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Research Article

RRx-001 Accelerates Low Dose Oxaliplatin-Induced Cytotoxicity in Triple-Negative Breast Cancer Cells via Synergistic Antitumor Mechanisms

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Abstract

Background: Triple-negative breast cancer (TNBC) represents an aggressive subtype that has few therapeutic options for targeting and is highly resistant to chemotherapy. The compound RRx-001 is a dinitroazetidine compound with both immune-modulating and epigenetic properties, whereas oxaliplatin elicits immunogenic cell death. ***Objective***: To evaluate the synergistic cytotoxic effects of RRx-001 combined with oxaliplatin in MDA-MB-231 TNBC cells. ***Methods***: Viability assays using the MTT assay were performed. The IC50 concentrations were derived from dose response curves. The drug interaction analysis was completed utilizing the Chou-Talalay method, along with CompuSyn software to calculate the combination index (CI) and dose reduction index (DRI). The intracellular reactive oxygen species (ROS) levels were assayed by performing a fluorometric assay with DCFH-DA. ***Results***: RRx-001 exhibited greater cytotoxicity (IC50=9.878 µM) than did oxaliplatin (IC50=202.2 µM). When the two drugs were used, the combination resulted in a significant decrease in the IC50 value for oxaliplatin (to 9.705 µM, ≈20.9-fold reduction). A strong synergy was seen (CI=0.0227 at Fa=0.05) with significant reductions in the doses required for each drug. Additionally, a combination of these two drugs significantly increases amounts of ROS compared to either drug alone. ***Conclusions***: RRx-001 has been shown to significantly enhance the cytotoxicity of oxaliplatin through synergistic and ROS-mediated pathways, suggesting that it may represent a viable option for a dose-sparing combinatorial therapeutic approach to treat TNBC.

Keywords: Chou–Talalay analysis; Combination index; Drug synergism; RRx-001; Triple-negative breast cancer.

يسرع RRx-001 من تسمم الخلايا الناتج عن الأكساليبلاتين بجرعة منخفضة في خلايا سرطان الثدي ثلاثية السلبية من خلال آليات متآزرة مضادة للأورام

الخلاصة

الخلفية: سرطان الثدي الثلاثي السلبي (TNBC) يمثل نوعا فرعيا عدوانيا لا يملك خيارات علاجية محددة للاستهداف ومقاوم بشدة للعلاج الكيميائي. المركب RRx-001 هو مركب دينيتروزيتيدين ذو خصائص مناعية وصفات فوق جينية، بينما يؤدي أكساليبلاتين إلى موت الخلايا المناعية. **الهدف**: تقييم التأثيرات السامة الخلوية التآزرية لـRRx-001 مع أوكساليبلاتين في خلايا MDA-MB-231 TNBC. **الطرائق**: تم إجراء اختبارات الجدوى باستخدام اختبار MTT. تم اشتقاق تراكيز IC_{50} من منحنيات استجابة الجرعة. تم إجراء تحليل تفاعل الأدوية باستخدام طريقة تشو-تالالاي، إلى جانب برنامج CompuSyn لحساب مؤشر التركيبة (CI) ومؤشر تقليل الجرعة (DRI). تم قياس مستويات أنواع الأكسجين التفاعلي داخل الخلايا (ROS) من خلال إجراء اختبار فلورومتري باستخدام DCFH-DA. **النتائج**: أظهر RRx-001 سمية خلوية أكبر (IC_{50}=9.878 ميكرومول) مقارنة بالأوكساليبلاتين (IC_{50}=202.2 ميكرومولار). عند استخدام الدواءين، أدى المزيج إلى انخفاض كبير في قيمة IC_{50} للأوكساليبلاتين (إلى 9.705 ميكرومولار، ≈انخفاض بمقدار 20.9 ضعف). لوحظ تآزر قوي (CI=0.0227 عند Fa=0.05) مع انخفاض كبير في الجرعات المطلوبة لكل دواء. بالإضافة إلى ذلك، فإن الجمع بين هذين الدواءين يزيد بشكل كبير من كميات ROS مقارنة بأي من الدوائين فقط. **الاستنتاجات**: ثبت أن RRx-001 يعزز بشكل كبير سمية الخلايا للأكساليبلاتين من خلال المسارات التآزرية والمتوسطة بواسطة ROS، مما يشير إلى أنه قد يمثل خيارا قابلا للتطبيق لنهج علاجي توافقي يوفر الجرعة لعلاج TNBC.

