In Vitro and in Vivo Antitumor Activity of ZENECA ZD0490, a Recombinant Ricin A-Chain Immunotoxin for the Treatment of Colorectal Cancer

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ABSTRACT

ZENECA ZD0490 is a recombinant ricin A-chain-containing immunotoxin that recognizes an antigen that is expressed on approximately 65% of colorectal tumors. The antigen CA242 is recognized by a mouse monoclonal antibody designated C242. C242 antibody was conjugated to recombinant ricin A-chain via a methyl-hindered disulfide linker which confers in vivo stability. ZD0490 was extremely potent against colorectal cell lines CoLo201 and CoLo205, which express the CA242 antigen. ZD0490 activity was determined in vitro by both protein synthesis inhibition (50% inhibitory concentrations of 1–20 ng/ml after 24-h exposure) and clonogenic assay (76–95% cell kill after 24-h exposure to a 50% inhibitory concentration for protein synthesis inhibition; >99.99% cell kill at 1000 ng/ml). This in vitro activity was translated to in vivo efficacy. ZD0490 activity of ZD0490 detailed in this paper show that this novel immunotoxin is worthy of clinical evaluation, which is currently under way in the United Kingdom.

INTRODUCTION

Antibody targeting of plant or bacterial toxins as an approach to the therapy of solid tumors and leukemias has been under investigation for several years, and immunotoxins have a number of unique properties when compared to conventional chemotherapeutic drugs or radiation therapy (1, 2). Notably, immunotoxins act through toxin-mediated prevention of protein synthesis and not DNA damage or replication inhibition. Hence, their action is not dependent on the proliferation status of the tumor cell. Additionally, provided that the relevant target antigen is not expressed, immunotoxins are not toxic to normal tissues. Despite these potential advantages, clinical success in the treatment of solid tumors with immunotoxins has been limited. Problems that have been encountered include low selectivity of the targeting antibody (3–5), capillary leak syndrome (3, 4, 6), rapid immunotoxin clearance from the plasma (7), and simply limited activity.

ZD0490 is a novel immunotoxin which comprises C242, a highly tumor-selective antibody which was generated by the immunization of mice with the CoLo205 human colorectal cell line. C242 has been demonstrated to bind to approximately 65% of more than 200 human colorectal tumor samples studied to date (8). The CA242 antigen that is recognized by C242 antibody is associated with the CANAG glycoprotein, as are the CA19–9 and CA50 antigens (9, 10). The CA242 antigen recognized by the C242 monoclonal antibody is frequently present in the serum of patients with colorectal carcinoma and is predictive of neoplastic disease having a higher sensitivity than carcinoembryonic antigen for Dukes grade B and C colorectal malignancy (10, 11). Thus, an immunotoxin targeted to the CA242 antigen could have potential in the effective management of human colorectal cancer.

To generate ZD0490, C242 monoclonal antibody was conjugated to recombinant ricin A-chain via the methyl-hindered disulfide linker first described by Worrell et al. (12). Aglycosyl or deglycosyl toxin A-chains and sterically hindered disulfide linkers have been shown to confer favorable pharmacokinetic and efficacy profiles in preclinical models (13, 14). Thus, hepatic clearance of the immunotoxin is reduced by the removal of carbohydrate residues from the toxin, and a stable disulfide bond enhances the in vivo persistence of the conjugate (13–15). In addition, disulfide stability limits the levels of free antibody that are released and which may compete with intact immunotoxin for tumor binding and uptake. Combination of a recombinant and hence aglycosyl toxin in ZD0490 with a stable linker has produced a conjugate with excellent pharmacokinetic properties, i.e., slow plasma clearance and a prolonged half-life (16).

The data reported here show that ZD0490 has potent activity against selected human colorectal cell lines in vitro at concentrations that can easily be achieved and maintained in plasma in mice after single-dose administration (16). The in vivo efficacy of ZD0490 was demonstrated using tumor growth delay assays and inhibition of [14C]leucine incorporation. In particular, the schedule and concentration/dose dependency of ZD0490 action was studied both in vitro and in vivo.

