Pharmacokinetic/Pharmacodynamic Study of ZD9331, a Nonpolyglutamatable Inhibitor of Thymidylate Synthase, in a Murine Model Following Two Curative Administration Schedules1

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ABSTRACT
ZD9331 is a nonpolyglutamatable antifolate inhibitor of thymidylate synthase currently in clinical development. This enzyme is crucial for DNA synthesis and catalyzes the reductive methylation of dUMP to form thymidylate, which is subsequently converted to dTTP. The pharmacokinetics of two curative antitumor doses of ZD9331 administered by either a single i.p. bolus injection (50 mg/kg) or by 24-h s.c. infusion (3 mg/kg) have been measured in a thymidine salvage-incompetent murine lymphoma model (L5178Y) using a sensitive and specific ELISA. To gain an understanding of the relationship between the pharmacokinetics of ZD9331 and antitumor activity perturbations in tumor, dTTP and dUMP concentrations were also determined. After bolus administration, ZD9331 was eliminated from plasma and tissues relatively rapidly, with terminal elimination (λz 0–24 h) of 4–6 h. Liver concentrations were 8-fold higher than those measured in the plasma. Kidney and lymphoma drug concentrations were similar to those of plasma, although there was evidence of a slower overall elimination of drug at later time points. Steady-state concentrations of ZD9331 were obtained 4–5 h after the start of the 24 h s.c. infusion. At the end of infusion, elimination rates were similar for plasma and tissues (~3.5 h) but appeared to be slower in the tumor at later time points. Liver concentrations were 4-fold higher, and kidney and tumor concentrations were similar to those in the circulation. Depletion of dTTP and elevation in dUMP in the tumor were consistent with inhibition of thymidylate synthase after both administration schedules, although the time for which dTTP was decreased was longer (~24 h) for the infusional route than for the bolus injection (~16 h). The results suggest that antitumor activity is dependent on attaining adequate drug concentrations to affect dTTP pools as well as on the duration of effective drug levels.

INTRODUCTION
In recent years, the development of inhibitors of TS3 as antitumor agents has been very fruitful (1). This enzyme catalyzes the reductive methylation of dUMP to form thymidylate, which is a rate-limiting step for DNA synthesis. Depletion of dTTP pools after TS inhibition is accompanied by an elevation of dUMP pools behind the enzyme block attributable to the loss of negative feedback control by dTTP. After TS inhibition, cell death occurs through prolonged depletion of dTTP pools (2) and/or misincorporation of uracil into DNA (3).

ZD9331 (Fig. 1), is a water soluble quinazoline-based antifolate inhibitor of TS (4), and it is currently being evaluated clinically (5, 6). Unlike some other inhibitors of this enzyme, e.g., RTX (ZD1694; Tomudex; Refs. 7 and 8), ZD9331 is not a substrate for folypolyglutamate synthetase although both these antifolates are transported into the cell by the reduced folate carrier. ZD9331 is a potent inhibitor of TS (Ki = ~0.4 nm) and of cell growth (IC50 for a range of human tumor cell lines, ~5–100 nm). Polyglutamation of antifolates normally serves to retain potent inhibitory species within cells; but in the case of ZD9331, drug efflux is not impaired in this way, and TS inhibition is rapidly reversed when cells are resuspended in drug-free medium. This has been demonstrated in vitro using the in situ TS inhibition assay (4) as well as by measuring dUMP/dTTP ratios (9, 10).

As expected for a compound that is unable to be polyglutamated, significant antitumor activity in mouse models was achieved when ZD9331 was administered by continuous infusion. Thus a 3mg/kg s.c. infusion of ZD9331 over 24 h (delivered via osmotic minipumps) was curative (4) using the L5178Y thymidine salvage incompetent (TK+/−) mouse lymphoma model (11). However, curative antitumor activity was also observed when the drug was administered by a single i.p. bolus

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3 The abbreviations used are: TS, thymidylate synthase; ZD9331, (S)-2-[(4-[(3,4-Dihydro-2,7-dimethyl-4-oxo-6-quinoxalinyl)-N-prop-2-ynylinamino]-2-fluorobenzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid; RTX, raltitrexed; HPLC, high-performance liquid chromatography; AUC, area under the concentration time curve; AUMC, area under the first moment curve; MRT, mean residence time.
injection of ZD9331 (25–50 mg/kg; Refs. 4 and 12). The purpose of the present study was to compare the pharmacokinetics of ZD9331 in this murine model using these two curative administration schedules. In addition, in an attempt to determine parameters important for antitumor activity, the extent to which dTTP and dUMP pools were perturbed in tumor tissue after ZD9331 administration was determined.

