Leucovorin Rescue from Raltitrexed (Tomudex)-induced Antiproliferative Effects: In Vitro Cell Line and in Vivo Mouse Studies

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ABSTRACT

Raltitrexed (RTX) is an antifolate thymidylate synthase (TS) inhibitor that is effective for the treatment of advanced colorectal cancer and other solid tumors. However, a small minority of patients receiving RTX monotherapy will experience grade III/IV gastrointestinal toxicity that can be life-threatening, particularly if copresenting with neutropenia. Lack of vigilance in recognition and treatment of symptoms of toxicity or violations of protocol have led to treatment-related deaths in some hospitals. The safety of RTX could be improved if an effective rescue agent was available. Leucovorin (LV) is a reduced folate cofactor that competes with RTX for transport and polyglutamation in both tumor and normal tissues and thus has potential as a rescue agent. In vitro cell studies are presented suggesting that the growth-inhibitory, and potentially cytotoxic, effects of RTX on populations of viable cells can be reversed by the delayed administration of LV. The mechanisms involved are inhibition of further drug uptake and polyglutamation and a redistribution and/or reduction in the concentration of preformed raltitrexed polyglutamates. A more clinically relevant in vivo mouse model was used to test the hypothesis further. BALB/c mice treated with 100 mg/kg/day × 4 days of RTX were used as a model for gastrointestinal and bone marrow toxicity. LV (200 mg/kg), which was given after the onset of severe weight loss and diarrhea (twice daily, days 5–7), prevented further weight loss and induced earlier recovery. This was accompanied by improvement in the histological appearance of the intestine (day 7) and the concentration of neutrophils and platelets in the blood (day 9). BALB/c mice could not tolerate 100 mg/kg daily × 5 days unless LV (200 mg/kg twice daily) was given on days 6–8. Measurement of RTX (polyglutamates) by RIA after 100 mg/kg RTX daily (days 1–4) showed less drug in plasma (3–4-fold), liver (8–11-fold), kidney (3–4-fold), and small intestinal epithelium (3–4-fold) on day 7 in LV-treated mice (100 or 200 mg/kg twice daily) compared with controls. A single injection of 100 mg/kg RTX (day 1) gave plasma levels of 3–4 pmol/ml on day 4 that are more clinically relevant. Administration of LV (100 or 200 mg/kg; twice daily on days 4–6) reduced the RTX concentration in the liver 2–4-fold on days 7, 9, and 11 compared with controls. A model is proposed where LV and/or its anabolic products can compete with RTX uptake into tissues and interfere with the homeostatic regulation of RTX polyglutamates. These data support the use of LV rescue in the small minority of patients treated with RTX who present with a severe pattern of antiproliferative toxicities. The use of LV is not recommended routinely because the antitumor activity of RTX may similarly be reversed.

INTRODUCTION

RTX5 (Tomudex; ZD1694) is a specific folate-based inhibitor of TS that is available in several countries for the first-line treatment of advanced colorectal cancer (1–5). Administration is by a 15-min infusion of 3 mg/m2 every 3 weeks. Response rates are generally considered to be equal to those with bolus 5-FU modulated with LV or infusional 5-FU (3–6). The incidence of grade III/IV neutropenia (6–18%) and mucositis (2–3%) is significantly lower than that seen with bolus 5-FU/LV, whereas that of diarrhea is similar (~10–14%; Refs. 4 and 5). The latter, however, may be life threatening if copresenting with neutropenia. In one Center, this has been reported as ~3% (4 of 127 patients, no deaths; Ref. 7). Although this toxicity is generally manageable with immediate and appropriate supportive care such as hydration and antibiotics, deaths have been reported in some studies. For example, a recent United Kingdom Medical Research Council study reported a 4% toxic death rate (6). Similarly, reports have highlighted the relationship between renal impairment and increased RTX plasma levels (8) and the danger of failing to reduce the dose in cases of renal impairment (9). Increasing experience with RTX and further clinician and patient education regarding the use of

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5 The abbreviations used are: RTX, raltitrexed; TS, thymidylate synthase; 5-FU, 5-fluorouracil; LV, leucovorin, 5-formyl tetrahydrofolate; MTX, methotrexate; DHFR, dihydrofolate reductase; FPGH, folylpolyglutamate hydrolase; dThd, thymidine.
this drug is likely to lower this incidence. Nevertheless, it is appropriate to examine the potential for rescue agents that may be given on presentation of these symptoms that may improve the management of these toxicities.

In considering potential rescue agents, it is necessary to understand the cellular biochemistry of RTX and other appropriate antifolates where rescue agents have been successfully used. An example of this is the administration of LV after high-dose MTX, usually at 24 h, to prevent otherwise lethal effects of the drug (10, 11). These protocols have been developed to promote penetration of MTX into pharmacological sanctuary sites and drug-resistant tumors and to selectively rescue normal proliferating tissues from the toxic effects of the drug. LV is commonly used later if patients on standard dose therapy have an unexpected or protracted toxic response. A number of mechanisms, probably acting in concert, have been suggested to explain LV rescue of MTX cytotoxicity at the cellular level (12). These include: (a) circumvention of DHFR inhibition by replenishment of the folate pool; (b) displacement, by FH₄ polyglutamates, of MTX from DHFR; and (c) in the continued presence of extracellular MTX, prevention of further drug uptake and polyglutamation.

