Recovery of Normal Hematopoietic Tissue and Tumor Following Chemotherapeutic Injury From Cyclophosphamide (CTX): Comparative Analysis of Biochemical and Clinical Techniques

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Simultaneous alterations in the incorporation of $^3$H-thymidine ($^3$H-TdR) into DNA are induced by CTX in normal host target tissues and L1210 ascites tumor. The timing of suppression and recovery of these nucleoside incorporation alterations was similar at the three CTX doses studied, but some evidence for a dose-response effect was seen as the magnitude of suppression of DNA synthesis increased with increasing dosage. A differential pattern of suppression and recovery of $^3$H-TdR incorporation in malignant and normal host tissues was observed. The pattern of suppression and recovery of the peripheral white blood count and bone marrow (BM) cellularity, two frequently studied clinical parameters of hematopoietic recovery, were out of phase with the recovery of BM-DNA synthesis and failed to accurately reflect the sensitivity of the BM to subsequent chemotherapeutic injury. In contrast, drug schedules based on the differential recovery patterns of the host tissues and tumor, reflected by their $^3$H-TdR incorporation into DNA, both reduced toxicity to normal mice and increased the survival of tumor-bearing animals.

Proper scheduling of cancer chemotherapy has been difficult because of a lack of understanding of the effect antineoplastic agents exert simultaneously on tumorous and normal host target tissues. As an outgrowth of the principle that the cytotoxic effects of cancer chemotherapeutic agents in normal and malignant tissue is in part a function of their proliferative stage, Young et al., studying cytosine arabinoside, and Rosenoff et al., studying BCNU (1,3, Bis-(2-chloroethyl)-1-nitrosourea), have investigated in vivo the alterations in DNA synthesis, as reflected in the incorporation of tritiated thymidine ($^3$H-TdR) into DNA, induced by these agents simultaneously in tumorous and host target tissues. They were able to demonstrate by this technique that a differential effect on recovery was produced between malignant and nor-
mal tissues which could be exploited through a nonemperic dose schedule which would increase survival of tumor-bearing animals.

In the present study, the effect of a range of doses of the clinically active\textsuperscript{1,2} and classic alkylating agent, cyclophosphamide (CTX), upon DNA synthesis as reflected in the incorporation of $\text{3H-TdR}$ into DNA, simultaneously studied in vivo in the bone marrow (BM), gastrointestinal mucosa (GI), and L1210 ascites tumor, has been investigated. In addition, we have compared the information provided from the biochemical determination of alterations in DNA synthesis with the more clinical techniques of serial white blood counts (WBC) and bone marrow cellularity after a single dose of CTX in an attempt to delineate those factors which would be most useful for the prediction of an optimal time for subsequent chemotherapy.

**MATERIALS AND METHODS**

BDF\textsubscript{1} female mice of an average weight of 20 g were obtained from Hazleton Laboratories (Falls Church, Va.) and used throughout these experiments. They were maintained in a constant-temperature environment, in plastic cages, and fed laboratory chow and water ad libitum. L1210 murine leukemia carried intraperitoneally (IP) was used as the tumor source and transplanted to recipient mice by the injection of $1 \times 10^7$ L1210 cells intraperitoneally. BDF\textsubscript{1} female mice of comparable age and weight were utilized as normal controls. Experiments were carried out during the same time period each day, and all mice were sacrificed by cervical dislocation.

