### Title

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# RRx-001 Acts as a Dual Small Molecule Checkpoint Inhibitor by Downregulating CD47 on Cancer Cells and SIRP-α on Monocytes/Macrophages

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## Abstract

RRx-001 is a pleiotropic anticancer agent in phase III clinical trials, which polarizes tumor-associated macrophages from a low phagocytic M2 phenotype to a high phagocytic M1 phenotype. One of the ways in which tumors promote M2 polarization and evade macrophage-mediated destruction is through upregulation of CD47 expression. As a myeloid-specific immune checkpoint, CD47 interacts with signal-regulatory protein alpha (SIRPα) on macrophages and monocytes to prevent phagocytosis. Herein, the effect of RRx-001 on CD47 and SIRPα expression was evaluated as well as its activity *in vivo* in macrophage-depleted tumors. *In vitro*, RRx-001 was found to decrease the expression levels of CD47 and SIRPα on tumor cells and monocytes/macrophages, respectively, reducing the phagocytosis inhibitory function of the CD47/SIRPα interaction. *In vivo*, macrophage depletion by clodronate in an A549 xenograft-bearing mouse model attenuated the ability of RRx-001 to suppress tumor growth, which suggests that the presence of infiltrated macrophages in the tumor microenvironment is a *sine qua non* condition for the antitumor activity of RRx-001. Furthermore, these *in vitro* effects translate into significant antitumor activity in mouse models of lung cancer. Importantly, unlike anti-CD47 antibodies, RRx-001, which has been evaluated in close to 300 patients in 9 clinical trials, is not associated with any hematologic toxicities. On the basis of demonstrated antitumor activity and minimal toxicity in phase II clinical trials, RRx-001 has received clearance from the FDA and the EMA for phase III, multicenter studies in subjects with relapsed/refractory solid tumors.

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## Introduction

CD47 (cluster of differentiation 47) is a glycoprotein of the immunoglobulin superfamily that is ubiquitously present on the surface of all cells, particularly tumors cells where its overexpression correlates with poor prognosis. The engagement of CD47 with signal regulatory protein-alpha (SIRPα) expressed on myeloid cells is referred to as an innate immune checkpoint[1] because it functions to prevent macrophage phagocytosis and render tumor cells less sensitive to innate immune surveillance.[2,3] In light of the overexpression of CD47 on cancer cells and its identification as an innate immune checkpoint, several CD47 targeted therapies including anti-CD47 antibodies, engineered receptor decoys, anti-SIRPα antibodies, and bispecific agents have been developed.[4] Since CD47/SIRPα blocking agents "release the brakes" on phagocytosis, antigen uptake, processing, and presentation are increased, which, in turn, stimulates acquired responses and links the innate and adaptive immune systems.[5] CD47/SIRPα blocking therapies may therefore enhance the anticancer effects of anti–PD-1, PD-L1, and CTLA-4 immune checkpoint inhibitors that target T-cell activation.

RRx-001, an anticancer agent with clearance to start phase III clinical trials, pleiotropically targets tumor-associated cells including

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macrophages and cancer stem cells.[6] The aim of these studies was to evaluate the effects of RRx-001 on CD47 expression and its interaction with SIRPα as well as macrophage-mediated phagocytosis of human tumor cells and downstream effects on c-myc and cancer stem cells. The experiments are described below.

## Materials and Methods

### *Ethics Statement*

All protocols were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, and conducted according to the Guide for the Care and Use of Laboratory Animals (US National Research Council, 2011).

### *Mice Management*

Female BALB/c nude mice (19.2 ± 1.7 g) were subcutaneously injected with $5 \times 10^6$ of A549 cells suspended in PBS and containing 50% Matrigel Matrix (Coining, 354234) to establish NSCLC xenograft models. Tumor-bearing mice were randomized into four cohorts after tumors had a volume ~100 $mm^3$. RRx-001, supernatant, and vehicle were intraperitoneal injected twice a week and once a day respectively. Tumor volume was calculated as length × width × width/2.

