A Novel Cytidine Analog, RX-3117, Shows Potent Efficacy in Xenograft Models, even in Tumors that Are Resistant to Gemcitabine

MI YOUNG YANG[1], YOUNG BOK LEE[1], CHANG-HO AHN[1], JOEL KAYE[2], TANIA FINE[2], RINA KASHI[2], OSNAT OHNE[2], KEES SMID[3], GODEFRIDUS J. PETERS[3] and DEOG JOONG KIM[1]

[1]*Rexahn Pharmaceuticals, Rockville, MD, U.S.A.;*
[2]*Teva Pharmaceuticals Industries LTD., Petach Tikva, Israel;*
[3]*Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands*

Abstract. *RX-3117 (fluorocyclopentenylcytosine) is a cytidine analog and this class of drugs, including gemcitabine, has been widely used for the treatment of various types of cancers. However, there is no oral formulation of gemcitabine and drug resistance to gemcitabine is common. In this study, the efficacy of orally-administered RX-3117 was examined in 9 different human tumor xenograft models (colon, non-small cell lung, small cell lung, pancreatic, renal and cervical), grown subcutaneously in athymic nude mice. In the Colo 205, H460, H69 and CaSki models, gemcitabine treatment resulted in 28% , 30%, 25% and 0% tumor growth inhibition (TGI), respectively, whereas oral treatment with RX-3117 induced 100%, 78%, 62% and 66% TGI, respectively. This indicates that RX-3117 may have the potential to be used for the treatment of tumors that do not respond to gemcitabine. RX-3117 was also evaluated in a single primary low-passage human pancreatic Tumorgraft™ CTG-0298 (TGI 76%), which is relatively resistant to gemcitabine (TGI 38%) and has a favorable RX-3117-activating enzyme profile. These studies demonstrated the therapeutic potential and anticancer efficacy of RX-3117.*

One class of drugs are cytotoxic anti-metabolites, which are widely used for the treatment of various types of cancer, both hematological and solid tumors (1). This class of compounds mimic physiological nucleosides in regard to uptake and metabolism and are incorporated into newly-synthesized RNA and DNA resulting in synthesis inhibition and chain termination. Within this class, the nucleoside analog gemcitabine (2'deoxy-2'2'-difluorocytidine monohydrochloride) is one of the most successful cytidine analogs used for the treatment of non-small cell lung cancer and pancreatic cancer (2). Gemcitabine, as a single drug, is the standard care for pancreatic cancer but in the other diseases, including lung and bladder, is used in combination with the chemotherapeutic cisplatin (3, 4). Gemcitabine is phosphorylated to its mononucleotide by deoxycytidine kinase (dCK) and subsequently by nucleotide kinases to its active metabolites, gemcitabine diphosphate and gemcitabine triphosphate. Gemcitabine triphosphate is then incorporated into RNA and DNA (5) with the latter resulting in masked chain termination (6). Although gemcitabine has been successfully used in cancer treatment, there are several disadvantages that require improved therapeutics. First, the investigated oral forms of gemcitabine (either gemcitabine itself or as a prodrug) were not successful (1, 7, 8) and, therefore, they are administered by the intravenous (*i.v.*) route. Second, drug resistance is common: it is hypothesized to be due to a loss of transporters or low expression of dCK responsible for the first phosphorylation step (9).

As a result of the attempts to find a therapeutic drug with a better pharmacological profile, RX-3117 was synthesized

Abbreviations: dCK, deoxycytidine kinase; UCK, uridine-cytidine kinase; hENT1, human equilibrative nucleoside transporter; RR, ribonucleotide reductase; DNMT, DNA methyltransferase; CDA, cytidine deaminase; TGI, tumor growth inhibition; SNP, single-nucleotide polymorphism; RX-MP, RX-3117 monophosphate; RX-DP, RX-3117 diphosphate; RX-TP, RX-3117 triphosphate; *s.c.*, subcutaneous; *i.v.*, intravenous; *p.o.*, per os; *i.p.*, intraperitoneal; t.i.w., three times a week; q.o.d., every other day; q3d, every three days; 5on/2off, once daily dosing for five days separated by two days of no dosing.

Correspondence to: Deog Joong Kim, Rexahn Pharmaceuticals, 15245 Shady Grove Rd. Suite 455, Rockville, MD, U.S.A. Tel: +1 3012685300 (ext. 306), Fax: +1 3012685310, e-mail: kimdj@rexahn.com

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and characterized (10, 11). Similar to other anti-metabolites, RX-3117 interferes with cell division and nucleic acid synthesis, causes cellular arrest in the G_1 phase and induces apoptosis. Cellular uptake of RX-3117 is mediated by the human equilibrative nucleoside transporter (hENT1). In order to be phosphorylated, RX-3117 requires activation by uridine-cytidine kinase (UCK) to its active phosphates. RX-3117 diphosphate would be reduced by ribonucleotide reductase (RR) to deoxyRX-3117 diphosphate, which can be converted to deoxyRX-3117 triphosphate and incorporated into DNA. DNA methyltransferase (DNMT) was inhibited, which was assumed to be either by the incorporation of RX-3117 into DNA by formation of a complex with the enzyme or directly by deoxyRX-3117 triphosphate (Figure 1). The inhibition of DNA synthesis by the active form of RX-3117 was more pronounced than that of RNA (11).

