The Schedule-Dependent Effects of the Novel Antifolate Pralatrexate and Gemcitabine Are Superior to Methotrexate and Cytarabine in Models of Human Non-Hodgkin's Lymphoma

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Abstract

Purpose: Methotrexate is known to synergize with cytarabine [1-β-D-arabinofuranosylcytosine (ara-C)] in a schedule-dependent manner. The purpose of this article is to compare and contrast the activity of pralatrexate (10-propargyl-10-deazaminopterin)/gemcitabine to the standard combination of methotrexate/ara-C and to determine if schedule dependency of this combination is important in lymphoma.

Experiment Design: Cytotoxicity assays using the standard trypan blue exclusion assay were used to explore the *in vitro* activity of pralatrexate and gemcitabine against a panel of lymphoma cell lines. Both severe combined imunodeficient beige and irradiated nonobese diabetic/severe combined imunodeficient mouse xenograft models were used to compare and contrast the *in vivo* activity of these combinations as a function of schedule. In addition, apoptosis assays were conducted.

Results: Compared with methotrexate-containing combinations, pralatrexate plus gemcitabine combinations displayed improved therapeutic activity with some schedule dependency. The combination of pralatrexate and gemcitabine was superior to any methotrexate and ara-C combination in inducing apoptosis and in activating caspase-3. *In vivo*, the best therapeutic effects were obtained with the sequence of pralatrexate → gemcitabine. Complete remissions were only appreciated in animals receiving pralatrexate followed by gemcitabine.

Conclusions: These data show that the combination of pralatrexate followed by gemcitabine was superior to methotrexate/ara-C *in vitro* and *in vivo*, and was far more potent in inducing apoptosis in a large B-cell lymphoma. These data provide strong rationale for further study of this combination in lymphomas where methotrexate and ara-C are used.

Antifolates and cytidine analogues have had a time-honored role in the treatment of many kinds of lymphoproliferative malignancies. For example, methotrexate, leucovorin, and cytarabine [1-β-D-arabinofuranosylcytosine (ara-C)] were major components of a combination chemotherapy regimen known as COMLA, which was the predecessor to the now standard CHOP (cyclophosphamide-Adriamycin-vincristine-prednisone) chemotherapy program (1). Doxorubicin supplanted the "MLA" once anthracyclines were found to be important drugs in aggressive lymphoma. Although many efforts have been made, and continue to be made, to integrate methotrexate and ara-C into "CHOP-like" regimens, the resulting myelotoxicity often limits the doses of individual drugs when given together in a single regimen. Today, the integration of methotrexate and ara-C are still used in select combination chemotherapy regimens, most notably in HyperCVAD/MA, which has been used in the treatment of acute lymphoblastic leukemia and mantle cell lymphoma (2, 3); PROMACE-cytaBOM for large cell lymphoma (4, 5); and the CODOX-M/IVAC regimen for Burkitt's lymphoma.

Pralatrexate (10-propargyl-10-deazaaminopterin) is the prototype of a new class of antifolates belonging to the class of molecules known as 10-deazaaminopterins. These compounds are structurally designed to have a much greater affinity for the reduced folate carrier (RFC-1) and foly-polyglutamyl synthase leading to enhanced intracellular accumulation and polyglutamylation in tumor cells (6, 7). For example, the K_m values for the RFC-1 for pralatrexate and methotrexate are 0.3 and 4.8 μmol/L, respectively, whereas the V_{max}/K_m values (i.e., rate

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of intracellular transport) are 12.6 and 0.9, respectively. These data suggest that the rate of pralatrexate influx is nearly 14-fold greater than for methotrexate. Similarly, the K_m for pralatrexate and methotrexate for foly-polyglutamyl synthase are 5.9 and 32.3 μmol/L, respectively, whereas the V_{max}/K_m for foly-polyglutamyl synthase is 23.2 and 2.2, respectively. These biochemical data suggest a much greater potential effect from pralatrexate on the determinants of folate metabolism compared with methotrexate. These determinants are also known to play a major role in mediating methotrexate resistance, which could conceivably be overcome to some degree by analogues with superior affinity for RFC-1 and foly-polyglutamyl synthase.

The effects of pralatrexate and methotrexate were evaluated in parallel in four non-EBV, nonvirally transformed non-Hodgkin's lymphoma cell lines by our group (8). These studies included the following cell lines: RL (a transformed follicular lymphoma overexpressing bcl-2), HT (a diffuse large cell lymphoma), Hs602 (B cell lymphoma derived from a mixed cell non-Hodgkin's lymphoma), and SKI-DLCL-1 (*de novo* diffuse large cell lymphoma overexpressing MUC-1). *In vivo*, pralatrexate was consistently found to be superior to methotrexate. In a nonobese diabetic/severe combined imunodeficient xenograft model of these lymphomas, complete regression of disease was observed in mice bearing the HT lymphoma following treatment with pralatrexate, whereas methotrexate-treated mice experienced only a 17% reduction in tumor growth. In the RL lymphoma xenograft model, mice treated with pralatrexate again exhibited significant tumor regression with two thirds of the mice in the pralatrexate-treated cohort experiencing a complete regression of their disease, whereas mice treated with methotrexate experienced only a modest growth delay compared with the control cohort. These data established the superior therapeutic activity of pralatrexate against human non-Hodgkin's lymphoma. In addition, they provided the basis for a single-agent phase I/II study of pralatrexate in patients with multiply relapsed and refractory aggressive non-Hodgkin's lymphoma and Hodgkin's disease, which is now ongoing. This phase I study has already begun to reveal intriguing activity of pralatrexate in methotrexate-resistant T-cell lymphomas that includes complete remissions in methotrexate-resistant disease (9).