MATERIALS AND METHODS

Culture of Human Colorectal Cell Lines. The human colorectal carcinoma cell lines CoLo201, CoLo205, HT29, and HCT116 were obtained from the American Type Culture Collection and were maintained in RPMI 1640 (Northumbria Biologicals, Ltd., Cramlington, Northumberland, United Kingdom) supplemented with 10% fetal bovine serum (Globepharm, Ltd., Esher, Surrey, United Kingdom) and 2 mM glutamine (GIBCO-BRL, Paisley, Scotland) and buffered with 7.5% sodium carbonate (v/v) (GIBCO-BRL). Cells were incubated at 37°C and gassed with 95% CO2 and 5% O2. Cells were subcultured at 4-day intervals to ensure that they did not become confluent. All tissue culture plasticware were supplied by Nunc GIBCO Life Technology.

ZD0490 and C242. ZD0490 was supplied as a solution by ZENECA Pharmaceuticals in different batches (for experimental use only) that were all characterized in terms of antibody to ricin A-chain ratios and ranged from 0.592–2.41 mg/ml of total protein. Unconjugated C242 antibody was also supplied by ZENECA Pharmaceuticals at a concentration of 1 mg/ml. The negative control MOPC21 immunotoxin was synthesized by ZENECA Pharmaceuticals using the same linker, recombinant ricin A-chain, and methodology as that for ZD0490.

Flow Cytometry of Cell Lines. Cells were trypsinized from flasks and 5 × 106 cells in 0.25 ml of Hanks’ buffer (ICN Flow High; Wycombe, Buckinghamshire, United Kingdom) were incubated with C242 antibody at a final concentration of 2 μg/ml. Cells were left to incubate at 4°C for 30 min in the dark, centrifuged at 500 × g for 5 min at 4°C, and washed twice with 250 μl of Hanks’ buffer. The cells were then incubated with goat anti-mouse IgG antibody conjugated to fluorescein isothiocyanate at 1 μg/ml (Sigma

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1 Supported by the North of England Cancer Research Campaign.

2 To whom requests for reprints should be addressed.
Chemical Co., Poole, Dorset, United Kingdom) for an additional 30 min. MOPC21 (Sigma) antibody (2 μg/ml) was used as a class-matched (mouse IgG1) negative control antibody. Cells were treated in an identical manner with this control antibody. All samples were run on a FACScan flow cytometer (Becton Dickinson). Propidium iodide (Sigma) was added to all samples, 0.1 μg per sample, prior to analysis to enable identification of dead cells. In all cases, dead cells accounted for less than 5% of total cell number. Cells were considered as CA242-positive if the binding profile was shifted when compared to the binding of the irrelevant MOPC21 antibody.

In Vitro Protein Synthesis Inhibition Studies. The activity of ZD0490 against human colorectal tumor cell lines was studied using [3H]leucine incorporation as a measure of protein synthesis. Cells (2 × 10⁵) were plated per well in 96-well plates (Nunc) in 100 μl of medium and were then left to adhere for 18 h prior to treatment with ZD0490. The medium was then removed, and ZD0490 was added in fresh medium at a range of concentrations from 0.0001 to 1000 ng/ml. Each concentration was repeated in 5 wells of the plate, and controls were treated with fresh medium. Cycloheximide (Sigma) was used as a positive control as a known, nonselective, inhibitor of protein synthesis. After a given exposure time (routinely 24 h), the immunotoxin was removed and replaced with labeling medium containing [3H]leucine (70 Ci/mmol; Amersham International PLC, Buckinghamshire, United Kingdom) in fresh medium at a specific activity of 1.5 μCi/well. After 6 h, the cells were washed and trypsinized from the plate using 100 μl of 2.5% trypsin (GIBCO) per well. The cells were then harvested onto glass fiber filter paper (Whatman, Maidstone, Kent, United Kingdom) using a cell harvester (Titertek ICFN Flow) and rinsed for 10 s with distilled water followed by 10 s with methanol. After the filter papers had dried in air, the amount of protein-incorporated [3H]leucine on the filters was quantified by the addition of 5 ml of Optipath scintillation fluid (LKB Scintillation Products) to the individual filter papers in 10-ml scintillation vials, which were then counted on a beta counter. Results were obtained as cpm. Results were analyzed using GraphPad software (San Diego, CA), and protein synthesis in treated cells was expressed as a percentage of control levels of [3H]leucine incorporation in untreated cells. A no nonlinear, four-parameter logistic equation was fitted to the data, which enabled the calculation of an IC₅₀. Experiments were also performed in the same way with the addition of unconjugated C242 or MOPC21 antibody at a concentration of 10,000 ng/ml in the medium during the 24-h incubation with ZD0490.

