Development of AO-176, a Next Generation Humanized Anti-CD47 Antibody With Novel Anti-Cancer Properties and Negligible Red Blood Cell Binding

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Running Title:

AO-176, A Next Generation Humanized Anti-CD47 For Cancer

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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 many 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 to induce tumor cell phagocytosis, but also induces tumor cytotoxicity in hematologic and solid human tumor cell lines, but not normal non-cancerous cells, by a cell autonomous mechanism (not ADCC). AO-176 also binds preferentially to tumor versus many normal cell types. In particular, AO-176 binds negligibly to RBCs in contrast to tumor cells, even at high concentrations up to 200 µg/ml and does not agglutinate RBCs up to 1 mg/ml in vitro. 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. When tested in cynomolgus monkeys, AO-176 was well tolerated with no adverse effects. Lastly, we show that AO-176 demonstrates dose-dependent anti-tumor activity in 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 in clinical development.
INTRODUCTION

CD47 is an integral membrane protein now recognized as an important immune checkpoint. The original description of CD47 as a “don't eat me” signal was based on the observation that CD47-null or deficient red blood cells (RBCs) injected into wild type (WT) mice were rapidly cleared from the circulation via phagocytosis by splenic macrophages (1). The phagocytosis of target cells was found to depend on a balance between pro-phagocytic and anti-phagocytic signals (2). Cell surface calreticulin, annexin-binding lipids and opsonized antibodies provide pro-phagocytic signals that are mediated by activating receptors such as low density lipoprotein receptor-related protein 1 (LRP) and Fc receptors while CD47 binding to SIRPα transmits a strong anti-phagocytic signal due to SIRPα activation of intracellular phosphatases such as SHP1 and SHP2 which then disable the actin-based phagocytic mechanism (3). An important corollary of the action of CD47 as a ”don't eat me” signal is its role as a “marker of self”. This provides a significant hindrance to phagocytosis of self and blocks a subsequent autoimmune response. Thus when certain autoimmune prone stains of mice are crossed with CD47 nulls, the resultant mice are severely and sometimes lethally autoimmune (4). The species-specific nature of the CD47/SIRPα interaction also allows for discrimination of foreign cells as non-self, providing a significant barrier to xenotransplantation (5). Cancer cells use CD47 to mask themselves in “selfness” consequently evading both the innate and adaptive immune systems. Anti-CD47 blockade has the potential to eliminate the acquired selfness of cancer cells.

The interaction of CD47 with SIRPα on innate immune cells such as macrophages and dendritic cells (DCs) has emerged as a viable target in cancer therapy. A large body of preclinical data (3, 6-14) has indicated that anti-CD47 antibodies that block the “don't eat me” signal sent by CD47 on cancer cells to SIRPα on innate phagocytes promote the phagocytosis and destruction of cancer cells and also prime the adaptive immune system to mount an effective anti-tumor response (15-18). While many cancer cells have elevated CD47 expression compared to their normal counterparts (10), the broad expression of CD47 on many normal cell types presents potential issues impacting the use of anti-CD47 agents in the clinic. Circulating blood cells including RBCs express CD47, presenting a large sink for CD47 antibodies. Furthermore,
blocking CD47 on RBCs has led to transient anemia (10, 19, 20). Although there are now several ongoing clinical trials with three different anti-CD47 antibodies and three other agents that target the CD47/SIRPα interaction (20, 21), there remains a need for next generation molecules that have unique properties to improve the clinical efficacy and safety profile (13, 21, 22).

Many groups have taken the approach of developing antibodies or constructs that solely block the CD47/SIRPα interaction (21, 22). However, there is a property of certain anti-CD47 antibodies that has yet to be leveraged in the treatment of cancer patients, the ability to exert a direct cytotoxic effect on cancer cells. Multiple groups have shown that particular anti-CD47 antibodies, including MABL-1, MABL-2, Ad22 and 1F7, exhibited the additional property of directly killing tumor cells in vitro via a cell autonomous mechanism that did not require the interaction with any immune effector cells including macrophages, DCs, natural killer (NK) or cytotoxic T cells (23-28). Importantly, MABL-1 and MABL-2 and their single chain derivates, that killed leukemia and multiple myeloma cells in vitro, were also efficacious in mouse xenografts in vivo (23, 27, 28).

Here we describe AO-176, a next generation humanized anti-CD47 antibody possessing a novel set of properties. In addition to inducing tumor phagocytosis through blocking the CD47-SIRPα interaction, AO-176 preferentially binds to tumor versus normal cells (particularly RBCs where binding is negligible) and directly kills tumor versus normal cells. We postulate that these differentiative properties will prove superior to other CD47/SIRPα axis targeting agents currently in clinical development.
MATERIALS and METHODS

Cell Culture

All human tumor lines (Jurkat T-ALL, Raji B cell lymphoma, OV90 ovarian carcinoma, HCC827 lung adenocarcinoma, SNU-1 gastric carcinoma and MDA-MB-231 triple negative breast cancer (TNBC) were purchased from the American Tissue Culture Collection (ATCC). OV10-315 ovarian carcinoma cells were transfected with and stably express human CD47 (29). Jurkat CD47 knockout (KO) cells were generated using CRISPR/Cas9 technology (30-31) employing guide RNA CACTGAAGTATACGTAAAGNGG and CTTGTTTAGAGCTCCATCAANGG (Genome Engineering and iPSC Center, Washington University, MO, USA). Jurkat wildtype, Jurkat CD47 KO, Raji, HCC827, MDA-MB-231 and OV10-315 were cultured in RPMI-1640 medium (GIBCO, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). OV90 cells were cultured in a 1:1 mixture of MCDB 105 medium containing a final concentration of 1.5 g/L sodium bicarbonate and Medium 199 containing a final concentration of 2.2 g/L sodium bicarbonate supplemented with 15% FBS and 1% P/S. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Human aortic endothelial cells (HAEC), human renal proximal tubule cells (RPTEC) and human skeletal muscle cells and myoblasts (SKMC) were purchased from Lonza (Basel, Switzerland). HAEC and SKMC cells were cultured in EGM-2 BulletKit media (Lonza, Basel, Switzerland). RPTEC cells were cultured in REGM Renal Epithelial cell growth medium (Lonza, Basel, Switzerland). All three lines were expanded at 37°C in a humidified CO₂ incubator and used between passages 1 and 3.

