Patterns of Elevation of Plasma 2'-Deoxyuridine, a Surrogate Marker of Thymidylate Synthase (TS) Inhibition, after Administration of Two Different Schedules of 5-Fluorouracil and the Specific TS Inhibitors Raltitrexed (Tomudex) and ZD9331


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ABSTRACT

5-Fluorouracil (5-FU) exerts cytotoxic effects through inhibition of thymidylate synthase (TS) and incorporation of metabolites into RNA. TS inhibition may be greater for infusional 5-FU, with bolus regimens more likely to cause RNA effects. Elevation of plasma 2'-deoxyuridine (dUrd) is a surrogate marker of TS inhibition. Nineteen patients were treated with continuous infusion (CI) 5-FU 300mg/m²/day or bolus 5-FU 425mg/m²/day plus leucovorin (LV) 20mg/m²/day days 1–5. Pretreatment (day 1) and day 2, 3, 4, 5, 8, 15, 22, and 29 plasma samples were assayed for dUrd by reverse-phase high-performance liquid chromatography. In patients treated with bolus 5-FU/LV, dUrd elevation at 24 and 48 h was 235 ± 125 and 254 ± 119%, respectively, falling to 138 ± 58%, 156 ± 89%, and 92 ± 25% on days 8, 15, and 22, respectively. dUrd elevation with CI 5-FU was 229 ± 86% at 24 h and 239 ± 86, 240 ± 98%, and 255 ± 109% at days 15, 22, and 29, respectively. Duration of dUrd elevation was generally less than 8 days for bolus 5-FU/LV. A single dose of raltitrexed (3 mg/m²) gave a similar profile to this regimen. ZD9331 (130 mg/m², days 1 and 8) gave dUrd elevation for 14 of 21 days, with some recovery prior to day 8. Thus, both 5-FU regimens inhibit TS, and prolonged TS inhibition is achieved by CI 5-FU without significant toxicity. This suggests that the mechanism of antiproliferative toxicity from bolus 5-FU/LV is partly non-TS mediated. These results clarify underlying pharmacodynamic processes and could guide scheduling of 5-FU and TS inhibitors.

INTRODUCTION

The fluorinated pyrimidine 5-FU3 has been used for the treatment of malignant disease for over 40 years and is still the mainstay of chemotherapy for colorectal cancer. In vitro studies show that 5-FU appears to have several effects on the dividing cell: its active metabolite FdUMP is a potent inhibitor of the enzyme TS, which is a critical enzyme in the de novo synthesis of thymidine-5'-monophosphate (thymidylate), a nucleotide vital for DNA synthesis. In addition, another active metabolite, 5-fluoro-UTP is incorporated into RNA, which may also lead to cytotoxicity. Clinical studies have shown that response rates to 5-FU may be improved by biochemical modulation with LV or by the use of prolonged infusions rather than by bolus administration (1, 2). In vitro studies suggest that TS inhibition may be more significant when cells are treated with a prolonged exposure to 5-FU, whereas the RNA effects may predominate when shorter exposures are used (3). Addition of LV to bolus 5-FU may increase the degree to which TS is inhibited by increasing levels of the cofactor 5,10-methylene-tetrahydrofolate, which augments the TS inhibitory activity of FdUMP (4, 5). In addition, a recent meta-analysis has suggested that schedules involving infusional 5-FU may have higher response rates than bolus regimens with a lower incidence of serious toxicity, suggesting a clinically relevant difference in mechanism of action according to the schedule used (6).