Soft Agar Clonogenic Assay. CoLo201, CoLo205, and HT29 cells were plated at 5 × 10³ cells/well in 6-well (35-mm) plates and left overnight to adhere. The medium was then removed and replaced with fresh ZD0490-containing medium at concentrations ranging from 0.0001 to 1000 ng/ml. After incubation with immunotoxin for fixed times ranging from 0.5-24 h, the cells were trypsinized using 0.25% trypsin and plated out in 0.25% Noble agar in 60-mm dishes using 2.5 ml of medium with 5% FCS per dish. The plates were incubated at 37°C for 1 week before they were inspected. Plates were considered to be positive when the survival fraction of the treated samples was 50% compared with the untreated control. Colonies were then stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 1 mg/ml in phosphate-buffered saline and incubated in an atmosphere (5% O₂, 5% CO₂, and 90% N₂) for 21 days to allow for the growth of same cell type were plated with the cells to aid colony growth at low cell density. Control cells have a surviving fraction of 100%. The plating efficiency was measured as the ratio of the number of colonies counted to the number of cells plated. Colonies were scored as positive when they contained more than 100 cells.

Determination of CA242 Expression by Flow Cytometry of Human Colorectal Cell Lines. Flow cytometry of the human colorectal cell lines used in these studies showed a range of binding levels for C242 monoclonal antibody. The fluorescence profiles of the CoLo201 and CoLo205 cell lines both demonstrated strong homogeneous binding of C242 (>95% of cells are positive), indicating a high level of expression of CA242 antigen. There was no detectable difference between the degree of C242 binding to CoLo201 and CoLo205 cell lines. The HCT116 human colorectal cell line gave a negative binding profile with C242 antibody, indicating no significant expression of C242 on the cell surface, and the HT29 colorectal cell line showed a heterogeneous profile for C242 binding. HT29 had a large population of positive-staining cells (approximately 60%) but also a significant number of negative cells. This heterogeneous antigen expression profile appears to be not uncommon in cultured cell lines and may be related to the expression of C242 by adenocarcinoma cell lines previously isolated from human colorectal tumors. CoLo201 and CoLo205. On exposure to ZD0490 for 24 h, protein synthesis was inhibited by 50% of the untreated control value.
Thus, in repeated experiments, CoLo201 proved to be between 3 and 8 times more sensitive to the effect of ZD0490. This difference was highly significant (Student t test, P = 0.0006). Protein synthesis inhibition in the HT29 cell line was more variable with IC_{50}s that ranged between 10 and 200 ng/ml ZD0490 (Fig. 2). Importantly, complete inhibition of protein synthesis could not be achieved, even at concentrations as high as 10,000 ng/ml of ZD0490, suggesting a subpopulation of CA242-negative cells as would be anticipated by the C242 binding profile seen on flow cytometric analysis. The HCT116 colorectal tumor cell line was relatively insensitive to ZD0490 in the protein synthesis assay such that 50% inhibition could not be achieved (Fig. 2). Incubation of CoLo205 cells with either recombinant ricin A-chain alone at concentrations up to 10 μg/ml or the irrelevant class-matched MOPC21 antibody-recombinant ricin A-chain conjugate at concentrations up to 1 μg/ml produced no inhibition in cellular protein synthesis.