Antibodies (AO-176, AO-104)

Mouse anti-CD47 monoclonal antibodies were generated by immunizing WT or CD47-deficient mice with purified human placental CD47. Following repetitive immunization, the spleen cells were fused with the non-secreting myeloma P3 x 63Ag8.653 (ATCC) and clones were screened for reactivity to human CD47. RNA was isolated from the hybridoma cells and immunoglobulin
cDNA was synthesized using and oligo dT primer and reverse transcriptase. Degenerate 5’ primers based on canonical nucleotide sequences encoding mouse heavy chain variable domain (VH) or light chain variable domain (VL) germline sequences and 3’ IgG1/2 or light chain constant region-specific primers were used to amplify the variable region coding sequences. Amplicons were analyzed on an agarose gel, purified, TOPO cloned and sequenced. Nucleotide sequence analysis of multiple clones for both variable regions was performed to verify the coding sequences. The variable domains were cloned into human Ig expression vectors to create chimeric mouse-human antibodies. Mouse clones 104 and 176 were humanized by rational design to create AO-104, with a wildtype kappa light chain and an IgG4 Fc, and AO-176, with a wildtype kappa light chain and a wildtype IgG2 Fc (Genbank accession # AAB59393, AH002839, Figure S1), which were expressed by Evitria AG and purified in house. AO-176 and AO-104 are cross reactive to human and cynomolgus monkey CD47 (see Supplemental Table 1). AO-176 is not cross-reactive to murine CD47 (Figure S2).

**Flow Cytometry Antibodies For T Cell Proliferation & Apoptosis**
Fluorochrome conjugated mouse anti-human Abs to CD3 (clone SK7), CD4 (clone OKT4), CD8 (clone SK1) antibody were purchased from eBioscience (CA, USA). Human TruStain Fc Block anti huCD16/huCD32/huCD64 blocking Ab and isotype-matched control murine IgG1 and IgG2a Ultra-LEAF were purchased from BioLegend (CA, USA). Human anti-Annexin V and 7-AAD were purchased from BD (NJ, USA).

**ELISA Assay For Measurement of Antibodies In Plasma**
A solid-phase CD47 ELISA was utilized to measure circulating plasma levels of both AO-176 and AO-104. Briefly, high binding plates were with the extracellular domain of human CD47 containing an N-terminal histidine tag (His-CD47, ACRO Biosystems, NJ, USA). The wells were washed with PBS containing 0.05% Tween-20, pH 7.4 (PBS+T) and blocked with casein blocking reagent (Pierce #37528). Plasma samples diluted in assay diluent (PBS+T, 0.5M NaCl, 5mM EDTA, 0.25% CHAPS, and 0.5% BSA). Bound antibody was detected using horseradish peroxidase conjugated goat anti-human IgG, Monkey Adsorbed (Bethyl Laboratories #A80-319P, TX, USA) using QuantaBlu substrate reagent (Thermo Scientific #15169, MA, USA) and
fluorescence at Em:320 nm and Ex: 405 nm. Antibody concentrations in serum samples were calculated by interpolation from a standard curve and multiplication by the dilution factor.

**CD47 Receptor Number**

Various human cell lines (tumor and normal cells), human immune cells and cynomolgus immune cells were quantified for CD47 cell surface antigen expression using QIFIKIT Dako kit (CA, USA). Briefly, cells were plated in triplicate and incubated with saturating concentrations of a mouse antibody against CD47 (Vx1000R mIgG2a, Arch Oncology) for 45 minutes at 4°C. Cells were washed twice in PBS/BSA/Azide (0.1% BSA/15 mmol/L Azide NaN3) and then cells and QIFIKIT beads were stained in parallel with F(ab')2 fragment of FITC-conjugated goat anti-mouse immunoglobulins (following manufacturer’s instructions) at saturating concentrations at 4°C for 45 minutes. Next, cells and beads were washed thrice in PBS/BSA/Azide and resuspended in the same buffer. All samples were assessed using a flow cytometer (Attune Nxt cytometer, Life Technologies/Thermo Fisher Scientific, MA, USA) and mean fluorescent intensities (MFIs) from beads were plotted to obtain a calibration curve. MFIs from each sample were interpolated from the calibration to obtain the number of CD47 receptor number expression on cells.

**Binding Assay With Tumor & Normal Cells**

Tumor cell lines or normal lines (HAEC, RPTEC and SKMC) were plated in duplicate in 96 well plates and incubated with various concentrations of AO-176 or an isotype control IgG2 (CrownBio) at 37°C for 1 hour. Cells were washed three times followed by incubation with a FITC-fluorescently-labeled goat anti-human whole IgG secondary antibody (Jackson ImmunoResearch Inc, PA, USA) and incubated at 37°C for 30 minutes. Cells were washed three times and resuspended in PBS containing 1% EDTA and 5% FBS. Samples from tumor binding assays were acquired on BD Accuri CS Sampler (BD Biosciences, NJ, USA) and samples from normal cells binding were acquired on Attune Nxt Cytometer (Life Technology/Thermo Fisher Scientific, Waltham, MA) to determine the median fluorescent intensity (MFI) at each antibody concentration. FACS data were analyzed using either the BD CS sampler software or the Attune Nxt Software.
**RBC, Platelet & PBMC Isolations**

Human RBCs were obtained from whole blood from 48 healthy donors (New York Blood Center) and cynomolgus monkey RBCs were obtained from whole blood (BioIVT, Westbury, NY), diluted 1:300 in phosphate buffered saline (PBS) containing 1mM ethylenediaminetetraacetic acid (EDTA) and washed twice with phosphate buffered saline PBS/1mM EDTA.

Human and cynomolgus monkey platelets were isolated from platelet rich plasma (PRP) by centrifugation of whole blood at 500 x g to remove RBCs, followed by secondary centrifugation of PRP at 2500 x g to pellet the platelets. The platelets were washed and incubated with chromPure human IgG Fc (Jackson ImmunoResearch, PA, USA) to block Fc receptors.

Peripheral blood mononuclear cells (PBMC) were isolated from freshly prepared buffy coats or whole blood from normal human healthy volunteers (n= 8; obtained from Interstate Blood Bank, Memphis, TN, USA) in heparinized bags and processed within 24 hours of collection. PBMCs were isolated by standard density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare, WI, USA). Cynomolgus monkey PBMCs were obtained frozen from IQ Biosciences (CA, USA).

