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Reid, Tony
Oronsky, Bryan
Abrouk, Nacer
et al.

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Original Article

The small molecule NLRP3 inhibitor RRx-001 potentiates regorafenib activity and attenuates regorafenib-induced toxicity in mice bearing human colorectal cancer xenografts

Tony Reid[1], Bryan Oronsky[1], Nacer Abrouk[1], Scott Caroen[1], Pedro Cabrales[2]

[1]*EpicentRx Inc., La Jolla, CA, USA;* [2]*Department of Bioengineering, University of California San Diego, La Jolla, CA, USA*

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Abstract: The multi-kinase inhibitor Regorafenib, approved for the treatment of metastatic colorectal cancer, is poorly tolerated with a Grade 3/4 drug related adverse event rate of 54% resulting in frequent dose reductions and discontinuations. RRx-001 is a minimally toxic NLRP3 inhibitor small molecule with macrophage-repolarizing properties in Phase 3 clinical trials. Studies have demonstrated the inhibitory impact of M2 macrophages on the activity of tyrosine kinases, suggesting that the repolarization of macrophages by RRx-001 may enhance the activity of TKIs. The purpose of these experiments was to determine whether RRx-001 demonstrated *in vitro* and *in vivo* synergy with regorafenib in colorectal cancer and whether RRx-001 attenuated the toxicity of regorafenib. Tumor-bearing mice were randomized into four cohorts: RRx-001 alone, regorafenib alone, RRx-001 + regorafenib and control. RRx-001 demonstrated *in vitro* and *in vivo* synergy with regorafenib with attenuation of toxicity in colorectal cancer cell lines. These results provide a rationale to treat colorectal cancer with RRx-001 plus another tyrosine kinase inhibitor like regorafenib.

Keywords: Chemotherapy, tyrosine kinase inhibitor, regorafenib, RRx-001, colorectal cancer

Introduction

Stage IV colorectal cancer is an aggressively morbid disease, with a poor prognosis: the median and 5-year survival rates are between 14 and 19 months and 10%, respectively, with treatment [1]. Over the last decade, the incidence of colorectal cancer has decreased slightly, due to improved screening programs and detection of precancerous polyps, however the burden of disease remains high, and disproportionate within demographic subpopulations [2]. The natural history of the disease is associated with rapid systemic dissemination predominantly to the liver although extrahepatic hematogenous spread to the lung, peritoneum and bone with significant morbidity is not uncommon [3].

From a dearth of therapeutic options before 1990 during which the only active agent for the treatment of metastatic colorectal cancer (mCRC) was 5FU, seven agents have been approved in the past 10 years, irinotecan, capecitabine, oxaliplatin, bevacizumab, cetuximab, regorafenib and TAS-102. Despite the apparent breadth of this therapeutic armamentarium, the treatment of mCRC is still palliative rather than curative and, hence, the identification of new treatment options remains a priority [4]. While single-agent therapy with either regorafenib or TAS-102 is approved in the USA as options after progression on initial FOLFOX or FOLFIRI-based regimens, they each have only modest overall survival (OS) and progression free survival (PFS) benefits compared with best supportive care [5]. In particular, regorafenib was approved on the basis of a median PFS of 1.9 months and a 1.4 median improvement in OS compared to placebo in the Phase 3 CORRECT trial, achieved at the expense of increased dermatologic and gastrointestinal toxicities, which has led to criticism from the medical community that the presumptive clini-

cal benefit is counterbalanced and perhaps even abrogated by these treatment morbidities [6].

RRx-001 is a first-in-class minimally toxic NLRP3 inhibitor small molecule with macrophage-repolarizing properties in clinical trials for the treatment of multiple tumor types, including a Phase 3 trial for the treatment of lung cancer and a recently completed Phase 2 trial for the treatment of colorectal cancer. In clinical trials, RRx-001 is used as monotherapy or in combination with chemotherapy, immunotherapy, radiation and targeted agents.

