

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



Pedro Cabrales

Department of Bioengineering, University of California San Diego, 9500 Gilman Dr., La Jolla, CA 92093, USA

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 (SIRP α) on macrophages and monocytes to prevent phagocytosis. Herein, the effect of RRx-001 on CD47 and SIRP α 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 SIRP α on tumor cells and monocytes/macrophages, respectively, reducing the phagocytosis inhibitory function of the CD47/SIRP α 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.

Translational Oncology (2019) 12, 626–632

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 (SIRP α) expressed on myeloid cells is referred to as an innate immune checkpoint¹ 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-SIRP α antibodies, and bispecific agents have been developed.⁴ Since CD47/SIRP α 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/SIRP α 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

Address all correspondence to: Pedro Cabrales, Department of Bioengineering, University of California San Diego, 9500 Gilman Dr., La Jolla, CA 92093, USA.
E-mail: authorscorrespondence@gmail.com

Received 1 October 2018; Revised 30 November 2018; Accepted 3 December 2018

© 2018 Published by Elsevier Inc. on behalf of Neoplasia Press, Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).
1936-5233/19

<https://doi.org/10.1016/j.tranon.2018.12.001>

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 SIRP α 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 \pm 1.7 g) were subcutaneously injected with 5 \times 10⁶ of A549 cells suspended in PBS and containing 50% Matrigel Matrix (Coating, 354234) to establish NSCLC xenograft models. Tumor-bearing mice were randomized into four cohorts after tumors had a volume \sim 100 mm³. RRx-001, supernatant, and vehicle were intraperitoneal injected twice a week and once a day respectively. Tumor volume was calculated as length \times width \times 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 μ g/ml streptomycin (Life Technologies, Inc.), and 10 μ g/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 \times penicillin/streptomycin, 250 mM glutamine, and 25 mM HEPES, all from Corning Inc.), bone

marrow cells were gathered and plated at 1 \times 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% CO₂. 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- γ (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 μ M RRx-001 for 24 hours. Phagocytosis of A549 cells by polarized M0, M1, or M2 macrophages treated with 2 μ M RRx-001 or the supernatant of previously treated cells with 2 μ M 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 antiCD47 (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 2×10^5 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, 1×10^5 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 2×10^5 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 \pm 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 SIRP α on Macrophages

To determine whether RRx-001 dually downregulates CD47 and SIRP α at the protein level, 1×10^5 A549 cells and 1×10^4 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 SIRP α compared to control (no treatment). A less dramatic reduction in CD47 and SIRP α levels occurred with the supernatant of RRx-001-treated macrophages, as shown in Figure 1. The decrease of CD47 and SIRP α expression suggests that RRx-001 disrupts CD47-SIRP α 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 \pm 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 SIRP α 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.

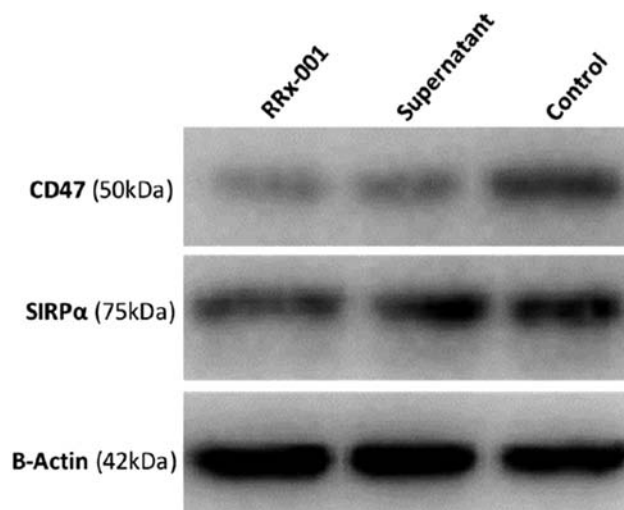


Figure 1. In the presence of RRx-001 and supernatant, CD47 and SIRP α levels are decreased.

