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Combination of Trabectedin With Irinotecan, Leucovorin and 5-Fluorouracil Arrests Primary Colorectal Cancer in an Imageable Patient-derived Orthotopic Xenograft Mouse Model

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Abstract. *Background/Aim: The aim of the present study was to determine the efficacy of trabectedin combined with FOLFIRI (irinotecan, leucovorin and 5-fluorouracil) on a colorectal cancer (CRC) patient-derived orthotopic xenograft (iPDOX) mouse model. Materials and Methods: A CRC tumor from a patient previously established in nude mice was implanted subcutaneously in transgenic green fluorescence protein (GFP)-expressing nude mice in order to label the tumor stromal cells with GFP. Mice were randomized into four groups: Group 1, untreated control; group 2, FOLFIRI; group 3, trabectedin alone; group 4, trabectedin plus FOLFIRI. Tumor width, length, and mouse body weight was measured twice every week. Results: All three treatment groups showed inhibited tumor growth compared to the untreated control group. Only the combination of FOLFIRI and trabectedin arrested tumor growth. No significant changes was observed in body weight in any group. Conclusion: These findings suggest that the combination of trabectedin plus FOLFIRI has clinical potential for patients with CRC.*

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Key Words: Colorectal cancer, trabectedin, irinotecan, leucovorin, 5-fluorouracil, FOLFIRI, PDOX, combination therapy.

In order to improve cancer therapy, we have developed the patient-derived orthotopic xenograft (PDOX) nude mouse model for many cancer types (1-10). The PDOX nude mouse model is advantageous compared to subcutaneous implantation patient-derived xenograft models in various aspects, in particular metastasis (10). We also developed the colorectal cancer (CRC) PDOX model to improve treatment for this disease (1-17).

Trabectedin has been used to treat many cancer types (18, 19). Trabectedin is highly active against many tumors resistant to alkylating agents (20). However, there are few studies on trabectedin in CRC. Izbicka *et al.* (19) and Twelves *et al.* (21) showed that patients with CRC could benefit from trabectedin. In a PDOX model of colon cancer, we recently showed that the combination of oxaliplaninum and 5-fluorouracil with trabectedin was highly effective (22). In PDOX models of sarcoma and pancreatic cancer, we have shown trabectedin to be an efficacious drug (23-28).

In this present study, we investigated the effectiveness of trabectedin on a CRC imageable PDOX mouse model in combination with irinotecan, leucovorin and 5-fluorouracil (FOLFIRI).

Materials and Methods

Mice. Non-transgenic and transgenic green fluorescence protein (GFP)-expressing athymic nude nu/nu mice (4- to 6-week-old) were obtained from AntiCancer Inc. (San Diego, CA, USA). All mice were fed under high efficiency particulate arrestance (HEPA)-filtered racks under standard conditions of 12 h light/dark cycles. All animal experiments were carried out in accordance with AntiCancer Inc. Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study, and in accordance with the principles and procedures outlined in the