The rationale for the combination of the first-in-class small molecule RRx-001 with regorafenib is multiplefold: (1) RRx-001 has anti-inflammatory properties mediated through NLRP3 inflammasome inhibition [7, 8]; (2) RRx-001 resensitizes to previous chemotherapy [9, 11] and (3) RRx-001 has macrophage repolarizing properties [10]. Studies have demonstrated the inhibitory impact of M2 macrophages on the activity of tyrosine kinases, suggesting that the repolarization of macrophages by RRx-001 may enhance the activity of TKIs [11].

In the Phase 2 randomized clinical trial called ROCKET (NCT02096354), 4 mg RRx-001 was combined with 180 mg/m^2 irinotecan vs. 160 mg regorafenib in 34 3rd/4th line colorectal cancer patients that were previously treated with- and progressed on-irinotecan. For the RRx-001 arm, the OS was 8.6 months and the PFS was 7.5 months compared to an OS of 4.7 months and a PFS of 1.9 months for regorafenib, which is similar to the OS and PFS seen in the Phase 3 CORRECT trial and the real-world REBECCA trial of 654 patients [12]. In addition, drug related adverse events were 62% for regorafenib, which is similar to the adverse event rate in other regorafenib clinical trials [13, 14], versus 16% for RRx-001 + irinotecan. As potential evidence of RRx-001-mediated chemoprotection, 0% of RRx-001 + irinotecan-treated patients experienced Grade 3/4 diarrhea or neutropenia, the two main dose limiting toxicities of irinotecan [15].

On the basis of these trial results and on the premise that regorafenib is minimally active and generally very poorly tolerated, despite recent dose optimization strategies [16], whereby regorafenib is slowly escalated from a starting dose of 80 mg to 160 mg, this study attempted to determine whether combination therapy with RRx-001 and regorafenib not only enhanced anticancer activity *in vitro* with HCT-116 and HCT-15 colorectal cell lines and *in vivo* with HCT-116 and HCT-15 xenografts but also attenuated the toxicity of regorafenib in these two xenografts.

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).

Cell culture and analysis of cytotoxicity

Colon cancer cell lines, HCT-116 and HCT-15 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and maintained according to the ATCC's instructions in a 37°C incubator at 5% CO_2. RRx-001 was provided by EpicentRx (Torrey Pines, CA) and regorafenib (BAY 73-4506, catalog No. S1178) was purchased from SelleckChem. Both agents were diluted with dimethylsulfoxide DMSO (Sigma).

The effect of regorafenib and RRx-001 on HCT-116 and HCT-15 cytotoxicity was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay [17] at indicated days. HCT-116 and HCT-15 cells were seeded at a density of 2×10^3 cells per well in a 96-well plate in 100 µl McCoy's 5A medium (Invitrogen, Carlsbad, CA) containing 10% FBS (HyClone, Logan, UT) and gentamycin.

Up to 10 µM RRx-001 and regorafenib were added to each well 24 h after plating (final concentration 0-100 µg/mL). The absorbance was measured at the wavelength of 570 nm. The measured optical density (OD) values were directly proportional to the number of viable cells. Then, dose-response curves were fitted to the data. All experiments were performed in triplicate and repeated thrice.

Mice management

Female 6-week-old Nu/Nu mice (Charles River, Wilmington, MA) were housed in a sterile environment with micro isolator cages and allowed access to water and chow *ad libitum*. Mice were

subcutaneously injected with 20 μL 1×10^6 cells/μL of HCT-116 cells or 20 μL 1×10^6 cells/μL of HCT-15 suspended in PBS and containing 50% Matrigel Matrix (Coining, 354234) to establish xenograft models. Tumor-bearing mice were randomized into four cohorts of 4 mice each (n = 4): RRx-001 alone, regorafenib alone, RRx-001 + regorafenib and control (no treatment). Regorafenib was dissolved in Cremephor EL/95% ethanol (50:50) as a 4× stock solution, and RRx-001 was diluted in DMSO to a 10 μM concentration. Both drugs were diluted to the final concentration with sterile water before use.