**Specific Activity of the DNA (dpm/µg DNA)**

Studies requiring the use of tumorous mice were initiated at day 6 of tumor growth. All treatments were given intraperitoneally at time zero. Six mice were left untreated and served as the zero-hour control group. At time 0, 1, 6, 12, 48, and 72 hr and at the various times indicated, six mice at each time point received 100 $\mu$Ci $\text{3H-thymidine}$ (specific activity, 6.7 Ci/m mole; New England Nuclear, Boston, Mass.) intraperitoneally, and 1 hr later\textsuperscript{7} were sacrificed. Ascites tumor was aspirated from the peritoneal cavity with a Pasteur pipette. Additional ascites was obtained by washing the peritoneal cavity with iced phosphate-buffered 0.85% NaCl, pH 7.4 (PBS). A 3-cm length of proximal duodenum was next removed from each mouse. It was gently agitated in iced PBS, blotted on absorbent paper to remove adherent ascitic tumor cells, and split lengthwise on a glass slide. The mucosa was stripped from the muscularis and serosal layers with a second slide and dispersed in iced PBS. The bone marrow was harvested from both tibias of each mouse by removing the tibias, stripping the muscle from the bone, sectioning the bone at each end, and expressing the marrow using a 25-gauge hypodermic needle and a syringe of iced PBS. Separate samples of ascites tumor, duodenal mucosa, and bone marrow from each of six mice were pooled into two groups of three. These pooled samples were spun at 800 g for 5 min in a refrigerated centrifuge ($4^\circ$C), the supernatant discarded, and the button of tissue frozen for further processing. DNA of each of the pooled specimens was extracted by a modification of the Schneider method.\textsuperscript{8} A 0.5-ml aliquot of final supernatant was added to 15 ml Aqua-sol (New England Nuclear, Boston, Mass.) and counted in a Packard Tri-Carb liquid scintillation spectrometer. A 0.5-ml aliquot of the final supernatant was processed by the Burton method\textsuperscript{5} for DNA determination. A standard quench curve for tritium in liquid scintillation fluid was used for determination of the quenching error. The efficiency of the tritium counting in the system was 29%. The results were then expressed as the disintegrations per minute (dpm) per microgram (µg) of DNA and graphed as a percentage of control over time. Variation in the incorporation of $\text{3H-TdR}$ into DNA among the control groups utilized in these studies was 10%–15%. Control data (pretreatment) used in these studies ± the standard error of the mean are as follows: bone marrow, 714 ± 110 dpm/µg DNA; gastrointestinal mucosa, 1128 ± 176 dpm/µg DNA; and ascites tumor, 42,921 ± 5034 dpm/µg DNA.
**L1210 Ascites Cell Counts and Cell Viability**

The numbers of ascitic cells remaining at various time intervals after 200 mg/kg CTX administered intraperitoneal was determined by sacrificing three mice at each time point and a group of three pretreatment mice which served as controls. The abdominal cavity of each animal was exposed, free ascitic fluid aspirated with a Pasteur pipette, and the peritoneal cavity washed three times with iced PBS. The cells from each animal were counted in a Coulter Model F (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). The cell counts were expressed as the means for each group ± the standard error and graphed over time. For determination of cellular viability, an aliquot of L1210 ascites in PBS was placed on a clear glass slide and incubated at room temperature for 5 min with 1 drop of trypan blue stain (0.4% in normal saline; Grand Island Biologic Company, Grand Island, N.Y.), covered with a cover slip, and examined under a light microscope. One hundred cells were counted per slide, and per cent viability was estimated as the per cent of cells excluding trypan blue. The data was graphed as the mean for the three determinations ± the standard error over time.

**Peripheral White Blood Counts (WBC)**

Blood was obtained from the tail veins of six mice at each time point and the WBC counted in a Coulter Model F. The WBC was expressed as cells/cu mm ± standard error of the mean (SEM) and graphed over time.

**Bone Marrow Cellularity**

Both femurs were removed from mice at each time point, fixed, decalcified, paraffin embedded, mounted on a glass slide, and stained with Giemsa. Animals receiving no therapy served as controls. Slides were numbered and studied by two independent observers in a double-blind manner. The cellularity was estimated as per cent of control on the basis of proportion of fat to hematopoietic cells.

**Toxicity Studies**

All mice received a single dose of 200 mg/kg CTX intraperitoneally at the start of the study (0 hr). Animals were then subdivided into treatment groups consisting of 30 mice per group. These varied only in the timing of the second dose of 200 mg/kg CTX. Treatment groups were as follows: 0 hr only (one dose); 0 hr only, two doses (400 mg/kg total dose); 0 hr and 24 hr; and 0 hr and other times as indicated in Fig. 5.

**Survival Studies**

BDF1, female mice bearing L1210 ascites tumor were treated on day 2 of tumor development with CTX 200 mg/kg intraperitoneally. Thirty mice remained untreated and served as controls. Treated mice were subdivided into groups of 30 each which varied only in the timing of a second (200 mg/kg) dose of CTX. These groups were 0 hr only, 0 hr and 12 hr, 0 hr and 24 hr, and 0 hr and other times as indicated in Table 1. The per cent of animals in each treatment group who survived 200% longer than the animals in the untreated control groups (ILS 200) are shown.