Previous studies demonstrated that coinubcation of mouse L1210 leukemia cells with RTX and LV reduces the growth-inhibitory activity of the drug primarily by competing for drug uptake and polyglutamation (1, 13). Polyglutamation of RTX has greater pharmacological consequences than that of MTX. RTX polyglutamates of high chain length (tri-hexa)glu form the majority of the intracellular drug pool and are approximately two orders of magnitude more potent inhibitors of TS than the parent drug (Ki RTX, ~60 nM; tetraglu, ~1 nM). Thus, cells expressing low levels of, or mutant FPGS are resistant to RTX (reviewed in Ref. 14). A property of RTX polyglutamates shared with MTX polyglutamates is a reduced rate of efflux giving rise to drug retention and cytotoxicity under short exposure conditions. Indeed, polyglutamation of RTX is so rapid that if LV is added to cultured L1210 cells just 4 h after the drug, it is considerably less effective in reducing the cytotoxicity (13).

Studies in mice have also shown rapid RTX polyglutamation in normal tissues and tumors (2, 15, 16), which is responsible for: (a) slow clearance of the drug from the tissues relative to plasma; and (b) activity by intermittent bolus dosing. Co-administration of LV (20 mg/kg twice daily) prevented both antitumour activity and toxicity (1). Studies have not investigated the effect of delaying LV administration by, for example, 4 or 24 h, or reducing the LV dose and examining any potential selective rescue effect that this may have for normal tissues. Moreover, the studies described below also do not address this issue but rather the role that LV may have in rescuing patients presenting with severe gastrointestinal toxicity/myelosuppression, which may be 8–15 days after RTX administration. This will be at a time when a proportion of the proliferating cell fraction will have either died or been committed to cell death pathways, but in contrast with cells in culture, a proportion should also have escaped damage by, for example, not being in an active phase of the cell cycle (G₀) during the time of maximal drug exposure. Such cells may still have accumulated low cytostatic or growth-inhibitory concentrations of RTX polyglutamates and may be sensitive to LV administration if it could reduce the concentration and stimulate cell division and tissue regeneration.

RTX polyglutamates are slowly lost from tissues (2, 16), believed to be partly through the action of FPGH. This enzyme hydrolyses high chain length folate or antifolate forms to shorter polyglutamates or parent drug that can be effluxed (17, 18). Recently, M. Rhee and J. Galivan have confirmed that raltitrexed tetraglutamate is a substrate for FPGH.⁶ It may be speculated that the hydrolysis products become substrates for repolyglutatation, thereby reducing the rate of drug loss. Thus, a dynamic situation may exist in tissues offering potential points of intervention for LV. For example, its metabolic products (reduced folate cofactors and polyglutamates) could compete or regulate the formation or hydrolysis of RTX polyglutamates already formed inside cells.

A further potential site for interaction of delayed LV is competition with RTX for drug influx via the RFC. RTX undergoes a third phase of elimination in mice and humans that is very slow (mice, ~3 h; humans, 257 ± 63 h; Refs. 8 and 16). During this time viable cells, or quiescent cells stimulated to re-enter the cell cycle, may be exposed for some days to cyto-static/growth-inhibitory concentrations that may inhibit normal tissue function. The concentration in human plasma 5 and 15 days after a standard dose of 3 mg/m² RTX is ~2–10 nM and 2–4 nM, respectively (19),⁷ a growth-inhibitory concentration for many cell lines in culture, and could therefore inhibit drug uptake and promote recovery.

To investigate whether delayed administration of LV might be a clinical option for patients presenting with a severe pattern of toxicity, in vitro and in vivo models have been used to study the effects of LV on RTX polyglutamation and cytotoxicity. Additionally, the effects of LV have been investigated on the drug-induced histopathological changes to BALB/c mouse small intestinal epithelium and the rate of recovery of body weight in mice. This mouse strain has been shown previously to be highly sensitive to the gastrointestinal effects of RTX (20) and provides a useful model for studying the potential of rescue agents. Data are presented herein that supports the clinical use of LV as part of more generalized supportive care, immediately after the relatively rare severe pattern of life-threatening toxicity is recognized.

MATERIALS AND METHODS

In Vitro Growth Inhibition. Full details relating to the growth of mouse L1210 leukemia and human W1L2 lymphoblastoid suspension cells have been published (21). Culture medium used was RPMI 1640 supplemented with 10% FCS. For experiments, duplicate cultures of 9.9 ml of cells in the logarithmic phase of growth (5 × 10⁶/ml) were treated with 100 nM RTX (0.1 ml of 10 μM solution in unsupplemented RPMI) continuously for 48 and 72 h for L1210 and W1L2 cells, respectively. At various times after the addition of RTX, 0.05 ml of 5 mM LV (10 mg/ml solution purchased from Lederle Laboratories Gosport, Hants, United Kingdom) was added. Total

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⁶ M. Rhee and J. Galivan, personal communication.

⁷ Unpublished data.
and viable cells were counted after trypan blue staining, as described previously (22).