For RRx-001 only: Two weeks after tumor cell implantation, blood from nu/nu mice was collected in citrate-phosphate-dextrose-adenine-one (CPDA) and then mixed with RRx-001. Mice were treated with the mixture (their own mouse blood + RRx-001) twice per week (through injection into the tail vein) for 2 weeks at 5 mg/kg. The blood-mix method of RRx-001 administration mimics clinical administration [18].

For regorafenib with or without RRx-001: Two weeks after tumor cell implantation, blood from nu/nu mice was collected in CPDA and then mixed with RRx-001 or vehicle. Mice were treated with vehicle or RRx-001 twice per week for 2 weeks at 5 mg/kg. After two weeks of treatment with RRx-001, each cohort of mice was treated with Regorafenib at 10 mg/kg/daily for at least 21/28 days via oral gavage for up to 16 weeks. Doses were skipped for up to 2 consecutive days in mice that either developed weight loss (>20% of baseline weight) or severe lethargy, and treatment was resumed when the affected animals regained the lost weight or resumed normal activity. Tumor growth was monitored by measuring tumor diameters every other day with a caliper and animal weights were monitored at the same time. The end point of this study was defined as the tumor load reaching 1700 mm^3. Tumor volume was calculated as length × width × width/2.

For control group: No treatment was administered.

Statistical analysis

Descriptive statistics (n, mean and std. deviation, std. error of the mean) were used for estimation and a comparative assessment of how the treatment groups may differ was carried out on available data using a one-way analysis of variance (ANOVA). Longitudinal graphical displays were created to allow for a visual inspection of group differences (viability relative to control mean profile by dose and treatment. Tumor volume, body weight percent change by dose and treatment).

The statistical significance ($P < 0.05$) of treatment groups differences employed the standard benchmark two-sided significance level of 0.05 (via ANOVA F test).

Results

In vitro cytotoxicity

To study the in vitro cytotoxicity, HCT-116 and HCT-15 cells were exposed to various concentrations of RRx-001 and regorafenib, and the cell viability was measured using the MTT assay. As shown in **Figure 1**, both RRx-001 and regorafenib exhibited dose-dependent cytostatic/cytotoxic activity, although RRx-001 was more cytotoxic in HCT-15 cells.

RRx-001 and regorafenib demonstrate enhanced antitumor effects when used in combination

To determine whether these *in vitro* observations were therapeutically significant, animal studies were performed. Tumor volumes and body weight changes (BWC) in HCT-116 and HCT-15 xenografted mice are shown in **Figure 2**, top and **Figure 2**, bottom respectively. Tumor growth was inhibited more in mice treated with RRx-001 and regorafenib alone than in the controls ($P < 0.01$). Furthermore, the antitumor activity of RRx-001 followed by regorafenib treatment was superior to that of either monotherapy. Regorafenib alone treated animals were observed to develop lethargy as well severe weight loss, up to 20%, after which doses were held. However, in regorafenib-treated animals that received RRx-001 weight loss was significantly attenuated ($P < 0.01$) and lethargy was not observed.

Discussion

With the exception of patients converted to curable surgical resection by systemic chemotherapy, patients with metastatic colorectal cancer