HPLC has been used previously to measure ZD9331 in biological samples (13). However, with a limit of detection of only 0.3 μM, this method of analysis was likely to lack sufficient sensitivity for the measurement of the drug after intra muscular administration and in longer-term time points after parenteral administration. For the work described here, a sensitive ELISA was therefore developed using a rabbit antiserum produced against a ZD9331-thyroglobulin immunogen.

**MATERIALS AND METHODS**

**Materials**

ZD9331 was supplied by Zeneca Pharmaceuticals. Stock solutions (10 μM) were prepared in 0.15 M sodium bicarbonate and stored at −20°C. For the mouse studies, the drug was formulated in 0.05 M sodium bicarbonate and the pH adjusted to 8.5–9.0. Acetaminol was from Fisons; porcine thyroglobulin and other chemicals were from Sigma Chemical Co. Horseradish peroxidase was obtained from Biozyme, Blaenavon, Gwent, United Kingdom.

**Mouse Pharmacokinetic Studies**

The L5178Y TK−/− mouse lymphoma was grown in tissue culture, harvested, and implanted (5 × 10⁶ cells) into the gastrocnemius muscle of female DBA2 mice 6–9 weeks of age as described previously (4, 11). Four to 7 days later, when the tumor measured ~8 mm, ZD9331 was administered to groups of three animals either by osmotic pumps (Alzet model 2001D; 3 mg/kg/24 h) or by a single i.p. injection (50 mg/kg; 0.1 ml/10 g). Some experiments were carried out in non-tumor-bearing animals. Blood was obtained by cardiac puncture under halothane anesthesia at various times over the next 48 h and placed in heparinized microtubes. Plasma was separated immediately from the blood by microcentrifugation (12,000 rpm for 2 min) and stored at −20°C until assayed. At each time point, the liver, kidney, and tumor were excised from the animals and immediately frozen on dry ice. Before drug assay, the tissues were weighed and homogenized in 2 volumes of 0.1 M Tris-HCl buffer (pH 10; Omni 2000 homogenizer used at ~10,000 rpm). Aliquots of homogenate (0.1–0.5 ml) were treated with an equal volume of acetonitrile, particulate material was removed by centrifugation (8,000 × g for 10 min), and the supernatant was aspirated for assay.

**Measurement of ZD9331 by ELISA**

**Antibody Production.** Two female New Zealand White rabbits (12–14 weeks of age; Regal Rabbits, Great Bookham, Surrey, United Kingdom) were immunized with a conjugate of ZD9331 and porcine thyroglobulin (molar ratio, 200:1) prepared using N-hydroxysuccinimide and dicyclohexyl carbodiimide as described previously (14). The immunogen was emulsified with Non-Ulcerative Freund’s adjuvant (Guildhay, Guildford, Surrey, United Kingdom) and used to immunize the rabbits following standard procedures. The priming injection, consisting of 2.5 mg conjugate and 100 μl *Bacillus Calmette-Guérin* vaccine (Evans Medical, Ltd., Horsham, United Kingdom), was followed 4 weeks later by a booster injection (1.25 mg without the addition of *B. Calmette-Guérin*). Serum obtained after the second immunization from both rabbits contained specific antibodies to ZD9331, but that from R8769 was of higher titer and was used for the development of sensitive ELISA methods.

**ELISA.** A coated-antigen ELISA, in which the wells of a microtiter plate were coated with a ZD9331-ovalbumin conjugate (molar ratio, 15:1), has been briefly described previously (2); but to reduce assay steps and overall assay time, a coated-antibody format was subsequently used. A purified immunoglobulin fraction from the whole antiserum was prepared by DEAE chromatography (14). ZD9331 was conjugated to horseradish peroxidase (20:1) using the same reaction as that used to prepare the immunogen and purified by gel filtration as described previously (14). Aliquots of enzyme conjugate were stored at −20°C or for daily use at 4°C. Optimized concentrations of both antiserum and enzyme conjugate were determined by checkerboard experiments.