### *Reagents*

All reagents were purchased Sigma-Aldrich. LysoTracker and MitoSox were purchased from Invitrogen. The primary antibodies used for Western analyses were purchased as follows: anti-LC3 (Cell Signaling Technology, 3868), anti–β-actin (Cell Signaling Technology, 3700), anti–caspase 9 (Cell Signaling Technology, 9502), anti–caspase 3 (Cell Signaling Technology, 9665), B-actin, rabbit monoclonal (Cell Signaling Technology), anti-CD47 (rabbit polyclonal; GeneTex), and anti–SIRP-α (rabbit polyclonal; GeneTex).

### *Cell Lines and Culture*

The following human cell lines were purchased from American Type Culture Collection (Manassas, VA): MCF-7 (breast adenocarcinoma, pleural effusion), AU-565 (adenocarcinoma, breast), HBL-100 (breast epithelial cells with transformed morphology), and MDA-MB-231 (adenocarcinoma). The cell culture medium used was RPMI 1640 (Life Technologies, Inc., Grand Island, NE) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc.), and 10 μg/ml ciprofloxacin. All cell lines used in the study were free from mycoplasma.

### *Cell Viability Assays*

Cells (approximately 5000 cells/well) were seeded into a 96-well plate and treated with variable concentrations of RRx-001 for 24 hours. Subsequently, the cells were labeled using a Cell Counting Kit-8 (CKK8) for 2 hours. Absorbance at 450 nm was measured with a plate reader, and cell viability was expressed as the percentage of the absorbance of treated cells *versus* untreated cells.

### *Mouse Bone Marrow–Derived Macrophage (BMDM) Preparation*

Mouse macrophages were obtained from 8- to 10- week-old mouse bone marrow. Briefly, mice were euthanized, and femurs and tibiae were isolated. The bones were kept in ice-cold PBS and sterilized in 70% ethanol. By flushing them with mouse macrophage medium (IMDM with 10% FBS, 1× penicillin/streptomycin, 250 mM glutamine, and 25 mM HEPES, all from Corning Inc.), bone marrow cells were gathered and plated at 1 × 107/ml in 25-mm Petri dishes in mouse macrophage medium.

### *Macrophage Polarization Procedure*

BMDMs were generated by isolating bone marrow from femurs and culturing in high-glucose Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) containing 10% (vol/vol) fetal bovine serum (Gibco) with macrophage colony-stimulating factor (15 ng/ml; Peprotech) at 37°C with 5% $CO_2$. Medium was replaced with fresh medium containing IL-4 (10 ng/ml) and IL-10 (20 ng/ml) to obtain M2 polarized BMDM, or with fresh medium containing IFN-gamma (20 ng/ml) and LPS (100 ng/ml) to obtain M1 polarized BMDM 24 hours. M0, M1, or M2 polarized BMDMs were incubated with vehicle or 2 μM RRx-001 for 24 hours. Phagocytosis of A549 cells by polarized M0, M1, or M2 macrophages treated with 2 μM RRx-001 or the supernatant of previously treated cells with 2 μM RRx-001 for 4 hours. Phagocytic index indicated the number of A549 cells phagocytosed per 100 macrophages.

### *Nuclear Fractionation and Western Blotting*

Cell lysates containing extracted proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked at room temperature for 1 hour in 5% fat-free milk and incubated overnight at 4°C with a primary antibody followed by incubation with the appropriate conjugated reporter antibody for 1 hour at room temperature.

### *Cytokine Assay*

Analysis of cytokines and chemokines in macrophage culture supernatants was performed in the Mouse 26-plex kits purchased from Affymetrix (Santa Clara, CA) and used according to the manufacturer's recommendations. Briefly, samples were mixed with antibody-linked polystyrene beads on 96-well filter-bottom plates and incubated at room temperature for 4 hours followed by overnight incubation at 4°C. Room temperature incubation steps were performed on an orbital shaker at 400 rpm. Plates were vacuum-filtered, washed twice with wash buffer, and then incubated with biotinylated detection antibody for 2 hours at room temperature. Samples were then filtered and washed twice as above and resuspended in streptavidin-PE. After incubation for 1 hour at room temperature, two additional vacuum washes were performed, and the samples were resuspended in reading buffer. Samples were measured in duplicate.