Treatment with drugs that deplete thymidylate (including methotrexate and TS inhibitors) causes a rise in intracellular pools of the TS substrate dUMP. This rise is partly attributable to the effect of TS inhibition in directly increasing levels of the substrate, and also to the release of the feedback inhibition exerted by dTTP on other enzymes such as deoxycytidylate deaminase, which catalyzes the conversion of dCMP to dUMP (7, 8). This elevation in dUMP is reflected in raised levels of the corresponding nucleoside dUrd, which is largely extracellular,
and can be measured in the plasma (9). Elevations of plasma dUrd have been shown to follow administration of TS inhibitors, and this is, thus, a surrogate marker of TS inhibition (10–12). Raltitrexed (ZD1694, Tomudex) and ZD9331 are quinazoline-based antifolates that are potent and specific TS inhibitors. They differ principally in their affinity for the enzyme folylpolyglutamate synthetase, which adds glutamate moieties to raltitrexed to form polyglutamated forms of the drug. These polyglutamates are retained intracellularly and have up to a 70-fold increased potency as TS inhibitors (13). ZD9331 is not a substrate for folylpolyglutamate synthetase and, therefore, is not retained within cells, but has similar potency to polyglutamated raltitrexed (14). Large randomized studies of raltitrexed have demonstrated equivalent response rates to 5-FU/LV in colorectal cancer, and reduced antiproliferative toxicity, particularly stomatitis and myelosuppression (15, 16). ZD9331 has recently entered Phase II study having demonstrated activity against a variety of tumor types including colorectal cancer in Phase I trials (17, 18).

The aim of this study was to show that 5-FU did indeed cause TS inhibition in vivo when given by 28-day continuous infusion, and whether this was also the case with bolus 5-FU given on a daily-for-5-days schedule modulated with LV. It was also planned to compare the plasma dUrd profiles measured after administration of these two regimens of 5-FU with those previously obtained from clinical studies of raltitrexed and ZD9331.

PATIENTS AND METHODS

All of the patients treated with 5-FU were treated under the auspices of the Gastrointestinal Unit of the Royal Marsden Hospital. Patients were recruited from the short adjuvant 5-FU and folinic acid study, a comparison between infusional and bolus 5-FU/LV study, a comparison between infusional and bolus 5-FU/LV in colorectal cancer (19). In this study, patients were randomized to receive either 5-FU 300 mg/m²/day by continuous infusion for 12 weeks or six cycles of the standard Mayo Clinic regimen of 5-FU 425 mg/m² and LV 20 mg/m² given as a daily bolus for the first 5 days of 28. Patients consenting to enter this study had blood taken prior to their first treatment on day 1 (first cycle only) and at days 2, 3, 4, 5, 8, 15, 22, and 29 after the start of therapy. Thus, the day 2–5 samples represent 24 h after the first, second, third, and fourth dosing in the bolus regimen. These samples were then analyzed for plasma dUrd.

dUrd elevation profiles had previously been obtained from patients recruited into an ongoing study examining factors involved in response to and toxicity from raltitrexed 3.0 mg/m² every 3 weeks in patients with advanced colorectal cancer, a dose-escalating Phase I and pharmacokinetic study of raltitrexed given every 2 weeks, and a two-center dose-escalating Phase I trial of ZD9331 in patients with advanced solid tumors. Data from patients treated at the dose of ZD9331 eventually selected for Phase II evaluation (130 mg/m² on days 1 and 8 of a 21-day cycle) were included in this study. All of the patients signed written consent to the studies, and all four of the trials were approved by local research ethics committees.

Sample Preparation. Blood was taken from the patients and immediately placed on ice. It was then centrifuged for 5 min at 2500 rpm at 4°C, and the supernatant was immediately frozen. Samples were stored at −70°C until analysis.

Sample Analysis. After protein removal by perchloric acid precipitation, samples were analyzed by reverse-phase high-performance liquid chromatography using a C18 column and 0.05% trifluoroacetic acid v/v in water mobile phase. dUrd was measured by UV detection at 261 nm using a photodiode array detector to confirm the dUrd peak purity and identity. Plasma dUrd was calculated with reference to a five-point standard curve over a concentration range corresponding to 25–400 pmol/ml Values for dUrd elevation were expressed as a percentage of pretreatment dUrd. This method has been described in detail previously elsewhere (20).