The ELISA was set up using Multiprobe automated liquid dispensing equipment (Canberra-Packard, Pangbourne, United Kingdom). The antiserum preparation was diluted in sodium barbitone solution [14.4 g/liter (pH 9.6)] and used to coat the wells of microtiter plates (Immulon II; Dynex, Billingshurst, United Kingdom; 1.5 μg protein/0.2 ml). The plates were incubated at 37°C in a moist chamber for 3 h and then washed three times with deionized water containing 0.1% Tween 20 using a Wellwash 4 plate washer (Labsystems, Cambridge, United Kingdom). A stock solution of ZD9331 (1 mg/ml) was stored at −20°C. A working standard (1 μM) prepared in assay buffer (8 g sodium chloride, 0.2 g KH₂PO₄, 2.9 g Na₂HPO₄, 12H₂O, 0.2 g KCl, 1.0 g gelatin, and 0.5 ml Tween 20/liter of deionized water) was stored frozen in aliquots sufficient for one assay. This ZD9331 solution was diluted further for each assay to give a range of standards (10–10,000 pm). Plasma samples and tissue extracts were assayed after dilution in assay buffer according to the expected drug concentration (at least twice for plasma samples and at least 1:20 for tissue extracts). Three dilutions of each sample were assayed. Standards and samples (50 μl) were added to the washed plates in duplicate with 100 μl assay buffer. Each plate included two uncoated wells (containing 150 μl assay buffer only) to determine nonspecific binding and two coated wells (also containing 150 μl assay buffer only) to determine total antibody binding in the absence of ZD9331 standard (Bo). ZD9331-horseradish peroxidase conjugate (50 μl of a 1:1000 dilution) was added to each well and the plate incubated again at 37°C for 2 h, rewashed, and 150 μl tetramethyl benzidine
substrate solution [0.1 mg/ml in citrate buffer (pH 5.0) containing 0.5 μl/ml hydrogen peroxide] added. After 30 min at 37°C, the reaction was stopped by the addition of 1 M hydrochloric acid (50 μl). The color in the wells was read at 450 nm (Multiskan Plus spectrophotometer, Labsystems, Cambridge, United Kingdom). ZD9331 concentrations were determined from the standard curve by data reduction using a curve fitting algorithm (four-parameter logistic plot; RiaSmart Canberra-Packard, Pangbourne, United Kingdom). ZD9331 concentrations were determined from the concentration time curve by log-linear regression using at least three to four time points. Clearance was determined from the dose and the AUC.

**RESULTS**

**ELISA**

The standard curve for ZD9331 is shown in Fig. 2. The mean coefficient of variation across the curve (10–10000 pm) was 10.3%, and the sensitivity of measurement was ~25 pm [significant (P < 0.0001) decrease in binding from Bo]. The addition of undiluted, drug-free plasma and tissue extracts caused a slight nonspecific effect on antibody binding that could be eliminated if samples were diluted before assay. Thus, plasma was diluted at least twice and tissue extracts at least 1:20, giving a limit of detection for plasma of 50 pM and for tissues of 3 nM (when all dilutions involved in homogenization and extraction are taken into account). Recovery of ZD9331 from plasma and tissue extracts was complete (Table 1). Three dilutions of each sample were included in the assay to ensure that at least two dilutions fell on the linear portion of the standard curve. Reproducibility assessed either within plate (679.5 ± 100.7 nm; 8.7 ± 0.6 nm) or between plate (576.7 ± 52 nm; 9.2 ± 0.8 nm) was <15%. The rabbit antiserum did not cross-react (<0.01%) with natural folates (folinic acid and methyl tetrahydrofolic acid) nor with the quinazoline antifolate, RTX. There was, however, almost complete cross-reaction with antifolate analogues with the same 7-CH₃,2-F-quinazoline structure but with different terminal γ-linked moieties in place of the tetrazole of ZD9331 [e.g., the dipeptides L-glu-γ-D-glu and L-glu-D-adipate (17) and the sterically hindered dipeptide L-glu-L-Me-glu (18)].

