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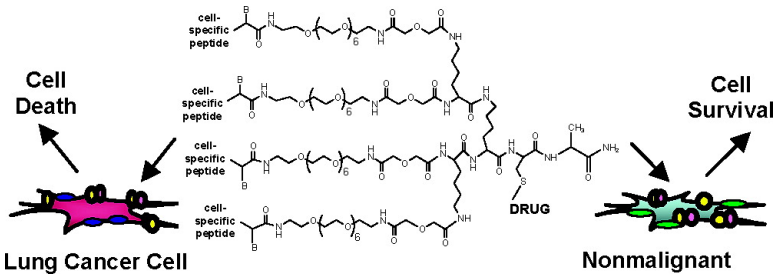


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# Cell-Specific Delivery of a Chemotherapeutic to Lung Cancer Cells

Xin Zhou, Ya-Ching Chang, Tsukasa Oyama, Michael J. McGuire, and Kathlynn C. Brown*

*Center for Biomedical Inventions and Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9185*

Received September 3, 2004; E-mail: Kathlynn.Brown@UTSouthwestern.edu

The nonspecific toxicity of anticancer drugs toward both cancerous and normal tissues can result in serious side effects, thereby limiting their clinical applications.[1] To overcome this obstacle, methods of delivering anticancer drugs preferentially to cancer cells have been sought. One approach is to conjugate a drug to a ligand that recognizes a known cell surface marker preferentially expressed on malignant cells. Most work in this field has focused on using antibodies as tumor-homing reagents, and monoclonal antibodies directed to tumor-associated antigens have been coupled to a variety of anticancer agents.[2]

While monoclonal antibodies display high affinity and specificity, they suffer from clinical limitations. To overcome the short comings of antibodies, peptides have been employed as targeting ligands. Peptides can be synthesized in large quantities, are amenable to derivatization, and are more accessible to solid tumors. A source of targeting peptides is from known peptidyl ligands that bind to cell surface receptors overexpressed in neoplastic cells.[3] When there is no known peptidic ligand for a desired cellular receptor, peptide libraries can be screened to isolate tumor-targeting ligands.[4] Phage display has been used to identify peptide ligands that bind to well-characterized tumor-associated cell surface receptors, including $\alpha_V\beta_3$ integrin,[5] HER2/neu,[6] transferrin receptor,[7] ErbB-2,[8] and ICAM-1.[9]

We recently reported the isolation of lung cancer-targeting peptides by panning a phage display library on intact cells.[10] Our approach requires no knowledge of the cellular receptor; thus, we can target tumor cells even when the appropriate tumor antigen is unknown. These peptides discriminate between cancerous and normal cells, suggesting that the peptides may have utility as delivery reagents in vivo. Here we report that the targeting peptides can be removed from the phage backbone and used to deliver a chemotherapeutic in a cell-specific fashion.

On the basis of the peptide sequences from our initial experiments, tetrameric peptide constructs were synthesized off a trilysine core using Fmoc chemistry[11] (Figure 1). Two peptide sequences were synthesized: TP H1299.1, which has cell-specific affinity for a large cell lung carcinoma cell line (H1299), and TP H2009.1, which binds to two lung adenocarcinoma cell lines (H2009 and H1648).[10,12] While the monomeric peptides bind to their target cell type, the tetrameric scaffold was chosen to increase the affinity of the ligand for its cell surface receptor by multivalent presentation of the peptides. Also, it is likely that the peptides must be multimerized to initiate cellular uptake, which is critical for intracellular drug delivery. A poly(ethylene glycol) (PEG) segment was added to each branch of the polymer to increase conformational flexibility and solubility of the drug conjugates.

To confirm that these peptides bind to their cellular receptor outside of the phage particle, the ability of these peptides to block uptake of their cognate phage was assessed (Figure 2). The TP H2009.1 tetrameric peptide inhibits phage uptake by >97% at 1 $\mu$M on both H2009 and H1648 cells, and the peptide exhibits phage