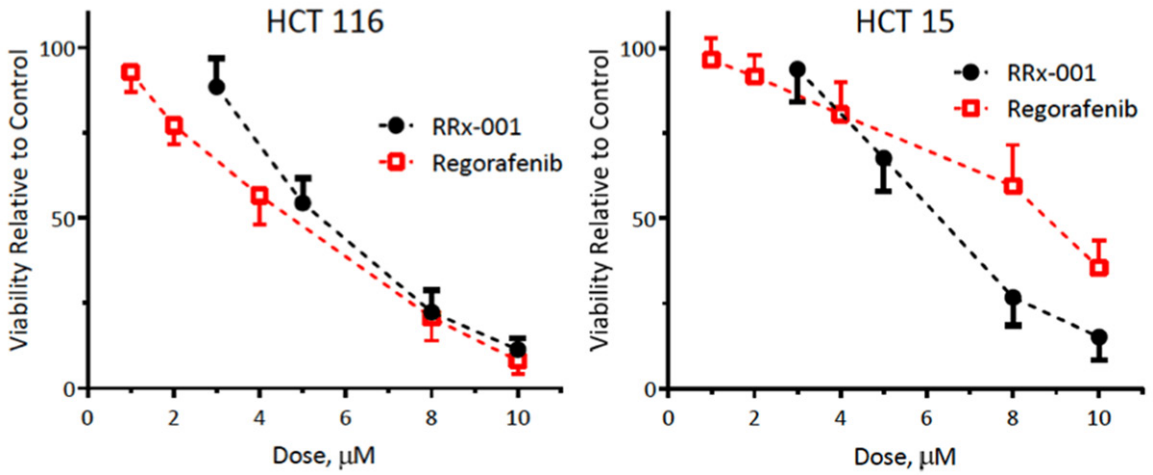

Figure 1. Cytotoxic effects of RRx-001 (black) and regorafenib (red) on (*left panel*) HCT-116 (*right panel*) HCT-15, expressed as the relative viability (percentage of untreated control), as determined by an MTT assay. *Bars*, mean + SE.

