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The HSP70 immune axis (HSPA1A, 1B and 1L) is a novel target for cancer immunotherapy – Development and selection of an Fc-enhanced anti-HSP70 IgG for the treatment of pre-clinical models of cancer

Abstract Number 1304

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Background

HSPA1A, -1B, and -1L (HSP70) are unique members of the HSP70 family, situated within the MHC-III genomic locus. These genes play a critical role in the innate and adaptive immune response. Many cancer types overexpress HSP70, leading to enhanced metastasis, protection from apoptosis, and secretion of HSP70. ADP-bound HSP70 is structurally distinct from ATP-HSP70. In the ADP-bound form it carries along with it tumor-derived proteins containing the repertoire of tumor neoantigens. Once processed by an APC, these antigens can be cross-presented via MHC-I or MHC-II complexes to elicit T-cell responses. Attempts for ADP-HSP70-based vaccines in clinical trials lacked robust clinical responses because of limited methods to purify material and successfully target APCs. Here, we report on the development of ASY-77A, a novel anti-HSP70 hu-IgG1 selectively recognizing the ADP-HSP70 neoantigen complex. The engineered Fc domain then redirects these complexes to the activating FcγRs on dendritic cells and macrophages in pre-clinical cancer models.

ASY-77A preferentially binds to ADP-HSP70

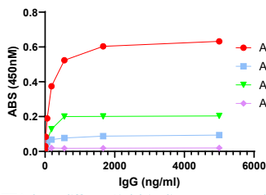


Figure 1. ASY-77A has differential binding compared to other antibodies. The ability of ASY-77A versus another anti-HSP70 antibody (CM170.1) to bind ADP-HSP70 or ATP-HSP70 was evaluated. ASY-77A bound preferentially to ADP-HSP70 over ATP-HSP70, while CM170.1 was agnostic to either. This has been shown with other antibodies as well (data not shown). This indicates that ASY-77A preferentially binds to HSP70-ADP-neoantigen peptides complexes upon release from tumor cells.

ASY-77A uniquely induces uptake of HSP70 by APC

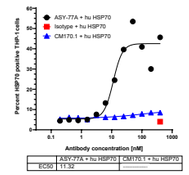


Figure 5. Cellular uptake of fluorescently tagged HSP70 into THP-1. THP-1, a human monocytic line, can internalize HSP70 via an antibody-mediated mechanism. Uptake via ASY-77A (circle), CM170.1 (triangle), or isotype (square) immune complexes (ICX) is shown. Antibody molarity was serially titrated in 2-fold step increments while HSP70 was held fixed at 50 nM. Percent HSP70 positive cells was plotted against the log of molar antibody concentration. Relative EC50 determined by nonlinear regression least squares fit. Due to lack of signal from CM170.1-mediated uptake, EC50 could not be determined.

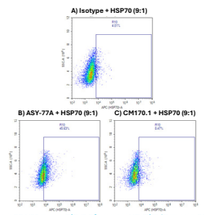


Figure 6. Scatter FACS plots of HSP70 uptake in THP-1 cells. HSP70 was labeled with CF647. Scatter plots of uptake for each antibody, from the highest antibody concentrations ([9:1] antibody:HSP70 molar ratio). Plot is side scatter against CF647 intensity, data corresponds to **Figure 5.** Plots for A) isotype, B) ASY-77A, or C) CM170.1 immune complex uptake

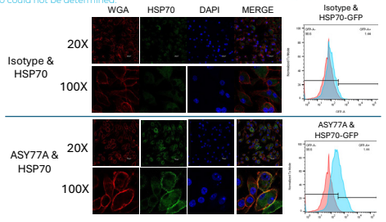


Figure 7. Uptake of HSP70 ICX By Murine DC Line. ICX formed with either ASY-77A or isotype. Product added to the murine dendritic cell line DC2.4, and uptake determined by FACS. Confocal microscopy performed in parallel. Wheat Germ Agglutin (WGA) was used to stain the cell membrane and a His-tagged HSP70 tracked by anti-HIS-Alexa-488, nuclei were stained with DAPI. Corresponding FACS histograms with MFI shift shown right of confocal images.

Single-agent ASY-77A shows control in E0771 model

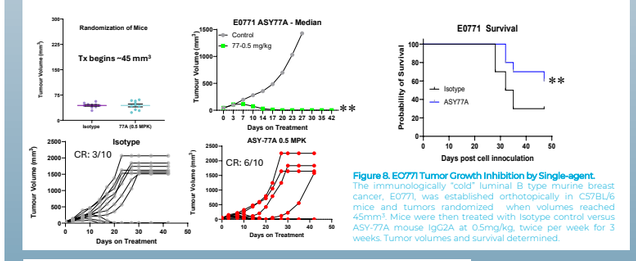


Figure 8. E0771 Tumor Growth Inhibition by Single-agent. The immunologically "cold" luminal B type murine breast cancer, E0771, was established orthotopically in C57BL/6 mice and tumors randomized when volumes reached 45mm³. Mice were then treated with Isotype control versus ASY-77A mouse IgG2A at 0.5mg/kg, twice per week for 3 weeks. Tumor volumes and survival determined.

