CURRENT RESEARCH

As of September 2018

1. EXO-DEPT Therapy

(see publication link for RELEVANT Mss)

In collaborative work, we have developed a gene-delivered enzyme prodrug therapy (GDEPT), which uses harmless prodrugs that are rendered toxic by a bacterial or viral gene-encoded enzyme. GDEPTs can avoid conventional chemotherapy problems (e.g., severe side effects/low tumor drug concentration/resistance development), provided the gene is specifically targeted to the cancer. GDEPT clinical trials have not as yet succeeded. Reasons include: insufficient gene delivery/expression, and enzyme potency; not ensuring sufficient bystander effect (BE) to kill the tumor; and use of potentially toxic viruses in non-directed DNA-based gene delivery. Our GDEPT has addressed these issues.

Our prodrug, CNOB, is activated by our improved humanized bacterial enzyme (HChrR6), generating MCHB, which is lethal, and fluorescent, facilitating observational assessment of its BE and tumor-specific activation in living mice (Fig. 1). We used HEK293 extracellular vesicles [EVs (aka exosomes)] displaying anti-HER2 scFv and loaded with HChrR6-encoding mRNA ["EXO-DEPT" EVs (Fig. 2)] for gene delivery. mRNA is superior to DNA for gene delivery, as it is translated directly upon cytosol entry; DNA in contrast has first to enter the nucleus, which is a highly inefficient process. EXO-DEPTs do not bind to HER2$^-$ cells but converted HER2$^+$ cells into CNOB activating entities (Actinomycin D-independent). In vitro, EXO-DEPT and CNOB dosage was optimized to specifically kill HER2$^+$ cells; the mRNA delivery amount, expression duration and the transfection ratio needed for this killing were established and guided in vivo studies. HER2$^+$ BT474 xenografts were orthotopically implanted in immune-compromised mice. When untreated, the tumors grew vigorously; treatment with CNOB + EXO-DEPTs nearly-completely arrested their growth (Fig. 3).

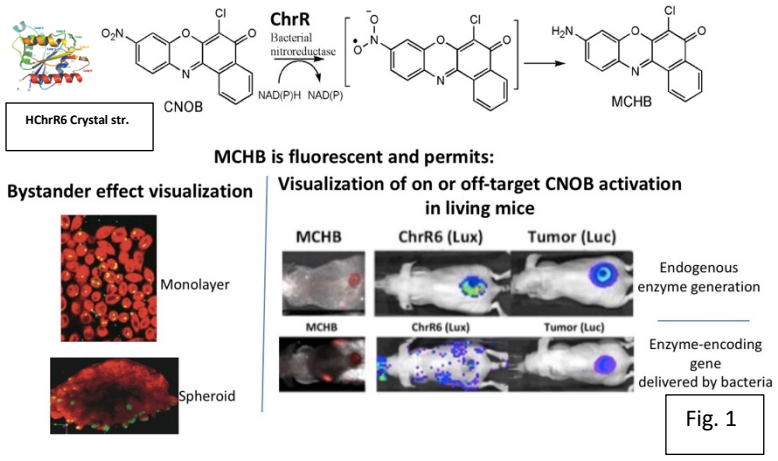


Fig. 1

Our enzyme also activates the prodrug tretazicar (CB1954); tretazicar + EXO-DEPT treatment also arrested the growth of the tumors (Fig. 4). Tretazicar has been used in clinical trials strengthening the potential of clinical transfer of this therapy. A provisional patent has been filed.

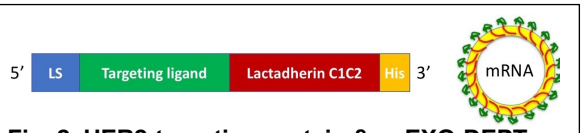


Fig. 2. HER2-targeting protein & anEXO-DEPT.

Our current research (in collaboration with several Stanford and extra-Stanford scientists) is aimed at clinical transfer of this regimen:

- Exploring whether and in what way, mobilization of immune-based antitumor effectors reinforces the therapy; we are using mice that spontaneously develop HER2+ve tumors.
- We have shown that exosomes can cross the blood brain barrier; thus, our therapeutic approach is being developed to treat brain metastasized cancers (other brain diseases) that overexpress a receptor

- IND-enabling non-clinical pharmacologic, pharmacodynamic and toxicological studies
- First-in-human, phase I clinical trial of HER2 scFv-directed, dendritic cell-derived EXO-DEPT EVs plus tretazicar in patients with HER2^{+} metastatic breast cancer that has progressed despite prior HER2-targeted therapies
- Generating a cell line that is largely devoid of markers of self, so essentially non-immunogenic exosomes could serve as the off-the-shelf source
- Upscaling EXO-DEPT production

