Effects of the Mammalian Target of Rapamycin Inhibitor CCI-779 Used Alone or with Chemotherapy on Human Prostate Cancer Cells and Xenografts

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Abstract

Selective inhibition of repopulation of surviving tumor cells between courses of chemotherapy might improve the outcome of treatment. A potential target for inhibiting repopulation is the mammalian target of rapamycin pathway. PTEN-negative tumor cells are particularly sensitive to inhibition of this pathway. Here we study the rapamycin analogue CCI-779, alone or with chemotherapy, as an inhibitor of proliferation of the human prostate cancer cell lines PC-3 and DU145. The PTEN and phospho-Akt/PKB status and the effect of CCI-779 on phosphorylation of ribosomal protein S6 were evaluated by immunostaining and/or Western blotting. Expression of phospho-Akt/PKB in PTEN mutant PC-3 cells and xenografts was higher than in PTEN wild-type DU145 cells. Phosphorylation of S6 was inhibited by CCI-779 in both cell lines. Cultured cells were treated weekly with mitoxantrone or docetaxel for two cycles, and CCI-779 or vehicle was given between courses. Growth and clonogenic survival of both cell lines were inhibited in a dose-dependent manner by CCI-779, but there were minimal effects when CCI-779 was given between courses of chemotherapy. CCI-779 inhibited the growth of xenografts derived from both cell lines with greater effects against PC-3 than DU145 tumors. CCI-779 caused mild myelosuppression. The activity of mitoxantrone or docetaxel was limited, but CCI-779 given between courses of chemotherapy increased growth delay of PC-3 xenografts. Our results suggest that repopulation of PTEN-negative cancer cells between courses of chemotherapy might be inhibited by CCI-779. (Cancer Res 2005; 65(7): 2825-31)

Introduction

The overall effect of chemotherapy on a tumor depends on the amount of tumor cell kill achieved with each course and the extent of repopulation of surviving tumor cells between courses of chemotherapy (1). There is evidence from experimental tumor models that the rate of proliferation of surviving tumor cells may increase after chemotherapy (2–5) and that the rate of repopulation after sequential treatments with chemotherapy may accelerate with time (6). Scheduling of short-acting agents that inhibit selectively the proliferation of tumor cells between courses of chemotherapy has potential to improve therapeutic index (1, 7).

A potential molecular target for inhibiting repopulation of tumor cells is the mammalian target of rapamycin (mTOR) pathway. mTOR regulates initiation of translation through phosphorylation and activation of ribosomal p70S6 kinase (S6K) and cap-dependent translation via eukaryotic initiation factor 4E (eIF4E; refs. 8, 9). Rapamycin and its derivative, CCI-779, down-regulate translation of specific mRNAs required for cell cycle progression from G1 to S phase (10–15). One important target is p70S6 (S6) ribosomal protein, which is involved in protein translation and is activated by p70S6 kinases (S6K; ref. 10). Signaling through the phosphatidylinositol 3-kinase (PI3K) pathway leads to an increase of S6K activity, concomitant with hyperphosphorylation of S6 (12, 13). These events are positively regulated by the kinase mTOR, although it is unclear whether mTOR directly phosphorylates S6K. The product of the PTEN tumor suppressor gene is a phosphatase that down-regulates the PI3K/Akt (PKB) pathway; it acts upstream of mTOR and has a negative effect on the phosphorylation of S6 (11). Loss of PTEN is correlated with up-regulated mTOR activity and with increased activity of S6 kinase (11, 14) and can render tumors particularly sensitive to mTOR inhibitors (15). Inactivation of mTOR by CCI-779 inhibits the proliferation of PTEN-negative tumor cells in vitro and in vivo and is associated with down-regulation of S6K activity (16). CCI-779 has shown antiproliferative activity against a wide range of cancers in preclinical models and is being evaluated in clinical trials (17–23). There is evidence that PTEN mutations render tumors particularly vulnerable to CCI-779 (15). It is also known that PTEN expression is inversely correlated with levels of Akt/PKB phosphorylation (24). Therefore, phospho-Akt/PKB may play a critical role in regulating cellular proliferation.

