Anti-Cancer Drugs, 1, pp. 37–44

Cell death in relation to cell cycle in a mouse ascites tumor growing *in vivo* after combined treatment with cisplatin and 5-fluorouracil

F Lewin,[CA] S Skog, B Tribukait and U Ringborg

F Lewin, S Skog and B Tribukait are at the Department of Medical Radiobiology, Karolinska Institute, S-104 01 Stockholm, Sweden. U. Ringborg is with the Department of General Oncology, Radiumhemmet, Karolinska Hospital, S-104 01 Stockholm, Sweden. F Lewin is also affiliated to the Department of General Oncology, Karolinska Hospital. Address requests for reprints to Dr Freddi Lewin, Department of Medical Radiobiology, Karolinska Institute, Box 60204, S-104 01 Stockholm, Sweden.

The interaction of 5-fluorouracil (5-FU) and cisplatin (CDDP) was studied in Bp8 ascites sarcoma cells growing in mice. By density gradient centrifugation in Percoll™ solution, non-viable cells were separated from viable cells. Single drug treatment with doses of 12 and 36 mg/kg body weight of 5-FU and 0.4 and 0.8 mg/kg body weight of CDDP did not yield non-viable cells during the time period studied (96 h). Combination of the drugs increased the non-viable cells to about 10–25% depending on the doses given. This indicates a supra-additive cytotoxic effect. Analysis of the distribution of viable cells in the different cell cycle phases by flow cytometry showed an accumulation of cells in the S-phase after 5-FU and in the S- and G_2-phases of the CDDP or combined 5-FU–CDDP treatment. Similar analysis of non-viable cells showed a similar cell cycle distribution, which suggests that supra-additive cytotoxicity is not cell cycle specific. By labeling the DNA of the tumor cells with [^{125}I]-deoxyuridine and using whole body measurement, the cell loss was studied. No changes in cell loss after single drug and combined treatment were found during the observation time. The molecular basis for the interaction between 5-FU and CDDP should be elucidated.

Key words: Cell death, cisplatin, 5-fluorouracil, combined treatment, ascites tumor, *in vivo*.

Introduction

The mechanisms that are responsible for the antitumor activity of 5-fluorouracil (5-FU) are not fully understood. One important mechanism is interference with the DNA replication by inhibition of the thymidylate synthase, an enzyme necessary for *de novo* synthesis of thymidine monophosphate.[1] Other mechanisms have been described, such as incorporation of the drug into the DNA molecule or into RNA.[2] Cisplatin (CDDP) induces cytotoxicity by binding to the DNA and, most probably, DNA–DNA crosslinks are the most important cytotoxic DNA lesions.[3,4] In an animal tumor system a supra-additive cytotoxic effect was obtained when 5-FU was administered in combination with CDDP.[5] In the treatment of patients with advanced squamous cell carcinoma of the head and neck a high response rate is obtained when CDDP is combined with 5-FU.[6] Although not tested, it is possible that the combination of the drugs causes a supra-additive cytotoxic effect. The antitumor effect consists of cell death and/or inhibition of cell progression through the cell cycle. In principle, two types of cell death can be distinguished, i.e. reproductive and interphase death, having different mechanisms. Reproductive cell death is connected with cell division and appears in the G_1-phase after mitosis while interphase cell death is non-cell cycle specific. Determination of the cell death induced by cytotoxic agents in relation to the cell cycle may therefore be of great value in order to understand the mechanism behind the cell kill.

Because there is a possible interaction between CDDP and 5-FU, which may result in an increased tumor cell killing, studies of this drug combination should be performed in experimental systems with the aim of identifying the mechanisms responsible for the supra-additive cytotoxicity.

We thank Bristol-Myers AB, Solna, Sweden and Roche AB, Skärholmen, Sweden for providing the drugs.

The investigation was supported by a grant from the Cancer Society of Stockholm and the Gustav Vs Jubilee Foundation.

[CA] Corresponding Author

© *1990 Rapid Communications of Oxford Ltd.*

Materials and methods

Experimental tumor and animals

Bp8 ascites tumor cells were administered every 10th day by i.p. injection into 3-month old male NMRI mice with a body weight of 20–25 g. Water and standard food were given *ad libitum*. At the start of the experiment (day 0), 20×10^6 cells in 0.2 ml saline were transplanted after appropriate dilution.

Cell cycle composition

The cellular DNA content was measured using the rapid flow cytofluorometric method previously described.[7] After correction for background, the proportion of cells in different phases of the cell cycle (G_1, early-S, late-S, G_2 + M) was determined from the areas of the histograms assuming a Gaussian function of the G_1 and G_2 + M maxima and attributing the remaining part of the DNA histograms to the cells of the S-phase, which was divided into two equal parts called early-S and late-S.

