Nitric oxide induces apoptosis in NALM-6, a leukaemia cell line with low cyclin E protein levels

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Received 1 November 2000; revision accepted 24 May 2001

Abstract. Intracellular nitric oxide levels may differ in resting and stimulated cells and contribute to the regulation of cell survival and proliferation through a variety of mechanisms and effects. We exposed two B-cell lines to a range of *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) concentrations in order to examine their susceptibility to exogenous nitric oxide and the participation of nitric oxide as modulator of cell proliferation. Although both FLEB and NALM-6 decreased their levels of thymidine incorporation, only NALM-6 cells were induced to undergo G₁ arrest, phosphatidyl serine exposure and DNA fragmentation when cultured in the presence of 250 µM SNAP. This higher sensitivity of NALM-6 coincided with initially low cyclin E protein levels which were increased 7.8-fold after culture for 24 h with 250 µM SNAP. In contrast, there was no difference in cyclins A and D3, Bcl-2 and actin levels, neither at the beginning nor at the end of the 24 h culture. Our study reveals that FLEB and NALM-6 exhibit different response to the same concentration of nitric oxide, that nitric oxide can simultaneously induce cell cycle alterations and apoptosis, and further suggests an association between these two processes, with the involvement of cell cycle regulatory molecules.

INTRODUCTION

Nitric oxide (NO) is a key molecule in various physiological and pathological processes. NO can promote cell proliferation and protect cells against cytotoxic stimuli (Nicotera *et al.* 1997), but it can also induce cell death (Messmer & Brune 1996). Most of the toxic effects of NO are mediated by nitrosylation and nitration of proteins and nucleic material, events which may lead to DNA damage, cell cycle arrest, apoptosis and necrosis (Ho *et al.* 1996; Li *et al.* 1997; Melino *et al.* 1997). However, because NO can target both pro- and anti-apoptotic molecules, the mechanisms by which NO regulates cell survival are complex and not fully understood (Brockhaus & Brune 1998; Chlichlia *et al.* 1998; Dimmeler *et al.* 1998).

Leukaemia and lymphoma B-cell lines are derived from a variety of haematological malignancies that differ in proliferation capacity and rates of accumulation in peripheral blood and lymphoid organs. Such B-cell lines are an important source of information that can be used to

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elucidate cell and molecular alterations contributing to tumour pathology and clinical progression. For instance, B-cell lines may be used as an *in vitro* model to analyse the effect of cytotoxic drugs currently being used to treat B-cell leukaemia and lymphoma patients. Cytotoxic drugs may trigger apoptosis in tumour cells, depending on various factors, including cell cycle position, redox status, survival gene products and NO levels (Swierniak *et al.* 1996; Gaunsage *et al.* 1997; Sarkar *et al.* 1997; Kroemer *et al.* 1998). Cytotoxic antibiotics (Adriamycin), microtubuli stabilizers (Taxol), alkylators (Melfhalan) and DNA synthesis inhibitors (cisplatin) (Ogura *et al.* 1998; Cook *et al.* 1997; Lind *et al.* 1997; Wang & Aggarwal 1997) are among those drugs in which NO may act either to prevent or to effectuate tumour cell killing.

An exquisite control of the cell cycle is achieved by timely regulated synthesis and accumulation of various cyclins, which form complexes with cyclin-dependent kinases (CDK), thus allowing cells to enter into a sequence of events that culminates in DNA replication and mitosis. The transient appearance of cyclin/CDK complexes drives cell progression, which is regulated by a third set of molecules known as CDK inhibitors. Agents that target cell-cycle regulatory molecules, causing biochemical alterations or their unscheduled activation may lead the cell into apoptosis (Meikrantz & Schlegel 1995). To investigate how NO may affect cell survival and proliferation of leukaemia B cells, we have studied its effects on two B-cell lines cultured in the presence or absence of a NO chemical donor and analysed their *in vitro* susceptibility to exogenous NO in terms of thymidine incorporation, alterations of the cell cycle and apoptosis. Our data suggest an association between cyclin E levels and sensitivity or resistance to NO induction of apoptosis in these two cell lines.