**RESULTS**

**Simultaneous Alterations Induced by CTX in DNA Synthesis in the BM, GI Mucosa, and Ascites Tumor In Vivo**

The simultaneous changes in DNA synthesis, as reflected by 3H-TdR incorporation into DNA in the tumor (ascites) and host target tissue (BM and GI mucosa) following a single dose of CTX of either 50, 100, or 200 mg/kg intraperitoneally, are presented in Fig. 1A, B, and C.

Following each of these doses, DNA synthesis in the BM, as reflected in the incorporation of 3H-TdR into DNA, shows a significant depression and reaches a nadir by 12 hr. This depression is dose related and reaches 62% of control at
Table 1. Survival of BDF<sup>1</sup> Mice With L1210 Leukemia<sup>*</sup> Treated With Cyclophosphamide<sup>†</sup>: Dose Schedule Variations

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Per Cent Achieving ILS 200</th>
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<tr>
<td>0 hr only</td>
<td>0</td>
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<tr>
<td>0 hr + 12 hr</td>
<td>67</td>
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<tr>
<td>0 hr + 24 hr</td>
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<tr>
<td>0 hr + day 9</td>
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<td>0 hr + day 10</td>
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<sup>*</sup>Day 2 L1210 ascites tumor.
<sup>†</sup>400 mg/kg intraperitoneally, total dose.
<sup>†</sup>Thirty mice in each treatment group.

The 50 mg/kg dose, 28% of control at the 100 mg/kg dose, and 14% of control at the 200 mg/kg dose. The time to recovery was 24 hr, with return to control by 36 hr at all three doses studied. This was followed by a period of “overshoot” of the control levels reaching a maximum at day 3, in the 200 mg/kg dose study (Fig. 1C), followed by a return to base-line levels by days 6–7 after therapy.

Fig. 1. Effect of a single dose of CTX on the incorporation in vivo of <sup>3</sup>H-thymidine into DNA of L1210 ascites tumor, normal murine bone marrow, and gastrointestinal mucosal cells: (A) CTX 50 mg/kg intraperitoneally, (B) CTX 100 mg/kg intraperitoneally, and CTX 200 mg/kg intraperitoneally. Each point represents the average of two pooled groups of three animals each.
The other normal host target tissue studied, GI mucosa, also showed a marked depression in $^3$H-TdR incorporation following CTX therapy. The nadir level of 54% of control was reached by 24 hr following the 50 and 100 mg/kg doses; whereas a more profound (32% of control) and early (12 hr) nadir of $^3$H-TdR incorporation was seen at the 200 mg/kg dose. Recovery was complete at all these doses by 36 hr, and "overshoot" of the control levels ensued, which was followed by a subsequent fall toward control levels (Fig. 1C).

DNA synthesis in the ascites tumor was suppressed by CTX within 1 hr at the 100 and 200 mg/kg dosages and by 6 hr at the 50 mg/kg dose. A dose-response effect was again noted as the nadir DNA synthesis for the 50, 100, and 200 mg/kg dosages was 24% of control, 15% of control, and 2% of control, respectively. Recovery was not noted during the 3 days of study at the 50 and 100 mg/kg doses and had only begun by days 6–8 (14% of control) at the 200 mg/kg.

Thus, a clear difference between the recovery pattern of the host target tissue (BM and GI mucosa) and the tumor was seen.

During the initial 1 hr of study, a rise above control levels was noted in the BM and tumor at the 50 mg/kg dose and in the GI at both the 50 and 100 mg/kg doses.