### *RNA Extraction and qRT-PCR*

Total cellular RNA was extracted with the TRIzolreagent (Invitrogen, USA), and cDNA was synthesized by using M-MLV reverse transcriptase (Life Technologies). Real-time PCR was performed with SYBR Green master mix (Applied Biosystems). To normalize the amount of total RNA in each reaction, β-actin cDNA was used as an internal control. Results are expressed as relative abundance. The purity of the amplified products was validated by the dissociation curve.

### *Flow Cytometry Analysis*

*Antibody Preparation, Flow Cytometry Analysis, and Cell Sorting.* The antihCD47 (B6H12) hybridoma was obtained from the ATCC. Hybridoma cells were cultured under standard conditions, and antibodies were purified by Protein G. For quantification of CD47 expression, cells were labeled with a saturating concentration of a 1:1 phycoerythrin-conjugated anti-CD47 antibody (BD Pharmingen) and

analyzed using a BD LSR Fortessa Analyzer. Tumor cells were incubated with 20 μM CFDA-SE using a Vybrant CFDA-SE Cell Tracer Kit (Invitrogen). Macrophages were incubated in serum-free medium for 2 hours with anti-CD11b APC-eFluor 780 (eBioscience) before the adding $2 \times 10^5$ of CFDA SE-labeled tumor cells. Macrophages and tumor cells were incubated for 2 hours, and subsequently, cells were washed twice. Gates were placed according to unstained and fluorescence-minus-one controls. Fluorescently labeled antibodies targeting macrophage and tumors were used. Macrophages that had successfully phagocytized tumor cells were positive for eFluor 780 and CFDA-SE stain. BD QuantiBRITE PE beads (BD Pharmingen) were analyzed at the same settings and conditions as the samples. Median absolute CD47 antibody binding for each sample was determined from a calibration curve constructed from the QuantiBRITE bead data using the FlowJo Data Analysis software calibration tool. BMDMs were trypsinized and incubated with phycoerythrin-labeled antibodies against mouse F4/80, CD80, and CD86 for 30 minutes on ice; washed with PBS; and analyzed using a FlowJo Data Analysis software calibration tool.

### *In Vitro Phagocytosis Assay*

Briefly, $1 \times 10^5$ macrophages were planted per well in glass-bottom cell culture dishes. According to the manufacturer's protocol, A549, MCF-7, AU-565, HBL-100, or MDA-MB-231 cells were labeled with 20 μM CFDA-SE using a Vybrant CFDA-SE Cell Tracer Kit (Invitrogen). Macrophages were incubated in serum-free medium for 2 hours before the adding $2 \times 10^5$ of CFDA SE-labeled tumor cells. Macrophages were repeatedly washed and subsequently imaged with confocal microscope. The phagocytic index was calculated as the number of phagocytosed carboxyfluorescein succinimidyl ester (CFSE)–positive cells per 100 macrophages.

### *In Vivo Treatment with A549 Xenografts and Clodrolip/Clodronate*

Dichloromethylene diphosphonate (clodronate, 2.5 g; Sigma) was encapsulated in liposomes formed by a 25:1 w/w ratio of phosphatidylcholine:cholesterol (Sigma), and the resulting liposomes were resuspended in 4 ml sterile PBS. Clodronate liposomes for syngeneic transfer experiments were synthesized or purchased from ClodronateLiposomes.org (the Netherlands). Mice bearing A549 were anesthetized by IP injection of 100 mg/kg ketamine and 10 mg/kg xylazine, and 50 μl of vehicle or clodronate-containing liposomes was injected. IV administration of clodronate depleted macrophages.

### *Statistical Analysis*

Values are expressed as means ± SD and were obtained from at least three independent experiments. Statistical analyses were performed using GraphPad. Student's *t* test (two-tailed) or one-way ANOVA was performed to determine significance when comparing data from different treatment groups. *P* values were calculated, and $P < .05$ was considered to represent significant difference.