MATERIALS AND METHODS

Reagents

RPMI-1640, HEPES, antibiotics, glutamine and fetal calf serum were purchased from Gibco BRL (Gaithersburg, MD, USA). *S*-nitroso-acetyl-D,L-penicillamine (SNAP) was acquired from Alexis (San Diego, CA, USA). Trypan blue, propidium iodide (PI), RNase type 1-A, phenyl-methylsulphonyl fluoride (PMSF) and sodium dodecyl sulphide (SDS) were purchased from Sigma (St Louis, MO, USA). Annexin-V-Fluos was obtained from Boehringer Mannheim Scandinavia AB (Bromma, Sweden). [³H]thymidine, Hybond ECL nitrocellulose membranes and horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibodies were acquired from Amersham (Stockholm, Sweden). Glass fibre filters and scintillation liquid were obtained from Wallac (Turku, Finland). The DC Protein Assay Kit was from Bio-Rad (Hercules, CA, USA). Mouse monoclonal antibody (mAb) anti-human cyclin E was from Pharmingen (San Diego, CA, USA). Rabbit polyclonal antibodies, anti-human cyclin A and anti-human cyclin D3, and goat polyclonal antibody against human actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal anti-Bcl-2 was purchased from Oncogene Science (Uniondale, NY, USA).

Cell lines and culture conditions

B-cell lines were maintained in culture with RPMI medium supplemented with 2 mM glutamine and antibiotics (100 IU penicillin and 100 µg streptomycin/mL). RPMI medium was supplemented with 10% fetal calf serum (FCS) to maintain FLEB (Epstein-Barr virus (EBV)-transformed pro-B-cell line) (Katamine *et al.* 1984) and with 5% FCS to maintain NALM-6 (pre B leukaemia cell line) (Hurwitz *et al.* 1979) in *in vitro* culture. Cell density was adjusted 24 h before the

experiments to 1×10^6 /mL to maintain the cells in a logarithmic phase of growth during culture in the presence or absence of NO. Experiments were performed by culturing > 95% viable cell suspensions newly adjusted to 1×10^6 cells/mL, in the presence or absence of 50 or 250 μ M SNAP. RPMI supplemented with antibiotics, glutamine and 0.5% bovine serum albumin (BSA), instead of FCS, was used to culture the cells during the experiments. SNAP was dissolved in 95% ice-cold ethanol and diluted in medium to minimize the amount of ethanol in the cultures. Control cultures received similar volume (0.005%) of ethanol. Cells were incubated at 37 °C in a 5% CO₂ atmosphere, harvested at 0, 12 and 24 h and stained with Annexin-V-Fluos and 2.5 μ g/mL PI to determine phosphatidyl serine (PS) exposure and cell permeabilization by flow cytometry (Fadok *et al.* 1992). Cell aliquots were also fixed with 70% ethanol and stored at 4 °C for analysis of the cell cycle phases and DNA fragmentation. Other aliquots were snap-frozen as cell pellets and stored at -70 °C for Western blot (WB) analysis.

Thymidine incorporation

Thymidine incorporation was determined at the same time points as the cell cycle phases, PS exposure and DNA fragmentation. To avoid the presence of growth factors that could influence the outcome of the experiment, cells were cultured at 10^5 cells/well in RPMI supplemented with 0.5% BSA instead of FCS, with or without SNAP in 96-microwell plates. Cells were pulsed with 37 kBq or 1 μ Ci [³H]thymidine during 6 h previous to 0, 12 and 24 h culture. The incorporation of thymidine was determined by harvesting on glass fibre filters, adding scintillation liquid and counting in a β counter. All measurements were done in triplicate and only experiments with a mean error deviation < 10% were accepted.

Flow cytometry

Freshly harvested cells were incubated for 10 min with Annexin-V-Fluos and PI and analysed by fluorescence-activated cell sorting (FACS). A Becton Dickinson FACSCalibur system was used to acquire 10 000 cells for each determination with an acquisition rate < 400 events/s. Other cell aliquots were harvested simultaneously, fixed with ethanol and stored at 4 °C. Afterwards, ethanol-fixed cells were washed, incubated with 500 μ g RNase in phosphate-buffered saline (PBS) for 30 min, stained with 5 μ g PI and analysed by FACS to identify cell cycle phases and the sub G0 fraction corresponding to DNA fragmentation (Nicoletti *et al.* 1991).

