Pergamon

European Journal of Cancer, Vol. 35, No. 5, pp. 796–807, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(98)00425-0

Original Paper

Cell Cycle Disturbances and Apoptosis Induced by Topotecan and Gemcitabine on Human Lung Cancer Cell Lines

C. Tolis, G.J. Peters, C.G. Ferreira, H.M. Pinedo and G. Giaccone

Department of Medical Oncology, Free University Hospital, Amsterdam 1007 MB, The Netherlands

Apoptosis is a major mode of cell death in response to cytotoxic drug treatment. A correlation between induction of apoptosis and chemosensitivity has been documented in some preclinical models. Topotecan (a topoisomerase I inhibitor) and gemcitabine (a deoxycytidine analogue), two active new drugs for the treatment of lung cancer, were evaluated for their growth inhibitory effect on human lung cancer cell lines and their effect on cell cycle perturbation, apoptosis and apoptosis-related genes. The cytotoxicities of topotecan and gemcitabine on the human lung cancer cell lines H460 (wild-type-*p53*) and H322 (mutant *p53*) were determined after 72 h drug exposure employing the MTT assay. The apoptotic index (AI) was assessed by three methods: analysis of morphological changes using May–Grünwald–Giemsa (MGG) staining, the TUNEL assay and FACS analysis. Cell cycle disturbances were studied by FACS and the number of cells expressing p53 and p21 were determined by immunohistochemistry. Both gemcitabine and topotecan had potent growth inhibitory effects in human lung cancer cell lines; combination treatment with these two drugs showed some additivity but no synergism. Induction of apoptosis after treatment was concentration- and time-dependent with both drugs and IC_{80} concentrations induced the highest values. The DNA histograms at 4, 24, 48 and 72 h indicate that topotecan at IC_{80} concentrations causes accumulation of cells in S and G2/M phases, whereas gemcitabine at IC_{80} concentrations causes, accumulation of cells in G1 phase. Both compounds induced p53 and p21 expression in the H460 cell line but not in the H322 cell line; the percentage of cells expressing p53 was highest at IC_{80} values, whereas the highest percentage of p21 positive cells could be induced with IC_{50} values. This could suggest that p53 induces cell cycle arrest at low drug concentrations, whereas p53 induces apoptosis at higher concentrations. In conclusion, p53-dependent and independent pathways of apoptosis exist in lung cancer cell lines. Activation of the p53 pathway depends on the induced cellular damage. Understanding the cell cycle disturbances induced by these drugs may help in the design of more rational treatment schedules. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: apoptosis, cell cycle, gemcitabine, lung cancer, topotecan
Eur J Cancer, Vol. 35, No. 5, pp. 796–807, 1999

INTRODUCTION

THE DEVELOPMENT or presence of resistance to chemotherapy is a major obstacle to the effective treatment of both small cell lung cancer (SCLC) and non-SCLC (NSCLC). The inability of a cell to undergo apoptosis has recently been proposed as a critical mechanism of drug resistance to a variety of cytotoxic agents [1]. Apoptosis is an active process of controlled, non-inflammatory cellular deletion that complements mitosis in the maintenance of cellular homeostasis. The *p53* tumour suppressor gene plays a central role in the regulation of the apoptotic machinery, suppressing growth of genetically damaged cells by causing a pause in the cell cycle [2, 3] and by promoting programmed cell death [4–6]. The status of the *p53* gene is a major determinant of response following DNA damage. *p53* mutations are the most common genetic changes found in human cancer and mutations in this gene result in loss of its function and inactivation [7, 8], suggesting its

Correspondence to G. Giaccone, e-mail: g.giaccone@azvu.nl
Received 3 Aug. 1998; revised 2 Nov. 1998; accepted 9 Nov. 1998.

pivotal role in human carcinogenesis and outcome of treatment [9]. *p53* is mutated in the majority of SCLC and in over 50% of NSCLC [10, 11].

Among new active compounds introduced in the treatment of lung cancer, two are of particular interest: topotecan (10-hydroxy-9-dimethylaminomethyl-(S)-camptothecin), a water soluble camptothecin analogue [12] and gemcitabine (2′,2′-difluoro-2′-deoxycytidine), a nucleoside analogue structurally related to cytosine arabinoside [13]. Topotecan exerts its action by interacting with the nuclear enzyme topoisomerase I. This enzyme unwinds the supercoiled double-stranded DNA during DNA replication and transcription, by temporarily binding and cleaving one of the strands. Topotecan stabilises the complex of cleaved DNA with topoisomerase I and as the replication fork progresses it interacts with the cleavable complex, resulting in double strand breaks [14]. Gemcitabine is activated to its difluorodeoxycytidine triphosphate that is incorporated into DNA, which leads to chain termination.

Limited information is available on the mechanisms of cell death in lung cancer cell lines exposed to topoisomerase I inhibitors and gemcitabine. In lung cancer, apoptosis has been shown to occur as a mechanism of cell death after exposure to cytotoxic drugs such as etoposide [15] and cisplatin [16] and also with fenretinide, a synthetic retinoid [17]. Apoptosis has also been shown to be induced in a number of leukaemic cell lines treated with camptothecin [18].

Although the cytotoxic action of gemcitabine has been shown to be correlated with the amount of drug incorporated into DNA and RNA [19], the pathway by which this drug causes cell death is unclear. The introduction of DNA damage by a second agent may enhance the incorporation of gemcitabine into DNA through repair synthesis. Gemcitabine and topotecan are suitable candidates for combination chemotherapy in lung cancer for three reasons: they are active as single agents, have different mechanisms of action and relatively non-overlapping toxicity profiles. The aim of our study was to determine the cytotoxic activity of topotecan and gemcitabine alone and in combination, against human lung cancer cell lines with wild-type and mutant *p53*, and to investigate further the effect of treatment on the distribution of the cell cycle and the expression of apoptosis-related genes.

MATERIALS AND METHODS

Cell lines

The human NSCLC cell lines NCI-H460 (expressing wild-type-*p53*) and NCI-H322 (expressing mutant *p53*) [20] were kindly provided by Dr A. Gazdar. All cells were grown in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (Gibco BRL, Life Technology, Breda, The Netherlands), 2 mM L-glutamine, 50 iu/ml penicillin and 50 μg/ml streptomycin. Cells from exponentially growing cultures were used for all experiments.

Drugs

Topotecan, a water soluble camptothecin analogue (SmithKline Beecham Pharmaceuticals, Hertfordshire, U.K.) was dissolved in water and stored at − 20°C until use. Gemcitabine, a new nucleoside analogue, provided by Eli Lilly Research Laboratories (Indianapolis, Indiana, U.S.A.) was dissolved in phosphorate buffered saline (PBS) and stored at − 20°C until use.

Cytotoxicity assay

Cytotoxicities to topotecan and gemcitabine were assessed with the MTT assay [21]. Cells were cultured at a density of 20 000 cells/well in 96-well plates (Costar, Cambridge, Massachusetts, U.S.A.), in 100 μl medium and grown overnight. Twenty-four hours later, drugs were made up in medium and eight different concentrations were added to the plates at a volume of 100 μl per well, and the plates were incubated for 72 h with the drugs. Then, 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemicals, St Louis, Missouri, U.S.A.) was added to each well and incubated for another 4 h at 37°C. The plates were then centrifuged at 1000 rpm at 4°C for 5 min and the medium was carefully discarded. The formazan crystals were dissolved in 100 μl dimethylsulphoxide (ACROS Organics, Geel, Belgium) and the absorbance was read at 540 nm on a plate spectrophotometer (Titertek Multiscan, MCC/340). Absorbance values were expressed as a percentage of untreated controls and an inhibition concentration 50 (IC_{50}) was calculated. The IC_{50} values represent the means of at least four independent experiments. We also tested the effect of combination treatment with topotecan and gemcitabine. For the combination experiments cells were exposed to topotecan or gemcitabine alone, or to the combination of the two drugs. Cells were exposed concomitantly or sequentially to the drugs. In the concomitant exposure experiments, drugs were added 24 h after seeding. In the sequential exposure experiments, cells were pretreated for 4 or 24 h with the IC_{15} or IC_{50} concentrations of one drug before the other drug was added, without removing the first one. Cultures were further incubated for 72 h after adding the second drug. One drug was added at a concentration which caused growth inhibition of 15–50% and the other drug was added at variable concentrations. The survival curves plotted represent the effect of one drug alone, the expected survival curve of the combination and the observed survival curve when the two drugs were combined. The expected curve was calculated by multiplying the growth inhibition of the single drug effect at each concentration by 0.85–0.50, for IC_{15}–IC_{50} drug concentrations of the second drug, respectively.

