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DNA synthesis after combined treatment with cisplatin and 5-fluorouracil of a mouse ascites tumor growing *in vivo*

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We examined whether an increase in the salvage and/or the *de novo* synthesis of thymidine (TdR) can explain the elevated DNA synthesis rate found up to 15–20 h after combined treatment with cisplatin and 5-fluorouracil (5-FU), compared with single-drug regimen. The salvage and the *de novo* pathways of TdR in Bp8 mouse ascites tumor cells were reduced equally after the combined treatment and the single-drug treatments. The inhibition of the *de novo* pathway of TdR was confirmed by a reduced thymidylate synthase activity, as measured in cell extract. A marked imbalance of the deoxyribonucleotide triphosphates were found, in particular between the deoxypyrimidines. These imbalances were similar between the 5-FU single-drug treatment and combined treatment. We conclude that neither the extracellular TdR salvage nor the *de novo* synthesis of TdR explain the relatively elevated DNA synthesis rate after combined treatment. We suggest that the supra-additive effect of the combined treatment is due to an interaction between the elevated DNA synthesis, the imbalanced deoxyribonucleotides and the cisplatin-induced DNA cross-links, and possibly also due to a higher concentration of 5-FU incorporated into DNA.

Key words: Ascites tumor, cisplatin, DNA synthesis, dNTP pools, 5-fluorouracil, *in vivo*, thymidylate synthase.

Introduction

Cytostatic treatment with the combination of cisplatin and 5-fluorouracil (5-FU) has effectively induced tumor responses in patients with head and neck cancer.[1] Although not compared with single-drug treatment in randomized clinical trials, it is possible that a supra-additive effect is achieved by the combined regimen. Supra-additive effects have been found after combined treatment of tumor-bearing animals, as measured by survival.[2] In a previous study on a mouse ascites sarcoma growing *in vivo*, we found a supra-additive effect on cell death.[3] In order to elucidate the mechanism behind this supra-additive effect, we determined the cell cycle kinetic effect of cisplatin and 5-FU, alone and in combination, on Bp8 ascites sarcoma cells growing in mice.[4] We found that the cell flow through the S-phase, which reflect DNA synthesis rate, was higher after the combined treatment than after single-drug treatment. The initiation of the DNA synthesis in G_1 was also elevated. At the same time, thymidylate synthase (TS) activity, determined by the amount of FdUMP bound to the enzyme, was almost completely inhibited.[5] These conflicting results may be due to an incomplete correlation between FdUMP binding and a reduced TS activity. If the TS activity is really inhibited, the lack of TdR via the *de novo* pathway can be compensated by the cell as an increase in the TdR salvage pathway.

The aim of the present study was to investigate whether the relatively higher DNA synthesis rate found after the combined treatment with 5-FU and cisplatin was due to an elevated salvage and/or *de novo* synthesis of TdR. For that purpose we determined the amount of TdR incorporated via both salvage and *de novo* pathways, using isotopic labelled TdR. The TS activity was measured in cell extract by determining the release of [^{3}H] H_2O from [^{3}H]5-dUMP during the formation of dTMP. In order to discuss any contributions from salvage and *de novo* pathways of TdR for the DNA synthesis, it is also necessary to determine the cellular concentrations of thymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP). The concentrations of these compounds were measured by high-performance liquid chromatography (HPLC).

Materials and methods

Experimental tumor and animals

BP8 ascites tumor cells were administered every 10 days by intraperitoneal injection into 3-month-old

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male NMRT 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 cytofluorometricmethod.[6] The percentage of G_1, S and $G_2 + M$ cells were calculated from the area of corresponding G_1, S and $G_2 + M$ peaks of the DNA histogram by planimetry.

Cytostatic treatment

On day 4 after transplantation, either cisplatin (0.5 mg/ml), 5-FU (25 mg/ml) or the drugs combined were injected intraperitoneally into the mice in a volume of 0.2 ml after proper dilution with normal saline solution to get the desired concentration of the drug. When combining the drugs, cisplatin was given 30 min before 5-FU. The doses of 25 μg of cisplatin/animal corresponding to about 0.8 mg/kg body weight and 0.9 mg 5-FU/animal corresponding to about 36 mg/kg body weight were used.