Cytostatic treatment

On day 4 after transplantation, cisplatin (CDDP) (0.5 mg/ml) and 5-fluorouracil (5-FU) (25 mg/ml) were injected i.p. into the mice at the exponential growth of the tumor, in a volume of 0.2 ml after proper dilution with normal saline solution. CDDP was given at the doses of 12.5 and 25 μg per animal corresponding to $\sim$ 0.4 and 0.8 mg/kg body weight respectively. 5-FU was given at doses of 0.3 and 0.9 mg per animal corresponding to about 12 and 36 mg/kg body weight respectively. When combining the two drugs, CDDP was given 30 min prior to 5-FU.

Density separation

10–20 $\times$ 10^6 cells were mixed with 10 ml of a Percoll™ solution without washing in order to prevent cell damage. The Percoll™ solution was prepared as follows. Nine parts of a Percoll™ stock solution (Pharmacia, Sweden) were diluted with one part of 1.5 mol/l NaCl. A mixture of 48% of the diluted Percoll™ solution and 52% saline (0.9%) was produced. In order to prevent cell aggregation, sodium methyl cellulose was added to a final concentration of 0.2%. To create the density gradient, the tubes were centrifuged at 15 000 rpm for 15 min using a JA-20 Beckman rotor head at 4°C. The gradient was harvested in 20 fractions of 0.5 ml by means of 75% sucrose introduced at the bottom of the tube, with a pump speed of 2.0 ml per min. The procedure was performed at 0°C. 12.5–25 μl from each density fraction was diluted with 10 ml Tris–NaCl–EDTA buffer (0.1 M Tris, 0.01 M NaCl, 0.005 M EDTA, pH 7.5) and the cells were counted twice in a Coulter counter™.

Whole-body measurement of [^{125}I]-deoxyuridine radioactivity

2 μCi [^{125}I]-deoxyuridine (45 000 c.p.m.) in 0.2 ml saline (0.9%) was i.p. injected into the mice at 30, 15 and 1 h prior to treatment. The radioactivity incorporated into the DNA of the cells of the mice was then measured at 6, 24, 48, 72, 96 and 174 h by whole body counting, using a Gamma camera GE 400.[8] The mice were kept in Plexiglas boxes allowing a minimum of movement.

Results

Density gradient centrifugation of cells after treatment with CDDP and 5-FU

For untreated cells and cells treated with CDDP or 5-FU one distinct peak was observed (Figure 1a, b) with only a few cells distributed in all parts of the gradient. The gradient should be divided into two parts. The first part contained the top three fractions of the tube; the second part contained the 4–18 fractions, corresponding to a density of less than 1045 and between 1060–1085 g/ml, respectively. The cells in part 1 and part 2 have previously been defined as non-viable and viable cells, respectively, according to a dye exclusion test and colony forming assay.[9] The density of single drug treated cells was unchanged during the observation period (Figure 1b). After treatment with the combination of CDDP and 5-FU the cell density was changed, resulting in a distribution of cells in other parts of the gradient (Figure 1c).

Cell cycle distribution after treatment with CDDP and 5-FU

The cell growth and the cell cycle composition after treatment with CDDP and 5-FU have been studied in detail elsewhere.[10,11] We found a good agreement with previous results. Thus, cells treated with 5-FU were transiently accumulated in early S-phase without any significant trapping in G_2-phase

