Activity of Anti-erbB-2 Recombinant Toxin OLX-209 on Lung Cancer Cell Lines in the Absence of erbB-2 Gene Amplification

Philip G. Kasprzyk,2 Terry L. Sullivan,3 Jay D. Hunt,4 Christopher T. Gubish, Cathy A. Scoppa,5 Mark Oelkuct,6 Robert Bird,7 Paul H. Fischer,8 Jill M. Siegfried,2 and C. Richter King9


ABSTRACT

The recombinant oncotoxin OLX-209 [e23(Fv)PE38KDEL] has been developed to target cancers with erbB-2 expression and is nearing a clinical trial. Important in clinical planning is the selection of patients on the basis of tumor expression of erbB-2. ErbB-2 gene amplification occurs in cancers of the breast, stomach, and ovary. Patients with these diseases and evident overexpression are candidates for OLX-209 therapy. In lung cancer, overexpression of erbB-2 is also frequent, but in most cases, it is not caused by gene amplification. This study demonstrates that OLX-209 has activity on lung cancer cells with varying levels of erbB-2 expression in the presence and absence of gene amplification. In vitro sensitivity of cell lines to OLX-209 is related to erbB-2 expression level. Normal bronchial epithelial cells were not sensitive. Effective treatment of lung cancer cell lines growing as xenografts in nude mice was shown with Calu-3 (a lung adenocarcinoma line with high levels of p185<sup>erbB-2</sup> caused by gene amplification) and three other lung adenocarcinomas (A549, NC1-H1466, and 201T) with lower levels of p185<sup>erbB-2</sup> and no gene amplification. The 201T cell line was isolated recently from a lung tumor with erbB-2 expression in the original tumor. The results of this study indicate that patients with erbB-2-positive, non-small cell lung cancer should be included in clinical trials of OLX-209.

INTRODUCTION

Lung cancer is the leading cause of death of all malignancies in the United States, and there is a clear need for improved treatments (1). One approach is to target the product of the erbB-2 (also known as HER-2/neu) proto-oncogene. Approximately 30% of cell lines and biopsy specimens of human lung cancer contain readily detectable levels of expression of p185<sup>erbB-2</sup>, which are higher than the surrounding lung epithelium (2, 3). In a report from China, more than 50% of primary non-small cell lung cancers expressed p185<sup>erbB-2</sup> (4). The contribution of this high level expression to malignancy is suggested by the association between erbB-2 expression and decreased survival (3, 5, 6). Frequent overexpression of erbB-2 has been observed also in breast and ovarian cancers (7, 8, 9). In these tumors, overexpression of erbB-2 is often a result of gene amplification. In contrast, lung cancer cell lines rarely contain measurable amplification (10). Lung cancer cell lines display a variety of erbB-2 protein expression levels that range from undetectable levels to quantities similar to those found in breast and stomach cancer cell lines that have amplified erbB-2 genes. These findings suggest that the underlying molecular biology of erbB-2 gene and protein expression may be different in lung cancers from that observed in the cases of breast, stomach, and ovarian cancers. The mechanisms of overexpression are being defined. In lung cancer, the mechanism leading to high levels of protein is linked apparently to increases in the amount of mRNA present (10). The exact mechanism leading to these increases is not clear. In cell lines derived from cancers of other tissues, transcriptional activation has been identified (11–13).

One approach to therapy of tumors with erbB-2 overexpression is to directly target the p185<sup>erbB-2</sup>-expressing tumor cell with a toxin. Recombinant molecules bearing an antibody-combining site fused to an enzymatic toxin have been created to target erbB-2 and other proteins on tumor cell surfaces. These agents, termed oncotoxins, have the potential advantages of extreme potency, small molecular size, and recombinant manufacture. In OLX-209 [e23(Fv)PE38KDEL], the specificity of an anti-erbB-2 antibody contained within a single-chain anti-
body domain (e23Fv) was coupled with a portion of *Pseudomonas* exotoxin (PE38KDEL), which has the ability to kill cells by inactivation of protein synthesis (14, 15). OLX-209 is undergoing advanced preclinical testing currently. Evidence of the binding of e23 to erbB-2 is derived from immunoprecipitation assays, enzyme-linked immunoassay results, and its ability to detect erbB-2 overexpression in immunohistochemical assays (16).