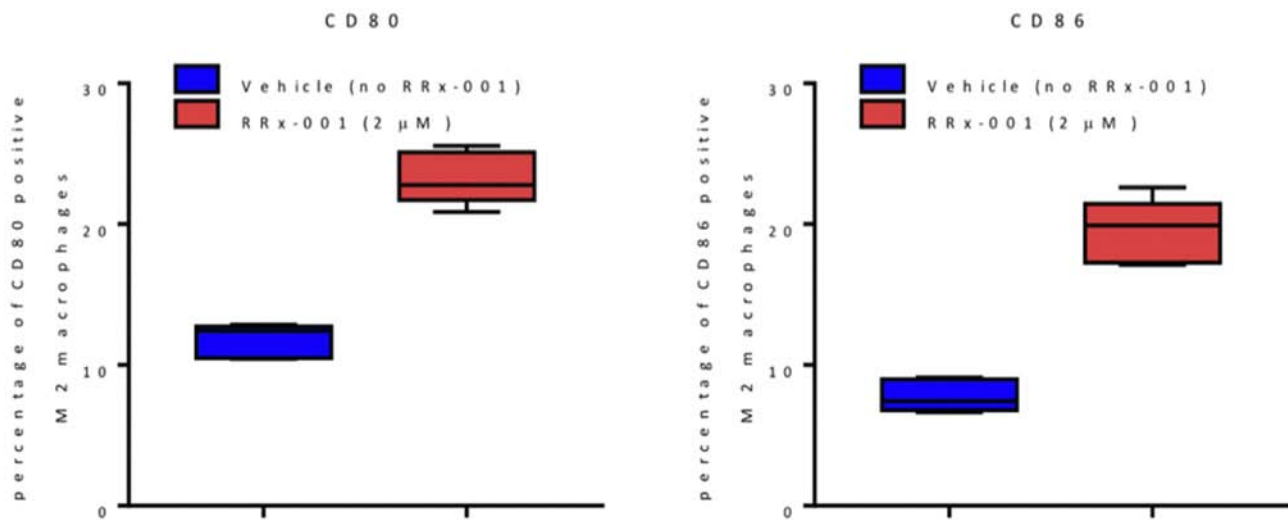


Figure 2. RRx-001 polarizes M2 macrophages to an M1 phenotype.

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 degree 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 ($n = 3$).

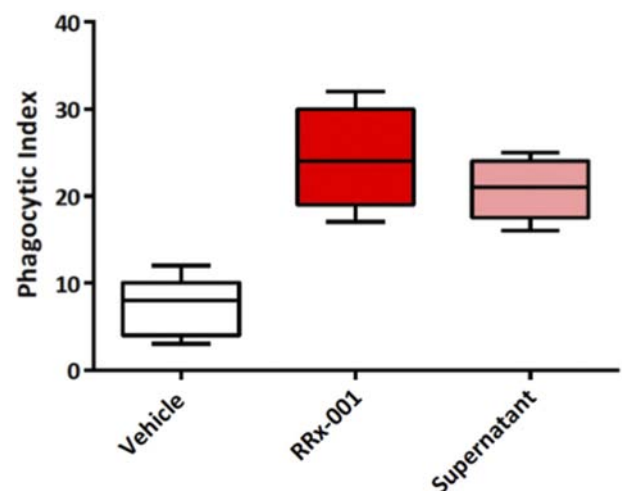


Figure 3. RRx-001 and the supernatant of previously RRx-001-treated macrophages promote *in vitro* phagocytosis of A549 cells.