Statistical Methods. Values for dUrd elevation at each time point are expressed as mean ± SD. When making statistical comparisons between different regimens, the measured dUrd value rather than the elevations were compared, and the median dUrd concentration at each time point were compared using the Mann-Whitney U test.

RESULTS

Bolus 5-FU. The mean pretreatment plasma dUrd in the bolus 5-FU/LV study (10 patients) was 43 ± 16 nM (median, 39 nM). The mean at 24 h (day 2) was 99 ± 44 nM (median, 94 nM), giving a mean dUrd elevation of 235 ± 125% (range, 146–556%). A similar or slightly higher level was seen throughout the rest of the treatment period (days 3–5; 24 h after the second, third, and fourth dose). Elevations were seen in 100% of these samples (range, 123–641%). Samples were not obtained 24 h after the fifth dose. The mean dUrd was 62 ± 19 nM (median, 63 nM; elevation, 138 ± 58% of pretreatment) on day 8 (72 h after fifth dose), and two of seven samples had returned to (or below) their pretreatment level (range, 82–260%). One patient had a day-8 measured dUrd elevation of 1232%, an order of magnitude greater than the other measurements at this date. This figure was thought to be incorrect and has been omitted from the analysis. Elevations were also low at day 15 (mean dUrd, 68 ± 32 nM; median, 60 nM; elevation, 156 ± 89%; range, 96–365%) and only three of eight samples still had dUrd elevation above 150%. By day 22, dUrd levels had fallen to near pretreatment values or below (mean dUrd, 41 ± 15 nM; median, 40 nM; elevation, 92 ± 25% range, 50–122).

Infusional 5-FU. Samples were obtained from nine patients treated with continuous infusion 5-FU, and the results are shown in Fig. 1. The mean pretreatment concentration was 55 ± 16 nM (median, 53 nM). A total of 61 posttreatment samples were analyzed for dUrd and only 3 of these (each from a different patient on different days) were below their individual pretreatment concentration. The mean dUrd was 122 ± 55 nM (median, 104 nM; elevation, 228 ± 86%; range, 133–357) at 24 h and remained at approximately this level for the entire 29-day period of study (Fig. 1).

Raltitrexed. The mean pretreatment concentration of dUrd in these 11 patients was 50 ± 25 nM (median, 42 nM). Mean dUrd concentration 24 h after the single treatment (day 2) was 144 ± 57 nM (median, 126 nM), giving a mean dUrd elevation of 349 ± 128% (range, 192–567; n = 10). Samples were not taken from all of the patients on days 3–5 (n = 5). The
level of dUrd elevation remained high on days 3 and 4 (373 ± 113\% and 310 ± 115\%, respectively) and fell to 215 ± 60\% on day 5 (range, 161–284). Samples were available from all 11 patients on day 6, and the mean dUrd concentration was 88 ± 40 nM (median, 81 nM), giving a mean elevation in dUrd of 186 ± 38\% (range, 110–225). The next sampling day was day 15, and about one-half of the patients still had plasma dUrd above pretreatment levels (mean dUrd, 60 ± 28 nM; median, 56 nM; mean dUrd elevation, 134 ± 87\%; range, 58–390). However, by day 22, the mean dUrd concentration had fallen to approximately pretreatment levels (57 ± 32 nM; median, 46 nM; mean dUrd elevation, 115 ± 33\%; Fig. 1).

