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Phase I pharmacodynamic study of time and sequence dependency of hydroxyurea in combination with gemcitabine: a California Cancer Consortium Trial

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Abstract Preclinical studies in our laboratory have demonstrated that prior exposure to hydroxyurea increases the percentage of cells in S phase, enhancing the cytotoxicity of subsequent gemcitabine treatment in human oropharyngeal KB cells. To evaluate the clinical implications of this time- and sequence-dependent potentiation, we performed a phase I trial of hydroxyurea given over 24 h followed by a 30-min infusion of gemcitabine in weeks 1 and 2 of a 3-week cycle. The dose of hydroxyurea was fixed at 500 mg orally every 6 h for four doses starting 24 h before each dose of gemcitabine. The initial dose level of gemcitabine was 250 mg/m^2 on days 2 and 9, and this was escalated stepwise to 1000 mg/m^2 on days 2 and 9. Gemcitabine pharmacokinetics were determined on days 2 and 9 of the first cycle. Of 27 patients enrolled (12 female, 15 male), 24 were evaluable for response and 23 were evaluable for toxicity. Their median age was 56 years (range 27–76 years). Tumor types included lung, head and neck, pancreas, breast, colon, prostate, stomach, ovary, esophagus, germ cell, thyroid, gallbladder, and unknown primary. A total of 80 cycles of treatment were completed. One patient (unknown primary) had an objective partial response lasting 21 months, and 12 patients had stable disease. All observed dose-limiting toxicities were related to myelosuppression. The gemcitabine maximum tolerated dose was established at 750 mg/m^2 on days 2 and 9. Hydroxyurea had no effect on the plasma pharmacokinetics of gemcitabine. These results suggest that hydroxyurea followed by gemcitabine can be safely administered and has activity on this schedule. We are presently developing a phase II trial of this regimen for patients with platinum-resistant head and neck cancer.

Keywords Ribonucleotide reductase · Drug sequence · Gemcitabine · Hydroxyurea

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Introduction

Gemcitabine (2,2′-difluorodeoxycytidine, dFdC) is a deoxycytidine (dC) analog that shows significant antitumor activity in solid tumors [1, 2]. For this prodrug to be cytotoxic, it must be converted to the triphosphate [3, 4]. Gemcitabine is phosphorylated by dC kinase to the monophosphate and further phosphorylated into the di- and triphosphates by other kinases intracellularly [4].

The incorporation of only one gemcitabine triphosphate (dFdCTP) molecule into the DNA strand can terminate DNA synthesis. After the active metabolite dFdCTP is added, only one more deoxynucleotide triphosphate molecule can be joined to the DNA strand before DNA elongation is prematurely terminated [5]. The proofreading activity of DNA polymerase cannot correct this blockage, and the cell is arrested in S phase, leading to cell death by apoptosis [4, 5, 6, 7].

The competition between gemcitabine and dC limits phosphorylation of gemcitabine and its eventual incorporation into DNA [6]. However, gemcitabine potentiates its own incorporation by several mechanisms. Gemcitabine diphosphate decreases competition by lowering deoxynucleotide production through direct inhibition of ribonucleotide reductase, the enzyme that converts ribose nucleotides to deoxyribose nucleotides [7, 8, 9]. Also, dFdCTP in high concentrations can cause dCTP depletion by direct inhibition of CTP synthetase [9, 10, 11]. dCTP is a cofactor that determines the activity of dCMP deaminase, which deaminates both dCMP and gemcitabine monophosphate (dFdCMP) [12]. As a result of lowering dCTP levels and reducing the rate of deamination of dFdCMP, gemcitabine's intracellular half-life is greatly extended, increasing the likelihood of incorporation into DNA [13].

Gemcitabine has been used to treat solid tumors such as pancreatic cancer, small-cell lung cancer, advanced ovarian cancer, squamous cell carcinoma of the head and neck, bladder cancer, renal cell carcinoma, non-small-cell lung cancer, advanced gastric cancer, metastatic malignant melanoma, and advanced colorectal adenocarcinoma [14, 15, 16, 17]. The therapeutic activity of gemcitabine can be enhanced when it is combined with other anticancer drugs and it can also sensitize various human carcinoma cells in vitro to radiation even at noncytotoxic concentrations [18, 19, 20, 21, 22, 23, 24, 25, 26, 27]. Gemcitabine has been found to have synergistic effects with cisplatin if exposure to gemcitabine occurs 4 h before or after exposure to cisplatin [20]. This phenomenon is seen both in vivo and in vitro. While the mechanism is still unknown, high response rates have been observed with this combination in non-small-cell lung cancer and gemcitabine in combination with hydroxyurea has also been examined [19, 20, 21].