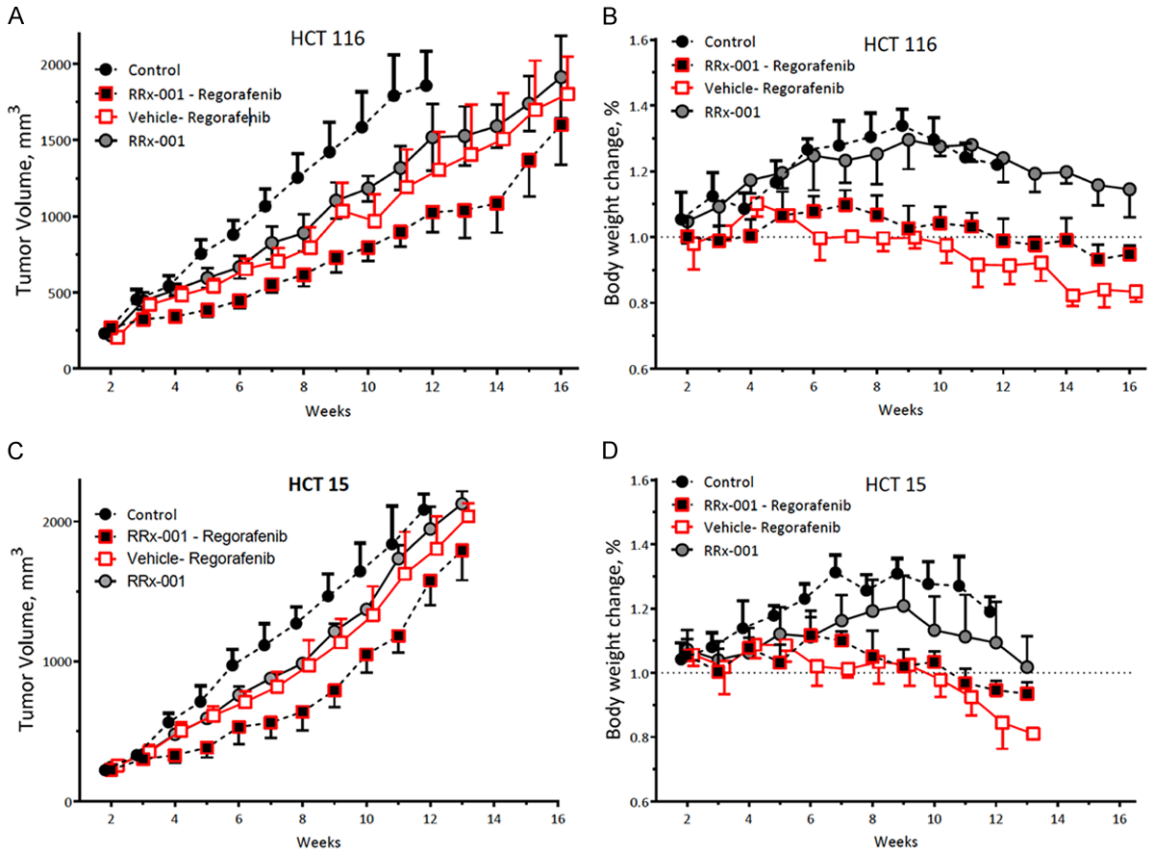

Figure 2. Tumor volume and body weight changes (BWC) in HCT-116 and HCT-15 xenografted mice after (A) control (no treatment) (B) RRx-001 alone twice per week for 2 weeks at 5 mg/kg (C) daily regorafenib alone 21/28 days for up to 16 weeks (D) RRx-001 twice per week for 2 weeks at 5 mg/kg followed by daily regorafenib 21/28 days for up to 16 weeks. Tumor volume changes and BWC in HCT-116 and HCT-15 xenografts are shown, respectively. Values indicate mean ± SD (n = 4).

progress through successive lines of therapy at an increasingly accelerated rate, due to the development of cumulative toxicities and multi-drug-resistance [19], until all standard thera-

pies have been exhausted and the only remaining options are palliative or experimental.

After progression of colorectal cancer on first and second-line oxaliplatin- and irinotecan-based regimens, the two main salvage options are regorafenib and TAS-102 [20]. Unfortunately, both are associated with short-term OS and PFS benefit; in addition, these therapies are poorly tolerated. Treatment with regorafenib results in a high rate (>50%) of hand foot skin reaction (HFSR), rash, fatigue, diarrhea and hypertension to the point that dose reductions and discontinuations are standard practice [16]; moreover, in light of how narrow its therapeutic index oncologists are generally reluctant to treat with regorafenib [21]. While better tolerability has been demonstrated with weekly dose escalation of regorafenib from 80 mg to 160 mg daily compared to 160 mg regorafenib daily, dose-limiting toxicities are still present [22], which may lead to suboptimal adherence, especially since regorafenib is an oral medication that is taken at home by patients, with poorer clinical outcomes i.e., disease progression, decreased quality of life and premature death as a result. In addition, as an oral agent, regorafenib presumably offers patients less access to and supervision by their healthcare providers compared with intravenous (IV) medications, since IV office visits are, by necessity, mandatory, and this also likely affects compliance especially when multi-morbidities and drug-drug interactions are present [23].

RRx-001 is an intravenous minimally toxic macrophage repolarizing agent in Phase 3 clinical trials that is associated with antitumor activity both alone and in combination with chemotherapy [24] and radiation as well as chemoprotection [25] and radioprotection [26]. However, the effect of RRx-001 on the activity and toxicity of tyrosine kinase inhibitors and regorafenib, in particular, has not been previously evaluated.

The results from these experiments demonstrate that 1) RRx-001 + regorafenib is more effective than either agent alone both *in vitro* and *in vivo* and that 2) the addition of RRx-001 to regorafenib attenuates the toxicity of regorafenib *in vivo*. RRx-001 is an IV medication that is dosed weekly, which may in itself promote better adherence to regorafenib, if it is administered concomitantly with RRx-001, which was not done in these experiments, since patients are likely to receive closer supervision and monitoring during clinic visits. The potential to positively impact survival and toxicity would be expected not only to result in better clinical outcomes such as longer survival and progression free survival, but also in better economic ones as well due to fewer visits to the emergency room and inpatient hospitalizations as well as less missed work days, which ultimately is more cost effective.

Conclusion

A clinical trial is planned to investigate the translational potential of the RRx-001 + regorafenib combination. Future experiments will determine whether RRx-001 also enhances the activity and decreases the toxicity of other tyrosine kinase inhibitors such as sorafenib, sunitinib, dasatinib, imatinib, lapatinib, and cabozantinib, all of which possess similar efficacy and safety profiles, not only in colorectal cancer but also other tumor types.

Disclosure of conflict of interest

None.

Address correspondence to: Bryan Oronsky, EpicentRx Inc., La Jolla, CA, USA. E-mail: boronsky@epicentrx.com

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