Measurement of RTX Polyglutamates in Human W1L2 Cells. Duplicate 10-ml cultures of WIL2 cells in the logarithmic growth phase (~3–5 × 10⁵/ml) were treated with 5-[³H]RTX (specific activity, 8.9–10.8 Ci/mmol) to give a final concentration of 100 nM. Full details have been published for RTX purification, treatment of W1L2 cells, extraction, and estimation of [³H] polyglutamate forms by an ion-pairing HPLC method (23).

LV (10 μM) was included in some experiments. This was added 4 h after RTX, and the cells were incubated for an additional 20 h. In one experiment, the RTX-treated cells were centrifuged, washed two times with fully supplemented RPMI 1640 at 37°C, resuspended in fresh medium containing 10 μM LV, and incubated for an additional 20 h. The cellular concentration is given in μM and was calculated taking into account the volume of the cells (1 × 10⁶ cells, 0.89 μl; Ref. 23).

RTX Administration to Mice. Male BALB/c mice, 7–9 weeks of age, were used as a sensitive model for RTX-induced gastrointestinal toxicity. Details of this experimental model have been published recently (20). RTX requires injection of relatively high doses over 4–5 days to induce a significant level of toxicity to the proliferating tissues (weight loss, diarrhea, and myelosuppression). This is attributable to the high levels of plasma thymidine and plasma folates in mice that partially mask the TS inhibitory effects (24, 25).

RTX (provided by AstraZeneca plc as a powder) was dissolved in 0.05 M NaHCO₃ at a concentration of 1 or 10 mg/ml and adjusted to pH ~9.0 with 1 M NaOH and injected i.p. into mice at a volume of 0.1 ml/10 g of body weight (10 and 100 mg/kg) at ~9.00 on days 1–4 or 1–5. Control mice received injections of 0.05 M NaOH NaHCO₃ or saline. LV (powder purchased from Lederle Labs Gosport) was dissolved in sterile water at 10 or 20 mg/ml. Mice received injections i.p. (0.1 ml/10 g of body weight) with LV (9.00 and 17.00 h), starting 24 h after the 4th or 5th injection of RTX (days 5 or 6) and continuing until day 7. To eliminate potential effects of administered fluid volume on recovery, saline was given in place of LV in some groups of RTX-treated mice. Daily weights of individual mice were recorded, and mice were inspected twice daily for signs of distress. Mice were sacrificed if they could not freely access food and water and/or had >30% body weight loss.

Histopathology of the Small and Large Intestine. Mice (six per group) received injections of 100 mg/kg RTX daily × 4 days (days 1–4) and saline, or LV was administered at a dose of 100 mg/kg (days 5–7) as described above. Mice were killed by cervical dislocation 24 h after the final RTX dose, which is also the starving time for the rescue (day 5). Mice from each “rescue” group were sacrificed 48 and 72 h after the start of rescue (days 7 and 8, respectively), and the whole length of the intestine was removed. Tissue was fixed, embedded in paraffin, sectioned, and stained with H&E as described previously (20).

Damage to the small intestine was graded in a blinded fashion as follows: 0, normal; +, minimal (minimal blunting and fusion of villi and vacuolated epithelium at the villi tips); ++, mild (some blunting and fusion of villi and decreased mitotic activity); +++, moderate (blunting and fusion of villi, decreased mitotic activity, and increased inflammatory cell infiltration of the lamina propria of the mucosa). Damage to the large intestine was graded as: 0, normal; +, minimal (occasional necrotic mucosal epithelial cells); and +++, mild (some necrosis of mucosal epithelium and increased inflammatory cells in mucosa). Higher grade damage was not seen to this organ.

Hematology. Mice (six per group) received injections of 100 mg/kg RTX and 200 mg/kg LV in the protocol described above. Control mice received saline. Blood was taken for full blood count and differential white cell count as described recently (20). In one experiment, 200 mg/kg LV were given instead of saline to control mice. There was no significant difference in the concentration of peripheral blood elements between these two control groups.

Measurement of Plasma and Tissue RTX (Polyglutamate) Levels by RIA. Mice (five or six per group) were injected with RTX and either saline or LV in the same schedule as described above. Blood was collected by open cardiac puncture under oxygen/halothane anesthesia (without recovery) into a 1-ml syringe and transferred to a 1.5-ml microcentrifuge tube containing 10 μl of heparin (50 units/ml; Leo Laboratories, Buckinghamshire, United Kingdom). This was immediately centrifuged at 13,000 rpm in a microfuge for 2 min to separate the plasma. Plasma was immediately frozen and stored at −70°C. Liver, kidneys, and intestine were removed immediately after exsanguination. The epithelium was scraped from the small intestine as described previously (15). All tissues were stored at −70°C until analysis.

The polyclonal RTX antibody (provided by AstraZeneca) was used to measure the concentration of plasma RTX or tissue RTX polyglutamates concentrations by RIA (16). This antibody does not cross-react with LV or natural folates.

