Therapeutic potential of AO-176, a next-generation humanized CD47 antibody, for hematologic malignancies

W. Casey Wilson, Myriam N. Bouchlaka, Benjamin J. Capoccia, Ronald R. Hiebsch, Michael J. Donio, Robyn J. Puro, Alun J. Carter, Prabir Chakraborty, Pamela T. Manning, Robert W. Karr, Vicki Sung and Daniel S. Pereira

Arch Oncology, 4320 Forest Park Avenue, St. Louis, MO 63108 and 2000 Sierra Point Parkway, Brisbane, CA 94005

Abstract

Inhibitors of adaptive immune checkpoints have shown promise as cancer treatments. CD47 is an innate immune checkpoint receptor broadly expressed on normal tissues and overexpressed on several tumors. Binding of tumor CD47 to signal regulatory protein alpha (SIRPα) on macrophages and dendritic cells triggers a "don't eat me" signal that inhibits phagocytosis enabling escape of innate immune surveillance. Blocking CD47/SIRPα interaction promotes phagocytosis reducing tumor burden in numerous xenograft and syngeneic animal models.

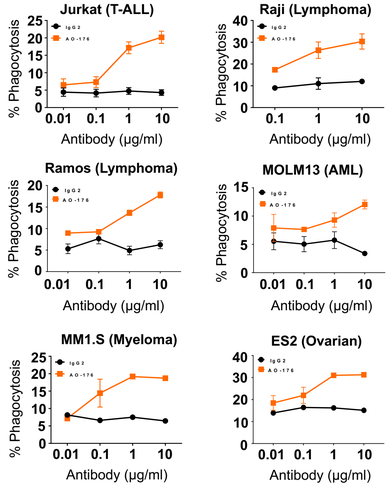
We have developed a next generation humanized anti-CD47 antibody, AO-176, that not only blocks the CD47/SIRPα interaction and induces phagocytosis of hematologic and solid tumor cells, but also exhibits several unique functional properties. The first property is the ability of AO-176 to induce direct tumor cytotoxic cell death in hematologic (ex. Jurkat, Raji and MOLT-4) as well as solid human tumor cell lines by a cell autonomous mechanism (not ADCC). Secondly, AO-176 exhibits preferential binding to tumor versus normal cells, including red blood cells (RBCs), T cells, endothelial cells, skeletal muscle cells and epithelial cells. A0-176 also does not affect the function of any of these primary cells when assayed ex vivo. The negligible binding of AO-176 to RBCs versus hematologic (ex. Jurkat, Raji or MOLT-4) or solid tumor cells is particularly profound and different from other reported anti-CD47 antibodies. AO-176 also does not induce hemagglutination of RBCs. These properties are expected not only to decrease the antigen sink, but also to minimize on-target clinical adverse effects observed following treatment with other reported RBC-binding anti-CD47 antibodies. Consistent with this attribute, AO-176 was well tolerated in cynomolgus monkeys with no adverse effects in general nor with respect to RBCs which was consistent with ex vivo results. A third novel property of AO-176 is its enhanced binding to tumor cells at acidic pH. Because the microenvironment of leukemic bone marrow and solid tumors has an acidic pH, this enhanced binding of AO-176 at low pH has the potential added advantage of tumor-specific targeting. Lastly, we show that AO-176 demonstrates dose-dependent anti-tumor activity in hematologic and solid tumor xenograft models.

Taken together, the unique properties and anti-tumor activity of our next generation anti-CD47 antibody, AO-176, distinguishes it from other CD47/SIRPα axis targeting agents as it progresses to clinical development.