Previously reported preclinical testing of OLX-209 has focused on tumors having very high levels of overexpression caused by gene amplification (15, 17). In this study, we show that OLX-209 activity is not limited to cell lines with erbB-2 gene amplification. We used cell lines with expression levels ranging from 8- to 500-fold greater than the normal level (Table 1; Ref. 10). Clear antitumor efficacy is observed in models with overexpression of erbB-2 as low as 8-fold compared with normal cells. Because many primary tumors also express moderate levels of p185 erbB-2, patients with erbB-2-positive lung cancer are candidates for OLX-209 therapy.

### MATERIALS AND METHODS

#### Cell Lines and Tissue Culture

Human tumor cell lines Calu-3 and A549 were obtained from the American Type Culture Collection (Rockville, MD) and cultured as recommended. NCI-H1466 cells were provided kindly by Dr. Herbert Oie (National Cancer Institute, Bethesda, MD), have been described previously (18), and were subcultured routinely in RPMI 1640 supplemented with 5–10% fetal bovine serum. The 201T cell line was established from a poorly differentiated adenocarcinoma using the method of Siegfried and Owens (19, 20). It has been described previously (12). Cultures were maintained in humidified incubators at 37°C in an atmosphere of 5% CO2 and 95% air. Cells were tested for mycoplasma by Safe Cells (Bionique Testing Laboratories, Inc., Saranac Lake, NY).

**Expression, Purification, and Characterization of OLX-209.** Expression and purification of the fusion protein OLX-209 in *Escherichia coli* was performed as described (15). Briefly, OLX-209 was expressed under the direction of a lac control element and was induced by isopropyl-1-thio-[3-D-galactopyranoside induction. The recombinant protein was isolated as described (15). OLX-209 is un-}

### Table 1 p185\(^{erbB-2}\) expression and OLX-209 activity in non-small cell lung cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor type</th>
<th>Gene amplification(^a)</th>
<th>Protein(^b) (ng/ml)</th>
<th>PSI, IC(_{50}) (ng/ml)</th>
<th>In vivo activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calu-3</td>
<td>Adenocarcinoma</td>
<td>8</td>
<td>100</td>
<td>2</td>
<td>++</td>
</tr>
<tr>
<td>NCI-H1466</td>
<td>Adenocarcinoma</td>
<td>1</td>
<td>62</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>A549</td>
<td>Bronchioalveolar</td>
<td>1</td>
<td>2.6</td>
<td>30</td>
<td>++</td>
</tr>
<tr>
<td>201T</td>
<td>Adenocarcinoma</td>
<td>ND(^c)</td>
<td>2.3</td>
<td>ND</td>
<td>++</td>
</tr>
<tr>
<td>NBE</td>
<td>Normal cells</td>
<td>1</td>
<td>0.3</td>
<td>&gt;1000</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) From Kern et al. (10).

\(^{b}\) Protein data determined by Western blotting (Fig. 2) Number is relative value compared with Calu-3.

\(^{c}\) ND, not determined.

and were substantially free of bacterial endotoxin. The activities of all preparations used in this study were verified by measuring PSI\(^{10}\) (see below) on the N87 cell line; all preparations used in this study had IC\(_{50}\) of 0.2 ng/ml.

**In Vitro Activity Measurement of OLX-209.** The activity of OLX-209 was determined by assaying the inhibition of protein synthesis as described (15). Briefly, cells were plated in 96-well plates and 24 h later were washed once with media before the addition of OLX-209 for 18 h. 

**In Vivo Antitumor Assay.** Tumor cells (5 \(\times\) 10\(^{6}\)/mouse) were injected s.c. into the flanks of athymic nude mice. In all experiments, tumors were allowed to establish and grow to an average volume of \(~100–200\) mm\(^3\). Three i.v. injections of OLX-209, one every 2 days, were given. Tumor weight and growth were monitored every 3–4 days. Tumor growth is reported as an average relative tumor volume, calculated as

\[ \frac{w^2 \times h}{2} \text{ (mm}^3\text{)}, \]

where \(w\) is the width and \(h\) is the length of the tumor, measured with calipers.