Western blot

Cell pellets were lysed in ice-cold buffer containing 1% Triton X-100, 0.1 M Tris hydrochloride, 0.15 M NaCl, 5 mM EDTA, pH 8.0, and 1 mM PMSF, followed immediately by the addition of 1/3 vol. modified 4 $\times$ Laemmli buffer (248 mM Tris hydrochloride pH 6.8, 8% SDS and 40% glycerol). After boiling for 10 min, cell lysates were centrifuged, the supernatant was transferred to a new tube and the protein concentration was determined using the DC protein assay kit. Seventy micrograms of protein obtained from each lysate were boiled for 5 min with 1/5 Vol. DB solution (1 M dithiothreitol (DTT) and 0.1% bromophenol blue, 3 : 2 vol. in ethanol), separated by 10% SDS-PAGE, and electroblotted onto Hybond ECL nitrocellulose membranes. The transfer efficiency was confirmed by Ponceau-S staining. Membranes were blocked with 5% non-fat milk and 0.05% Tween-20 in PBS. Anti-human cyclins A, D3 and E, as well as anti-Bcl-2 antibodies were diluted 1 : 400, and the horseradish peroxidase-conjugated anti-mouse IgG was diluted 1 : 5000 with blocking solution. Films were developed using the ECL system. The mean optical density of the areas was obtained by scanning and analysed using the NIH image software.

Figure 1. Thymidine incorporation of FLEB and NALM-6 cells exposed to 50 μM (filled square) or 250 μM SNAP (filled circle). Control cultures without NO were regarded as 100% proliferation in order to normalize the values.

Statistics

Student's *t*-test for unpaired samples was used to analyse data from NALM-6 as compared to FLEB, or from the same cell line, either NALM-6 or FLEB when cultured under different culture conditions. The coefficient of correlation was used to analyse correlations between independent observations and two-tailed statistical significance was determined.

RESULTS

Thymidine incorporation

Five independent experiments were performed to compare thymidine incorporation in the two cell lines at 0, 12 and 24 h exposure to 50 and 250 μM SNAP in order to investigate the effect of NO on cell proliferation. Thymidine incorporation in the non-SNAP-treated controls was considered to be 100% cell proliferation at 0, 12 and 24 h in order to determine higher or lower levels of proliferation in SNAP-treated cells. Both cell lines significantly reduced their proliferation after 24 h exposure to 250 μM SNAP. FLEB decreased from $237\,685 \pm 10\,004$ – $140\,740 \pm 34\,788$ and NALM-6 from $142\,150 \pm 21\,368$ – $50\,266 \pm 23\,514$ c.p.m., which was in both cases, statistically significant ($P < 0.05$). Differences were also significant between NALM-6 and FLEB exposed to 250 μM SNAP when compared to their 24 h nonexposed controls ($P < 0.05$; Fig. 1).

PS exposure and membrane permeabilization

Cells were exposed to SNAP in a range of concentrations between 10 and 1000 μM to determine the minimal concentrations that could affect cell survival in the two studied cell lines. Cells were harvested at 0, 12 and 24 h, stained with Annexin-V-Fluos and PI and analysed using FACS (Fig. 2). By using a dot plot with mean fluorescence intensity (MFI) of FL1 on the *x*-axis and FL3 on the *y*-axis, we could recognize intact (not stained either with Annexin or PI) cells

Figure 2. FACS analysis of NALM-6 cells during 24 h culture in the presence or in the absence of SNAP at various concentrations. Dot plots show the population of intact viable cells in the lower left quadrant, cells having phosphatidyl serine exposure in the lower right quadrant, and directly permeabilized (necrotic) cells in the upper left quadrant. The upper right quadrant contains a mixed population of directly permeabilized (necrotic) cells and secondary (to apoptosis) necrotic cells. PS exposure was determined by Annexin-V-Fluores binding (x-axis) while permeabilization was determined by staining with propidium iodide (y-axis).

in the lower left gate, permeabilized (PI stained) cells in the upper gates and purely apoptotic (Annexin- but not PI-stained) cells in the lower right gate. The population of cells in the lower right gate is widely recognized as non-permeabilized, apoptotic cells having PS exposure (Fadok *et al.* 1992), while the population of permeabilized cells in the upper right gate is composed of non-apoptotic, directly permeabilized cells (also in the left upper gate) and a population of apoptotic cells that becomes permeabilized afterwards. A larger number of cells with PS exposure and plasma membrane permeabilization were found at higher SNAP concentrations, as was evident with NALM-6 cells during 24 h exposure to SNAP (Fig. 2). The SNAP effect was also time-related as longer exposure times produced more apoptotic and more permeabilized NALM-6 cells in comparison to the controls (Fig. 2).