Time course experiments using both the CoLo201 and CoLo205 cell lines showed that maximal inhibition of protein synthesis by ZD0490 could be achieved by exposing cells to ZD0490 for 24 h, when protein synthesis was determined 21.6 h later (total exposure time, 24 h). For example, the IC_{50} after a 2.4-h exposure to ZD0490 was 5.5 ng/ml for the CoLo205 cell line as compared to 4.2 ng/ml after 24 h exposure. Addition of 10,000 ng/ml of unconjugated C242 antibody reduced the sensitivity of CoLo205 cells to ZD0490 by 18-fold and CoLo201 cells by 70-fold. The addition of the class-matched irrelevant antibody MOPC21 at 10,000 ng/ml did not affect the potency of ZD0490 in either cell line.

**Determination of ZD0490 Activity in Vitro using Soft Agar Clonogenic Assay.** The soft agar clonogenic assay was used to demonstrate that ZD0490-induced protein synthesis inhibition resulted in cytotoxicity and to investigate further the exposure time dependency of cytotoxicity. The clonogenic assay confirmed the relative insensitivity of the HT29 and HCT116 cell lines, i.e., incubation for 24 h with 1000 ng/ml ZD0490 resulted in less than 1 log of cell kill. With both the CoLo201 (Table 1) and CoLo205 (Table 2) cell lines, 24 h exposure to ZD0490 at a concentration of 1000 ng/ml resulted in greater than 99.99% cell kill. Concentrations that approximated the IC_{50} for protein synthesis inhibition, 1 ng/ml and 10 ng/ml for CoLo201 and CoLo205, respectively, gave a more modest effect of 76 and 95% cell kill. Time course experiments with both CoLo201 and CoLo205 cells demonstrated that cytotoxicity was induced after only 30 min exposure to ZD0490 (Tables 1 and 2). Indeed, a concentration of 1000 ng/ml ZD0490 was capable of inducing greater than 99.9% cell kill after just 30 min exposure in CoLo201 cells. Thus, cytotoxicity was both concentration and time dependent in both cell lines. To illustrate this relationship, Fig. 3 shows the product of ZD0490 concentration and exposure time plotted against the surviving fraction of cells for the CoLo201 cell line. A very strong correlation between cytotoxicity and the concentration × exposure time product was observed (r^2 = 0.98; Fig. 3). A similar relationship is also obtained with the CoLo205 cell line (r^2 = 0.93).

**In Vivo Tumor Growth Delay Studies.** Having demonstrated the concentration, time, and antigen expression dependency of ZD0490 activity in *vitro*, experiments were performed to define the *in vivo* efficacy of the conjugate. A pilot study in BALB/c mice produced a 10% lethal dose of 5.4 mg/kg (95% confidence limits, 4.9–5.8 mg/kg) as a single i.v. dose (data not shown). The *in vivo* experiments were then performed using a dose of approximately 50% of the 10% lethal dose, i.e., 2.5 mg/kg. Prolonged tumor growth inhibition was observed in all CoLo201 ZD0490-treated mice. In CoLo205 tumors, transient tumor regression was observed with a median growth delay of 16 days. However, it should be noted that the rate of growth of the CoLo201 tumors in vivo was considerably slower than for the CoLo205 tumors. Despite this, when specific growth delays were calculated by dividing the growth delay observed (in days) by the doubling time of the tumor (in days), the CoLo201 tumors retained their greater sensitivity to ZD0490 with a median specific growth delay of 6.6 as compared to 4 in the CoLo205 model. Administration of either a MOPC21-recombinant ricin A-chain conjugate or unconjugated C242 and recombinant ricin A-chain at equimolar doses had no effect on CoLo205 tumor growth.

