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MONITORING TUMOR RESPONSE TO THERAPEUTIC TR4 FUSION PROTEIN VIA *IN VIVO* IMAGING

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INTRODUCTION

The Notch signaling pathway has been associated with breast, cervical and lung cancer, neuroblastomas and acute T-cell lymphoblastic leukemia and is strongly implicated in tumorigenesis. [1] Notch genes encode large single pass transmembrane proteins that regulate cell fate. Upon activation, Notch undergoes cleavage, releasing its intracellular domain NICD and translocates into the nucleus for transcriptional activation of its downstream target genes. [2] Discovery of this pathway has prompted many pharmaceutical companies to develop novel anti-Notch therapeutics. We (Trojantec Ltd) have generated a novel fusion protein TR4 that inhibits the Notch pathway at the transcriptional level and can potentially reduce tumor growth *in vivo*. TR4 consists of the truncated version Mastermind-like (MAML) that behaves in a dominant negative fashion and inhibits Notch activation and the cell penetrating peptide Antennapedia (Antp). The fusion protein is able to translocate into the cell nucleus and suppress Notch activation thus reverting the transformed phenotype, inhibiting the anchorage dependent growth and inducing apoptosis in highly metastatic epithelial human breast cancer cells.

Utilizing a state-of-the-art *in vitro* pharmacokinetics and toxicology program, in combination with innovative imaging techniques such as *in vivo* flow cytometry and whole body fluorescence reflectance imaging, we have investigated the pharmacokinetic and pharmacodynamic behavior of the TR4 protein in order to define its mode of action and establish safety and efficacy in treating tumors in animal models of breast adenocarcinoma.

METHODS

A comprehensive *in vitro* and *in vivo* pharmacokinetics study that included apoptotic, toxicology and immunogenicity assays was undertaken through administration of various concentrations of TR4 in 10 immuno-competent mice in order to determine the effects of TR4 and define the mechanism(s) of action. Blood samples were collected for toxicological assays and to monitor the immune response by ELISA whereas major organs were excised for immunohistochemistry to determine tissue toxicity and induction of apoptosis.

The dynamics of the TR4 fusion protein were investigated in animal cancer models, through the successful conjugation of the TR4 to proprietary fluorescent markers (Cy5.5). Molecular modeling studies were performed to identify appropriate conjugation sites on the TR4 molecule to ensure no loss of the membrane transfer capabilities of the fusion protein. A group of fluorescent dyes was prepared, with maleide groups appropriate for bioconjugation to thiols on the TR4 molecule. TR4-Cy5.5 conjugates were chosen based on their optical properties (absorption 600-650 nm, emission 650-700 nm) and suitability for the *in vivo* imaging study. Conjugated TR4 was purified using proprietary methods to ensure protein solubility and avoid excessive aggregation and was further tested to ensure that the protein retained the ability to translocate in breast cancer cells.

The *in vivo* pharmacodynamic behavior of the fluorescent TR4-Cy5.5 was investigated using fluorescence-based *in vivo* flow cytometry and whole body imaging. These molecular imaging techniques have the ability to monitor and quantify fluorescently labeled vehicles in circulation, to detect and image the biodistribution and incorporation of therapeutic agents in cells and organs of both healthy and tumor-bearing mice, as well as to provide a quantifiable assessment of tumor growth over time. Following intravenous administration of the TR4-Cy5.5 in non-tumor bearing mice (n=5) the non-specific uptake of the protein by circulating blood cells was assessed via *in vivo* flow cytometry, while the biodistribution of the protein accumulating in different organs/tissues was imaged using whole body reflectance imaging. The accumulation of TR4 in various

major organs (liver, kidney, lung and brain) was further verified by Western blot analysis.

An orthotopic mouse model for breast cancer disease was developed via injection of $7x10^6$ MDA-MB-231 GFP labeled cells in SCID mice, in order to enable the imaging and assessment of tumor growth *in vivo*. Animals that expressed a detectable fluorescent signal from established orthotopic tumors were selected and divided into two (n=5) groups: untreated (control) and treated with TR4 (day 0). Mice were monitored for 2 weeks via *in vivo* imaging and were then euthanized at the end of the experiment (day 14).

RESULTS

The pharmacokinetics studies indicated that TR4 did not induce any overt toxicity, notable death of cells, organ dysfunction, or any toxic manifestations that were predicted materially to reduce the life span of the animals. The immunogenicity tests indicated that animals do not raise an antibody response against the injected TR4 protein. Finally, no murine antibodies against full-length TR4 were detected by ELISA in mice serum.

TR4-Cy5.5 conjugates prepared via molecular modeling studies (Fig. 1A) and selected based on their optical characteristics (Fig. 1B) were first validated *in vitro* for their membrane translocation ability (Fig. 1C) prior to their use for the *in vivo* part of the study.