**ZD9331.** The mean pretreatment plasma dUrd in the 19 patients included in the analysis was 65 ± 22 nM (median, 64 nM). At 24 h (day 2), the mean dUrd after ZD9331 administration was 203.6 ± 98 nM (median, 160 nM; elevation, 342 ± 170\%; range, 114–728\%). One patient had a very low pretreatment value (10 nM) that may have artificially given the very high value of 1600\% at 24 h and, therefore, was excluded from all of the analyses. Although levels slowly declined over the next few days, a high mean dUrd elevation was still seen on day 5 (mean dUrd, 120 ± 73 nM; median, 100 nM; elevation, 212 ± 167\%). The range in values for elevation was wide (78–789\%) but 8 of 17 still had values above 150\% of pretreatment values. By day

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**Fig. 1** Median dUrd concentration and mean percentage (±SD) dUrd elevation versus time for bolus 5-FU/LV (A); continuous infusion 5-FU (B); raltitrexed (C), and ZD9331 (D).
8, the mean plasma dUrd had fallen farther (mean, dUrd 93 ± 45 nM; median, 95 nM; elevation, 143 ± 61%). Nevertheless, 5 of 14 patients still had a level >150% of pretreatment (range, 63–280%). The second dose of ZD9331 was given on day 8 (data available for 10 patients). This led to a further rise in dUrd on day 9 (303 ± 146%; range, 180–632) that again slowly declined over the next few days (Fig. 1) to a level of 225 ± 41% on day 12 (n = 8; range, 175–297%). Data are available only for four and three patients on days 15 and 22, respectively, giving mean values of 194 ± 63 and 103 ± 53%. Although these small numbers make the data difficult to interpret, it appears that dUrd is still elevated on day 15, at least in these four patients (range, 133–258%).

Comparisons between Treatments: Statistical Analyses. The mean pretreatment dUrd concentration was similar for all of the treatment groups. Elevations in plasma dUrd were observed at 24 h (day 2) in all of the patients irrespective of treatment. However, the median values were higher in the raltitrexed (126 nm) and ZD9331 patients (160 nm) compared with the bolus 5-FU patients (94 nm; P = 0.11 and 0.003, respectively). These medians were also higher than for infusional 5-FU (104 nm; P = 0.50 versus raltitrexed and P = 0.02 versus ZD9331). No significant difference was seen between the two 5-FU treatments (P = 0.39).

The day-3 (48 h) plasma dUrd was higher in the raltitrexed and ZD9331 patients compared with either of the 5-FU groups of patients. Plasma dUrd remained high during the bolus × 5 day 5-FU treatment (days 1–5). Although a day-6 value (24 h after the fifth dose) is not available, it would be expected to be at a similar level. Infusional 5-FU gave dUrd elevations similar to bolus 5-FU during this period. In contrast, after the single dosing of raltitrexed and the first dose of ZD9331, levels declined (from days 2 or 3). However, because the peak dUrd was generally higher in the raltitrexed and ZD9331 patients, the median day-5 levels were similar for all of the treatment groups: raltitrexed, 105 nm; ZD9331, 100 nm; bolus 5-FU, 90 nm; and infusional 5-FU, 113 nm.

Three days after the last dose of bolus 5-FU (day 8), the median plasma dUrd had fallen to 63 nm (138% of pretreatment, similar to that for ZD9331 at 143%). Day-8 dUrd was higher in patients on infused 5-FU (92 nm) than with bolus 5-FU (P = 0.008). The measured value of dUrd was actually higher for ZD9331 at this time point (95 nm), although the elevation was greater for infused 5-FU (200% versus 143%). Data for raltitrexed at this time are not available, but extrapolation (Fig. 2) suggests that the mean value is in the region of 150% and, therefore, is also less than for infused 5-FU.

The plasma dUrd levels were still elevated above pretreatment on day 15 in some patients treated with either bolus 5-FU or raltitrexed; median for day-15 dUrd was 60 nm for bolus 5-FU and 56 nm for raltitrexed, giving median elevations of 119% and 123%, respectively. However, these values were significantly lower than those for infused 5-FU (113 nm, 246%; P = 0.015 compared with bolus 5-FU and P = 0.003 compared with raltitrexed). Interestingly, because the ZD9331 schedule includes a second dose on day 8, the day-15 dUrd elevation was similar to that of infusional 5-FU (193%). However, by day 22, only the infused 5-FU patients had elevated dUrd.