In the CoLo201 tumor model using growth delay analysis, it was not possible to determine if there was a significant difference between the effect of a single 2.5 mg/kg dose of ZD0490 compared with a 5-day schedule at 0.5 mg/kg. With the single 2.5 mg/kg dose of ZD0490, 5 of 10 mice had no detectable tumor deposits, and a sixth mouse gave a growth delay of 38 days. For the repeated 0.5 mg/kg administration of ZD0490, 4 of 10 mice had no detectable tumor deposits, whereas an additional three mice gave growth delays of 30, 37, and 52 days. The remaining mice from each group were killed prematurely due to loss of body weight. With the CoLo205 tumor...
were exposed to a range of ZD0490 concentrations for 0.5, 2.4, or 24 h, followed by the effect of single and repeated dose administration. In this model, it was the product of the exposure time and ZD0490 concentration is plotted against surviving fraction. For further details, see "Materials and Methods."

Cells had a plating efficiency of 55% and are designated a surviving fraction of 100%. The lack of activity in the HCT116 and HT29 models confirms that the anti-tumor effect that was observed in the CoLo201 and CoLo205 tumors was a specific effect and was not caused by general toxicity.

Growth following ZD0490 treatment, despite incurring similar reductions in body weight. The median growth delays being — 1 day for the single 2.5 mg/kg dose was 17 days and for the daily 5X 0.5 mg/kg schedule, the median delay was 15 days. Some body weight loss was observed in all of the ZD0490-treated animals, but this toxicity was not the cause of the delay in tumor growth. Thus, the CA242-negative cells was unlikely to be responsible for the regrowth of the tumor. Immunohistochemistry of CoLo201 tumors remaining after treatment with ZD0490 revealed that there was still extensive and homogeneous expression of CA242 antigen; hence, a population of antigen-negative cells was unlikely to be responsible for the regrowth of the tumor.


Table 1 Effect of exposure time on ZD0490-induced cytotoxicity in CoLo201 cells

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Percentage$^a$ of surviving CoLo201 cells at ZD0490 concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>2.4</td>
<td>92</td>
</tr>
<tr>
<td>24</td>
<td>98</td>
</tr>
</tbody>
</table>

* Control cloning efficiency is designated as 100%; ND, not determined.

Table 2 Effect of exposure time on ZD0490-induced cytotoxicity in CoLo205 cells

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Percentage$^a$ of surviving CoLo205 cells at ZD0490 concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>2.4</td>
<td>ND</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
</tr>
</tbody>
</table>

* Control cloning efficiency is designated as 100%; ND, not determined.

Fig. 3. The effect of ZD0490 exposure time on cytotoxicity in CoLo201 cells. Cells were exposed to a range of ZD0490 concentrations for 0.5, 2.4, or 24 h, followed by incubation for approximately 21 days after plating in agar for colony formation. Untreated control cloning efficiency is designated as 100%; ND, not determined.

Twenty-four h after a single i.v. dose of 2.5 mg/kg ZD0490, protein synthesis in CoLo201 tumors was reduced to 48 ± 19% (20–58%; n = 7; Fig. 4) and 58 ± 17% (19–116%) in CoLo205 tumors (n = 26; Table 3). Administration of a 2.5 mg/kg dose of the irrelevant immunotoxin (MOPC21 antibody conjugated to recombinant ricin A-chain) did not influence tumor protein synthesis (90 ± 18% of control levels; n = 4). $^{[14C]}$Leucine incorporation in all other tissues studied (liver, kidney, and brain) was unaffected by ZD0490 administration. Administration of the nonselective protein synthesis inhibitor cycloheximide (100 mg/kg) to mice caused a reduction in protein synthesis of up to 90% in all tissues studied (Fig. 4). A range of doses of ZD0490 from 0.5 to 5 mg/kg were studied in the CoLo205 tumor model (Table 3).

Fig. 4. The effect of cycloheximide and ZD0490 on $^{[14C]}$leucine incorporation in normal tissues and CoLo201 tumors. Columns, the mean of samples from six or seven mice; bars, SD. Cycloheximide was administered i.v. at 100 mg/kg 1 h prior to $^{[14C]}$leucine (total exposure time, 7 h). ZD0490 was administered i.v. at 2.5 mg/kg 18 h prior to $^{[14C]}$leucine (total exposure time, 24 h).