Hydroxyurea is an S phase-specific inhibitor of ribonucleotide reductase with a broad spectrum of preclinical and clinical antitumor activity [28, 29, 30]. As a standard first-line agent in chronic myelogenous leukemia [31], and as a radiosensitizer [32, 33, 34], large clinical experience has established the schedule-dependency of its biological effects. Antitumor effects and radio sensitization are greater with prolonged schedules of administration, which is characteristic of the majority of S phase-active agents. Ribonucleotide reductase is generally considered the "gate" between RNA and DNA precursor metabolism, because it is the only enzyme that converts ribonucleotides (normally present at two logs greater concentration than deoxyribonucleotides) to deoxyribonucleotides. As an inhibitor of ribonucleotide reductase, hydroxyurea depletes DNA precursors without affecting RNA and protein synthesis, causing G_1/S cell cycle arrest and cytotoxicity via "unbalanced growth". Lately, in in vitro studies investigating the interaction between gemcitabine and hydroxyurea we have shown that the combination has an enhanced effect when gemcitabine is administered 8 h following hydroxyurea (unpublished data). The decrease in colony formation ability and incorporation of [^{3}H]gemcitabine both peak when gemcitabine treatment is initiated after 8 h exposure to hydroxyurea. A decrease in RRM2 subunit mRNA, protein, and activity is noted after between 4 and 8 h of hydroxyurea exposure. The subsequent depletion of the dCTP pool allows increased incorporation of dFdCTP into DNA, resulting in higher levels of cytotoxicity than either treatment alone. This phase I study was conducted to validate the preclinical observations and results summarized here.

Patients and methods

Patient selection

Between February 1998 and October 1999, 27 patients were entered into this phase I trial. All patients had histologically verified advanced malignancies, unresponsive to previous chemotherapeutic regimens, or for which no "standard" chemotherapeutic regimen existed. Patients were required to have a Karnofsky performance status of ≥60%, age ≥18 years, an expected survival of at least 2 months, adequate renal function defined by serum creatinine ≤ 2.0 mg/dl or 24-h creatinine clearance of ≥50 ml/min, adequate bone marrow function defined by an absolute neutrophil count (ANC) ≥1200/dl and a platelet count ≥100,000/μl, and adequate hepatic function defined by a serum bilirubin ≤ 3.0 mg/dl with aspartate aminotransferase and alanine aminotransferase within five times the upper limit of normal. Prior radiation or chemotherapy must have been completed at least 4 weeks before beginning treatment on this protocol. There was no limit to the number of prior courses of chemotherapy. Pregnant female patients were excluded. All patients gave their voluntary informed consent and signed a consent document that had been reviewed and approved by the City of Hope National Medical Center Institutional Review Board. This trial was also approved by the Cancer Therapy Evaluation Program of the National Cancer Institute (NCI).

Pretreatment evaluation

All patients had a complete history and physical examination, including documentation of weight, Karnofsky performance status, evaluation for the presence of measurable or evaluable disease, baseline laboratory blood tests, chest radiograph, electrocardiogram, urinalysis, pregnancy test if indicated, and computed tomographic scans of the chest, abdomen, and pelvis as needed to document measurable or evaluable disease. Patients with measurable disease were required to have radiographic procedures for analysis of measurable disease repeated after two cycles of therapy.

Treatment plan

Hydroxyurea was administered orally at 500 mg every 6 h for four doses on days 1 and 8 of each cycle. Gemcitabine was administered as a 30-min infusion 6 h after the fourth dose of hydroxyurea (i.e., on days 2 and 9). Patients were required to have a platelet count

≥75,000/μl and an ANC ≥1000/dl on day 8 to receive chemotherapy. Patients received the chemotherapy as outpatients. Granulocyte colony stimulating factor (G-CSF) was administered beginning on day 10 of all cycles of therapy, including the first cycle. G-CSF was started 24 h after completion of hydroxyurea/gemcitabine administration at a dose of 5 μg/kg per day as a single daily subcutaneous injection, and was continued for at least 7 days and until the white blood cell count was greater than 10,000/μl.