ASY-77A activates antigen presentation

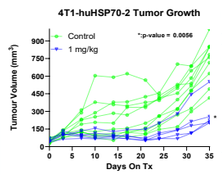
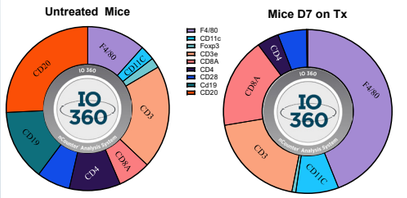
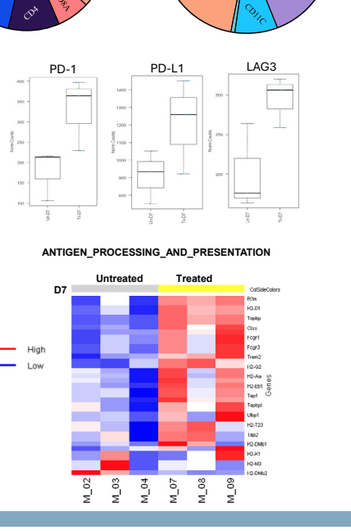
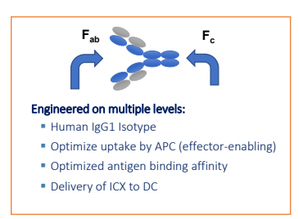


Figure 10. Molecular Profiling of ASY-77A Treatment. Established orthotopic 4T1 tumors were treated with isotype versus ASY-77A twice per week for 3 weeks. A subset of tumors were collected at 7 days on treatment, and the nCounter PanCan IO 360 Panel used to analyze the RNA extracted from tumors. Analysis performed to understand changes in microenvironment and the immune response. Data was analyzed and displayed below as pie charts for isotype versus ASY-77A. Boxplots depict expression by group for genes identified as differentially expressed. Heatmaps show gene increased or decreased expression for the Antigen_Processing_And_Presentation pathway.





Engineering humanized IgG1 ASY-77A to enhance human DC uptake



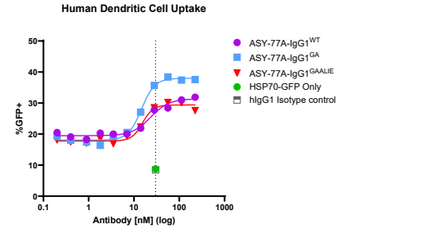
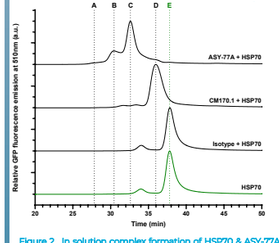


Figure 11. ASY-77A humanization into a tailored human IgG1 isotype. Fc-engineering was deployed to modify binding to the Human FcγR family, with a focus on the activating receptor FcγRIIA. This FcR is primarily found on professional antigen presenting cells, i.e. dendritic cells and macrophages. Three variants were generated as: wild-type (**WT**) hIgG1, the G236A mutation (**GA**) to enhance binding to FcγIIA, or mutations G236A/A330L/I332E (**GAALIE**) to enhance binding to FcγIIA and FcγIIIA. Immature human dendritic cells were generated from peripheral blood monocytes using treatment with IL-4 and GM-CSF. HSP70-GFP was incubated with the ASY-77A human IgG1 variants to form immune complexes (ICX) at a 1:1 molar ratio. The ICX mixes were then added to the each of the DC. Percentage of cells with uptake was determined by FACS.

Screening shows no off-target binding of ASY-77A Human IgG1-GA

Figure 12. Antibody Off-Target Binding Analysis Performed by Retrogenix (Now CRL). The Cell Microarray Technology expresses over 6,500 proteins, including human plasma membrane monomers, heterodimers, and secreted proteins, was used to evaluate the human IgG1 ASY-77A candidate on live human cells. Further evaluation by FACS was performed as a confirmatory screen of expression array findings. Positive controls (black) of expression array are shown, which included FcγR. A total of 6 potential weak binders were identified. Validation by FACS of these weak binders showed no validated interaction. Finding of array conclusively shown as only background.

ASY-77A forms unique immune complexes (ICX)



Protein	Peak #	Retention Time (min)	Approx. MW (kDa)*	Peak Area %
ASY-77A ICX	A	27.84	~802	2.2
ASY-77A ICX	B	30.45	~473	21.1
ASY-77A ICX	C	32.62	~305	68.8
CM170.1 ICX	D	35.98	~155	93.6
Isotype ICX	E	37.83	~106	90.1
HSP70		34.00	~231	10.1
HSP70	E	37.81	~107	89.8

Table 1. Retention time, MW, and peak area of profiles from Figure 2 Data represents the signal of only HSP70-GFP-containing molecules. *MW for each peak determined from MW standard curve (data not shown)

Figure 2. In solution complex formation of HSP70 & ASY-77A. Comparison of aSEC profiles (310 nm fluorescence emission) after co-incubation of HSP70 with various antibodies. Separation using dual column SEC and PBS as the mobile phase. Traces normalized to respective highest intensity.