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INTRODUCTION

According to the International Agency for Research on Cancer (IARC), breast cancer is the most commonly occurring malignant neoplasm in females worldwide. In 2024, it is projected that there will be 310,720 new instances of invasive breast cancer and approximately 42,250 fatalities in the United States [1,2]. Triple-negative breast cancer (TNBC), characterized by the lack of estrogen receptors, progesterone receptors, and HER2 expression, is one of the most dangerous subtypes [3]. Triple-negative breast cancer (TNBC) disproportionately affects younger women and is associated with higher recurrence rates, greater metastatic potential, and decreased overall survival relative to other breast cancer subtypes [4]. Although TNBC initially responds to chemotherapy, the lack of druggable molecular targets and the swift development of chemoresistance highlight the urgent necessity for innovative therapeutic approaches [5]. The current standard of care for non-metastatic TNBC involves chemotherapy combined with surgery, utilizing platinum-based compounds, anthracyclines, taxanes, and alkylating agents [5,6]. However, resistance frequently develops through cancer stem cell renewal and adaptive tumor mechanisms, necessitating innovative or repurposed therapeutic approaches [7]. Oxaliplatin, a third-generation platinum compound primarily used in gastrointestinal cancers, has demonstrated direct cytotoxic activity against TNBC cell lines such as MDA-MB-231 in preclinical studies [8]. Notably, oxaliplatin possesses a unique capacity among platinum drugs to trigger immunogenic cell death (ICD) [9,10], by inducing the release of damage-

associated molecular patterns (DAMPs), including calreticulin, ATP, and HMGB1, which recruit and activate antigen-presenting cells and promote antitumor T-cell responses [9,10]. This immunogenic property makes oxaliplatin particularly attractive for combination strategies that aim to harness both direct cytotoxicity and immune activation [11]. RRx-001 is a novel dinitroazetidine derivative currently in advanced clinical development (Phase 3) as a radio- and chemosensitizer [12,13]. RRx-001 has demonstrated a favorable safety profile in preclinical and clinical studies, supporting its development as a chemosensitizer [14,15]. Structurally, RRx-001 integrates two pharmacophores linked via a stable amide bond: a bromoacetyl targeting moiety and a dinitroazetidine payload, resembling an antibody-drug conjugate design [13]. The α-bromoacetamide moiety selectively alkylates accessible thiolate anions ($R\text{-}S^-$) through nucleophilic substitution, with reactivity governed by the local pKa environment of target cysteine residues [16]. This selective covalent binding underpins the remarkably favorable safety profile of RRx-001 [14], which lacks the classical chemotherapy-associated toxicities such as myelosuppression, alopecia, and severe nausea observed with conventional agents [13,15]. RRx-001 exerts its antitumor effects through multiple complementary mechanisms. It primarily targets the NLRP3 inflammasome by covalently binding to cysteine 409 within the NACHT domain, thereby blocking inflammasome assembly and inhibiting NF-κB signalling [15, 17]. Additionally, RRx-001 acts as a nitric oxide donor in hypoxic tumor environments, generating peroxynitrite ($ONOO^-$) and reactive nitrogen species that produce tumor-selective nitro-oxidative stress and cytotoxicity [18]. RRx-001 also demonstrates significant epigenetic regulatory activity, promoting DNA hypomethylation and histone modifications that can restore the expression of silenced tumor suppressor genes, potentially reversing chemoresistance [19,20,35]. Furthermore, RRx-001 modulates the tumor immune microenvironment by reprogramming tumor-associated macrophages from an immunosuppressive M2 phenotype to a tumoricidal M1 phenotype [21] and by downregulating CD47 on cancer cells, thereby enhancing macrophage-mediated phagocytosis [21]. These combined effects facilitate T-cell activation and infiltration into the tumor microenvironment, bolstering the antitumor immune response [17]. Given the complementary mechanisms of Oxaliplatin (direct DNA damage and ICD induction) and RRx-001 (epigenetic modulation, oxidative stress induction, and immune activation), we hypothesized that their combination would produce synergistic cytotoxicity in TNBC cells while enabling significant dose reductions. The objective of this study was to assess and quantify the synergistic anticancer effects of RRx-001 in combination with low-dose oxaliplatin in MDA-MB-231 TNBC cells using the Chou–Talalay median-effect principle. The hypothesis of this study is based on their respective mechanisms of action. We hypothesized that the combination of RRx-001 with oxaliplatin would be capable of inducing a synergistic cytotoxic response against TNBC cells while allowing for a substantial reduction in doses. Based on this rationale, RRx-001 would prime cancer cells so as to sensitize them to platinum-based chemotherapy by virtue of its effects on gene expression, oxidative stress, and the cellular immune environment. The study aimed to evaluate and quantify how combining RRx-001 with lower doses of Oxaliplatin would have a synergistic anti-cancer effect on TNBC (triple-negative breast cancer) MDA-MB-231 cells by using the Chou-Talalay median-effect principle. Additionally, the study investigated if the combined treatments could produce synergistic cytotoxicity while allowing for significant dose reductions of each drug.