**Binding Assays With Human & Cynomolgus RBCs, Platelets & Primary T Cells**

Washed RBCs were incubated with increasing concentrations of AO-176 or AO-104 at 37°C for 1 hour, washed and incubated with FITC-labeled goat anti-human IgG secondary antibody (Jackson ImmunoResearch Inc, PA, USA) and incubated at 37°C for 30 minutes. Samples were analyzed using an Accuri flow cytometer.

Isolated platelets were incubated with AO-176, AO-104 or a human isotype control IgG at 37°C for 1 hour, washed and incubated at 37°C for 30 minutes with an Alexafluor 647 (AF647)-labeled goat anti-human IgG secondary antibody. Cynomolgus monkey platelets were further stained with Pacific Blue-conjugated anti-human CD41 (cynomolgus monkey reactive, BioLegend, CA, USA) and FITC-conjugated anti-human CD63 (cynomolgus monkey reactive, BioLegend, CA, USA). Samples were analyzed using an Accuri or Attune flow cytometer gating on CD41⁺ CD63⁺ cells for cynomolgus monkey samples and CD41⁺ CD62⁺ cells for human samples.

Unstimulated (naïve) cynomolgus monkey and human naïve and activated PBMCs were incubated with increasing concentrations of AO-176, AO-104 or a human isotype control IgG at
37°C for 1 hour. Cells were washed followed by incubation with a FITC- or AF647-fluorescently-labeled goat anti-human IgG secondary antibody (Jackson ImmunoResearch Inc, PA, USA), incubated at 37°C for 30 minutes and washed. Human PBMCs were incubated with AF450 conjugated anti-human CD3 at 4°C for 20 minutes, washed twice in FACS buffer and resuspended in FACS buffer for flow cytometry analysis. Cynomolgus monkey PBMCs were further stained with PE-conjugated anti-human CD45 and APC-conjugated anti-human-CD3 (both cynomolgus monkey reactive). Samples were analyzed using an Attune flow cytometer, gating on CD3^+ cells.

**T Cell Activation**

For T cell activation, 96 well plates were coated with 1µg/ml of anti-CD3 (clone OKT3, eBiosciences) at 4°C for 16 hours. The plates were washed with PBS to remove excess anti-CD3 antibody. PBMCs (2 x10^5 cells/well) were added to the anti-CD3 pre-coated plates followed by addition of 0.2 µg /well of soluble anti-human CD28 (clone 28.8, eBiosciences) in AIM-V media containing AlbuMax (BSA) (GIBCO, MA, USA). The cells were cultured up to 3 days at 37 °C in a humidified CO_2 incubator. Prior to the binding assay, naïve and activated PBMCs were incubated for 15 minutes with True stain human Fc block (BioLegend, CA, USA).

**T Cell Proliferation & Apoptosis Assays**

T cells were activated as described above. For T cell proliferation, PBMCs (2 × 10^5/well) were labeled with 1 µM carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies, CA, USA) for 15 minutes. CFSE staining was neutralized with RPMI (GIBCO, MA, USA) and cells were subsequently washed with PBS. CFSE-labeled PBMCs were plated on day 0 into plates pre-coated with anti-human CD3 and anti-human CD28 was added in soluble form to PBMCs in AIM-V media containing AlbuMax (BSA). On day 2 of T cell activation, AO-176 and isotype control antibody were added in soluble form, at different concentrations, to activated T cells and incubated for another 24 hours at 37 °C in a humidified CO_2 incubator.

For apoptosis assays, cells from each group were collected and stained with fluorochrome conjugated anti-human CD3, Annexin V and 7ADD (BD) to determine the percent of late apoptotic cells (Annexin V^+ /7AAD^+) on CD3^+ T cells by flow cytometry. To measure T cell proliferation, cells were collected on day 3 and stained with fluorochrome conjugated anti-human CD3 , anti-human CD4 and anti-human CD8 (BioLegend, CA, USA) to determine the
percent of proliferating (diluted CFSE) CD3⁺ T cells, CD3⁺CD4⁺ T cells or CD3⁺ CD8⁺ T cells by flow cytometry. Flow cytometry was performed with an Attune Nxt FACS machine (Life Technology/Thermo Fisher Scientific, MA, USA) and data were analyzed using Attune Nxt software.

**Human Tumor & Normal Cell Killing Assays**

Human normal cells (HAEC, SKMC and RPTEC) or tumor cell lines were plated in 96 well plates at 1x10⁵ cells/well and incubated with various concentrations of AO-176, human IgG2 isotype control, media or 6 µM Camptothecin (CPT) as a positive control (Sigma Aldrich) for 24 hours at 37°C. Cells were harvested, washed once with PBS and once with Annexin V Binding Buffer (BD Biosciences, NJ, USA). Cells were stained with Annexin V and 7-AAD dye in Annexin V buffer for 30 minutes at room temperature, washed, resuspended in Annexin V buffer and analyzed immediately for the percent of cells that stained positive for cell surface Annexin V and 7-AAD nuclear incorporation by flow cytometry (Accuri C6, Becton Dickinson, NJ, USA). Early apoptotic cells defined as Annexin V⁺ and 7-AAD⁻ or late apoptotic cells defined as Annexin V⁺ and 7-AAD⁺.

**Hemagglutination Assay**

Human RBCs were collected by venipuncture and washed three times with PBS containing 1mM EDTA. Increasing concentrations of anti-CD47 mAbs (up to 200 µg/ml) were added to wells containing RBCs and the plates were incubated for 4 hours at 37°C. A diffuse hazy pattern indicates hemagglutination (HA) whereas a small punctate circle in the well indicates no HA.

**SIRPα Inhibition Assay**

Human SIRPα-Fc (R and D Systems Catalog # 4546SA) was fluorescently labeled with Alexa Fluor 647 using reagents from Molecular Probes (Catalog #A20173) as per the manufacturer’s instructions. Cultured cells were incubated in growth medium containing CD47 antibodies at the indicated concentrations and incubated for 30 minutes at 37°C. Fluorescently-labeled SIRPα was added to a final concentration of 10 µg/ml and cells were incubated for an additional 60 minutes. Cells were harvested by centrifugation at 1000 x g for 5 minutes and washed once with PBS.
Washed cells were resuspended in PBS and analyzed by flow cytometry using a BD Accuri C6 flow cytometer (BD Biosciences, NJ, USA).