**Protein Binding Studies**

Aliquots of pooled mouse plasma obtained during these studies and drug-free plasma fortified with ZD9331 (0.1 and 1 μM) were filtered in Amicon Centrifree microfilters (Millipore, Watford, Hertfordshire, United Kingdom) using centrifugation at 4°C for 45 min at 1800 × g in a fixed-angle rotor. The concentration of unconbound drug in the ultrafiltrate was measured by ELISA.

**Measurement of TTP and Immunoreactive dUMP Pools in L5178Y TK−/− Lymphomas**

DBA2 mice, bearing the L5178Y TK−/− tumor, were treated with ZD9331 as described above. At the appropriate time points, tumor tissue was excised rapidly and placed immediately in preweighed, cooled containers containing 0.5% PCA, homogenized (Omni 2000 used at ~10,000 rpm), and frozen (~80°C). Before assay, tissue weight was obtained by reweighing. After neutralization and treatment with sodium periodate, dTTP pools and immunoreactive dUMP pools were measured by RIA as previously described (9).

**Pharmacokinetic Analysis**

Pharmacokinetic parameters were derived using standard procedures (15, 16) and the computer program WinNonlin (SCI Software, Lexington, KY) using noncompartmental models as described below. The linear trapezoidal method was used for calculation of the AUC. The AUMC was also obtained using the linear trapezoidal rule. MRT was calculated from AUMC/AUC procedures (15, 16) and the computer program WinNonlin (SCI Research).
Pharmacokinetics

i.p. Bolus Administration (50 mg/kg). Plasma concentrations in DBA2 mice following i.p. administration were similar in tumor and non-tumor-bearing animals (data not shown), and results have been combined for clarity. Plasma, liver, and kidney concentrations of ZD9331 from 1–48 h after administration (50 mg/kg i.p.) are shown in Fig. 3. The highest drug concentrations were measured in the liver. At 1 h, the hepatic concentration was 69.3 ± 26.0 μM compared with 7.75 ± 5.2 and 7.60 ± 1.8 μM in plasma and kidney, respectively. Subsequent elimination from liver was similar to that in the plasma, although hepatic concentrations were ~7-fold higher than plasma throughout the time course. Kidney ZD9331 concentrations were also similar to those in plasma at least up to 8 h (mean kidney to plasma ratio, 1.03). After this time point, clearance from the kidney was apparently slower than from the plasma (mean kidney:plasma ratio from 16–48 h was 2.6). Because drug concentrations at early time points (<1 h) were not measured in this study, noncompartmental analysis was used to estimate the terminal (λz) half-life and to compare AUC, AUMC, MRT, and clearance of ZD9331 in plasma, liver, and kidney (Table 2). Overall agreement between observed and predicted concentrations (r) was >0.88 when the 48 h time point was included, but this was improved (r > 0.92) if the 0–24 h data only was used. The AUC_{0–24 h} and AUC_{0–48 h} in liver was ~10-fold higher than in either plasma or kidney, which had similar values. The AUC increased by ~2% for plasma and liver between 24 and 48 h but by ~8% for kidney, again supporting and apparent slower overall clearance from this organ. Terminal elimination half-life (λz) determined using all of the data points was similar for plasma and tissues, i.e., 4–6 h up to 24 h and 12–13 h up to 48 h, respectively. However, the λz for kidney calculated using late time points only (8–24 h) was longer (7.74 h) compared with plasma and liver (5 h). MRT for kidney was also longer than that for plasma and liver. Elimination of drug between 24 and 48 h appeared to be slow. Indeed ZD9331 was still detectable in plasma 7 days after drug administration (data not shown).

ZD9331 concentrations in the L5178Y TK−/− lymphoma are shown in Table 3 with the tumor:plasma ratios. At 4–6 h, the tumor:plasma ratio was <1; but this ratio increased to 1.6 at 16 h and to 4.2 at 24 h, demonstrating an apparently slower clearance from the tumor than from plasma.