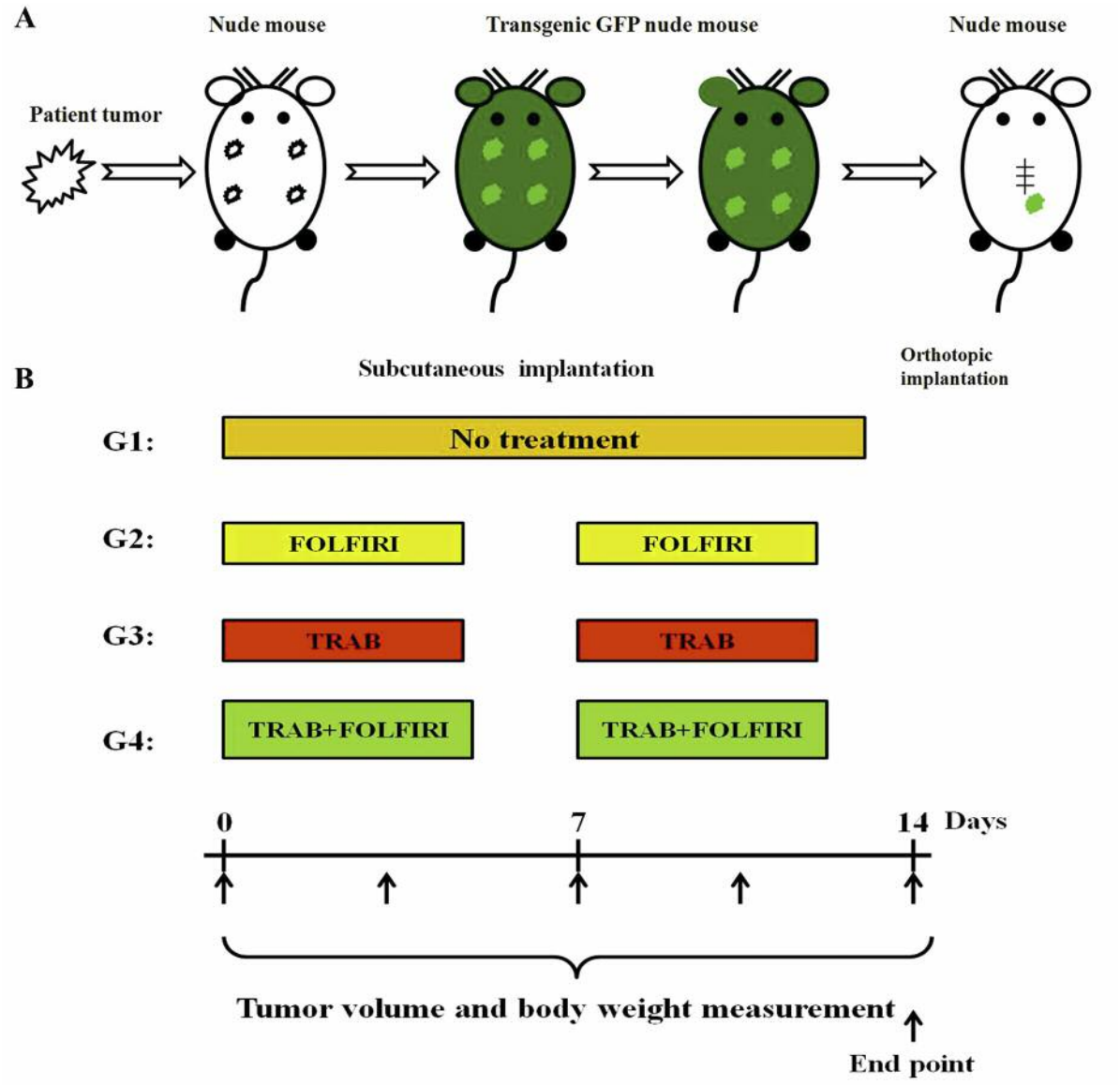

Figure 1. *Establishment of an imageable patient-derived orthotopic xenograft (iPDOX) model and drug treatment schema. A: Schematic illustration of the surgical orthotopic implantation for establishment of iPDOX models of human colorectal cancer. B: Treatment regime and quantitative drug efficacy. G1: Untreated control; G2: treated with FOLFIRI (24 mg/kg irinotecan i.p., 90 mg/kg leucovorin i.p., 50 mg/kg 5-fluorouracil i.p., weekly for 2 weeks); G3: 0.15 mg/kg trabectedin i.v., weekly for 2 weeks; G4: trabectedin plus FOLFIRI at the above doses, weekly for 2 weeks. n=7 mice/per treatment group. All treated mice were sacrificed on the end-point, and tumors were resected for histological analysis. GFP: Green fluorescent protein.*

National Institutes of Health Guidelines for care and Use of Animals under Assurance Number A3873-1 (8). Mouse housing, feeding, and surgical procedures were performed as previously described (29, 30). Mice were humanely sacrificed.

Patient-derived tumor. The primary tumor was previously obtained from a patient with CRC at the Division of Surgical Oncology, University of California, San Diego, USA (16, 31). The patient did not receive any chemotherapy or radiotherapy before surgery. Fresh tumor tissues were obtained from patient surgery with informed patient consent and Institutional Review Board approval. Using the surgical orthotopic implantation technique, the CRC imageable PDOX mouse model (iPDOX) was established, as previously reported (32).