## Results

### *Treatment with RRx-001 and the Supernatant of RRx-001–Treated Macrophages Downregulates CD47 on Tumor Cells and SIRPα on Macrophages*

To determine whether RRx-001 dually downregulates CD47 and SIRPα at the protein level, $1 \times 10^5$ A549 cells and $1 \times 10^4$ A549 macrophages were plated per well, incubated with 2 μM RRx-001, and analyzed with Western blotting. RRx-001 was shown to decrease the protein levels of CD47 and SIRPα compared to control (no treatment). A less dramatic reduction in CD47 and SIRPα levels occurred with the supernatant of RRx-001–treated macrophages, as shown in Figure 1. The decrease of CD47 and SIRPα expression suggests that RRx-001 disrupts CD47-SIRPα interaction, leading to phagocytosis.

### *Treatment with RRx-001 Polarizes Macrophages from an M2 Phenotype to M1*

In the tumor microenvironment, macrophages are able to dynamically switch between two distinct phenotypes: M1 classically activated macrophages, which are proinflammatory and antitumor, and M2 alternatively activated macrophages, which are anti-inflammatory and immunosuppressive.[7] Based on clinical biopsies, RRx-001 has been shown to reorient or reprogram M2 tumor-associated macrophages (TAMs) to the M1 phenotype.[8]

BMDMs from C57/BL6 mice were incubated with IL-4 (10 ng/ml) and IL-10 (20 ng/ml) for 24 hours to polarize them to an M2 phenotype. CD80 and CD86 were selected as M1 macrophage markers, and CD206 and CD163 as M2 macrophage markers.[9]

These M2-polarized BMDM were then incubated with vehicle or 2 μM RRx-001 for 24 hours. The mRNA expression levels of M1 markers CD80 and CD86 were found to significantly increase with RRx-001 treatment compared to vehicle control (Figure 2).

The mRNA expression levels of M1 markers, CD80 and CD86, as determined by reverse transcription–quantitative polymerase chain reaction, were significantly increased with RRx-001 treatment, as shown on the box-and-whisker plot. These data indicate that RRx-001 preferentially polarizes macrophages to an M1 phenotype. Data are mean ± SD for three independent experiments.

### *RRx-001, the Supernatant of Previously RRx-001–Treated Macrophages, and RRx-001-Cys-Ox Reduce the Expression of CD47 and SIRPα on A549 Cells*

Previous work on the metabolites of RRx-001 has identified an oxidized cysteine adduct with anticancer activity. This adduct is referred to as RRx-001-Cys-Ox.