Here we study the potential of CCI-779 as an inhibitor of repopulation of surviving tumor cells between courses of chemotherapy, using the two human prostate cancer cell lines PC-3 and DU145. PC-3 cells were reported PTEN negative, whereas DU145 cells were reported to have high PTEN levels (14). We also compare concurrent and sequential use of CCI-779 with chemotherapy.

Materials and Methods

Cell lines. Human prostate cancer cells PC-3 and DU145 were purchased from the American Type Culture Collection (Manassas, VA). PC-3 cells were maintained in Ham’s F-12K medium, and DU145 cells were cultured in α-MEM. Both media contained 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1% penicillin and streptomycin.

Drugs and preparation. CCI-779 (Wyeth-Ayerst Laboratories, Pearl River, NY) was stored as a dry powder at 4°C and suspended in 100% ethanol on the day of use. A stock solution of CCI-779 was diluted to a concentration of 2 mmol/L using 5% Tween 80 (Sigma, St. Louis, MO) and 5% polyethylene glycol 400 (Sigma; refs. 16, 18). Mitoxantrone (Wyeth-Ayerst, Canada, Inc., Montreal, Quebec, Canada) and docetaxel (Aventis Pharmaceuticals, Inc., Bridgewater, NJ) were obtained from the hospital pharmacy.

Evaluation of PTEN status. The PTEN status of tumor xenografts was examined using immunohistochemical staining (25). We did antigen retrieval with 0.4% pepsin for 5 minutes. Peroxidase activity was quenched with 3%...
hydrogen peroxide in water. Primary mouse anti-human PTEN monoclonal antibody (Cascade, Bioscience, Winchester, MA) was diluted to 1:200 in TBS and applied overnight at 4°C, followed by biotinylated anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) at 1:200 dilution for 30 minutes and streptavidin-peroxidase (DS Labs, Inc., London, Ontario, Canada) for 30 minutes. Novolaid substrate kit (Vector Laboratories) was used to visualize specific antigen localization for PTEN. Slides were counterstained with hematoxylin.

**Evaluation of phosphorylation of Akt/PKB and ribosomal protein S6.** DU145 and PC-3 cells were cultured either in medium containing 10% fetal bovine serum or serum-free (starved) overnight. Total Akt/PKB and phospho-Akt/PKB were evaluated by Western blotting analysis (10, 15). In xenografts, phospho-Akt/PKB proteins were detected by using immunohistochemistry (26). Primary mouse anti-human phospho-Akt/PKB monoclonal antibody (Upstate, Inc., Charlestown, MA) was diluted to 1:400 in TBS. 

Phosphorylation of ribosomal protein S6 was determined with anti-P-S6 ribosomal protein Ser235/236 antibody (New England Biolabs Ltd., Mississauga, Ontario, Canada) dissolved at 1 μg/mL in TBS at 0.1% Tween 20 containing 5% milk (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Mice with tumors were treated with a single dose of CCI-779 (50 mg/kg) or vehicle solution. One hour later, the animals were killed and each tumor was dissected from the surrounding tissue and cut in several pieces (~3 x 3 x 3 mm). Pieces from different regions of the tumor were lysed for 1 hour on ice in 1.5 mL of lysis buffer and analyzed as described below.

For Western blotting, the cells were washed twice in ice-cold PBS, and 650 μL of lysis buffer [50 mmol/L HEPES (pH 8.0), 10% glycerol, 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 1.5 mmol/L MgCl2, 100 mmol/L NaF, 10 mmol/L Na3PO4, 1 mmol/L Na2VO4, and 1 tablet/7 mL protease inhibitor cocktail from Roche Diagnostics, Mannheim, Germany] was added to each dish. Lysis took place for 1 hour on ice. The lysates were cleared from insoluble material and the resulting extracts were assayed for total protein content (bicinchoninic acid protein assay, Pierce Biotechnology, Rockford, IL). Equivalent amounts of protein were separated by SDS-PAGE 10% gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore Co., Bedford, MA) and incubated overnight with the primary antibody at 4°C. Then, they were exposed to the secondary antibodies for 1 hour at room temperature (anti-mouse and anti-rabbit from Amersham Biosciences, Buckinghamshire, United Kingdom). Proteins were detected using an enhanced chemiluminescence kit (Amersham Biosciences). Blotting for α-tubulin (Oncogene Research Products, Calbiochem, San Diego, CA) was used to control for protein loading.