METHODS

Chemicals

Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, and amphotericin B were obtained from Capricorn Scientific (Germany). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin, and dimethyl sulfoxide (DMSO) were obtained from EuroClone (Italy). Oxaliplatin was supplied by MSD Pharmaceutical Co. (Hangzhou, China). RRx-001 was acquired from Takeda Pharmaceutical Co. RRx-001 was solubilized in DMSO due to its hydrophobic nature, whereas oxaliplatin was dissolved in the medium. MTT was diluted in PBS to a concentration of 5 mg/mL to prepare an MTT stock solution. All chemicals were sterilized via filtration using a 0.22 µm Millipore syringe filter.

Cell culture

The human TNBC cell line MDA-MB-231 (ATCC, Manassas, VA, USA) was cultured in DMEM with 10% FBS and 1% antibiotic-antimycotic solution at 37 °C in a humidified environment of 5% CO_2. Cells were passaged at ~75% confluency, medium changed every three days and routinely screened for mycoplasma contamination.

In vitro cell cytotoxicity assay

The cytotoxicity was evaluated by the MTT test [22,33]. Cells have been cultivated in 96-well plates (5×10^3 cells/well) and allowed to attach overnight. RRx-001 and oxaliplatin alone or in combination were tested at successive concentrations (1.95–1000 µM) over 24, 48, and 72 h. After treatment, MTT reagent (20 µl; Invitrogen, USA) was applied to each well and incubated for 4 h at 37°C. Formazan crystals were dissolved in DMSO (100 µl), and absorbance was read at 560/600 nm in a GloMax® Microplate Reader (Promega, USA). IC_{50} values were determined by nonlinear regression using GraphPad Prism 9.2. Cell viability and death were calculated as:

$$Cell\ Viability\% = \frac{OD560,600\ (sample)}{OD560,600\ (control)} \times 100 \quad \text{Eq. (1)}$$

$$Cell\ death\% = 100 - Cell\ viability\% \quad \text{Eq. (2)}$$

All experiments were performed in triplicates across at least three independent replicates. Controls received 0.1% DMSO as a vehicle.

Detection of ROS by fluorometric assay

DCFH-DA fluorometric test for quantification of reactive oxygen species (ROS) [23]. Cells were seeded in 24-well plates (5 × 10 4 cells/well) and treated with RRx-001 (9.87 µM), oxaliplatin (202.2 µM), their combination, or vehicle for 24 h. Then cells were treated with 0.10 µM DCFH-DA in serum-free media for 30 min at 37°C in the dark, rinsed with PBS, trypsinized and centrifuged (1000 × g, 5 min). The oxidized DCF was measured for fluorescence at 488/525 nm excitation/emission with a fluorescent plate reader and represented as relative fluorescence units normalized to untreated controls.