In the present study, we examined the efficacy of RX-3117 in several cancer xenograft models, including gemcitabine-resistant ones, since a previous study displayed the possibility of potent efficacy of RX-3117 (10). Not only has RX-3117 demonstrated potent efficacy in several cancer xenograft models but oral treatment with RX-3117 results in dose-dependent tumor growth inhibition (TGI), even in tumors that are only moderately sensitive or resistant to gemcitabine.

Materials and Methods

Materials. [6-^{3}H]-RX-3117 ([Cytosine-6-^{3}H]RX-3117): specific activity 90.5 mCi/mmol) was synthesized by Aptuit, Kansas city, MO, USA and provided by TEVA Pharmaceuticals, Petach Tikva, Israel. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Mice. Female nude mice (*nu/nu*, Harlan) were 9-10 weeks old and had a body weight (BW) range of 17.8-25.8 g on day 1 of the study. The mice were housed on irradiated Enrich-o'Cobs™ Laboratory Animal Bedding in static microisolators on a 12-hour light cycle at 21-22 ˚C (70-72˚F) and 40-60% humidity (Charles River Discovery Research Services Seattle, WA, USA). At Champions Oncology (Hackensack, NJ, USA), the mice between 4-6 weeks of age were housed on irradiated paper twist-enriched 1/8" corncob bedding (Sheperd, Milford, NJ, USA) in individual HEPA ventilated cages (Innocage® IVC, Innovive, San Diego, CA, USA) on a 12-hour light-dark cycle at 20-23˚C (68-74˚F) and 30-70% humidity. Both companies specifically comply with the recommendations of the *Guide for Care and Use of Laboratory Animals* with respect to restraint, husbandry, surgical procedures, feed and fluid regulation and veterinary care. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, which assures compliance with accepted standards for the care and use of laboratory animals.

Tumor cell culture. HCT116, HT29, H460, H69, Caki-1, CaSki, MiaPaca2, BxPC3 and Colo 205 cells (ATCC, Manassas, VA, USA) were cultured according to ATCC's instruction. The tumor cells were cultured in tissue culture flasks in a humidified incubator at 37˚C, in an atmosphere of 5% CO_2 and 95% air.

Primary low passage human TumorGraft™. A piece of the patient's living tumor, which was removed during surgery or biopsy was implanted in mice by Champions Oncology. By implanting the tumor together with its microenvironment, TumorGrafts continue to very closely resemble the patient's tumor.

Cancer cells implantation and measurement. The cells were harvested during exponential growth and re-suspended with phosphate buffered saline. Each test animal received a subcutaneous (*s.c.*) injection of 5×10^6 tumor cells into the right flank and tumor growth was monitored as the average tumor size approached the target range of 80-120 mm^3. When tumors reached the target size, mice were randomized into several groups (n=10) and treatment with various regimens of RX-3117 or gemcitabine was initiated. Tumors and body weights were measured regularly until the study was terminated. Tumors were measured with a caliper and tumor volumes calculated using the formula $L\times W^2\times0.5$ and presented as the mean±SEM. Response to treatment was evaluated for tumor growth inhibition (TGI%). TGI was defined as the formula: %TGI=[(Ct – C0) – (Tt – T0)]/(Ct – C0) ×100, where Tt=median tumor volume of treated at time t, T0=median tumor volume of treated at time 0, Ct=median tumor volume of control at time t and C0=median tumor volume of control at time 0.

Mice were treated with gemcitabine according to optimal schedules described earlier (12). Treatment with gemcitabine was given intraperitoneally (*i.p.*) according to the every three days (q3d) ×4 (occasionally at q3d ×10) schedule at doses varying from 80-120 mg/kg depending on the mice and the tumor. Paclitaxel was given intravenous (*i.v.*) at 30 mg/kg every other day (q.o.d.) ×5. For RX-3117, a number of schedules, both *i.p.* and per os (*p.o.*) were tested in order to optimize dosing, scheduling and route of administration.