More than 20 years ago, a variety of *in vitro* and *in vivo* experiments showed that sequential methotrexate and ara-C was synergistic compared with the single agents or alternative schedules. As early as 1973, Hoovis and Chou (10) showed that methotrexate and ara-C exhibited marked synergy in a murine leukemia cell line (L5178Y) when used in a sequence-dependent fashion. The authors showed that the pretreatment with methotrexate significantly increased the level of 1-β-D-arabinofuranosylcytosine 5′-triphosphate independent of DNA synthesis, which led to increased incorporation into RNA and enhanced cytotoxicity. Cadman and Eiferman (11) similarly showed the sequence dependency of methotrexate and ara-C in the L1210 model of myeloid leukemia, confirming the preferential accumulation of 1-β-D-arabinofuranosylcytosine 5′-triphosphate in methotrexate-pretreated cells. Using the classic isobologram method of Steel and Peckham, Akutsu et al. (12) recently showed that the ordered treatment of methotrexate followed by ara-C is truly synergistic in a mathematical analysis, whereas other schedules of administration (e.g., given together) were potentially antagonistic. The recent emergence of a novel cytidine analogue gemcitabine (2′,2′-difluorodeozycytidine) and its documented activity in very drug-resistant aggressive lymphoma, with an overall response rate of ~20%, raises the interesting possibility that combinations of gemcitabine and pralatrexate could be exploited, given their likely greater single-agent activity in lymphoma, in similar synergistic fashion (13, 14). What is not clear is whether such schedule dependency will be important for these drugs as has been shown for methotrexate and ara-C. As such, this is the first report to show that pralatrexate and gemcitabine interact in a potentially additive or better fashion, that they are likely to be schedule dependent, and that the combination of pralatrexate and gemcitabine are superior to that of methotrexate and ara-C in inducing apoptosis in lymphoma cell lines.

Materials and Methods

Materials. All reagents and chemicals were obtained from Sigma (St. Louis, MO). Pralatrexate was obtained from Allos Therapeutics, Inc. (Westminster, CO). Methotrexate (XanoDyne, Newport, KY), ara-C (Bedford Laboratories, Bedford, OH), and gemcitabine (Gemzar; Lilly, Indianapolis, IN) were all purchased from the in house pharmacy.

SKI-DLCL-1 (diffuse large B-cell lymphoma) cell line established from the ascitic fluid of a patient at our institution, which has been previously described (15, 16). Suspension cultures of all cell lines were grown in RPMI supplemented with 15% fetal bovine serum, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, 1.5 g/L bicarb, and penicillin/streptomycin, prepared and provided from the Memorial Sloan Kettering Cancer Center Media Laboratory. All cultures were maintained at 37°C in an atmosphere of 5% CO_2 and 100% humidity. Collected cells were always washed twice with Dulbecco's PBS before preparing any assay.

Cytotoxicity assays. The trypan blue exclusion assay was done by aliquoting 5×10^5 SKI-DLCL-1 cells into each well of a 24-well plate with a final volume of 1 mL. Each drug was added in duplicate to the plate wells at 10 nmol/L for 48 hours. This concentration was selected because it approximates the $EC_{10\text{-}25}$ for each drug and allows for a comparison of equimolar concentrations to evaluate differences in potency. For the scheduled administration of drug, drug A alone was dispensed in additional duplicate wells. After 24 hours, drug B was added to these wells and incubated for an additional 24 hours. At the end of incubation, 20 μL of each cell culture was added to 20 μL of trypan blue, after which 10 μL was added to a hemocytometer (Hausser Scientific, Horsham, PA), where viable cells were counted based on the presence (dead cells) or exclusion (live cells) of dye. Repeat performance of all assays was conducted in replicate on a separate day, the collection of which was submitted for statistical evaluation.

Apoptosis assays. Apoptosis was assessed using two well-standardized techniques. In the first (YO-PRO), cell membrane permeability was determined by measuring fluorescence of treated and control cells. SKI-DLCL-1 cells (2×10^7) were treated with either methotrexate, ara-C, gemcitabine, or pralatrexate alone or combinations of drug given together or scheduled. All drugs were again assayed at equimolar concentrations (i.e., 10 nmol/L). The incubation conditions were modeled after the *in vitro* cytotoxicity experiments. Single-agent drugs or combinations of all drugs studied without schedule considerations were incubated for 48 hours, whereas drugs that were studied in some schedule were introduced in a 24 + 24 incubation scheme (i.e., drug B added 24 hours following drug A, then incubated for an additional 24 hours for a total incubation time of 48 hours). Cells were then collected, washed twice, and resuspended at a concentration of 1×10^6/mL in Dulbecco's PBS. Membrane permeability was detected via the differential cellular permeability of the fluorescent dyes YO-PRO-1 and propidium iodide by flow cytometry as per the guidelines

of the manufacturer (Vybrant apoptosis kit 4, Molecular Probes, Eugene, OR). In addition to monitoring apoptosis, cell cycle kinetic analyses were also done on all treated cells based the propidium iodide binding. Apoptotic cells were noted to be permeable to the green fluorescent dye YO-PRO-1 (em 530, FL-1) and impermeable to the red fluorescent dye propidium iodide (em 575, FL-3). Typically, necrotic cells allow the entrance of both dyes, whereas living cells are impermeable to both dyes.

The second assay for apoptosis included an evaluation of caspase-3 activation. Caspase-3 activity was tested by EnzChek Caspase-3 Assay kit 2 (Molecular Probes) based on the fluorometric detection of caspase-dependent Z-DEVD-R110 cleavage [rhodamine 110 bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide), a nonfluorescent bisamide], which, via a two-step process, first yields the monoamide product (weakly fluorescent) and then the fluorescent product R110 (rhodamine 110, strongly fluorescent). Cells were treated and prepared in the same manner as that described for the YO-PRO-1 assay. Detection was done on a Fluoroskan Ascent FL fluorescent plate reader (Thermo Lab Systems, Helsinki, Finland) according to the guidelines of the manufacturer. All assays were conducted in duplicate on two separate occasions. This complete data set was submitted for statistical analysis.