Synergistic effects analysis

Drug interactions were evaluated by the Chou–Talalay median-effect principle in CompuSyn software v1.0 (CompuSyn Inc., NJ, USA) [24]. Drugs were mixed in a fixed molar ratio of 1:20 (RRx-001: Oxaliplatin) based on their 24-h IC50 ratio at five dose levels (25%, 50%, 100%, 200%, and 400% of each IC50). The combination index (CI) was calculated to be [24]:

$$Combination\ Index\ (CI) = \frac{D1}{(Dx)1} + \frac{D2}{(Dx)2} \quad \text{Eq. (3)}$$

Where D1 and D2 are the doses of each drug in combination to produce x% an effect, and Dx1 and Dx2 are the doses of each drug alone to have the same effect. CI < 1, = 1, and > 1 mean synergism, additivity, and antagonism, respectively. The dose reduction index (DRI) was calculated to assess the degree of dosage reduction for each drug in the synergistic combination. Fa-dose graphs, CI-Fa plots, isobolograms, and DRI-Fa plots were constructed for effect levels of Fa= 0.05 to 0.90.

Ethical considerations

All experimental approaches were conducted in accordance with protocols approved by the Research Ethics Committee of the College of Pharmacy, Mustansiriyah University, Baghdad, Iraq.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Group differences were analyzed by one-way ANOVA followed by Tukey's post hoc test. Statistical significance was defined as a p-value < 0.05. All analyses were performed with GraphPad Prism 9.2 (GraphPad Software Inc., USA) except drug interaction analysis was performed using the Chou–Talalay method with combosyn.com (CompuSyn Inc., Paramus, NJ, USA).

RESULTS

Dose-response curves for RRx-001 and oxaliplatin were initially constructed utilizing the MTT cell proliferation test to determine the exact IC50 values for these agents in the current investigation. Dose-response curves for the evaluated drugs in MDA-MB-231 cells were established at an initial concentration of 1.95 µM over 24, 48, and 72 hours. The findings indicated IC50 values (24 h) of 202.2 µM for oxaliplatin and 9.878 µM for RRx-001, respectively (Figure 1).

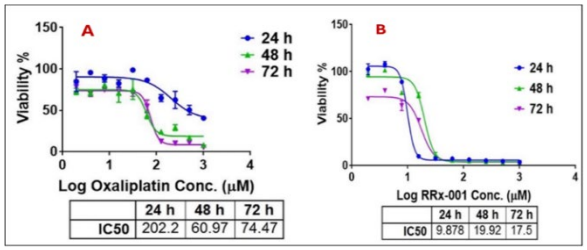

Figure 1: Dose-response curves and IC50 for RRx-001 and Oxaliplatin in breast cancer cells. MDA-MB-231 cells were treated with **(A)** Oxaliplatin and **(B)** RRx-001 at different concentrations for 24h, 48h, and 72h, followed by an MTT cytotoxicity assay. Data are presented as mean±SD of three independent experiments performed in triplicate. IC50, half-maximal inhibitory concentration.

Oxaliplatin had the half-maximal inhibitory concentrations (IC50)values of 202.2 µM at 24 h, 60.97 µM at 48 h, and 74.47 µM at 72 h (Figure 1A).The half-maximal inhibitory concentrations (IC_{50}) were calculated based on dose-response curves at 24 h, 48 h, and 72 h. RRx-001 was shown to be highly active with an IC50 value of 9.878 µM at 24 h, 19.92 µM at 48 h, and 17.5 µM at 72 h (Figure 1B). While the concentrations employed to evaluate the IC50 of the individual drug in the breast cancer cell line guided the application of a fixed combination ratio of RRx-001 to oxaliplatin, hence facilitating the synergistic inhibitory effects of both agents. Cell viability was measured at 24, 48 and 72 hours at various concentrations (0–1000 µM) to determine the cytotoxic effects of RRx-001, oxaliplatin and their combination **(**Tables 1-3**)**.

Table 1: Cell viability at 24 h

Conc. (µM)	RRx-001 (%)	Oxaliplatin (%)	Combo (%)
0	90	100	91
1.95	102	83	89
3.91	108	95	86*
7.81	87	87	70*#
15.62	9	81	7*
31.25	6	100	5*
62.5	6	85	5*
125	5	63	4*
250	4	60	3*
500	4	50	2*
1000	2	39	2*

Values of RRx-001 and oxaliplatin as single agents and in combination in MDA-MB-231 TNBC cells at 24 h incubation evaluated by MTT test. Values are means ± SD of three independent experiments. Values are the mean % viability (read from graph. Original also has error bars (±SEM). *Represent significant difference between combination and oxaliplatin, # represent significant differences between combination and RRx-001. Oxa: oxaliplatin, combo: combination.