Definitions of dose-limiting toxicities and the maximum tolerated dose

Hematologic dose-limiting toxicity (DLT) was defined as a platelet count < 75,000/μl on day 8, or a platelet count < 25,000/μl lasting seven or more days, an ANC < 1000/μl on day 8 or an ANC < 500/μl lasting seven or more days. Hepatic DLT was defined as bilirubin 4.5–6.0 mg/dl or transaminase 8.0–20.0 times normal (grade 3), and bilirubin > 6.0 mg/dl or transaminase more than 20.0 times normal (grade 4). Other toxicities were graded according to the NCI Common Toxicity Criteria Version 1.0. Dose escalations and determination of the maximum tolerated dose (MTD) were based on DLT occurring in the first cycle. MTD was defined as the highest dose tested at which none or only one patient experienced DLT when at least six patients were treated at that dose and were evaluable for toxicity. Patients received additional cycles of therapy until unacceptable toxicity required discontinuation of treatment, the patient requested discontinuation, or disease progression occurred.

Pharmacokinetic methods

Blood samples for determination of plasma gemcitabine were collected on days 2 and 9 of the first cycle. Samples were collected at the following times: before gemcitabine, just prior to the end of the 30-min infusion, and 5, 10, 15, 30, and 45 min after the end of the infusion. Blood samples were collected in heparinized tubes and kept on ice until plasma could be separated by centrifugation (within 30 min). Plasma was transferred to polypropylene tubes containing tetrahydrouridine to inhibit cytidine deaminase and stored at –70°C until analysis.

Gemcitabine in plasma was measured by a novel reversed-phase HPLC assay that was developed and validated in the City of Hope Analytical Pharmacology Core Facility. Following addition of the internal standard (IS), 2′3′-dideoxycyditine (Sigma Chemical Company, St. Louis, Mo.), gemcitabine was extracted from plasma by solid-phase extraction using a 1-ml aromatic sulfonic acid cartridge (JT Baker, Phillipsburg, N.J.). Separation of gemcitabine and IS was achieved on a 25×4.6 mm C18 column (Beckman Instruments, Fullerton, Calif.) by gradient elution with a mobile phase consisting of 2–20% methanol in 50 m*M* KH_2PO_4, pH 7.0, at a flow rate of 1 ml/min. Detection was performed by UV absorbance at 275 nm. The standard curve was linear for gemcitabine over the range 200 to 10,000 ng/ml. The inter- and intraday coefficient of variation across the entire range of the standard curve were < 10%, and the accuracies were ± 7%. The lower limit of quantitation of the assay was 5 ng/ml.

Plasma gemcitabine data were analyzed by compartmental methods using ADAPT II software (USC Biomedical Simulations Resource, Los Angeles, Calif.). Data were fitted to a two-compartment model with first-order elimination. Secondary pharmacokinetic parameters (CL_{sys}, V, and $t_{1/2}$) were determined and compared within and among patients.

Statistical methods

This study was designed as a standard phase I trial to establish the MTD and the DLTs of hydroxyurea and gemcitabine administered in combination. The dose schema is outlined in the treatment section. Secondary pharmacokinetic parameters determined on days 2 and 9 were compared using paired two-tailed *t*-tests.

Results

Patient characteristics

Enrolled in the study were 27 patients (12 female, 15 male) and 80 cycles of treatment were completed (Table 1). The median age was 55.4 years (range 27–76 years), and the median Karnofsky performance status was 80% (range 60–100%). The predominant tumor types included: adenocarcinoma of the colon or rectum (four), pancreatic cancer (four), non-small-cell lung cancer (three), and head and neck cancers (tonsilar and larynx) (three). Five patients had no prior chemotherapy and the other patients had received one to five prior chemotherapy treatments; 11 patients (41%) had received prior radiation. Patient characteristics are summarized in Table 1.