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

- Humanized IgG2 monoclonal antibody
- 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
- Greater binding affinity at acidic pH
- Direct killing of tumor cells (non-ADCC) via a Programmed Cell Death Type III mechanism and immunogenic cell death
- Anti-tumor efficacy in human xenograft models
- Promotes immune cell recruitment and release of cytokines and chemokines

AO-176 Induces Phagocytosis of Hematological and Solid Tumor Cells

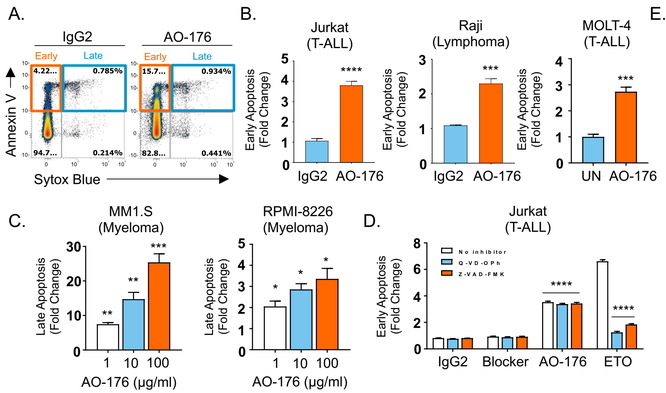


B.

Cell Line	Indication	Δ% Phagocytosis	EC_{50} (µg/ml)
Jurkat	T-ALL	45	0.72
Raji	NHL	21	0.43
MM1.S	MM	22	0.05
Ramos	NHL	18	1.00
MOLM13	AML	12	1.00
ES2	Ovarian	18	0.21
OV90	Ovarian	16	0.30
OVCAR3	Ovarian	16	3.30
OV56	Ovarian	17	0.22
UWB1.289	Ovarian	10	0.09
FaDu	HNSCC	17	0.16
CAL27	HNSCC	29	0.19

(A) CFSE-labeled human Jurkat T cell leukemia, Raji lymphoma cells, Ramos lymphoma cells, MOLM13 acute myeloid leukemia cells, MM1.S multiple myeloma cells or ES2 ovarian carcinoma cells were cultured with human macrophages in the presence of 0.01-10 µg/ml of either IgG2 control antibody or AO-176 at 37ºC for 2 hours. Cells were harvested and additionally stained for CD14 to delineate macrophages. Phagocytosis was determined by the percentage of $CFSE^+CD14^+$ cells using flow cytometry. **(B)** Table listing % phagocytosis observed with hematological and solid tumor cell lines treated with AO-176, with corresponding EC_{50} values.

AO-176 Possesses Direct Tumor Killing Activity via a Caspase-Independent Mechanism: Programmed Cell Death Type III

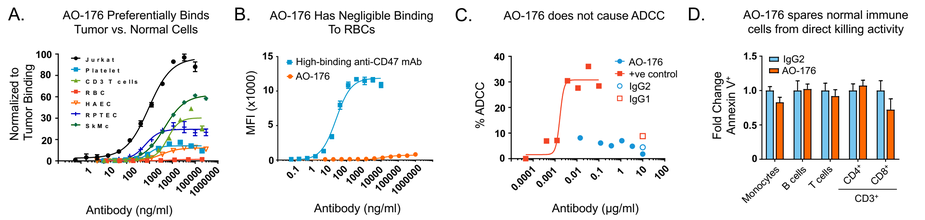


E.

Cell Line	Indication	% Killing (Ann V+)	EC_{50} (µg/ml)
Jurkat	T-ALL	33	11
MOLT4	T-ALL	18	1.0
Raji	NHL	18	10
MM1.S	MM	15	10
RPMI-8226	MM	20	10
NCI-H929	MM	12	10
OV90	Ovarian	29	11
SKOV3	Ovarian	21	5
FADU	HNSCC	13	30
CAL27	HNSCC	16	30
NCI-H1437	NSCLC	35	11
NCI-H1975	NSCLC	14	31
CFPAC	PDAC	49	12

