# AO-176, A Next-Generation CD47 Antibody, Induces Immunogenic Cell Death

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## Abstract

Recent success in cancer immunotherapy has targeted immune checkpoints such as PD-1, PD-L1, and CTLA-4 to enhance the cytotoxic activity of the adaptive T cell immune response. While the clinical response to these therapies has been dramatic for some, many others have shown partial or even no response, highlighting the need for alternative or synergistic approaches that activate innate immunity. Disruption of the interaction between SIRPα and CD47, an innate checkpoint inhibitor, using anti-CD47 antibodies, for example, is known to enhance innate immunity by increasing the phagocytosis of tumor cells by macrophages and dendritic cells (DCs) leading to processing and presentation of tumor antigens. Recently, we described AO-176, a next generation anti-CD47 antibody that blocks the CD47/SIRPα interaction, induces phagocytosis and causes a direct tumor cell-autonomous death and with the advantageous property of negligibly binding RBCs.

Here, we characterize the ability of AO-176 to induce Immunogenic cell death (ICD) and Damage Associated Molecular Patterns (DAMPs) in tumor cells and to potentiate chemotherapy-induced ICD/DAMPs. ICD is a process whereby an agent induces cell surface exposure and release of DAMPs from dying cells which stimulates DCs and adaptive immune responses.

Tumor cells were treated in vitro with AO-176 either alone or in combination with chemotherapeutics followed by assessment of ICD/DAMPs using flow cytometry and biochemical assays. NanoString was also performed on cells undergoing AO-176 mediated ICD/DAMP induction to better understand how CD47 inhibition may lead to ICD.

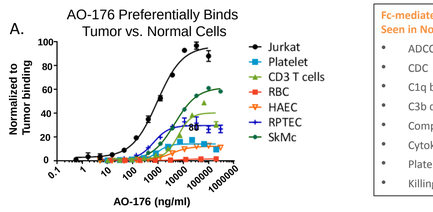
AO-176 and other CD47 antibodies, developed by Arch Oncology, caused mitochondrial stress and loss of outer-membrane integrity, typically observed prior to cells undergoing programmed cell death type III (PCDIII). In addition, CD47 antibody treatment induced a significant ER stress response at the genetic level resulting in the surface exposure of ER chaperone proteins calreticulin, Hsp90, and PDIA3. Concomitantly, our CD47 antibodies increased autophagy and JAK/STAT signaling which resulted in both ATP and HMGB1 release, respectively, characteristic of ICD like responses. Finally, we demonstrated that in combination, our antibodies potentiated the effects of ICD/DAMP-inducing chemotherapy (e.g. Doxorubicin).

Here, we describe the unique ability of a specific subset of next generation CD47 antibodies, such as AO-176 to induce ICD/DAMPs. RNAseq analysis of treated cells also revealed alteration of several pathways, including those where DAMPs play a role. In summary, next generation CD47 antibodies such as AO-176 may provide a novel approach to enhancing the current landscape of checkpoint immunotherapy by enhancing both the innate and adaptive immune responses against tumors.

## AO-176: A Next-Generation Humanized anti-CD47 mAb

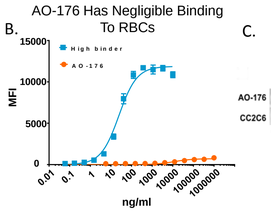
- Humanized IgG2
- Blocks CD47/SIRPα interaction to induce phagocytosis of tumor cells
- Selectively and potently binds to human CD47 on tumor cell lines
- Reduced binding to normal cells, negligible binding to human RBC, no hemagglutination
- Direct killing of tumor cells (non-ADCC); programmed cell death type III and Immunogenic cell death characterized by DAMP induction
- Potentiation of ICD-inducing chemotherapy
- Anti-tumor efficacy in human xenograft models
- Greater binding affinity at acidic pH (TME is pH ~ 6-6.5; potential tumor targeting mechanism)
- Very well tolerated in IND enabling toxicology studies

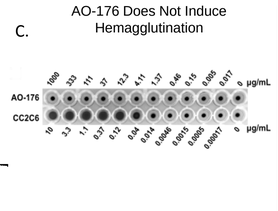
## AO-176 Preferentially Binds Tumor vs. Normal Cells and Negligibly Impacts RBC or Other Normal Cells