RESULTS

Rescue of RTX-induced Growth Inhibition in Vitro. In a continuous exposure growth inhibition assay, the IC₅₀ for RTX in L1210 (48 h assay) was ~10 nM. This growth-inhibitory effect was prevented if 25 μM LV was added to L1210 cells up to 18 h after the addition of the drug (data not shown). However, growth inhibition induced by a 10-fold higher concentration of RTX could only be prevented if LV was added simultaneously. Further studies have characterized the effect of delaying the LV addition on cell growth using trypan blue exclusion as a viability measurement at 48 h. Incubation with RTX (100 nm) alone reduced both the total number of cells and the number of viable cells compared with controls by ~90 and ~97%, respectively (Fig. 1A). Furthermore, the number of viable cells had fallen to <50% of the initial cell count (t = 0), consistent with a cytotoxic response. Studies not reported here have shown that when cell cycle distribution is measured by flow cytometry, an apoptotic sub-G₁ population is present at this time. When 25 μM LV was added simultaneously with RTX there was no drug-induced growth inhibition, but when it was delayed for 4 or 8 h, the total cell number was ~50 and 20% of control, respectively. Nevertheless, the cell population had expanded during the 48 h and remained largely viable. Delaying the LV for 18 or 24 h markedly reduced both the growth rate of the culture (~10% of control by 48 h) and the number of these cells remaining viable at 48 h (~20–30% of population). In contrast, if dThd was
added at 4 or 8 h, the cell numbers were 100 and 50% of control, respectively, at 48 h, and all were scored as viable (data not shown).

Similar experiments were performed in the slower growing human W1L2 lymphoblastoid cell line, but cell counting and viability studies were performed after 72 h [however, the total number of control cell doublings (~4) was the same as for the L1210 cells]. Seventy-two h of incubation with 100 nM RTX led to almost complete growth inhibition and loss of cell viability (Fig. 1B). Furthermore, this effect was characterized by the presence of apoptotic cells and the loss of clonogenicity (data not shown). Coincubation with LV gave a pattern of results similar to that seen for L1210 cells with no reduction in culture growth rate or viability at 72 h. If LV was added after 4 or 8 h, a reduction in the viable cell population was observed (~70 and 25% of control, respectively, at 72 h). Although the addition of LV at 18 h reduced the cell number to <20% of control, there was nevertheless an expansion in the population of viable cells over the 72-h period (approximately one population doubling), and the number of viable cells was three times greater than with RTX alone. Rescue effects were not seen when LV was added after 24 h and particularly after 48 h. In contrast, dThd could be added 18–24 h after the addition of 100 nM RTX without affecting viability (~90%), although the number of cells was ~50% of controls, consistent with growth inhibition before the addition of dThd, i.e., one cell doubling (data not shown).

**Polyglutamation of RTX in W1L2 Cells.** The rate of drug uptake and polyglutamation during continuous exposure to 100 nM RTX is shown in Fig. 2. RTX polyglutamates accumulated inside cells rapidly so that a 40-fold higher intracellular concentration was measured at 4 h compared with the extracellular environment (~4 μM). Mono-diglutamates accounted for just 2% of the total drug pool, and the predominant drug forms were tetraglutamates and pentaglutamates. Accumulation continued more slowly after this time, giving levels of RTX polyglutamates of ~6.5 and 11 μM at 18 and 48 h, respectively. The
rate of conversion to polyglutamates appeared to slow with time as the mono and diglutamate fractions increased to 9 and 15% of the total drug pool, respectively. The ratio of the cellular RTX polyglutamates (>diglu) to the extracellular parent RTX pool at 48 h was 110. These data represent uptake in a dividing cell population because dThd was present during the incubation to prevent cell death. Data not shown demonstrated an intracellular:extracellular RTX ratio of 240 when the extracellular concentration was 10 nm (99% >diglu). dThd was not included in these experiments because growth was only inhibited by exclusion of trypan blue.

Effect of LV on RTX-induced Toxicity and Leucovorin Rescue

RTX (100 mg/kg daily days 1–4) induced “mild” (+ +) to “moderate” (+ ++ +) changes to the small intestinal epithelium on days 5 and 7, which included some blunting and fusion of villi, a marked decrease in mitotic activity, and an inflammatory cell infiltrate (data not shown). Saline or LV rescue (200 mg/kg twice daily) was given days 6–8) responded well and gained weight from day 8 or 9 (74% of initial weight). LV-treated mice had higher body weights than saline-treated mice on days 8–12 that were statistically significant. However, excessive weight loss (>30%) was seen in some mice in both groups; therefore, these mice were culled: 6 of 20 saline-rescued mice and 3 of 21 LV-rescued mice.

The same dose of RTX was given in a daily × 5 days schedule, but this was not well tolerated, and mice had to be culled on day 10 (Fig. 4B). However, six of seven mice given LV (200 mg/kg twice daily, days 6–8) responded well and gained weight from day 8 or 9 (74% of initial weight). This clearly demonstrates that LV can rescue from potentially lethal doses of RTX in mice.

Effect of LV on RTX-induced Body Weight Loss in BALB/c Mice

Data not shown demonstrated that mice treated with RTX (100 mg/kg daily × 4), followed by saline 0 and 8 h on days 5–7 inclusive, lost the same amount of body weight as those not receiving saline. Furthermore, the rate of weight recovery was the same. Results from three separate experiments (20 mice in total) are combined in Fig. 4A. Mice given 100 mg/kg/day of RTX for 4 days (days 1–4) and saline “rescue” on days 5–7 lost body weight from day 2 with a day 8–9 nadir (~75% of starting weight). Weight gain was rapid after this time, with the mice reaching 95% of their starting weight by day 14 (Fig. 4A). Mice that had received 100 mg/kg of LV twice a day on days 5–7 lost the same amount of weight as the saline “rescued” mice up to day 7 (~78% of starting weight). In contrast, however, further weight loss did not occur in the LV-treated mice, and significant weight gain was seen on day 9 (~83% of starting weight) with recovery to at least 95% of starting weight by day 12. LV-treated mice had higher body weights than saline-treated mice on days 8–12 that were statistically significant. However, excessive weight loss (>30%) was seen in some mice in both groups; therefore, these mice were culled: 6 of 20 saline-rescued mice and 3 of 21 LV-rescued mice.

