**A Phase II Immuno-gene therapy CLINICAL TRIAL EVALUATING *In situ* Vaccination of MALIGNANT PLEURAL MESOTHELIOMA with INTRAPLEURAL delivery of ADENOVIRAL-MEDIATED INTERFERON-ALPHA (Ad.hIFN-2b) IN COMBINATION WITH High-Dose Celecoxib and systemic chemotherapy**

**Supplemental Methods**

**Definitions of Dose Limiting Toxicity**

The primary endpoint of the clinical trial was to identify new toxicities and the safety of two doses of intrapleural AdIFN in combination with standard of care chemotherapy for MPM. Enrolled subjects were evaluated for dose-limiting toxicity (DLT) from the start of celecoxib and the first dose of Ad.IFN to 14 days after the first round of chemotherapy (approximately Day 30). Dose limiting toxicities (DLTs) were defined as any of the following treatment-related adverse events (AEs) as per the Common Terminology Criteria for Adverse Events (CTCAE v.3.0) adopted by the National Cancer Institute:

* Any Grade 4 toxicity (except isolated Grade 4 lymphopenia lasting ≤ 7 days after the last dose of AdIFN).
* Grade 3 hypotension, disseminated intravascular coagulation (DIC) or allergic reaction/hypersensitivity.
* Grade 3 non-hematologic toxicity persisting for >7 days except for cytokine release syndrome (CRS) within 6-48 hours after AdIFN dosing.
* Persistent CRS starting within 48-72 hours of dosing and lasting up to 10 days after last dose of AdIFN.
* Grade 3 hematologic toxicity persisting for > 7 days after last vector dose (except isolated lymphopenia)

If a DLT occurred during the infusion, AdIFN administration was stopped, and no further study drug was to be administered. If a DLT occurred between Day 1 and Day 3, the second dose of study drug would not be administered.

The protocol stopping rules stipulate that if two (2) DLTs occurred within the first treatment group, enrollment in the study was to be halted pending a review of the data and discussion with the FDA and IRB about de-escalation.

In addition, the protocol specified that subjects may be withdrawn from the study prior to the expected completion if, among other events, the subject experiences a DLT or serious adverse event, or if a chemotherapy cycle is delayed more that 3 weeks from scheduled cycle due to lack of resolution of toxicities.

**Procedures**

**Immunoblotting**

To detect induced humoral responses against tumor antigens, we performed immunoblotting against purified mesothelin and extracts from allogeneic mesothelioma cell lines. Purified mesothelin was purchased from Raybiotech (Norcross, GA). Cell lines were derived from patient pleural fluid samples from previous clinical trials and were grown in culture as previously described (Sterman et al. 2010). Extracts from cells or purified proteins were prepared and immunoblotted with patient serum (diluted at 1:1500) from time points before treatment, and 6 weeks after treatment as previously described (Sterman et al., 2010). Multiple exposures were obtained and comparisons were made on the exposures in which the major bands detected on pre-treatment blots were of equal intensity in post-treatment blots. Two independent, blinded observers visually scanned each blot to detect new bands or bands that appeared markedly increased in the post-treatment serum and came to a consensus score. The blots were semi-quantitatively scored as follows: 0= no change in any bands; 1= minimal changes (i.e. increased intensity in one or two bands); 2= clear increases in >2 bands or appearance of new bands. A sample showing each scoring category is shown in **Supplemental Figure 1**.

**Flow Cytometry**

Cryopreserved peripheral blood mononuclear cells (PBMC) collected prior to treatment, 2 days after Ad.INF instillation (before the second dose) and Day 15 days after the first vector dose (before chemotherapy) were thawed, and natural killer cell (NK) and T cell subsets and their activation status, were assessed with mAbs against CD3, CD4, CD25, FoxP3, CD8, CD56, CD16, CD69, CD38, HLA-DR, Ki67, Bcl2, ICOS, NKG2D, NKG2A, and NKp30. All mAbs were from BD Biosciences (San Diego, CA) and R&D systems (Minneapolis, MN, USA). PBMCs from a set of six patients who responded the therapy and 6 patients who did not were studied (**Supplemental Table 2**).