**Fc-mediated and direct AO-176 Activities Not Seen in Normal Cells**

- ADCC
- CDC
- C1q binding
- C3b deposition
- Complement mediated hemolysis
- Cytokine release
- Platelet activation
- Killing of non-tumor cells



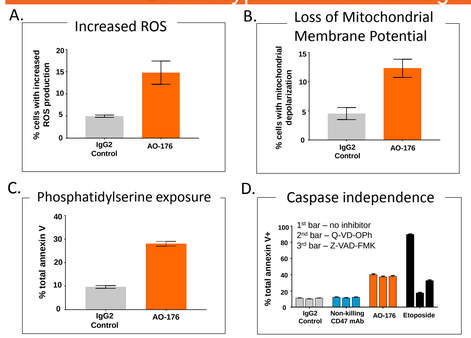




**AO-176 IND Enabling Toxicology Study**

- Dosed cynomolgus monkeys at 5, 50, 100 mg/kg IV once weekly x 5
- Transient, minor changes in hematology, only after first dose
  - No dose response observed
- Very well tolerated NOAEL = 100 mg/kg

(A) Comparison of binding of AO-176 to tumor cells (Jurkat) with reduced binding to human platelets, CD3 T cells, RBC, human aortic endothelial cells (HAEC), renal proximal tubule epithelial cells (RPTEC), and skeletal muscle cells (SkMC). (B) Negligible binding of AO-176 to human RBC compared to a high binding anti-CD47 mAb defined as a CD47 mAb that binds equally well to both tumor and normal cells. (C) In vitro washed RBC hemagglutination assay showing agglutination with positive control CC2C6 (CD47 mAb) and no agglutination with AO-176.

## AO-176 Mediates Caspase-Independent Programmed Cell Death Type III in Hematologic and Solid Tumor Cell Lines

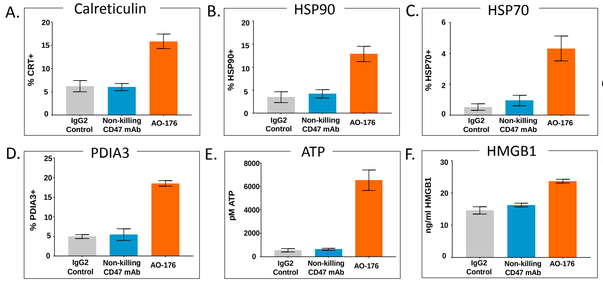



E.

| Cell Line | Tumor Type | % AV+ $IC_{50}$ | Max %AV+ |
|---|---|---|---|
| Jurkat | Acute lymphoblastic leukemia | 0.63 µg/ml | 45.6 |
| Raji | B cell lymphoma | 10 µg/ml | 32 |
| OV90 | Ovarian carcinoma | 5.1 µg/ml | 52 |
| Detroit562 | Pharyngeal carcinoma | 7.5 µg/ml | 52.9 |
| FaDu | Squamous cell carcinoma | 18.5 µg/ml | 20.3 |
| NCI-H1437 | Lung adenocarcinoma | 9.9 µg/ml | 34.4 |
| NCI-H1975 | Lung adenocarcinoma | 12.6 µg/ml | 14 |
| CFPAC-1 | Pancreatic adenocarcinoma | 1 µg/ml | 45 |

Jurkat T-ALL cells were incubated with 30 µg/ml AO-176 for 6 hours at 37°C. The percent of cells that (A) produce ROS and (B) have a loss of mitochondrial potential was detected by flow cytometry. Jurkat cells were treated with 30 µg/ml IgG2 control antibody or AO-176 for 24 hours then stained with Annexin V. Cells were analyzed by flow cytometry for the percentage of cells that had (C) flipped PS to the outer membrane as determined by total Annexin V. (D) Jurkat cells were treated for 30 minutes with the caspase inhibitors Q-VD-OPh or Z-VAD-FMK then treated for 24 hours with a non-killing CD47 antibody, AO-176, or etoposide. Cells were stained with Annexin V. (F) Hematologic and solid tumor cell lines were treated with AO-176 at various concentrations for 24 hours and analyzed for total Annexin V+ cells. The IC50 for % Annexin V+, max % Annexin V+ cells are shown for each cell line tested.