Determination of thymidylate synthase activity

The TS activity was determined as described by Navalgund *et al.*[7] Cell extract, 10×10^6 cells, was washed twice in ice-cold Tris–buffer, resuspended in Tris–buffer (Tris–HCl 10 mM, pH 7.5) containing 0.5% (v/v) Tween 80, 1 mM dithiothreitol (DTT) and 1 mM β-mercaptoethanol, and sonicated for 20 s, 30 W at 4°C (Ultrasonic System, Model 1000). The sonicated cell suspension was centrifuged at 48 000 ***g*** for 15 min at 4°C.

Assay: 50 μl of the cell extract were added to 200 μl of a mixture containing 150 mM Tris–HCl (pH 7.65) 60 mM NaF, 5 mM DTT, 0.052% formalin, 0.1 mM tetrahydrofolate, 0.05 mM dUMP, 1.3 μCi [5-^{3}H]dUMP (specific activity 10.9 Ci/mmol) and 0.56% bovine serum albumin. After incubation at 37°C for 30 min, the reaction was stopped by boiling for 4 min, followed by the addition of 180 μl of a charcoal suspension (100 mg/ml). After 1 h the suspension was centrifuged at 14 400 ***g***. From the supernatant, 150 μl were removed and mixed with 4.5 ml scintillation fluid (Ultima gold, Packard). The radioactivity was measured in a scintillation counter (Packard). The enzyme activity was expressed as dUMP converted to dTMP/S-phase cell/min (expressed in $\times 10^{-18}$ mol).

Measurement of DNA synthesis rate

At 6, 15, and 24 h after cytostatic treatment, 1.0 μCi [^{3}H]TdR ([methyl ^{3}H]TdR (specific activity 25 Ci/mmol; Amersham International, Amersham, UK)) was injected intraperitoneally into the animals. The animals were killed by cervical dislocation 7 min after the injection of the isotope. The cell suspension was removed from the animal and centrifuged at 2800 ***g*** for 5 min at 4°C. After washing twice in Tris buffer (0.1 M Tris, 0.07 M NaCl, 0.005 M EDTA; pH 7.5), the cells were precipitated in 0.2 M PCA and washed once in 0.2 M PCA. The radioactivity (^{3}H) incorporated into DNA, was measured in an Emulsifier 299 (Packard 300C).

The DNA synthesis rate was determined from the radioactivity in the PCA insoluble fraction and expressed as mole dTTP incorporated into DNA/S-phase cell/min. To determine the true DNA synthesis rate, however, correction has to be made for the labelled/unlabelled ratio of dTTP in the cell, i.e. the specific activity of [^{3}H]dTTP.[8] The dTTP was analysed by HPLC (see below). The HPLC fraction corresponding to the dTTP peak was collected and the radioactivity of this fraction was determined. The specific activity of [^{3}H]dTTP was calculated as dpm per gram dTTP of this fraction (dpm/10^{-9} g). The DNA synthesis rate calculated in this way is referred to as the total DNA synthesis rate, i.e. incorporation of thymidine from both the salvage and the *de novo* pathways.

The contribution of TdR via salvage pathway to DNA synthesis was calculated from the amount of [^{3}H]dTTP incorporated into DNA and corrected for the specific activity of [^{3}H]TdR in the ascites fluid of the animal. This value was calculated from the known amount of radioactivity injected into the ascites fluid, the measured concentration of TdR in the ascites fluid and the total volume of the ascites fluid, measured earlier.[8] The amount of TdR was determined by HPLC.[5] The concentration of TdR in the ascites fluid of untreated control and after 5-FU, cisplatin and combined treatment as found in the present study was similar to results of an earlier investigation,[5] and therefore the results are not shown here. The DNA synthesis resulting from the *de novo* pathway was calculated by

subtracting the salvage DNA synthesis value from the total DNA synthesis.