Details of the cell preparation and staining have been previously published (Stevenson et al., 2013). Analysis was done by collecting 100,000 live lymphocytes (defined by size and granularity in FSC and SSC). Dead cells were excluded by manual gating in FSC/SSC. Detection thresholds were set according to isotype-matched negative controls. Results were expressed as Mean Fluorescent Intensity (MFI) and percent (%) of lymphocytes, NK cells (Lin3-CD56dimCD16+), CD3+CD4+ or CD3+CD8+ T cells. Data analysis was performed using FloJo software (Tree Star, San Carlos, CA).

**Immunohistochemistry**

Formalin-fixed paraffin-embedded sections from original surgical biopsies or previous surgery were available from 18 patients. After deparaffinization and antigen retrieval, these were stained by the Penn Cancer Center Pathology Core for T cells (using anti-CD8 antibody) and macrophages (using anti-CD68 antibody). Sections were also stained for anti-PD-L1 by Merck using a proprietary antibody (clone 22C3). Sections were scored for quantity of PD-L1 expression by a pathologist in a blinded fashion on a 0 to five scale: 0= negative, 1= trace/rare, 2= low, 3= moderate, 4=high, and 5= very high.

Tissue sections were also used for RNA analysis using Nanostring analysis. Prior to making the cell lysate or isolating the RNA, tissue sections were deparaffinized in xylene for 3 x 5 min and then rehydrated by immersing consecutively in 100% ethanol for 2 x 2 min, 95% ethanol for 2 min, 70% ethanol for 2 min and then immersed in dH2O until ready to be processed. Tissue was lysed on the slide by adding 10-50ul of PKD buffer (Qiagen catalog #73504). Tissue was scraped from the slide and transferred to a 1.5 ml eppendorf tube. Proteinase K (Roche Prot-K catalog # 03115836001) was added at no more than 10% final volume and the RNA lysate was incubated for 15 min at 55ºC and then 15 min at 80ºC. The RNA lysate or total RNA was stored at -80ºC until gene expression profiling was performed using the NanoString nCounter™ system. 50ng of cellular lysate or total RNA per sample, in a final volume of 5ul, was mixed with a 3′ biotinylated capture probe and a 5′ reporter probe tagged with a fluorescent barcode, from the desired gene expression codeset. Analysis of 600 immune-related genes were selected based on a PubMed literature review. Probes and target transcripts were hybridized overnight at 65 ºC for 12-16 hours as per manufacturers’ recommendations. Hybridized samples were run on the NanoString nCounter™ preparation station using their high sensitivity protocol where excess capture and reporter probes were removed and transcript-specific ternary complexes were immobilized on a streptavidin-coated cartridge. The samples were scanned at maximum scan resolution capabilities using the nCounter™ Digital Analyzer. Data analysis was performed using quantile normalization in which relative ranks of genes (across all genes on the Nanostring codeset) within each sample were replaced by values having the same relative rank from the pooled distribution (from all samples and genes in the dataset). All quantile normalized data underwent subsequent log10 transformation.

**Flow Cytometry Statistics**

Data were described as medians, 25th and 75th percentiles. Comparisons between responders (n=6) and non-responders (n=6) at each time point were done using Wilcoxon Kruskal-Wallis tests (Rank Sums). Differences between time points for all patients (n=12) were tested using Wilcoxon Signed-Rank or paired t-tests depending on data distribution, while differences between time points in responders (n=6) and in non-responders (n=6) were tested using Wilcoxon Signed-Rank. p values that were less than 0.05 were considered statistically significant. All statistics used JMP Pro11.

**References**

Sterman DH, Reico A, Haas AR, et al. A phase I trial of repeated Intrapleural adenoviral-mediated interferon-beta gene transfer for mesothelioma and metastatic pleural effusion. *Mol Ther* 2010; **18** : 852-60.

Stevenson JP, Kindler HL, Papasavvas E, et al. Immunological effects of anti-transforming growth factor-beta (TGF-beta) antibody GC1008 in cancer patients with malignant pleural mesothelioma (MPM). *Oncoimmunology* 2013; **8** :e26218.