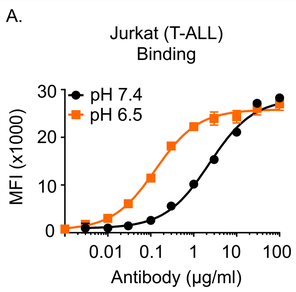
(A) FACS plots representative of Annexin V and Sytox Blue staining. **(B)** Jurkat T-ALL, Raji lymphoma, or MOLT-4 T-ALL cells were incubated with 30 µg/mL IgG2 control antibody or AO-176 for 24 hours. Early apoptosis was measured by Annexin V^+ and Sytox $Blue^-$ ($7AAD^-$ for MOLT-4) staining, with fold change compared to IgG2 (or untreated "UN" for MOLT-4) quantified. **(C)** MM1.S and RPMI-8226 multiple myeloma cell lines were incubated with 1, 10, or 100 µg/mL AO-176, and stained with Annexin V and 7AAD to assess early and late apoptosis. Fold changes in Annexin V^+ $7AAD^+$ (late apoptosis) cells are shown, compared to IgG2 control (not shown). **(D)** To evaluate caspase-dependence of direct tumor cell killing by AO-176, Jurkat cells were treated with 30 µg/mL IgG2 control, 30 µg/mL of non-killing anti-CD47 antibody (Blocker), 30 µg/mL AO-176, or 1µM etoposide (ETO) for 24 hours in the presence of Caspase inhibitors. **(E)** Table listing % killing (Annexin V+) observed with hematological and solid tumor cell lines treated with AO-176, with corresponding EC_{50} values. *p<0.05, **p< 0.01, ***p<0.001 and ****p< 0.0001 vs. IgG2.

AO-176 Preferentially Binds Tumor vs. Normal Cells With Negligible Impact on Red Blood Cells and Normal Cells



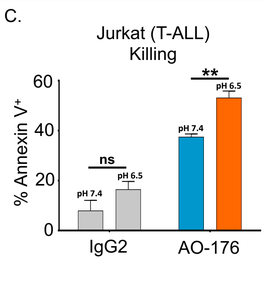
(A) Binding of AO-176 to tumor cells (Jurkat) with reduced binding to human platelets, CD3 T cells, RBC, human aortic endothelial cells (HAEC), renal proximal tubule epithelial cells (RPTEC), and skeletal muscle cells (SkMC). **(B)** Negligible binding of AO-176 to human RBCs compared to a high binding anti-CD47 mAb. **(C)** Calcein AM-loaded human dermal microvascular endothelial cells (HMVEC) were incubated with antibodies and NK cells at an Effector:Target ratio of 10:1 for 4 hours, then % lysis was measured by release of Calcein as a surrogate for ADCC. **(D)** Isolated human PBMCs were incubated with 100 µg/mL AO-176 or IgG2 control for 24 hours, then % Annexin V^+ quantified in monocytes ($CD14^+$), B cells ($CD20^+$), total T cells ($CD3^+$), CD4 T cells ($CD3^+CD4^+$) or CD8 T cells ($CD3^+CD8^+$), with fold change of AO-176 treated cells calculated relative to IgG2.

AO-176 Binding of CD47 and Direct Killing Activity Increases at Acidic pH



B.

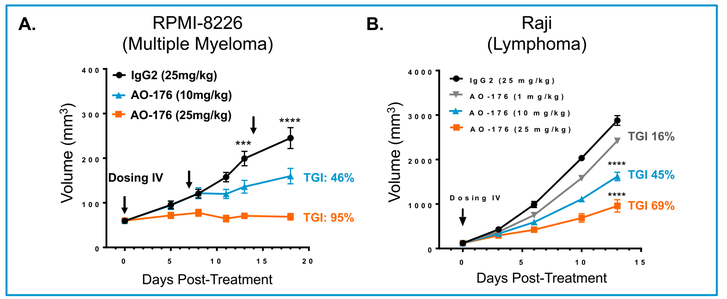
Cell Line	Tumor Type	EC_{50} pH 7.4	EC_{50} pH 6.5	Fold Change in Binding
Jurkat	Acute lymphoblastic leukemia (ALL)	0.39 µg/mL	0.07 µg/mL	6
Raji	B cell lymphoma	1.91 µg/mL	0.039 µg/mL	49
OV10-315	Ovarian cancer	0.25 µg/mL	0.054 µg/mL	6
HCC827	Lung adenocarcinoma	0.39 µg/mL	0.084 µg/mL	5
HCT116	Colorectal 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