DNA labelling and flow cytometric analysis

To quantitate apoptosis, a flow cytometric analysis using propidium iodide (PI) (Sigma Aldrich, Deisenhofen, Germany) was performed as described previously [22]. Cells which were less intensively stained than G_1 cells (sub-G_1 cells) in flow cytometric histograms were considered apoptotic cells. H460 and H322 cell lines were seeded in 25 cm^2 flasks (Nalge Nunc™ International, Naperville, Illinois, U.S.A.) with 4 ml medium and left overnight to attach to the substrate. The cells were then treated for 72 h with the IC_{50} and IC_{80} concentrations of topotecan and gemcitabine. At 4, 24, 48 and 72 h, cells from parallel cultures were harvested after trypsinisation, including the floating cells. Aliquots containing 1×10^6 cells were centrifuged at 800 rpm for 5 min, the pellet was gently resuspended in 1 ml hypotonic PI solution (PI, 100 μg/ml Sigma, 0.1% sodium citrate, 0.1% Triton X-100, 0.1 mg/ml ribonuclease A) in round-bottomed tubes. The tubes were wrapped in aluminium foil, placed on ice and analysed immediately on a FACScan flow cytometer (Beckton Dickinson, Mountain View, California, U.S.A.). At least 5000 cells were analysed, using the lysys program (Becton Dickinson, San Jose, California, U.S.A.). At least three experiments were performed for each cell line.

Cell cycle analysis was performed using the same experimental conditions and distributions were determined using the CellFit program (Becton Dickinson). All measurements were carried out under the same instrumental settings.

May–Grünwald–Giemsa (MGG) staining

Cells were treated for 72 h with the IC_{50} and IC_{80} concentrations of topotecan or gemcitabine. At 4, 24, 48 and 72 h cells from parallel cultures were harvested and cytospins were prepared. The slides were dried overnight, then wrapped in aluminium foil and stored at $-80°C$ until use. Before use, the slides were thawed and then stained: an apoptotic index (AI) was determined as the percentage of apoptotic cells from at least 400 counted cells, by light microscopy. Apoptotic cells were identified by criteria previously described [23].

TUNEL assay

The assay was performed as described by Gorczyca and colleagues [24]. The slides were prepared as described above and the cells were fixed with freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4), for 30 min at room temperature. The slides were rinsed with PBS and incubated with permeabilisation solution (0.1% Triton X-100 and 0.1% sodium citrate), for 1 min at 4°C, then rinsed twice with PBS and 5 µl TUNEL reaction mixture (*In Situ* Cell Death Detection Kit, Boehringer-Mannheim BV Biochemicals, Almere, The Netherlands) was placed on the slide. The slides were covered with parafilm to avoid evaporation and to ensure homogenous spread of the TUNEL mixture. The samples were incubated for 60 min in a humidified chamber at 37°C, then rinsed three times with PBS and finally analysed under a fluorescence microscope. An AI was given as the percentage of positive cells, from at least 400 counted for each sample.

Immunohistochemistry

Immunohistochemistry was performed using the avidin–biotin–peroxidase complex, with primary antibodies raised against p53 (DO-7, DAKO, diluted 1:100) and p21 (p21-Pharmingen, diluted 1:100). Briefly, cells were fixed in acetone for 10 min, then washed with PBS and preblocked with normal rabbit serum at a 1:50 dilution for 30 min and the primary antibody described above was added and incubated for 60 min. After washing with PBS, the samples were incubated for 30 min at room temperature, with the secondary rabbit antimouse immunoglobulin. The avidin–biotin complex was applied for 1 h. Finally, the samples were rinsed with PBS, developed with diaminobenzidine tetrahydrochloride (DAB) for 4 min, counterstained with haematoxylin, dehydrated in an alcohol gradient and mounted with Depex. Simultaneous incubations of slides where the first antibody was omitted were used as negative controls for both antibodies. Positivity was scored based on the percentage of positively stained cells from at least 400 counted. The intensity of the staining was not taken into consideration.

Western blot analysis

Whole cell pellets (3×10^6 cells) were resuspended in 50 µl PBS and lysed by the addition of 50 µl lysis solution (50 µM Na_3VO_4, 500 µM NaF, 250 µM Na_2HPO_4, 5 µl NP-40, 20 µl protease cocktail inhibitor in PBS) per 1×10^6 cells. Lysates were boiled for 5 min and the proteins quantified using the Biorad protein estimation assay (BioRad Laboratories, Richmond, California, U.S.A.). Equal amounts of protein (25 µg/sample) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto a nitrocellulose membrane (Amersham Life Science). The blots were stained in Ponceau's red to ensure transfer and then blocked with TBS-Tween and 5% milk powder for 1 h at room temperature. The blots were then probed at 4°C overnight with the appropriate dilution of the primary antibodies (p53/DO7, DAKO, in 1:200 and β-actin, in 1:3000). The blots were again washed with TBS-Tween and developed by enhanced luminescence (ECL). Autoradiographs were quantitated by densitometry (BioRad, Model GS-690, imaging densitometer).

RESULTS

Cytotoxicity

The IC_{50} and IC_{80} values for both drugs after 72 h continuous drug exposure are shown in Table 1. The IC_{50} of gemcitabine was lower than that of topotecan for both cell lines and H322 (mutant *p53*) cells were more resistant than H460 (wild type *p53*) for both compounds. We also tested the effect of combining topotecan and gemcitabine with different treatment schedules. Concomitant treatment with a low concentration of one of the compounds (IC_{25}–IC_{50}) plus a range of concentrations of the other drug resulted in an additive effect (Figure 1). Sequential exposure to low-doses (IC_{25}–IC_{50}) of topotecan or gemcitabine followed by the addition of the second drug also resulted in a modest additive effect (Figure 1). However, synergism was not observed with any of the schedules tested.

Cell cycle distributions

The kinetics of the cell cycle distribution of cells treated with topotecan and gemcitabine are shown in Table 2. The untreated H460 and H322 control cells showed progressive accumulation in G0/G1, with a parallel decrease of the population in S and G2/M phase, with increasing culture time, reflecting the effect of growth factor deprivation on progressively confluent cultures. Changes to the distribution of cells in the cell cycle could be observed by 4 h after drug exposure, especially for IC_{80} drug concentrations.

For gemcitabine IC_{50} concentrations, a gradual increase of cells in S phase was observed for both cell lines. However, at IC_{80} drug concentrations cells predominantly accumulated in G0/G1 and S phase for the H460 (Figure 2) and the H322 cell lines, respectively. For both cell lines, the IC_{50} concentrations of topotecan induced a slower passage through S phase and an increase of the G2/M population. For the H460 cell line, the arrest in G2/M was even more striking with IC_{80}

Table 1. *Sensitivity of human lung cancer cell lines to topotecan and gemcitabine. Sensitivity is expressed as the concentration of drug to achieve* 50% *or* 80% *growth inhibition (*IC_{50} *or* IC_{80}*), after treatment for* 72 *h. Values are means of six experiments*

	Topotecan		Gemcitabine	
Cell lines	IC_{50} (nM ± S.D.)	IC_{80} (nM ± S.D.)	IC_{50} (nM ± S.D.)	IC_{80} (nM ± S.D.)
H460	14.3 ± 11.4	147 ± 60	4.3 ± 3.7	260 ± 114
H322	31.4 ± 14.5	900 ± 114	25.0 ± 13.3	1040 ± 145

S.D., standard deviation.

concentrations, especially at 48 and 72 h. In the H322 cell line, at these drug concentrations there was accumulation in S and G2/M phase at 24 and 48 h, but after 72 h there was a dramatic depletion of cells in these phases of the cell cycle with a concurrent rise of the G0/G1 population. One interpretation could be that cells in S and G2/M phase died and only the remaining viable cells (present in G0/G1) were analysed.