Establishment of the CRC iPDOX model. CRC tissues were cut into 5 mm^3 fragments and seeded subcutaneously in nude mice. The CRC tumors grown in nude mice were harvested, cut into 5 mm^3 fragments, and implanted subcutaneously in GFP-expressing nude mice. After two passages in GFP-expressing nude mice, CRC tumors stably containing GFP-expressing stromal cells, were harvested and cut into 5 mm^3 fragments. After non-GFP-expressing nude mice were anesthetized (20 mg/kg ketamine, 15.2 mg/kg xylazine, and

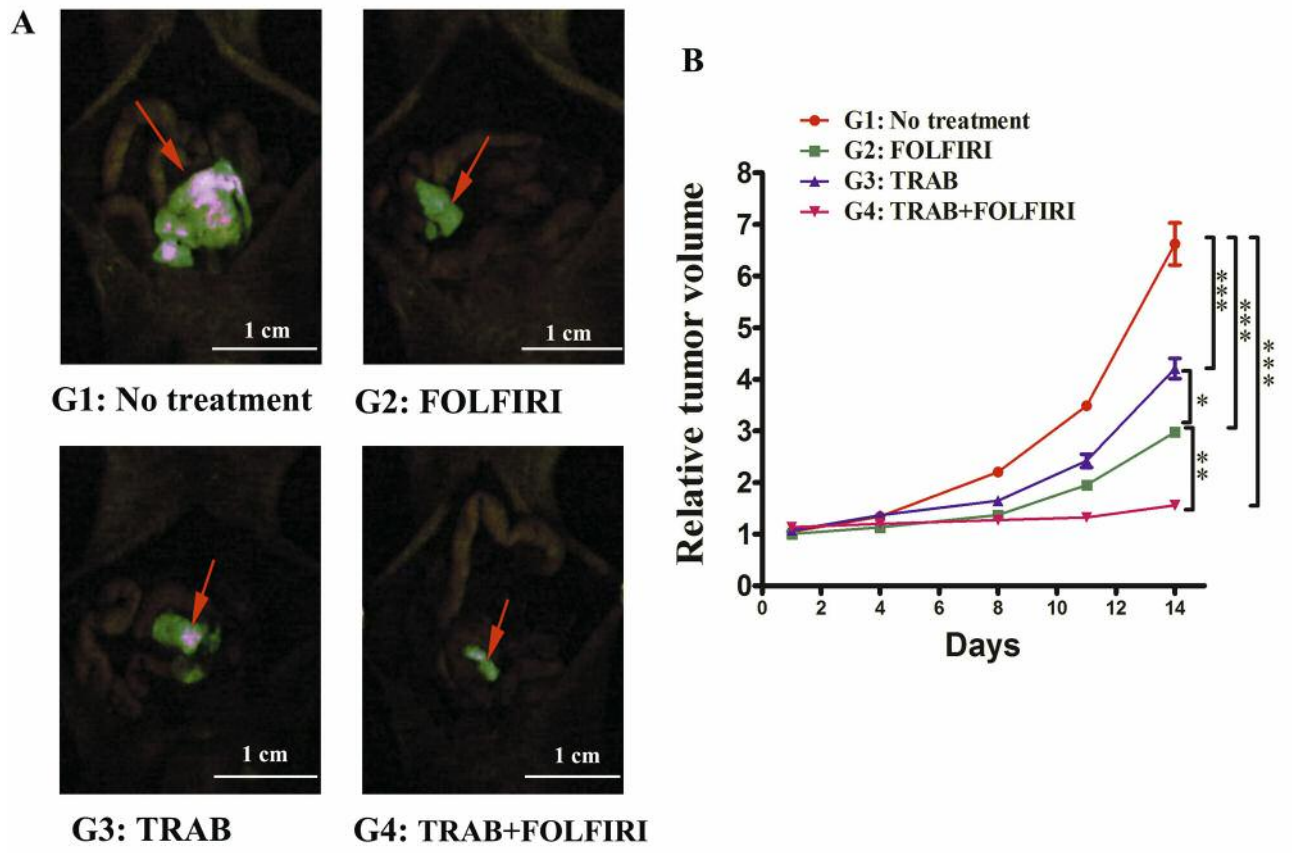

Figure 2. *Imaging of patient-derived orthotopic xenograft (PDOX) tumors treated with each drug, and quantitative efficacy of chemotherapy. A: Images of representative iPDOX mouse models from each treatment group at the end-point. Arrows indicate green fluorescent protein (GFP)-expressing tumors. The FluorVivo imaging System was used. B: Line graphs indicate relative tumor volume (ratio of tumor volume at each time point to volume at initiation of treatment) for each drug and control group. n=7 mice/group. Significantly different at *p<0.05, **p<0.01, ***p<0.001. TRAB: Trabectedin.*