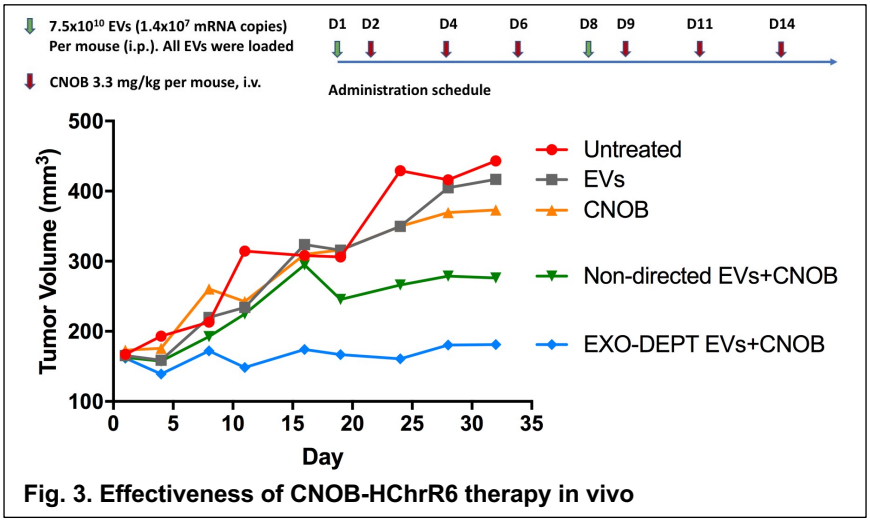


Fig. 3. Effectiveness of CNOB-HChrR6 therapy in vivo

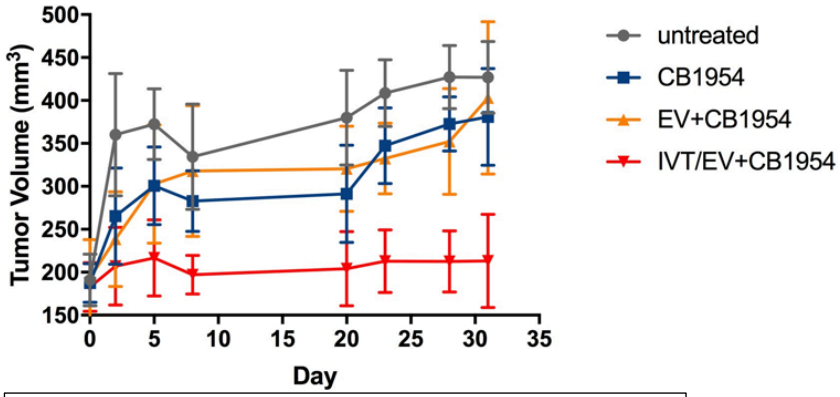


Fig. 4. Effectiveness of CB1954-HChrR6 therapy in vivo

2. Bacterial antibiotic resistance.

Project goal. Bacterial antibiotic resistance is a serious problem, which is not being adequately addressed. Our finding that oxidative stress generated by bactericidal antibiotics greatly augments their lethality affords a novel way to increase their effectiveness, namely, using a small compound to inhibit a sigma factor that controls bacterial antioxidant defense.

*Background***.** It is known that bactericidal antibiotics, aminoglycosides (e.g., gentamicin), quinolones (e.g., norfloxacin) and β-lactams (e.g., ampicillin) kill bacteria by interfering with protein, DNA, and cell wall synthesis, respectively.

Specificity of gene transcription in bacteria depends on the RNA polymerase-sigma factor combination. Our work (1) had showed that σ^s-RNA polymerase transcribes genes that make bacteria resistant to disinfectants and other stresses (e.g., H_2O_2, ethanol, low pH, hyperosmosis). The proteins involved in this defense protect biomolecules, e.g., molecular chaperones (proteins/other biomolecules), UvrABC endonuclease (DNA), D-alanine carboxylase (cell wall) etc. Since the antibiotics also act by damaging biomolecules, we reasoned that σ^s should protect bacteria also against them (2).

We have tested this hypothesis in uropathogenic *Escherichia coli*, AMG1 (UPEC; isolated in the Medical School clinic), the causative agent of urinary tract infections (UTI). Deletion of the *rpoS* gene did indeed render UPEC highly sensitive to the above drugs, (Fig. 1). Proteomic analysis showed that, upon antibiotic treatment, compared to the wild type, the Δ*rpoS* strain showed depressed levels of antioxidant defense proteins; it also generated greater reactive oxygen species (ROS), as well as showed greater induction of the SOS response, of protein carbonylation, and DNA damage, all being consequences of oxidative stress. Co-administration of the quencher N-acetyl cysteine (NAC) diminished Gm-mediated killing, as did anaerobiosis, which prevents ROS formation.

