DRUG-LOADED AND EGFR-TARGETED NANO-CONSTRUCTS SUCCESSFULLY TREAT INTRACRANIAL GLIOMA IN MOUSE MODELS

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DISCLOSURES

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Cell type specific drug targeting of cancer cells is a far better chemotherapeutic vehicle to avoid the normally seen harmful side effects of the same drugs given systemically.

In collaboration with Rice University, we have developed a EGFR-targeting nanosyringe system for the treatment of glioma. The nanosyringe can be loaded with dyes, allowing visualization of payload transfer into cells, or with drugs for efficacy studies.

EGFR-targeting nanosyringes have a high affinity for their ligand. Binding of the nanosyringe to EGFR results in the activation/auto-phosphorylation of EGFR. The nanosyringes are internalized after EGFR activation, and dyes/drugs are transferred from the nanosyringe interior into the cells during both cell surface binding and when internalized in endosomes. Nanosyringe binding is competitive with EGF and with anti-EGFR antibodies. The internalization of the nanosyringes can be arrested by using EGFR inhibitors like erlinotinib or inhibitors of coated-pit internalization, like the compound PitStop.

Our aim is to create a specific tyrosine-kinase targeted nanoconstruct that is capable of both, carrying a large drug payload and releasing the payload into cancer cells, upon internalization following tyrosine kinase activation.

Here, we demonstrate that our targeted nanosyringes, loaded with doxorubicin are able to transfer this flurophoric drug from their interior, into gliomal cytosol, where it then partitions into nuclei.

We also show, in an intracranial U87-EGFR xenograft model that nanosyringe carried doxorubicin and vinblastine are far better chemotherapeutic agents than are the same drugs given in normal, pharmaceutical, authentic form.
Targeted nanosyringes are made by oxidative fracturing of single-walled nanotubes, using sulphur trioxide dissolved in sulphuric/nitric acid (a). The resulting particles are called hydrophobic/hydrophilic carbon clusters (HCC) and are graphene sheets with a large number of carboxylates (b). HCC’s are made highly aqueously soluble by amide-linkage to amino/azido PEG5000 (c). The resulting PEG-HCC have a large hydrophobic core, capable of being loaded with a range of drugs/dyes; for instance a single construct can carry 5,500 vinblastine drug molecules. The ability to target EGFR is imparted by affixing a modified version of the GE11, peptide. The GE11 peptide has been previously identified from phage display as a ligand for EGFR. We use a GE11 peptide that has been modified with a terminal lysine that bares an ε-amino amidic ethynyl group. The combination of azido-PEG and ethynylated-peptide allows the two to be coupled, covalently, using Cu catalyzed ‘click’ chemistry (d). The loading of the EGFR-targeting nanosyringe with a huge range of hydrophilic compounds is easily accomplished. The hydrophobic drug/dye is dissolved in methanol, slowly added to an aqueous nanosyringe solution and the hydrophobic compound spontaneously loads into the interior of the nanosyringe (e).
**Results 1:** Fig 1. Binding, internalization and dye transfer from nanosyringe into U87-EGFR

**(a)** U87-EGFR cells were incubated with Nile Red-loaded EGFR-targeted nanosyringes, fixed, washed, labeled with DAPI and imaged by fluorescence microscopy.

**(b)** The levels of Nile Red per cell (n=6). PepEGFR-PEG-HCC nanosyringe has an equilibrium binding constant KD of 19.4 nM.

**(c)** Dye transfer from Nile Red loaded nanosyringes is slowed by the presence of 450 pM rhEGF.

**(d)** Traces of gliomal Nile Red fluorescence showing 450 pM EGF drops nanosyringe dye transfer by ≈50%

**(e)** Co-localization of nanosyringe delivered Nile Red and FITC-labeled rhEGR (FITC-EGF) at 5 minutes of incubation.

**(f)** Competition between EGF and nanosyringe demonstrated using a pulse chase technique, glioma pre-incubated with FITC-EGF, followed by Nile Red nanosyringe.