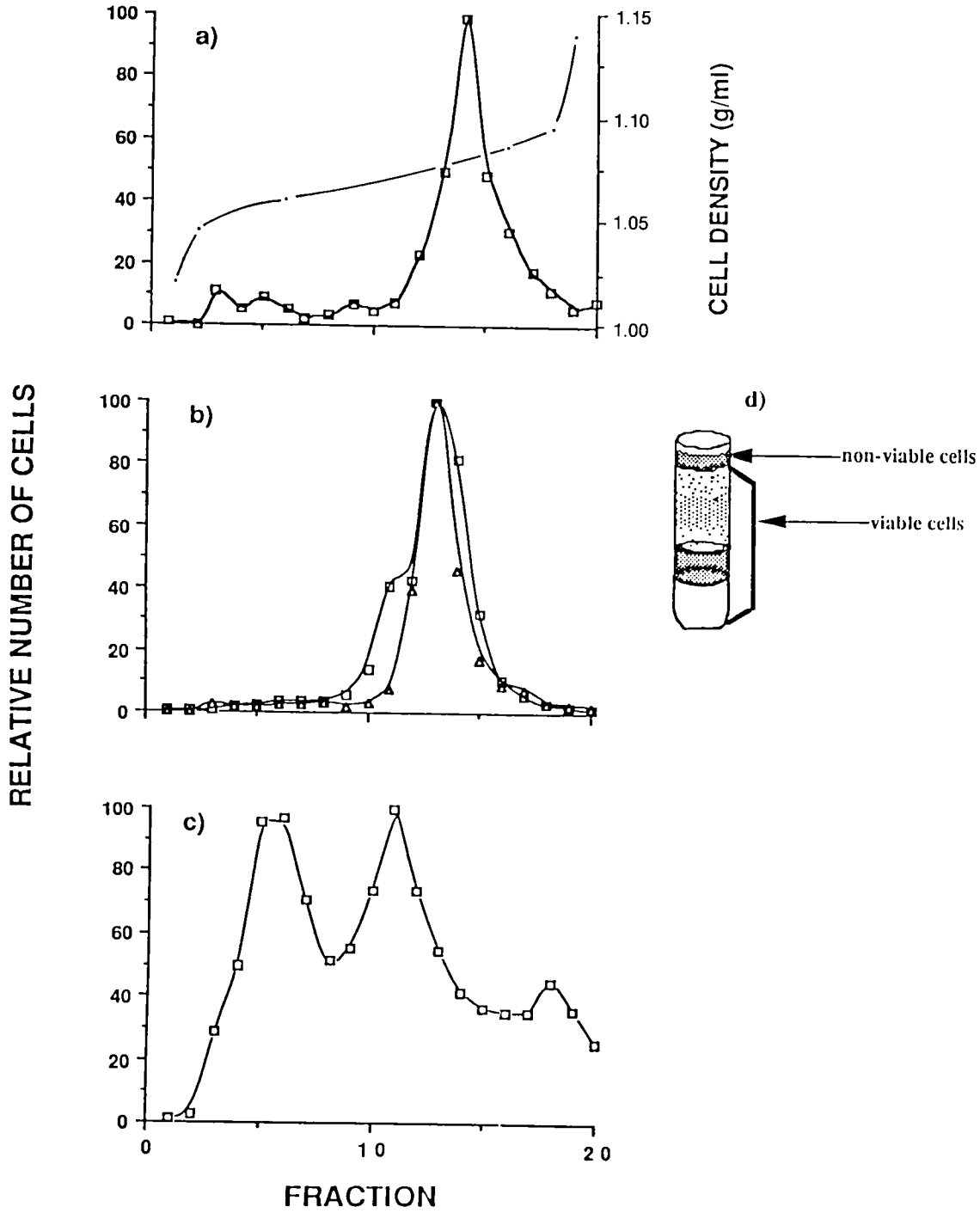


Figure 1. Cellular density of: (a) untreated cells, (–□–) number of cells in each fraction, (–•–) shape of the gradient; (b) cells 24 h after treatment with 5-FU (–△–) and CDDP (–□–); and (c) cells treated with a combination of the same doses of 5-FU and CDDP. (d) A depiction of the gradient tube. The fraction containing the highest number of cells was put as 100%. The results are representative examples of 6 animals for each group.

(Figure 2a). Cells treated with CDDP were accumulated in both S- and G_2-phases (Figure 2). Combination of the drugs resulted in accumulations corresponding to single drug treatments, i.e. in S- and G_2-phases, the later stage somewhat pronounced.

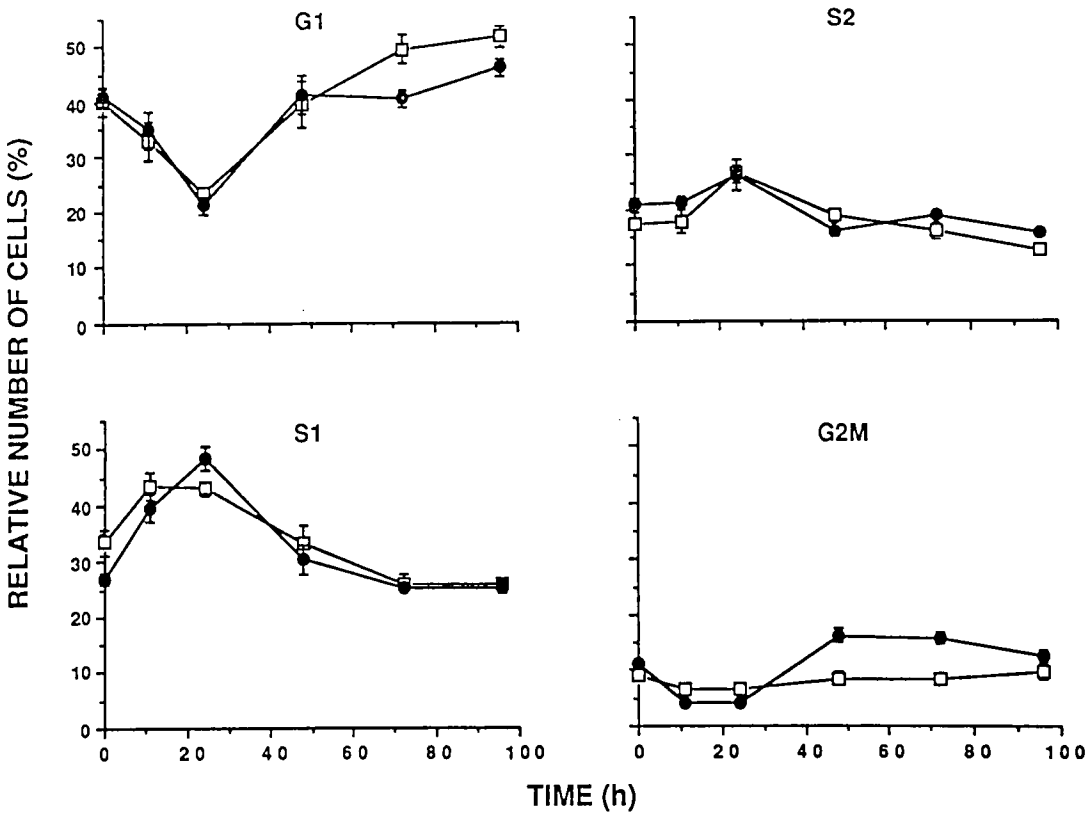
Analysis of dead cells after treatment with CDDP and 5-FU