Alterations in Cell Count and Per Cent Viability of L1210 Ascites Tumor After 200 mg/kg CTX (Fig. 2)

The effect of CTX on the total ascites tumor number and the viability of cells within the ascitic population was studied utilizing trypan blue exclusion as a measure of cellular viability. The results appear in Fig. 2. The control level (pretreatment) for trypan blue exclusion was 93% ± 1.9 (SEM). Significant depression was not noted until 48 hr after CTX therapy and only reached a nadir of 74% at 72 hr. This was followed by a gradual return to control levels over the remaining 4 days of the study. During the same time period that the
cellular viability remained between 93% and 74% (days 0–3), CTX had produced a greater than one log kill of the ascitic tumor population (Fig. 2). It would appear then that dead tumor cells are cleared extremely rapidly from the intraperitoneal compartment under in vivo conditions. In addition, the marked early fall in DNA synthesis in the tumor which is severe by 12 hr after CTX therapy is not manifest in the total tumor cell count until much later, with nadir ascites cell counts at 3 days after initial therapy.

Alterations in the Peripheral WBC and BM Cellularity Affected by a Single Dose of CTX (Fig. 3)

Following a single dose of 200 mg/kg of CTX, the BM cellularity initiated its’ decline at 12 hr, continued to decline to a nadir by 48 hr, and subsequently recovered to control cellularity by day 6. The peripheral WBC was also seen to fall during the first 24 hr, reached its nadir of 1100 cells/cu mm on day 4 (2 days following the BM cellularity nadir), and returned toward pretreatment levels over the next 3 days. Thus, the nadir peripheral blood count and recovery followed the pattern of bone marrow suppression and recovery, but was delayed approximately 2 days. These manifestations of CTX injury occurred much later than the alteration in DNA synthesis in the bone marrow which, at this dose, reached nadir at 12 hr and had recovered to pretreatment levels by 24–30 hr.

The Effect of Dose Schedule on Lethal Toxicity of CTX on Normal Mice (Fig. 4B)

As therapy with single doses of CTX had demonstrated a pattern of late suppression and recovery of the clinical hematopoietic parameters of the peripheral WBC and BM cellularity as well as the early alterations reflected in 3H-TdR incorporation into BM-DNA, a toxicity study was performed to explore the possibility that a knowledge of these alterations would have therapeutic impor-

![Fig. 3. The effect of a single intraperitoneal 200 mg/kg dose of CTX on the peripheral white blood count (each point represents the mean of six determinations ± the standard error) and the bone marrow cellularity (each point represents the mean of three observations). Peripheral white blood count (□□□□) and bone marrow cellularity (○○○○○○).]
Fig. 4. (A) The effect of 200 mg/kg intraperitoneally of CTX on the incorporation in vivo of $^3$H-thymidine into DNA in normal bone marrow (each represents the mean of two pooled groups of three animals each). Values are expressed as percent of control of the dpm/μg of DNA for each of the three separate tissues over time. BM-$^3$H-TdR-DNA, Δ—Δ. (B) The effect of varying dose schedules on the lethal toxicity of 400 mg/kg intraperitoneally of CTX total dose, given in various divided dose schedules. The lower horizontal bar represents the toxicity of a single dose of 200 mg/kg CTX, while the upper horizontal bar represents the toxicity of two single doses given simultaneously at time 0 (400 mg/kg of CTX). The vertical bar appears at the time of the second dose of CTX and represents the toxicity of the individual double-dose schedules. Thirty mice were used for each schedule and lethality determined after 30 days.

Recovery from CTX Injury

In BDF$_1$ mice, a single dose of 200 mg/kg CTX is an LD$_{10}$, whereas two doses given simultaneously at time 0 (400 mg/kg) is an LD$_{50}$. When a single dose (200 mg/kg) is given at time 0 and a second 200 mg/kg 12 hr later, the LD$_{50}$ effect of the 400 mg/kg dose is reduced to an LD$_{25}$. This scheduling difference is significant ($p < 0.001$). When the second dose of 200 mg/kg of CTX is delayed until 24, 36, or 48 hr after the first dose, the toxicity increases to approximately an LD$_{50}$. This schedule difference is also significant ($p < 0.05$). If the second dose is delayed further until 72 hr after the first, the toxicity rises to an LD$_{50}$. Further delay in the administration of the second dose of 200 mg/kg of CTX is accompanied by a reduction from this peak toxicity to approximately an LD$_{50}$ at days 4 and 5 and approximately an LD$_{20}$ if one waits to day 6 or 7 to administer the second dose.