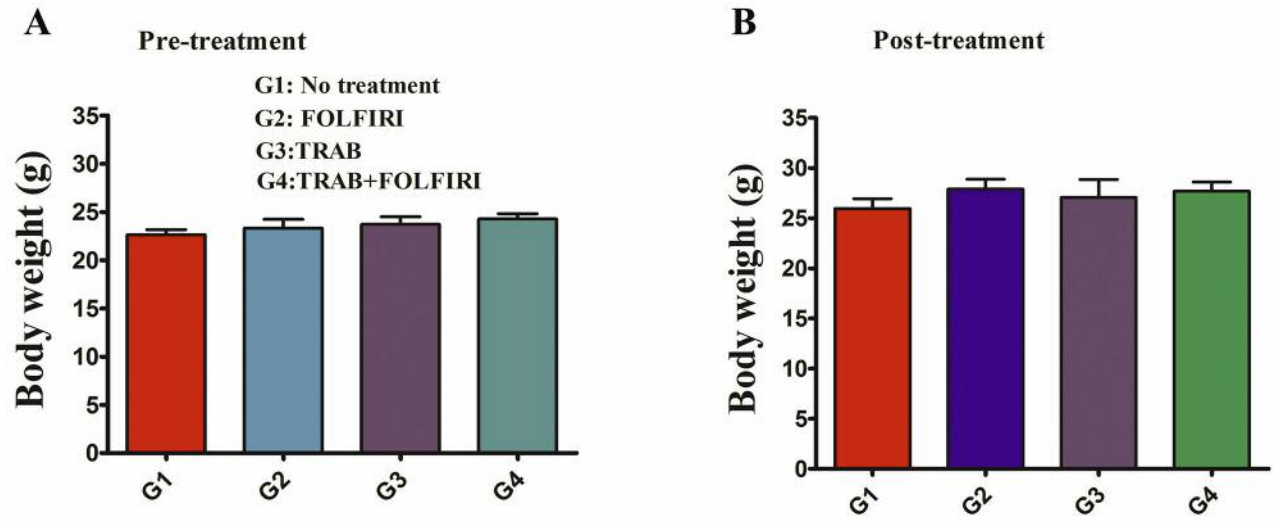

Figure 3. *Effect of treatment on mouse body weight. Bar graphs show body weight for each group at pre-treatment (A) and 2-weeks post-treatment (B). There were no significant differences between any group. TRAB: Trabectedin.*