In vivo *tumor model*. Five- to seven-week-old SCID beige mice (CBSCBG-MM double) were obtained from Taconic Laboratories (Germantown, NY). Mice were injected with 1×10^7 SKI-DLBCL-1 cells in the posterior flank via s.c. route. When tumors approached 300 mm^3, mice were divided into nine groups of five animals and were treated twice weekly with four i.p. doses at the maximum tolerated dose of methotrexate (40 mg/kg), ara-C (300 mg/kg), pralatrexate (60 mg/kg), and gemcitabine (60 mg/kg), or a combination of the drugs, each given at one half or one quarter the maximum tolerated dose. Schedule-dependent treatment was also determined by pretreating animals with pralatrexate or methotrexate followed 24 hours later with gemcitabine or ara-C, respectively. One cohort of animals was treated with gemcitabine followed by pralatrexate. In all cases, the control animals received i.p. injections of normal saline.

Tumor-bearing mice were assessed for weight loss and tumor volume at least twice weekly for the duration of the experiment. The data are expressed as the average tumor volume (mm^3) per group as a function of time. Tumors were assessed by measuring the two largest perpendicular axes (l = length, w = width) with standard calipers. Tumor volume was calculated using the formula $4/3\Pi r3$, where $r = (l + w) / 4$. Tumor-bearing mice were assessed for weight loss and tumor volume at least twice weekly for the duration of the experiment. Animals were sacrificed when one-dimensional tumor diameter exceeded 2.0 cm, or after loss of >10% of their body weight in accordance with institutional guidelines. Animals were housed in standard shoe box cages in temperature and humidity constant rooms on a 12-hour light and dark cycle. Food and water were supplied *ad libitum*. Animals were maintained in core animal facilities under an institute approved animal protocol. All experiments were done in accordance with the Principles of Laboratory Animal Care (NIH publication no. 85-23 revised 1085).

Statistical methods. Viable cells were counted using the trypan blue exclusion assay and YoPro apoptotic activity measurements are modeled as binomial proportions and displayed with SE. Treatment group comparisons were made using pair-wise Fisher's exact tests. Xenograft data were analyzed by computing the relative tumor volume, defined as tumor volume at each measurement divided by the baseline tumor volume of that mouse. This establishes an internal control to account for baseline differences. Statistical calculations are based on the relative tumor volume curve (time versus log 10) from which the area under the time-relative volume curve for each mouse was calculated. This is considered as a measure of the total tumor burden over the experiment. We finally divided the area under the curve by the number of days the mouse was under observation, which can be interpreted as an average daily tumor burden. The average daily tumor burden was compared across groups using pair-wise Wilcoxon rank-sum tests. Exact reference distributions were used to account for small samples.

Results

***Cytotoxicity of pralatrexate and methotrexate combinations* in vitro.** To determine the best concentrations of individual drugs to use in combination with pralatrexate, a series of concentration-effect curves were established using an *in vitro* trypan blue exclusion assay (data not shown) with a variety of conventional antineoplastic agents. Based on the single-agent data presented in Fig. 1, concentrations less than the estimated EC_{25} were selected for combination studies. Previous experiments exploring other favorable drug combinations with pralatrexate showed that gemcitabine was the most active drug in combination with pralatrexate compared with other agents, including irinotecan, vincristine, and doxorubicin. Note that these assays are intentionally exploiting relatively subtherapeutic concentrations of drugs to try and identify the most active combinations relative to the single-agent data. Figure 1 presents the results of a cytotoxicity assay comparing the activity of methotrexate, ara-C, pralatrexate, gemcitabine, and the combinations. This figure suggests that the combination of pralatrexate → gemcitabine was the most cytotoxic, with methotrexate → ara-C being the second most cytotoxic combination, although this difference was not statistically significant for the *in vitro* data. Similarly, there was no statistically significant difference between the data in the methotrexate → ara-C– and pralatrexate → ara-C–treated cells. These data seem to support previous observations that methotrexate → ara-C is superior to methotrexate + ara-C ($P < 0.01$; ref. 12). The cytotoxicity seen with the sequenced combinations (methotrexate → ara-C or pralatrexate → gemcitabine) seems to be much greater than the benefit anticipated by the additive combination of the two single drugs. This pattern of activity was uniformly evident across other lymphoma cell lines studied. Interestingly, gemcitabine may not substitute for ara-C in the methotrexate → ara-C combinations, as methotrexate → gemcitabine is significantly worse ($P = 0.003$). Although some of the differences are small, there is a suggestive pattern that the alternative controls (pralatrexate → ara-C; methotrexate → gemcitabine) were inferior to the methotrexate → ara-C– and pralatrexate → gemcitabine–treated cells, suggesting that the cross-substitution of drugs, the cytidine analogues in this case, may not produce the same benefit.