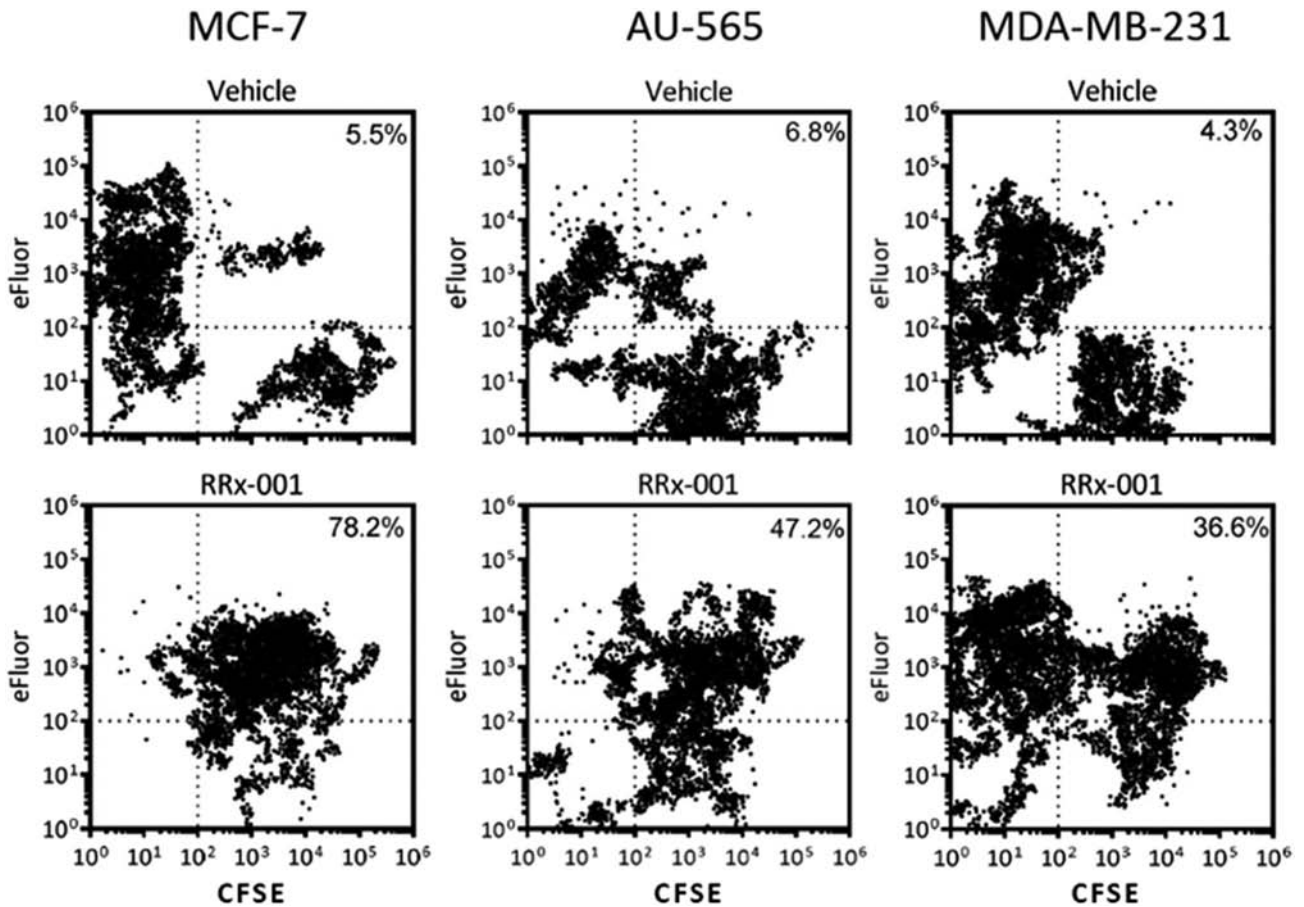


Figure 4. RRx-001 increases phagocytosis of breast cancer cells by bone marrow macrophages.

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

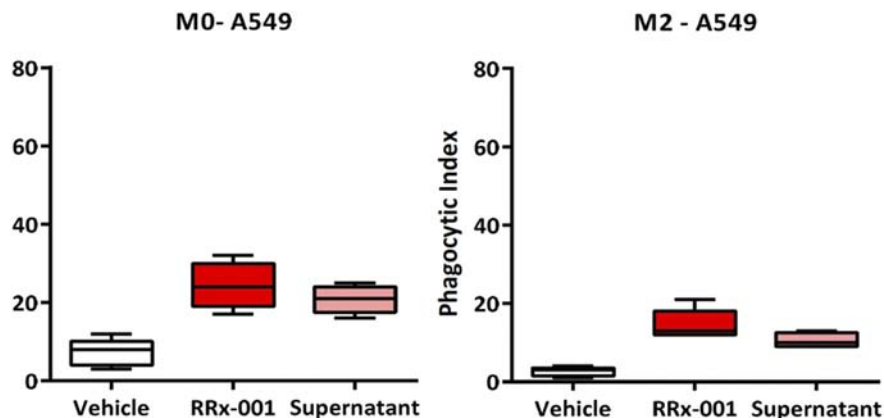


Figure 5. RRx-001 and the supernatant of previously RRx-001–treated macrophages promote *in vitro* phagocytosis of A549 cells by M0 and M2 macrophages.

