

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

Benjamin J. Capoccia, Ronald R. Hiesch, Michael J. Donio, Alun J. Carter, Robyn J. Puro, W. Casey Wilson, Daniel S. Pereira, Pamela T. Manning and Robert Karr  
Arch Oncology, 4320 Forest Park Avenue, St. Louis, MO 63108 and 2000 Sierra Point Parkway, Brisbane, CA 94005

## Abstract

### Background

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 our CD47 antibodies such as 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.

### Methods

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

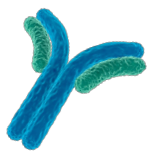
### Results

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 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).

### Conclusions

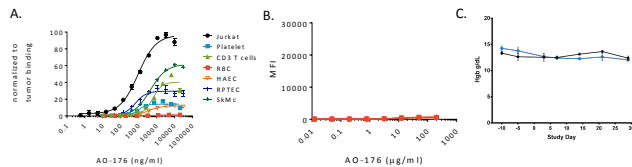
Here, we describe the unique ability of a specific subset of next generation CD47 antibodies, such as AO-176 to induce ICD/DAMPs. RNAseq analysis of treated cells also revealed alteration of several pathways, including those where DAMPs play a role. In summary, next generation CD47 antibodies such as 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



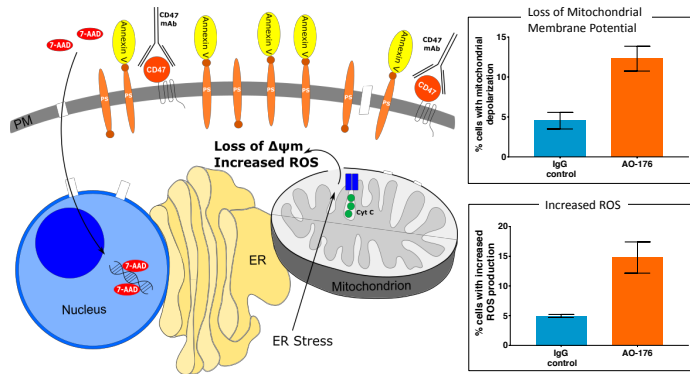
- Humanized IgG2
- Blocks CD47/SIRPα interaction to induce phagocytosis of tumor cells
- Novel PCDIII, direct killing of tumor cells (non-ADCC), and DAMP induction
- Potentiation of ICD-inducing chemotherapy
- Negligible binding to human RBCs and reduced binding to normal cells
- Minimal impact on red cells in non-GLP toxicology studies
- Greater affinity at acidic pH (potential tumor cells (non-ADCC), and DAMP induction)
- Anti-tumor efficacy in human xenograft models

## AO-176 Preferentially Binds Tumor vs. Normal Cells and Negligibly Impacts RBC and Hemoglobin in Cynomolgus Monkey



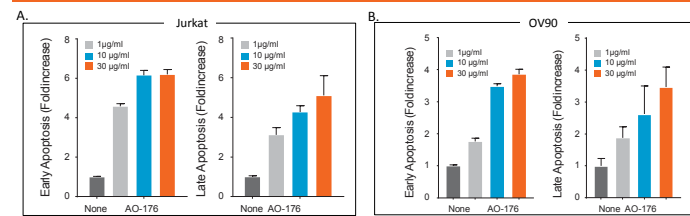
Binding of AO-176 to human CD47. (A) Comparison of binding of AO-176 to tumor cells (Jurkat, Platelet, CD3+ T cells, H460, H1975, H127, H1975, Detroit562, FaDu) and normal cells (RBC, H1975, H127, H1975, Detroit562, FaDu). (B) Negligible binding of AO-176 to human RBC. (C) AO-176 tolerability was examined in a four-week exploratory safety study in cynomolgus monkeys, where drug was given as weekly IV infusions (first dose of 5 mg/kg followed by 3 weekly doses of 50 mg/kg). AO-176 treatment resulted in minimal reduction in hemoglobin. These data are consistent with the reduced RBC binding observed with AO-176.

## AO-176 Mediates Programmed Cell Death Type III



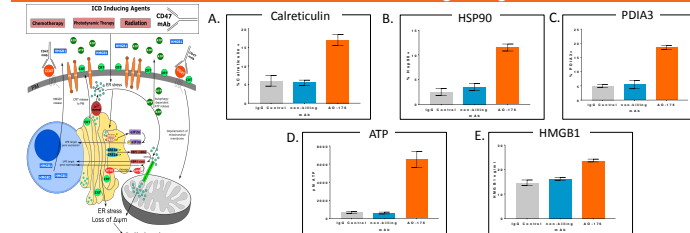
Programmed cell death type III is a caspase-independent process that involves mitochondrial damage and loss of membrane integrity. PCDIII induced by AO-176 was measured in Jurkat T-ALL cells incubated with 30 μg/ml of AO-176 for 6 hours at 37°C. Cells were stained with MitoSOX to measure the production of mitochondrial reactive oxygen species (ROS), and TMRM dye was used to measure the loss of mitochondrial membrane potential. The percent of cells that produce ROS and have a loss of mitochondrial potential was detected by flow cytometry. The data are shown as percent of total population for cells treated with IgG control antibodies or AO-176.