## AO-176 Induces DAMP Signaling in Hematologic and Solid Tumor Cell Lines




G.

| Cell Line | Tumor Type | Calreticulin | PDIA3 |
|---|---|---|---|
| OV90 | Ovarian carcinoma | ✓ | ✓ |
| HCC70 | Breast carcinoma | ✓ | ✓ |
| CFPAC-1 | Pancreatic adenocarcinoma | ✓ | ✓ |

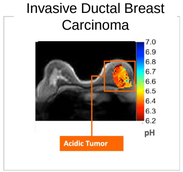
Addition of 10 µg/ml AO-176 or non-killing CD47 mAb to Jurkat cells for 24 hours. DAMP signaling as detected by externalization of (A) calreticulin (CRT+), (B) heat shock protein 90 (HSP90+), (C) heat shock protein 70 (HSP70+), (D) Protein Disulfide Isomerase Family A Member 3 (PDIA3), measured by flow cytometry and release of (E) ATP measured by bioluminescence assay and (F) HMGB1 by ELISA. In contrast, a non-killing antibody that binds with high affinity to CD47 and causes phagocytosis had no effect on the induction of DAMPs. (G) Additional cell lines that demonstrated increases in CRT and PDIA3 exposure are shown.

## AO-176 Enhanced Binding and Function At Acidic pH (Tumor Microenvironment)

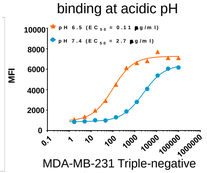
**AO-176 Shows Enhanced Binding At Acidic pH (Tumor Microenvironment)**

- Extracellular tumor microenvironment generally acidic due to high rates of metabolism and poor perfusion
- Expected to increase selectivity to tumor cells in acidic microenvironment

Patient with High-Grade Invasive Ductal Breast Carcinoma




AO-176 shows enhanced binding at acidic pH

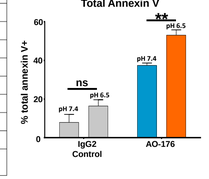



MDA-MB-231 Triple-negative breast cancer

B.

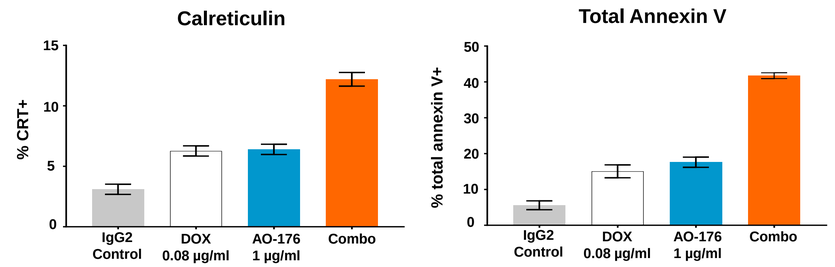
| Cell Line | Tumor Type | $EC_{50}$ pH 7.4 | $EC_{50}$ pH 6.5 | Fold Change in Binding |
|---|---|---|---|---|
| Jurkat | Acute lymphoblastic leukemia (ALL) | 0.39 µg/mL | 0.07 µg/mL | 6 |
| Raji | B cell lymphoma | 1.91 µg/mL | 0.039 µg/mL | 49 |
| OV10-315 | Ovarian cancer | 0.25 µg/mL | 0.054 µg/mL | 6 |
| HCC827 | Lung adenocarcinoma | 0.39 µg/mL | 0.084 µg/mL | 5 |
| HCT116 | Colorectal carcinoma | 3.62 µg/mL | 0.77 µg/mL | 5 |
| OV90 | Ovarian carcinoma | 0.13 µg/mL | 0.078 µg/mL | 1.6 |
| SkOV3 | Ovarian carcinoma | 1.27 µg/mL | 0.064 µg/mL | 20 |
| NCI-1975 | Lung adenocarcinoma | 2.56 µg/mL | 0.14 µg/mL | 18 |
| MCF7 | Breast carcinoma | 0.74 µg/mL | 0.10 µg/mL | 7 |
| MDA-MB-231 | Triple-negative breast cancer | 2.70 µg/mL | 0.11 µg/mL | 25 |