Quantification of the cellular deoxyribonucleotide triphosphates

After removing the cells from the ascites fluid, the cells were precipitated in 0.3 M TCA and washed once in 0.3 TCA. From the TCA-soluble fraction the dTTP, the dCTP, deoxyadenine triphosphate (dATP) and deoxyguanine triphosphate (dGTP) were quantitated by HPLC method.[8] Since the ribonucleotides are in much greater amount than the deoxyribonucleotides, with similar retention times, the ribonucleotides were removed by periodate oxidation (20 mM sodium periodate, 170 mM metylamine, 40 mM rhamnose, pH 7.4, 37°C, 1 h) before quantitation of the deoxyribonucleotide triphosphates. The compounds were expressed as mol/cell.

Results

DNA synthesis

The DNA synthesis rate was measured at 6, 15 and 24 h. The reason for this choice of times was the findings in a previous cell kinetic study, where a higher DNA synthesis rate during the first 24 h following the combined treatment with 5-FU and cisplatin was found compared with a single-drug treatment.[1]

Single-drug treatment with 5-FU and cisplatin inhibited the DNA synthesis rate up to 15 h by about 75 and 95% of untreated control, respectively. When combining these drugs, the DNA synthesis rate was almost completely reduced (95%) at 6 h, but only partly reduced (40%) at 15 h. However, 9 h later, the DNA synthesis rate after the combined treatment was inhibited approximately as much as after the single-drug treatment (Figure 1). In single-drug and combined treatment, the major TdR pathway supporting DNA synthesis was the *de novo* pathway. The TdR salvage pathway accounted for only less than 1% (Table 1).

Thymidylate synthase activity

The TS activity was measured at 30 min, 15 h and 24 h after single-drug and combined treatment. The TS activity of untreated control cells was about 12 × 10^{18} mol dUMP converted to dTMP/S-phase

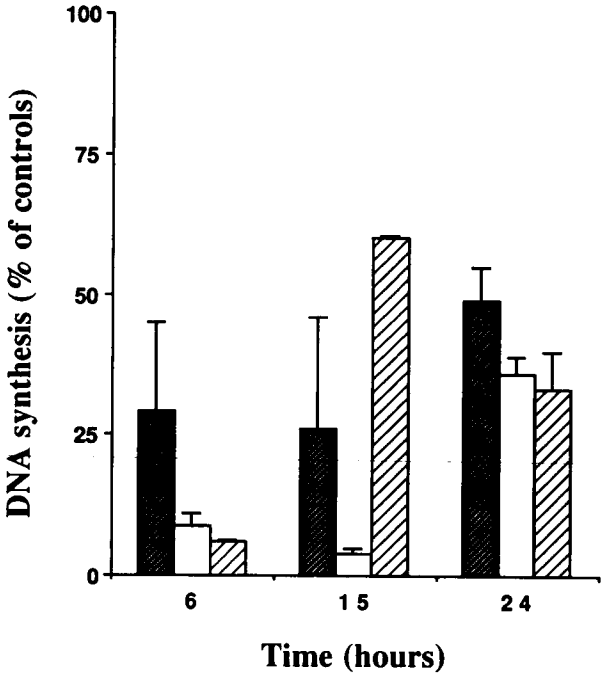

Figure 1. DNA synthesis rate of Bp8 ascites tumor after treatment with 36 mg 5-FU/kg body weight (▨); 0.8 mg cisplatin/kg body weight (□); and the combination of the two drugs (▨). The drugs were injected intraperitoneally at day 4 after transplantation. Mean values from four animals ±SEM.

cell/min. 5-FU and combined treatment reduced the TS activity at 30 min by about 80%. This inhibition was further augmented at 15 h and 24 h following 5-FU treatment (90%), but was less pronounced after the combined treatment (70%) (Figure 2). TS activity after cisplatin treatment was unchanged initially, but increased markedly at 15 h by 250%. The enzyme activity returned to approximately normal value at 24 h (Figure 2).