**Flow Cytometry-Based Phagocytosis Assay**

Human macrophages were derived from heparinized whole blood from healthy donors. PBMCs were isolated by Ficoll-Paque Plus (GE Healthcare, WI, USA) by density gradient and plated in 24 well plates in AIM-V media for 1 hour at 37° C in 5% CO₂ to allow for monocyte adherence to the plastic. Floating cells were removed, wells were replenished with fresh AIM-V media and incubated at 37°C for 7 days to allow monocytes to differentiate into macrophages. Fresh AIM-V media was added to macrophages every 3 days. PBMC-derived human macrophages were co-cultured for 2 hours at 37°C in 5% CO₂ with CFSE-labeled Jurkat or Raji tumor cells (1:4 ratio of macrophages to tumor cells) in the presence of various concentrations of either a human IgG isotype control or AO-176. Each sample was assessed in triplicate for each antibody concentration. Phagocytosis of Jurkat or Raji was determined by the percentage of CFSE⁺CD14⁺ cells using flow cytometry.

**AO-176 Effects On Tumor Xenograft Models**

Female NSG (NOD-Cg-PrkdscidI12rgtm1Wjl/SzJ) mice were supplied by Jackson Laboratory (ME, USA). Mice were received at 5 weeks of age, allowed to acclimate and housed in microisolator cages (Lab Products; DE, USA) maintained under specific pathogen-free conditions. The mice were fed Teklad Global Diet® 2920x irradiated laboratory animal diet (Envigo; Indianapolis, IN) and provided autoclaved water *ad libitum*. All procedures were carried out under the institutional guidelines of Translational Drug Development Institutional Animal Care and Use Committee, AZ, USA.

For Raji lymphoma xenograft models, mice were inoculated subcutaneously in the right flank with 5x10⁶ cells/mouse in 0.1ml of a 70% RPMI/30% Matrigel™ (BD Biosciences, MA, USA) mixture containing a suspension of Raji tumor cells. At the time of inoculation, the mice were 11 weeks old. Seven days following inoculation, tumors were measured using a digital caliper. Calipers were used to measure width and length diameters of the tumor. Tumor volumes were calculated utilizing the following formula: Tumor volume (mm³) = (a x b²/2) where ‘b’ is the smallest diameter and ‘a’ is the largest diameter. When tumors were palpable mice were...
randomized into four treatment groups of eight mice each, by random equilibration. Each group resulted in a mean tumor volume of approximately 50 mm$^3$ on Day 0. Mice were treated once weekly by intravenous administration of 1, 10 or 25 mg/kg of AO-176 or human IgG2 control antibody (25 mg/kg) for 4 weeks. Tumor volumes and body weights were recorded twice weekly. Gross observations were noted daily. Mean tumor growth inhibition (TGI) was calculated for Day 22 utilizing the following formula:

$$\text{TGI} = \left[1 - \left(\frac{\bar{X}_{\text{Treated (Day 22)}}}{\bar{X}_{\text{Control (Day 22)}}}\right)\right] \times 100\%$$

For MDA-MB-231 TNBC xenograft model, mice were inoculated orthotopically in the mammary fat pad with $2 \times 10^7$ cells/mouse in a 0.2 ml of a 70% RPMI/30% Matrigel™ mixture containing a suspension of MDA-MB-231 tumor cells. At the time of inoculation, the mice were 6 weeks old. Nineteen days following inoculation, tumors were measured as described above. When tumors were palpable mice were randomized into four treatment groups of eight mice each. Each group resulted in a mean tumor volume of approximately 100 mm$^3$ on Day 0. Mice were treated 5X/week by intraperitoneal administration of 15 mg/kg AO-176 or vehicle control (PBS) for 5 weeks. Tumor volumes and body weights were recorded twice weekly. Gross observations were noted daily. Mean tumor growth inhibition (TGI) was calculated utilizing the formula above.

For SNU-1 gastric carcinoma xenograft model, mice were inoculated subcutaneously in the right flank with $5 \times 10^6$ cells/mouse in 0.1 ml of a 70% RPMI/30% Matrigel™ mixture containing a suspension of SNU-1 tumor cells. Eight days following inoculation, tumors were measured as described above. When tumors were palpable mice were randomized into two treatment groups of ten mice each. Each group resulted in a mean tumor volume of approximately 87 mm$^3$ on Day 0. Mice were treated once weekly by intraperitoneal administration of 25 mg/kg AO-176 or hIgG2 control for 6 weeks. Tumor volumes and body weights were recorded twice weekly. Gross observations were noted daily. Mean tumor growth inhibition (TGI) was calculated utilizing the formula above.

For OV90 ovarian carcinoma xenograft model, mice were inoculated subcutaneously in the right flank with $5 \times 10^6$ cells/mouse in 0.1 ml of a 70% RPMI/30% Matrigel™ mixture containing a
suspension of OV90 tumor cells. At the time of inoculation, the mice were 6 weeks old. Ten days following inoculation, tumors were measured as described above. When tumors were palpable mice were randomized into two treatment groups of ten mice each. Each group resulted in a mean tumor volume of approximately 80 mm$^3$ on Day 0. Mice were treated 5X/week by intraperitoneal administration of 10 mg/kg AO-176 or hIgG2 control for 6 weeks. Tumor volumes and body weights were recorded twice weekly. Gross observations were noted daily. Mean tumor growth inhibition (TGI) was calculated utilizing the formula above.

**Effect of AO-176 On Cynomolgus Monkeys**

Naïve female cynomolgus, weighing 2.0 – 3.5 kg, were used to assess the effects of AO-176 and AO-104 administration on hematological parameters including RBCs and hemoglobin and on circulating antibody concentrations. All procedures were carried out under the institutional guidelines of Charles River Laboratories, NV, USA. Animals were assigned to 3 animals/group and treated with either vehicle (PBS) or with 5 mg/kg or 15 mg/kg of either AO-176 or AO-104 on Days 1 and on day 18, respectively, by 1 hour intravenous infusions. Blood samples were collected via the femoral vein prior to and at various times following antibody administrations. Data from two separate studies where PBS control 1 corresponds to the control group for AO-176 and PBS control 2 corresponds to the control group for AO-104.