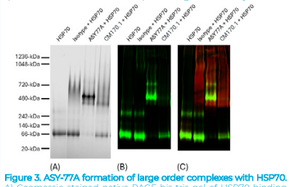


Figure 3. ASY-77A formation of large order complexes with HSP70. A) Coomassie stained native-PAGE bis-tris gel of HSP70-binding antibodies and antigen. LICOR immunoblot of (B) Detection of HSP70 (C92F3A-5; **green**) and (C) detection of mouse IgG1 antibodies (**red**) from native-PAGE in Panel A. Note, the HSP70 detection antibody and CM170.1 demonstrate overlapping binding epitopes to HSP70.

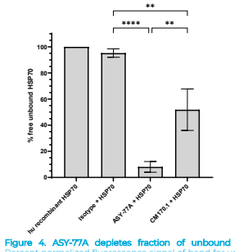


Figure 4. ASY-77A depletes fraction of unbound HSP70. Percent normalized fluorescence signal of band for unbound HSP70 (~66-kDa) from **Figure 3.** Statistical analysis and comparisons performed using one-way ANOVA from replicate studies (n=3; replicate native-PAGE not shown). **p<0.005, ***p<0.001

ASY-77A sensitizes tumors to α-PD1

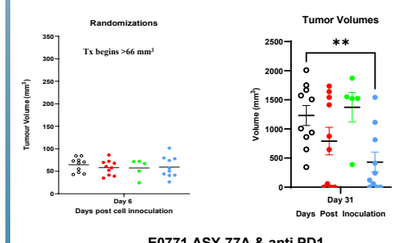


Figure 9. E0771 Tumor Growth Inhibition With ASY-77A in ICP Combo. E0771 was established orthotopically in C57BL/6 mice and tumors grown until large, established, tumors could be detected. Mice were then randomized and treated with ASY-77A mouse IgG2A (0.5mg/kg), anti-PD1 (RMP1-14)(5 mg/kg) or the combination (ASY-77A for 3 weeks). Tumor volumes are shown longitudinally and individual volumes at day 31.

ASY-77A synergizes with α-CTLA4 in B16 melanoma

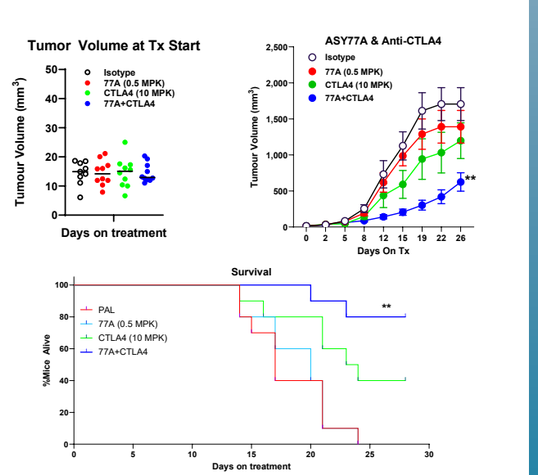
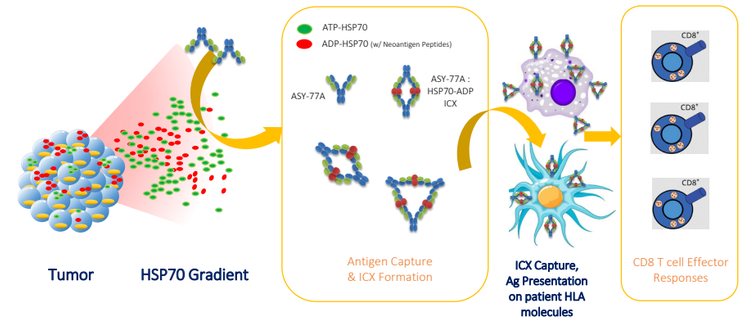


Figure 11. Tumor Growth Inhibition of B16F1 Tumors in a ICP Combination B16F1 (500,000 cells) was injected SC into C57BL6 mice, and tumors randomized on day 2 to 15mm³ for each group. Mice were then treated as indicated with ASY-77A mouse IgG2A (0.5mg/kg), anti-CTLA4 (9D9) or the combination twice per week for 3 weeks. Tumor volumes are shown longitudinally and survival.

Graphical Overview: ASY-77A *in vivo* mechanism of action



Conclusion

Our data demonstrates ASY-77A as a novel immunotherapy for cancer, having single-agent efficacy and demonstrated synergy with ICP inhibitors. Mechanistic data is supportive of rational combinations for ICP. These findings have broad applicability in cancer treatment due to the ubiquitous expression of and reliance on HSP70 in a multitude of cancer types.

Acknowledgements

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