Table 1. Incorporation of thymidine into DNA of untreated controls and after treatment with 5-FU, cisplatin and the drugs combined, expressed as the total incorportion from both the salvage and the *de novo* pathways of thymidine (the incorporation of thymidine from the salvage pathway is given as separate values)

	Salvage		Total incorporation	
	15 h	24 h	15 h	24 h
Control	0.0136	0.034	11.9	18.6
5-FU	0.0016	0.0016	3.1	8.9
Cisplatin	0.0012	0.0138	0.5	6.7
5-FU + cisplatin	0.0025	0.0011	7.1	6.0

All values are expressed as ($\times 10^{-18}$ mol dTTP/S-cell/min). Mean values of four animals. For deviations, see Figure 1.

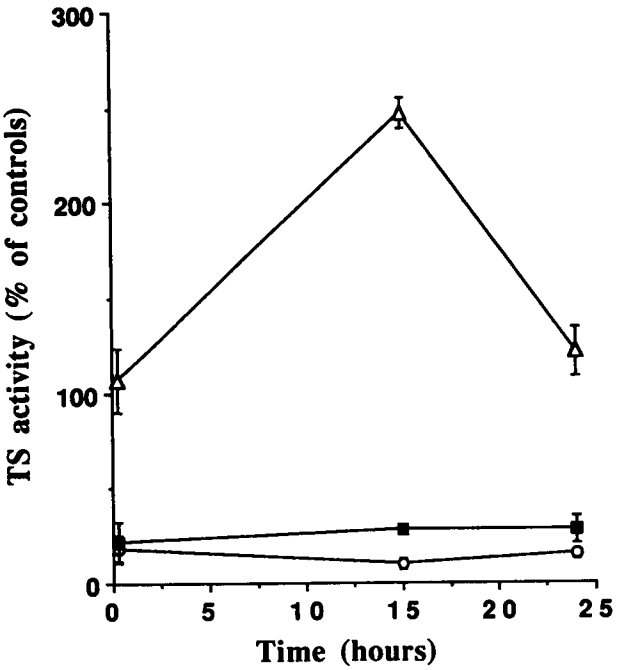

Figure 2. Thymidylate synthase activity of Bp8 ascites tumor treated with 36 mg 5-FU/kg body weight (○); 0.8 mg cisplatin/kg body weight (△); and the combination of the two drugs (■). The drugs were injected intraperitoneally at day 4 after transplantation. Mean values of four animals ±SEM.

Cellular concentration of deoxyribonucleotide triphosphates

The deoxyribonucleotide triphosphate (dTTP, dCTP, dATP, dGTP) levels were measured at 6, 15 and 24 h after single-drug and combined treatment. The results are shown in Figure 3 and Table 2.

The dTTP pool decreased by 75–85% at 6 h after 5-FU and combined treatment and remained at this level up to 24 h. At the same time, the dCTP pool increased by about 200%. The cellular concentration of dATP increased at 6 h (145% after 5-FU and 309% after drug combination), was normal (5-FU) or slightly increased (combination) at 15 h and elevated to almost double values at 24 h. The dGTP pool was reduced by about 25–50% at 15 and 24 h.

After treatment with cisplatin, only minor changes in the cellular concentration of the deoxyribonucleotide triphosphates were found. At 24 h, however, dCTP and dATP levels increased markedly.