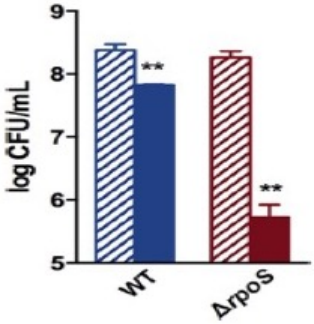
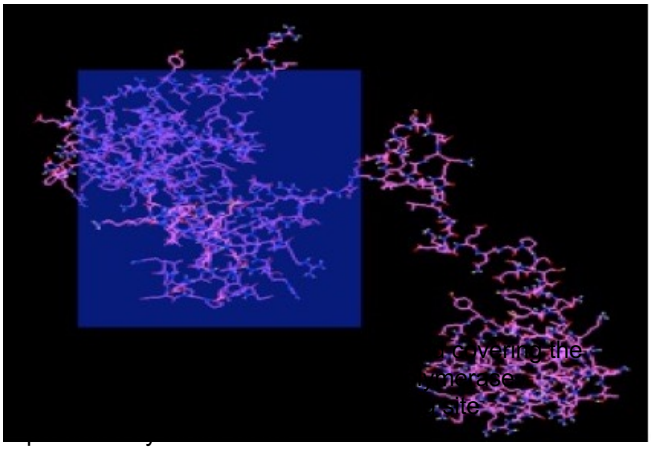


Fig. 1. Loss of σ^s increases Gm (16 μg/ml; MIC of sensitivity of UPEC; similar results with other drugs

Deletion of genes encoding antioxidant proteins also resulted in greater sensitivity. Mutants missing genes that encode e.g., superoxide dismutase A and B, and hydroperoxidase, which decompose ROS, were more sensitive to the drugs. Loss of genes of the pentose phosphate pathway, the main source of NADPH, also increased drug sensitivity. (Examples: glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase or transketolase A): NADPH provides electrons for the ROS decomposers. These mutants also generated more ROS and stronger SOS response upon drug treatment, which was reversed by NAC.

In a female mouse urinary tract infection model, gentamicin inhibited bladder colonization by the *rpoS* mutant but not when NAC was co-administered; oxidative stress had thus a role also *in vivo*.



Ongoing work. Our current work is based on the premise that small molecule inhibitors of proteins of antioxidant defense will enhance the effectiveness of the drugs and minimize their side effects, such as nephrotoxicity caused by gentamicin. We have initiated bioinformatic work to identify such compounds. This has included RpoS and superoxide dismutase (SodA). Inhibition of both may prove synergistic, **but the present emphasis is on RpoS**, since its inhibition will jeopardies not only antioxidant defense but also resistance to other insults bacteria

encounter *in vivo*, e.g., acid stress and dearth of nutrients.

High throughput structure-based virtual screening. High-resolution crystal structure of RpoS and SodA were downloaded from Protein Data Bank; ~1 million (1019477) small inhibitor compounds were downloaded from the ZINC database (3). MetaPocket 2.0 software was used to predict the ligand binding sites on the surface of RpoS and SodA.

As mentioned above, RpoS must bind to RpoC to be effective. Inhibition of this binding will inactivate σ^s-RNA polymerase holoenzyme. Uniprot database identified the RpoS amino acid binding positions to RpoC.

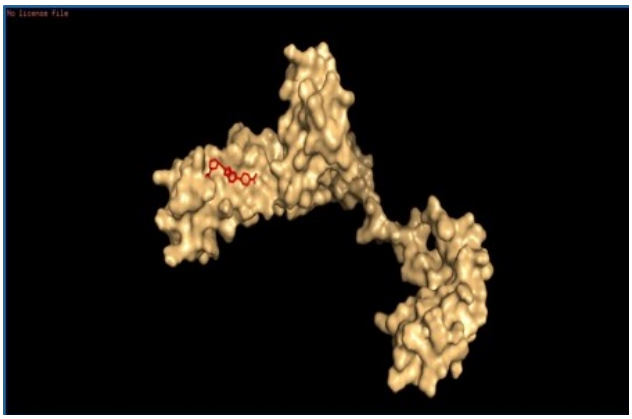


Fig. 3. RpoS bound to ZINC14997580

To carry out docking of the compounds, PyRx python pipeline was used to minimize the binding energies of selected compounds and to convert all the compounds into AutoDock Vina (4) input format. Screening was carried out by AutoDock Vina software (server edition Stanford computing facility). AutoDock is a molecular docking algorithm; its Auto Grid module was used to build a grid around potential binding sites; Fig 2 illustrates this for RpoS.

The screening resulted in 1,019,477 RpoS-Compound docked files and 1,019,823 SodA-compound docked files for each individual compound with its free binding energy as well as up to 9 different binding poses with RpoS and SodA. To analyze ~1 million Auto Dock output files, a python script was written to automatically read the output files and select the best compounds based on their highest free energy; five of the best were chosen. Fig. 3 illustrates RpoS bound to a small inhibitor compound.

Using gentamicin MIC, we have tested the effect of co-administration of this compound with the drug. Very preliminary results suggest that it tends to increase gentamicin sensitivity of the wild type to approach that of the mutant.

Phenotyping resistance with single cell resolution. Since heterogeneity of the degree of drug sensitivity in a bacterial population can often not identify the resistant fraction, we have also developed a microfluidic system that permits enables the quantitative phenotyping of heterogeneous resistance with single-cell resolution (5).

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4. Trott, O. and Olson, A. J. (2010), AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem., 31: 455–461. doi:10.1002/jcc.21334
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