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

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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 phagocytosis, but also induces tumor cytotoxicity in hematologic and solid human tumor cell lines, but not normal noncancerous 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. Finally, we show that AO-176 demonstrates dose-dependent antitumor activity in tumor xenograft models. Taken together, the unique properties and antitumor 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 (RBC) 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, whereas 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 (DC) 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 antitumor response (15–18). Although many cancer cells have elevated CD47 expression compared with 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 patients with cancer, the ability to exert a direct cytotoxic effect on cancer cells. Multiple groups have shown that particular anti-CD47 antibodies, including MAB1-1, MAB1-2, 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 derivatives, which killed leukemia and multiple myeloma cells in vitro, were also efficacious in mouse xenographs 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 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 CTACTGAAGATCAGTGAAANGGG and CTTGGTTAGAGCTCCATCAANGG (Genome Engineering and iPSC Center, Washington University, MO). Jurkat WT, Jurkat CD47 KO, Raji, HCC827, MDA-MB-231, and OV10-315 were cultured in RPMI1640 medium (GIBCO) supplemented with 10% 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. HAEC and SKMC cells were cultured in EGM-2 BulletKit media (Lonza). RPTEC cells were cultured in REGM extended human placental epithelial cell growth medium (Lonza). 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 nonsecreting myeloma P3 × 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, TOP0 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 humанизed by rational design to create AO-104, with a WT kappa light chain and an IgG4 Fc, and AO-176, with a WT kappa light chain and a WT IgG2 Fc (GenBank accession nos. AAB59393, AH002839; Supplementary Fig. 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 (Supplementary Table S1). AO-176 is not cross-reactive to murine CD47 (Supplementary Fig. S2).

Flow cytometry antibodies for T-cell proliferation and apoptosis

Fluorochrome conjugated mouse anti-human Abs to CD3 (clone SK7), CD4 (clone OKT4), CD8 (clone SK1) antibody were purchased from eBioscience. Human TruStain Fc Block anti huCD16/huCD32/huCD64 blocking Ab and isotype-matched control murine IgG1 and IgG2a Ultra-LEAF were purchased from BioLegend. Human Annexin V and 7-AAD were purchased from BD Biosciences.

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). 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, 0.5 M NaCl, 5 mmol/L EDTA, 0.25% CHAPS, and 0.5% BSA). Bound antibody was detected using horseradish peroxidase conjugated goat anti-human IgG, Monkey Adsorbed (Bethyl Laboratories; #A80-319D) using QuantaBlu substrate reagent (Thermo Fisher Scientific; #15169) 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. Briefly, cells were plated in triplicate and incubated with saturating concentrations of a mouse antibody against CD47 (Vx1000R mIgG2a; Arch Oncology Inc.) 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 control IgG2a Ultra-LEAF, and IgG2a Ultra-LEAF were purchased from BioLegend. Antibody concentrations in serum samples from tumor binding assays were acquired on BD Accuri CS Sampler (BD Biosciences) and samples from normal cells binding were acquired on Attune Nxt}

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Cytofluorometer (Life Technology/Thermo Fisher Scientific) 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, and 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 (BioVIT), diluted 1:300 in PBS containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and washed twice with PBS/1 mmol/L EDTA.

Human and cynomolgus monkey platelets were isolated from platelet rich plasma (PRP) by centrifugation of whole blood at 500 × g to remove RBCs, followed by secondary centrifugation of PRP at 2,500 × g to pellet the platelets. The platelets were washed and incubated with chromPure human IgG Fc (Jackson Immunoresearch) 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) in heparinized bags and processed within 24 hours of collection. PBMCs were isolated by standard density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare). Cynomolgus monkey PBMCs were obtained frozen from IQ Biosciences.

**Binding assays with human and cynomolgus RBCs, platelets, and 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.), 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 Alexa Fluor 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) and FITC-conjugated anti-human CD63 (cynomolgus monkey reactive; BioLegend). 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.), 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 proliferation and apoptosis assays**

T cells were activated as described above. For T-cell proliferation, PBMCs (2 × 10^5/well) were labeled with 1 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) for 15 minutes. CFSE staining was neutralized with RPMI ( Gibco) and cells were subsequently washed with PBS. CFSE-labeled PBMCs were plated on day 0 into plates precoated with anti-human CD3 and anti-human CD28 was added in soluble form to PBMCs in AIM-V media containing AlbuMax (BSA) for 1 hour. 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 fluorescein conjugated anti-human CD3, Annexin V, and 7AAD (BD Biosciences) 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 fluorescein conjugated anti-human CD3, anti-human CD4, and anti-human CD8 (BioLegend) 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) and data were analyzed using Attune Nxt software.