It appears that topotecan and gemcitabine have different effects on cell cycle progression, and also that the variable effects on the two cell lines point to p53-dependent and p53-independent regulation of the cell cycle.

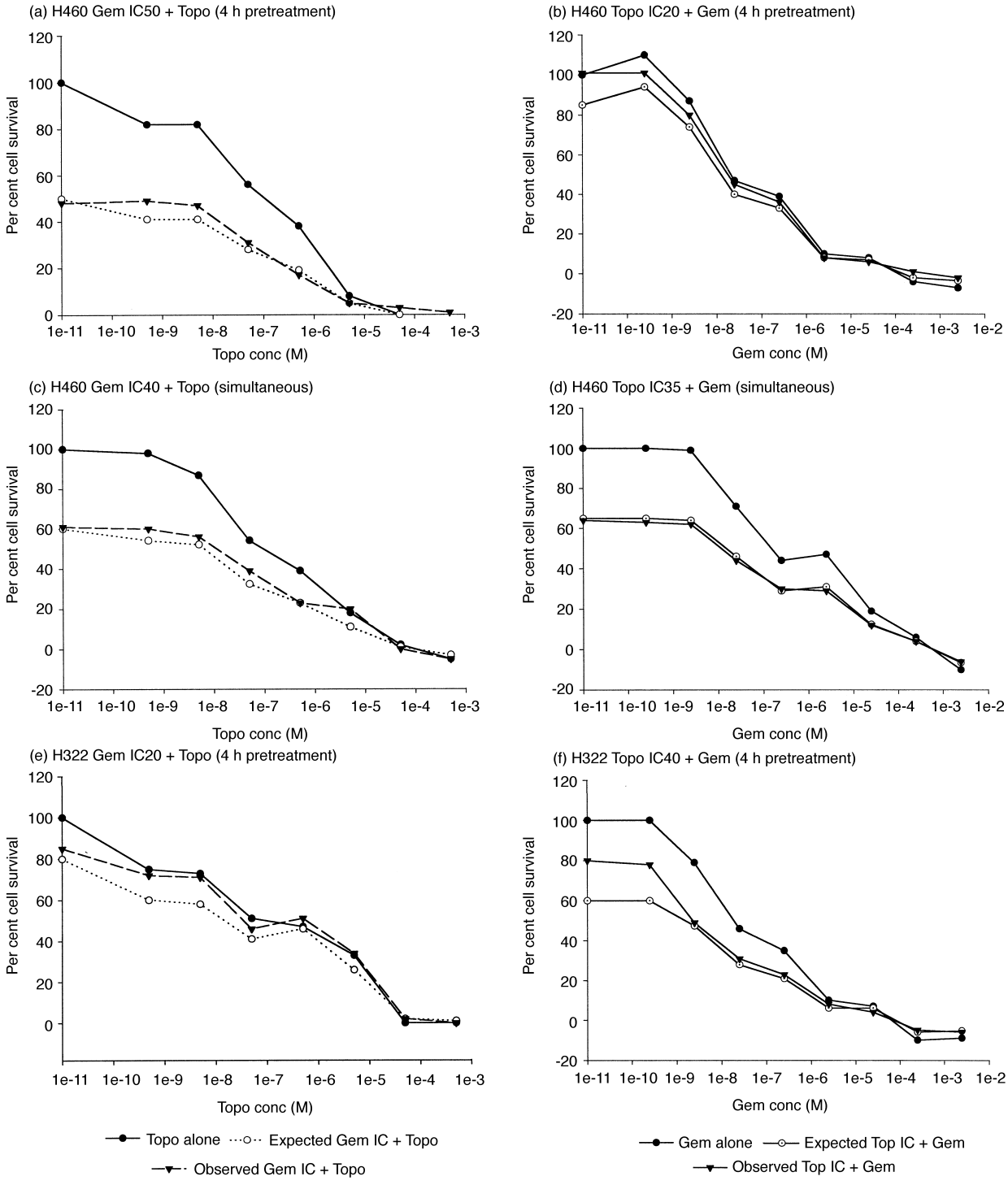

Figure 1. Growth inhibition curves of topotecan (Topo) or gemcitabine (Gem), the expected effect of combining topotecan and gemcitabine and the observed growth curve of the combination of the two drugs on the H460 and H322 cell lines. Cells were treated concomitantly or sequentially with the two drugs according to the MTT assay. From the values of topotecan or gemcitabine alone, the expected curve was calculated (see Materials and Methods). Single representative experiments for each condition tested are shown. At least three experiments for each condition were performed. Standard deviations (S.D.) were 5–10%.

Table 2. Cell cycle distribution determined by FACS analysis. H460 and H322 cells treated with the IC_{50} and IC_{80} concentrations of topotecan (Topo) or gemcitabine (Gem) were harvested, stained with propidium iodide and analysed at 4, 24, 48 and 72 h. Values are the means of four experiments for each time point

Phase of the cell cycle	Controls (% of cell cycle)		Gem IC_{50} (% of cell cycle)		Gem IC_{80} (% of cell cycle)		Topo IC_{50} (% of cell cycle)		Topo IC_{80} (% of cell cycle)	
	H460	H322	H460	H322	H460	H322	H460	H322	H460	H322
G0/G1										
4 h	43.4	46.5	45.9	42.3	60.0	60.8	36.5	41.4	55.6	56.8
24 h	52.5	48.3	41.9	36.4	64.4	63.3	32.4	12.4	18.2	37.7
48 h	63.5	55.9	49.4	60.1	73.1	67.9	44.1	14.7	57.6	37.0
72 h	74.0	61.7	68.0	61.6	87.0	62.0	56.1	47.5	60.8	70.6
S										
4 h	49.5	40.1	44.0	50.5	39.2	33.1	49.7	47.0	38.1	36.2
24 h	33.3	33.7	48.3	56.9	30.3	30.0	47.4	69.5	78.9	47.6
48 h	23.3	32.0	39.4	34.5	22.2	24.4	32.4	52.4	11.7	38.6
72 h	13.5	26.9	22.7	34.1	9.8	32.0	24.3	32.9	5.3	22.0
G2/M										
4 h	11.8	13.4	10.5	7.3	2.6	6.1	17.6	11.7	1.2	6.8
24 h	14.1	18.1	9.8	6.7	5.3	6.8	20.2	18.6	3.0	14.7
48 h	13.1	12.1	11.2	5.4	4.6	7.7	23.5	32.8	24.7	24.3
72 h	12.6	11.5	9.3	4.8	3.8	6.1	19.6	19.6	33.8	7.5