Table 2: Cell viability at 48 h

Conc. (μM)	RRx-001 (%)	Oxaliplatin (%)	Combo (%)
0	93	100	94
1.95	97	72	81#
3.91	101	70	66#
7.81	76	80	55*#
15.62	75	70	10*#
31.25	10	75	5*
62.5	5	43	4*
125	5	23	4*
250	4	29	3*
500	3	17	3*
1000	2	7	2

MTT test of MDA-MB-231 TNBC cells at 48 h of treatment with RRx-001 and oxaliplatin as single agents and in combinations. Values are means ± SD of three independent experiments. Values are the mean % viability (read from graph. Error bars are original (± SEM). *Represent significant difference between combination and oxaliplatin, # represent significant differences between combination and RRx-001. Oxa: oxaliplatin, combo: combination.

Table 3: Cell viability at 72 h

Conc. (μM)	RRx-001 (%)	Oxaliplatin (%)	Combo (%)
0	100	100	100
1.95	70	78	66*
3.91	80	70	62#
7.81	63	74	45*#
15.62	49	72	7*#
31.25	7	68	4*
62.5	4	54	4*
125	4	10	3
250	3	9	3
500	3	10	2
1000	3	5	2

MTT assay values of RRx-001 and oxaliplatin as single agents and in combination in MDA-MB-231 TNBC cells at 72 h incubation. Data are expressed as mean ± SD of three independent experiments. Values are the mean % viability (read from graph. Error bars (±SEM) same as original. * Represent significant difference between combination and oxaliplatin, # represent significant differences between combination and RRx-001. Oxa: oxaliplatin, combo: combination.

RRx-001 as a single agent exhibited a strong cytotoxicity at relatively low doses. The viability declined dramatically at 15.62 μM and reached near full cell death (≤10%) at concentrations ≥31.25 μM at all time points. Oxaliplatin produced a more protracted concentration and time-dependent decrease in cell viability, with significant cytotoxicity visible at higher concentrations and longer time points, causing significant cell death at 125–1000 μM by 72 h. Importantly, at multiple concentrations and all time points, the combination of RRx-001 with oxaliplatin (RRx-001+Oxa) resulted in considerably higher cytotoxicity than either agent alone ($p<0.05$ to $p<0.001$), suggesting a synergistic interaction. This increased cytotoxic effect was most noticeable at low-intermediate concentrations (3.91-62.5 μM) when the combination consistently decreased viability below that reached by either agent alone. These data suggest that RRx-001 augments the anti-tumor action of oxaliplatin in a time- and concentration-dependent manner. As shown in Figure 2, the amount of reactive oxygen species (ROS) is present in the MDA-MB-231 cells after they have been treated with RRx-001, oxaliplatin and both drugs compared to untreated controls.

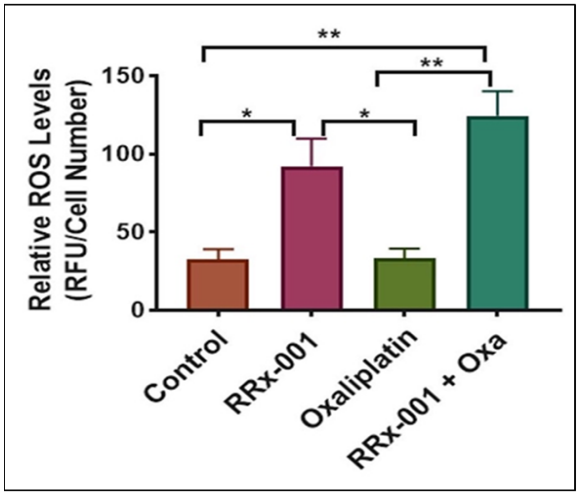

Figure 2: Effect of RRx-001, oxaliplatin, and their combination on intracellular ROS levels in MDA-MB-231 cells after 24 h treatment measured by the DCFH-DA assay. Data are presented as mean±SD (*$p< 0.05$, and **$p< 0.01$).