C. AO-176 shows enhanced killing at acidic pH



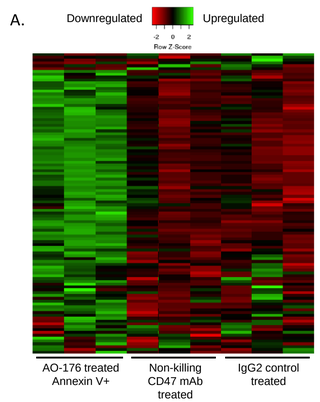

Binding of AO-176 to tumor cells at pH 7.4 and pH 6.5 was determined by flow cytometry. (A) Acidic tumor microenvironment of a ductal breast carcinoma. AO-176 binding to Jurkat cells at pH 7.4 and pH 6.5. (B) Hematological and solid tumor cell lines were analyzed for differential binding of AO-176 at pH 7.4 and pH 6.5. (C) Jurkat cells were treated with IgG2 control or AO-176 antibody in media buffered to pH 7.4 or 6.5 for 2 hours at 37°C without CO2. Cells were stained with Annexin V.

## AO-176 Potentiates ICD-inducing Chemotherapy




AO-176 potentiates chemotherapy induced ICD. Jurkat T-ALL cells were treated with IgG control, AO-176, doxorubicin (DOX) alone, or AO-176 in combination with doxorubicin for 24 hours. Cells were stained with calreticulin to measure ER stress and Annexin V to measure externalization of phosphatidylserine (Annexin V+). Signal intensity of calreticulin+ (CRT+) and Annexin V+ (AV+) cells was detected by flow cytometry.

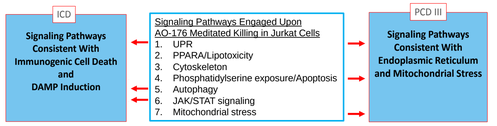
## NanoString Sequencing of Cells Undergoing AO-176 Mediated Cell Death Confirms Engagement of PCDIII and ICD Genetic Pathways




B.

| CD47 Signaling Pathways | Genes upregulated out of 5 probes representing each pathway (FC≥1.2, p≤0.05) |
|---|---|
| UPR | 4 |
| PPARA | 4 |
| Cytoskeleton | 4 |
| PS/Apop | 4 |
| Autophagy | 4 |
| JAK/STAT | 4 |
| Mito Stress | 3 |

C.




Jurkat cells were treated with 10 µg/ml of AO-176 before magnetic bead separation of cells based upon the surface exposure of phosphatidylserine (PS) (cells undergoing AO-176 mediated cell death). RNA was harvested from AO-176 treated PS+ (Annexin V+), IgG2 control treated, and non-killing CD47 mAb treated cells and sequenced by NanoString. (A) Heat map of gene expression in AO-176 treated Annexin V + cells compared to IgG2 control and non-killing CD47 antibody treated cells (10 µg/ml). (B) Pathway enrichment analysis of genes upregulated in AO-176 treated Annexin V+ cells compared to IgG2 control antibody treated cells. (C) Signaling pathways engaged upon CD47 mAb mediated killing are consistent with known ICD and PCDIII pathways.

## Conclusions

- In addition to promoting phagocytosis, AO-176 induces PCDIII as well as immunogenic cell death (ICD) in an ADCC independent manner in both hematologic and solid tumor cell lines
- AO-176 mediated PCDIII and ICD is characterized by genetic patterns of cellular stress
- In line with ICD, AO-176 induces DAMPs which wed and bolster anti-tumor innate and adaptive immunity
- AO-176 potentiates ICD-inducing chemotherapy
- AO-176 demonstrates preferential binding to tumor versus normal cells especially RBCs
- AO-176 shows enhanced binding and function at acidic pH levels seen in the tumor microenvironment, a potential tumor targeting mechanism
- AO-176's unique killing profile coupled with phagocytosis induction and preferential binding to tumor versus normal cells suggest that AO-176 will have an improved therapeutic index compared to current clinical candidates and supports further clinical investigation