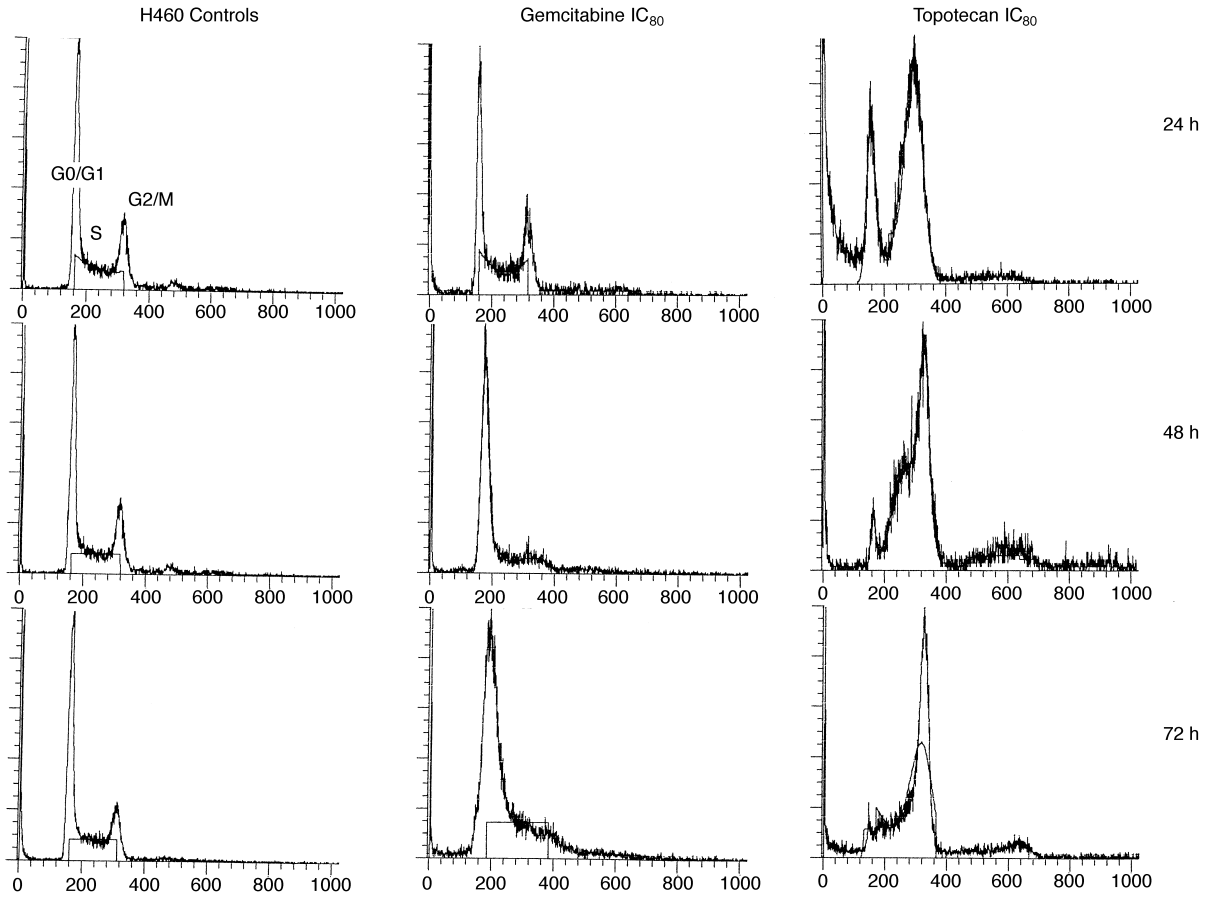

Figure 2. Characteristic DNA histograms of H460 cells, showing the progressive cell cycle changes observed after 24, 48 and 72 h of treatment with topotecan or gemcitabine IC_{80} concentrations, as compared with the untreated controls. Treated cells and controls were stained with propidium iodide, measured by FACS and cell phase distributions were determined using the Cell-Fit™ program (see Materials and Methods).

Apoptosis induction

The induction of apoptosis was investigated after treatment for 72 h with IC_{50} and IC_{80} of topotecan and gemcitabine. The response was evaluated at 4, 24, 48 and 72 h. We employed three different methods to estimate the induction of apoptosis: of these, FACS analysis gave the highest AI, the TUNEL assay the lowest (Figures 3 and 4). After 72 h drug exposure at IC_{80} values, a significant number of cells had morphological characteristics of necrosis. As these cells may represent truly necrotic or terminally apoptotic cells, an accurate AI could

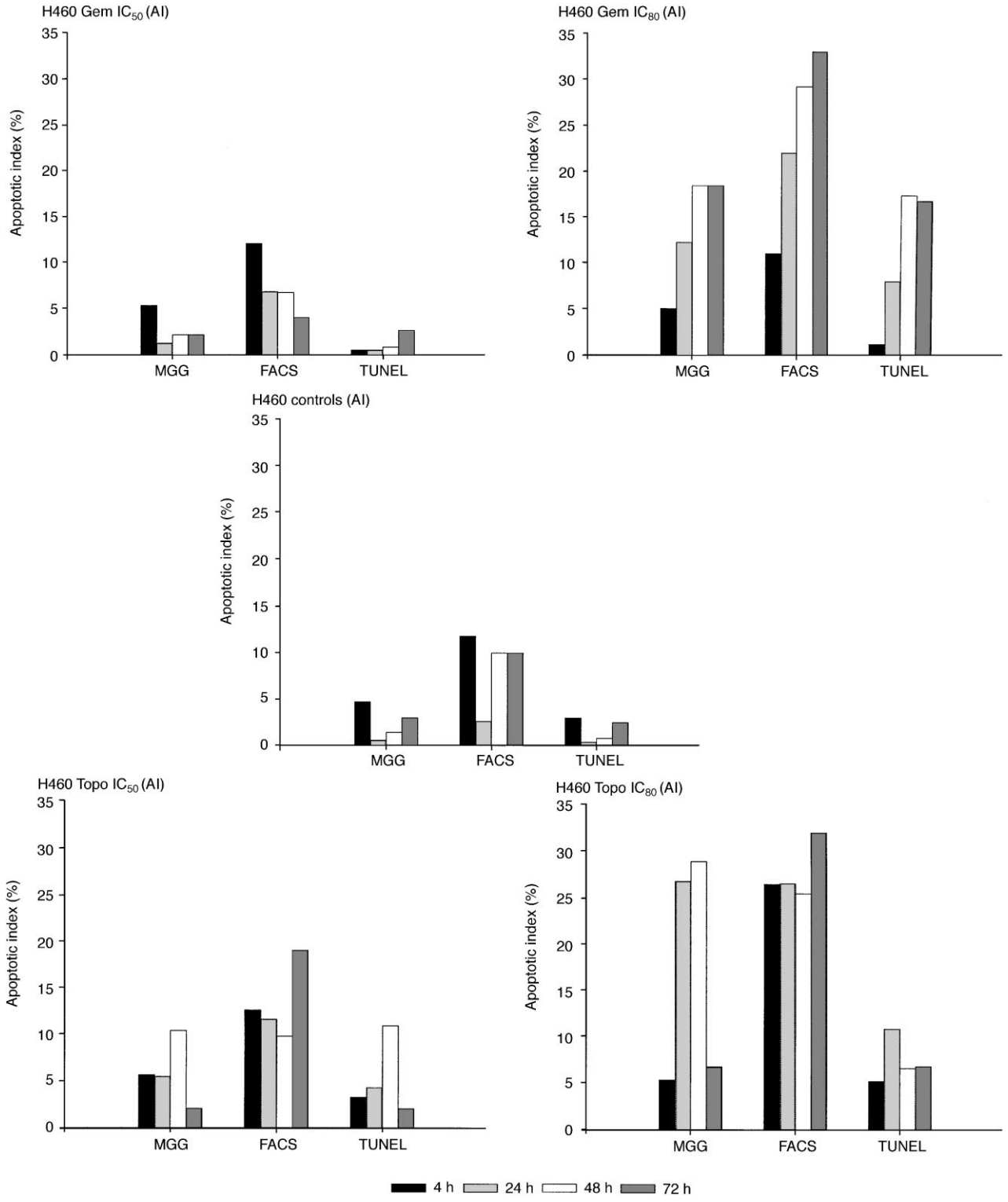

Figure 3. The induction of apoptosis was determined by three methods: May–Grünwald–Giemsa (MGG) staining, FACS analysis and the TUNEL assay. H460 cells treated with topotecan (Topo) and gemcitabine (Gem) at IC_{50} and IC_{80} concentrations were analysed at 4, 24, 48 and 72 h. For MGG and TUNEL, 400 cells per sample were counted and the percentage of positive cells was given as the apoptotic index (AI). Cells stained with propidium iodide were analysed by FACS and the sub-G1 population of cells (apoptotic cells) was estimated. Three experiments were performed for each time point.

not be determined for these samples. Basal levels of apoptosis were observed in the untreated cells, which ranged from 0.5 to 8%, and these were higher in the wild type *p53* H460 cell line and time-dependent: the highest values were observed at 72 h when the cultures became confluent and the cells were deprived of growth factors. Treatment with IC_{50} concentrations of topotecan and gemcitabine resulted in only moderate induction of apoptosis with levels of 5–18% and 4–14% for topotecan and gemcitabine, respectively. However, higher drug concentrations (IC_{80}) induced apoptosis more efficiently, and levels were highest at 48 and 72 h. Interestingly, higher levels of apoptosis were observed in H460, which also had higher basal levels of apoptosis. There was marked difference in the ability of cells to activate the apoptotic path-