**Statistical analysis**

Statistical analysis was performed using Prism7 software (Graph Pad Prism). For analysis of three or more groups, the nonparametric ANOVA test was performed with Bonferroni’s post-test. Data were expressed as mean ± SEM. Values of $p$ less than 0.05 were considered significant.
RESULTS

AO-176 specifically binds to CD47 on a variety of human cancer cells with reduced binding to CD47 on normal cells

Specific binding of AO-176 to CD47 was shown using cell lines that either express (Jurkat WT) or lack (Jurkat knockout) CD47 on the cell surface. AO-176 (and AO-104, another high affinity CD47 antibody) bound with high affinity to Jurkat WT but not to Jurkat KO cells, demonstrating the specific binding of AO-176 to human CD47 (Figure S3). Additionally, species specificity was assessed both for AO-176 and AO-104, a CD47 antibody with cross-species binding specificity. AO-176 bound equally well to both human and cynomolgus monkey CD47 (Table S1), with no discernable binding to mouse CD47 (Figure S2). Binding of AO-176 to cancer cells was determined by flow cytometry compared with a human IgG2 isotype control. AO-176 binds with high affinity to hematological (Jurkat T-ALL and Raji B lymphoma) and solid tumor cell lines (OV90) (Figure 1A) and OV10-315 ovarian carcinoma, MDA-MB-231 TNBC, SNU-1 gastric carcinoma and HCC827 lung adenocarcinoma with an EC50 range of 130-2700 ng/ml (0.84 – 18 nM) (Figure 1B).

We next determined whether AO-176 preferentially bound to cancer cells compared to normal cells. To do this we compared the binding of AO-176 to AO-104, a high affinity CD47 antibody with broad binding specificity for many cell types expressing CD47, including RBCs. Of particular interest was whether AO-176 retained strong binding to human RBCs, a property of all CD47 antibodies characterized thus far. As shown in figure 1C (LEFT panel), AO-104 binds with high affinity to RBCs from six different blood donors while AO-176 binding to RBCs from these same donors was virtually undetectable. Data for the Bmax binding of the two antibodies to RBCs obtained from 48 healthy donors (Figure 1C RIGHT panel) further illustrated that the negligible binding of AO-176 to human RBCs is a general property compared to AO-104 and not a donor-specific phenomenon.

We also compared the binding of AO-176 and AO-104 to freshly isolated human T cells (Figure 1D). While AO-104 bound with high affinity to naïve/resting T cells, AO-176 binding was as much as 50-fold weaker. PBMCs were plated on immobilized anti-CD3 or immobilized anti-CD3 and soluble anti-CD28 to activate T cells. While the level of binding of both AO-104 and AO-176 slightly increased to activated T cells, the relative difference in the apparent affinities of
the two antibodies was even more pronounced on activated T cells. AO-176 displayed markedly reduced affinity for CD47 on both naïve and activated T cells. Comparison of AO-176 and AO-104 binding to three normal (non-tumor) cell lines similarly revealed reduced binding with 13-50 fold weaker binding as measured by EC₅₀ and reduced Bₘₐₓ of AO-176 compared to AO-104 (Table 1).

Preferential binding of AO-176 to tumor cells is depicted by its increased binding to Jurkat T-ALL cells versus that of three types of circulating human normal cells (platelets, T cells and RBC) and endothelial cells of the vessel wall (Figure 1E). AO-176 binds to all of these normal cells with significantly reduced affinity and with a range in Bₘₐₓ of 645-43521 and an EC₅₀ range of 1000-26000 ng/ml (6-173 nM, Table 1) compared to the Jurkat cells with an observed Bₘₐₓ of 29902 and EC₅₀ of 390 ng/ml (2.6 nM, Figure 1B). These data, and particularly the negligible binding to RBCs suggest that the “vascular sink” that has been shown to reduce CD47 mAb exposures at low dosing regimens in both non-human primates and humans (19, 32, 33) may not hinder access of AO-176 to CD47 on cancer cells. It should be noted that differences in binding of AO-176 to normal and cancer cells was not due to differences in receptor number (Table 1).

**AO-176 kills tumor cells but not normal cells in vitro**

In the development of AO-176, a main goal was to replicate the cell-autonomous tumor cell killing of antibodies such as MABL-1 and -2, 1F7 and Ad22. The Sarfati group reported that monoclonal antibody B6H12, regarded by most as a “blocking only” (i.e. non-killing antibody), could kill certain leukemic cells when immobilized on plastic, thus presenting a highly valent surface to the cells in question (34). In contrast, antibody 1F7 killed Jurkat T cells when added to cells in soluble form (25). We thus tested AO-176 tumor toxicity by adding it to cells in soluble form and assessing Annexin V binding, here designated as early apoptosis and in addition, permeabilization of the cells to 7-AAD, designated late apoptosis. Figure 2A shows that soluble AO-176 at a saturating concentration of 10 μg/ml induces death of Jurkat (T-ALL, Figure 2A, left panel) and OV90 (ovarian carcinoma, Figure 2A, right panel) cells. The concentrations responsible for AO-176 induced cell death of a variety of other hematologic and solid tumor cell lines was determined to be in the range of 1 – 18.5 μg/ml (see Supplemental Table 2).
Since anti-CD47 antibodies, such as CC2C6, have been described to induce hemagglutination (HA) of hRBCs (35), we assessed whether AO-176, despite negligible binding to RBCs, was capable of causing HA. In contrast to CC2C6, AO-176 did not cause agglutination of washed human RBCs in vitro (Figure 2B). To demonstrate that the induction of cell death was selective for tumor cells, we also tested the effect of soluble AO-176 and an isotype matched human IgG2 control antibody on normal endothelial cells (HAEC), human skeletal muscle cells (SKMC) and renal tubular epithelial cells (RPTEC) (Figure 2C). At concentrations of 10, 30 and 100 µg/ml, AO-176 failed to induce death of these cells while camptothecin, a known apoptotic agent, induced substantial cell death.