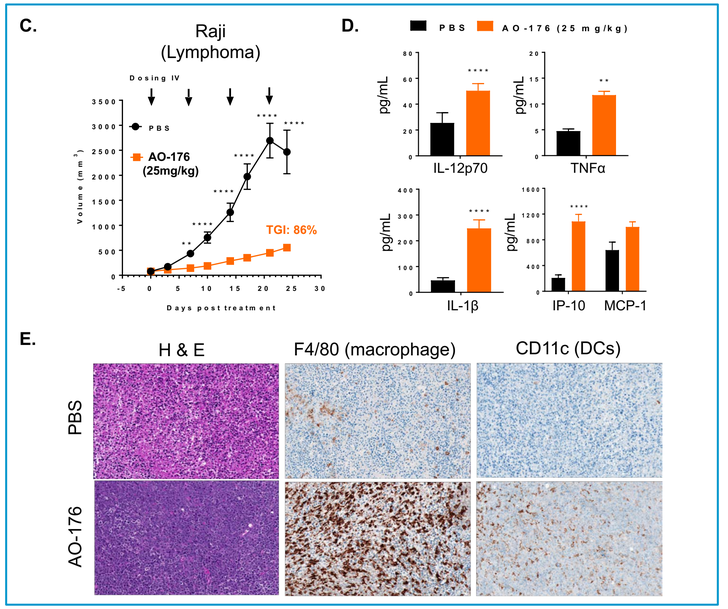
(A) Jurkat T-ALL cells were incubated with 0.001-100 µg/mL AO-176 in media buffered to pH 7.4 (black line) or 6.5 (orange line). Binding curves were then generated by staining cells with anti-human secondary antibody and measuring MFI by flow cytometry. **(B)** Table listing EC_{50} values of AO-176 binding observed with hematological and solid tumor cell lines at pH 7.4 and pH 6.5, with corresponding fold changes in binding. **(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. Cells were then stained with Annexin V and analyzed by flow cytometry. **p<0.01 pH 6.5 vs. pH 7.4.

AO-176 Inhibits Hematological Tumor Growth *In Vivo* And Promotes Macrophage/Dendritic Cell Recruitment and Cytokine Induction

Dose Escalation and Efficacy



Increased Immune Infiltrate



AO-176 –Treated Lymphoma Tumors Exhibit Increased

(A) Human RPMI-8226 multiple myeloma or **(B)** Raji B cell lymphoma cells were implanted subcutaneously into NSG mice (N= 10/group). **(A)** Mice received IgG2 or AO-176 intravenous (IV) on days 0, 7, & 14 for RPMI model.(N=10/ group) **(B)** In Raji model, mice received IgG2 or AO-176 as a single dose on Day 0 by IV injections. **(C)** Human Raji B cell lymphoma cells were injected subcutaneously into NSG mice. PBS vehicle control or AO-176 at 25 mg/kg was administered on days 0, 7, 14 & 21 by intravenous injections (N=10/group). **(D-E)** Tumors were excised on Day 28 and **(D)** tumor cytokines and chemokines were quantified in tumor microenvironment (N=5 mice /group) or **(E)** stained for H&E (Hematoxylin and Eosin), F4/80+ macrophages or mouse CD11c+ DCs (dendritic cells). **p< 0.01, ***p<0.001, and ****p< 0.0001 vs. vehicle control group. TGI: tumor growth inhibition.

Conclusions

AO-176 is a next generation CD47 antibody that is differentiated from current clinical agents targeting the CD47 axis as follows:

- AO-176 efficiently induces phagocytosis of a variety of hematological tumor cell lines, and direct killing activity.
- 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 mechanism for enhanced tumor targeting.
- AO-176 has shown significant tumor growth inhibition *in vivo*, likely driven by recruitment of macrophages and DCs.
- Recruitment of these innate immune cells leads to the release of cytokines and chemokines within the tumor microenvironment that may aid anti-tumor efficacy.
- 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.