Thus, the toxicity of spaced doses of CTX has a relationship to the observed alterations in $^3$H-TdR incorporation in the bone marrow as well as the gastrointestinal mucosa. Minimal toxicity is achieved with the administration of a second dose of CTX at 12 hr after the first at a time when $^3$H-TdR in marrow is at nadir levels. Further reduction in toxicity can be achieved by waiting until $^3$H-TdR incorporation has returned to baseline after its period of “overshoot” from days 2–5. In contrast, information obtained from the peripheral blood counts or bone marrow cellularity patterns (Fig. 3) would suggest that marrow and peripheral blood counts have passed their nadir and are recovering from injury on days 3–5. One might be tempted to readminister chemotherapy at this point, but toxicity (Fig. 4B) is actually shown to be maximal over this period.
The Effect of Dose Schedule Upon the Survival of Mice Bearing L1210 Leukemia (Table 1)

The single-dose studies with CTX have all shown differential recovery patterns of DNA synthesis between the normal tissues and the tumor, in each instance showing more profound and persistent suppression in the tumor. This suggested that the proper timing of the second dose at a point when the tumor was beginning recovery, but normal tissues had either attained their nadir \(^3\)H-TdR incorporation into DNA or had completed recovery, might result in an improved chemotherapeutic effect.

To study the effect of varying dose schedules of CTX administration upon the survival of animals with L1210 leukemia, mice bearing a 2-day growth of L1210 leukemia were initially treated with CTX 200 mg/kg intraperitoneally and later received a second dose of CTX 200 mg/kg at a time dictated by the particular treatment group (Table 1). A single 400 mg/kg dose of CTX resulted in no animals achieving an increased life span of 200% of control (ILS). If the 400 mg/kg dose was divided, so that 200 mg/kg was administered at time 0 and another 200 mg/kg 12 hr later, 67% of mice achieved an ILS\(_{200}\). If one delays the second 200 mg/kg dose of CTX to 24 or 36 hr, the increased survival achieved with the 0-hr and 12-hr schedule is lost, and only 19% of the animals achieved an ILS\(_{200}\). Further delay in the second 200 mg/kg dose of CTX to days 3, 4, 5, 6, 7, 8, 9, or 10 also increased survival slightly over that seen with the 24- and 36-hr second doses, but never achieved the therapeutic effect of the 0-hr and 12-hr schedule, as only 29% of the animals achieved a 200% increase in the life span.

DISCUSSION

Bruce and his co-workers,\(^{3,4,9}\) utilizing the technique of lymphoma and bone marrow colony formation, and Young et al.,\(^{10}\) employing the assay of the specific activity of DNA, have stressed that a knowledge of differences inherent in the cell kinetics of tumorous tissues as compared to the cell kinetics of normal host target tissues may be exploited to optimize the selection and schedule of chemotherapeutic agents.

In the present studies, the technique for the determination of the specific activity of DNA (dpm/\(\mu\)g DNA) after the administration of \(^3\)H-TdR, has been utilized, which allows one to follow simultaneously the alterations in DNA synthesis in tumor and normal host target tissues in vivo. This system has been compared to the frequently used clinical determinations of the peripheral WBC and BM cellularity as to their ability to reflect the sensitivity of the host tissues to subsequent chemotherapy and as to their value in the rational design of chemotherapeutic schedules for the treatment of tumors in vivo.

We have demonstrated in these studies that, following a dose of CTX, a pattern of suppression and recovery of DNA synthesis occurs in the host target tissues (BM and GI mucosa) which is different from that seen in the tumorous tissue studied (Fig. 1). The time course of the suppression and recovery of \(^3\)H-TdR incorporation in the BM and GI mucosa was similar. The onset of suppression of DNA synthesis in both tissues was 6 hr after CTX treatment, maxi-
Recovery from CTX Injury

Mal suppression was noted by 12 hr for the BM and by 24 hr for the GI mucosa, recovery was complete by 36-48 hr for both tissues, and thereafter a period of "overshoot" of control levels of DNA synthesis ensued. Although the timing of suppression and recovery of DNA synthesis was similar at the three doses studied, a dose-response effect was seen to the extent that the magnitude of suppression of DNA synthesis in BM and GI mucosa was greater as the dose of CTX was increased.