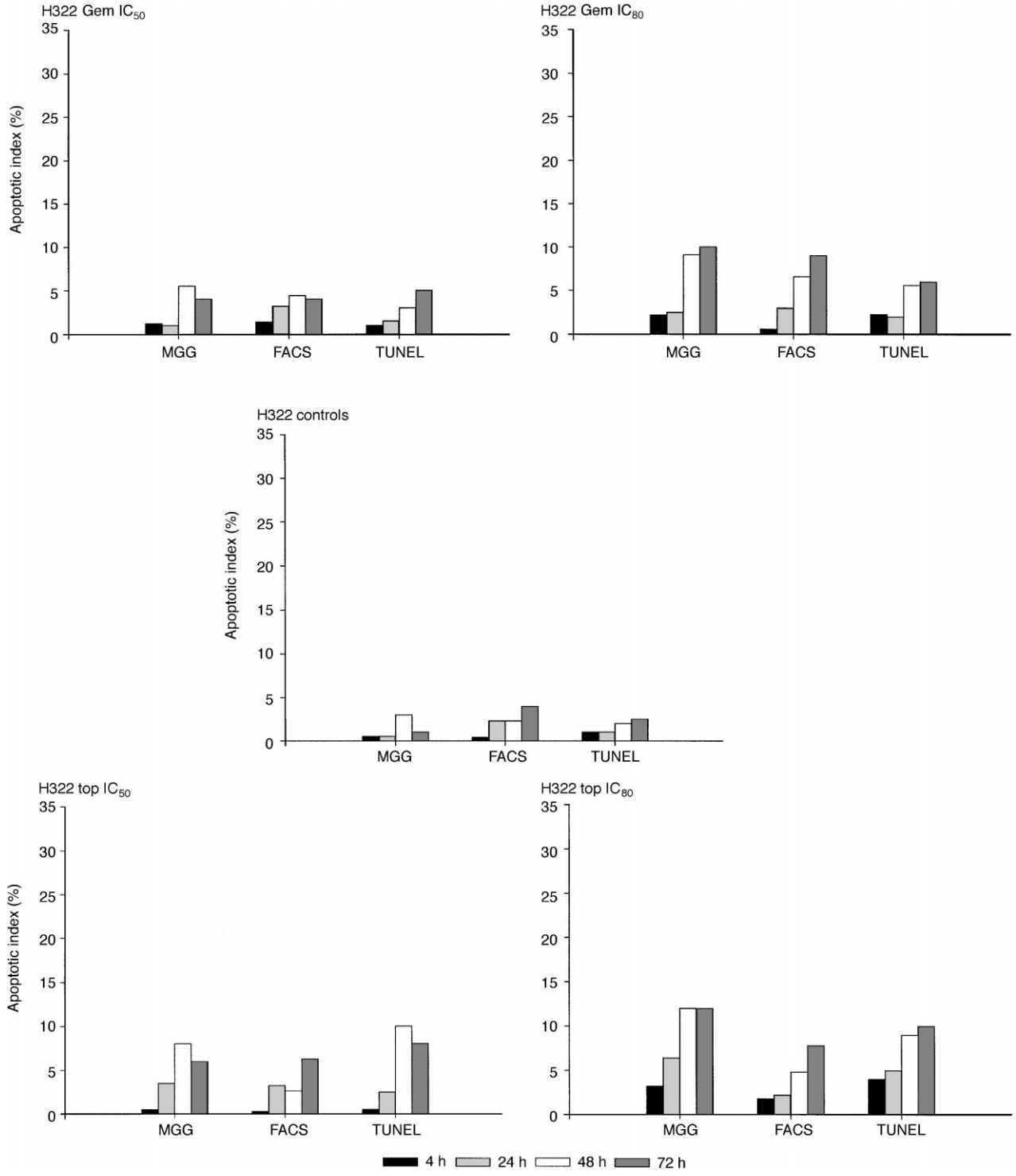

Figure 4. The induction of apoptosis was determined by three methods: May–Grünwald–Giemsa (MGG) staining, FACS analysis and the TUNEL assay. H322 cells treated with topotecan (Topo) and gemcitabine (Gem) at IC_{50} and IC_{80} concentrations were analysed at 4, 24, 48 and 72 h. For MGG and TUNEL, 400 cells per sample were counted and the percentage of positive cells was given as the apoptotic index (AI). Cells stained with propidium iodide were analysed by FACS and the sub-G1 population of cells (apoptotic cells) was estimated. Three experiments were performed for each time point.

way, depending on *p53* status: the AI for the wild type *p53* cell line H460 was 3- to 4-fold higher as compared with the mutant *p53* cell line H322. Nevertheless, apoptosis could also be induced in the H322 cell line.

Immunohistochemistry

The percentage of cells expressing p53 and p21 after treatment was determined by immunohistochemical staining of cytospins. H460 control cells were negative for p53 staining at 4 h, but showed induction of p53 and p21 (5–8% positive cells) as cultures became confluent (Figure 5). After treatment with both tested drugs, a marked increase in cells expressing p53 and p21 was observed, and this effect was time- and dose-dependent. The percentage of p21 positive cells was at all time points higher than that of p53 positive cells (for IC_{50} values), probably reflecting the shorter half-life of the p53 protein, rather than an alternative induction pathway of p21. At IC_{80} values the increase in the p21 expressing cells was minimal, as compared with p53 positive cells which clearly increased at this concentration. The higher percentage of cells expressing p53 at IC_{80} concentrations of topotecan and gemcitabine parallels the increased AI observed at these