Patient evaluability

Of the 27 patients, 24 were evaluable for response and 23 for toxicity and the determination of the MTD. At the dose of 250 mg/m^2 of gemcitabine on days 2 and 9, a DLT did not permit the continuation of cycle 1 in one patient. As a result, the patient never completed a cycle of therapy and was inevaluable for response. At the dose of 750 mg/m^2 of gemcitabine, one patient was replaced because the patient did not receive a complete cycle due to a pleural effusion, and became inevaluable for both toxicity and response. Another patient at this dose was not treated on days 8 and 9 of cycle 1 due to a grade 3 rash, but completed a second cycle. This patient was replaced in the evaluation of toxicity, but

Table 1. Patient characteristics

Number of patients	
Total	27
Male	12
Female	15
Age (years)	
Median	55.4
Range	27–76
Karnofsky performance status	
Median	80
Range	60–100
Primary site (no. of patients)	
Breast	1
Larynx	2
Tonsil	1
Lung	3
Colon/rectum	6
Esophagus	1
Ovary	1
Pancreas	4
Stomach	1
Unknown primary	2
Gallbladder	1
Prostate	2
Testis	1
Thyroid	1

was included in the evaluation of response. At the dose of 875 mg/m^2, one patient was not treated on days 8 and 9 of cycle 1 due to a urinary tract infection, but eventually completed five cycles of therapy. This patient was replaced in the evaluation of toxicity also, but was included in the evaluation of response. Similarly, at the dose of 1000 mg/m^2, one patient was not treated on days 8 and 9 of cycle 1 due to urinary tract infection, but completed a second cycle. This patient was also included in the evaluation of response, but not of toxicity. One patient at the same dose completed one cycle, was evaluable for toxicity evaluation, but did not have measurable disease for response evaluation. The rest of the patients were evaluable for both toxicity and response.

Toxicities of therapy

The number of patients treated and the toxicities for the first cycle are summarized in Table 2. Overall, myelosuppression was the DLT. After DLT (grade 4 neutropenia) was observed in two patients at a gemcitabine dose of 1000 mg/m^2, a dose level of 875 mg/m^2 was tested. At 875 mg/m^2, grade 3 neutropenia developed in one patient on day 8 and grade 4 neutropenia developed in another patient. Therefore, the MTD for gemcitabine in this combination of drugs was estimated to be 750 mg/m^2 on days 2 and 9. A detailed toxicity profile of the eight patients treated at the MTD dose is presented in Table 3.

Treatment response

In this study, 24 patients were evaluable for response summarized by dose level in Table 2. One patient who had an unknown primary (retroperitoneal mass and adenopathy) achieved a partial response of 21 months duration. This patient was treated with gemcitabine 750 mg/m^2. A further 12 patients (50%) had stable disease, three with head and neck cancer, two with lung cancer, two with pancreatic cancer, one with prostate cancer, three with colon cancer and one with breast cancer. Four patients with stable disease were treated with 875 mg/m^2 of gemcitabine, three patients with

Table 2. Dose levels, dose-limiting toxicities, and response data (*PLT* thrombocytopenia, *ANC* neutropenia)

Gemcitabine dose (mg/m^2)[a]	Patients treated	First cycle			All cycles				
		Inevaluable for toxicity	DLT		Inevaluable for response	Cycles completed/ cycles started	Best response to therapy		
			Number	Description			Stable disease	Progressive disease	Partial response
250	6	0	1	PLT	1	22/23	3	2	
500	3	0	0		0	4/4	1	2	
750	8	2	1	ANC[b]	1	26/28	3	3	1
875	5	1	2	ANC[c]	0	17/17	4	1	
1000	5	1	2	ANC[d]	1	11/11	1	3	
Total	27	4	6	5 ANC, 1 PLT	3	80/83	12	11	1

[a]30-min infusion on days 2 and 9. The dose of hydroxyurea was fixed at 500 mg every 6 h for four doses on days 1 and 8
[b]One grade 4 neutropenia (absolute neutrophil count $< 500/\mu l$) lasting more than 7 days
[c]One grade 3 neutropenia on day 8 and one grade 4 neutropenia lasting more than 7 days
[d]Two grade 4 neutropenias lasting more than 7 days

Table 3. Treatment-related toxicities of grade 3 or 4 during cycle 1 at 750 mg/m^2. The one DLT on this arm was the grade 4 neutropenia in patient 7. Patient 5 (primary site gallbladder) with pleural effusion, ascites, and liver nodules made attribution of the gastrointestinal toxicity to the drug unlikely

Toxicity	Patient number at this dose								Total grade 3/4
	1	2	3	4	5[a]	6[a]	7	8	
Hematology									
Anemia	3		3				3		3/0
Leukopenia					3		3	3	3/0
Neutropenia					3		4		1/1
Thrombocytopenia	3	3	3	3				3	5/0
Liver								3	1/0
Dermatology/rash						3			1/0
Gastrointestinal					3				1/0
Neurological					3				1/0
Total									16/1