The effect on the tumor was more profound. DNA synthesis in the ascites tumor fell to below 30% of control levels by 24 hr after CTX administration at all three doses. 3H-TdR incorporation into the ascites tumor DNA was still markedly depressed (14% of control) 8 days after CTX therapy, at a time when the normal tissues had already been suppressed, recovered, "overshot," and returned toward normal levels.

The manifestation of CTX injury to the ascites tumor was monitored with total ascites cell counts and cell viability studies. Nadir ascites cell counts occurred on day 3 after therapy, while profound suppression of DNA synthesis in the tumor had been manifest as early as 12 hr after treatment. Ascites tumor cell viability studies revealed a relatively small number of dead cells persisting in the ascites (nadir viability 73% at 72 hr) at the time of a greater than one log cell kill, and therefore there must be a rapid clearance mechanism for dead cells from the ascites fluid under in vivo conditions. This finding is important for the interpretation of the 3H-TdR incorporation studies because dead cells could contribute to an artifactual depression of the specific activity of DNA through a dilution of living-cell DNA by the dead-cell DNA. Under in vivo circumstances, the dead cells within the ascites fluid appear to be very rapidly cleared, however.

The present studies reveal CTX-induced alterations in the 3H-TdR incorporation into BM-DNA, as well as alterations induced in peripheral WBC and bone marrow cellularity. The time relationships of each of these aspects of marrow recovery are presented in Fig. 5. Although a pattern of suppression and recovery is seen with either the peripheral WBC or BM cellularity, their nadir levels occurred during the period of greatest BM activity as reflected by BM-DNA synthesis. Toxicity studies showed minimal marrow toxicity with a second dose administered at 12 hr (nadir BM-DNA synthesis) or days 6-7, when DNA synthesis has returned to baseline after overshoot. In contrast, when looking at marrow recovery as manifest by peripheral WBC or BM cellularity, recovery from nadir levels was seen over days 3-5. Second doses of CTX at these times actually produced excessive toxicity (LD95, Fig. 4B). It would appear then that neither the peripheral WBC nor the BM cellularity, the two frequently studied indexes of hematopoietic system recovery, accurately reflect the sensitivity of the BM to subsequent chemotherapy with CTX.

In a previous study, simultaneous comparisons of 3H-TdR incorporation into BM-DNA and bone marrow colony-forming units (BM-CFU-C) have been performed after CTX therapy to test the relative strengths of these two assays of the proliferative state of the marrow. While both techniques are better than the peripheral WBC or BM cellularity, BM-DNA synthesis proved to be the easiest and most accurate in predicting proper sequences of chemotherapy.
The toxicity experiments illustrate that the proliferative state of the BM, following antineoplastic therapy, as assayed by BM-DNA synthesis, was an accurate index of the toxicity of subsequent therapy, and we therefore endeavored to utilize this knowledge to increase the therapeutic index of CTX in the treatment of mice bearing L1210 leukemia through nonimperic dose scheduling. The results in Table 1 indicate that the 0–12 hr schedule, which had been demonstrated to be advantageous in terms of toxicity, produces the maximal prolongation of survival (67% of animals have ILS200). If the second dose of therapy is delayed to 24–36 hr, one achieves the poorest therapeutic enhancement (19% of animals have ILS200), and the difference is highly significant (p < 0.001).

If one delays the second dose of CTX to the point of maximal toxicity as shown in the double-dose toxicity study (Fig. 4), only 29% of mice achieve an ILS200 as compared to the 67% achieved with the 0-hr and 12-hr schedule (p < 0.001). These latter two losses in therapeutic effect were most likely related to increasing toxicity of the dose schedules (Fig. 4). When the second dose of CTX is administered after the return of BM-DNA synthesis to baseline and decreasing toxicity (Fig. 4), therapeutic effect cannot be regained (29% of animals achieving an ILS200), this time due to tumor regrowth in the interval between doses (Table 1). Thus, a nonimperic schedule, predicated on a period of reduced host tissue sensitivity and nadir BM-DNA synthesis (0 hr and 12 hr), allowed the design of an optimal intermittent schedule for CTX therapy which minimized toxicity and enhanced antitumor effect.

REFERENCES
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