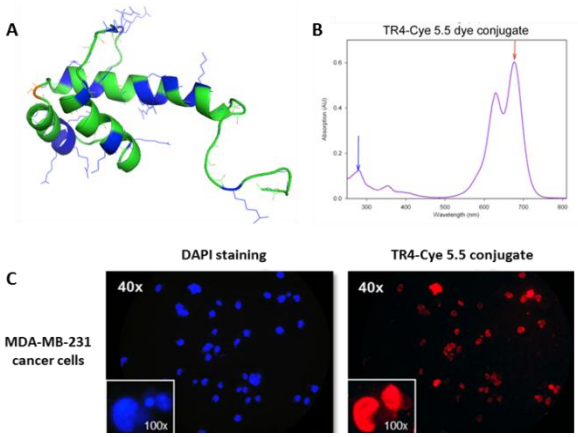

Figure 1: (A) The TR4 model containing 24 arginine residues (green), 9 lysine residues (blue) and 4 cysteine thiol residues (orange). (B) UV-vis spectra showing the quality of the conjugation and the peak absorbance shifts. (C) Cell penetration of TR4-Cy5.5 in MDA-MB-231 cancer cells.

In vivo flow cytometry in mice, injected i.v. with the TR4-Cy5.5 conjugates, showed that free, non-internalized protein was rapidly cleared from circulation, as the background (noise) signal collected from the blood vessels did not substantially increase. Furthermore, fluorescently labeled circulating cells that may have internalized the TR4 protein were not detected at any time point. Whole body imaging showed that TR4-Cy5.5 accumulated in some of the major organs/tissues imaged and was cleared within 24 hrs via the gastrointestinal tract (Fig. 2A-B). This is in agreement with the Western blotting analysis of excised liver and kidney (Fig. 2C).

Assessment of tumor burden via *in vivo* imaging demonstrated a reduction in tumor growth over time in mice treated with TR4-Cy5.5 compared to untreated mice (Fig. 3). The figure shows an overall increase of less than 50% in the relative intensity of fluorescence signal in the treated mice (right panel) compared to a 3-fold increase in signal and thus tumor size in untreated mice by day 14 (left panel).

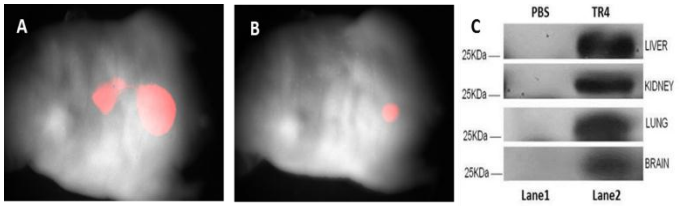

Figure 2: The TR4 protein biodistribution at (A) 2h and (B) 24h post injection. (B) Western blot analysis of excised mouse liver, kidney, lung and brain.

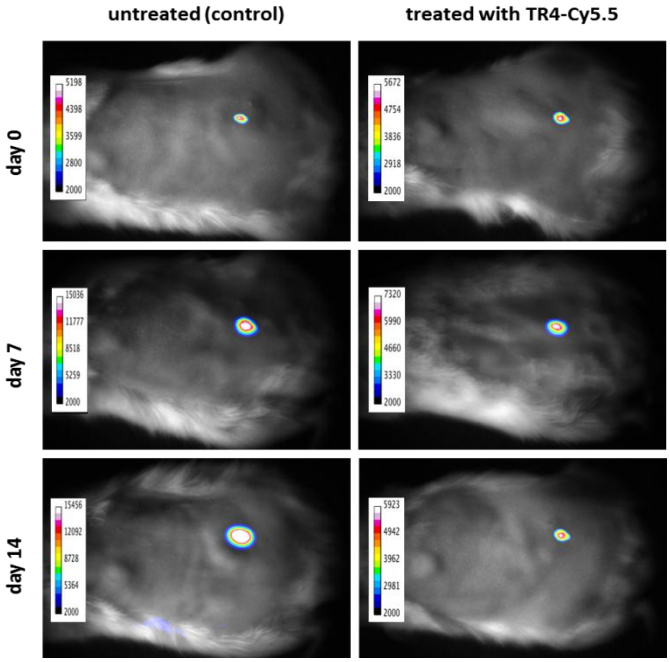

Figure 3: Tumor response in untreated (control) and treated with TR4-Cy5.5 mice at day 0, 7 and 14 post injection (representative images of the n number of mice tested).

DISCUSSION

In this report we have performed preclinical studies to validate TR4 as a promising therapeutic strategy for breast adenocarcinoma. The dynamics of the drug molecule have been investigated in an *in vivo* setting in order characterize its therapeutic efficacy in an animal tumor model. Mechanisms of action have been investigated via the application of innovative imaging modalities with the aim of guiding the technology closer to the market by completing the necessary preclinical studies in order to optimize the TR4 fusion protein as a novel therapy for invasive cancer disease.

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