A primary goal of immunotherapy is the establishment of a durable adaptive, T cell-mediated immune response (36). Therefore, we tested the potential killing effect of AO-176 along with the IgG2 control, on anti-CD3 and CD28 activated human T cells. PBMCs from healthy donors were activated on plates coated with anti-CD3 and treated with soluble anti-CD28 for 2 days at which time AO-176 or the IgG2 control antibody were added in soluble form. Activated T cells were not killed by AO-176 (Figure 2D) even at concentration as high as 30 µg/ml in contrast to tumor cells (Figure 2A). To further assess the effect of the antibodies on the activated T cells, we examined the proliferation of the activated T cells of three classes, CD3, CD8 and CD4 positive, using a CFSE dilution assay. As seen in Figure 2D, soluble AO-176 (at concentrations up to 30 µg/ml) did not have an inhibitory effect on the proliferation of any of the activated T cells. Thus, normal activated T cells are not susceptible to killing by AO-176.

**AO-176 promotes phagocytosis of cancer cells**

The in vivo anti-tumor effects of CD47 antibodies have thus far been demonstrated using antibodies that simply block the interaction of CD47 with the inhibitory receptor SIRPα thus promoting phagocytosis of the tumor cells (10, 37, 38). First, we show that AO-176 directly inhibited the binding of fluorescently labeled SIRPα to CD47 on Jurkat cells with an IC₅₀ of 0.78 - 0.87 µg/ml (5.1-5.7 nM) (Figure 3A). We then tested the effect of AO-176 on the phagocytosis of Jurkat cells by human macrophages. AO-176 promoted the phagocytosis of both Jurkat T-ALL (Figure 3B), Raji B cell lymphoma cells (Figure 3C) and several solid tumor cell lines including OV90, Detroit 562 and FaDu cells (Supplemental Table 3) in a concentration-dependent fashion. It is unlikely that stimulation of phagocytosis was due to opsonization of the
cancer cells with AO-176 since it is an IgG2 antibody with limited affinity for Fc receptors (39, 40). Thus, not only does AO-176 kill tumor cells, as expected it also leads to tumor cell phagocytosis by blocking the CD47 “don't eat me” signal.

**In vivo studies of AO-176 confirm anti-tumor activity**

We tested the in vivo anti-tumor activity of AO-176 in NSG mice. For Raji lymphoma xenografts, mice with established subcutaneous tumors of 50 mm$^3$ were treated with 1, 10 or 25 mg/kg AO-176 dosed once weekly intravenously (iv). Tumor volumes were assessed twice per week. The lowest dose of AO-176 inhibited tumor growth by 25% relative to control IgG2 antibody and the two higher doses inhibited tumor growth more than 50% (73% and 82% TGI at 10mg/kg and 25mg/kg, respectively) without notable overt clinical observations or weight loss (Figure 4A). To further explore the efficacy of AO-176 in solid tumor xenografts, we assessed in vivo anti-tumor efficacy in 3 additional models: an orthotopic MDA-MB-231 TNBC model (Figure 4B) where 83% TGI was observed after 4 weeks of dosing at 15 mg/kg, 5 times/week, ip; SNU-1 gastric carcinoma model (Figure 4C) where 64% TGI was observed after 6 weeks of dosing at 25 mg/kg, weekly, ip and an OV90 ovarian carcinoma model (Figure 4D) where 52% TGI was observed after 6 weeks of dosing at 10 mg/kg, 5 times/week, ip.

**AO-176 is well tolerated in cynomolgus monkeys**

To assess tolerability, including hematologic changes of AO-176, we performed studies in cynomolgus monkeys. We first established that the reduced binding of AO-176 to human RBCs, platelets and T cells relative to other CD47 antibodies was similar to that of cynomolgus monkey cells. As seen in Figure 5 A-C, AO-176 binds with lower maximal binding and affinity to cynomolgus RBCs, platelets and CD3$^+$ T cells compared to AO-104, similar to the data obtained with human cells (Figure 1 and Table 1). This reduced binding of AO-176 to RBCs in vitro was also reflected by a reduced effect on RBC parameters in vivo and was in contrast to a reduction in these parameters caused by AO-104 and as reported with other blocking CD47 antibodies (20). Figures 5D and 5E illustrate that the RBC number and hemoglobin values remain relatively stable after administration of two doses of AO-176 (5 mg/kg and 15 mg/kg i.v. on days 0 and 18 respectively). However, the high affinity RBC binding antibody AO-104 caused the aforementioned significant but transient reduction in RBCs (Figure 5D) and hemoglobin values.
These data indicate that the reduced binding of AO-176 to RBCs observed in vitro translates to a lack of transient anemia in vivo. Furthermore, the reduced binding of AO-176 to all normal circulating cells and endothelial cells predicts that AO-176 should exhibit increased or prolonged exposure in the vascular compartment. As seen in Figure 5F and consistent with that prediction, AO-176 was present and measurable in the circulation for 18 days following administration of the 5 mg/kg dose in contrast to AO-104 which was detected for the first 48 hours following administration but at no timepoints thereafter. AO-176 was well tolerated after both doses and no adverse side effects were observed. An additional study was also conducted with AO-176 administered by iv infusion, with a first dose of 5 mg/kg followed by 3 weekly doses of 50 mg/kg. Similar results were obtained as described above showing that AO-176 was well tolerated following repeat dose administration and that minimal changes in hematological parameters occurred (Figure S4) (41).

**DISCUSSION**

The CD47/SIRPα “don't eat me” axis is a target of a number of immunotherapeutic agents currently in development. Targeting either side of this axis is sufficient to activate phagocytosis driven by the engagement of several different prophagocytic ligands on target cells with receptors such as LRP and FcRs on the phagocyte, either macrophage or DC (3, 42). Enabling phagocytosis and thus removal of cancer cells not only serves to “debulk” the cancer, but also serves to prime the adaptive immune response leading to the generation of tumor targeted cytotoxic T cells (often CD8+ T cells) (15-18). At present, antibodies that only block CD47 have the potential to boost the function of the innate immune system to increase its role in recognition of cancer’s “non-selfness” and thus prime an adaptive response. Our next generation anti-CD47 antibody, AO-176, also blocks the CD47/SIRPα interaction but brings additional efficacy through direct, selective killing of cancer cells. In this report, we showed that AO-176 killed solid and liquid tumor lines in vitro in a cell autonomous manner (not ADCC, Figure S5) and exhibited anti-tumor activity in vivo (both sub-cutaneous and orthotopic xenograft models).