Subcutaneous Infusion Administration (3 mg/kg over 24 h)

ZD9331 concentrations in plasma, liver, kidney, and L5178Y TK−/− lymphoma during and for 24 h after a s.c. infusion (3 mg/kg over 24 h) are shown in Fig. 4. Pharmacokinetic parameters (Table 4) were obtained by noncompartmental analysis. Drug concentrations during infusion were 4- and 2-fold higher for liver and kidney than for plasma, respectively, although tumor concentrations were similar to those in plasma. Steady-state concentrations were achieved by 4 h in plasma and by 5 h in the tissues. At the end of infusion, elimination rates were similar for plasma and tissues (~3.5 h) but appeared to be slower in the tumor at later time points. Also, the tumor:plasma ratio was 1.2 at the end of the infusion compared with 2.3 at 4 h and >10 at 17–24 h after the end of the infusion. MRT and AUC in liver, kidney, and L5178Y TK−/− lymphoma were approximately 4, 2, and 1.5 times greater than that in plasma.

ZD9331 concentrations in plasma and tissues were also measured after increasing doses administered via s.c. infusion. Plasma- but not tumor-drug concentrations increased in a linear fashion, resulting in tumor:plasma ratios at steady state (~17 h into the 24 h s.c. infusion) of 1.0, 1.4, 0.6, and 0.5 at 1, 3, 10, and 20 mg/kg ZD9331.

Plasma Binding

In drug-free human and mouse plasma fortified with ZD9331 (1 μM and 0.1 μM), plasma binding was 94.4 and 92.2% (n = 2), respectively. These values were similar to that determined in plasma from ZD9331-treated mice (91.8 ± 6.8%; n = 4).

Deoxynucleotide Pools in L5178Y TK−/− Lymphomas

Previous experiments have shown that exposure of mouse and human tumor cells to TS inhibitors in vitro causes a rapid depletion of dTTP pools with a concomitant increase in dUMP pools (9, 10). In an attempt to compare the pharmacodynamic effects of ZD9331 in vivo, dTTP and dUMP pools in the L5178Y TK−/− tumors were determined at various times after administration of the drug by either the i.p. bolus (50 mg/kg) or infusional (3 mg/kg) routes using RIAs. To avoid degradation of deoxynucleotides, tissues were excised rapidly and immediately extracted with ice-cold PCA as described in “Materials and Methods.” No significant differences in measured dTTP and dUMP were observed using this method compared with freeze-clamping of tumors before excision (data not shown). dTTP and

Table 2: Pharmacokinetic parameters for ZD9331 after administration of 50 mg/kg i.p. to DBA2 mice using noncompartmental analysis

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0–24 h} (μM h)</td>
<td>37.44</td>
<td>38.40</td>
<td>395.15</td>
</tr>
<tr>
<td>MRT_{0–24 h} (h)</td>
<td>2.66</td>
<td>3.41</td>
<td>1.48</td>
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<tr>
<td>AUMC_{0–24 h} (h)</td>
<td>99.6</td>
<td>131.02</td>
<td>584.41</td>
</tr>
<tr>
<td>λ_{z (0–24 h)} (h⁻¹)</td>
<td>4.39 (0.96)</td>
<td>12.95 (0.86)</td>
<td>5.19 (0.98)</td>
</tr>
<tr>
<td>λ_{z (48–96 h)} (h⁻¹)</td>
<td>5.02 (0.92)</td>
<td>15.4 (0.88)</td>
<td>4.93 (0.88)</td>
</tr>
<tr>
<td>λ_{z (0–24 h)} (h⁻¹)</td>
<td>16.93 (0.93)</td>
<td>15.4 (0.95)</td>
<td>7.43 (0.98)</td>
</tr>
<tr>
<td>Clearance (liters/h)</td>
<td>0.050</td>
<td>0.048</td>
<td>0.0047</td>
</tr>
</tbody>
</table>

Note: Results were analysed using data from 0–24 h and 0–48 h.
dUMP concentrations in untreated controls were 3.19 ± 1.99 pmol/mg and 2.99 ± 2.1 pmol/mg, respectively (mean of 13 determinations obtained in four experiments).