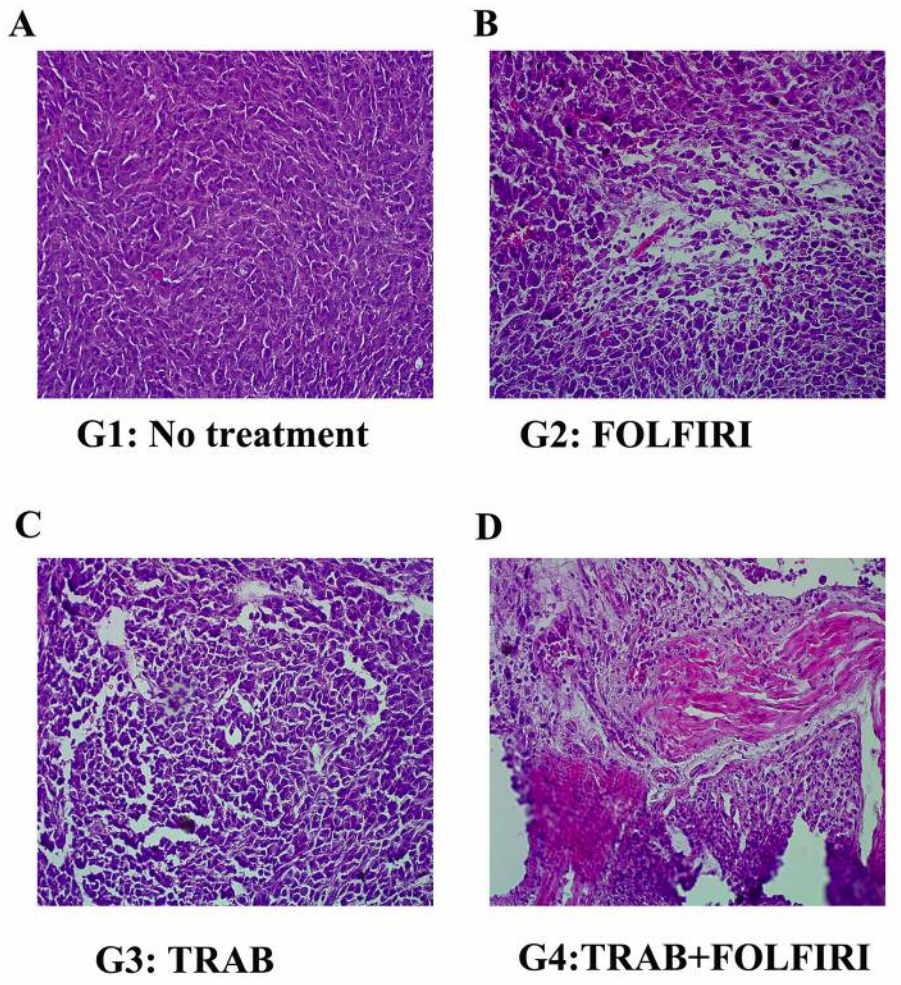

Figure 4. *Histology of imageable colorectal cancer a patient-derived orthotopic xenograft (iPDOX) mouse model in treated and untreated tumors. Hematoxylin and eosin-stained sections. Microscope magnification is 200×. TRAB: Trabectedin.*

0.48 mg/kg acepromazine maleate), an approximately 1 cm skin incision was made at the middle of the abdomen. The 5 mm^3 tumor tissue was sutured on the cecum. The incision was closed using 6-0 nylon sutures as previously described (11). The schematic diagram for establishing the CRC iPDOX model is shown in Figure 1A.

Treatment study design in the CRC iPDOX model. Six weeks after orthotopic implantation of CRC-GFP tumors, the abdomen of the PDOX mice was opened to assess tumor growth. The iPDOX mice were randomized into four groups (7 mice/per treatment group): Group 1: Control group (no treatment); group 2: FOLFIRI: 24 mg/kg irinotecan *i.p.*, 90 mg/kg leucovorin *i.p.*, 50 mg/kg 5-fluorouracil *i.p.*, weekly for 2 weeks; group 3, 0.15 mg/kg trabectedin *i.v.*, weekly for 2 weeks; group 4: trabectedin plus FOLFIRI at the above doses, weekly for 2 weeks) (Figure 1B). Tumor length, width and mouse body weight were measured twice per week. Tumor volume was calculated with the following formula: Tumor volume (mm^3)=tumor length (mm) × tumor width (mm) × width (mm)/2. Data are presented as the mean±standard deviation (SD). iPDOX mice treated with each drug were imaged with the FluorVivo imaging system (INDEC, Bio System, Santa Cruz, CA, USA) (33).

Histological analysis. Before sectioning and staining, fresh tumor samples were fixed in 10% formalin and embedded in paraffin. Tumor tissue sections of 4-μm were made and deparaffinized in xylene and rehydrated in an ethanol series. Hematoxylin and eosin (H&E) staining was performed according to a standard protocol. After staining, specimens were observed under a BHS system microscope and images were acquired with INFINITY ANALYZE software (Lumenera Corporation, Ottawa, Canada) (34).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) with Tukey's *post hoc* test was used when more than two groups were compared. The paired *t*-test was used for the parametric test to compare the means between two related groups. The data are expressed as the mean±SD. A *p*-value of less than or equal to 0.05 is considered statistically significant.

Results

Efficacy of tested drugs on CRC-iPDOX. To test the efficacy of FOLFIRI and trabectedin, alone and in combination on the CRC iPDOX mouse model, 6 weeks following orthotopic implantation, mice with tumors were randomized into four groups for treatment (Figure 1B). The tumor volumes at the endpoint of the experiment were: Untreated control: 662.2±90.8 mm^3; FOLFIRI: 297.6±11.5 mm^3; trabectedin: 421±43.9 mm^3; FOLFIRI plus trabectedin: 156±16.8 mm^3 (Figure 2A). The control group tumors grew more than five times larger compared to their size at initiation of treatment (tumor-volume ratio=6.32±1.87). In the FOLFIRI-treated group, at the end point, there was significant inhibition in tumor growth compared to the control group (tumor-volume ratio=2.96±1.36, $p<0.001$). In the trabectedin-treated group, at the end point, there was significant inhibition in tumor growth compared to the control group (tumor-volume ratio=3.93±4.58, $p<0.001$). The combination of FOLFIRI and trabectedin arrested tumor growth (tumor-volume ratio=1.37±1.4, $p<0.001$) compared to the control (Figure 2B). The combination resulted in a greater inhibition of tumor growth than FOLFIRI and trabectedin alone (Figure 2B).