Table 1 shows mean $\pm$ SE of three experiments analysing PS exposure and permeabilization during culture of both cell lines in the presence or in the absence of SNAP at concentrations up to 250 μ M. Different sensitivity to SNAP was observed in cells cultured for 24 h under sub-optimal conditions. We found that 250 μ M SNAP induced PS exposure in NALM-6 ($P < 0.05$) but not in FLEB. The SNAP effect in FLEB was only seen in a relatively low percentage (11.7 ± 2.3 , $P < 0.05$) of PI positive cells (Table 1).

Table 1. Induction of cell death by SNAP treatment

SNAP		PS exposure ^a		Permeabilization ^b	
		12 h	24 h	12 h	24 h
FLEB		6.3 ± 0.4 ^c	6.7 ± 0.8	6.3 ± 0.5	5.5 ± 0.6
	50 µM	8.0 ± 2.1	7.7 ± 1.3	5.3 ± 1.2	7.3 ± 1.8
	250 µM	10.7 ± 0.9	10.3 ± 1.7	6.0 ± 1.0	11.7 ± 2.3 ^d
NALM-6		2.3 ± 0.5	2.7 ± 0.5	3.5 ± 0.7	4.3 ± 1.4
	50 µM	3.7 ± 1.2	6.0 ± 0.6	5.0 ± 1.0	8.3 ± 1.4
	250 µM	15.7 ± 1.7 ^d	28.3 ± 4.9 ^d	6.3 ± 1.4	27.3 ± 7.2

^aDetermined by Annexin-V binding.

^bDetermined by PI staining.

^cMean ± SE.

^d*P* < 0.05.

Figure 3. Percentages of cell cycle phases in cells after culture for 24 h with or without 50 and 250 µM SNAP. Histograms show DNA staining and the sub G₀ fraction of NALM-6 at 24 h culture in the presence or in the absence of SNAP.

Cell cycle phases and DNA fragmentation

Aliquots of ethanol-fixed cells were used to determine the percentage of cells in G₁, S and G₂ + M cell cycle phases, and the sub G₀ fraction, corresponding to DNA fragmentation, a late event in apoptosis. Figure 3 shows data from representative experiments for FLEB and NALM-6 after 24 h of culture in the presence of 0, 50 and 250 µM SNAP. The histograms correspond to NALM-6, which also had a positive correlation (*r* = 0.86, *P* < 0.05) between the number of permeabilized (PI-positive) and DNA fragmented cells (Table 1 and Fig. 3).

Cyclin expression

Cell aliquots were collected at the same time points as the samples analysed by thymidine incorporation and FACS in order to analyse cyclin expression by WB in three independent experiments. We observed a higher initial cyclin E protein level in FLEB cells by comparing its area × OD values (427.4 ± 159.8) at the beginning of the culture with those from NALM-6

Figure 4. Western Blot analysis of cyclin D3, cyclin E and cyclin A in FLEB and NALM-6 cells after culture for 0, 12 and 24 h with or without 50 and 250 μ M SNAP. Figure shows mounted computer scans of original blots from one out of three independent experiments with similar results.

Figure 5. Western Blot analysis of Bcl-2 in FLEB and NALM-6 cells after culture for 0, 12 and 24 h with or without 50 and 250 μ M SNAP. Figure shows mounted computer scans of original blots from one out of three independent experiments with similar results.

(170.8 ± 53.4) at the same point in time. Cyclin E levels were augmented in both FLEB and NALM-6 with a 1.6- and 7.8-fold increase, respectively, after 24 h of exposure to 250 μ M SNAP. A representative experiment is shown in Fig. 4, with initially high cyclin E levels in FLEB that coincided with no induction of PS- or PI-positive cells, which was in contrast with initially low cyclin E protein levels and higher susceptibility of NALM-6 24 h exposure to 250 μ M NO (Table 1 and Fig. 4).

An extra, low MW band that can be observed in Fig. 4 represents one of the five cyclin E isoforms, with MW between 43 and 52 kDa, which have been previously described (Ohtsubo *et al.* 1995; Scuderi *et al.* 1996). In this experiment, the extra band is a faint one, as recognized by our anti-cyclin E antibody, and does not appear to be relevant for the interpretation of our results. Actin was run in parallel as a control for cell loading and protein levels of a housekeeping gene. Protein levels of cyclins A and D3 were studied in parallel, and found that they remained unchanged during the experimental time course, in both the presence and absence of SNAP (Fig. 4) which suggests that they do not participate in the outcome of the experiment.

Bcl-2 expression

To investigate whether differences in the susceptibility to NO of the studied cell lines could be due to different levels of survival gene products, we tested Bcl-2 expression before and after exposure to SNAP. Figure 5 shows mounted computer scans of the original blots from one out

of three independent experiments with similar results. There was no difference in Bcl-2 protein levels between these cell lines and there was no significant change in such levels during 24 h exposure to 50 and 250 μM SNAP.