Pralatrexate/gemcitabine induces apoptosis more efficiently than methotrexate/ara-C. Given that the combination of methotrexate → ara-C has been shown to have the greatest therapeutic benefit when scheduled with the antifolate preceding the cytidine analogue, it was an early objective to determine if these results could be extended to show a difference in the induction of apoptosis in a similar schedule-dependent manner with pralatrexate. In these assays, methotrexate and ara-C combinations were found to be inferior to pralatrexate and gemcitabine both as single agents and in combination in SKI-DLCL-1 (Figs. 2 and 3). SKI-DLCL-1 was selected for the majority of these experiments given our historical experience that this particular line seems to be the more pralatrexate resistant than most of the other lymphoma cell lines in the

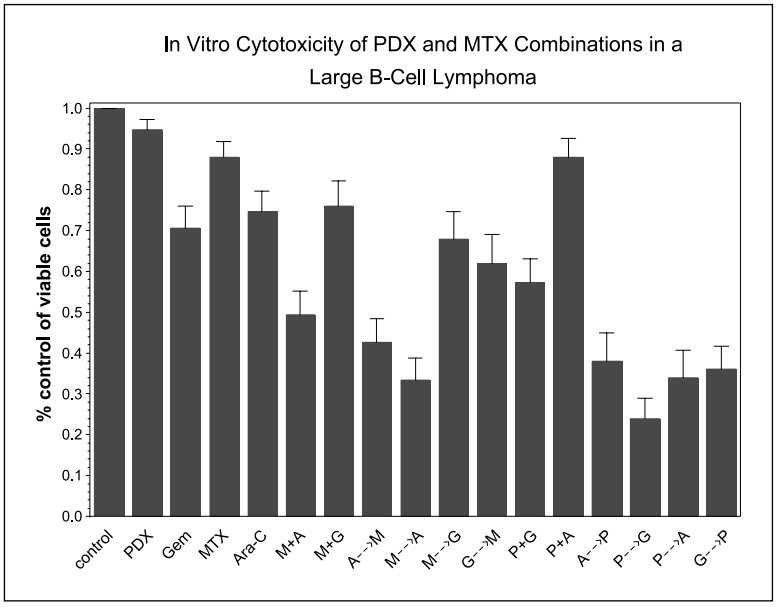

Fig. 1. Cytotoxicity assay (trypan blue exclusion) of SKI-DLBCL-1 treated with methotrexate (*MTX*, *M*), ara-C (*A*), pralatrexate (*PDX*, *P*), and gemcitabine (*Gem*, *G*) alone and in combination. In all cases, cells were exposed to the first drug for 24 hours followed by the addition of the second drug for another 24 hours. Assays were conducted in duplicate on two separate occasions with a total incubation time of 48 hours. Experiments were conducted using concentrations of drugs that approximates the $EC_{10\text{-}25}$. The combination of pralatrexate → gemcitabine was the most cytotoxic combination overall, although methotrexate → ara-C was the second most cytotoxic combination. Columns, percentage control of viable cells; bars, SE.

library studied to date. For example, this line is known to have the lowest level of RFC-1 expression on its surface as shown by reverse transcription-PCR (rendering it more resistant than lines with greater RFC-1 expression; ref. 8). Because this line was felt to be more resistant to pralatrexate than other B-cell lines, we concentrated our studies on this line to identify strategies that might be useful under these theoretically more difficult treatment scenarios.

Figure 2 shows that the pralatrexate and gemcitabine combinations effected the most robust induction of apoptosis compared with any methotrexate and ara-C combination. Whereas there are little differences between any of the single agents (no statistically significant differences between any single-agent cohorts), mostly because such low concentrations of drug are used, the combinations of pralatrexate and gemcitabine consistently produce more apoptosis in a fashion that seems synergistic. In fact, the combination of pralatrexate → gemcitabine was statistically different from every cohort, except the ara-C → pralatrexate cohort ($P = 0.08$), whereas the gemcitabine → pralatrexate cohort was statistically different from every other comparator ($P \ll 0.05$), including methotrexate → ara-C ($P < 2 \times 10^{-12}$), methotrexate → gemcitabine ($P < 0.00015$), pralatrexate → ara-C ($P < 0.009$), and pralatrexate + gemcitabine ($P < 5 \times 10^{-8}$). These data show that the sequence of pralatrexate and gemcitabine may be important given the inferior results seen with the simultaneous combination of pralatrexate + gemcitabine, and that methotrexate and ara-C cannot directly substitute for pralatrexate or gemcitabine. These results are consistent with the *in vitro* data concerning the importance of schedule with pralatrexate and gemcitabine, and the superiority of the pralatrexate and gemcitabine combinations above that seen with methotrexate and ara-C.

To further explore other markers of the apoptotic cascade, we assayed the activity of caspase-3 in SKI-DLCL-1–treated

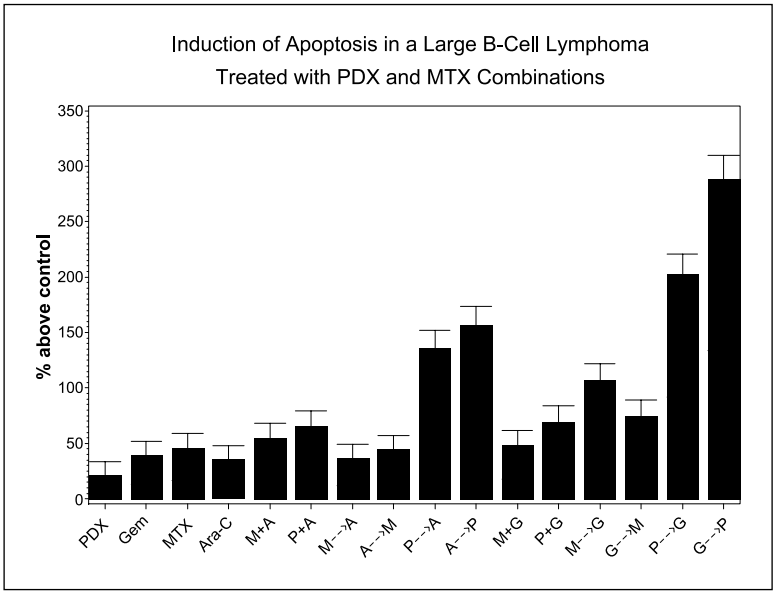

Fig. 2. Induction of apoptosis (Yo-Pro) in SKI-DLCL-1 following exposure to methotrexate, ara-C, pralatrexate, and gemcitabine in combined and scheduled exposures. In all cases, the pralatrexate and gemcitabine combinations seem to exhibit the highest levels of apoptosis induction compared with any methotrexate and ara-C combination. The scheduled administration of pralatrexate and gemcitabine seems superior to the simultaneous exposure. Columns, percentage above controls; bars, SE.

