Hydroxycyclophosphamide) have been found to elevate c-*myc* mRNA transcripts and/or c-*myc* oncoprotein levels (27, 30, 31), the present study was undertaken to examine the levels of c-*myc* mRNA transcripts in Mafosfamide-treated HL-60 cells undergoing apoptosis.

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Materials and Methods

Cell culture and Mafosfamide-exposure. HL-60 human promyelocytic leukemic cells (ATCC passage 17, Rockville, MD, USA) were seeded in RPMI 1640 medium supplemented with fetal calf serum, L-glutamine and Penicillin/Streptomycin antibiotics (GIBCO, Madison, WI, USA). Cells were maintained at 37°C in a humidified atmosphere of air and 5% CO_2. Mafosfamide crystalline powder (MW=500.5) was a kind gift from Dr. J. Pohl, ASTA PHARMA, Pr Brock, Asta-Werke, Bielefeld, West Germany. Fresh Mafosfamide solution was prepared in RPMI 1640 medium immediately prior to use at final concentration ranging from 0.1 μmg/ml to 20 μmg/ml.

DNA fragmentation analysis. At the conclusion of the incubation period the cells were pelleted at 4°C and prepared for subsequent fragmentation analysis (32). The pellet was resuspended in 70μl of lysis buffer (100mM EDTA; 50mM Tris-HCl, pH=8.0; 0.5% sodium lauryl sarkosinate; 0.5mg/ml proteinase K) and incubated for 1 hour at 50°C. A 10μg/ml aliquot of 1mg/ml ribonuclease A was added to each sample prior to a further 1 hour incubation at 50°C. Samples were then heated to 70°C, after which the reaction was terminated by the addition of 20μl of loading buffer (10mM EDTA, pH=8.0; 0.25% bromophenol blue; 40% sucrose). Extracts were loaded into the wells of a 2% agarose gel and electrophoresed in a phosphate buffer (2mM EDTA; 800mM Trisphosphate; pH=7.8). Both the gel and the buffer contained 1μg/ml of ethidium bromide. BglI/HinfI cleaved pBR328 served as molecular weight markers.

c-myc mRNA expression. c-*myc* mRNA expression was investigated using standard techniques (33). Extracted total RNA (10ug) was size-fractionated by 1.2% agarose-formaldehyde gel electrophoresis, and then trans-

31 λλλ 4 ργ λ.114 1 0,1 05 05 93

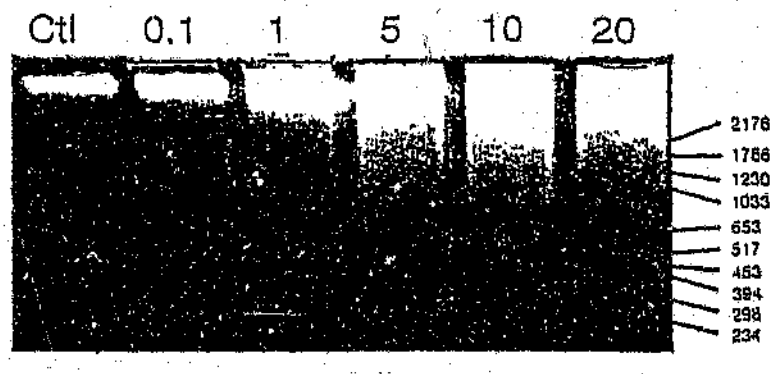

Figure 1. *Agarose gel electrophoresis of total DNA extracted from control and Mafosfamide-treated (ugind) HL-60 cells at 6 hours and 24 hours after drug exposure. The sizes in bases of molecular weight markers are shown.*

ferred and UV-crosslinked to nylon membrane. Hybridization at 42°C for 16 hours was to the [γ-^{32}P]dCTP (Amersham, England) random primer-labelled 1.6Kb SstI fragment from the 5' end of the human c-*myc* gene (ATCC, USA). Following stringency washes at 42°C and at room temperature, the membrane was exposed at -70°C to film using image-intensifying screens. Finally the mebrane was stripped before rehybridization either to the [α_{32}P] dCTP random primer-labelled 1.9Kb BamHI fragment of ß-actin (ATCC, USA), or to the [γ_{32}P] ATP (Amersham, England) end-labelled 40mer oligonucleotide antisense GAPDH (Oncogene Science, USA). ß-actin and GAPDH served as internal controls for constitutive gene expression.

All other reagents were of analytical grade and were from standard commercial sources.