Table 3 Effect of a single dose of ZD0490 on protein synthesis in CoLo205 tumors

<table>
<thead>
<tr>
<th>Dose of ZD0490 (mg/kg)</th>
<th>No. of tumors</th>
<th>Mean (% control)</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>4</td>
<td>89%</td>
<td>28%</td>
<td>NS</td>
</tr>
<tr>
<td>1.0</td>
<td>6</td>
<td>74%</td>
<td>12%</td>
<td>.044</td>
</tr>
<tr>
<td>1.25</td>
<td>6</td>
<td>89%</td>
<td>11%</td>
<td>NS</td>
</tr>
<tr>
<td>2.5</td>
<td>26</td>
<td>58%</td>
<td>17%</td>
<td>.0001</td>
</tr>
<tr>
<td>5.0</td>
<td>13</td>
<td>55%</td>
<td>18%</td>
<td>.0001</td>
</tr>
</tbody>
</table>

Data are expressed as a % of control rate of protein synthesis at 24 h after administration of ZD0490. P gives the level of significance of the inhibition compared with control tumors.
Table 4 Effect of ZD0490 dose schedule on protein synthesis inhibition in CoLo205 tumors

<table>
<thead>
<tr>
<th>Dose of ZD0490 (mg/kg)</th>
<th>Schedule (to total dose of 2.5 mg/kg)</th>
<th>No. of tumors</th>
<th>Mean (% control)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>Single dose</td>
<td>26</td>
<td>58%</td>
<td>17%</td>
</tr>
<tr>
<td>0.5</td>
<td>Daily X 3</td>
<td>10</td>
<td>55%</td>
<td>16%</td>
</tr>
<tr>
<td>0.83</td>
<td>Days 1, 3, and 3b</td>
<td>4</td>
<td>52%</td>
<td>23%</td>
</tr>
</tbody>
</table>

*a 0.5mg/kg given daily for 5 days.
*b 0.83mg/kg given on alternate days for three doses.

Table 5 Level of protein synthesis in CoLo205 tumors 24, 48, 72, and 96 h after 2.5mg/kg ZD0490

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>No. of tumors</th>
<th>Mean (% control)</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>26</td>
<td>58%</td>
<td>17%</td>
<td>.0001</td>
</tr>
<tr>
<td>48</td>
<td>12</td>
<td>50%</td>
<td>19%</td>
<td>.0001</td>
</tr>
<tr>
<td>72</td>
<td>9</td>
<td>60%</td>
<td>16%</td>
<td>.0002</td>
</tr>
<tr>
<td>96</td>
<td>4</td>
<td>58%</td>
<td>9%</td>
<td>.0009</td>
</tr>
</tbody>
</table>

*a Level of significance of the inhibition compared with control tumors.

In vitro and in vivo activity of ZD0490

However, no marked inhibition of total tumor protein synthesis was observed at doses below 2.5 mg/kg. In addition, there was no significant increase in the protein synthesis inhibitory effect of ZD0490 when the dose was doubled to 5 mg/kg. Similarly, dividing the dose of ZD0490 immunotoxin administration over 5 days did not significantly improve the protein inhibitory effect that was achieved (Table 4). Administration of the nonbinding MOPC21-recombinant ricin A-chain immunotoxin at a single bolus dose of 2.5 mg/kg did not cause a significant reduction in tumor protein synthesis (tumor protein synthesis, 90 ± 12% of controls) at 24 h.

When protein synthesis was studied at later time points after a single dose of 2.5 mg/kg ZD0490 in the CoLo205 tumor model, there was a prolonged reduction in protein synthesis for 96 h after a single dose of 2.5 mg/kg, the latest time point studied (Table 5). The level of protein synthesis did not go below approximately 50% of control values after the administration of 2.5 mg/kg ZD0490, which is in contrast to the effect of cycloheximide where protein synthesis decreased to less than 20% of control levels (Fig. 4).