DISCUSSION

In this study, we started testing a number of B-cell lines, exposing them to a range of SNAP concentrations (not shown). Then, two B-cell lines and a concentration high enough to induce cell death in the sensitive (NALM-6) but not in the resistant (FLEB) cell line were selected. With this model, we analysed the effect of exogenous NO (SNAP) when added to cultures in concentration up to 250 μM , in comparison to control cultures without SNAP. Higher inhibition of thymidine incorporation coincided with larger numbers of cells in the G_1 cell cycle phase and cell death of NALM-6 cells during 24 h of exposure to SNAP. Initially low cyclin E expression was also found in NALM-6, although such expression was greatly increased during exposure. All of this was in contrast to the stronger initial cyclin E expression and less effect in all the other parameters during FLEB cells exposure to SNAP.

Under our experimental conditions, cell death was mainly due to apoptosis, which is a highly regulated suicide programme, characterized by distinctive morphological, cytoplasmic and nuclear features (Wyllie 1997; Lincz 1998). As it may have multiple targets, NO has been associated with opposite effects leading to antagonistic pathways that end in the promotion or prevention of apoptosis. Depending on the NO concentration, the cell *milieu* and the molecules being targeted, opposite effects in cell proliferation may also be observed (Kröncke *et al.* 1997).

The ability of NO to induce DNA damage (Sandau *et al.* 1997) provides a scenario for intimate associations between the cell cycle and apoptosis, which may involve p53 activation. NALM-6 has wild type p53 (Filippini *et al.* 1998), and the exposure to SNAP could produce DNA damage, increased levels of p53 and G1 arrest, leaving the cell with the dilemma of repairing its DNA or dying (Lane 1992). DNA damaging agents have been reported to increase cyclin E levels (Lauricella *et al.* 1998). Our study suggests that elevated constitutive cyclin E levels as observed in FLEB cells, may be more important for leukaemia B cells to resist NO induction of apoptosis, allowing them to continue going through G_1 to S phase. The following cyclin E increase in the sensitive NALM-6 cells might only indicate an unsuccessful attempt to forward the cell cycle into the S phase and to escape apoptosis. On the other hand, cyclin E over-expression may produce increased levels of CDK2–cyclin E complexes that unbalance the fine coordination of cyclins and CDK molecules and disrupt the cell cycle progression.

It is also possible that NO directly affects and interferes with the normal function of one or several cell cycle regulatory molecules. Cyclin D–CDK4/6 and cyclin E–CDK2 activation is required to progress to late G_1 , while cyclin A–CDK2 activation is necessary to exit G_1 and start DNA replication (Sherr 1994). Cyclin A participation in the cell cycle is exceptional because it is also involved in the induction of mitosis (Pines & Hunter 1990). In our study, cyclin D3 and cyclin A protein levels from FLEB and NALM-6 remained unchanged, and thus they seem to have been irrelevant to NO induction of apoptosis in these B cell lines under our experimental conditions. We did not find differences in Bcl-2 protein levels, but other survival gene products may be involved in the resistance versus development of apoptosis in our model, and we have yet to analyse the activation of cyclins, CDK and CDK inhibitors during exposure of these B

cell lines to NO. Further studies on the activity and interactions of these three types of molecules will clarify their participation either as NO targets or molecules that act to compensate for NO cell damage.

ACKNOWLEDGEMENTS

This study was supported by funds from The Cancer Society in Stockholm (98:100 and 99:143) and Karolinska Institute.