A relative number of dead cells in the untreated cell population was measured by the density gradient centrifugation and was found to be less than 3%. Single drug treatment did not significantly increase cell death (Figure 3a, b). Combination of the two drugs increased the number of dead cells. For the combination of 0.3 mg 5-FU and 12.5 μg CDDP an increased cell death of about 10% of the population was seen at 48 h (Figure 3c). For cells treated with the combination of 0.9 mg 5-FU and 25 μg CDDP, the dead cells were about 25% of the total cell population at 96 h (Figure 3d).

Disintegration of cells is not detected with density gradient centrifugation. We therefore labeled the tumor cells with [^{125}I]-deoxyuridine and measured the total radioactivity of the tumor by whole body counting in a scanner. In untreated cells, radioactivity declined about 30% during the first 18 h followed by another 20% gradual decline during the observation period of 174 h (Table 1). These values correspond to a cell death rate of 1.7%/h during the first 18 h and a cell death rate of 0.2%/h thereafter. No significant difference was observed between the treated and untreated cells. treatment due to disintegration, was observed.

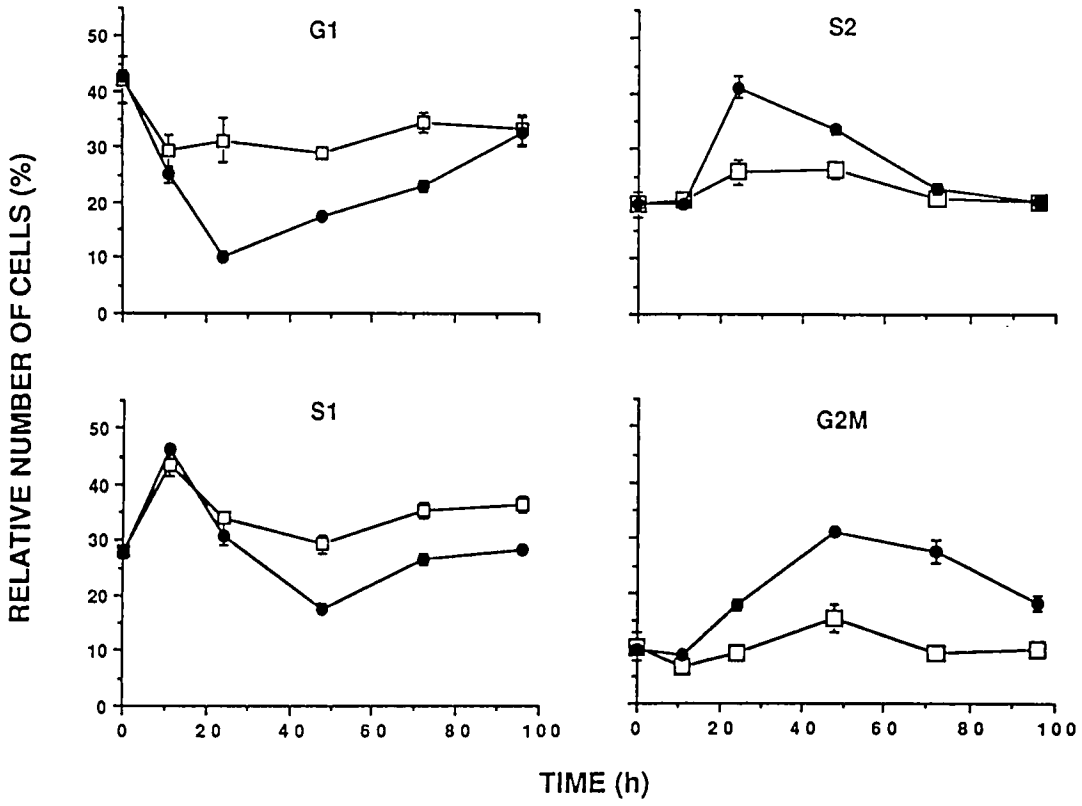
Cell cycle distribution of dead and living cells after treatment with CDDP and 5-FU

The cell cycle composition of dead and living cells of untreated normal growing tumor was essentially the same during the observation time (Table 2.) For cells treated with 5-FU the cell cycle distribution was essentially the same in non-viable and viable cells (Figure 2a). A slightly higher percentage of dead cells in the G_1-phase at 72–96 h after treatment was, however, found, as well as some decrease of dead cells in the G_2 + M-phase.