Relationship between dUrd and Plasma Raltitrexed Levels. To assess the relationship of dUrd to drug levels, dUrd elevations were compared for the group of patients treated with raltitrexed (3 mg/m²), from whom blood was drawn from day 1 to day 22, as detailed above, and eight patients treated on a dose-escalating Phase I study of raltitrexed given on a 14-day schedule. Samples analyzed on this latter group of patients (treated at doses from 1.0 to 2.5 mg/m²) were taken at early time points, from 30 min to 8 h after treatment. These two sets of dUrd elevations were then compared with plasma drug levels in the same samples measured by RIA (21). The data from these two studies are shown in Fig. 2. It is striking that at the later time points, at which median raltitrexed concentration was 4.1 nm (range, 0.42–18.6), there appeared to be an association between drug levels and dUrd elevation. At early time points, however, at which raltitrexed levels were approximately 20-fold higher (median, 113 nm; range, 9.5–310 nm), although a significant rise in dUrd was seen, there was no such correlation with drug level. These data suggest that, whereas at lower raltitrexed concentrations, dUrd elevation is related to drug levels, above a given concentration of drug, a plateau is reached, and no additional dUrd elevation occurs.

DISCUSSION

Although the small sample size means that any statistical analysis of these data should be treated with caution, and in particular, the comparison between the patients with advanced disease treated with specific TS inhibitors and those treated with 5-FU in the adjuvant setting, nevertheless there were some clear patterns. The theory that there are different mechanisms of action for bolus and infusional 5-FU regimens is founded on in vitro studies demonstrating this to be the case in at least one cell line (3), and the clinical observation that the dose-limiting toxicities and maximum tolerated dose of 5-FU are highly schedule dependent (22). Clinical efficacy has also been shown to be schedule dependent, with poor response rates seen in patients treated with short (10–20 min) infusions of 5-FU, when compared with true bolus (2–4 min) administration (23). In addition, studies of a murine model have shown that more prolonged TS inhibition appears to result in improved antitumor effect without increased toxicity (24). Thus the scheduling of 5-FU may affect specificity for the TS target and duration of TS inhibition, which may in turn impact on response and toxicity. To provide clinical evidence for this hypothesis we have measured plasma dUrd, a surrogate marker of TS inhibition, after treatment with bolus (daily for 5 days, modulated with LV) and continuous infusion 5-FU. Although the latter was given continuously for 3–6 months, monitoring of dUrd was for the first month only.

Patients receiving infusional 5-FU appear to demonstrate some degree of TS inhibition throughout their treatment. Certainly there was no evidence of any lessening of the degree of dUrd elevation over the 4-week period studied. In contrast, bolus 5-FU/LV gave a similar increase in dUrd during the 5-day treatment period. However, this returned to pretreatment levels soon afterward, and the duration of dUrd elevation was generally less than 8 days. In addition, the peak values for dUrd concentration achieved after bolus 5-FU in this study were, if
anything, lower than those seen with the infused 5-FU regimen, although the elevations were comparable. This study confirms that the preclinical observation that TS is inhibited by both infusional and bolus regimens of 5-FU also holds true in clinical practice. The observation that TS may be inhibited for at least 4 weeks by an infusional regimen with less toxicity than is seen with bolus therapy is more evidence of an alternative mechanism for toxicity from bolus 5-FU. Indeed, the previously reported pattern of increased toxicity with the bolus as compared with infusional regimens was also seen in this study (19). This, coupled with the improved response rates seen with such regimens, and supported by the in vitro data from the murine model, implies that improved therapeutic indices might result from regimens causing prolonged inhibition of TS.