REFERENCES

- BROCKHAUS F, BRUNE B (1998) U937 apoptotic cell death by nitric oxide: Bcl-2 downregulation and caspase activation. *Exp. Cell Res.* **238**, 33.
- CHLICHLIA K *et al.* (1998) Caspase activation is required for nitric oxide-mediated CD95 (APO-1/Fas)-dependent and independent apoptosis in human neoplastic lymphoid cells. *Blood* **91**, 4311.
- COOK JA *et al.* (1997) Nitric oxide enhancement of melphalan-induced cytotoxicity. *Br. J. Cancer* **76**, 325.
- DIMMELER S *et al.* (1998) Nitric oxide inhibits APO-1/Fas-mediated cell death. *Cell Growth Differ.* **9**, 415.
- FADOK VA *et al.* (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* **148**, 2207.
- FILIPPINI G *et al.* (1998) A novel flow cytometric method for the quantification of p53 gene expression. *Cytometry* **31**, 180.
- GAUNSAGE S *et al.* (1997) Exogenous but not endogenous, nitric oxide increases proliferation rates in senescent human fibroblasts. *FEBS Lett.* **410**, 160.
- HO YS, WANG YJ, LIN JK (1996) Induction of p53 and p21/WAF1/CIP1 expression by nitric oxide and their association with apoptosis in human cancer cells. *Mol. Carcinogenesis* **16**, 20.
- HURWITZ R *et al.* (1979) Characterisation of a leukemic cell line of the pre-B phenotype. *Int. J. Cancer* **23**, 174.
- KATAMINE S *et al.* (1984) Epstein-Barr virus transforms precursor B cells even before immunoglobulin gene rearrangements. *Nature* **309**, 369.
- KROEMER G, DALLAPORTA B, RESCHE-RIGON M (1998) The mitochondrial death/life regulator in apoptosis and necrosis. *Ann. Rev. Physiol.* **60**, 619.
- KRÖNCKE KD, FEHSEL K, KOLB-BACHOFEN V (1997) Nitric oxide: cytotoxicity versus cytoprotection – how, why, when, and where? *Nitric Oxide* **1**, 107.
- LANE DP (1992) Cancer. p53, guardian of the genome. *Nature* **358**, 15.
- LAURICELLA M *et al.* (1998) Increased cyclin E level in retinoblastoma cells during programmed cell death. *Cell. Mol. Biol.* **44**, 1229.
- LI J *et al.* (1997) Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochem. Biophys. Res. Comm.* **240**, 419.
- LINCZ LF (1998) Deciphering the apoptotic pathway: all roads lead to death. *Immunol. Cell Biol.* **76**, 1.
- LIND DS *et al.* (1997) Nitric oxide contributes to adriamycin's antitumor effect. *J. Surg. Res.* **69**, 283.
- MEIKRANTZ W, SCHLEGEL R (1995) Apoptosis and the cell cycle. *J. Cell. Biochem.* **58**, 160.
- MELINO G *et al.* (1997) S-nitrosylation regulates apoptosis. *Nature* **388**, 432.
- MESSMER UK, BRUNE B (1996) Nitric oxide (NO) in apoptotic versus necrotic RAW 264.7 macrophage cell death: the role of NO-donor exposure, NAD⁺ content, and p53 accumulation. *Arch. Biochem. Biophys.* **327**, 1.
- NICOLETTI I *et al.* (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* **139**, 271.
- NICOTERA P, BRUNE B, BAGETTA G (1997) Nitric oxide: inducer or suppressor of apoptosis? *TIPS* **18**, 189.
- OGURA T *et al.* (1998) Suppression of anti-microtubule agent-induced apoptosis by nitric oxide: possible mechanism of a new drug resistance. *Jap. J. Cancer Res.* **89**, 199.
- OHTSUBO M *et al.* (1995) Human cyclin E, a nuclear protein essential for the G₁-to-S phase transition. *Mol. Cell Biol.* **15**, 2612.

- PINES J, HUNTER T (1990) Human cyclin A is adenovirus E1A-associated protein P60 and behaves differently from cyclin B. *Nature* **346**, 760.
- SANDAU K, PFEILSCHIFTER J, BRUNE B (1997) Nitric oxide and superoxide induced p53 and Bax accumulation during mesangial cell apoptosis. *Kidney Int.* **52**, 378.
- SARKAR R *et al.* (1997) Dual cell cycle-specific mechanisms mediate the antimitogenic effects of nitric oxide in vascular smooth muscle cells. *J. Hypertens.* **15**, 275.
- SCUDERI R *et al.* (1996) Cyclin E overexpression in relapsed adult acute lymphoblastic leukemias of B-cell lineage. *Blood* **87**, 3360.
- SHERR CJ (1994) G₁ phase progression: Cycling on cue. *Cell* **79**, 551.
- SWIERNIAK A, POLANSKI A, KIMMEL M (1996) Optimal control problems arising in cell-cycle-specific cancer chemotherapy. *Cell Prol.* **29**, 117.
- WANG Y, AGGARWAL SK (1997) Effects of cisplatin and taxol on inducible nitric oxide synthase, gastrin and somatostatin in gastrointestinal toxicity. *Anticancer Drugs* **8**, 853.
- WYLLIE AH (1997) Apoptosis: an overview. *Br. Med. Bull.* **53**, 451.