**Immunoblot of p185\(^{erbB-2}\).** Cells were grown to confluence, washed twice with PBS, and extracted by scraping in 0.5 ml 1\% sample buffer [0.05 m Tris (pH 6.8), 1\% SDS, 10\% glycerol, and 0.01 g/ml bromophenol blue], followed by sonication. Protein was determined by the BCA method (Pierce Biochemicals). The samples were adjusted to contain 1\% \(\beta\)-mercaptoethanol and boiled. Samples were then loaded onto SDS-PAGE gradient gels (8–16%; Novex) and separated in 1\% Tris-glycine SDS running buffer. Gels were transferred to nitrocellulose. The blots were blocked in 1\% milk and 1\% BSA. Antibody incubation was conducted at 6.25 \(\mu\)g/mL (OM-11–

\(^{10}\) The abbreviations used are: PSI, protein synthesis inhibition; IC\(_{50}\), 50\% inhibitory concentration; NBE, normal bronchial epithelium; PE, *Pseudomonas* exotoxin.
RESULTS

Previous studies have reported differing expression of erbB-2 mRNA and protein among human lung tumor cell lines (10). The overall objective of our studies was to determine the sensitivity of these lung cancer cell lines to OLX-209 both in vitro and as tumors in nude mice.

OLX-209 Activity in Vitro on Lung Cancer Cell Lines. OLX-209 contains the portion of PE responsible for enzymatic inactivation of protein synthesis by ADP ribosylation of elongation factor 2 (21, 22). As a result, the activity of OLX-209 can be measured by its ability to inhibit protein synthesis, determined by the rate of incorporation of radioactive leucine into the protein. Fig. 1 shows protein synthesis inhibition by OLX-209 on three human lung cancer cell lines and NBE cells, which differ in erbB-2 expression level. The activity of OLX-209 on lung cancer cell lines is similar to that reported previously for cell lines of different tissue origin (23). The lowest IC_{50} values are observed in cell lines that have the highest erbB-2 levels, such as Calu-3 [similar to the breast carcinoma cell line SK-BR-3, known to have an amplified erbB-2 gene (7) and to the N87 human gastric cell line (15, 16)]. Interestingly, NCI-H1466 cells are very sensitive to OLX-209 and have high levels of p185_{erbB-2} (Table 1) but have no evidence of gene amplification. The higher values are obtained for cell lines that have lower-level erbB-2 expression, such as A549. Measurements of erbB-2 expression in human lung cancer cell lines indicate a range of mRNA levels from 12- to 220-fold above the NBE (10). These studies show that when cell lines were analyzed by Western blot, erbB-2 expression was approximately proportional to levels of mRNA (10).

Western blots of protein in cell line isolates used in this study confirm the relative level of erbB-2 expression among cell lines (Fig. 2). As seen in Fig. 2, Lane 1, Calu-3 cells, which have a copy number of eight genes, express high levels of p185_{erbB-2}. Compared with NBE cells (Lanes 2 and 5), Calu-3 cells express 500 times more p185_{erbB-2}. In the NBE cells, no protein was detectable using 25 μg protein extract (Lane 2); p185_{erbB-2} was detected with 50 μg protein extract (Lane 5). The other two lung tumor cell lines expressed more p185_{erbB-2} than NBE cells, but it was still considerably lower than Calu-3. Lanes 6 and 7 show that 201T and A549 cells express comparable levels of p185_{erbB-2}. Comparison of Lanes 4 and 7 shows the reproducibility of the determination using two preparations of 50 μg A549 extract.