**(g)** FITC-EGF added to glioma an imaged at 70 minutes.

**(h)** An identical experiment carried out in the presence of PitStop, an inhibitor of EGFR internalization.

**(i)** Nile Red loaded nanosyringe added to glioma an imaged at 70 minutes. (j) Nile Red loaded nanosyringe internalization is a slowed by PitStop.
**Results 2:** Fig 2 A & B. Endocytosis of nanosyringe visualized with immunoochemistry

A. (a) Increase in red-phosphoT678 with time and internalization of membrane-bound EGFR following incubation of U87-EGFR cells with nanosyringe. PhosphoT678 is a late-marker of activated EGFR and typically signals that the EGFR will be recycled following endocytosis.  
(b) is high resolution images taken at 90 min showing colocalization of the two signals, along with the DAPI stained nucleus.  
(c) The increase in phosphoT678 per cell relative to EGFR with time demonstrate that the targeted-nanosyrings not only bind to EGFR, but activate EGFR

B. (a) Shows the increase in green-PEG staining with time showing internalization of the nanosyringes via re-clathrin-mediated endocytosis.  
(b) High-resolution images taken at 90 min showing colocalization of the green-PEG and red-clathrin. The colocalization of PEG, from the nanosyringe, and clathrin shows the nanosyringe is internalized within the glioma.  
(c) The increase in PEG signal is compared to a constant level of clathrin over time. The increase in PEG shows continuous uptake of nanosyringes during the incubation.
Results 3: Fig 3 Modulation of the rate of transfer of Coumarin-6 into U87-EGFR cells

(a) Transfer of coumarin-6 from EGFR-targeted nanosyringe into U87-EGFR cells, as a function of time. Unlike the more hydrophobic Nile Red, this dye can be seen in cytosol as well as in membranes,

(b) If U87-EGFR cells are preincubated with EGFR inhibitor Erlotinib before the addition of nanosyringe loaded with coumarin-6 then localization and kinetics of dye transfer is altered, with no endocytosis.

(c) High resolution images of gliomal cells taken at 60 minutes show cells incubated with nanosyringe loaded with Coumarin-6 in the absence and presence of Erlotinib. In the absence of the EGFR inhibitor much of the dye signal is cytosolic, within endosomes, whereas in the presence of the inhibitor the dye is localized on the cell surface.

(d) A one hour time course showing the transfer of Coumarin-6 from loaded, EGFR-targeted, nanosyringes into U87-EGFR cell. In the lower panels dyes transfer is slowed due to the preincubation of anti-EGFR IgG.
**Results 4:** Fig 4 Transfer of doxorubicin into primary gliomal cells and in intercranial model

**A.** Transfer of doxorubicin into primary gliomal cells using EGFR-targeted Nanosyringes.

High-resolution images of accumulation of 30 nM nanosyringe Doxorubicin in EGFR-expressing primary human gliomal cells over time (indicated) upper panels. The lower panels show higher resolution views of the nuclei in a cell cluster and show the slow transfer of doxorubicin from the cytosol to nucleus.

**B.** Authentic chemotherapeutics and chemotherapeutics loaded into EGFR-targeted nanosyringe efficacy in a U87-EGFR intracranial model

Intracranial, U87-EGFR, tumors in a nude mouse model were treated using doxorubicin or vinblastine, either as in the Kolliphor® emulsification form or in the form of EGFR-targeted nanosyringes. As is the case when treating cancer patients, authentic Doxorubicin was highly toxic at 3.9mg/kg; resulting in animal discomfort and the deaths of 3/8 animals. Discomfort and off-target toxicity is unfortunately seen in the treatment human cancer patients. When Doxorubicin loaded nanosyringes were injected at identical drug concentrations there was no animal distress. Targeted nanosyringe delivered chemotherapeutics outperform the conventional emulsion versions in efficacy between 4 and 17 fold, and with less toxicity.
The targeting of drugs to cancer cells will increase their efficacy in treatment and also reduce systemic toxicity.

We have developed a nanosyringe with very good drug/dye load carrying capacity.

Peptides previously identified by biopanning are used for targeting.

Coating the surface of the nanosyringe with EGFR-binding peptides gives rise to very high binding affinities, due to high avidity (a property of multiligand systems).

EGFR-targeted nanosyringes are taken up by gliomai cells expressing EGFR, via the normal clathrin coated pit mechanism.

Binding and uptake of the drug/dye laden nanosyringes allows the transfers of the hydrophobic payload into the membranes of the targeted cell.

We show that using nanosyringes, loaded with vinblastine or doxorubicin, that these chemotherapeutics are not only delivered to intracranial tumors, increasing anti-tumor efficacy, but that systemic toxicities associated with these drugs is greatly mollified.
Summary

Using drug loaded nanosyringes, we measured a >4-fold increase in the efficacy of doxorubicin, without the toxicity associated with this drug.

The efficacy of vinblastine loaded nanosyringes is even better, >15-fold more than standard clinical drug delivery.

We conclude that the peptidyl-targeted nanosyringe system is able to bind to specific tyrosine kinases, triggering their activation, and internalize the nanosyringes via a coated-pit mechanism.

We have demonstrated that the nanosyringe drug/dye payload is mobilized, both on binding to the cell surface and upon internalization, and from their these dyes/drugs can partition into the nucleus.