**Human tumor and normal cell killing assays**

Human normal cells (HAEC, SKMC, and RPTEC) or tumor cell lines were plated in 96-well plates at 1 × 10^5 cells/well and incubated with various concentrations of AO-176, human IgG2 isotype control, media, or 6 μmol/L 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). 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 stained positive for cell surface Annexin V and 7-AAD nuclear incorporation by flow cytometry (Accuri C6; Becton Dickinson). 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 1 mmol/L 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 no. 4546SA) was fluorescently labeled with Alexa Fluor 647 using reagents from Molecular Probes (catalog no. 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 1,000 × 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).

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) 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 cocultured 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-PrkdcscidIl2rgtm1Wjl/SzJ) mice were supplied by The Jackson Laboratory. Mice were received at 5 weeks of age, allowed to acclimate, and housed in microisolator cages (Lab Products) maintained under specific pathogen-free conditions. The mice were fed Teklad Global Diet 2920x irradiated laboratory animal diet (Envigo) and provided autoclaved water and ad libitum. All procedures were carried out under the institutional guidelines of Translational Drug Development Institutional Animal Care and Use Committee. For Raji lymphoma xenograft models, mice were inoculated subcutaneously in the right flank with 5 × 10^6 cells/mouse in 0.1 mL of a 70% RPMI/30% Matrigel (BD Biosciences) 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 × b²)/2, where "a" is the smallest diameter and "b" is the largest diameter. When tumors were palpable mice were randomized into four treatment groups of 8 mice each, by random equilibration. Each group resulted in a mean tumor volume of approximately 100 mm³ on Day 0. Mice were treated 5×/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 TGI was calculated utilizing the formula above.

For SNU-1 gastric carcinoma xenograft model, mice were inoculated subcutaneously in the right flank with 5 × 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 10 mice each. Each group resulted in a mean tumor volume of approximately 87 mm³ on Day 0. Mice were treated once weekly by intraperitoneal administration of 25 mg/kg AO-176 or IgG2 control for 6 weeks. Tumor volumes and body weights were recorded twice weekly. Gross observations were noted daily. Mean TGI was calculated utilizing the formula above.

For OV90 ovarian carcinoma xenograft model, mice were inoculated subcutaneously in the right flank with 5 × 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 10 mice each. Each group resulted in a mean tumor volume of approximately 80 mm³ on Day 0. Mice were treated 5×/week by intraperitoneal administration of 10 mg/kg AO-176 or IgG2 control for 6 weeks. Tumor volumes and body weights were recorded twice weekly. Gross observations were noted daily. Mean TGI was calculated utilizing the formula above.

Effect of AO-176 on cynomolgus monkeys

Naïve female cynomolgus, weighing 2.0 to 3.5 kg, were used to assess the effects of AO-176 and AO-104 administration on hematologic parameters including RBCs and hemoglobin and on circulating antibody concentrations. All procedures were carried out under the institutional guidelines of Charles River Laboratories. Animals were assigned to 3 animals/group and treated with either vehicle (PBS) or with 5 or 15 mg/kg of either AO-176 or AO-104 on Day 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 posttest. Data were expressed as mean ± SEM. Values of P < 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 (Supplementary Fig. S3). In addition, species specificity was assessed both for AO-176 and AO-104, a CD47 antibody with cross-species binding specificity. AO-176
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bound equally well to both human and cynomolgus monkey CD47 (Supplementary Table S1), with no discernable binding to mouse CD47 (Supplementary Fig. 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 hematologic (Jurkat T-ALL and Raji B lymphoma) and solid tumor cell lines (OV90; Fig. 1A) and OV10-315 ovarian carcinoma, MDA-MB-231 TNBC, SNU-1 gastric carcinoma, and HCC827 lung adenocarcinoma with an EC50 range of 130 to 2,700 ng/mL (0.84–18 nmol/L, Fig. 1B).