The single dosing of raltitrexed induced a duration of effect that was similar to that of bolus 5-FU in most patients. This supports the preclinical evidence for drug retention through polyglutamation (25, 26). Day 2 plasma dUrd levels were higher in the raltitrexed patients compared with either of the 5-FU regimens, and this may suggest more rapid and/or sustained TS inhibition after dosing. Day-3 levels were also higher in the raltitrexed patients, but statistical significance was not attained. The relationship between raltitrexed levels and dUrd elevation was further investigated. Although comparison of the two groups studied is not possible because of the fact that the doses and time points differed in the two studies, the data could be interpreted as showing that there is a plasma drug level above which no further elevation in dUrd is seen. It may be that it is not possible to inhibit TS above a given level or that the degree of TS inhibition and plasma dUrd are not linearly related. A further complication is that the percentage elevation in dUrd is related to the pretreatment levels, with higher increases being seen in patients with low pretreatment levels (data not shown). This is currently being investigated further. Ideally, additional preclinical studies need to explore the correlation between dUrd elevation, TS inhibition, and depletion of thymidine nucleotide pools. Thus, until this is more fully understood, dUrd measurements should be used to assess duration of effect rather than degree of inhibition. In addition, when evaluating the role of dUrd elevation as a surrogate marker of TS inhibition, it should be taken into consideration that the vast majority of measured dUrd is likely to be released from normal tissues and, therefore, may not bear a close relationship to alterations in tumor nucleotide pools. However, studies in the L5178Y mouse lymphoma cell line suggest that treatment with a TS inhibitor does lead to decreases in cellular thymidine nucleotides and a corresponding moderate increase in intracellular dUMP (27). In addition, in vivo studies using a murine model demonstrate a good correlation between decreases in tumor dTTP and increases in both tumor dUMP and plasma dUrd.

Treatment with the non-polyglutamatable TS inhibitor, ZD9331, gave a pattern of dUrd elevation after the first dose very similar to that of raltitrexed. However, in contrast to raltitrexed, this prolonged inhibition of TS is caused by sustained drug exposure as a result of slow plasma clearance (28). The ZD9331 schedule incorporates a second day-8 dose and, therefore, immediately after recovery or near recovery of TS (as judged by plasma dUrd elevations), a second period of TS inhibition is induced. Recovery varied between patients but appeared to be generally between days 15 and 21. With the next dose of ZD9331, given on day 22, it is clear that TS inhibition is more sustained using this schedule than with either bolus 5-FU or raltitrexed. The near continuous effect on TS with ZD9331 is obtained without the need for continuous infusion.

Fig. 2 dUrd concentration (nM) and elevation (%) versus raltitrexed concentration at time points (A) 0–24 h and (B) 24 h–21 days.

4 G. W. Aherne and A. L. Jackman, unpublished observations.
The data described in this study have potential to help clinicians both to select optimum 5-FU schedules and to guide scheduling of future TS inhibitors in early clinical study. A critical question to be addressed is whether or not prolonged inhibition of TS is reflected in improved tumor response rates. Clinical studies (16) have not shown significant differences in response rates between raltitrexed and 5-FU/LV regimens, but, as our data show, the duration of TS inhibition achieved with raltitrexed is likely to be similar to that of 5-FU/LV. A meta-analysis of trials in colorectal cancer has suggested that both response rates and survival are improved in patients treated with infusional 5-FU schedules when compared with bolus regimens, which suggests a therapeutic benefit from more prolonged TS inhibition (6). The recommended dose for further evaluation from the Phase I trial of raltitrexed on a 14-day schedule, as described above, is 2.0 mg/m² every 2 weeks (29). It will be interesting to compare results from patients treated with such a regimen with the standard 3-weekly schedule, because the dose intensities are identical, but TS inhibition should be maintained for at least 2 of every 3 weeks on treatment with the 14-day schedule. Currently ongoing Phase II trials with ZD9331 on the day-1 and -8 schedule will also provide further information on the effects of more prolonged treatment with a specific TS inhibitor.

ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of Eileen Murphy, Catharine Noyce, Helen Farrah, Simon Joel, Joanne Kellaway, and the Royal Marsden IV team to this study.

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