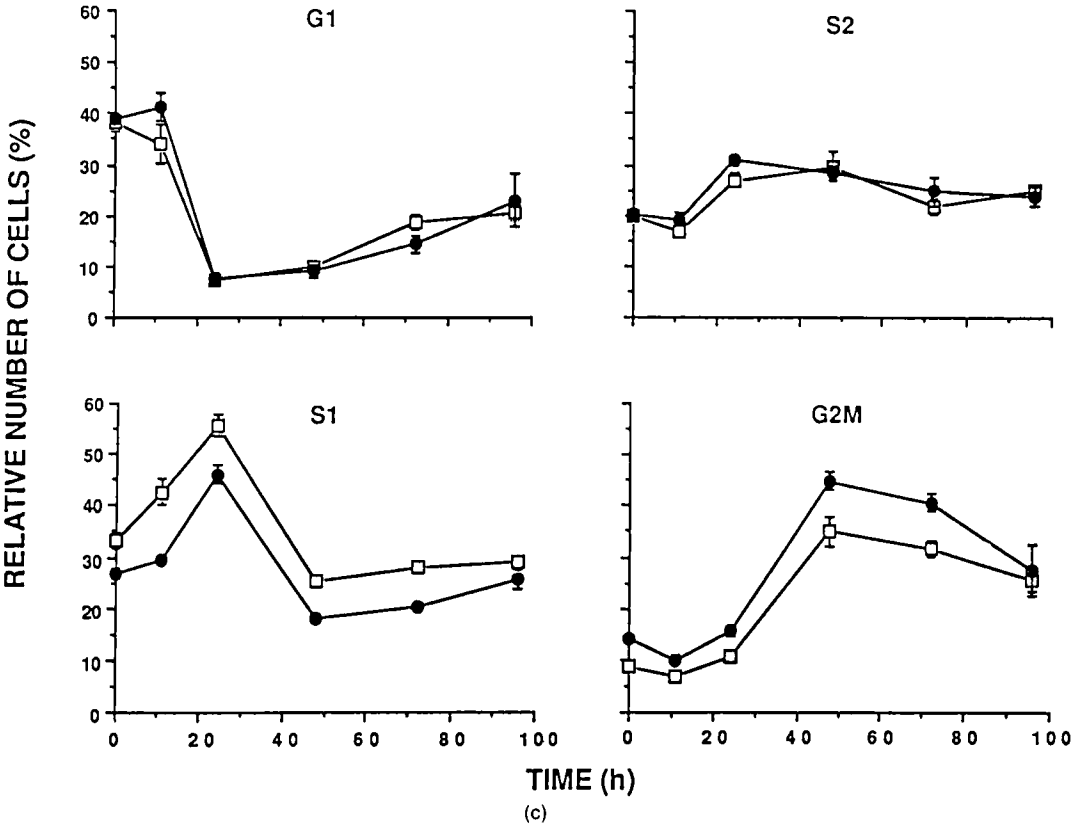


(a)

Figure 2. Cell cycle distribution of G_1, S-phase and G_2 + M cells of dead cells (–□–) and living cells (–●–) after treatment with 5-FU and CDDP and a combination of the two drugs. Mean values of 5–6 mice ± SE. (a) 0.9 mg 5-FU; (b) 25μg CDDP; (c) 0.9 mg 5-FU + 25 μg CDDP.



(b)



(c)

Figure 2 (*continued*)

