Characterization of Death Receptor 5 Targeting Nanoring Cancer Drugs

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Introduction

Death Receptor 5 (DR5) is a good target for drug delivery due to its overexpression on cancer cells. A cyclic ligand that was previously found to bind DR5 was modified for this study into a linear ligand, and both the cyclic and linear variants were attached to a dimeric species of Dihydrofolate reductase (DHFR-DHFR), forming two types of fusion proteins. Each type of fusion protein was constructed through attachment of the DR5 peptide to either the C-terminus or N-terminus of the DHFR-DHFR protein (Figure 1). The fusion proteins were manipulated to form either dimeric or octameric nanorings upon the addition of methotrexate, a toxic drug (Figure 2). The rings were further manipulated to release the methotrexate after cellular internalization, leading to enhanced cell-killing (Figure 3) .

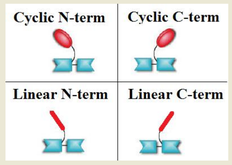

Figure 1. The four ligands studied (red) attached to DHFR-DHFR (blue)

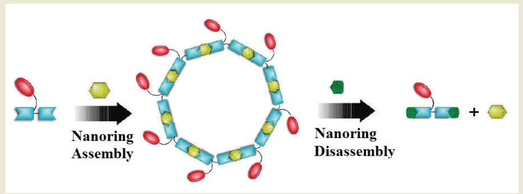

Figure 2. Self assembly of octameric nanorings upon the addition of methotrexate (yellow) and disassembly upon the addition of trimethoprim (green).

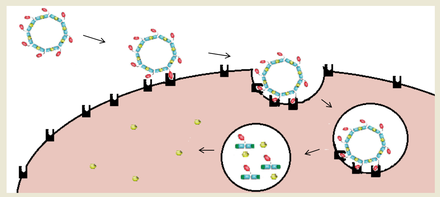

Figure 3. Endocytosis occurs when the nanoring binds to DR5 on the cell. Methotrexate is released as the ring is disassembled.

Methods

1. Protein Preparation and Purification: Mutagenesis was performed on a previously constructed plasmid to modify the ligand. The plasmids were expressed in E. coli and the protein was collected from the cell lysate and purified with methotrexate-agarose and DEAE columns.
2. In vivo cytotoxicity: 2500 cells (prostate and pancreatic) were plated in triplicate under conditions of varying drug concentration. Trimethoprim was added after 2 hours to disassemble the nanorings, unless otherwise noted. After 72 hours, cell viability was determined using a dye measuring cell metabolism. Percent Cell Viability was calculated relative to cells that received no treatment.
3. Visualization of drug delivery with confocal microscopy: The protein drug was stained with a fluorescent dye and incubated with cells for 2 hours. Cells were then fixed and stained with a dye that bound to nucleic DNA. An Olympus FV1000 confocal microscope measured the fluorescence of cells through a depth scan.

Results

Cytotoxicity:

Drug IC_{50} (μM)

	Pancreatic Cancer	Prostate Cancer
Dimer - Cyclic Ligand (C-term)	0.6	1.8
Dimer – Cyclic Ligand (N-term)	0.3	0.9
Octamer – Cyclic Ligand (C-term)	1.8	2
Octamer - Cyclic Ligand (N-term)	0.3	0.4
Dimer - Linear Ligand (C-term)	Inconclusive (>10)	Inconclusive (>10)
Dimer – Linear Ligand(N-term)	Inconclusive (>10)	Inconclusive (>10)
Oligomers - no disassembly (no trimethoprim)	Inconclusive (>10)	Inconclusive (>10)

Table 1. Comparison of the $IC_{50's}$ of drugs with cyclic ligands and linear ligands, and the role of disassembly. An IC_{50} was deemed inconclusive if the minimum cell viability did not reach 50% at the maximum concentration of drug tested.

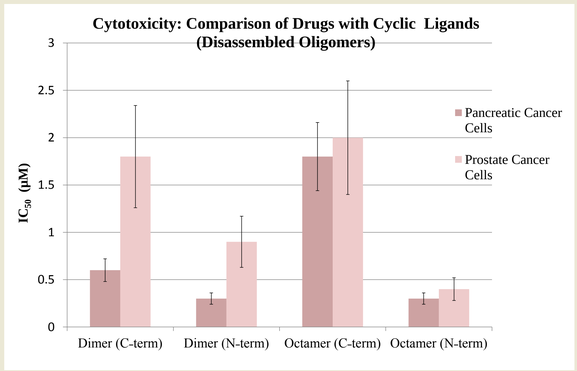

Figure 4. IC_{50} trends for drugs with cyclic ligands in pancreatic and prostate cancer cell lines.