The ROS levels are based on relative fluorescence units per cell number (RFU/cell). Untreated cells in the control group had very low amounts of ROS being produced, as indicated by RFU values of about ~30, which represents the normal oxidative status of non-treated cells. A single treatment with RRx-001 resulted in a large increase in ROS production, as evidenced by a value of approximately ~90 RFU, and was found to be significantly higher than the control group ($p< 0.05$). The results suggest that RRx-001 induces oxidative stress in TNBC cells. In contrast to RRx-001, single treatments of oxaliplatin resulted in only minor increases in ROS production (~30 – 35 RFU), indicating that it does not produce a significant alteration in oxidative status as compared to the control group. Interestingly, the combination of RRx-001 and oxaliplatin resulted in the greatest amount of ROS produced (approximately ~120 – 130 RFU) and was significantly higher than both single treatments ($p< 0.01$) and also higher than the control group. The results demonstrate a synergistic impact in the augmentation of oxidative stress when the two drugs are administered together. The pharmacological interaction between RRx-001 and oxaliplatin was quantified using the Chou–Talalay median-effect model. The Chou–Talalay technique has become a benchmark in preclinical drug development for identifying effective drug combinations and optimizing dosage [25]. Table 4 shows determined values of combination index (CI) and dosage reduction index (DRI). The results demonstrated synergism for the RRx-001/oxaliplatin combination in MDA-MB-231 cells at low to moderate levels of impact with CI values ranging from 0.0227 to 0.8107 (Table 4). The DRI analysis also suggested a significant dose decrease for both RRx-001 and oxaliplatin due to their synergistic interaction. At the fixed combination ratio of 1:20, DRI values at Fa = 0.5 suggested a 1.31-fold dosage reduction for RRx-001 and a 3.38-fold dose reduction for oxaliplatin (Table 4).

Table 4: Combination Index (CI) and Dose Reduction Index (DRI) for Oxa and RRx-001 at a ratio of 1:20

Total dose (μM)	Fa	CI Value	DRI (RRx-001)	DRI (OXa)
0.21086	0.05	0.0227	82.73	93.95
1.09411	0.10	0.0594	28.91	40.40
3.03238	0.15	0.1081	15.07	23.95
6.53293	0.20	0.1702	9.23	16.16
12.3145	0.25	0.2480	6.16	11.68
21.4249	0.30	0.3450	4.32	8.79
35.4290	0.35	0.4661	3.14	6.79
56.7214	0.40	0.6181	2.32	5.34
89.0701	0.45	0.8107	1.74	4.23
138.602	0.50	1.0583	1.31	3.38
215.680	0.55	1.3824	0.99	2.69
338.684	0.60	1.8167	0.74	2.14
542.228	0.65	2.4173	0.55	1.68
896.648	0.70	3.2826	0.40	1.30
1560.01	0.75	4.6013	0.28	0.98
2940.59	0.80	6.7800	0.19	0.71
6335.17	0.85	10.8557	0.11	0.48
17558.3	0.90	20.3311	0.06	0.28
91107.7	0.95	56.2911	0.03	0.14
293999.	0.97	116.567	0.002	0.06

Abbreviations: CI, combination index; DRI, dose reduction index; Fa, fraction affected (proportion of cells inhibited); Oxa, oxaliplatin; RRx-001, the dinitroazetidine study compound. CI < 1 indicates synergism; CI = 1 indicates additivity; CI > 1 indicates antagonism. Total dose is expressed as the sum of both drug doses (μM) at the fixed molar ratio of 1:20 (RRx-001: Oxa).

Importantly, DRI values were higher at lower effect levels, for example, at Fa = 0.05, DRI increased to 82.73-fold for RRx-001 and 93.95-fold for oxaliplatin (Table 4 and Figure 3A)**.** Synergism between RRx-001 and oxaliplatin was further validated by isobologram analysis, especially at low to moderate effect levels, with combination points located below the line of additivity (Figure 3B). CI-Fa analysis indicated strong synergism at low levels of effect consistently, with CI values close to additivity at high doses. The combination at the maximum dose demonstrated an almost additive impact with a CI value of about 1.06 (Table 4 and Figure 3C).