The colon was affected to a lesser extent by RTX, and changes were characterized only as “mild” (day 7) necrosis of the mucosal epithelium and some evidence of the presence of inflammatory cells with normal by day 8. LV administration reduced the effects on day 7 from grade + + to +.
Effect of LV on RTX-induced Myelosuppression in Mice. Peripheral blood elements were measured on days 8 and 9. Data not shown demonstrated insignificant changes on day 5 when mice were given 100 mg/kg RTX × 4 days. However, this dosing schedule induced a fall in the neutrophil and platelet count to 20 and 45% of control, respectively, on day 8 with some recovery to 50 and 64%, respectively, by day 9 (Table 2). LV (200 mg/kg days 1–4) did not significantly increase these values on day 8 but did on day 9 (147 and 75% for neutrophils and platelets, respectively). In a second experiment (day 9 only), the neutrophil and platelet count was again higher in the LV group but only attained statistical significance for the neutrophil count.

The Effect of LV on Plasma and Tissue RTX Levels in Mice. Initial experiments were performed at the 100 mg/kg RTX daily × 4 schedule, with LV being given at either 100 or 200 mg/kg twice daily on days 5–7. In mice receiving no rescue agent (saline), the plasma RTX level on day 5 (24 h after the 4th RTX injection) was 440 pmol/ml (242 ng/ml), which slowly declined to 39 and ~2 pmol/ml (21 and ~1 ng/ml) by days 7 and 15, respectively (Fig. 5A). Liver RTX levels were considerably higher (11 and 3 nmol/g on days 5 and 7, respectively), and a concentration of ~1 nmol/g was detected from days 8 to 15 (Fig. 5B). Thus, the liver:plasma ratio increased from ~25 on day 5 to ~400 on day 15. Mice that received 200 mg/kg LV had statistically significantly lower plasma (2–4-fold) and liver (4–13-fold) drug levels on days 7–15 compared with mice that had received saline (Fig. 5). The day 7 results are included in Table 3, together with those obtained after administration of 100 mg/kg LV. Compared with their respective saline-rescued controls, the reduction in RTX in the plasma and RTX (polyglutamates) liver was similar in both the 100 and 200 mg/kg LV-
rescued groups of mice. Measurements are also shown for small intestinal epithelium and kidney at this time. There were 3–4-fold lower levels in the intestinal epithelium and kidney at both 100 and 200 mg/kg LV, respectively.

Similar experiments were carried out with a lower dose of 10 mg/kg RTX (days 1–4) and 30 mg/kg LV (days 5–7). Day 5 plasma levels before LV injection were ~30 pmol/ml. Plasma, liver, and kidney levels of RTX on day 7 were 22 pmol/ml, 1200 pmol/g, and 290 pmol/g, respectively. These were 3–4-fold lower than seen at the higher 100 mg/kg daily dose. LV significantly reduced (P < 0.05) the levels in these tissues by 4-, 10-, and 3-fold for plasma, liver, and kidney, respectively (data not shown). Intestinal epithelial levels were not measured.

Additional experiments were performed with a single injection of 100 mg/kg RTX (day 1), with LV (100 mg/kg) being administered from days 4 to 7. This single administration is well tolerated by mice because of the high concentration of plasma dThd; therefore, this study is not in the context of that of a toxicity model. At the start of the LV rescue (day 4), the RTX plasma and liver levels were ~4 and 1000 pmol/g, respectively. The plasma, liver, and kidney levels of RTX on day 7 were 4 pmol/ml, 630 pmol/g, and 80 pmol/g, respectively, which is ~20-, 8-, and 15-fold lower, respectively, than that seen in the 100 mg/kg daily schedule. After LV, no differences in the RTX levels were observed in plasma and kidney on day 7 (two experiments) and days 9 and 11 (one experiment; data not shown). However a 2–3-fold lower level was measured in the liver (Table 4). Similarly, a 4-fold lower liver concentration was observed when mice were given 200 mg/kg LV. Small intestinal epithelial levels were not measured because previous experiments had shown that the concentration of RTX is rapidly “diluted” in this tissue when noncytotoxic drug doses/schedules

Fig. 4 Effect of the delayed administration of LV on BALB/c mouse body weight loss induced by repeated administration of 100 mg/kg raltitrexed. A, 100 mg/kg/day RTX × 4 days (1–4) and saline twice daily × 3 days (5–7; ■) or 200 mg/kg LV twice daily × 3 days (5–7; ▲); bars, SE (n = 14–21, three separate experiments). Mice that were culled because of moribundity are indicated (+). B, 100 mg/kg/day RTX × 5 days (1–5) and saline twice daily × 3 days (6–8; ■) or 200 mg/kg LV twice daily × 3 days (6–8; ▲); bars, SE (n = 6 and 7, respectively).
DISCUSSION