[a]Patients 5 and 6 missed day 8 of therapy (pleural effusion and rash, respectively) and were replaced for the calculation of DLT/MTD. Patient 6 was eventually able to complete two cycles, and patient 5 was unable to continue

750 mg/m^2, three patients with 250 mg/m^2, and one patient each with 1000 mg/m^2 and 500 mg/m^2. Of the 12 patients with stable disease, 4 survived more than 15 months and 3 were alive at the last evaluation, 33 months, 7 months and 5 months from the date of initial treatment. The remaining 11 patients developed progressive disease within 2 months.

Gemcitabine pharmacokinetics

Day 2 gemcitabine pharmacokinetics were studied in 17 patients and the data from 14 of the 17 patients for both days 2 and 9 were evaluable. Plasma gemcitabine data were best described by a two-compartment model with first-order elimination. Secondary gemcitabine pharmacokinetic parameters determined on day 2 are summarized in Table 4. The mean values for CL_{sys}, V_c, and $t_{1/2}$ estimated in this trial are in good agreement with previously published data for both single-agent gemcitabine and gemcitabine used in combination with other anticancer drugs [35, 36, 37]. As shown in Fig. 1, there was no apparent effect of administered dose on gemcitabine CL_{sys} within the range of doses used here. Furthermore, gemcitabine CL_{sys} varied by about sixfold within a single dose level.

Table 5 summarizes the secondary parameters determined in patients on both day 2 and day 9. As shown in Table 5 and Fig. 1, there were no significant differences seen in gemcitabine pharmacokinetic parameters determined on day 2 and day 9. However, day-9 pharmacokinetic parameters could not be predicted from the day-2 results (data not shown), highlighting the significant interpatient variability.

Table 4. Gemcitabine pharmacokinetics: cycle 1, day 2

Patient	Dose (mg/m^2)	CL_{sys} (l/h/m^2)	V_c (l/m^2)	$t_{1/2\ alpha}$ (min)	$t_{1/2\ beta}$ (min)
1	250	109.4	6.1	1.9	15.8
2	250	106.1	6.9	2.4	13.6
3	250	153.9	21.9	4.8	22.6
4	250	151.9	15.1	2.9	14.0
5	500	45.4	3.0	2.4	10.8
6	750	102.4	28.1	6.9	15.7
7	750	200.6	42.9	4.5	29.0
8	750	36.2	1.3	1.2	14.7
9	750	172.1	2.6	0.3	10.0
10	750	46.6	3.3	2.7	17.8
11	750	31.6	0.9	0.8	12.2
12	875	95.8	1.1	0.3	12.2
13	875	93.4	16.7	4.9	16.5
14	875	126.0	11.0	2.5	18.5
15	1000	74.7	7.4	2.9	17.5
16	1000	109.3	17.4	1.6	15.0
17	1000	86.0	4.3	1.8	11.1
Mean		102.4	11.2	2.7	15.7
SD		49.8	11.5	1.8	4.9

Discussion

This phase I trial demonstrated the feasibility of combining hydroxyurea and gemcitabine when hydroxyurea was administered in four doses beginning 24 h before gemcitabine on days 1 and 8 of a 21-day cycle. The MTD for gemcitabine was determined to be 750 mg/m^2 on days 2 and 9 in this sequence with a fixed dose of hydroxyurea (500 mg every 6 h for four doses on day 1 and four doses on day 8). The DLT for this combination was neutropenia, which occurred in two patients treated with 875 mg/m^2 of gemcitabine on days 2 and 9 and in two patients treated with 1000 mg/m^2 of gemcitabine on days 2 and 9. Pharmacokinetic data indicated that hydroxyurea had no apparent effect on gemcitabine plasma pharmacokinetics when given at this dose and in

Table 5. Gemcitabine pharmacokinetics: cycle 1, day 2 vs day 9 (values are means ± SD)

Parameter	Day 2	Day 9	P value[a]
CL_{sys} (l/h/m^2)	107.1 ± 52.0	97.2 ± 74.3	0.7
V_c (l/m^2)	12.0 ± 12.5	9.0 ± 9.4	0.5
$t_{1/2\ alpha}$ (min)	2.6 ± 1.9	2.4 ± 1.4	0.7
$t_{1/2\ beta}$ (min)	15.2 ± 5.3	17.6 ± 7.3	0.4