**DISCUSSION**

ZD0490 is a recombinant ricin A-chain immunotoxin with high potency against CA242-positive human colorectal tumor cell lines. The CoLo201 and CoLo205 cell lines were indistinguishable by flow cytometry in terms of CA242 expression, and both showed complete inhibition of protein synthesis after in vitro treatment with ZD0490 for 24 h (Fig. 1). However, the CoLo201 cell line was routinely more sensitive than the CoLo205 cell line with mean IC50 of 2.2 and 14 ng/ml, respectively. The HT29 cell line showed heterogeneous CA242 antigen expression as determined by flow cytometry and was relatively insensitive to ZD0490 (Fig. 2). Heterogeneous CA242 expression and consequently, reduced immunotoxin potency, has been reported previously by Debinski et al. (17) in studies with a *Pseudomonas* exotoxin-C242 conjugate. The HCT116 cell line was entirely negative for CA242 expression and was insensitive to ZD0490 at concentrations as high as 10,000 ng/ml (Fig. 2). These data suggest that CA242 expression is a primary requirement for ZD0490 sensitivity, and this conclusion is supported by the observation that the addition of excess unconjugated C242 antibody caused an 18–70-fold reduction in the IC50 for the two sensitive cell lines. However, the number of cell lines that have been demonstrated to be positive for CA242 expression does not appear to reflect the situation seen with human colorectal tumor samples. Immunohistochemistry of colorectal tumor samples reveals that approximately 65% are positive for CA242 expression (8). This discrepancy may be due to the loss of CA242 expression during the culture-adaptation process of cell lines, or it may reflect a reduced ability for CA242-expressing cells to become adapted to immortal cell lines.

Debinski et al. (17) also conducted experiments to investigate whether the *Pseudomonas* exotoxin-C242 conjugate could be displaced by unconjugated C242 antibody. These authors reported that an excess of 500-fold of C242 antibody was capable of totally blocking conjugate activity. The data presented here show a more modest effect. However, C242 specificity was further verified by the observation that MOPC21, a class-matched but nonbinding antibody, was not capable of diminishing ZD0490 activity at all. All cell lines studied were sensitive to a ricin A-chain Fib75 immunotoxin (18), indicating no innate resistance to the action of ricin A-chain.

In studies using the CoLo205 cell lines, a 24-h exposure to ZD0490 resulted in equivalent activity to a 24-h exposure when protein synthesis was determined 24 h later. This result indicates that antigen-antibody binding occurs very rapidly in this system. The occurrence of rapid extracellular binding would be in agreement with most other immunotoxins studied to date (19).

In clonogenic assays, low concentrations of ZD0490 produced extensive cell kill at concentrations comparable to those that can be achieved after a single nontoxic dose in mice (16), i.e., after a single 2.5-mg/kg dose of ZD0490 to BALB/c mice, plasma levels of greater than 20 μg/ml were achieved for 1 h and greater than 1 μg/ml for 96 h. Indeed, the results from the clonogenic assay suggest that the in vitro protein synthesis inhibition assay may slightly underestimate the effect of ZD0490. Disparity between protein synthesis inhibition and cytotoxicity has been reported previously as a feature of ricin A-chain immunotoxins (19), and Sung et al. (19) conclude from their studies that the kinetics of protein synthesis inhibition do not necessarily correlate with the efficiency of cell kill. This may in part explain why the CoLo205 cell line appeared less sensitive to ZD0490 in the protein synthesis inhibition assay, whereas the difference was not apparent using the clonogenic method.