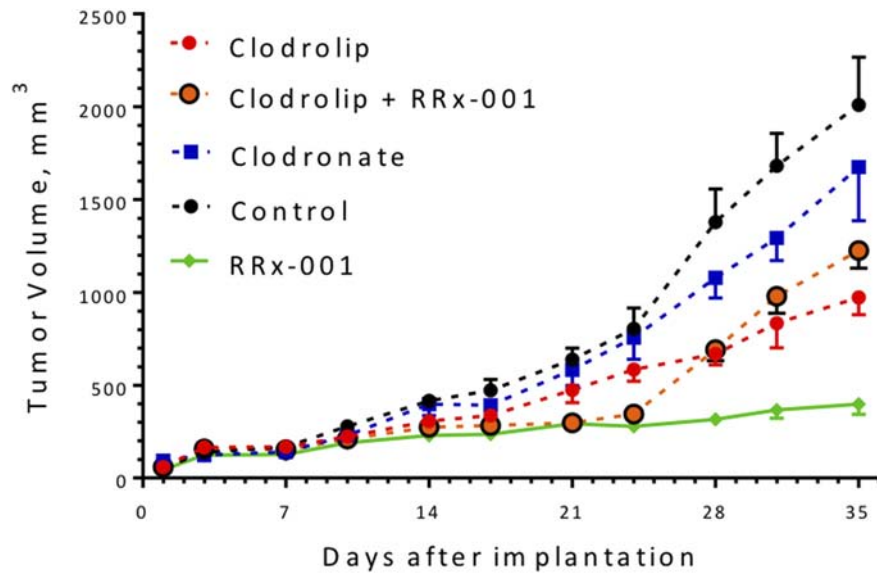


Figure 6. Clodronate-induced macrophage elimination attenuates the anticancer activity of RRx-001 in A549 cells.

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 SIRP α was examined since disruption of the CD47-SIRP α axis is known to promote M1 polarization.¹³

These experiments identify an RRx-001-dependent surface decrease of CD47 on tumor cells and of SIRP α on macrophages. Rather than a CD47 or SIRP α 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-SIRP α 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, SIRP α , on myeloid cells; this interaction serves as a primary regulatory checkpoint¹⁸ 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 SIRP α 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.

References

- [1] Liu X, Kwon H, Li Z, and Fu Y (2017). Is CD47 an innate immune checkpoint for tumor evasion? *J Hematol Oncol* **10**, 12. <https://doi.org/10.1186/s13045-016-0381-z>.
- [2] Zhang X, Fan J, Wang S, Li Y, Wang Y, Li S, Luan J, Wang Z, Song P, and Chen Q, et al (2017). Targeting CD47 and autophagy elicited enhanced antitumor effects in non-small cell lung cancer. *Cancer Immunol Res* **5**(5), 363–375. <https://doi.org/10.1158/2326-6066.CIR-16-0398> [Epub 2017 Mar 28].
- [3] Matlung HL, Szilagyi K, Barclay NA, and van den Berg TK (2017). The CD47-SIRP α signaling axis as an innate immune checkpoint in cancer. *Immunol Rev* **276**(1), 145–164.
- [4] Weiskopf K (2017). Cancer immunotherapy targeting the CD47/SIRP α axis. *Eur J Cancer* **76**, 100–109. <https://doi.org/10.1016/j.ejca.2017.02.013> [Epub 2017 Mar 10].
- [5] Veillette A and Chen J (2018). SIRP α -CD47 immune checkpoint blockade in anticancer therapy. *Trends Immunol* **39**(3), 173–184.
- [6] Reid T, Oronsky A, Caroen S, Carter C, and Cabrales P (2018). Brief report: RRx-001 is a c-Myc inhibitor that targets cancer stem cells. *Oncotarget* **9**(34), 23439–23442.
- [7] Martinez FO and Gordon S (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* **6**, 13. <https://doi.org/10.12703/P6-13>.
- [8] Jha S, Summers T, Zeman K, Brzezniak C, Carter C, Ferry L, Sciscinski J, Oronsky B, Caroen S, and Trepel J, et al (2017). Phase II clinical trial patient responses to the macrophage activating agent RRx-001 correlate to TGF- β pathway activation and markers for fibrosis. *AACR Annual Meeting 2017*; April 1–5, 2017; Washington, DC; 2017.
- [9] Bouhlef MA, Derudas B, Rigamonti E, Dièvert R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, and Marx N, et al (2007). PPAR γ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab* **6**, 137–143.
- [10] Liu J, Wang L, Zhao F, Tseng S, Narayanan C, Shura L, Willingham S, Howard M, Prohaska S, and Volkmer J, et al (2015). Pre-clinical development of a