DISCUSSION

Management of Triple-Negative Breast Cancer (TNBC) remains a significant challenge in oncology due to its elevated metastatic potential, heightened recurrence rates, biological heterogeneity, and limited targeted treatment options [26,27]. In response to the need to address the limitations of chemotherapy, particularly drug resistance, this study investigated the potential for achieving synergistic cytotoxic effects through the combination of the dinitroazetidine compound RRx-001 and the third-generation platinum agent oxaliplatin. The dose-response curves and IC_{50} values of oxaliplatin and RRx-001 in MDA-MB-231 TNBC cells revealed that these agents demonstrated time- and concentration-dependent cytotoxicity, respectively, albeit with distinct potency profiles. Oxaliplatin exhibited relatively modest cytotoxic activity at early time points ,which reflects the time-dependent nature of its mechanism of action through DNA-DNA crosslink formation that interferes with DNA replication and transcription, ultimately inducing apoptosis [6]. The progressive increase in cytotoxic potency over time is consistent with the accumulation of irreparable DNA lesions, a well-established characteristic of platinum-based agents [29].

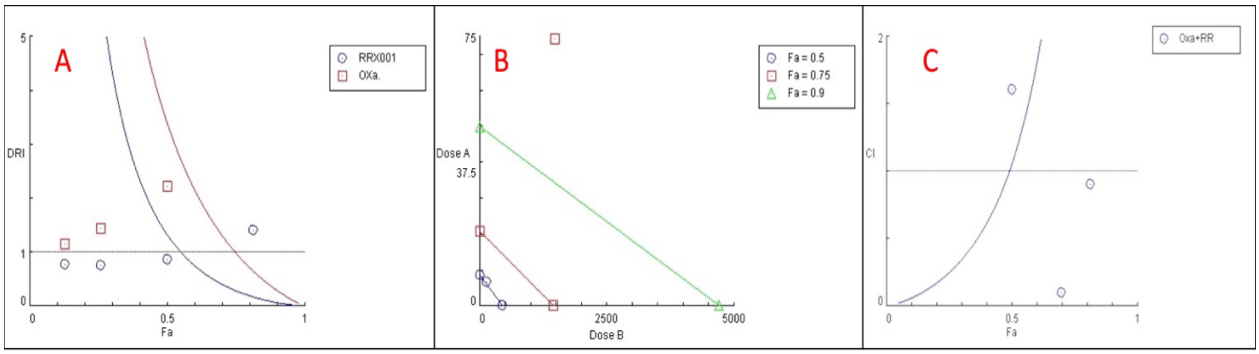

Figure 3: Chou–Talalay analysis of the interaction between RRx-001 and Oxaliplatin in MDA-MB-231 cells. **(A)** Dose Reduction Index (DRI) plot that shows the number of fold reductions in drug dose which can be achieved using both agents together as opposed to single agent treatment. **(B)** Isobologram showing interaction between RRx-001 and Oxaliplatin at different effect levels. **(C)** Combination Index (CI)-Fa plot that evaluates the interaction between drugs based on the CI-Fa values; if CI<1 is considered as synergism; if CI=1 as additive effects; if CI>1 as antagonism. Taken collectively these studies indicate a synergistic interaction between RRx-001 and Oxaliplatin in the triple negative breast cancer cells.

Conversely, RRx-001 showed significantly higher intrinsic cytotoxic potency at all time points evaluated. This finding is consistent with the role of RRx-001 as a rapid redox modulator that generates mitochondrial superoxide and glutathione depletion soon after administration [18]. Together these findings illustrate the complementary cytotoxic characteristics of the two agents: Oxaliplatin has predominantly a delayed DNA damage mechanism, and RRx-001 works fast via production of oxidative stress [6,18]. This molecular complementarity provides a scientific rationale for the combination of both drugs to improve anticancer activity in TNBC. The reference concentrations for further assessments were considered to be the 24 h IC50 of RRx-001 (9.878 μM) and oxaliplatin (202.2 μM). Oxaliplatin showed lower IC50 values at 48 and 72 h however the 24-h time point was utilised for both agents to ensure methodological consistency, to illustrate direct pharmacological action prior to cellular adaptation and to reduce overcrowding artefacts in prolonged 96-well plate cultures. This enabled systematic dose stratification at 0.5x, 1.0x, and 2.0x IC50 and consequently direct comparison between medicines throughout the study. An interesting finding