We next determined whether AO-176 preferentially bound to cancer cells compared with 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 Fig. 1C (left), AO-104 binds with high affinity to RBCs from six different blood donors whereas 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 (Fig. 1C, right) further illustrated that the negligible binding of AO-176 to human RBCs is a general property compared with 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 (Fig. 1D). Although AO-104 bound with high affinity to naive/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. Although 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 (nontumor) cell lines similarly revealed reduced binding with 13- to 50-fold weaker binding as measured by EC50 and reduced Bmax of AO-176 compared with 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 (Fig. 1E). AO-176 binds to all of these normal cells with significantly reduced affinity and with a range in Bmax of 645–4352 and an EC50 range of 1,000 to 26,000 ng/mL (6–173 nmol/L, Table 1) compared with the Jurkat cells with an observed Bmax of 29902 and EC50 of 390 ng/mL (2.6 nmol/L, Fig. 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 MAR1-1 and MAR1-2, 1F7, and Ad22. The Safiati group reported that mAb B6H12, regarded by most as a “blocking only” (i.e., nonkilling 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, Fig. 2A, left) and OV90 (ovarian carcinoma, Fig. 2A, right) 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 to 18.5 μg/mL (see Supplementary Table S2).

Because anti-CD47 antibodies, such as CC2C6, have been described to induce 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 (Fig. 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 RPTEC (Fig. 2C). At concentrations of 10, 30, and 100 μg/mL, AO-176 failed to induce death of these cells whereas CPT, 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 (Fig. 2D) even at concentration as high as 30 μg/mL in contrast to tumor cells (Fig. 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, producing a CFSE dilution assay. As seen in Fig. 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 antitumor 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 to 0.87 μg/mL (5.1 to 5.7 nmol/L, Table 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 (Fig. 3B), Raji B-cell lymphoma cells (Fig. 3C) and several solid tumor cell lines including OV90, Detroit 562, and FaDu cells (Supplementary Table S3) 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 antitumor activity

We tested the in vivo antitumor activity of AO-176 in NSG mice. For Raji lymphoma xenografts, mice with established subcutaneous tumors of 50 mm3 were treated with 1, 10, or 25 mg/kg AO-176 dosed once weekly intravenously. 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 10 and 25 mg/kg, respectively).
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, n = 6; right, B_{max} comparison of 48 donors from binding performed up to 10 and 200 µg/mL for AO-104 and AO-176, respectively). D, Binding of AO-176 and AO-104 to naive 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, naive CD3 T cells, RBCs, human aortic endothelial cells (HAEC), human skeletal muscle cells (SkMC), and human RPTEC.
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Table 1. Summary of CD47 expression levels and binding properties of AO-176 and AO-104 to CD47 expressed on normal cells.

<table>
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<tr>
<th>Species</th>
<th>Cell type</th>
<th>Mean CD47 RN</th>
<th>E&lt;sub&gt;50&lt;/sub&gt; AO-176 (ng/mL)</th>
<th>E&lt;sub&gt;50&lt;/sub&gt; AO-104 (ng/mL)</th>
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<td></td>
<td>Naïve CD3&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>85,477</td>
<td>1,600–6,000</td>
<td>2–120</td>
<td>6,032–14,245</td>
<td>22,492–54,972</td>
</tr>
<tr>
<td></td>
<td>Activated CD3&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>N.D.</td>
<td>3,000–26,000</td>
<td>89</td>
<td>4,200–43,521</td>
<td>21,996–91,383</td>
</tr>
<tr>
<td></td>
<td>Endothelial</td>
<td>48,944</td>
<td>3,000–7,100</td>
<td>9–40</td>
<td>3,994–29,762</td>
<td>168,816–264,242</td>
</tr>
<tr>
<td></td>
<td>Epithelial</td>
<td>115,784</td>
<td>3,300</td>
<td>5–20</td>
<td>8,743</td>
<td>46,956–250,699</td>
</tr>
<tr>
<td></td>
<td>Skeletal</td>
<td>85,661</td>
<td>2,275</td>
<td>0.9</td>
<td>2,260</td>
<td>226,204</td>
</tr>
<tr>
<td>Cynomolgus monkey</td>
<td>RBCs</td>
<td>26,087</td>
<td>5,000–10,000</td>
<td>12–18</td>
<td>675–1,751</td>
<td>3,951–7,183</td>
</tr>
<tr>
<td></td>
<td>Platelets</td>
<td>34,728</td>
<td>1,000–5,800</td>
<td>17–58</td>
<td>5,852–13,781</td>
<td>18,866–40,265</td>
</tr>
<tr>
<td></td>
<td>CD3&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>35,800</td>
<td>3,180–5,093</td>
<td>3–7</td>
<td>3,652–8,925</td>
<td>13,721–26,609</td>
</tr>
</tbody>
</table>