- humanized anti-CD47 antibody with anti-cancer therapeutic potential. In: Bunting KD, editor. *PLoS ONE*, 10(9); 2015. p. e0137345.
- [11] Oronsky B, Paulmurugan R, Foygel K, Scicinski J, Knox S, Peehl D, Zhao H, Ning S, Cabrales P, and Summers Jr T, et al (2017). RRx-001: A systemically non-toxic M2-to-M1 macrophage stimulating and prosensitizing agent in phase II clinical trials. *Expert Opin Investig Drugs* **26**(1), 109–119.
- [12] Jha S, Summers T, Zeman K, Brzezniak C, Carter C, Ferry L, Scicinski J, Oronsky B, Caroen S, Trepel J, Cabrales P, and Day R (2017). Abstract 966: phase II clinical trial patient responses to the macrophage activating agent RRx-001 correlate to TGF- β pathway activation and markers for fibrosis. *Cancer Res* **77**(13 Supplement), 966.
- [13] Zhang M, Hutter G, Kahn SA, Azad TD, Gholamin S, Xu CY, Liu J, Achrol AS, Richard C, and Sommerkamp P, et al (2016). Anti-CD47 treatment stimulates phagocytosis of glioblastoma by M1 and M2 polarized macrophages and promotes M1 polarized macrophages in vivo. *PLoS One* **11**, e0153550.
- [14] Uno S, Kinoshita Y, Azuma Y, Tsunenari T, Yoshimura Y, Iida S, Kikuchi Y, Yamada-Okabe H, and Fukushima N, et al (2007). Anti-leukemic activity of a monoclonal antibody against CD47 in xenograft models of human leukemia. *Oncol Rep* **17**, 1189–1194.
- [15] Liu J, Wang L, Zhao F, Tseng S, Narayanan C, Shura L, Willingham S, Howard M, Prohaska S, and Volkmer J, et al (2015). Pre-clinical development of a humanized anti-CD47 antibody with anti-cancer therapeutic potential. *PLoS One* **10**, e0137345.
- [16] Chen P and Bonaldo P (2013). Role of macrophage polarization in tumor angiogenesis and vessel normalization: implications for new anticancer therapies. *Int Rev Cell Mol Biol* **301**, 1–35.
- [17] Sica A and Mantovani A (2012). Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest* **122**, 787–795.
- [18] Gordon SR, Maute RL, Dulken BW, Hutter G, George BM, McCracken MN, Gupta R, Tsai JM, Sinha R, and Corey D, et al (2017). PD-1 expression by tumor-associated macrophages inhibits phagocytosis and tumor immunity. *Nature* **545**(7655), 495–499. <https://doi.org/10.1038/nature22396>.
- [19] Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, Traver D, van Rooijen N, and Weissman IL (2017). CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid Phagocytosis. *Cell* **138**(2), 271–285.
- [20] Pietsch EC, Dong J, Cardoso R, Zhang X, Chin D, Hawkins R, Dinh T, Zhou M, Strake B, and Feng PH, et al (2017). Anti-leukemic activity and tolerability of anti-human CD47 monoclonal antibodies. *Blood Cancer J* **7**(2), e536. <https://doi.org/10.1038/bcj.2017.7>.