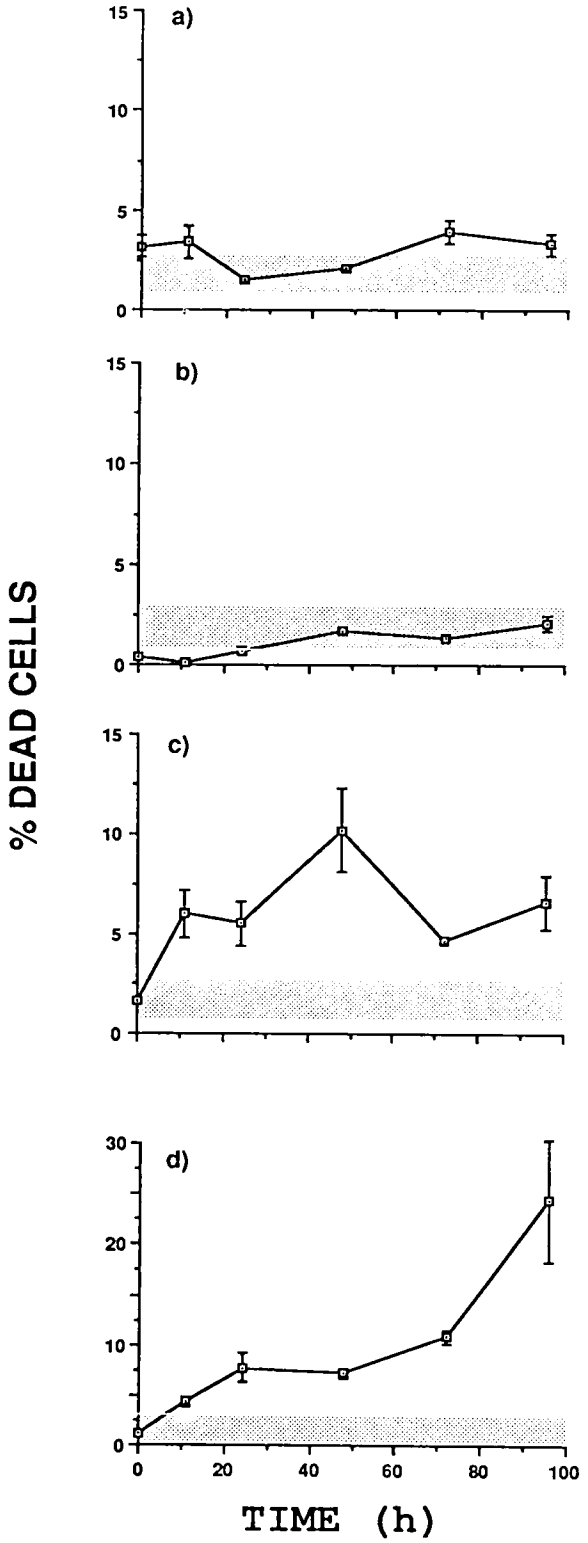


Figure 3. Relative number of dead cells after treatment with (a) 0.9 mg 5-FU; (b) 25 μg CDDP; and combinations of these two drugs: (c) 0.3 mg 5-FU and 12.5 μg CDDP; (d) 0.9 mg 5-FU and 25 μg CDDP. The dotted areas indicate the mean percentage of dead cells in the untreated animals. Mean values of 5–6 mice ± SE.

Table 1. Relative radioactivity in mice—not treated, and treated with 5-FU, CDDP and a combination of the two drugs[a]

Time (h)	Controls	5-FU	CDDP	5-FU + CDDP
6	100	100	100	100
24	69 ± 7	65 ± 4	77 ± 7	74 ± 10
48	61 ± 4	59 ± 4	72 ± 7	66 ± 11
72	56 ± 5	54 ± 5	66 ± 6	59 ± 11
96	55 ± 8	52 ± 6	66 ± 9	58 ± 10
174	46 ± 7	43 ± 3	49 ± 7	43 ± 5

[a] The mice were injected i.p. with 0.2 ml [^{125}I]-deoxyuridine (45 000 c.p.m.).
Values are percentage of initial values. Each value represents the mean of 5 mice for each treatment ± SE.

The cells treated with CDDP showed an increased percentage of dead cells in G_1- and early-S-phases from 12 h after treatment (Figure 2b). A decrease in the amount of dead cells in the late-S and G_2 + M-phases was also observed.

For cells treated with the combination of CDDP and 5-FU less marked differences were noted. There was a slight increase in the percentage of dead cells in the early part of the S-phase and a minor decrease in the percentage of dead cells in the G_2 + M-phases as compared to living cells (Figure 2c). Thus, the combination of CDDP and 5-FU induces a supra-additive cytotoxicity, but the cell death obtained is not cell cycle specific.

Discussion

Cell death can be studied by different methods, which measure various steps of the processes of the dying cells. The colony forming assay measures decreased ability to divide. A bias in this technique may be cells reversibly arrested in their growth. The dye excluding methods identify cells with affected cell membrane functions. The reason may be changes in the cellular metabolism which results in cell death. But altered membrane functions may also be caused by transient membrane damages. Isotope techniques, like labeling cellular DNA with [^{125}I]-deoxyuridine, measure cell disintegration over time as thymidine from breakdown of DNA from dying cells is rather inefficiently reincorporated into DNA of living cells.[8] The cell death rate expected in untreated Bp8 ascites cells is presumed to be about the same as for Ehrlich ascites tumor cells. In these cells the rate of cell death during exponential growth has been estimated to 0.3%/h.[12] The decline in radioactivity seen in our experiment in untreated cells after one day would represent a cell death rate of about 1.7%/h. A de-iodination of