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 5A–C, AO-176 binds with lower maximal binding and affinity to cynomolgus RBCs, platelets, and CD3<sup>+</sup> T cells compared with AO-104, similar to the data obtained with human cells (Figure 1, Table 1). This reduced binding of AO-176 to RBCs in vivo 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). Figure 5D and E illustrate that the RBC number and hemoglobin values remain relatively stable after administration of two doses of AO-176 (5 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 (Fig. 5D) and hemoglobin values (Fig. 5E). These data indicate that the reduced binding of AO-176 to RBCs observed in vivo 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 intravenous 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 (Supplementary Fig. S4; ref. 41).

**Discussion**

The CD47/SIRP<sub>α</sub> “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 phagocytic 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 CD<sup>8+</sup> T cells; refs. 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/SIRP<sub>α</sub> 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 vivo in a cell autonomous manner (not ADCC, Supplementary Fig. S5) and exhibited antitumor activity in vivo (both subcutaneous 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/SIRP<sub>α</sub> 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 vivo and had antitumor effects in vivo in xenograft models (23, 27, 28). Lindberg and colleagues (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 (ΔΨm), 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 of this axis is sufficient to activate phagocytosis driven by the engagement of several different phagocytic 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 CD<sup>8+</sup> T cells; refs. 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/SIRP<sub>α</sub> 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 vivo in a cell autonomous manner (not ADCC, Supplementary Fig. S5) and exhibited antitumor activity in vivo (both subcutaneous and orthotopic xenograft models).

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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 nonlytic 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...
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; ****, 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 × 10⁶ Raji tumor cells/mouse (n = 10/group). When tumors reached an average of ~100 mm³, mice were treated once weekly intravenously 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 × 10⁵ MDA-MB-231 tumor cells/mouse (n = 8/group). When tumors reached an average of ~100 mm³, mice were treated five times/week intraperitoneally with 15 mg/kg AO-176 or vehicle control (PBS) for 5 weeks. C, Female NSG were inoculated in the right flank with 5 × 10⁵ SNU-1 tumor cells/mouse (n = 10/group). When tumors reached an average of ~100 mm³, mice were treated once weekly intraperitoneally with 25 mg/kg AO-176 or IgG2 control for 6 weeks. D, Female NSG were inoculated in the right flank with 5 × 10⁵ OV90 tumor cells/mouse (n = 10/group). When tumors reached an average of ~100 mm³, mice were treated 5 ×/week intraperitoneally 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.
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 and colleagues reported that although 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 (e.g., RBC erythrocyte membrane protein 4.2 and/or components of the Rh complex; ref. 47) or is dependent on a CD47 interacting protein (e.g., integrin; ref. 48) for its binding. Because CD47 can be heavily glycosylated with five potential NXT/S sequences in its extracellular IgV domain (44) and/or modified by addition of glycosaminoglycans (49), 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 versus 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 with other antibodies that bind to RBCs 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 with AO-104. The reduced binding to both cynomolgus monkey and human RBCs predicts that a similar lack of hematologic 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/SSIRPα, 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 HA. Taken together, we expect that these unique and differential 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.

Disclosure of Potential Conflicts of Interest

R.R. Hiebsch is a Scientist at Arch Oncology Inc. P.T. Manning is an employee and consultant during previous 3-year period; and also has ownership interest (including patents) in Arch Oncology Inc. W.A. Frazier is a consultant at Arch Oncology Inc. R.W. Karr is an employee and consultant at Arch Oncology Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.J. Donio, P.T. Manning, R.W. Karr

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.J. Puro, M.N. Bouchlaka, R.R. Hiebsch, B.J. Capoccia, M.J. Donio, P.T. Manning, R.W. Karr

Writing, review, and/or revision of the manuscript: R.J. Puro, M.N. Bouchlaka, R.R. Hiebsch, B.J. Capoccia, P.T. Manning, W.A. Frazier, R.W. Karr, D.S. Pereira

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.J. Puro

Study supervision: R.J. Puro, P.T. Manning, D.S. Pereira

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AO-176, a Next-Generation Humanized Anti-CD47 for Cancer


Molecular Cancer Therapeutics

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

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