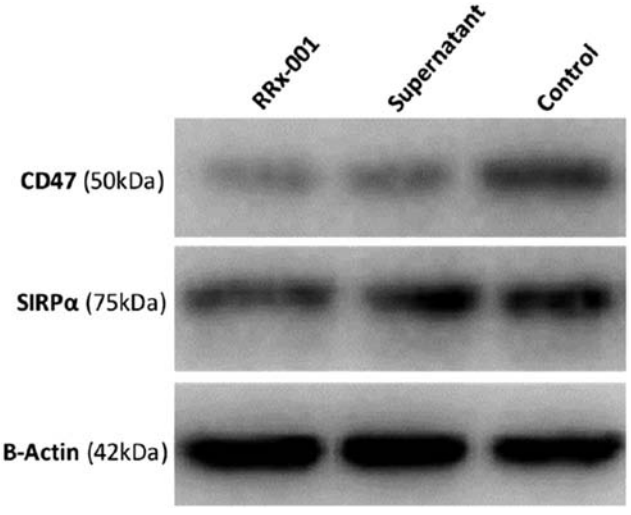


**Figure 1.** In the presence of RRx-001 and supernatant, CD47 and SIRPα levels are decreased.

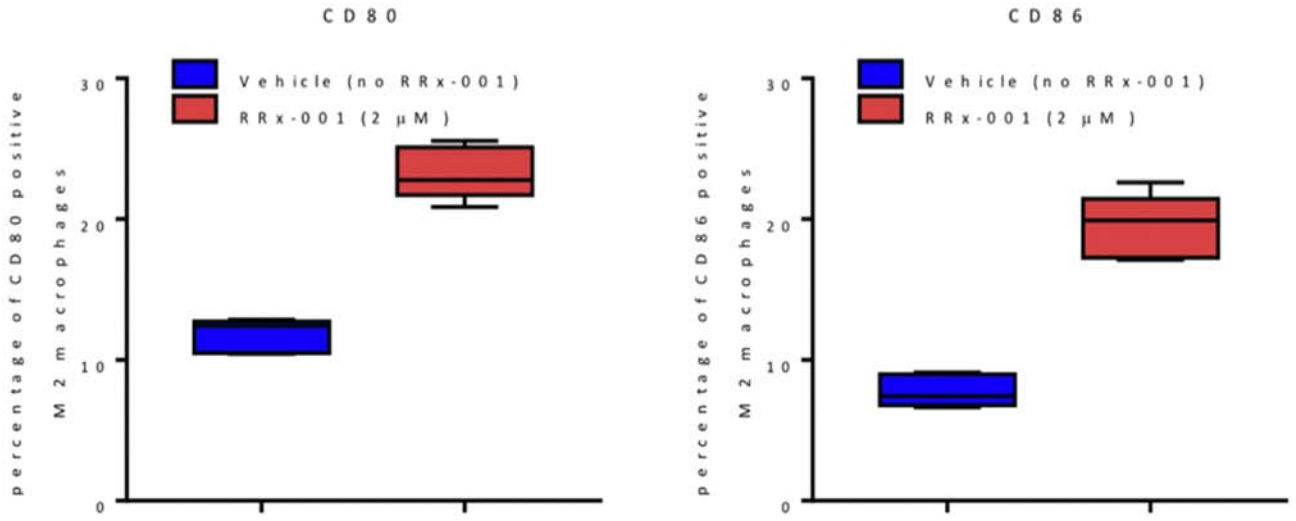


**Figure 2.** RRx-001 polarizes M2 macrophages to an M1 phenotype.

### *RRx-001 and the Supernatant of Previously RRx-001–Treated Macrophages Promote the Phagocytosis of CD47-Overexpressing A549 Cells*

Blocking CD47 with anti-CD47 monoclonal antibodies leads to the phagocytosis of cancer cells.[10] On this basis, it was investigated whether RRx-001 would enhance the phagocytosis of A549 lung cancer cells. Macrophages derived from mouse bone marrow cells phagocytosed A549 cells at a low frequency when treated with 0.1% DMSO vehicle. However, treatment with 2 μM and 5 μM RRx-001 or the supernatant of previously treated cells with 2 μM and 5 μM RRx-001 for 2 hours significantly increased the phagocytosis of A549 cells (Figure 3). Incubation for 8 hours of human breast cancer cells AU-565, MCF-7, and MDA-MDB-231 that overexpress CD47 and mouse bone marrow macrophages in the presence of 2 μM RRx-001 dramatically increased the phagocytosis of the cancer cells *vs.* vehicle control (Figure 4).

CFSE-labeled lung cancer A549 cells were incubated with mouse macrophages and 2 μM RRx-001 or the supernatant of previously treated cells with 2 μM RRx-001 for 2 hours and examined by immunofluorescence microscopy to detect phagocytosis. Phagocytic index, represented on the box-and-whisker chart, was calculated as the mean number of A549 cells phagocytized per 100 macrophages according to the following formula: phagocytic index = number of phagocytized A549 cells/100 macrophages. The error bars represent mean ± SD ($n$ = 3).

Flow cytometric analysis of eFluor-labeled bone marrow macrophages were incubated at 37°C with CFSE-labeled breast cancer cells AU-565, MCF-7, and MDA-MDB-231. The percentage value represents the proportion of phagocytic cells. Mean ± SD ($n$ = 3). Scattergram was generated by combining eFluor fluorescence with CFSE fluorescence, and the double-positive population (indicating phagocytosis of CFSE-labeled cancer cells by eFluor 670–labeled macrophages) is shown in the upper right quadrant. FACS analysis indicated the presence of CD47 in breast cancer cells (Figure 4). Cell lines used in this study showed abundance of CD47. Exposure to RRx-001 induced apoptosis in breast cancer. Incubation of breast cancer cells with RRx-001 increased annexin V binding in a dose-dependent manner. The percentage of annexin V–positive cells was used to determine the decree of apoptosis.