The changes observed in dTTP and dUMP pools after administration of ZD9331 (50 mg/kg) by i.p. bolus administration are shown in Fig. 5. By 4 h, dTTP pools had significantly decreased to 32.2% of those in the untreated tumor (P < 0.0001). At 6 h, dTTP pools were 46.8% controls (P < 0.02) and continued to rise so that at 24 h they were ~2-fold higher than controls. This paradoxical rise in dTTP pools has been observed previously in vitro after the release of TS inhibition (9). After bolus administration of ZD9331, dUMP pools were increased to 125% and 150% (P = 0.005) at 4 and 6 h, respectively; although by 16–24 h, no significant difference from untreated controls was observed. Thus, it appears that TS in the L5178Y TK–/– lymphomas cells was partially inhibited for a period of time <16 h after bolus administration.

In contrast, the infusional administration protocol resulted in a significant depletion of dTTP pools during and for 4 h after the end of the 24-h infusion (Fig. 6). This was accompanied by a 1–2-fold increase in dUMP pools for at least 16 h. At the end of the infusion and 4 h later, dUMP pools were less than those in tumors from untreated controls, although dTTP pools were apparently still depleted. dTTP depletion was also determined after different doses of ZD9331 (1, 3, 10, and 20 mg/kg s.c. over 24 h). At steady state (≈17 h into infusion), there was no significant difference in the extent to which dTTP pools were reduced. However, there was a trend for dTTP to be depleted less at 4, 17, and 24 h after 1 mg/kg compared with 3 mg/kg (data not shown).

**DISCUSSION**

ZD9331 is a nonpolyglutamatable inhibitor of TS currently in clinical development. It was originally expected that because of the nonpolyglutamatable nature of ZD9331, infusional protocols would be required to maintain TS inhibition for antitumor efficacy. However, antitumor activity has been observed after a single bolus schedule of administration in the L5178Y TK–/– mouse model. The aims of this study were to describe the pharmacokinetics of ZD9331 at two curative doses in this murine model in relation to depletion of dTTP pools that can be used as a pharmacodynamic marker of response to TS inhibition.

Drug concentrations were measured using a reproducible and sensitive immunoassay. The ELISA was suitable for measuring the compound over long periods of time and may provide an alternative technique for monitoring drug levels in clinical samples. Experience has shown that antibodies to folates and antifolates are highly specific (19–21). The rabbit antibodies described here did not cross-react with natural folates, although, as expected, there was a large degree of cross-reaction with antifolate analogues of ZD9331 that contained the 7-CH quinazoline and 2’-F benzyl substituents (17, 18). As shown for its dipeptide analogues (22), ZD9331 does not appear to be subject to hydrolysis in the plasma, and significant metabolism of ZD9331 has not yet been observed (12). It is unlikely, therefore, that there is metabolic interference in the ELISA, and results obtained by immunoassay compared well with those obtained by HPLC (data not shown).

ZD9331 has been measured previously using HPLC (13). This assay was suitable for the measurement of plasma concentrations in mice for 6–8 h after administration of bolus doses of 50–200 mg/kg. The sensitivity of the immunoassay has now allowed the pharmacokinetics of ZD9331 over 48 h to be described. Terminal elimination of ZD9331 estimated using noncompartmental analysis in the present study was longer than reported previously (13), although this may be because of the longer sampling periods used in this study. When the data up to 24 h was used, the plasma terminal half-life of ZD9331 was 4–5 h, but longer (15–16 h) if the results from the 48-h time point were also included. Interestingly, immunoreactivity was measurable until day 7 (data not shown).