[a]Two-tailed paired *t*-test

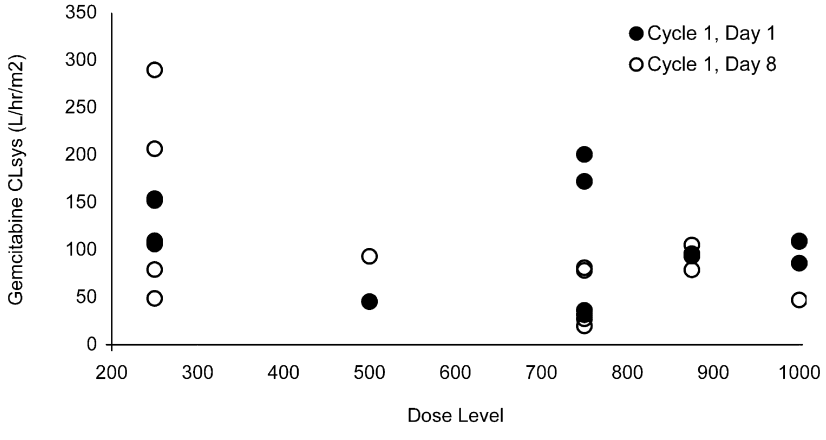

Fig. 1. Scatter plot of total gemcitabine clearance versus dose level (*closed circles* data for day 2 of cycle 1, *open circles* data for day 9 of cycle 1)

this schedule. Stabilization of disease was observed mainly in head and neck and lung cancer patients.

The sequence of hydroxyurea followed by gemcitabine was rationally based on the mechanisms of action of the two drugs. A noncytotoxic dose of hydroxyurea can arrest cells at the G_1/S phase boundary. Kinsella et al, reported significant cytotoxicity from hydroxyurea followed by radiation based on increased radiosensitivity due to accumulation of cells in early S phase when hydroxyurea preceded radiation within 6 h [33]. The radiation treatment caused G_2 delay within 6–18 h. The combination of hydroxyurea and radiation resulted in a low G_1 population for 30 h. The enhancement in cytotoxicity was schedule-dependent, only occurring when hydroxyurea preceded radiation. Our preclinical studies on the sequencing of hydroxyurea and gemcitabine resulted in a similar observation.

In our in vitro studies, we exposed human KB cells to 0.1 m*M* hydroxyurea over a period of 8 h followed by the addition of 0.3 μ*M* gemcitabine and noted significantly enhanced cytotoxicity when compared to gemcitabine alone, gemcitabine followed by hydroxyurea, and concomitant treatment with hydroxyurea and gemcitabine in a colony-forming assay (unpublished data). Treating KB cells with radiolabeled gemcitabine at various times following hydroxyurea treatment demonstrated that the incorporation of dFdCTP into DNA was increased sixfold over cells incubated with gemcitabine alone. Our molecular evidence suggests that hydroxyurea temporarily decreases hRRM2 mRNA and protein, and depletes dNTP pools, particularly dCTP, resulting in S phase arrest. Decreased dCTP and S phase arrest would "set-up" cells for sensitivity to gemcitabine as a consequence of recovery from S phase arrest in the presence of intracellular dFdCTP.

Similar experience has been reported for the combination of hydroxyurea and gemcitabine by Rodriguez et al. [21]. Hydroxyurea (500–3500 mg) beginning 6 h before a fixed dose of gemcitabine (1000 mg/m^2) was associated with promising response rates and dose-limiting myelosuppression. The schedule-dependency of hydroxyurea favors treatment with hydroxyurea prior to the administration of S phase-active agents. There is evidence for synergy on this schedule with cytosine arabinoside, bleomycin, doxorubicin and amsacrine [28, 31]. The unifying mechanism of sensitization would appear to be cell cycle arrest at the G_1/S phase boundary, and a decrease in multiple enzymes resulting from ribonucleotide reductase inhibition in the replitase complex, with cross-inhibition of DNA polymerase and thymidylate synthase [38]. A similar mechanism is likely to pertain to the combination of hydroxyurea and gemcitabine. Therefore, hydroxyurea followed by gemcitabine may be more efficacious than either drug alone and merits further elucidation. Currently, we are testing this combination for the treatment of head and neck cancer patients in a phase II trial.

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