### *RRx-001 and the Supernatant of Previously RRx-001–Treated Macrophages also Promote M2 Macrophages to Phagocytose A549 Cells*

The same *in vitro* phagocytosis assay was used to evaluate whether RRx-001 induced mouse M2 macrophages to phagocytose CD47-overexpressing A549 cells. BMDMs from C57/BL6 mice were incubated with IL-4 (10 ng/ml) and IL-10 (20 ng/ml) for 24 hours to polarize them to an M2 phenotype. CD206 and CD163 were selected as M2 macrophage markers. M2 macrophages did not phagocytose tumor cells when treated with 0.1% DMSO vehicle. However, treatment with 2 μM RRx-001 or the supernatant of previously treated cells with 2 μM RRx-001 for 2 hours significantly increased the phagocytosis of A549 cells (Figure 5). Incubation for 8 hours with A549 cells and M2-derived macrophages in the presence of 2 μM RRx-001 and supernatant significantly increased the mean phagocytosis rate of the M2 macrophages. The error bars represent mean ± SD ($n$ = 3).

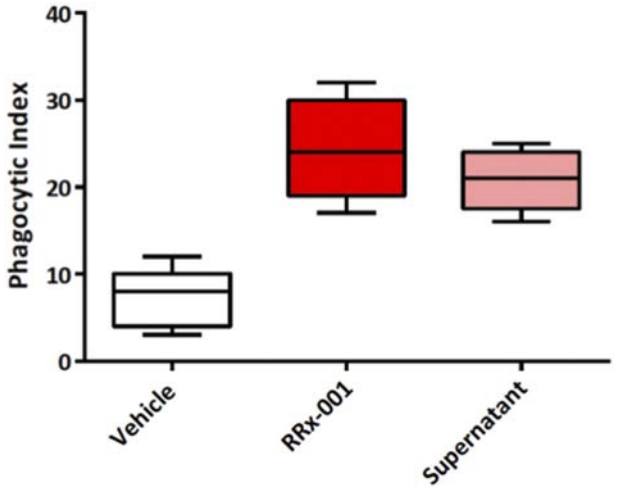


**Figure 3.** RRx-001 and the supernatant of previously RRx-001–treated macrophages promote *in vitro* phagocytosis of A549 cells.