Discussion

In a previous study, when investigating the DNA synthesis rate with cell kinetic methods, we found a relative increase in the DNA synthesis rate up to about 20 h[4] after combined treatment with 5-FU and cisplatin, compared with single-drug treatments. To examine the mechanisms behind this effect, we decided to determine the contribution of both the salvage and the *de novo* pathways of TdR to the DNA synthesis. We used isotope-labelled TdR, since its incorporation into DNA reflects the synthesis rate of the DNA. The use of labelled TdR also made it possible to study the salvage and the *de novo* pathways. Thus, the results from the previous cell cycle kinetic investigation[4] were reproduced in the present study. However, there were some minor differences. The degree of inhibition of the DNA synthesis rate at 6 h was equal for single-drug and combined treatments. Furthermore, the difference in the DNA synthesis rate between single-drug and combined treatments at 10–15 h found with the cell cycle kinetic method was less marked in the present study. In the cell cycle kinetic method,[4] where the DNA synthesis rate was determined by calculating the travers of cells through the S-phase, the DNA synthesis rate was expressed as a mean value for a certain time period. For example, the 6 h value given for the DNA synthesis is a mean value for the time period of 0–12 h. However, the determination of the DNA synthesis rate in the present study was done at specific times, for example at 6 h, using incorporation of [^{3}H]TdR into DNA. Thus, the disagreement between the DNA synthesis rates found in these two studies concerning the first 6–12 h is probably explained by the differences in technique. Although we have corrected the DNA- incorporated TdR for changes in the specific activity of [^{3}H]dTTP in the cell, it is still possible that other factors exist, for example compartmentation of the dTTP pool. If the dTTP pool consists of more than one compartment, and only one is used for DNA synthesis, the determined specific activity would be wrong. However, there is no convincing evidence in the literature for a compartmentation of the dTTP pool.[9–12]

There are different possible mechanisms behind the elevated DNA synthesis rate after the combined treatment. In this study we examined whether the *de novo* and/or the salvage pathways of TdR were increased. The extent of the *de novo* synthesis was determined by calculating the amount of TdR incorporated into DNA via this pathway. No increase was found. This was also confirmed by the reduced TS activity, although some higher activity values were found after the combined treatment. However, this higher TS activity is not enough to explain the higher DNA synthesis rate obtained. The amount of dTTP incorporated into DNA after single-drug treat-