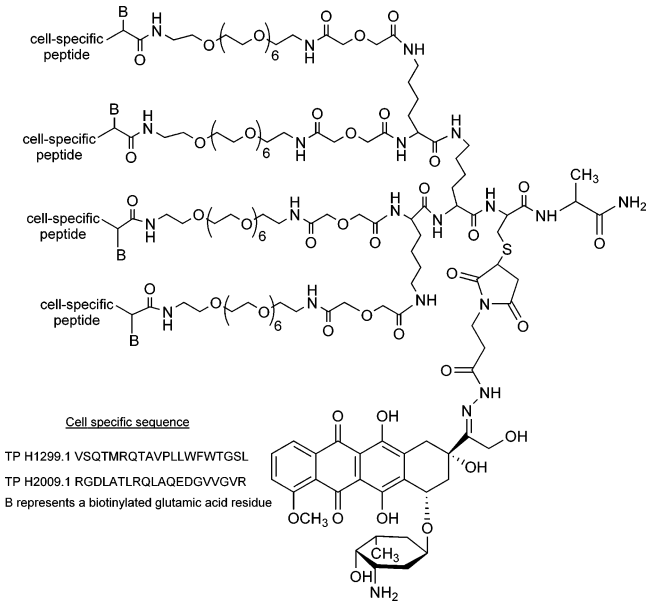


***Figure 1.*** Structure of the tetrameric peptide−doxorubicin conjugates, 1299.1M4-Dox, and 2009.1M4-Dox. The phage-blocking experiments were performed with the unconjugated peptides in which the cysteine was protected as an acetamidomethyl group.

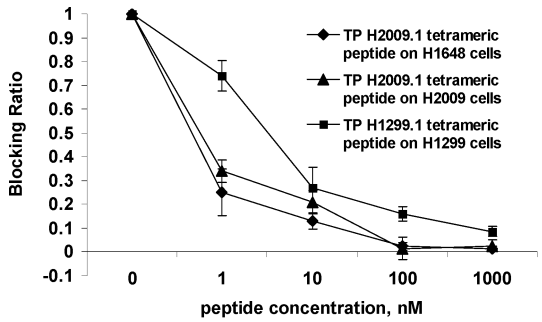


***Figure 2.*** Blocking of phage uptake by tetrameric peptides. The blocking ratio is the ratio of output phage to input phage in the presence of peptide normalized to the output/input ratio with no peptide added.

blocking at concentrations as low as 1 nM. Similar results are observed for the TP H1299.1 tetrameric peptide on H1299 cells, although less phage blocking is observed at 1 nM. The monomeric versions of both peptides show significantly reduced ability to block phage uptake,[10] supporting the need for multimerization of the peptide. We previously reported the synthesis of the tetrameric TP H2009.1 peptide without the PEG linker and its ability to bind H2009 cells.[10] Addition of the linker does not alter the affinity of the peptide scaffold for the cells as determined by this assay. However, it increases peptide solubility in aqueous solutions. This aids in purification and ease of handling of the peptides. We were unable to synthesize and purify the H1299.1 tetrameric peptide without the PEG linker due to insolubility.



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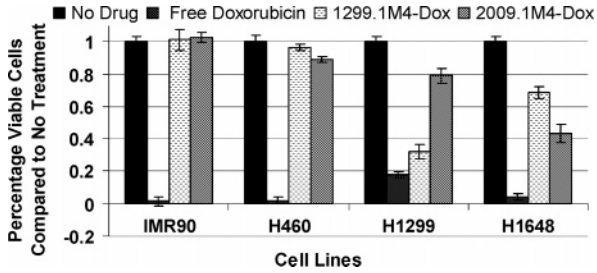


***Figure 3.*** Viability of cells treated with 10 $\mu$M doxorubicin, 1299.1M4-Dox, and 2009.1M4-Dox. Cells were exposed to the drug for 2 days followed by a 3 day recovery. Cell viability was determined by quantitation of ATP, a measure of viable, metabolically active cells. Cell viability is normalized against the untreated cells. Similar results are observed when the cells are exposed to the drug conjugates for 5 days.

As the cell-targeting tetrameric peptides bind their cellular target, we sought to determine if they could deliver an active therapeutic in a cell-specific fashion. The chemotherapeutic doxorubicin was coupled to the tetrameric peptides via a $\beta$-maleimidopropinoic linker at a unique cysteine placed before the branch point of the polymer (Figure 1).[13] The hydrazone linkage between the peptide linker and doxorubicin was chosen so that the drug could be released from the peptide under the acidic environment of the endosome.[14] To confirm that the drug could be released from the targeting moiety, the stability of the conjugate was assessed at acidic pH. The peptide−doxorubicin conjugate was found to release >50% doxorubicin at 37 °C within 24 h at pH 4, while <7% release was observed at neutral pH.