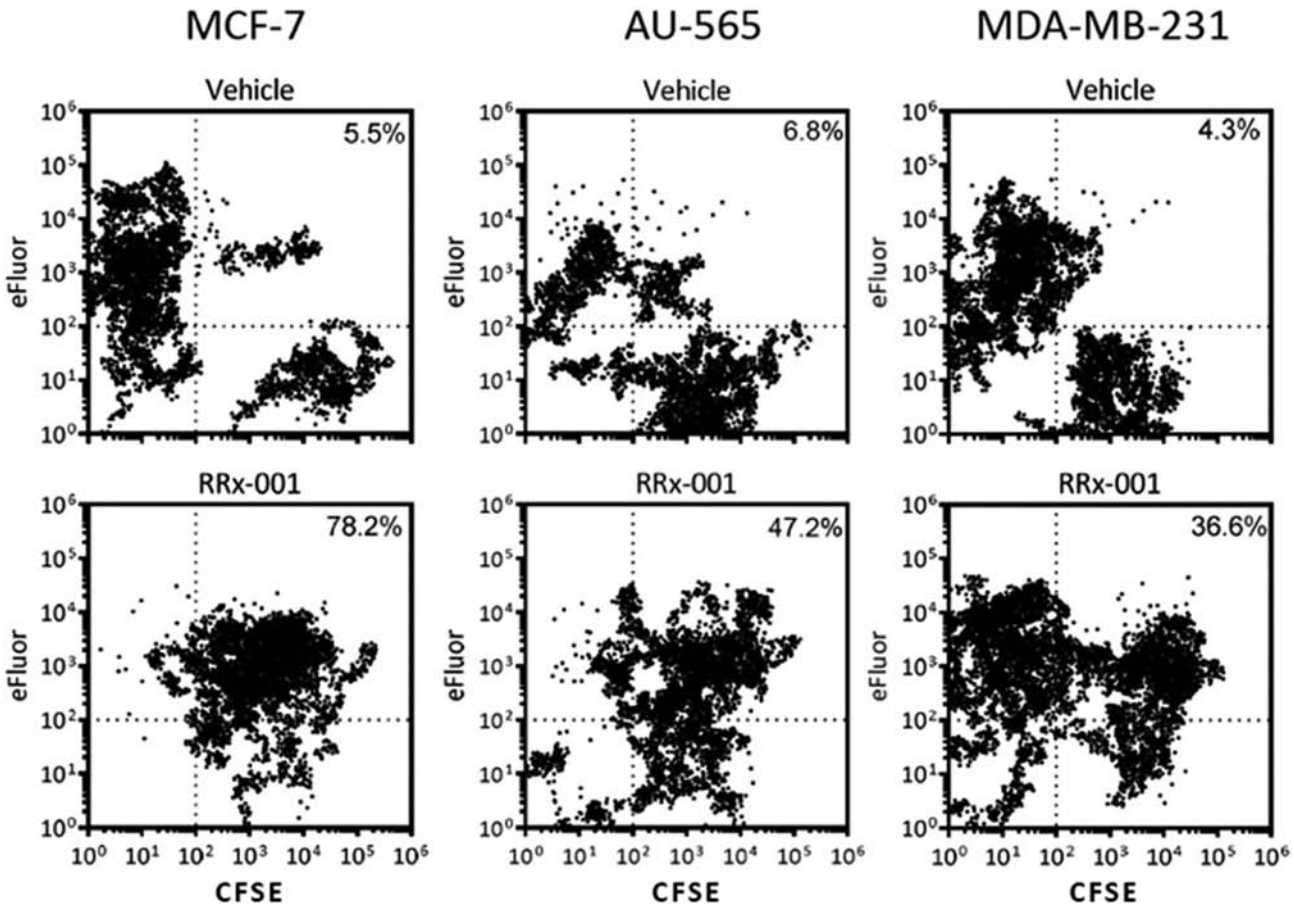


**Figure 4.** RRx-001 increases phagocytosis of breast cancer cells by bone marrow macrophages.

### *TAM Depletion with Clodronate or Clodrolip Attenuates RRx-001 Antitumor Activity in A549 Xenografts*

To determine whether the anticancer activity of RRx-001 on the A549 lung cancer model is dependent on the presence of TAMs in tumor tissue, A549-bearing nude mice were randomized into five groups ($n$ = 4 per group), as follows:

1. IV clodrolip: 0.5 g/kg clodrolip (clodronate emulsion)
2. IV clodrolip + IV RRx-001: 0.5 g/kg clodrolip and 10 mg/kg of RRx-001
3. IP 1 g/kg clodronate in PBS
4. IV RRx-001
5. Control: no treatment

The results from the experiment are shown in Figure 6. Treatment with RRx-001 resulted in the most significant tumor growth retardation, followed by clodrolip + RRx-001, clodrolip alone, and clodronate alone, both of which reduced tumor progression through macrophage depletion. These data indicate that reduction of resident

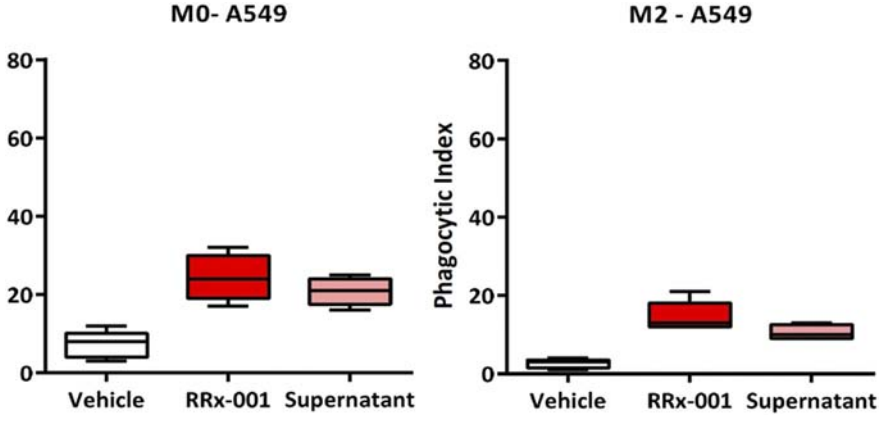


**Figure 5.** RRx-001 and the supernatant of previously RRx-001–treated macrophages promote *in vitro* phagocytosis of A549 cells by M0 and M2 macrophages.