The in vitro data obtained with ZD0490 demonstrated sufficient activity to warrant in vivo experiments. In vivo studies were performed with ZD0490 using two different end points to measure activity, tumor growth delay and tumor protein synthesis inhibition. The results of administration of 2.5 mg/kg of ZD0490 to both CoLo201 and CoLo205 tumor-bearing athymic mice demonstrated significant growth delays. However, in a comparison between a single 2.5-mg/kg dose of ZD0490 and 5 daily doses of 0.5 mg/kg ZD0490, no significant difference was observed between the two schedules. More markedly, in the study reported here using the CoLo205 cell line, it was not possible to differentiate between a single dose of 0.5 mg/kg and 2.5 mg/kg in their antitumor effect. It was this lack of sensitivity in the growth delay assay, also reported by Debinski et al. (17) using a *Pseudomonas* exotoxin-C242 conjugate, that led to the development of a new in vivo assay that potentially allows a more quantitative measure of efficacy by evaluating tumor protein synthesis inhibition.

In interpreting the in vivo efficacy data, the effect of CA242 antigen shed into the plasma has also been considered. It can be demonstrated that, in these mouse studies, the amount of ZD0490 present in the plasma after the doses used will be greatly in excess of the amount of circulating antigen, assuming stoichiometry between antibody and antigen (data not shown). However, this may not be the case in early clinical studies where initially low doses of ZD0490 will be used. However, dose escalation should ultimately allow cytotoxic free ZD0490 concentrations to be achieved.

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Following treatment with ZD0490, protein synthesis inhibition was observed in both the CoLo201 (Fig. 4) and CoLo205 tumors with no concurrent inhibition in the normal tissues that were studied. As a further reflection of the selective in vivo protein synthesis assay, administration of an equivalent dose of MOPC21-ricin A-chain immunoconjugate did not result in a significant reduction in tumor protein synthesis. In the in vivo protein synthesis assay, a dose-dependent effect on protein synthesis could be observed, with single doses of less than 2.5 mg/kg failing to produce a significant reduction in tumor protein synthesis. At the 2.5-mg/kg ZD0490 dose, there was a consistent reduction in tumor protein synthesis by 40–60%. Interestingly, however, the level of protein synthesis in tumors after the administration of ZD0490 was not reduced by more than 60%, and there are several possible reasons that may underlie this observation.

Due to their high molecular weight, penetration of immunotoxin molecules into the tumor mass may be problematic. The greater tumor protein synthesis inhibition induced by the administration of cycloheximide is consistent with this suggestion. Data produced by Shockley et al. (20, 21) suggest that antibody penetration into tumors of 4–12 mg in size may be a problem; however, the affinity of the antibody for the antigen can also influence tumor uptake. The concept of a binding-site barrier due to high affinity antibody binding has been described previously (22, 23). However, the prolonged plasma persistence of immunotoxins, such as that observed with ZD0490 in mice, may help penetration of the immunotoxin into the tumor despite such a barrier (20).

Another possible explanation for the residual protein synthesis that was observed after ZD0490 treatment may be the presence of a large number of host mouse cells within the tumors. These would also be more sensitive to the nonselective agent cycloheximide. Autoradiographic and histology studies need to be performed to define why complete inhibition of protein synthesis could be achieved in vitro but not in vivo.

Following a single dose of 2.5 mg/kg ZD0490, tumor protein synthesis inhibition was maintained for at least 96 h, the latest time point studied. Prolonged protein synthesis inhibition may be a reflection of the long plasma half-life of ZD0490, which results in the maintenance of cytotoxic immunotoxin concentrations. Alternatively, the prolonged effect may be due to an extended period of target cell protein synthesis inhibition prior to inducing a reduction in tumor size. Notably, reports suggesting immunotoxin activity in clinical trials indicate that where activity is observed, it develops, in some cases, more slowly than is usually observed with conventional cytotoxic agents (6).

In conclusion, the in vitro and in vivo data that are presented here (summarized in Table 6) demonstrate that ZD0490 is a highly potent and selective recombinant ricin A-chain anti-colorectal tumor immunotoxin. These results, in conjunction with pharmacokinetic (16) and toxicity data, provide preclinical support for the clinical study of ZD0490 currently being performed in the United Kingdom.

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