RTX is an antifolate drug for the treatment of advanced colorectal cancer, which also has potential in combination with other drugs for colorectal cancer and other various types of cancer (5, 26). Management of occasional life-threatening RTX toxicity, presenting as diarrhea complicated by myelosuppression, is standard supportive care such as rehydration and administration of broad-spectrum antibiotics. Studies in mice suggest that although the etiology of the gastrointestinal toxicity may be complicated, the slow clearance of RTX polyglutamates from tissues may be a contributing factor (20). The terminal phase of clearance of RTX from mouse and particularly human plasma is also slow, giving potentially growth-inhibitory drug levels for prolonged periods (8, 19). Polyglutamation is a property of the drug that contributes to its profile of antitumour activity (1, 13, 14). Nevertheless, it may be hypothesized that some patients may express a pattern of gene expression in their proliferating tissues that under certain circumstances may lead to increased toxicity. For example, low TS or FPGH, or high FPGS expression either individually or combined, may lead to increased sensitivity to the drug. Alternatively, some patients may have increased sensitivity to the cytotoxic effects of the drug for other reasons. Experiments in BALB/c mice suggested that there may

Table 1  Effects of LV and saline on RTX-induced histological damage in Balb/c mice

Mice were treated (six per group) with 100 mg/kg RTX daily × 4. LV (200 mg/kg) or saline was injected on days 5–6 inclusive (for day 7 samples) and days 5–7 (day 8 samples). The number of mice/total number of mice treated is in parentheses.

<table>
<thead>
<tr>
<th>Grade of damage</th>
<th>Day 7</th>
<th>Day 8</th>
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<tr>
<td><strong>Small intestine</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Controls</td>
<td>0 (6/6)</td>
<td>0 (6/6)</td>
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<tr>
<td>RTX + saline</td>
<td>++ (6/6)</td>
<td>0 (2/6)</td>
</tr>
<tr>
<td>RTX + LV (200 mg/kg)</td>
<td>++ (6/6)</td>
<td>0 (3/6)</td>
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<tr>
<td><strong>Large intestine</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Controls</td>
<td>0 (6/6)</td>
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<tr>
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<tr>
<td>RTX + LV (200 mg/kg)</td>
<td>+ (6/6)</td>
<td>0 (6/6)</td>
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<sup>a</sup> Damage to the small intestine was graded as follows: 0, normal; +, minimal (minimal blunting and fusion of villi and vacuolated epithelium at the villi tips); ++, mild (some blunting and fusion of villi and decreased mitotic activity); and ++++, moderate (blunting and fusion of villi, decreased mitotic activity, and increased inflammatory cell infiltration of the lamina propria of the mucosa).

<sup>b</sup> Damage to the large intestine was graded as follows: 0, normal; +, minimal (occasional necrotic mucosal epithelial cells); and +++, mild (some necrosis of mucosal epithelium and increased inflammatory cells in mucosa).

Table 2  The effect of 200 mg/kg LV or saline (twice daily, days 5–7) on peripheral blood elements of Balb/c mice treated with 100 mg/kg RTX daily × 4 days (days 1–4)

<table>
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<tr>
<th>Controls + saline</th>
<th>RTX + saline</th>
<th>RTX + LV</th>
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<tr>
<td><strong>A. Day 8</strong></td>
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<tr>
<td>Total WBC&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>3.5 ± 1.2</td>
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<tr>
<td>Neutrophils</td>
<td>1.2 ± 0.45</td>
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<td>Lymphocytes</td>
<td>6.9 ± 0.72</td>
<td>3.0 ± 1.2</td>
</tr>
<tr>
<td>Platelets</td>
<td>1206 ± 61</td>
<td>547 ± 74</td>
</tr>
<tr>
<td><strong>B. Day 9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total WBC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.9 ± 1.7</td>
<td>6.8 ± 1.7</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.7 ± 0.55</td>
<td>0.86 ± 0.37</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>6.2 ± 1.4</td>
<td>5.0 ± 1.6</td>
</tr>
<tr>
<td>Platelets</td>
<td>1081 ± 154</td>
<td>695 ± 194</td>
</tr>
</tbody>
</table>

<sup>c</sup> × 10<sup>9</sup>/liter.
<sup>b</sup> Mean ± SD.
<sup>c</sup> Significantly increased over ZD1694 + saline.

are given to mice. For example, if day 5 drug levels are compared in the two schedules, plasma, liver, and kidney were ~30-fold lower in the single administration schedule. However, the small intestinal epithelial concentration was nearly 200-fold lower at this time (data not shown).
be an undefined effect of RTX on intestinal functional or an increased rate of the engagement of apoptotic pathways (20). Cancer patients may also differ widely in their pharmacological handling of drugs such as RTX. Indeed, renal impairment in humans is associated with reduced renal clearance of RTX and increased toxicity (8). Occasional violations of protocol, particularly for dose modification for renal impairment, have led to severe toxicity or death (9). Whatever the etiology, reversal of the drug action on presentation of severe symptoms could speed the rate of recovery and increase drug safety.