Table 2. Cell cycle composition of dead (D) and living (L) untreated cells

Time (days)	%G_1-phase		%S-phase		%G_2 + M-phases	
	L	D	L	D	L	D
4	40.5 ± 8.4	37.3 ± 4.9	46.5 ± 4.8	53.3 ± 4.5	12.8 ± 4.2	8.3 ± 0.8
5	44.5 ± 7.3	47.2 ± 5.7	44.3 ± 4.0	46.0 ± 5.5	11.0 ± 3.7	7.0 ± 1.7
6	41.3 ± 5.5	41.2 ± 8.0	45.8 ± 3.8	49.8 ± 6.7	12.8 ± 2.8	9.0 ± 3.3
7	48.2 ± 2.0	45.7 ± 5.6	40.5 ± 2.8	44.7 ± 3.9	11.7 ± 1.2	9.0 ± 1.8
8	49.0 ± 4.0	45.2 ± 2.8	39.0 ± 2.1	46.0 ± 2.8	12.3 ± 2.2	8.7 ± 2.2

Mean values of 5–6 mice ± SD.

deoxyuridine is known to occur.[8] This might partly explain the decrease in radioactivity seen in both controls and treated cells during the first 18 h. Another, maybe more plausible, explanation might be that in this experiment the last portion of [^{125}I]-deoxyuridine was injected only 7 h before the first whole body counting. Some of this [^{125}I]-deoxyuridine was probably not incorporated into the cells but rather, to a large extent, excreted from the animal (Table 1). The method used to study cell cycle related cell death was density gradient centrifugation in combination with flow cytometry.[9] By the density gradient centrifugation technique potentially dead cells can be separated from viable cells due to cell membrane damage, which changes the density of the cells. It is obvious that such a method detects only part of the dead or dying cells. Further, cell cycle related studies of dead cells will be inconclusive if the number of disintegrated cells is significant and if disintegration occurs with different rates after different treatments. However, by means of [^{125}I]-deoxyuridine labeling we have not found any significant increase in disintegration of cells after the cytostatic treatment during the time of the experiment, as compared to controls.

There was no significant increase in the relative number of dead cells following treatment with 5-FU or CDDP as compared to control cells. In earlier studies, when Bp8 sarcoma cells were treated with 5-FU and CDDP, we showed a dose dependent transient arrest in the S-phase up to 24 h and for cells treated with CDDP there was also an accumulation of cells in G_2.[10,11] From these results it is reasonable to predict an elevated death of these arrested cells. However, no such increase was found during the time when cells were arrested. Instead, an increasing relative number of dead G_1 cells was observed 24 h after the CDDP treatment. At that time the cell cycle progression was reduced but there still was an outflow of cells from M, allowing cells to divide and therefore enter the G_1 phase. However, it has been reported that cells in G_1 treated with alkylating agent show a higher sensitivity to the drug than cells in other phases of the cell cycle.[13,14] It is therefore impossible to determine if the cell death after CDDP treatment occurs before or after mitosis.

Also, for 5-FU treated cells, a slightly increased distribution of dead cells in the G_1-phase was found. An increase of dead cells in G_1 might well be due to cells dying after the first mitosis after treatment, i.e. reproductive cells death after 5-FU treatment. It has been previously shown that 5-FU, in this dose range, gives rise to DNA strand breaks, predominantly double strand breaks (DSB).[15]

CDDP induces DNA cross-links[3,4,15] that probably generate both single and double strand breaks in the DNA when repaired. Repair of double strand breaks is believed to result in chromosomal aberrations of various types. One of the most likely mechanisms responsible for reproductive cell death is chromosomal aberrations resulting in less genetic material in one of the two new daughter cells. As cell death after single drug treatment with the dosages used did not differ significantly from the untreated cells, it might be hazardous to speculate if the reproductive cell death is the general path of death in cells treated with CDDP or 5-FU.

Cell death following the combination of the drugs showed more than additive effect and was elevated from 2–3% in the single drug treatment to about 25% in the combination. Even with a combination of the two drugs with lower drug concentrations, at least an additive number of dead cells was found. In contrast to single drug administration no significant predominance of any cell cycle phases for the dead cells were found. Furthermore, the increase in the number of dead cells had already started after 12 h. These two observations speak in favor of the hypothesis that the elevated cell death caused by the combined treatment is due to interphase cell death. It is possible when cell death reaches maximum levels at

96 h, that cells may be recycling in the cell cycle with a mixture of reproductive and interphase cell death. However, this probably would result in an increased amount of dead cells in the G_1 phase at 96 h, an increase that we did not find. Our findings suggest a different mechanism of cell death when combining the two drugs, as compared to the mechanism responsible for the cell death after single drug treatment. The mechanism behind the supra-additive cytotoxic effect by combined 5-FU–CDDP treatment has not yet been determined. Imbalanced growth has to be considered as one possible mechanism of cell death. An imbalance in the protein:DNA ratio and/or in the deoxynucleoside triphosphate pools may cause cell death by induction of DNA-strand breaks.[15–17] It is also possible that the addition of 5-FU to CDDP either increases the amount of DNA cross-links induced by CDDP or decreases the removal of such cross-links; or that the 5-FU may influence the DNA repair process other than by modifying the level of CDDP induced DNA cross-linking. We also found that the cells as a consequence of the combined 5-FU–CDDP treatment continuously increase their cellular density. This may be a result of a general reduced metabolic activity due to the processes of death or caused by a specific cellular membrane damage due to the combined drug treatment.