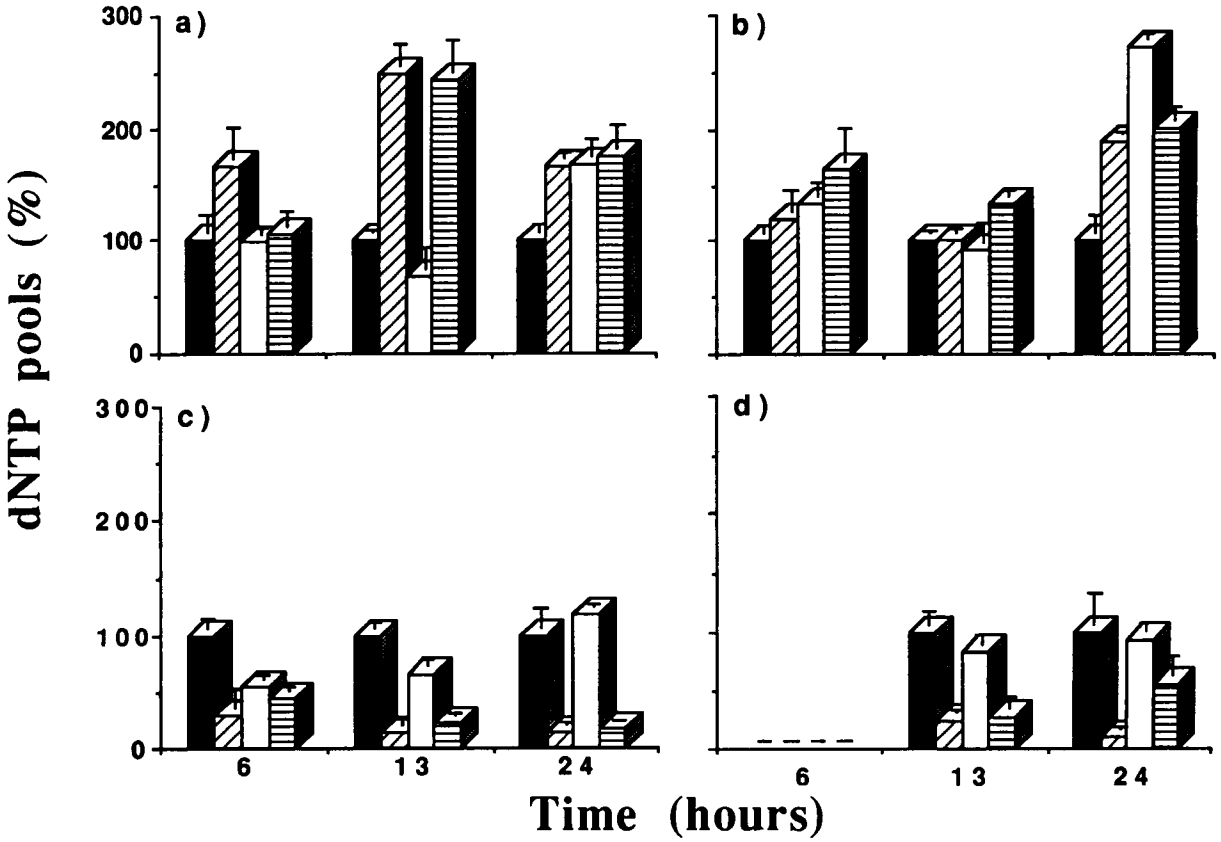

Figure 3. dNTP pool sizes of Bp8 ascites tumor untreated (■) and treated with 36 mg 5-FU/kg body weight (▨); 0.8 mg cisplatin/kg body weight (□); and the combination of the two drugs (▤). (a) dCTP, (b) dATP, (c) dTTP and (d) dGTP. The concentration of dGTP at 6 h was too low to allow detection with the technique used. The drugs were injected intraperitoneally at day 4 after transplantation. Mean values of four animals ±SEM.

ment with 5-FU was about 3×10^{-18} mol/S-cell per min with a corresponding TS activity of about 2.5×10^{-18} mol dUMP converted to dTMP/S-cell per min. After the combined treatment, the DNA synthesis rate increased to about 7×10^{-18} mol dTTP incorporated into DNA/S-cell per min. The TS activity increased only to approximately 3.5×10^{-18} mol/S-cell per min. The slight increase in the TS activity after the combined treatment might be explained by the presence of cisplatin. Single treatment with cisplatin elevated the TS activity markedly (about 250%), accompanied by a corresponding increase in the extracellular concentration of TdR.[5] These results speak in favour of the excretion of TdR being markedly elevated. Similar results were obtained when 3T6 mouse fibroblast cells were treated with aphidicoline, a DNA synthesis inhibitor.[9] These investigators suggested that the cell is able to regulate the deoxyribonucleotide pools not only via ribonucleotide

Table 2. Amount of deoxyribonucleotide triphosphates of untreated controls and after treatment with 5-FU, cisplatin and the drugs combined ($\times 10^{-18}$ mol/cell)

	dCTP			dTTP			dATP			dGTP		
	6 h	13 h	24 h	6 h	13 h	24 h	6 h	13 h	24 h	6 h	13 h	24 h
Controls	1.3	6.2	5.2	6.9	21.9	13.3	2.2	17.4	11.4	*	5.4	6.9
5-FU	2.2	12.6	8.9	1.7	3.0	2.0	2.8	17.4	22.1	*	1.4	1.0
Cisplatin	1.3	4.3	9.0	3.1	15.4	15.3	3.0	16.5	31.9	*	4.6	6.6
5-Fu + cisplatin	1.5	15.2	9.3	3.0	4.8	2.4	3.7	23.7	23.7	*	1.5	3.8

Mean values of four animals. For deviations, see Figure 3.
* Undetectable levels (less than 0.01×10^{-18} mol/cell).

reduction, but also by excretion of deoxyribonucleosides. Thus, instead of decreasing the synthesis of deoxypyrimidine to keep the pools of dTTP and dCTP at constant levels when the DNA synthesis is inhibited by cisplatin, the treated cell regulates these levels by excretion of TdR via conversion of dUMP to dTMP catalyzed by TS.