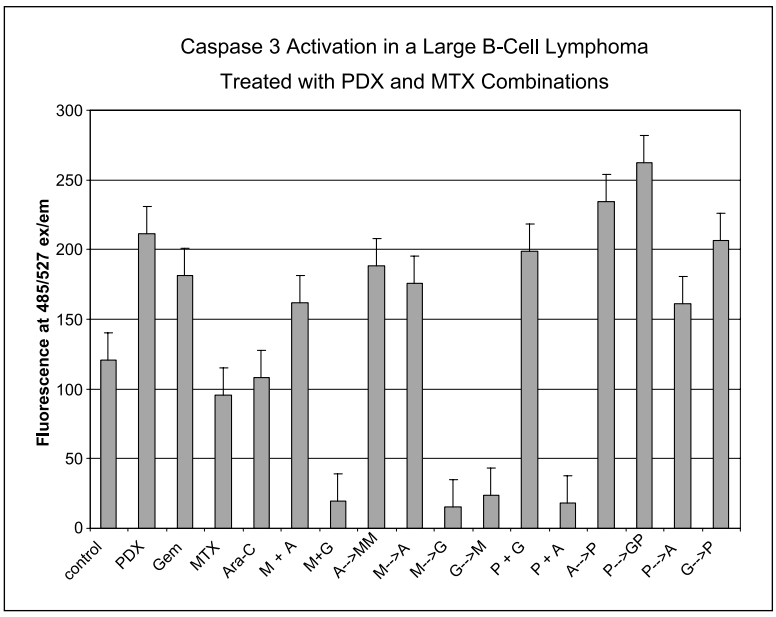

Fig. 3. Caspase-3 activation in SKI-DLCL-1 following exposure to methotrexate, ara-C, pralatrexate, and gemcitabine in combined and scheduled exposures. Pralatrexate → gemcitabine seems superior to any other combination and is superior to the simultaneous exposure of these two drugs. These data also suggest that pralatrexate and gemcitabine cannot substitute for methotrexate or ara-C methotrexate → ara-C cohort, respectively. Pralatrexate alone is also better than methotrexate alone or the best combination of methotrexate → ara-C combination. Columns, fluorescence at 485/527 excitation/emission; bars, SE.

cells as shown in Fig. 3. The results of the initial apoptosis assay are corroborated in a very similar pattern in the caspase-3 assay. Again, uniformly, pralatrexate and gemcitabine combinations were superior to any methotrexate or ara-C combination. Interestingly, pralatrexate is more potent in inducing caspase-3 activation than any of the other single agents studied. The sequence of pralatrexate → gemcitabine seems nearly 1.5-fold better than the result seen with methotrexate → ara-C, as the scheduled sequence of pralatrexate and gemcitabine seems superior to the simultaneous exposure. Once again, it seems that methotrexate cannot be substituted for pralatrexate in that the methotrexate → gemcitabine combination is inferior to the pralatrexate → gemcitabine sequence. Similarly, ara-C cannot substitute for gemcitabine as the pralatrexate → gemcitabine sequence is superior to the pralatrexate → ara-C sequence. Collectively, these *in vitro* assays support the hypothesis that combinations of pralatrexate and gemcitabine are superior to methotrexate and ara-C and that the optimal antitumor effects in this representative large cell lymphoma are likely to be schedule dependent, with the antifolate pralatrexate preceding the cytidine analogue gemcitabine.

Pralatrexate followed by gemcitabine induces complete cell cycle arrest compared with methotrexate and ara-C. SKI-DLBCL-1 cells were exposed to the same set of treatment scenarios as discussed in Figs. 2 and 3 above. Following a 48-hour incubation, cells were treated with propidium iodide and analyzed by flow cytometry. Figure 4 shows that, for the most part, all the drug combinations examined yielded essentially the same percentage of cells in G_1 arrest. However, the fraction of cells in S phase was variable, with the fewest in those samples treated with gemcitabine. Remarkably, whereas the number of cells in G_2 is relatively similar among all the groups (9-15%), there are no cells in G_2 in the group treated with pralatrexate followed by gemcitabine (0%). For example, the percentage of cells in G_2 in the control were ~12%, which was not significantly different from that noted in cells treated with methotrexate, pralatrexate, gemcitabine, or ara-C alone (9-15%). The cells treated with the scheduled administration of pralatrexate → gemcitabine were the only ones to show a complete absence of cells in G_2, with a proportionately greater number of cells exhibiting S-phase arrest compared with any other treatment condition. This accumulation of cells in S phase in the pralatrexate → gemcitabine–treated cells conceivably enhances the sensitivity of the cell to the cytotoxic effect of the cytidine analogue. These data suggest that this particular combination (pralatrexate → gemcitabine) is more effective at inducing a G_1-S arrest compared with other treatment exposure groups, which may explain the greater degree of apoptosis noted in the earlier experiments.