**Effects of CCI-779 and of chemotherapy on prostate cancer cells.** Exponentially growing cells were trypsinized and 10^5 cells were seeded into multiple 25-cm² flasks. Different doses of CCI-779 ranging from 0 to 10 μmol/L were added immediately to each flask. After 3 days of treatment, the cells were trypsinized, and counted using a Coulter counter (2 Series 9914591-C, Beckman Coulter, Inc., Miami, FL).

Survival of prostate cancer cells following various treatments was also determined in a colony-forming assay. Exponentially growing cells were exposed to varying doses of mitoxantrone or docetaxel for 24 hours, or to CCI-779 for 3 days. Following this treatment, the cells were washed and trypsinized. Serial dilutions were plated in 6-well plates in 5 mL medium. The plates were incubated for 10 days at 37°C in an atmosphere containing 5% CO2 at 90% humidity. The plates were then stained with methylene blue and colonies containing >50 cells were counted.

The effect of CCI-779 on cellular repopulation was determined following one and two treatments with chemotherapy, given at 7-day intervals. Two experimental conditions were included: (1) mitoxantrone or docetaxel (5 or 10 ng/mL for 24 hours, respectively) was given weekly, and cells were exposed to fresh medium during the intervals between treatments; fresh medium was replaced every 3 days (2). Mitoxantrone or docetaxel was given weekly, and medium containing 100 μmol/L CCI-779 was applied for 3 days after chemotherapy and replaced by drug-free medium. After each treatment with chemotherapy, the cells were washed thrice with PBS. The cells were also washed after CCI-779 treatment to remove the remaining drug. The total number of cells was measured before and after each treatment with mitoxantrone or docetaxel and clonogenic assays were done to determine the number of colony-forming cells.

**Generation and treatment of xenografts.** Male, 4 to 5 weeks old, athymic nude mice were purchased from Harlan Sprague-Dawley (Madison, WI) laboratory animal center and acclimatized in the animal colony for 1 week before experimentation. The animals were housed in microisolator cages, five per cage, in a 12-hour light/dark cycle. The animals received filtered sterilized water and sterile rodent food ad libitum.

For generation of xenografts, cells were implanted in matrigel (Becton Dickinson, Bedford, MA); matrigel was stored at −20°C and then thawed on ice at 4°C for 3 hours before use. Cells were gently resuspended in 1 mL of PBS and incubated on ice for 5 minutes. A prechilled pipette was used to transfer cells to the tube containing 1 mL of matrigel (on ice; ref. 27), and the cell concentration was adjusted to 3 x 10^6/μL. The cells (3 x 10^6/μL) were injected s.c. into both flanks of mice using a 25-gauge needle. When xenografts grew to a size of about 5 mm in diameter, animals were randomly divided into 10 mice. The following experiments were conducted:

1. Mice bearing PC-3 tumors were treated with CCI-779 (1, 5, 10, and 20 mg per kg per day), or vehicle solution for 3 or 5 days per week for 3 weeks; (4) chemotherapy followed by CCI-779.

2. Mice bearing PC-3 tumors received the following treatments: (a) control, vehicle solution for CCI-779; (b) chemotherapy alone, mitoxantrone 1.5 mg/kg or docetaxel 10 mg/kg was injected i.p. weekly for 3 doses; (c) CCI-779 alone, 5 or 10 mg/kg was injected i.p. daily, three times a week for 3 weeks; (d) chemotherapy followed by CCI-779.

The largest and perpendicular diameters of tumors were measured twice weekly, and animals were coded using ear tags so that the observer was unaware of their treatment history. Tumor volume was estimated and plotted against time to evaluate response to treatment.

**Figure 1.** Phosphorylation of Akt/PKB in cultured cells and xenografts. A, PTEN-negative PC-3 cells exposed to serum-free medium overnight exhibited little change in PKB/Akt phosphorylation. In contrast, exposure of PTEN-positive DU145 cells to serum-free medium abolished PKB/Akt phosphorylation. B, in xenografts, expression of phospho-PKB/Akt in PC-3 tumors was strong, whereas in DU145 tumors the expression was very weak.
**Evaluation of tumor cell proliferation.** Tumors were excised for immunohistochemical staining to evaluate cell proliferation by quantifying expression of Ki67.