Potential rescue agents include dThd and LV. dThd, which circumvents TS inhibition induced by RTX through the dThd salvage pathway, is not available as a registered agent (1). However, LV is available because it is used clinically as a rescue agent with high-dose MTX and as a promoter of the cytotoxicity of 5-FU (9, 27). The different function of TS compared with DHFR in folate metabolism and the importance of RTX polyglutamates as potent TS inhibitors suggest that delayed LV rescue may be less effective for RTX than MTX. Nevertheless, LV has been shown to reverse the growth-inhibitory effects of RTX in vitro when added to the culture medium at the same time as RTX because of the prevention of the formation of RTX polyglutamates (13). This has now been explored further to gain insight into the relationship between the time interval between RTX and LV and the effects on cell viability. Even when 25 μM LV was given 8 h after RTX to L1210 or W1L2 cells, the cell population was able to expand (although not at control rate), with all cells remaining viable 48 or 72 h later. In the slower growing W1L2 cells, the LV could even be added as late as 18 h, and some population expansion was still observed.

W1L2 cells were also used to examine the effect of the delayed addition of LV on RTX polyglutamation and on the stability of preformed RTX polyglutamates. Polyglutamation of [3H]RTX was rapid over the first 4 h, as described previously. However, because polyglutamation continued over the following 20 h, albeit at a slower rate, the addition of LV at 4 h prevented further accumulation of the drug as high chain length polyglutamates. In turn, this appeared to promote drug loss through efflux of low chain length polyglutamates or parent drug. In wash-out experiments, when the RTX-containing medium was exchanged at 4 h for LV-containing medium, loss of polyglutamates was greater than that seen without LV. Although the mechanism(s) involved are unknown, it can be hypothesized that expansion of the intracellular folate pool through the administration of LV inhibits FPGS (either by competition or feed-back inhibition) so that any hydrolysis of RTX occurring through the action of FPGH leads to the efflux of RTX from the cells. However, the in vitro loss of polyglutamates after the addition of LV was not large and may relate to low expression of FPGH in cultured cells compared with either tumor cells in vivo or some normal tissues (17). Indeed, our own studies have failed to detect significant enzyme levels in tissue culture compared with tissues such as liver and small intestine (data not shown). This may lead to relatively high polyglutamate stability in vitro compared with in vivo. Overall conclusions from these in vitro studies are that, in cycling cells, the delayed addition of LV may: (a) reduce further uptake of RTX; and (b) reduce the level of preformed RTX polyglutamates. These results are useful in explaining results in context of an in vivo animal model.

A previously described BALB/c mouse model (20) was used to study the effect of delayed administration of LV on mouse body weight and tissue drug levels after RTX administration. Toxicity (weight loss and diarrhea) continued after completion of the course of daily 100 mg/kg injections of RTX (days 1–4). LV rescue, which commenced 24 h later (day 5), led to less weight loss and more rapid recovery of weight. Histological

Table 3  Effect of delayed administration of LV on the concentration of RTX (polyglutamates) in plasma, liver, kidney, and intestinal mucosa (day 7) after administration of 100 mg/kg/day RTX × 4 days (days 1–4).

Mice (five or six/group) received injections of 100 mg/kg/day RTX on days 1–4. Rescue was given twice daily inclusively on days 5–7. Results are the pooled results from two separate experiments, except where indicated, and given as mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Liver</th>
<th>Gut mucosa</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.11 ± 0.059</td>
<td>6.4 ± 3.8</td>
<td>1.8 ± 1.3</td>
<td>1.0 ± 0.39</td>
</tr>
<tr>
<td>100 mg/kg LV</td>
<td>0.03 ± 0.023</td>
<td>0.76 ± 0.35</td>
<td>0.51 ± 0.14</td>
<td>0.31 ± 0.12</td>
</tr>
<tr>
<td>100 mg/kg LV</td>
<td>0.039 ± 0.033</td>
<td>3.2 ± 2.5</td>
<td>1.7 ± 1.2a</td>
<td>1.32 ± 0.82b</td>
</tr>
<tr>
<td>200 mg/kg LV</td>
<td>0.011 ± 0.007</td>
<td>0.30 ± 0.33</td>
<td>0.44 ± 0.39a</td>
<td>0.36 ± 0.14a</td>
</tr>
</tbody>
</table>

* Mann-Whitney test.
* One experiment only.

Table 4  Effect of delayed administration of LV on the concentration of RTX (polyglutamates) in the liver after a single dose of 100 mg/kg RTX.

LV was administered twice daily on days 4–7 inclusively. Results are given as mean ± SD of five or six mice.

<table>
<thead>
<tr>
<th>RTX (polyglutamate) concentration, nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
</tr>
<tr>
<td>Day 7</td>
</tr>
<tr>
<td>Saline</td>
</tr>
<tr>
<td>100 mg/kg LV</td>
</tr>
<tr>
<td>200 mg/kg LV</td>
</tr>
</tbody>
</table>

* Pooled results of two experiments.
* Mann-Whitney test.
examination of the intestine confirmed that this effect was attributable, at least in part, to effects on the intestinal epithelium. Furthermore, the potentially lethal dose of 100 mg/kg RTX daily × 5 (days 1–5) were prevented by LV administration (commenced day 6). LV also effectively stimulated bone marrow recovery, as shown by recovery of peripheral neutrophils and platelets.