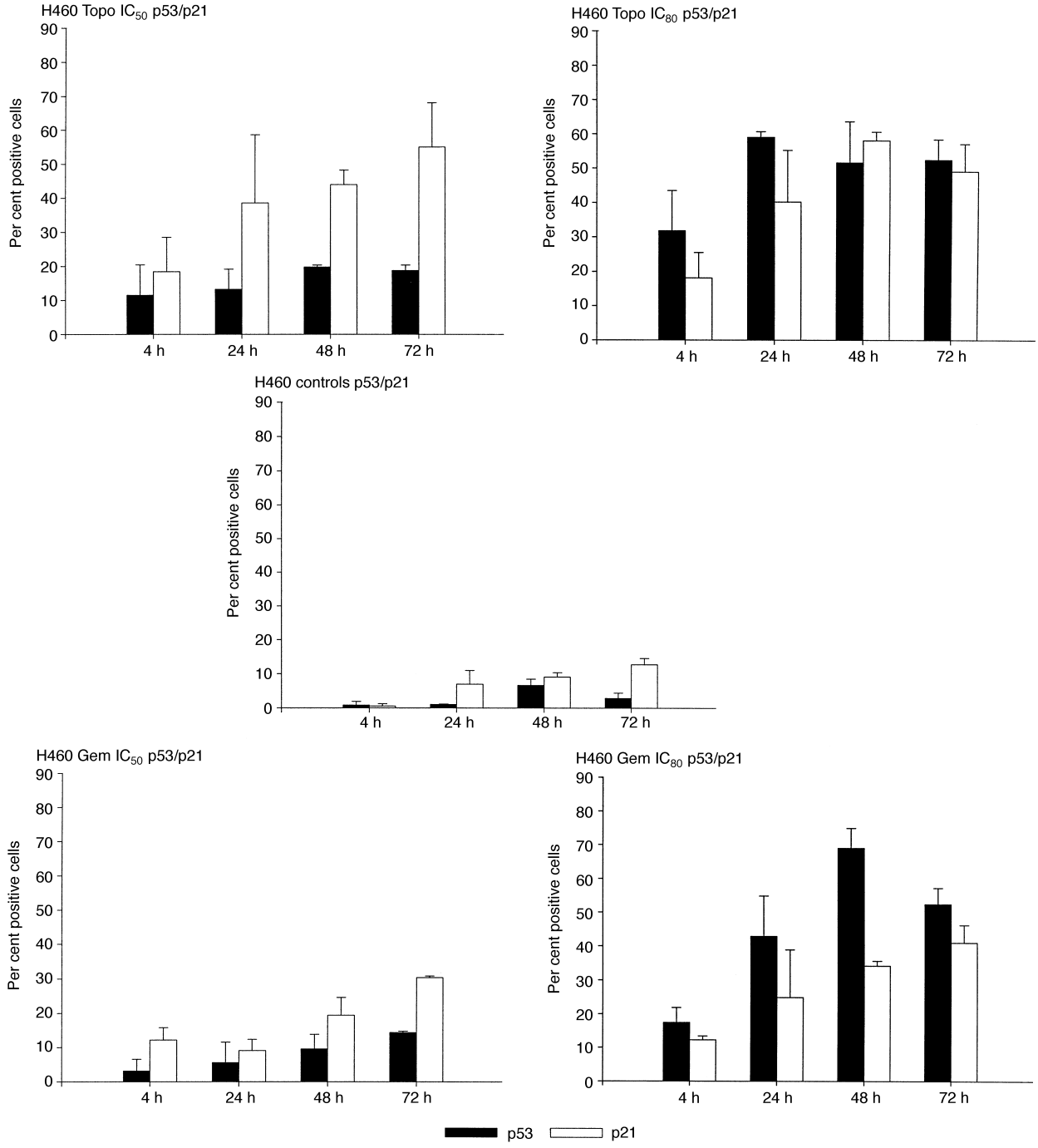

Figure 5. Effect of topotecan (Topo) and gemcitabine (Gem) on p53 and p21 expression on the H460 cell line. The results were assessed by immunohistochemistry. Treated cells were fixed and stained with antibodies raised against p53 and p21. Approximately 400 cells per slide were counted and an index (%) of positive staining cells was given (see Materials and Methods).

concentrations. 30 to 40% of H322 control cells stained positive for p53, but even with IC_{50} concentrations of topotecan and gemcitabine at 4 h 100% of the cells expressed a strong nuclear staining, concordant with the increased half-life of the mutant p53 protein. No p21 staining could be detected in this cell line (Figure 6). In the H460 cell line, the apoptotic cells did not show any p53 or p21 staining, indicating that the expression of these genes is an early event, before the morphological characteristics of apoptosis can be observed.

Western blot analysis

Concentration- and time-dependent upregulation of the expression of p53 was observed in the H460 cell line, after treatment with topotecan and gemcitabine. p53 expression in the H322 cell line was high at all time points and was not affected by treatment (data not shown). We used β-actin as a control for the loading of protein. However, β-actin in this case was not a good marker to quantitate protein concentrations as it was also affected by the apoptotic changes observed. In fact, actin is the substrate of CPP-32/apopain(-like)

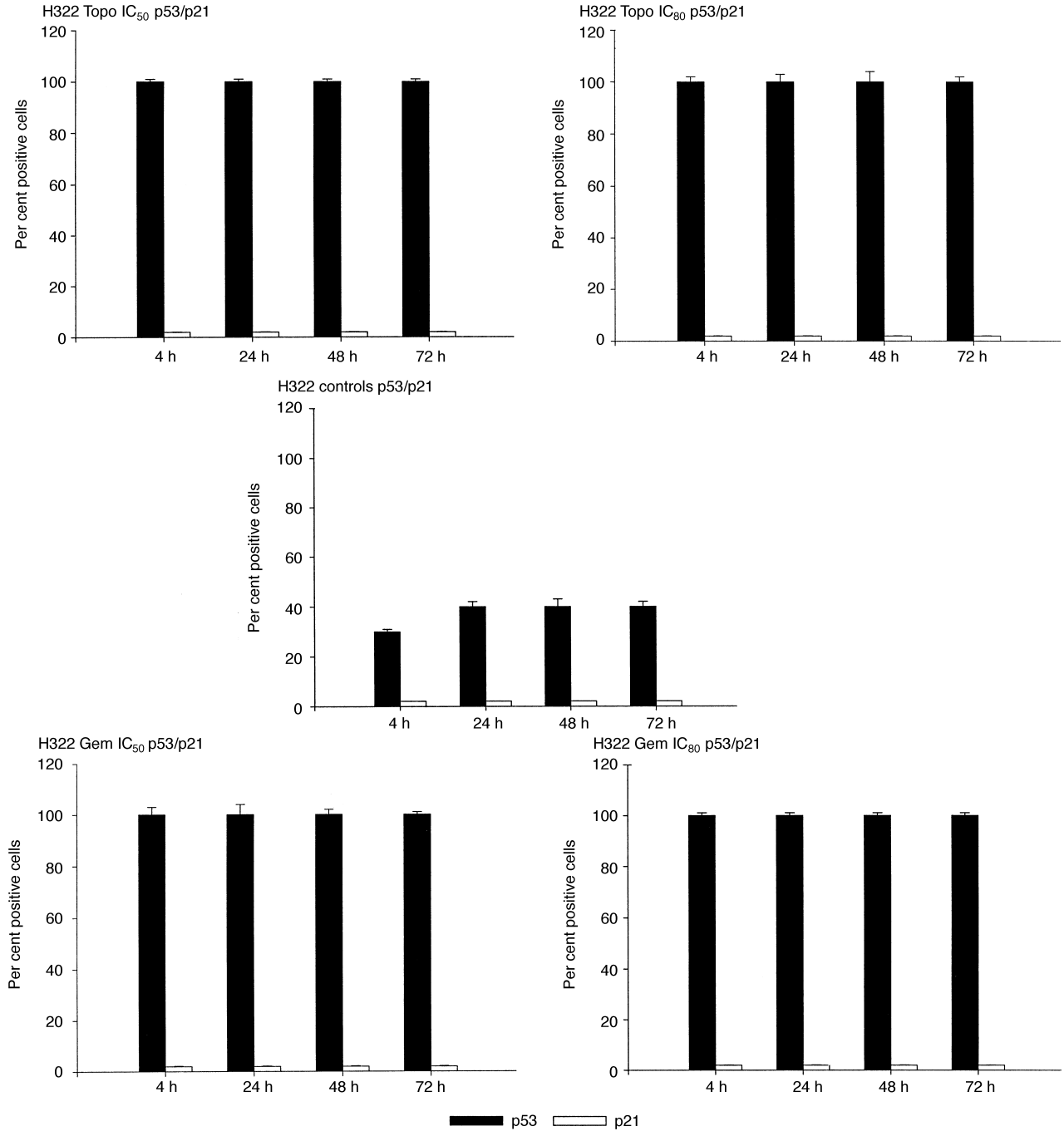

Figure 6. Effect of topotecan (Topo) and gemcitabine (Gem) on p53 and p21 expression on the H322 cell line. The results were assessed by immunohistochemistry. Treated cells were cytocentrifuged on slides fixed and stained with antibodies raised against p53 and p21. Approximately 400 cells per slide were counted and an index (%) of positive cells was given (see Materials and Methods).

protease both *in vitro* and *in vivo* and a role for actin in the control of cell growth and apoptosis has been suggested [25, 26].

DISCUSSION

Apoptosis has been shown to be a significant mode of cell death following cytotoxic drug treatment in a variety of tumour types. In this process *p53* plays a key role, as mutations of this gene have been correlated with increased resistance to chemotherapy, and restoration of normal *p53* function results in apoptotic cell death [27, 28]. In our study, the presence of wild type *p53* in the human lung cancer cell line H460 correlated with increased chemosensitivity to topotecan and gemcitabine treatment, as compared with the mutant *p53* cell line H322. These two compounds have shown activity in various preclinical models and in clinical studies [13, 29–31]. Favourable interaction of topotecan with other compounds, such as flavopiridol [32] or etoposide [33, 34] or irradiation [35] has been observed, although scheduling of the treatment seems to play an important role in optimising the effectiveness of the combinations. Topotecan cytotoxicity was shown to be enhanced mainly by combination with certain DNA damaging agents (melphalan, bis[chloroethyl]nitrosourea [BCNU], 4-hydroperoxycyclophosphamide and cisplatin), than by combination with antimetabolites (fluorouracil, methotrexate, or cytarabine) or antimicrotubule agents (vincristine or paclitaxel) [36]. Gemcitabine, a new nucleoside analogue, exhibits potent antitumour activity both *in vitro* and *in vivo* and has promising therapeutic effects in the treatment of certain solid tumours including ovarian cancer and NSCLC [37–40]. In preclinical models, the cisplatin–gemcitabine combination suggested synergy between the two drugs [41]. In phase I–II studies in advanced NSCLC, response rates were as high as 54% when gemcitabine was combined with cisplatin [42, 43]. It has also been shown that gemcitabine is a potent radiosensitiser, when drug incubation preceded radiation exposure [44, 45]. Radiosensitisation increased with increasing gemcitabine concentration and duration of exposure. In our study, however, the combination of topotecan and gemcitabine did not result in a synergistic effect, irrespective of the treatment schedule, although an additive effect could be observed. A plausible explanation for this observation is the different effect on cell cycle distribution induced by these drugs. Arrest of cells at G1 by gemcitabine minimises the population of cells that will enter S and G2/M phase, where topotecan is active and vice versa. None the less, the rationale for combining drugs is still unresolved, as combinations are often still purely empirical.