## AO-176 Mediates Cell-Autonomous Early and Late Apoptotic Killing of Tumor Cells



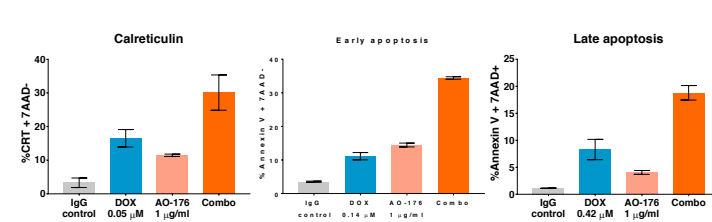
Cell autonomous killing by AO-176 was measured in (A) Jurkat (B) OV90 ovarian carcinoma cells incubated with various concentrations of AO-176 for 24 hours at 37°C. Cells were stained with Annexin V to measure externalization of phosphatidylserine (Annexin V+), a marker of early apoptosis, and uptake of 7-AAD dye, an indicator of apoptotic cell death. Signal intensity of Annexin V+ (AV+) and 7AAD+ cells was detected by flow cytometry. The data are shown as fold-change in early apoptotic (Annexin V+/7AAD negative) over untreated cells (none) and fold-change late apoptotic (Annexin V+/7AAD double positive) over untreated cells. Additional cell lines that demonstrate similar findings include RAJI, MOLT-4, SK-OV-3, CFPAC, H-1437, H-1975, Detroit562, CAL27, FaDu.

## AO-176 Induces DAMP Signaling



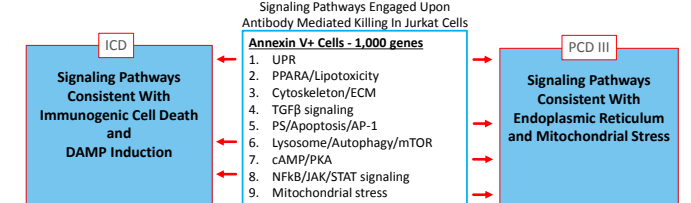
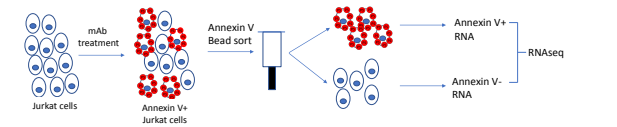
Induction of Damage Associated Molecular Patterns (DAMPs), endogenous danger signals which are markers of cell stress and promote inflammatory responses, were assessed in Jurkat cells. Addition of AO-176 for 24 hours induced DAMP signaling as detected by externalization of (A) calreticulin (CRT+), (B) heat shock protein 90 (HSP90), (C) Protein Disulfide Isomerase Family A Member 3 (PDIA3), measured by flow cytometry and release of (D) ATP measured by bioluminescence assay and (E) 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. Additional cell lines that demonstrated increases in at least one DAMP include RAJI, OV90, HCC70, PANC-1, CFPAC, H-1975.

## AO-176 Potentiates ICD-inducing Chemotherapy



ICD inducing CD47 mAbs potentiate chemotherapy induced ICD. Jurkat T-ALL cells were treated with 1 μg/ml IgG control, AO-176, 0.05, 0.14 and 0.42 μM doxorubicin (DOX) alone, or AO-176 in combination with doxorubicin for 24 and 48 hours. Cells were stained with calreticulin to measure ER stress at 48 hours, Annexin V to measure externalization of phosphatidylserine (Annexin V+), a marker of early apoptosis, and uptake of 7-AAD dye, an indicator of apoptotic cell death at 24 hours. Signal intensity of calreticulin+ (CRT+), Annexin V+ (AV+) and 7AAD+ cells was detected by flow cytometry.

## RNAseq of Cells Undergoing CD47 Mediated Cell Death Confirm Engagement of PCDIII and ICD Genetic Pathways



Jurkat cells were treated with 10 μg/ml of a killing CD47 antibody before magnetic bead separation of cells based upon the surface exposure of phosphatidylserine (PS) (cells undergoing CD47 mediated cell death). RNA was harvested from PS+ (Annexin V+) and PS- cells and sequenced. COMPBIO analysis (GTAC, Washington University School of Medicine) identified the most abundantly represented molecular pathways represented by the genes that were enriched in Annexin V+ cells. These pathways are considered characteristic of cells undergoing CD47 mediated cell death.

## Conclusions

- In addition to promoting phagocytosis, AO-176 induces tumor cell autonomous stress and causes a PCDIII as well as immunogenic cell death (ICD) in an ADCC independent manner
- 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 mediated PCDIII and ICD is characterized by genetic patterns of cellular stress
- AO-176 demonstrates preferential binding to tumor versus normal cells especially RBCs
- AO-176's unique killing profile coupled with its innate 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