The measurement of RTX (polyglutamates) by RIA after 100 mg/kg RTX days 1–4 demonstrated lower levels in plasma, liver, kidney, and small intestinal epithelium in LV-treated mice. This difference was as much as an order of magnitude in the liver. These data are consistent with LV interrupting homeostatic regulation of RTX polyglutamates and promoting recovery of TS activity.

Although these preclinical data support the role of LV as a rescue agent, it is worth considering the relevance of the experimental model. The LV was given as a 3-day course starting 24 h (day 5) after the final (day 4) RTX injection. This timing is largely determined by the narrow “window” in which mice continue to lose weight before recovering naturally. However, because mice require high doses of RTX (100 mg/kg daily × 4) to induce the level of toxicity required, plasma drug levels at the start of the “rescue” were still ~400 pmol/ml (0.5 μM), despite the fact that the vast majority of the drug has been eliminated from the plasma. This compares with ~2–10 pmol/ml in humans 5 days after administration of a standard 3 mg/m² dose (19). The question therefore arises whether, in the mouse model, LV is largely preventing further drug uptake and polyglutamation and consequently preventing greater damage rather than reversing the effects of preformed RTX polyglutamates. To address this question, two different experimental designs were used. The RTX dose was reduced to 10 mg/kg daily × 4 (days 1–4). This resulted in plasma levels of ~30 pmol/ml on day 5. LV (30 mg/kg twice daily, days 5–6) significantly reduced the concentration of RTX in the plasma, liver, and kidney on day 7. A single injection of 100 mg/kg (not sufficient to induce toxicity) was given to mice on day 1, and LV was given from days 4 to 6. The plasma and liver concentrations of RTX at the start of the LV rescue were 3–4 and 1000 pmol/g, respectively. These are values similar to those observed in human plasma and liver 5 days after treatment (Ref. 19 and data not shown). LV administration reduced the RTX concentration in the liver by ~3-fold (days 7, 9, and 11) but not in the plasma and kidney. Unfortunately, intestinal epithelial measurements could not be made because previous experiments had shown that a single injection of RTX does not cause marked gastrointestinal toxicity in mice, and hence the rapid proliferation of the intestinal epithelium “dilutes” out the RTX in this tissue. These data suggest that LV can reduce tissue levels of RTX in mice when the plasma concentration at the start of the rescue are of the same magnitude as those that may be seen in patients presenting with toxicity.

Although the exact mechanisms of the LV effect and relevance of the preclinical model have some uncertainties, the fact remains that LV can rescue mice from toxicity at a time when considerable damage has been done to the small intestinal epithelium and suppressive effects are being seen in the bone marrow. This observation is therefore consistent with some cell populations in the intestinal epithelium or bone marrow, e.g., stem cells, being “resistant” to the drug by being quiescent for example. If quiescent Chinese hamster ovary cells in culture are exposed to RTX for 24 h before being stimulated to proliferate they are relatively resistant to the effects of the drug. This may be attributable to low activity of either the RFC or FPGS during quiescence, and previous reports concerning the effects of MTX in quiescent tumor cells demonstrated reduced MTX polyglutamate formation during this period (28, 29). Thus, quiescent cells may evade the cytotoxic effects of the transiently high drug concentrations seen in the plasma after bolus injection of RTX. However, these cells may still be exposed to, or have accumulated, growth-inhibitory concentrations of the drug that could still manifest as toxicity. Intervention with LV has the potential to overcome this effect and stimulate growth more quickly.

At 100 mg/kg RTX daily × 4 days, the toxicities observed in BALB/c mice are gastrointestinal toxicity combined with myelosuppression. Experiments described herein suggest that LV can prevent the lethal combination of toxicities by preventing further RTX uptake into cells and/or promoting RTX efflux. Although extrapolation from the preclinical models is difficult, it is nevertheless recommended that LV be given alongside vigilant supportive care in patients presenting with the life-threatening combination of gastrointestinal and bone marrow toxicities. LV rescue should be given earlier if protocol violations, such as failure to reduce dose in patients with poor renal function, are recognized soon after treatment. It is difficult to recommend a clinical dose of LV to use because the dose of RTX used in the mouse experiments (100 mg/kg × 4 days) was necessarily higher than that in humans (3 mg/m² × 1; equivalent to ~1 mg/kg × 1). For this reason, high concentrations of LV were also used (100 and 200 mg/kg twice daily × 3 days). Although this translates into a human dose of 300–600 mg/m², it is probably not necessary to give equivalent high doses to humans. It is therefore recommended to give a similar LV dose to that used for high-dose MTX, e.g., 25 mg/m² every 6 h and continue until symptoms resolve. Finally, it should be emphasized that although LV has a place in the rescue of toxicity, its use should not be advocated routinely because it is likely to induce similar rescue effects in tumors. The possibility of selective effects on normal proliferating tissues versus tumors, perhaps at lower LV doses, should be the subject of further investigation.

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Leucovorin Rescue from Raltitrexed (Tomudex)-induced Antiproliferative Effects: *In Vitro* Cell Line and *in Vivo* Mouse Studies

D. C. Farrugia, G. W. Aherne, L. Brunton, et al.