Paraffin sections were dewaxed in five changes of xylene and exposed sequentially to decreasing concentration of ethanol (finally to water). Sections were then microwaved in 10 mmol/L citrate buffer at pH 6.0 in a pressure cooker for about 20 minutes. Endogenous peroxidase and biotin activities were blocked respectively using 3% hydrogen peroxide and avidin/biotin blocking kit (Vector Laboratories). Sections were treated for 10 minutes with Protein Blocker (Signet Laboratories, Inc., Dedham, MA) and then incubated for 1 hour with monoclonal antibody against human Ki67 (clone MIB-1; Dako, Carpinteria, CA) at 1:100 in a moist chamber. This was followed by 30 minutes each with biotinylated horse anti-mouse IgG (Vector Laboratories) and horseradish peroxidase-conjugated Ultra Streptavidin (Signet Laboratories). Color development was undertaken with freshly prepared NovaRed solution (Vector Laboratories) and counterstained with hematoxylin.

The extent of proliferation was represented by the percentage of viable tumor area occupied by positive nuclei (called the Ki67 index), as described previously (6).

**Toxicity of CCI-779 to mouse bone marrow.** Male BALB/c mice weighing 18 to 20 g were treated with CCI-779 10 mg/kg i.p., using the same schedule as for treatment of tumors. On days 0, 7, 14, 21, and 28, blood samples (0.3-0.5 mL per mouse) were collected from the heart under anesthesia with isoflurane using an anesthetic machine (Benson Medical Industries, Inc., Markham, Ontario, Canada). Blood counts, including hemoglobin, total WBC count, and differential, and platelets were evaluated by using an automated cell counter.

**Data analysis.** All experiments were repeated at least once. Total cells, clonogenic cells, mouse blood counts, Ki67 labeling index, and tumor volumes were represented as mean ± SE. The paired t test for independent samples of equal variance was done to compare sample means. Statistical significance was based on two-sided Ps < 0.05 (z = 0.05).

**Results**

**PTEN status and phosphorylation of Akt/PKB in cultured cells and xenografts.** DU145 cells were confirmed PTEN positive and PC-3 cells PTEN negative. The total level of Akt/PKB in the cell lines was similar when cultured in medium supplemented with 10% fetal bovine serum. However, under serum-free conditions, phospho-Akt/PKB was detected in PTEN-negative PC-3 cells but not in PTEN-positive DU145 cells (Fig. 1A). PTEN mutant PC-3 xenografts were positively stained for phospho- Akt/ PKB when using immunohistochemistry, whereas PTEN wild-type DU145 xenografts were not (Fig. 1B).

**Effect of CCI-779 on phosphorylation of ribosomal protein S6.** The phosphorylation of ribosomal S6 protein in both cell lines and in tumors derived from them decreased after treatment with CCI-779 (Fig. 2, left, in vitro; right, in vivo). Inhibition of phosphorylation of PTEN-positive DU145 cells seemed to be greater than that of PTEN-negative PC-3 cells. In xenografts, S6 phosphorylation in both tumors was inhibited completely by a single dose of CCI-779 and returned to normal levels when evaluated 30 days after treatment with CCI-779. If the tumors were re-treated, S6 phosphorylation was again inhibited, to a greater extent in DU145 than in PC-3 tumors (data not shown).

**Effects of CCI-779 on cell growth and clonogenic survival.** The growth of both cell lines was inhibited in a concentration-dependent manner by CCI-779 (Fig. 3A). The effect of various doses of CCI-779 to reduce the number of colony-forming cells is shown.
In Fig. 3. Following a 3-day exposure to 100 nmol/L CCI-779, the numbers of colony-forming PC-3 and DU145 cells were 0.18 ± 0.09 and 0.37 ± 0.03, respectively, compared with controls; this concentration of CCI-779 was used in subsequent experiments.