Effect of treatment on body weight. To determine whether the drug treatment had any effect on body weight, we measured the mouse body weight pre- and post-treatment. There was no significant difference observed in body weight in all four groups (Figure 3). In the group treated with trabectedin alone, the tail of nude mice became necrotic because of the intravenous injection of trabectidin. In both groups treated with FOLFIRI, several nude mice developed symptoms of diarrhea. There were no other observable side-effects or animal deaths in any group.

Histology of CRC iPDOX. To better understand the relationship of histological findings with drugs tested, we compared the tumor histology of treatment groups with the control group. Representative histological images of tumors with H&E staining from each group are shown in Figure 4. Tumors in the untreated control group mainly comprised viable cancer cells and tumor tissue structure was dense (Figure 4A). In the tumors treated with drugs, cancer-cell density was lower than that of the control group (Figure 4B-D). The cancer-cell density was lower in the group treated with FOLFIRI alone than trabectedin alone (Figure 4B and C). The strongest efficacy was observed when CRC iPDOX tumors were treated with the combination of trabectedin and FOLFIRI; cancer cell density was the lowest among all four groups (Figure 4D).

Discussion

The FOLFIRI regimen has increased the effectiveness of treatment of advanced CRC in the clinic (35). In European countries, the FOLFIRI regimen is even used as first-line chemotherapy (36). However, the overall and progression-free survival of patients with CRC has not greatly improved with FOLFIRI therapy (35). In order to develop an effective curative strategy, we assessed the combination of trabectedin and FOLFIRI using a CRC iPDOX mouse model. In the present study, we found that the combination of trabectedin and FOLFIRI was more effective compared to either alone. Trabectedin was originally extracted from a marine tunicate, *Ecteinascidia turbinate,* and has complex mechanisms of action. Trabectedin binds to the minor groove of double-stranded DNA, resulting in double-strand breaks (37, 38). It also affects the cell cycle, causing death of cancer cells, and down-regulates transcription factors related to cell proliferation (38). Trabectedin interacts directly with components involved in nucleotide excision repair, thus inhibiting repair of specific substrates and forms a cell death complex (39). Trabectedin has been used to treat solid tumors, such as ovary, breast, prostate, renal cancer and lung cancer (18, 19). It is highly active against many tumors resistant to alkylating agents (20). Trabectedin was shown to re-sensitize resistant cells in some tumors (40). In sarcoma (41-44), recurrent ovarian cancer (45), metastatic breast cancer (46), juvenile myelomonocytic leukemia, and chronic myelomonocytic leukemia (47), trabectedin has also been shown as efficacious. Trabectedin was effective in PDOX mouse models of sarcoma and pancreatic cancer (23-28) and colon cancer (22).

Although, several studies have revealed that trabectedin may be effective against CRC (19, 21, 48), no studies have examined the efficacy of the combination of trabectedin and FOLFIRI on CRC. In the present study, we showed that the combination of trabectedin and FOLFIRI was highly effective against a PDOX mouse model of CRC, suggesting the use of this combination in the clinic. Our experimental results suggest the improved clinical prospect of patients with CRC and also show the importance of PDOX models for individualized therapy. The sensitivity of the iPDOX model was increased by initial growth of the tumor in transgenic nude mice expressing a fluorescent protein (49-51). It should be emphasized that orthotopic mouse models of cancer make drug-sensitivity studies clinically relevant (52-54). Our Experimental results suggest that trabectedin may be applied in neoadjuvant chemotherapy for advanced CRC.

Conflicts of Interest

GZ, YS and RMH are unpaid affiliates of AntiCancer Inc. MZ, YT and QH are employees of AntiCancer Inc. AntiCancer Inc uses PDOX models for contract research. The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

GZ and JY designed the study and wrote the draft article; GZ performed the experiments; MZ, QH, YS, and YT assisted in the experiments; GZ, JY, MZ, QH, YS, YT, MB, BC and RMH analyzed the data. SRS and RMH revised the article. All Authors approved the final article.

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