of this study was the considerable drop in the IC50 of oxaliplatin in combination with RRx-001, suggesting a significant chemosensitizing impact that reduces the quantity of medicine needed to suppress the proliferation of cancer cells. Similar chemosensitizing effects have been reported in MDA-MB-231 combination models [30,34]. This dose-sparing effect is clinically relevant, as it provides a strategy for minimizing the systemic toxicity associated with platinum-based drugs [28,29]. The reason for this effect appears to be related to RRx-001's ability to prime cancer cells through its epigenetic and immunomodulatory actions, thereby rendering them more sensitive to platinum-based chemotherapy [19,21]. The increased ROS generated by the drug combination likely contributes to enhanced cytotoxicity through oxidative damage to cellular components, thereby promoting apoptosis. While oxaliplatin is a strong DNA alkylator that generates ROS secondary to DNA damage-induced apoptosis over a longer kinetic window [31], RRx-001 has been demonstrated to be a rapid redox modulator causing mitochondrial superoxide production and glutathione depletion almost immediately upon administration [18]. This suggests that RRx-001 may prime the intracellular environment for redox-sensitive DNA repair pathways prior to the genomic activity of oxaliplatin, thereby amplifying the overall cytotoxic response that may the cause why the combination produce the greatest amount of ROS. Chou-Talalay analysis confirmed strong synergism between RRx-001 and oxaliplatin, particularly at lower drug concentrations, representing a therapeutically advantageous window where significant tumor inhibition can be achieved with reduced doses of cytotoxic agents [24,25]. The bell-shaped synergy profile indicates that the combination has the greatest therapeutic potential at the lowest doses, where the risk of toxic side effects is minimal. The Dose Reduction Index (DRI) further highlights the practical clinical benefit of this combination, as the large potential for reducing oxaliplatin doses is particularly important given the dose-limiting toxicities associated with platinum-based agents, including peripheral neuropathy and myelosuppression [29]. The observed synergy is likely the result of the complementary biological mechanisms of both drugs. Oxaliplatin is uniquely distinguished among platinum compounds for its ability to trigger immunogenic cell death (ICD), inducing DNA damage and cellular stress that results in the liberation of damage-associated molecular patterns (DAMPs), including calreticulin, ATP, and HMGB1, which recruit and activate the immune system against the tumor [10,32]. RRx-001 enhances this effect through several pathways: it induces DNA hypomethylation and histone modifications that can restore the expression of silenced tumor suppressor genes and reverse chemoresistance [20]; it shifts the tumor microenvironment from an immunosuppressive M2 phenotype to a pro-inflammatory, tumoricidal M1 phenotype [21]; and by covalently binding to cysteine 409 of the NLRP3 inflammasome and inhibiting NF-κB signaling, it reduces tumor-promoting inflammation and enhances T-cell infiltration [17]. Together, the combination essentially creates a dual attack: oxaliplatin kills cancer cells and activates immune signaling, while RRx-001 removes the epigenetic and immunosuppressive brakes imposed by the tumor microenvironment [20].

Study Limitations

This study is limited to an in vitro assay utilizing a single human TNBC cell line (MDA-MB-231) that does not account for the entire biological diversity of triple-negative breast cancer and therefore cannot be directly linked to patients' treatment outcome(s). The reactive oxygen species was evaluated only at 24 hours; however, the peak of oxaliplatin-induced ROS-mediated DNA damage occurs at approximately 48 – 72 hours. Additionally, no cellular system exists to replicate the immunomodulatory characteristics of RRx-001, including the induction of M1 / M2 macrophages, the activation of T cells, and the down-regulation of CD47; hence, all of these effects remain undetermined. Lastly, the numerous molecular pathways by which it is hypothesized that the synergistic effect will occur (i.e., NLRP3 inhibition, NF-kappa B signaling suppression, epigenetic reprogramming) have been neither confirmed nor invalidated in the present study.

Conclusion

This study showed that RRx-001 potentiated the cytotoxic effect of oxaliplatin in MDA-MB-231 triple-negative breast cancer cells. The combination demonstrated a higher decrease of cell viability than either drug alone and a notable dose-reduction effect as per Chou-Talalay analysis. The combination also resulted in a considerable rise of intracellular ROS levels compared to single-agent treatment, suggesting that oxidative stress contributed to the amplified cytotoxic response. “Overall, these results support the potential utility of combining RRx-001 with oxaliplatin as a dose-sparing strategy in TNBC.” These results require confirmation by additional investigations employing other TNBC models and in vivo validation.

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Conflict of interests

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Data sharing statement

The data that supports the findings of this study are available from the corresponding author upon a reasonable request.

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