***Pralatrexate followed by gemcitabine is superior to all other treatment groups* in vivo.** Previous experiments by our group have clearly shown the superiority of pralatrexate compared with methotrexate in *in vivo* lymphoma models across many different cell lines including HT, RL, SKI-DLCL-1, and a Burkitt's cell line (Raji). Here, again, given the experience that SKI-DLCL-1 is one of the more pralatrexate-resistant lines in the panel, it was selected for these *in vivo* combination experiments. In all xenograft experiments, animals in the control group showed rapid progression of disease and had to be sacrificed early secondary to advanced tumor growth. All animals were treated with the maximum tolerated dose of each drug, save the combination studies. A preliminary xenograft experiment using irradiated nonobese diabetic/severe combined imunodeficient mice with s.c. tumors of SKI showed that mice treated with pralatrexate or gemcitabine at 60 mg/kg initially responded to treatment but by day 44 experienced rapid progression of disease. Those animals treated with methotrexate at 40 mg/kg (maximum tolerated dose) or ara-C at 300 mg/kg (maximum tolerated dose) or at the maximum tolerated dose of the combination (i.e., methotrexate at 20 mg/kg and or ara-C at 150 mg/kg) displayed modest growth delay compared with the control cohort, with all animals in these groups having to be sacrificed due to excessive tumor volume by week 3. Animals treated in the combination experiments with one half the maximum tolerated dose of pralatrexate and gemcitabine (30 mg/kg each) experienced significant reduction (63.1%) of their initial tumor volume, with three animals achieving a complete remission and one a

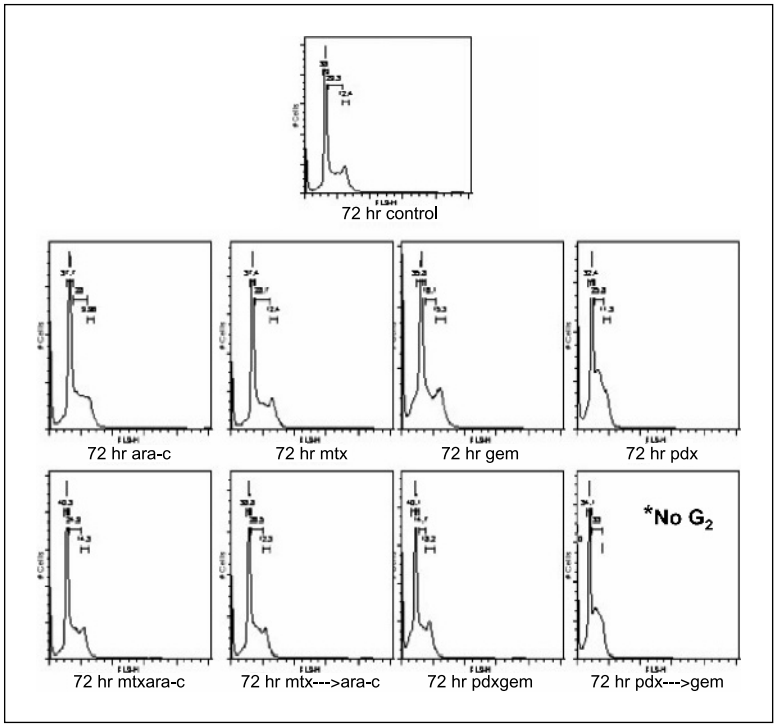

Fig. 4. Flow cytometric analysis of SKI-DLCL-1 treated with methotrexate, ara-C, pralatrexate, and gemcitabine in combined and scheduled exposures.

partial remission, whereas one developed disease progression (data not shown). Complete remissions were confirmed by resecting the tumor site and evaluating for the presence of viable residual lymphoma.

To explore the lowest dose of pralatrexate and gemcitabine that could be used before losing the potential "synergistic" interaction, a second xenograft experiment was conducted using one-quarter of the maximum tolerated dose of pralatrexate and gemcitabine (15 mg/kg; Fig. 5). A similar dose reduction for methotrexate and ara-C was not done given the lack of activity seen at the higher doses. Interestingly, the patterns appreciated in the earlier experiment were recapitulated in the second experiment. Again, control groups had to be sacrificed early due to excessive tumor growth, as did animals receiving methotrexate (60 mg/kg) alone, ara-C (300 mg/kg) alone, or both drugs in combination. These data showed that even using one quarter of the maximum tolerated dose of pralatrexate and gemcitabine given in a scheduled manner was significantly more efficacious that any methotrexate- or ara-C-treated cohort (P = 0.016). Interestingly, gemcitabine alone was statistically superior to methotrexate → ara-C (P = 0.016), whereas pralatrexate alone trended toward statistical significance (P = 0.06) compared with methotrexate → ara-C. Although fewer complete remissions were noted, one complete remission was only observed in those animals receiving pralatrexate → gemcitabine (Fig. 5), whereas no complete remissions were ever appreciated in any methotrexate-treated cohort. These *in vivo* experiments clearly establish the marked superiority of pralatrexate and gemcitabine when given in a sequential fashion even in a cell line noted to be historically among the most resistant in the collection to pralatrexate and methotrexate.

Immunohistochemical staining of resected lymphoma reveals more caspase-3 activation in pralatrexate → gemcitabine–treated mice. Tumors resected from all mice receiving methotrexate, ara-C, pralatrexate, or gemcitabine in a schedule-dependent manner revealed minimal activation of caspase-3 in the tissue