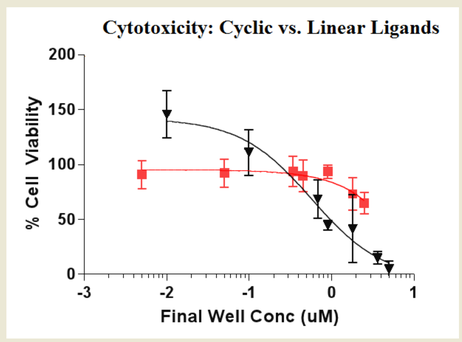

Figure 5. A representative dose-response curve. The shape of the curve for the dimer of the C-term linear ligand protein (red squares) was similar to that of the other linear ligand proteins and non-disassembled octamers, with an inconclusive IC_{50}. The dimer of the C-term cyclic ligand protein (black triangles) had a shape representative of the cyclic ligand proteins.

Confocal Results:

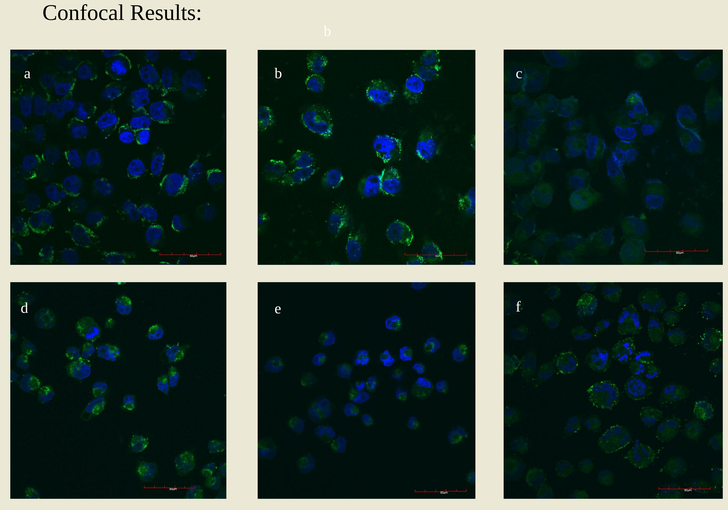

Figure 6. Internalization study with pancreatic cancer cells. Cells were stained blue and each drug was stained green. Monomeric drugs, including the C-term cyclic ligand drug (a), N-term cyclic ligand drug (b), and C-term linear ligand drug (c), contained a different fluorescent tag than the oligomeric drugs: the octameric N-term cyclic ligand drug (d), dimeric N-term cyclic ligand drug (e), and octameric N-term linear ligand drug (f). Scale bars are 50 μm long. Internalization is indicated by the small dots of green.

Discussion

Overall, the octameric N-term cyclic ligand protein performed the best, showing the highest cytotoxicity and internalization. Since the original ligand modified for testing was N-term cyclic, this was expected. The C-term proteins with cyclic ligands were less toxic than the N-term proteins with cyclic ligands (Figure 4). Drugs with linear ligands, and drugs whose nanorings were not disassembled, showed $IC_{50's}$ higher than the maximum concentration of the assay, much higher than the $IC_{50's}$ of the cyclic ligand drugs. It appeared that the mechanism for cell killing was dependent on the methotrexate, since toxicity increased when oligomers were disassembled in cells, releasing the methotrexate. Confocal microscopy experiments showed qualitatively that in monomeric form, the N-term proteins with cyclic ligands were internalized well, whereas the C-term proteins with cyclic ligands were internalized to a lesser extent. Internalization of the monomeric protein with linear ligands was quite low and could not be readily discerned from the background noise, seen as faint green in Figure 4c. Octameric proteins were internalized better than dimeric proteins, regardless of the ligand type, though this may be due to different steric interactions with the fluorescently tagged methotrexate linker.

Conclusions

Due to the moderately high degree of cell-killing and internalization seen by the octameric N-term fusion protein, it is a good candidate for further delivery studies. Since cytotoxicity was greatly increased upon disassembly of the oligomeric proteins, the mechanism for cell killing is presumed to be through the delivery of methotrexate, and not by the binding of the fusion protein to DR5, and the subsequent activation of the death receptor's apoptosis pathway.

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1. Chemically Self-Assembled Antibody Nanorings (CSANs): Design and Characterization of an Anti-CD3 IgM Biomimetic
Qing Li, Christopher R. So, Adrian Fegan, Vivian Cody, Mehmet Sarikaya, Daniel A. Vallera, and Carston R. Wagner
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