Conclusion

A supra-additive effect on cell death was seen when combining 5-FU and CDDP in the treatment of Bp8 mouse ascites tumor. For cells treated with 5-FU or CDDP, a slightly increased distribution of dead cells in the G_1-phase was found. No such cell cycle specificity of dead cells was seen when the drugs were combined.

Acknowledgment—The authors wish to acknowledge the assistance of Mr P-O Schnell for the whole body counting of the mice.

References

1. Berne MHO, Gustavsson BG, Almersjö O, Spears PC, Frösing R. Sequential methotrexate/5-FU: FdUMP formation and TS inhibition in a transplantable rodent colon adenocarcinoma. *Cancer Chemother Pharmacol* 1986; **16**: 237.
2. Valeriote F, Santelli G. 5-Fluorouracil (Fura). *Pharmac Ther* 1984; **24**: 107–132.
3. Roberts JJ. Bacterial, viral and tissue culture studies on neutral platinum complexes. In: Connors TA, Roberts JJ eds. *Recent Results in Cancer Research*, Berlin: Springer-Verlag, 1974: 79–97.
4. Roberts JJ, Pascoe JM, Plant JE, Sturrock JE, Crathorn AR. Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells. The effect on HeLa and Chinese hamster cell survival of alkylation of cellular macromolecules. *Chem–Biol Interactions* 1971; **3**: 29–47.
5. Schabel FM, Trader MW, Laster WR, Corbett TH, Griswold DP. Cis-dichlorodiammineplatinum(II): Combination chemotherapy and cross-resistance studies with tumors of mice. *Cancer Treat Rep* 1979; **63**: 1459–1473.
6. Kish JA, Ensley JF, Jacobs J, Weaver A, Cummings G, Al-Sarraf M. A randomized trial of cisplatin (CACP) + 5-fluorouracil (5-FU) infusion in CACP + 5-FU bolus for recurrent and advanced squamous cell carcinoma of the head and neck. *Cancer* 1985; **56**: 2740–2744.
7. Tribukait B, Moberger G, Zetterberg A. Methodological aspects on rapid-flow cytofluorometry for DNA analysis of human urinary bladder cells. In: Haanen CAM, Hillen HFP, Wessels JMC eds. *Pulse-cytometry*, Ghent: European Press Medikon, 1975: 50–60.
8. Quackenbush RC, Shields AF. Local re-utilization of thymidine in normal mouse tissues as measured with iododeoxyuridine. *Cell Tissue Kinet* 1988; **21**: 381–387.
9. Skog S, Tribukait B. Irradiation induced cell death as related to cell cycle. *Acta Radiol Oncol* 1985; **24**: 87–93.
10. Lewin F, Skog S, Tribukait B, Ringborg U. Effect of 5-fluorouracil on the cell growth and cell cycle kinetics of a mouse ascites tumour *in vivo*. *Acta Radiol Oncol* 1987; **26**: 125–131.
11. Lewin F, Skog S, Tribukait B, Ringborg U. Effect of cis-diammino-dichloro-platinum (II) on cell growth and cell cycle progression of a mouse ascites tumor growing *in vivo*. *In Vivo* 1989; **3**: 237–242.
12. Skog S, Tribukait B. Analysis of cell flow and cell loss following X-irradiation using sequential investigation of the total number of cells in the various parts of the cell cycle. *Cell Tissue Kinet* 1985; **18**: 427–444.
13. Barlogie B, Drewinko B. Cell cycle related induction of cell progression delay. In: Drewinko B, Humphrey RM eds. *Growth Kinetics and Biochemical Regulation of Normal and Malignant Cells*, Baltimore, MD: Williams and Wilkins, 1977: 315–327.
14. Meyn RE, Murray D. Cell cycle effects of alkylating agents. *Pharmac Ther* 1984; **24**: 147–163.
15. Yoshioka A, Tanaka S, Hiraoka O, Koyama Y. Deoxyribonucleoside-triphosphate imbalance death: deoxyadenosine induced dNTP imbalance and DNA double strand breaks in mouse FM3A cells and the mechanism of cell death. *Biochem Biophys Res Comm* 1987; **146**: 258–264.
16. Ross DW. Unbalanced growth and increased protein synthesis induced by chemotherapeutic agents. *Blood Cells* 1983; **9**: 57–68.
17. Sawecka J, Golos B, Malec J. Mechanism of unbalanced growth induced cell damage. II. A probable relationship between unbalanced growth, DNA breakage and cell death. *Chem Biol Interactions* 1986; **146**: 47–55.

(Received 16 July 1990; accepted 29 August 1990)