Considering the salvage pathway of TdR, no increase was found. The part of the salvage pathway we examined was the extracellular TdR. Intracellular salvage from degradation of DNA to thymine, followed by phosphorylation to dTTP is also a possibility to be considered. Such a study is in progress.

Another hypothesis is that the pool size of dTTP is still large enough to enable the cell to proceed with the DNA synthesis. The dTTP pool size was decreased to about the same level after treatment with 5-FU as after the combined treatment. Cisplatin treatment reduced the dTTP pool only slightly. The changes in the dNTP pools, except for dATP, are explained on a regulatory basis, starting with the decrease in the dTTP pool due to the inhibited TS activity. However, if the cells use the existing dTTP pool more efficiently after the combined treatment, the remaining dTTP pool size should have been much smaller than found, because of a higher turnover rate.

So far, the elevated DNA synthesis rate after combined treatment can be explained neither by an increased *de novo* synthesis nor by an extracellular salvage synthesis of TdR or by a more efficient use of the remaining dTTP pool. It is also possible that the combined drug treatment exerts its effect on the DNA polymerase enzyme level. Elevated DNA synthesis rate has also been found when normal mouse fetus cells *in vivo* and mouse lymphoma cells (P388) *in vitro* have been treated with a combination of 5-FU and X-irradiation.[13] These results may indicate that 5-FU plays an important role in the mechanism of the elevated DNA synthesis in combined treatments.

The balance between the dNTP pools after single-drug and combined treatments was disturbed significantly, particularly the balance between the dTTP and the dCTP pools after the 5-FU and the combination treatment. It has been discussed in several studies that imbalance in dNTP pools can be of significant importance for the survival of the cell. It has been shown that such an imbalance can result in mutations and that DNA strand breaks with lethal potential for the cell.[14,15] An increase in the dCTP pool has especially been correlated to an increase in mutations.[16] The imbalance in the dCTP/dTTP ratio was, however, equal for 5-FU treatment and combined treatment. As 5-FU treatment in single-drug regimen did not cause a significant cell death at the dose used,[4] other factors have to be accounted for in order to explain the increased cell death seen after the combined treatment. The relative increase in the DNA synthesis rate found might lead to misincorporation of precursors together with an impaired proofreading leading to possibly lethal mutations.[17] The increased DNA synthesis rate at a time when the DNA harbors almost maximum amount of cisplatin-induced DNA cross-links could in itself explain the increased cytotoxicity. Similar conclusions were drawn from experiments where human T lymphoma cells were treated with a low concentration of hydroxyurea.[18,19] No increase in DNA single-strand breaks (SSB) or cell death were obtained in the presence of the drug. However, when the drug was removed, the DNA synthesis rate increased markedly, followed by an elevated frequency of SSB and an increase in cell death. It has also been shown that 5-FU is incorporated into DNA to a higher extent after the combined treatment than after single-drug treatment.[20] This may result in a defect transcription of DNA and thus disturbed synthesis of proteins, for example enzymes for DNA repair, leaving the drug-induced DNA damage unrepaired or insufficiently repaired.[21]

From our results we suggest the following mechanisms for the supra-additive toxicity found after the combined treatment with 5-FU and cisplatin. The elevated DNA synthesis rate in the presence of imbalanced dNTP pools and cisplatin-induced DNA cross-links leads to further DNA damages (single- and double-strand breaks, deletions, etc) and misincorporation of dNTP into DNA. The increased dCTP pool found would result in dCTP being the main dNTP misincorporated into DNA. This misincorporation could then result in mutations and give rise to defective DNA repair and/or other severe changes in the cellular metabolism. These effects together lead to a type of cell death, which has been characterized as interphase death.[4] To confirm this hypothesis, studies on RNA and protein synthesis, repair of DNA cross-links and induction of other DNA damages as well as misincorporations of dCTP and increased frequency of mutations are in progress.

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