Influence of CCI-779 on repopulation of PC-3 cells during chemotherapy. The surviving fraction of PC-3 cells after 24 hours of exposure to mitoxantrone (5 ng/mL) and docetaxel (10 ng/mL) was 0.16 ± 0.02 and 0.12 ± 0.01, respectively. The total number of cells and the number of clonogenic cells during weekly cycles of chemotherapy, with or without CCI-779 present between treatments are shown in Fig. 4. Repopulation of PTEN mutant PC-3 cells between treatments with either mitoxantrone (Fig. 4A) or docetaxel (Fig. 4B) was not inhibited significantly by CCI-779.

Effects of CCI-779 on growth of xenografts. Growth of both prostate cancer xenografts was inhibited by CCI-779. Growth of PC-3 tumors was inhibited in a dose-dependent manner (Fig. 5A) and growth inhibition was greater than for DU145 tumors (Fig. 5B). Mean delay in growth to a volume of 500 mm³ was 39 ± 5 and 17 ± 3 days, respectively, following treatment with CCI-779 (20 mg/kg i.p.) five times weekly for 3 weeks. Following regrowth after treatment with CCI-779, PC-3 tumors responded to re-treatment with CCI-779 (data not shown).

Effects of CCI-779 on proliferation of PC-3 and DU145 cells in xenografts. The nuclei of PTEN-negative PC-3 cells in tumors treated with CCI-779 became smaller and condensed (Fig. 6A), whereas there were no obvious changes in DU145 tumors. Ki67 immunostaining showed that the relative area occupied by positive nuclei decreased following treatment of both types of xenograft with CCI-779 (Fig. 6B). The effect was greater for PC-3 tumors, where the Ki67 index was much lower following treatment with CCI-779 (7 ± 2%) than that of control tumors (42 ± 3%, P < 0.001). The Ki67 indices of DU145 tumors treated with CCI-779 or vehicle solution were 29 ± 2% and 78 ± 3%, respectively (P < 0.001).

Administration of CCI-779 between treatments with chemotherapy. Mitoxantrone (1.5 mg/kg) or docetaxel (10 mg/kg) alone had limited effects on growth of either tumor. Low doses of CCI-779 (5 mg per kg per day, 3 days per week for 3 weeks) given between courses of mitoxantrone increased the growth delay of PC-3 tumors and the effects seemed additive to those from chemotherapy (Fig. 7A). CCI-779 (10 mg/kg, 3 days per week for 3 weeks) given between treatments with docetaxel resulted in greater tumor growth delay than that due to docetaxel alone (Fig. 7B).

Toxicity of CCI-779 in mice. Mice receiving CCI-779 seemed healthy even after the highest doses of 20 mg/kg, 5 days per week, and no life-threatening toxicity was observed during and after treatment for 3 weeks. The body weight of the mice did not change significantly. Effects on blood counts during and after treatment in Fig. 3B. Following a 3-day exposure to 100 nmol/L CCI-779, the numbers of colony-forming PC-3 and DU145 cells were 0.18 ± 0.09 and 0.37 ± 0.03, respectively, compared with controls; this concentration of CCI-779 was used in subsequent experiments.

Effects of CCI-779 on the total number of PC-3 cells and of clonogenic PC-3 cells between courses of treatment with either mitoxantrone (5 ng/mL × 24 hours) or docetaxel (10 ng/mL × 24 hours) on days 0 and 7. Mitoxantrone or docetaxel was followed by CCI-779 for 3 days, control, CCI-779. Points, means from three experiments; bars, ± SE. Neither the total number of cells nor number of clonogenic cells on days 7 and 8 are significantly different for treatments with and without CCI-779 (P > 0.05).

Effects of CCI-779 on proliferation of xenografts. The nuclei of PTEN-negative PC-3 cells in tumors treated with CCI-779 became smaller and condensed (Fig. 6A), whereas there were no obvious changes in DU145 tumors. Ki67 immunostaining showed that the relative area occupied by positive nuclei decreased following treatment of both types of xenograft with CCI-779 (Fig. 6B). The effect was greater for PC-3 tumors, where the Ki67 index was much lower following treatment with CCI-779 (7 ± 2%) than that of control tumors (42 ± 3%, P < 0.001). The Ki67 indices of DU145 tumors treated with CCI-779 or vehicle solution were 29 ± 2% and 78 ± 3%, respectively (P < 0.001).