The sensitivities of human tumor cell lines and NBE cells with varying degrees of erbB-2 expression to OLX-209 are summarized in Table 1, along with published information on the erbB-2 gene copy number (10). The data indicate that sensitivity in vitro to OLX-209 is related directly to the amount of p185_{erbB-2} expressed. The degree of PSI by OLX-209 toward...
various human cell lines derived from non-lung-derived tissues has been presented previously (15, 17, 23). Also, as reported previously, this potent activity is highly specific; the inhibition of protein synthesis on N87 and SK-BR-3 cells can be blocked by the addition of a 30–3000-fold excess of e23 antibody with no interference detected by a nonspecific IgG (23). Similar results were obtained with the A549 lung cancer cell line, in which no gene amplification is detected (data not shown). These data indicate that OLX-209 requires binding to the erbB-2 target. Consistent with this are PSI data showing little activity of OLX-209 on NBE cells, which have little erbB-2 expression (Fig. 1 and Table 1). As shown, protein synthesis remained above 50% of control even when OLX-209 was present at a concentration of 0.1 μg/ml. These data indicate that cancer cells in culture are 20–500 times more sensitive to OLX-209 than are their normal counterparts.

OLX-209 Antitumor Activity. To examine antitumor activity against lung cancer cell lines with differing levels of erbB-2 expression and in vitro sensitivity, we examined the effect of OLX-209 on the growth of four established human lung cancer cell lines in immunocompromized mice. These lines have a 50-fold range in erbB-2 expression and a 15-fold range in sensitivity to OLX-209 as measured by in vitro PSI (Table 1). These lines also represent examples with and without gene amplification. All experiments were conducted using established tumors, with tumor shrinkage considered the primary end point. As shown in Fig. 3, regressions in established tumor volume were observed with doses of OLX-209 of 43 μg/kg or more for all lung cancer cell line-derived tumors. The same schedule of administration (three i.v. doses, one every 2 days) was used in all experiments for ease of comparison. The LD<sub>10</sub> for OLX-209 on this schedule is approximately 90 μg/kg/dose;
to link our data on established cell lines more closely with the tissues they were derived showed moderate expression of erbB-2. In an attempt to test for the presence of erbB-2 in cell lines from primary lung tumors, we examined a cell line, 201T, that was recently isolated from a stage I, poorly differentiated adenocarcinoma. The 201T cells used in this study are 15–20 passages from the primary sample. 201T cells were identical with the 86-μg/kg dose, tumor volumes were reduced by about 50% by day 22. Thus, detectable expression by immunohistochemical staining in patient material may predict susceptibility to OLX-209 in vivo.

**DISCUSSION**

The cell surface presentations of functionally relevant proteins such as epidermal growth factor, fibroblast growth factor, and v-epidermal growth factor and their receptors have attracted much interest as targets for new cancer therapies (24–27). The erbB-2 gene product is a particularly promising example of such a cell surface protein, because it is found at high levels only in malignant cells. The high levels of erbB-2 protein that accompany gene amplification in cancers of the breast, ovary, and stomach are well documented (7–9). Accumulating evidence demonstrates that the enhanced erbB-2 expression seen in lung cancers is not associated with gene amplification in the majority of the cases. To plan the clinical testing of OLX-209 appropriately, it is important to know whether there is a rationale for its use in this setting. Our results show that the erbB-2-directed activity of OLX-209 can be very active in inhibiting protein synthesis in a variety of human lung cancer cell lines. As expected, cell lines with the highest levels of erbB-2 are most sensitive to OLX-209 in vitro. Antitumor activity in vivo does not require the very high levels of erbB-2 seen in association with gene amplification. Although the best antitumor responses are seen with the Calu-3 cell line, OLX-209 can produce clear antitumor responses in mice bearing three different human lung cancers without associated gene amplification. In fact, the response of A549 and 201T cells to OLX-209 was similar to the other lung cell lines expressing moderate amounts of erbB-2; at the 86-μg/kg dose, tumor volumes were reduced by about 50% by day 22. Thus, detectable expression by immunohistochemical staining in patient material may predict susceptibility to OLX-209 in vivo.