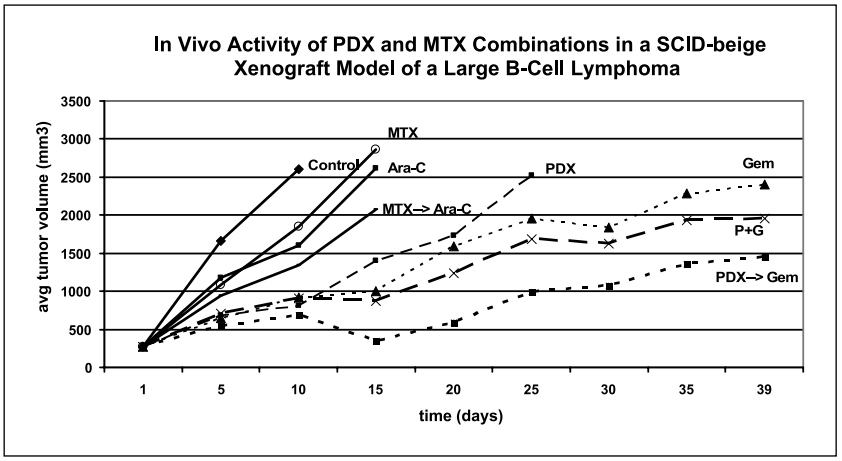

Fig. 5. *In vivo* SCID beige xenograft model of SKI-DLCL-1. Methotrexate is given at 40 mg/kg i.p., ara-C was given at a dose of 300 mg/kg; pralatrexate and gemcitabine were both given at a dose of 60 mg/kg. All drugs were dosed twice weekly for 2 weeks (i.e., on days 1 and 4 and 8 and 11) followed by a 1 week rest for a total of two cycles of therapy in all cases. In cases where ordered administration was used, the second drug was given 24 hours after the first drug. In the case where methotrexate and ara-C are given in combination, the doses of drugs used were 20 and 150 mg/kg, respectively. In the cases where pralatrexate and gemcitabine were used, the doses were 15 mg/kg for both agents.

of mice treated with saline (i.e., control), any single agent, and the doublet of methotrexate and ara-C. For illustrative purposes, we have only depicted the slides for the control, methotrexate →A, and pralatrexate → gemcitabine (Fig. 6). Interestingly, the only significant staining for activated caspase-3 occurred in those samples of tumor in mice treated with pralatrexate → gemcitabine. These data are concordant with the observations from the *in vitro* experiments and apoptosis assays, which also showed the most significant activation of caspase-3 in cells treated with pralatrexate followed by gemcitabine.

Discussion

The pharmacologic concept behind pralatrexate that differentiates it from other efforts to develop improved antifolates revolves around its designed affinity for RFC-1 (17–19). Rather than designing the drug to have superior affinity for other determinants of antifolate metabolism like foly-poly-glutamyl synthase or dihydrofolate reductase, pralatrexate was designed to overcome some of the transport liabilities that can accompany other antifolates. A drug with improved affinity for RFC-1 with improved membrane transport and improved affinity for the other determinants of antifolate pharmacology should have improved antitumor effects. RFC-1, in fact, turns out to be an excellent target for drug development because it is an oncofetal protein that has been shown to be almost exclusively expressed in fetal and neoplastic tissue, which seems fitting given the intrinsic proliferative state of these tissues and their demand for natural folate pools. Hence, pralatrexate may offer several advantages over other antifolates like methotrexate, including a better therapeutic index.

Whereas methotrexate has proved to be an active agent in the treatment of lymphoproliferative malignancies, it is clear

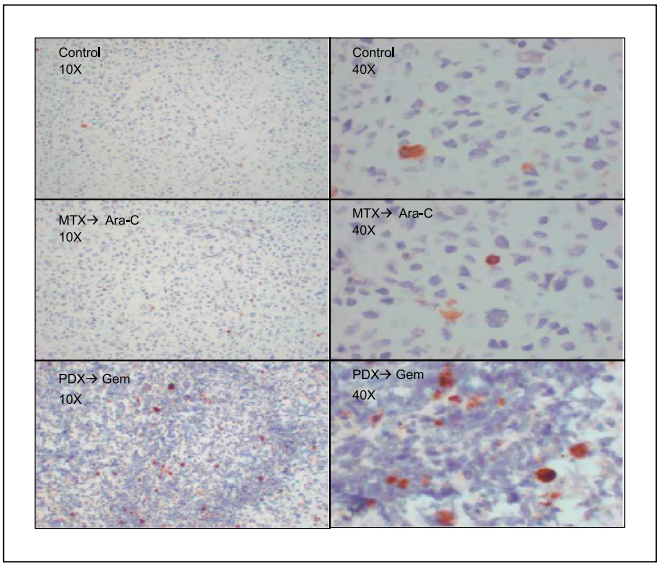

Fig. 6. Immunohistochemistry staining for caspase-3 from resected tumors taken from mice treated with saline (control), methotrexate → ara-C, and pralatrexate → gemcitabine. These data show that the most significant amount of activated caspase-3 was shown in those samples of tumor taken from mice treated with pralatrexate → gemcitabine, whereas little to no activation could be shown in any other specimen stained.

that resistance to methotrexate can be significant and that its activity is not universal across all subtypes of lymphoma. Improvements in the activity of methotrexate have been shown by integrating it into various chemotherapy regimens with cytidine analogues, based on the mechanistic rationale advanced by Hoovis and Chu (10) and Cadman and Eiferman (11). Such approaches, like that used in the HyperCVAD regimen, also exploit cytokinetic principles for the use of antimetabolites in cytoreduced states as part of a strategy to treat minimal residual disease. Based on the single-agent activity of pralatrexate in our previously reported studies, integration of pralatrexate with other new generation cytidine analogues seemed to represent a logical extension of these previously established concepts.