To evaluate the ability of the peptide conjugates to deliver active doxorubicin in a cell-specific manner, a cytotoxicity screen was conducted for four cell lines: IMR 90, H460, H1299, and H1648. IMR 90, a normal lung fibroblast cell line, and H460, a large cell lung carcinoma cell line, serve as negative controls as neither peptide displays significant affinity for these cells (Supporting Information). The H2009 cells were not tested in this assay as they are resistant to doxorubicin, even at high concentrations. Among the four cell lines, cell viability in the presence of free doxorubicin is <20%, and the normal lung fibroblasts are affected as well as the cancer cell lines (Figure 3). In contrast, cell viability of H1299 in the presence of 1299.1M4-Dox was 32 ± 4.5%, while no cell death was observed for H460 and IMR 90 cells. The cell viability of H1648 cells was slightly reduced (69 ± 3.6%) when exposed to 1299.1M4-Dox. This is not unexpected as TP H1299.1 peptide has a moderate affinity for H1648 cells. A similar pattern of cell viability was observed for 2009.1M4-Dox, with cell viability being reduced to 43 ± 4.5% for H1648 cells, but with little effect observed on IMR 90, H460, and H1299 cells. Treatment with unconjugated tetrameric peptides did not affect the growth of the cells, indicating that the decrease in cell viability is not due to the addition of the targeting peptide. Furthermore, addition of unconjugated tetrameric peptides had no effect on the toxicity of free doxorubicin.

The $IC_{50}$ values of doxorubicin, 1299.1M4-Dox, and 2009.1M4-Dox on the four cell lines were determined (Table 1). The $IC_{50}$ value of 1299.1M4-Dox toward H1299 cells is similar to that of free doxorubicin, but the peptide−drug conjugate is less toxic to the other three cell lines than free doxorubicin. The 2009.1M4-Dox is also more toxic to H1648 than to the other three cell lines. However, the H2009.1M4-Dox conjugate is 13-fold less potent than free doxorubicin on H1648 cells. Similar decreases in potency have been observed in other drug conjugates.[15] This may stem from low drug uptake, inefficient drug release from the peptide carrier, or incorrect cellular trafficking. These problems can be overcome by increasing the drug load of the conjugate or by changing the linkage of the drug to the targeting agent. Nonetheless, the increase in the therapeutic window observed suggests that higher concentrations of the peptide−drug conjugate may be employed in order to achieve the same drug efficacy while decreasing cell toxicity to normal cells.

***Table 1.*** Cytotoxicity of Doxorubicin and Peptide−Doxorubicin Conjugates against IMR 90, H460, H1299, and H1648 Cells ($IC_{50}$, $\mu$M)

| cell line | doxorubicin | 1299.1M4-Dox | 2009.1M4-Dox |
|---|---|---|---|
| IMR 90 | 1.0 ± 0.06 | ND[a] | ND[a] |
| H460 | 0.096 ± 0.007 | ND[a] | ND[a] |
| H1299 | 2.3 ± 0.02 | 4.2 ± 0.03 | >10[b] |
| H1648 | 0.56 ± 0.005 | >10[b] | 7.2 ± 0.03 |

[a] Less than 10% growth inhibition at 35 $\mu$M; $IC_{50}$ not determined. [b] Extrapolated from the growth inhibitory data. The $IC_{50}$ values were obtained from polynomial-fitted curves of cell viability versus concentration.

Our data indicate that these targeting peptides can deliver an active anticancer agent in a cell-specific fashion. Conjugation of cell-permeable drugs to the targeting peptides renders them cell-impermeable, except to the target cells. This results in an increase of the therapeutic index of the targeted drug compared to systemic delivery. The efficacy of the peptide conjugate correlates to the phage binding for a particular cell line. Thus, cell-specific targeted drugs can be synthesized, even when the cell surface target is unknown. Efforts to increase the drug load of the peptide conjugates and to test these targeted drugs in animals are underway.

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**Supporting Information Available:** Experimental methods for peptide and conjugate synthesis, cell viability assays, peptide selectivity assays, and $IC_{50}$ determinations. This material is available free of charge via the Internet at http://pubs.acs.org.

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