Several important studies of clinical lung cancer have detected erbB-2 overexpression using immunohistochemistry of formalin-fixed, paraffin-embedded samples (3, 4). In an attempt to link our data on established cell lines more closely with the responsiveness of primary lung tumors, we examined a cell line, 201T, that was recently isolated from a stage I, poorly differentiated adenocarcinoma. The 201T cells used in this study are 15–20 passages from the primary sample. 201T cells were selected for the study because the primary tumor from which they were derived showed moderate expression of erbB-2 by immunohistochemistry. In addition, the 201T cell line forms tumors in nude mice with approximately the same growth kinetics as the established cell lines tested (Fig. 1), allowing for a direct comparison of the data. In 7 of 10 primary lung cancer samples evaluable by histochemistry, the level of erbB-2 was equal to or greater than the level found in 201T. Five of these p185\(\text{erbB-2}\)-positive tumors were adenocarcinomas, and 2 were squamous cell carcinomas (data not shown). The level of erbB-2 expression in the 201T cell line was determined by Western blotting and compared with that in the available long-term lung cancer cell lines. Protein expression was comparable to cell lines A549 and approximately 12-fold greater than normal epithelium and 50-fold less than Calu-3 cells (Table 1). Fig. 3D shows that the response of 201T cells to OLX-209 was similar to the other lung cell lines expressing moderate amounts of erbB-2; at the 86-μg/kg dose, tumor volumes were reduced by about 50% by day 22. Thus, detectable expression by immunohistochemical staining in patient material may predict susceptibility to OLX-209 in vivo.

**Fig. 4** Therapy of tumors formed in mice by the human breast adenocarcinoma cell line MDA-MB-231, which contains low or undetectable levels of erbB-2 protein. Tumors were established by injection of 1 × 106 cells in athymic mice. Therapy was initiated 12 days following injection of cells. Treatment with OLX-209 (five mice/group) was given i.v. three times, once every 2 days. This tail vein injection was given with ~1% human serum albumin/PBS. Measurements were conducted externally with calipers. Tumor growth is reported as an average relative tumor volume.

The LD50 is approximately 160 μg/kg.11 Doses of 43 μg/kg on a schedule of three doses, one every 2 days, are routinely safe, producing no deaths or changes in weight. Apparent cures (defined as no evidence of tumors 4 months after treatment) of the Calu-3-derived tumors were identified in some animals. In the experiment shown (Fig. 3A) for Calu-3, one of five animals had only a residual tumor at day 36 at the 129 μg/kg dose. At the 86-μg/kg dose, there was also one apparent cure. There were no animal deaths in this experiment at any dose level. Tumors had only a residual tumor at day 36 at the 129 μg/kg dose. At the 86-μg/kg dose, tumor volumes were reduced by about 50% by day 22. Thus, detectable expression by immunohistochemical staining in patient material may predict susceptibility to OLX-209 in vivo.

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may be susceptible to therapies such as OLX-209 that target p185\textsuperscript{erbB-2}. The results of this study suggest that OLX-209 can have activity against cells that have moderate expression of p185\textsuperscript{erbB-2} and this could have implications for the normal tissue toxicity of this agent. Our results, which show that NBE cells have little sensitivity to OLX-209, are somewhat reassuring; however, it will be important to monitor for effects on tissues that have detectable levels of p185\textsuperscript{erbB-2}.

Immunohistochemical analysis of primary lung tumor material is the simplest method for evaluation of erbB-2 expression. We show that antitumor efficacy can occur for a recently isolated cell line, 201T, in which the original tumor had detectable but moderate expression of erbB-2. The immunohistochemical staining methods of this study were similar to those used in a reported analysis of more than 300 cases of lung cancer in which erbB-2 expression was detected in 30–50% of these cases (2–5). Therefore, it seems likely that the level of expression found in 201T would be judged positive in the previous studies. An appropriate cutoff point that predicts responsiveness to OLX-209 is not obtainable outside of clinical testing. Nevertheless, our results in animal models of human lung cancers with erbB-2 expression suggest the potential for OLX-209 as a therapy for lung cancer, perhaps in combination with other therapeutic agents. There is an urgent need for new therapeutics for this disease. The results of this study indicate that patients with erbB-2-positive, non-small cell lung cancer should be included in clinical trials of OLX-209.

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REFERENCES

Activity of anti-erbB-2 recombinant toxin OLX-209 on lung cancer cell lines in the absence of erbB-2 gene amplification.


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