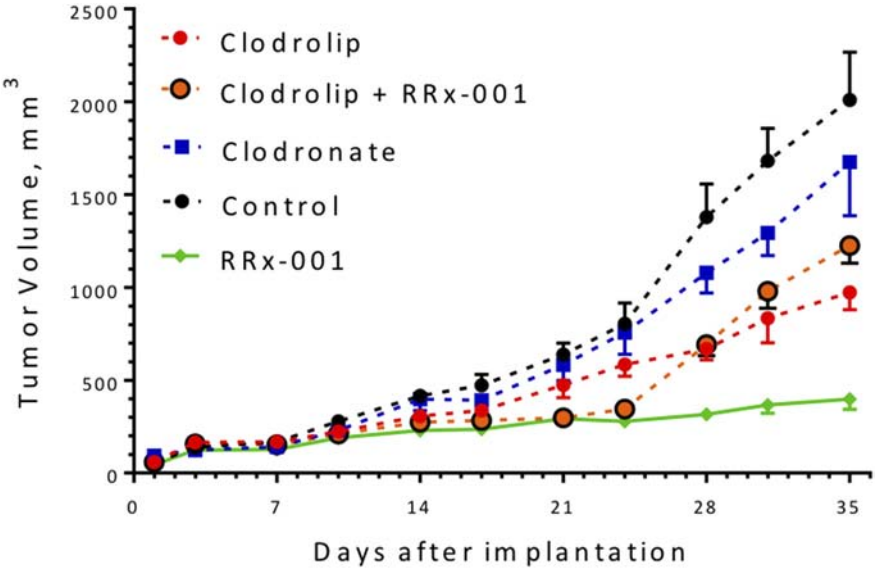


**Figure 6.** Clodronate-induced macrophage elimination attenuates the anticancer activity of RRx-001 in A549 cells.

macrophages in tumor-bearing mice attenuates the antitumor activity of RRx-001.

## Conclusion

Elsewhere, it has been demonstrated that RRx-001 polarizes M0 and M2 macrophages to the M1 phenotype.[11,12] To dissect the mechanism by which RRx-001 skews macrophage differentiation and stimulates phagocytosis, its effect on the expression of CD47 and SIRPα was examined since disruption of the CD47-SIRPα axis is known to promote M1 polarization.[13]

These experiments identify an RRx-001–dependent surface decrease of CD47 on tumor cells and of SIRPα on macrophages. Rather than a CD47 or SIRPα blocking antibody, which is associated with platelet aggregation, red blood cell hemagglutination,[14] and dose-limiting hemolytic anemias,[15] RRx-001 is a well-tolerated, minimally toxic, and nonmyelosuppressive small molecule that downregulates the CD47-SIRPα axis and is not associated with hemolytic anemia.

Within the tumor microenvironment, macrophages, which are abundant,[16] acquire two opposing phenotypes: proinflammatory, antitumor M1 and anti-inflammatory, protumor M2.[17] To subvert M1 macrophage–mediated cytotoxicity, cancer cells overexpress the cell surface receptor CD47, which binds to its counter-receptor, SIRPα, on myeloid cells; this interaction serves as a primary regulatory checkpoint[18] through the delivery of an inhibitory "do not eat" antiphagocytosis signal. CD47 upregulation, which has been reported in multiple solid and hematological malignancies as well as cancer stem cells,[19] correlates with poor clinical outcomes.[20]

Herein, the effect of RRx-001 on malignant cells was examined *in vitro* and *in vivo*; in addition, the effect of the supernatants of macrophages either treated or not with RRx-001 was evaluated. *In vitro* studies demonstrate that RRx-001 and supernatant promote macrophage-mediated phagocytosis of tumor cells. *In vivo* studies demonstrate that RRx-001 potently inhibits antitumor growth in preclinical xenograft models; this inhibitory effect is diminished when TAM density is depleted with clodronate.

Collectively, these data support the conclusion that RRx-001 is a minimally toxic dual checkpoint inhibitor of CD47 and SIRPα that primes macrophages both *in vitro* and *in vivo* to attack cancer cells and suggest the potential for enhanced antitumor efficacy in combination with immune checkpoint inhibitors and other immunotherapies.

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