Conventional chemotherapeutics act directly on cancer cells, often targeting some aspect of their enhanced proliferation. The most successful of these also prime the adaptive response to cancer by making available neoantigens within cancer cells. Ideally, one would like to enhance both the
innate and adaptive response to cancer by killing cancer cells while also facilitating, or at least not inhibiting, the T cell response to the neoantigens present in cancer cells. Although a small number of mouse CD47 antibodies that exhibit killing potential have been identified, to date those being pursued in early clinical development simply block the CD47/SIPRα interaction and promote phagocytosis of cancer cells. These killing antibodies, MABL-1, MABL-2, Ad22 and 1F7 were reported by multiple groups (23-26, 43). MABL-1, MABL-2 and dimerized single chain variants of MABL-2 induced cell death of human leukemia and multiple myeloma cell lines, but not normal cells in vitro and had anti-tumor effects in vivo in xenograft models (23, 27, 28). Lindberg et al (44), characterized 1F7 along with other blocking CD47 antibodies including B6H12. 1F7 and B6H12 bound to epitopes on CD47 that were overlapping, however B6H12 had only CD47 blocking activity with no cytotoxic activity toward cancer cells (25, 45). The killing mechanism of the cytotoxic antibodies (MABL-1, MABL-2, 1F7, Ad22) appeared to be the same and involved an indirect attack on mitochondria leading to loss of mitochondrial potential (ΨΔm), the generation of ROS, and loss of ATP generating capacity (25). An early effect of these antibodies was also to display negatively charged lipids on the cell surface detected by the binding of annexin V, followed by disruption of the plasma membrane leading to release of cellular contents, potentially including exposure of neoantigens. In this sense, the killing was not classical apoptosis, which is non-lytic and thus immunologically silent. Taken together, these features are typical of programmed cell death III. We are currently investigating the underlying mechanism for the direct killing exhibited by AO-176 that will be the topic of another manuscript.

These early reports indicated that normal cells, including hematopoietic progenitor cells, endothelial cells and naïve mouse or human T cells were not killed by the antibodies, but that upon activation, T cells were killed (24, 25). However, those studies tested the T cells on surfaces coated with both the activating antibody (anti-CD3) and the CD47 antibody (immobilized antibodies). Surface coating renders even B6H12 capable of killing some cancerous leukocytes (34, 46) suggesting that the valency of the antibody binding sites is an important feature required for killing. Supporting this notion, Kikuchi et al. reported that while a single chain (ScFv) fragment of their intact killing antibody did not kill Raji cells, several divalent forms including intact IgG or the ScFv either dimerized or crosslinked by a secondary antibody exhibited killing activity (23, 43). In contrast, we report here that AO-176 did not kill
either naïve or activated human T cells. In addition, we have found no normal, untransformed cell type that is killed by AO-176.

An unexpected property of AO-176 that was identified during its development was cell type binding selectivity with greatly reduced binding to CD47 on RBCs and several other normal cell types. A potential explanation for this selectivity is that CD47 associates with different membrane proteins (in cis) on different cells. Thus, it is possible that the AO-176 epitope is either masked by a CD47 binding partner in different cells (ex. RBC erythrocyte membrane protein 4.2 and/or components of the Rh complex (47)) or is dependent on a CD47 interacting protein (ex. integrin (48)) for its binding. Since CD47 can be heavily glycosylated with 5 potential NXT/S sequences in its extracellular IgV domain (44) and/or modified by addition of glycosaminoglycans (47), differing patterns or extents of carbohydrate additions in different cell types could also explain the differential binding of AO-176. A further explanation for the differential binding of AO-176 to cancer vs normal cells lies in potential differences in surface mobility of CD47 on different cell types (50) or different densities or distribution of CD47 in lipid rafts (51).

Regardless of the cause of the reduced binding of AO-176 to RBCs and other normal cells, this property should result in a more favorable pharmacokinetic and safety profile in humans compared to other antibodies that bind to with high affinity to RBC and other normal cells. When tested in cynomolgus monkeys, AO-176 was found to be well tolerated and devoid of hematologic effects in cynomolgus monkeys following repeat dose administration. This is in contrast to other CD47 blocking antibodies, including AO-104, that exhibit high binding to RBCs and show transient anemia. In addition, following low dose administration of 5 mg/kg of AO-176 and AO-104, the reduced binding to normal cells of AO-176 resulted in extended duration of exposure compared to AO-104. The reduced binding to both cynomolgus monkey and human RBCs predicts that a similar lack of hematological effects as well as greater exposure at low doses may be observed in patients.

In summary, we show that AO-176, a next generation humanized anti-CD47 antibody, blocks CD47/SIRPα, induces phagocytosis, directly kills cancer cells in a cell autonomous manner (not-ADCC) and preferentially binds and kills tumor versus normal cells and particularly RBCs, where AO-176 shows negligible binding and does not induce hemagglutination. Taken together, we expect that these unique and differentiative features of AO-176 will translate to improved
exposure, efficacy, tolerability and therapeutic index in comparison to other CD47/SIRPα axis targeting agents under development and currently in clinical testing.

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The authors thank the hardworking and thoughtful members of Arch Oncology that did not appear on this paper, but that significantly contributed.

REFERENCES


Table 1 Summary of CD47 expression levels and binding properties of AO-176 and AO-104 to CD47 expressed on normal cells

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell Type</th>
<th>Mean CD47 RN</th>
<th>EC$_{50}$ AO-176 (ng/ml)</th>
<th>EC$_{50}$ AO-104 (ng/ml)</th>
<th>B$_{\text{max}}$ AO-176</th>
<th>B$_{\text{max}}$ AO-104</th>
</tr>
</thead>
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<td>Human</td>
<td>RBCs</td>
<td>32,086</td>
<td>Negligible</td>
<td>4-20</td>
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<td></td>
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<td>1000</td>
<td>7-20</td>
<td>2118-4301</td>
<td>12613-69415</td>
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<td>Naïve CD3+ T cells</td>
<td>83,477</td>
<td>1600-6000</td>
<td>2-120</td>
<td>6032-14245</td>
<td>22492-54972</td>
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<tr>
<td></td>
<td>Activated CD3+ T cells</td>
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<td>3000-26000</td>
<td>89</td>
<td>4200-43521</td>
<td>21996-91383</td>
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<tr>
<td></td>
<td>Endothelial</td>
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<td>9-40</td>
<td>3994-29762</td>
<td>168816-264242</td>
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<tr>
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<tr>
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<td>2275</td>
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<td>226204</td>
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<tr>
<td>Cynomolgus Monkey</td>
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<td>CD3+ T cells</td>
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<td>3180-5093</td>
<td>3-7</td>
<td>3652-8925</td>
<td>13721-26609</td>
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</table>
FIGURE LEGENDS