Results

DNA fragmentation analysis. Apoptosis was not seen in Mafosfamide-treated cells after 1 and 6 hours of exposure (data not shown). At 24 hours, however, there was morphological evidence of apoptosis (chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies) in Mafosfamide-treated cells (data not shown). This was confirmed ultrastructurally (data not shown), and by DNA electrophoresis which showed the pattern of double-stranded DNA fragments that result from the activation of an endogenous endonuclease (Figure 1).

italics insert comma

c-myc *mRNA expression.* There was no change in c-*myc*, ß-actin and GAPDH mRNA expression after 1 hour of Mafosfamide-exposure in HL-60 cells (data not shown). After 6 hours and 24 hours, however, there was a dose-dependent decrease in the expression of these genes in Mafosfamide-treated cells (Figure 2). c-*myc* mRNA expression was reduced more than either the expression of ß-actin or GAPDH (Figure 2 and Figure 3).

Discussion

We recently found that the apoptotic pathway was activated in HL-60 cells following exposure to the prodrug Mafosfamide, a derivative of the alkylating cancer chemotherapeutic agent cyclophosphamide (25). Since the expression of the proto-oncogene c-*myc* has been strongly implicated in the regulation of the apoptotic cascade (1-10), we undertook to examine the effect of Mafosfamide on c-*myc* mRNA levels in HL-60 cells. In the current study there was no change in c-*myc* mRNA levels after 1 hour of Mafosfamide-exposure, whereas after 6 hours and 24 hours, there was a dose-dependent decrease in c-*myc* mRNA transcripts. insert comma

These findings suggest that in the context of Mafosfamide treated HL-60 cells, upregulation of c-*myc* mRNA transcription was not fundamental for the induction of apoptosis. This is consistent with the down-regulation of the c-*myc* gene that has been found to precede IL-6-induced apoptosis of Y6 cells (8), steroid-induced apoptosis of 697 pre-B lymphocytes (9), and phorbol ester-induced apoptosis of U-937 cells (10). Our findings are in contrast with the elevated c-*myc* mRNA levels seen in rat thymocytes undergoing apoptosis following dexamethasone treatment (3), and with the evidence that c-*myc* is a potent inducer of apoptosis in rat fibroblasts (6). Our data are also inconsistent with the observations that c-*myc* expression is a requirement for activation-induced apoptosis of T-cell hybridomas (5), for apoptotic regression of estrogen-treated MCF-7 human mammary cancers (7), and for apoptosis elicited in c-*myc*-amplified cell lines by the DNA topoisomerase II inhibitor VM-26 (1).

In the context of anticancer agents, it is of interest that in Colo320HSR cells treated with another cyclophosphamide derivative (4-hydroperoxycyclophosphamide), c-*myc* mRNA levels increased after 1 hour of exposure but decreased subsequently, raising the possibility that alkylating agents may suppress the expression of specific transforming genes (27). Further evidence for this comes ~~for~~ *in vitro* experimentation which demonstrated that alkylating agents including nitrogen mustard and melphalan, but not 4-S-(propionic acid)-sulfidocyclophosphamide, terminated c-*myc* transcription in a sequence-specific, drug-specific manner (28). In addition mithramycin, which binds to G-C-rich sequences in the DNA minor groove, has been found to inhibit c-*myc* from

(and other genes which have G-C rich promoter sequences)