The cell kill mechanism of these two drugs is still unknown, although apoptosis has been shown to play a role in certain cell types with both drugs [46–49]. Topoisomerase I inhibitors induce enzyme-linked DNA breaks, but the steps leading to cell death after the cleavable complex is formed are, as yet, not well understood. Inhibition of DNA replication with, for example, a DNA polymerase inhibitor that blocks double strand break production, reduces topotecan cytotoxicity. Topotecan is, therefore, considered to be a S phase specific drug, producing preferential toxicity in proliferating cells [50, 51]. However, in another study, topotecan was highly active against CLL cells, showing no evidence of DNA synthesis, strongly arguing against a requirement for S phase progression in this cell type [52]. Since *p53* influences the apoptotic response, we investigated the induction of apoptosis of H460 and H322 cells with treatment with topotecan and gemcitabine. Numerous methods have been developed to distinguish apoptotic from necrotic cells. These methods are based on changes in cell morphology, plasma membrane structure and transport function, function of cell organelles, DNA stability to denaturation and endonucleolytic DNA degradation (reviewed in [53]). We employed three different assays to investigate apoptosis: MGG staining, the TUNEL assay and FACS analysis using PI. In this study, there was variability in the results depending on the method used. Assessment of morphological changes consistent with apoptosis by the MGG method seemed to reveal the most consistent and reproducible results. The cellular effects of IC_{80} concentrations of both drugs tested were so dramatic that the AI was difficult to score, since a high proportion of cells expressed features of necrosis, which could not be distinguished from secondary effects on terminally apoptotic cells. The calculation of the AI does not precisely estimate the real number of apoptotic cells because it does not take into consideration the different growth rate of cells, but it gives an indication of the capacity to activate the apoptotic pathway. There was a definitely higher tendency of the wild type *p53* H460 cells to undergo apoptosis as compared with the mutant *p53* H322 cells. However, H322 cells also retained the capability to die by apoptosis, but this process must probably be executed by an alternative *p53*-independent pathway, as described by others [23, 54, 55]. p53-initiated G1/S cell cycle arrest is primarily mediated by the upregulation of the cyclin-dependent kinase inhibitor p21 [56], but there are conflicting reports of the role of p21 in inducing apoptosis. In irradiated cells, p21 regulates the cell cycle and arrests the cell at the G1 checkpoint. p21 positive cells proceed to apoptosis or DNA repair in cell lines [57] and in another model it was shown that p21 is not an inducer of apoptosis in that ionising radiation induces p53-dependent apoptosis in $p21^{-/-}$ cells from $p21^{Waf1}$ gene knockout mice [58]. p53 has also been shown to have a role in regulating the G2/M checkpoint [59, 60], but the role of p21 in the G2/M transition is still unclear. We found that in the H460 cell line p53 expression could be induced in a time- and concentration-dependent manner, irrespective of the cytotoxic compound used. Although treatment with topotecan resulted in G2/M phase accumulation and treatment with gemcitabine resulted in G1 arrest, wild-type *p53* could be detected in both circumstances, indicating a role of *p53* in controlling both checkpoints. What determines whether *p53* directs the cell to G1/S or G2/M arrest is unknown at present. In our study, the pattern of p21 expressing cells paralleled p53, but the percentage of cells expressing p21 was higher at IC_{50} drug concentrations than that of p53. The percentage of p21 expressing cells could not be further increased at high concentrations (IC_{80}). Cells that showed features of apoptosis did not express p53 or p21, suggesting at least the role of p53 in the early events of apoptosis and also indicating the distinct function of p21 in cell cycle arrest [61].

In conclusion, the current study showed that topotecan and gemcitabine exert their antitumour effect by the activation of the apoptotic machinery. Although apoptosis could more easily be induced in the H460 (wild-type *p53* cell line), the process was also inducible in the H322 (mutant *p53*) cell line, indicating the presence of alternative, p53-independent pathways. The *p53* gene, apart from its well-documented

functions in G1 checkpoint control, may also regulate the G2/M transition by the upregulation of p21. The distinct patterns of cell cycle perturbations induced by these two drugs may be important in designing more rational and effective treatment schedules.

1. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995, **267**, 1456–1462.
2. Di Leonardo LA, Linke SP, Clarkin K, Wahl GM. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev* 1994, **8**, 2540–2551.
3. Kagawa S, Fujiwara T, Hizuta A, *et al.* p53 expression overcomes p21WAF1/CIP1-mediated G1 arrest and induces apoptosis in human cancer cells. *Oncogene* 1997, **15**, 1903–1909.
4. Caelles C, Helmberg A, Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes (see comments). *Nature* 1994, **370**, 220–223.
5. Fan S, el-Deiry WS, Bae I, *et al.* p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res* 1994, **54**, 5824–5830.
6. Haupt Y, Rowan S, Shaulian E, Kazaz A, Vousden K, Oren M. p53 mediated apoptosis in HeLa cells: transcription dependent and independent mechanisms. *Leukemia* 1997, **11**(Suppl. 3), 337–339.
7. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991, **253**, 49–53.
8. Hollstein M, Shomer B, Greenblatt M, *et al.* Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. *Nucleic Acids Res* 1996, **24**, 141–146.
9. Kovach JS, Hartmann A, Blaszyk H, Cunningham J, Schaid D, Sommer SS. Mutation detection by highly sensitive methods indicates that p53 gene mutations in breast cancer can have important prognostic value. *Proc Natl Acad Sci USA* 1996, **93**, 1093–1096.
10. Takahashi T, Suzuki H, Hida T, Sekido Y, Ariyoshi Y, Ueda R. The p53 gene is very frequently mutated in small-cell lung cancer with a distinct nucleotide substitution pattern. *Oncogene* 1991, **6**, 1775–1778.
11. Casey G, Lopez ME, Ramos JC, *et al.* DNA sequence analysis of exons 2 through 11 and immunohistochemical staining are required to detect all known p53 alterations in human malignancies. *Oncogene* 1996, **13**, 1971–1981.
12. Ardizzoni A, Hansen H, Dombernowsky P, *et al.* Topotecan, a new active drug in the second-line treatment of small-cell lung cancer: a phase II study in patients with refractory and sensitive disease. The European Organization for Research and Treatment of Cancer Early Clinical Studies Group and New Drug Development Office, and the Lung Cancer Cooperative Group. *J Clin Oncol* 1997, **15**, 2090–2096.
13. Thatcher N, Hopwood P, Anderson H. Improving quality of life in patients with non-small cell lung cancer: research experience with gemcitabine. *Eur J Cancer* 1997, **33**(Suppl. 1), S8–S13.
14. Binaschi M, Zunino F, Capranico G. Mechanism of action of DNA topoisomerase inhibitors. *Stem Cells (Dayt)* 1995, **13**, 369–379.
15. Okamoto-Kubo S, Nishio K, Heike Y, Yoshida M, Ohmori T, Saijo N. Apoptosis induced by etoposide in small-cell lung cancer cell lines. *Cancer Chemother Pharmacol* 1994, **33**, 385–390.
16. Fujiwara T, Grimm EA, Mukhopadhyay T, Zhang WW, Owen-Schaub LB, Roth JA. Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type p53 gene. *Cancer Res* 1994, **54**, 2287–2291.
17. Kalemkerian GP, Slusher R, Ramalingam S, Gadgeel S, Mabry M. Growth inhibition and induction of apoptosis by fenretinide in small-cell lung cancer cell lines (see comments). *J Natl Cancer Inst* 1995, **87**, 1674–1680.
18. Shimizu T, Pommier Y. Camptothecin-induced apoptosis in p53-null human leukemia HL60 cells and their isolated nuclei: effects of the protease inhibitors Z-VAD-fmk and dichloroisocoumarin suggest an involvement of both caspases and serine proteases. *Leukemia* 1997, **11**, 1238–1244.
19. Ruiz van Haperen VW, Veerman G, Vermorken JB, Peters GJ. 2′,2′-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. *Biochem Pharmacol* 1993, **46**, 762–766.
20. Winter SF, Minna JD, Johnson BE, Takahashi T, Gazdar AF, Carbone DP. Development of antibodies against p53 in lung cancer patients appears to be dependent on the type of p53 mutation. *Cancer Res* 1992, **52**, 4168–4174.
21. Campling BG, Pym J, Baker HM, Cole SP, Lam YM. Chemosensitivity testing of small cell lung cancer using the MTT assay. *Br J Cancer* 1991, **63**, 75–83.
22. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991, **139**, 271–279.
23. Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 1994, **73**, 2013–2026, 3108.
24. Gorczyca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 1993, **53**, 1945–1951.
25. Brown SB, Bailey K, Savill J. Actin is cleaved during constitutive apoptosis. *Biochem J* 1997, **323**, 233–237.
26. Levee MG, Dabrowska MI, Lelli JLJ, Hinshaw DB. Actin polymerization and depolymerization during apoptosis in HL-60 cells. *Am J Physiol* 1996, **271**, C1981–C1992.
27. Liu TJ, el-Naggar AK, McDonnell TJ, *et al.* Apoptosis induction mediated by wild-type p53 adenoviral gene transfer in squamous cell carcinoma of the head and neck. *Cancer Res* 1995, **55**, 3117–3122.
28. Gomez-Manzano C, Fueyo J, Kyritsis AP, *et al.* Adenovirus-mediated transfer of the p53 gene produces rapid and generalized death of human glioma cells via apoptosis. *Cancer Res* 1996, **56**, 694–699.
29. Peters GJ, Bergman AM, Ruiz van Haperen VW, Veerman G, Kuiper CM, Braakhuis BJ. Interaction between cisplatin and gemcitabine *in vitro* and *in vivo*. *Semin Oncol* 1995, **22**, 72–79.
30. Ardizzoni A, Hansen H, Dombernowsky P, *et al.* Topotecan, a new active drug in the second-line treatment of small-cell lung cancer: a phase II study in patients with refractory and sensitive disease. *J Clin Oncol* 1997, **15**, 2090–2096.
31. ten Bokkel Huinink W, Carmichael J, Armstrong D, Gordon A, Malfetano J. Efficacy and safety of topotecan in the treatment of advanced ovarian carcinoma. *Semin Oncol* 1997, **24**, S5–S25.
32. Bible KC, Kaufmann SH. Cytotoxic synergy between flavopiridol (NSC 649890, L86-8275) and various antineoplastic agents: the importance of sequence of administration. *Cancer Res* 1997, **57**, 3375–3380.
33. Bonner JA, Kozelsky TF. The significance of the sequence of administration of topotecan and etoposide. *Cancer Chemother Pharmacol* 1996, **39**, 109–112.
34. Whitacre CM, Zborowska E, Gordon NH, Mackay W, Berger NA. Topotecan increases topoisomerase II alpha levels and sensitivity to treatment with etoposide in schedule-dependent process. *Cancer Res* 1997, **57**, 1425–1428.
35. Lamond JP, Mehta MP, Boothman DA. The potential of topoisomerase I inhibitors in the treatment of CNS malignancies: report of a synergistic effect between topotecan and radiation. *J Neurooncol* 1996, **30**, 1–6.
36. Kaufmann SH, Peereboom D, Buckwalter CA, *et al.* Cytotoxic effects of topotecan combined with various anticancer agents in human cancer cell lines (see comments). *J Natl Cancer Inst* 1996, **88**, 734–741.
37. Carmichael J, Allerheiligen S, Walling J. A phase I study of gemcitabine and carboplatin in non-small cell lung cancer. *Semin Oncol* 1996, **23**, 55–59.
38. Giaccone G. New drugs in non-small cell lung cancer. An overview. *Lung Cancer* 1995, **12**(Suppl. 1), S155–S162.
39. Seiter K, Feldman EJ, Halicka HD, *et al.* Phase I clinical and laboratory evaluation of topotecan and cytarabine in patients with acute leukemia. *J Clin Oncol* 1997, **15**, 44–51.
40. Perez-Soler R, Fossella FV, Glisson BS, *et al.* Phase II study of topotecan in patients with advanced non-small-cell lung cancer previously untreated with chemotherapy. *J Clin Oncol* 1996, **14**, 503–513.
41. van Moorsel C, Veerman G, Bergman AM, *et al.* Combination chemotherapy studies with gemcitabine. *Semin Oncol* 1997, **24**, S7–S23.

42. Crino L, Scagliotti G, Marangolo M, *et al*. Cisplatin-gemcitabine combination in advanced non-small-cell lung cancer: a phase II study. *J Clin Oncol* 1997, **15**, 297–303.
43. Pedersen AG, Kanzawa F, Saijo N, *et al*. Phase I studies of gemcitabine combined with carboplatin or paclitaxel. *Semin Oncol* 1997, **24**, S7–S68.
44. Shewach DS, Lawrence TS. Gemcitabine and radiosensitization in human tumor cells. *Invest New Drugs* 1996, **14**, 257–263.
45. McGinn CJ, Shewach DS, Lawrence TS. Radiosensitizing nucleosides. *J Natl Cancer Inst* 1996, **88**, 1193–1203.
46. Huang P, Plunkett W. Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. *Cancer Chemother Pharmacol* 1995, **36**, 181–188.
47. Santini V, Bernabei A, Gozzini A, *et al*. Apoptotic and antiproliferative effects of gemcitabine and gemcitabine plus Ara-C on blast cells from patients with blast crisis chronic myeloproliferative disorders. *Haematologica* 1997, **82**, 11–15.
48. Traganos F, Seiter K, Feldman E, Halicka HD, Darzynkiewicz Z. Induction of apoptosis by camptothecin and topotecan. *Ann N Y Acad Sci* 1996, **803**, 101–110.
49. Planchon SM, Wuerzberger S, Frydman B, *et al*. Beta-lapachone-mediated apoptosis in human promyelocytic leukemia (HL-60) and human prostate cancer cells: a p53-independent response. *Cancer Res* 1995, **55**, 3706–3711.
50. Holm C, Covey JM, Kerrigan D, Pommier Y. Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I and II inhibitors in Chinese hamster DC3F cells. *Cancer Res* 1989, **49**, 6365–6368.
51. Seiter K, Feldman EJ, Halicka HD, *et al*. Phase I clinical and laboratory evaluation of topotecan and cytarabine in patients with acute leukemia. *J Clin Oncol* 1997, **15**, 44–51.
52. Jonsson E, Fridborg H, Csoka K, *et al*. Cytotoxic activity of topotecan in human tumour cell lines and primary cultures of human tumour cells from patients. *Br J Cancer* 1997, **76**, 211–219.
53. Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 1997, **27**, 1–20.
54. Lanni JS, Lowe SW, Licitra EJ, Liu JO, Jacks T. p53-independent apoptosis induced by paclitaxel through an indirect mechanism. *Proc Natl Acad Sci USA* 1997, **94**, 9679–9683.
55. Midis GP, Shen Y, Owen-Schaub LB. Elevated soluble Fas (sFas) levels in nonhematopoietic human malignancy. *Cancer Res* 1996, **56**, 3870–3874.
56. el-Deiry WS, Tokino T, Velculescu VE, *et al*. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993, **75**, 817–825.
57. el-Deiry WS, Harper JW, O'Connor PM, *et al*. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 1994, **54**, 1169–1174.
58. Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ. Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* 1995, **377**, 552–557.
59. Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci USA* 1995, **92**, 8493–8497.
60. Stewart N, Hicks GG, Paraskevas F, Mowat M. Evidence for a second cell cycle block at G2/M by p53. *Oncogene* 1995, **10**, 109–115.
61. Attardi LD, Lowe SW, Brugarolas J, Jacks T. Transcriptional activation by p53, but not induction of the p21 gene, is essential for oncogene-mediated apoptosis. *EMBO J* 1996, **15**, 3693–3701.

Acknowledgements—This work was supported by a grant from the European Cancer Centre.