This study has shown that ZD9331 has similar plasma pharmacokinetic properties to another structurally related nonpolyglutamatable antifolate, CB30900 (23). In this case, the plasma elimination curve was described as triphasic (α, β, and γ half-lives of 2.8 min, 19.1 min, and 4.1 h, respectively). Previous studies have measured ZD9331 concentrations in plasma at earlier time points than 1 h, and the disappearance of ZD9331 in murine models was described as biphasic (12, 13). Attempts were made to fit the present data to both a 2- and 3-compartmental model, but this was not successful because precision was poor. To aid data modeling, early time points (<1 h) from these previous studies were combined with the present data (1–24 h). In this way, it was possible to show that the disappearance of ZD9331 from the plasma of DBA2 mice subsequent to i.p. dosing (50 mg/kg) over 24 h was best described as triphasic (T_{α} = 5 min, T_{β} = 29.4 min, and T_{γ} = 4.3 h). Binding of ZD9331 to plasma proteins is relatively high.
(compared with that in tissue culture medium (~50%)), and, if this is only slowly reversible, it may account for the low concentrations that remain over a period of several days. The contribution to antitumor activity that the prolonged plasma elimination phase makes remains to be determined. Interestingly, the plasma pharmacokinetics of ZD9331 in mice are similar to those of RTX (20, 24), although, in this case, the prolonged terminal elimination is thought to be attributable to the retention and slow release from tissues of polyglutamated forms of the drug.

Although kidney and tumor concentrations of ZD9331 were similar to those of plasma, drug concentrations were eight times higher in the liver than those in the circulation. The tissue AUC to plasma AUC ratios obtained with ZD9331 were very similar to those reported for the same dose of the structurally similar compound CB30900 (22). This is in contrast to RTX, where, because it is highly metabolized to polyglutamated species, plasma/tissue levels are much higher (≈10-fold in liver and ~50-fold in kidney; Refs. 20 and 24). However, for ZD9331 at time points later than 8 h, there was evidence of a slower elimination of drug from the kidney and tumor tissue than from plasma. Thus, although efflux of ZD9331 from cells grown in culture is extremely rapid,4 clearance from kidney and tumor appears to be less rapid.

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4 G. W. Atherne, unpublished data.

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Table 4 Plasma pharmacokinetics of ZD9331 after s.c. infusion of 3 mg/kg over 24 h in DBA2 mice determined by noncompartmental analysis

<table>
<thead>
<tr>
<th></th>
<th>Plasma (μM) (nmole/ml)</th>
<th>Liver (nmole/g)</th>
<th>Kidney (nmole/g)</th>
<th>L5178Y TK−/− lymphoma (nmole/g)</th>
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<tr>
<td>C₀ (μM)</td>
<td>~0.63</td>
<td>~2.56</td>
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<td>T1/2 (h)</td>
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<tr>
<td>Clearance</td>
<td>0.007</td>
<td>0.002</td>
<td>0.003</td>
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</table>

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Fig. 5 dTTP (■) and dUMP (□) concentrations were measured in implanted i.m. L5178Y TK−/− lymphoma at 4, 6, 16 and 24 h after ZD9331 administration (50 mg/kg i.p.). Results were compared with untreated controls obtained in each experiment. TTP pools were significantly different from controls 4 h (P < 0.001) and 6 h (P = <0.02) after injection of drug. At 6 h, dUMP pools were significantly different from controls (P = 0.005).

Fig. 6 dTTP (■) and dUMP (□) concentrations were measured in implanted i.m. L5178Y TK−/− lymphoma during and 4 h after a 24-h s.c. infusion of ZD9331 (3 mg/kg) delivered by osmotic pump. Results were compared with untreated controls obtained in each experiment. dTTP pools were significantly different from untreated controls (P < 0.03) at all time points. dUMP pools were not significantly different from untreated controls except 4 h after the end of the infusion (P = 0.001).

The significance of the apparent retention of ZD9331 in the kidney at a bolus dose of 50 mg/kg is not known. Although at a higher dose (200 mg/kg i.v.) reversible nephrotoxicity was attributed to hugely elevated drug concentrations in the kidney in DBA2 mice (12) no renal toxicity was observed at 50 mg/kg ZD9331 administered either by i.v. or i.p. bolus.