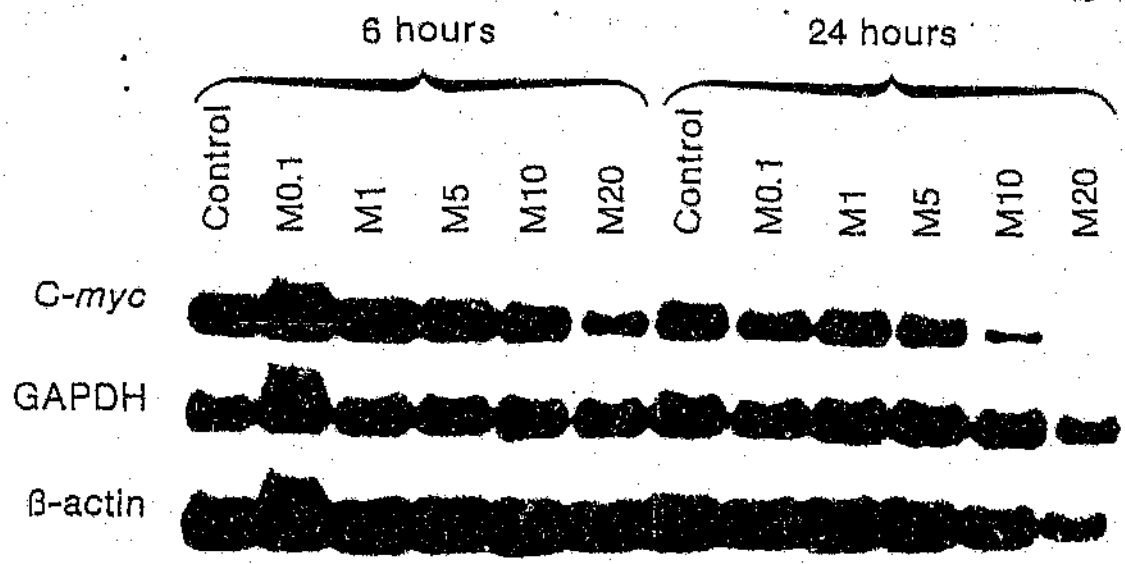

Figure 2. c-myc, *β-actin, and GAPDH mRNA expression at 6 hours and at 24 hours in control and in Mafosfamide-treated (μg/ml) HL-60 cells (10μg total RNA loaded per lane). The results were reproduced on one further occasion.*

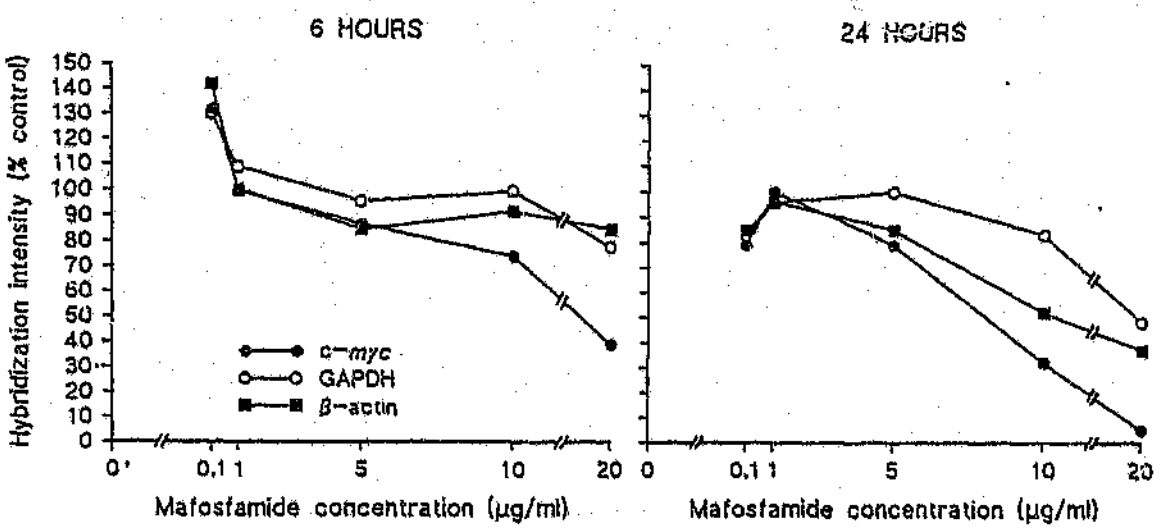

Figure 3. *Hybridization intensities at 6 hours and at 24 hours in Mafosfamide-treated HL-60 cells are shown relative to that in untreated control cells (normalised to 100%). The results were quantitated by densitometric scanning of the autoradiogram.*

transcription in HeLa cells ~~(and other genes which have G-C rich promoter sequences)~~ by preventing the formation of the transcription initiation complex (29). It is of interest, however, that increased c-*myc* protein has been observed to accumulate in a lymphoblastoid cell line in response to certain alkylating agents (Dimethylsulphonate, Ethylmethane sulphonate and Mitomycin C) (30), and that elevated c-*myc* mRNA levels have been reported in SV40-transformed human keratinocytes in response to ultravoilet irradiation (20), and in rat embryonic cells exposed to ionizing irradiation (21). (34) (35)

It is clear that, although c-*myc* expression has been strongly implicated in both apoptosis and in the cellular response to DNA-damaging agents, the precise nature of its regulation of and/or participation in such processes still requires elucidation. To address the issue more comprehen-

sively in the future, c-*myc* mRNA expression should be evaluated not only at the levels of RNA and of protein, but also at the level of DNA, since c-*myc* gene-specific DNA repair may itself be differentially affected according to the eliciting insult (36). Finally, it would be worthwhile to examine the transcription of mammalian genes termed *gadd* (growth arrest and DNA damage inducible genes that specifically code for transcripts which are increased by DNA damage) (37), and to examine their role, if any, in the apoptotic response to cancer chemotherapeutic drugs, particularly the alkylating agent, Mafosfamide

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