Figure 1: AO-176 binding to human tumor and normal cells. (A) Binding of AO-176 to Jurkat T-ALL, Raji B lymphoma and OV90 ovarian carcinoma cells was determined by flow cytometry. (B) Tabulated tumor cell binding, receptor number (RN) and B_{max} of tumor lines was determined using AO-176 and AO-104. (C) Binding of AO-176 and AO-104 to freshly isolated RBCs from healthy donors (left panel n=6, right panel B_{max} comparison of 48 donors from binding performed up to 10 µg/ml and 200 µg/ml for AO-104 and AO-176, respectively) and (D) Binding of AO-176 and AO-104 to naïve CD3 T cells or anti-CD3 activated T cells or anti-CD3/CD28 (3 days activated). (E) Comparison of binding of AO-176 to Jurkat tumor cells and normal cells with reduced binding to human activated platelets, naïve CD3^+ T cells, RBCs, human aortic endothelial cells (HAEC), human skeletal muscle cells (SkMc) and human renal proximal tubular epithelial cells (RPTEC).

Figure 2: AO-176 effects on hematologic and solid tumor lines and normal cells. (A) Cell autonomous killing of tumor cells by AO-176 was measured in Jurkat, Raji and OV90 ovarian carcinoma cells incubated with 10 µg/ml of AO-176 or IgG2 isotype control antibody for 24 hours at 37°C. Cells were stained with Annexin V and 7-AAD to measure the percent of early apoptotic cells (Annexin V^+ and 7AAD^−) or late apoptotic cells (Annexin V^+ and 7AAD^+) measured by flow cytometry. (B) In vitro washed RBC HA assay showing agglutination with positive control CC2C6 and no agglutination with AO-176. (C) Lack of killing of normal cell lines by AO-176 in contrast to positive control chemotherapeutic camptothecin (CPT), determined by assessment of Annexin V staining and Annexin V^+ and 7AAD^+ by flow cytometry. (D) Lack of killing assessed as in Figure 2A and lack of effects on proliferation of activated T cells by 30 µg/ml AO-176 and determined by CFSE-labeling and flow cytometry. Error bars represent mean ± SEM of duplicate samples. Bar graph statistics determined using one-way ANOVA comparing AO-176 to untreated control for each corresponding antibody concentration, *P<0.05, **P<0.005, *** P<0.0005 and ****P<0.0001.
Figure 3: Blocking CD47 and SIRPα binding by AO-176 leads to in vitro phagocytosis of human tumor cells. (A) Inhibition of CD47 binding to SIRPα by AO-176 and an IgG2 control antibody was assessed in vitro using a fluorescently-labeled SIRPα-Fc fusion protein. Data are representative of two independent experiments. (B) PBMC-derived human macrophages were co-cultured for 2 hours at 37°C with CFSE-labeled Jurkat or (C) Raji cells in the presence of various concentrations of a human isotype control or AO-176. Phagocytosis of Jurkat or Raji determined by the percentage of CFSE+CD14+ cells using flow cytometry. Error bars represent mean ± SEM of duplicate samples. Bar graph statistics determined using one-way ANOVA comparing AO-176 vs IgG control for each corresponding antibody concentration, *P<0.05, ***P<0.001 and ****P<0.0001.

Figure 4: AO-176 inhibits tumor growth in hematologic and solid tumor xenograft models. (A) Female NSG were inoculated subcutaneously in the right flank with 5 x10^6 Raji tumor cells/mouse (n= 10/group). When tumors reached an average of ~100 mm^3, mice were treated once weekly iv with various doses of AO-176 or IgG2 control (PBS) for 4 weeks. Tumor volumes were assessed twice/week. (B) Female NSG were inoculated orthotopically in the mammary fat pad with 2 x10^7 MDA-MB-231 tumor cells/mouse (n= 8/group). When tumors reached an average of ~100 mm^3, mice were treated 5 times/week ip with 15 mg/kg AO-176 or vehicle control (PBS) for 5 weeks. (C) Female NSG were inoculated in the right flank with 5 x10^6 SNU-1 tumor cells/mouse (n= 10/group). When tumors reached an average of ~100 mm^3, mice were treated once weekly ip with 25 mg/kg AO-176 or IgG2 control for 6 weeks. (D) Female NSG were inoculated in the right flank with 5 x10^6 OV90 tumor cells/mouse (n= 10/group). When tumors reached an average of ~100 mm^3, mice were treated 5X/week ip with 10 mg/kg AO-176 or IgG2 control for 6 weeks. Tumor volumes were assessed twice/week. Error bars represent mean ± SEM of tumor volumes.

Figure 5: Reduced binding of AO-176 to cynomolgus monkey RBCs, platelets and CD3+ T cells in vitro correlates with minimal reduction in RBC and hemoglobin in cynomolgus monkeys treated with AO-176. Binding to isolated cynomolgus monkey (A) RBCs, (B) platelets, and (C) CD3+ T cells (n=3) was determined using flow cytometry by measuring median fluorescent intensity (MFI) to increasing concentrations of AO-176 or a high anti-CD47
binding antibody (AO-104). Each antibody concentration for each donor was run in triplicate and mean illustrated. Female cynomolgus monkeys (3 animals/group) were treated with vehicle (PBS) or with 5 mg/kg or 15 mg/kg of either AO-176 or AO-104 on Days 1 and on day 18, respectively, by 1-hour intravenous infusions. Data from two separate studies where PBS control 1 corresponds to the control group for AO-176 and PBS control 2 corresponds to the control group for AO-104. Blood samples were obtained at various times after the infusions and assessed for hematological parameters including (D) RBCs and (E) hemoglobin. A transient reduction in both hemoglobin and RBCs occurred following treatment with AO-104, while minimal reduction occurred following treatment with AO-176, consistent with the reduced RBC binding to cynomolgus RBCs in vitro (A). (F) Exposure levels of AO-176 and AO-104 in cynomolgus monkey plasma following 5 mg/kg iv administration.
Figure 4

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

D

![Graph D](image)
Molecular Cancer Therapeutics

Development of AO-176, a Next Generation Humanized Anti-CD47 Antibody With Novel Anti-Cancer Properties and Negligible Red Blood Cell Binding

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