The first important observation revolves around the importance of schedule dependency. Like that shown for methotrexate and ara-C (10–12), there seems to be a consistent demonstration of schedule dependency with pralatrexate and gemcitabine. These observations are supported by the *in vitro* cytotoxicity experiments, the apoptosis assays, as well as the *in vivo* xenograft experiments. Even more important are the observations that more complete remissions seem to be recorded when these drugs are given in a schedule-dependent manner and not when given in a simultaneous exposure. The sequence of pralatrexate → gemcitabine was five times more effective in inducing apoptosis compared with the simultaneous exposure of pralatrexate + gemcitabine. Despite the well-established position of methotrexate and ara-C in the treatment of lymphoma, there seems to be remarkably little induction of the apoptotic cascade compared with the results seen with pralatrexate and gemcitabine. Another interesting feature of these data is the idea that antifolates (pralatrexate or methotrexate) and cytidine analogues (gemcitabine or ara-C) do not seem to be interchangeable. Despite the relatively nominal concentrations used in the *in vitro* cytotoxicity assay, there does not seem to be any immediate benefit in substituting one antifolate or cytidine analogue for the other. However, in the apoptosis assays, there may be a benefit in favor of those combinations containing pralatrexate (i.e., pralatrexate → ara-C) and gemcitabine (i.e., methotrexate → gemcitabine) relative to the standard combinations of methotrexate → ara-C. Whereas simultaneous exposure to pralatrexate and gemcitabine is inferior to the scheduled combination, it is not entirely clear that the combination of gemcitabine → pralatrexate is all that inferior to the alternative schedule, although the former schedule was substantially more toxic in the mouse modes even at substantially reduced doses.

More recently, others have tried to recapitulate this phenomenon with other new generation antifolates, including the multitargeted antifolate pemetrexed (LY231514). In one example, Tesei et al. (20) studied the cytotoxicity of the pemetrexed alone or in combination with gemcitabine using different exposure schedules in three different colon cancer cell lines. They found that, among the different combinations tested, the combination of gemcitabine followed by pemetrexed was synergistic in all three cell lines tested, whereas the combination of pemetrexed followed by gemcitabine was synergistic in two of the three lines (WiDR and LoVo) and additive in the third (LRWZ). The combined treatment of multitargeted antifolate and gemcitabine without consideration of schedule was antagonistic in two of three lines (LRWZ and

WiDR) and additive in the LoVo cell line. Similarly, Giovannetti et al. (21) have recently shown that in a pancreatic cell line model, the combination of pemetrexed followed by gemcitabine produced the most synergistic interaction, with a substantially greater induction of apoptosis in this exposure group compared with any other. They also showed that pemetrexed increased the fraction of cells in S phase, rendering them more sensitive to gemcitabine. Similar to what has been shown here, the combination of pralatrexate and gemcitabine in a schedule-dependent manner effected greater cell cycle arrest with an accumulation of cells in S phase, with a greater induction of apoptosis being appreciated in the sequence-treated cell lines.

The links between inhibition of the thymidylate cycle, cell cycle arrest, p53 status, and the induction of apoptosis have been well established (21–26). In the face of DNA damage, p53 can either trigger cell cycle arrest to permit time for adequate DNA repair or mediate the induction of apoptosis (27). Both the inhibition of DNA replication and the block at G_1-S transition allow time for cells to repair the damage, which, if considered exceptionally high at the cellular level, may trigger apoptosis in a p53-dependent manner. In the cell lines studied here, both SKI-DLCL-1 and RL are both known to accumulate p53 based on immunohistochemistry. Many lines of evidence have shown that inhibition of the thymidylate cycle with antifolates and thymidylate synthase inhibitors can result in DNA strand breaks after imbalances in the deoxynucleotide triphosphate pools and inhibition of DNA synthesis, triggering apoptosis (22–24). What remains interesting in the data presented here is the degree of difference in the induction of the apoptotic cascade in a presumably p53-independent manner that is more pronounced with scheduled pralatrexate and gemcitabine compared with methotrexate and ara-C. The fact that schedule matters likely relates to the observation that pretreatment of cells with an antifolate causes greater degrees of nucleotide pool imbalance with more accumulation of the cytidine analogue triphosphate.

Although not specifically addressed in this article, another possible mechanism of apoptosis mediated by pralatrexate could revolve around the regulation of Fas. The coupling of Fas by Fas ligand leads to a death-inducing complex and activation of the initiator caspase, caspase-8/FADD-like interleukin 1β-converting enzyme, which in turn activates downstream caspases such as caspase-3, caspase-6, and caspase-7 (20, 21). Longley et al. (25) have shown that the Fas death receptor is highly up-regulated in response to 5-fluorouracil and antifolates like raletrexed, and have clearly shown that Fas is an important mediator of apoptosis in response to fluoropyrimidines and antifolates, although the sensitivity of particular cell lines to Fas agonists and antifolates was more dramatic in cells with wild-type p53. Recently, Backus et al. (28) have shown that inhibition of thymidylate synthesis triggers apoptosis via activation of caspase-8 and caspase-9 in a p53-independent manner, and that the regulation of the downstream apoptotic processes is independent of p53.

Regardless of the precise mechanism, these data consistently show that the combination of pralatrexate → gemcitabine is significantly superior to the combination of methotrexate and ara-C, a well established and still actively used drug combination used in the treatment of lymphoproliferative malignancies. Importantly, it also shows that schedule of pralatrexate and gemcitabine may be as important as has been shown for methotrexate and ara-C. To date, a phase I/II study of pralatrexate in lymphoma has been remarkable for significant activity in a variety of non-Hodgkin's lymphoma but with particular activity being shown in methotrexate-resistant T-cell lymphoma, including durable complete remissions (9). Interestingly, this is a disease where single-agent gemcitabine has found widespread use as well. The results presented here raise the possibility that pralatrexate, with or without the scheduled administration of gemcitabine, could provide a new platform for the treatment of select lymphoproliferative malignancies. Further preclinical studies will continue to explore the mechanistic basis for the schedule-dependent interaction between pralatrexate and gemcitabine, while ongoing clinical studies establish the spectrum of activity and toxicity across a panoply of lymphoma subtypes.

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