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with CCI-779 are shown in Table 1. WBC and platelets decreased during the first 1 to 2 weeks, showed partial recovery even during continued treatment, and returned to normal 10 days post-treatment. Levels of hemoglobin showed minimal changes.

**Discussion**

Repopulation during courses of chemotherapy is an important cause of drug resistance in patients. Our hypothesis is that short-acting agents that selectively inhibit the proliferation of tumor cells are likely to improve the effectiveness of chemotherapy by inhibiting repopulation of surviving tumor cells between courses of cytotoxic treatment (1). Here we describe experiments that were designed to evaluate whether CCI-779, an analogue of rapamycin, might lead to inhibition of repopulation between courses of chemotherapy.

CCI-779 is known to inhibit mTOR kinase activity and results in inhibition of the S6 kinase (10, 20). The S6 kinase activates the S6 protein, which is an important downstream translational regulator, and its inhibition decreases the translation of proteins essential for cell cycle progression from G1 to S phase. Surprisingly, we found that phosphorylation levels of S6 decreased to a greater extent in PTEN-positive DU145 cells after treatment with CCI-779, compared with PTEN-negative PC-3 cells; however, inhibition of phosphorylation of S6 was similar and complete in xenografts derived from these cell lines (Fig. 2). In contrast, growth of PC-3 xenografts was markedly inhibited by CCI-779 compared with DU145 xenografts. This differential sensitivity to CCI-779 is clearly not due to differences in S6 phosphorylation. Dysregulation of cap-dependent translation because of alterations in the 4E-BP-eIF4E pathway, in addition to activation of S6K, is associated with human cancer (8, 9). The effect of CCI-779 on this
growth factor (29). This effect might explain why CCI-779 decreased response of endothelial cells to vascular endothelial production of vascular endothelial growth factor and to a signaling pathways, rapamycin and its analogues are reported effects on PC-3 cells in culture. Besides targeting the mTOR pathway, which is also involved in regulation of cell proliferation (28). As the two cell lines, PC-3 and DU145, are not isogenic, we recognize that differences in sensitivity to CCI-779 may be due to multiple factors other than those relating to PTEN status.

Our results show much greater effects of CCI-779 to inhibit growth of PC-3 xenografts than would be predicted by its effects on PC-3 cells in culture. Besides targeting the mTOR signaling pathways, rapamycin and its analogues are reported to have antangiogenic activity linked to a decrease in production of vascular endothelial growth factor and to a decreased response of endothelial cells to vascular endothelial growth factor (29). This effect might explain why CCI-779 inhibited tumor growth of both xenografts. However, the growth delay of PTEN mutant PC-3 tumors was greater than that of PTEN normal DU145 tumors, suggesting that direct antiproliferative effects are also important.

Mitoxantrone and docetaxel were selected for study because they are used to treat hormone-resistant prostate cancer (30–32). However, both agents had limited effects against PC-3 and DU145 cells and xenografts. Administration of CCI-779 during courses of treatment with either mitoxantrone or docetaxel in vitro did not increase the effects of chemotherapy in cultured PC-3 cells, perhaps because chemotherapy led to a low rate of proliferation after treatment, and the rate of repopulation was low.

In vivo, mitoxantrone (1.5 mg/kg) and docetaxel (10 mg/kg) had limited effect on growth delay of PC-3 xenografts. The effect of CCI-779 alone in doses of 20 or 10 mg/kg, daily, 5 days per week, was overwhelmingly more effective than chemotherapy in inhibiting the growth of PC-3 xenografts in nude mice. The effect of lower doses was more limited. When a lower dose of 5 or 10 mg per kg per day was given during the intervals between chemotherapy, we found additive effects. In addition to an effect to inhibit repopulation, CCI-779 may be affecting the "recruitment" of quiescent cells from entering the cell cycle after cytotoxic reduction of the tumor burden (33).

The doses of CCI-779 used in the current study caused minimal hematologic toxicity. A high proportion of human prostate cancers are PTEN mutant (34), and hormone-resistant prostate cancer is moderately sensitive to mitoxantrone and docetaxel (30–32). If CCI-779 has similar effects to inhibit the proliferation of PTEN-negative human prostate cancer, then it has potential to inhibit selectively the repopulation of tumor cells between courses of chemotherapy.

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