Steady-state concentrations of ZD9331 during a 24-h s.c. infusion (3 mg/kg) were achieved relatively rapidly (within 4–5 h) and were maintained for the duration of the infusion. At the end of the infusion, drug elimination occurred with a half-life of 3–5 h. As observed after i.p. bolus administration, ZD9331 concentrations were highest in liver (4-fold greater than plasma). Drug concentrations in the kidney and tumor were similar to those in plasma, at least during the infusion, although drug concentrations seemed to decline more slowly from the tumor (7.5 h). After administration of ZD9331 at a range of doses (1–20 mg/kg s.c.), tumor concentrations did not increase in proportion to those in plasma. This may indicate that drug uptake into the lymphoma was concentration dependent.

dTTP depletion, subsequent dUMP accumulation (9), and cell cycle arrest (25, 26) are early events after TS inhibition, although prolonged inhibition of TS is required to produce cytotoxicity (2, 27). In some cell lines, fraudulent misincorporation of uracil into DNA also causes DNA damage and cytotoxicity (28–30). dTTP pools in the L5178Y TK−/− tumor were depleted within 4 h after both the infusional and bolus doses of ZD9331 used in this study, although the time for which this perturbation persisted was shorter for the bolus dose (~16 h) than for the infusional protocol (>16 h). The reduced pools of both dTTP and dUMP observed at the end of the infusion and 4 h after the infusion, when plasma drug levels were falling, may be attributable to a loss of viability in tumor cells. As has been reported previously in in vitro experiments using L5178Y TK−/− cells (31), only modest increases in dUMP pools were measured in the lymphoma cells in vivo, and it is unlikely that uracil misincorporation has a major role to play in the loss of cell viability. It has been shown previously that some human tumor cell lines accumulate little or no dUTP after inhibition of
TS, and this was associated with high expression and activity of deoxuryridine triphosphatase (32).

Clonogenic assays in vitro using the L5178Y TK−/− cell line have suggested that exposure times >4 h are required for ZD9331 to exert a significant effect on growth inhibition and cytotoxicity. A 24-h exposure to 1 μM and 0.1 μM ZD9331 resulted in a 5 and 3.5 log loss of colony formation, respectively (4). The pharmacokinetic/pharmacodynamic relationship in vivo is more complex. (33). Pharmacokinetic parameters such as maximum concentrations (Cmax), drug exposure (AUC), and time above a threshold plasma concentration have been used to relate pharmacokinetics to response. For anticancer drugs, tumor pharmacokinetics are also important, but they are difficult to measure. The pharmacokinetic/pharmacodynamic relationship with the mouse lymphoma model used here may also be confounded by an immune component (11). The importance of the apparent long terminal elimination phase of ZD9331 to cytotoxicity is not known.

In summary, steady-state ZD9331 plasma and tumor concentrations during the infusion schedule (3 mg/kg) above 0.5 μM (a concentration within the range that causes significant loss of viability) were achieved for ~24 h. This resulted in reduced dTTP pools for at least 16 h after s.c. infusion. It is interesting that after an infusional dose of 1 mg/kg (shown previously to produce antitumor activity and cures in some animals (8 of 15; Ref. 4), dTTP pools were also significantly depleted by 4 h. However the extent of depletion was marginally less than that seen at the curative dose of 3 mg/kg. However, after the i.p. bolus injection of ZD9331, a concentration of 0.5 μM was achieved in the plasma for <16 h, and in the lymphoma tissue this concentration was exceeded only for <6 h. The apparent result of this is that dTTP depletion occurred for a shorter time and had fully recovered by 16 h. Although the time above a threshold (effective) concentration was greater in the infusional protocol, total plasma drug exposure as measured by AUC was greater (~5-fold) after the bolus dose, resulting in ~2-fold greater tumor AUC (estimated by extrapolation to time 0). This may have resulted in significant depletion of dTTP earlier than the 4-h time point measured in this study.

Thus no clear pharmacokinetic reason has emerged for the equal antitumor activity of an infusional and bolus dose of ZD9331, although, because a 25 mg/kg bolus dose is also curative, exposure above a critical concentration as well as duration of effective drug levels are important factors. Ongoing studies using the murine model described here aim to relate pharmacodynamic events in the tumor (dTTP and dUMP pools) with plasma and tumor pharmacokinetics information and will include the measurement of plasma deoxuryridine (34) as another pharmacodynamic measure of TS inhibition. As the direct measurement of tumor dTTP pools is not feasible in the clinical situation, such studies may provide useful information for optimizing clinical schedules.

REFERENCES


Pharmacokinetic/Pharmacodynamic Study of ZD9331, a Nonpolyglutamatable Inhibitor of Thymidylate Synthase, in a Murine Model Following Two Curative Administration Schedules


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