Activity of uridine-cytidine kinase (UCK). Measurement of the activity of UCK was performed as described earlier (11). Briefly, tumors were snap frozen in liquid nitrogen and subsequently stored at –80˚C. The frozen tumors were dissociated with a micro-dismembrator and taken up in assay buffer (13). The reaction volume consisted of 70 μl, while the reaction was started by the addition of the substrate mix consisting of ATP (final concentration, 5 mM), 10 mM $MgCl_2$ and the nucleoside. The activity of UCK was assayed at 10 and 100 μM [6-^{3}H]-RX-3117 (90.5 mCi/mmol) (11). For tumors, we assessed linearity in time and protein. The assay was terminated by heating for 5 min at 95˚C; denatured proteins and DNA were removed by centrifugation and the supernatant was analyzed by spotting 5 μl of the reaction mixture on polyethyleneimine-coated plates. Separation was achieved by simple water elution, as described earlier (13). Spots were visualized by adding extra non-labeled substrate to the reaction vial after termination of the assay. The formed nucleotide remained on the origin; the nucleoside had an R_f value of about 0.9. Spots were cut out and, after elution of the radioactivity from the plate, scintillation fluid was added and radioactivity was measured.

Results

Orally-administered RX-3117 showed potent efficacy across a broad variety of types of tumors in animal models. RX-3117 was evaluated in various xenografts and tested at various schedules and doses. HCT116 human colorectal tumors were

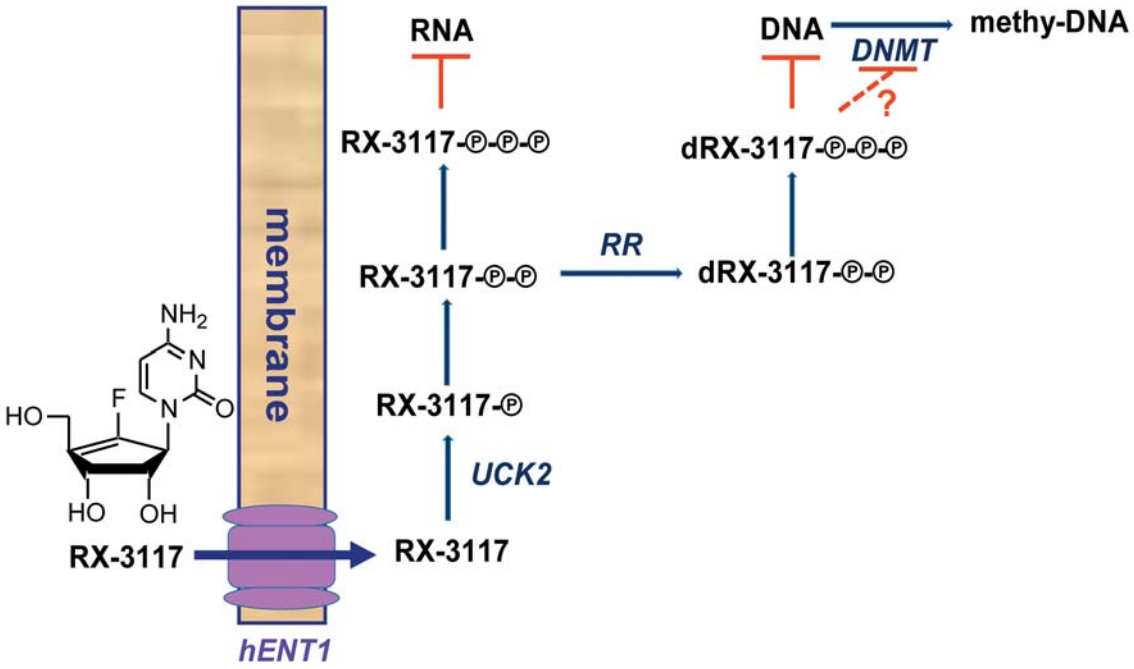

Figure 1. *Putative mechanism of action of RX-3117. After its uptake mediated by hENT1, RX-3117 is phosphorylated by UCK2 to its active form, which can be incorporated into RNA. Ribonucleotide reductase (RR) can reduce RX-3117 diphosphate to deoxyRX-3117 diphosphate, which can be converted to its triphosphate and incorporated into DNA. DNA methyltransferase (DNMT) is inhibited either by the incorporation of RX-3117 into DNA by formation of a complex with the enzyme or directly by the triphosphate.*

highly responsive to the positive control, paclitaxel, with tumor regression. The efficacy of RX-3117 at *i.v.* and oral treatment was comparable and were both highly effective in HCT116 tumors (Figure 2A). RX-3117 was also tested at different oral schedules, such as intermittent, at which a similar efficacy was observed (Figure 2A). Mean body weight loss was negligible. The efficacy of RX-3117 was examined in HT29 human colon cancer, Caki-1 human renal cancer, MiaPaca2 and BxPC3 human pancreatic cancer xenograft models. In HT29 xenograft both gemcitabine and RX-3117 showed a strong antitumor effect (Figure 2B). However, gemcitabine treatment resulted in more weight loss in contrast to RX-3117, indicating a better therapeutic index of RX-3117 (Figure 2C). In the Caki-1 renal cancer xenograft, RX-3117 showed its anti-tumor effect in a dose-dependent manner, resulting in 31%, 81% and 87% TGI in 150 mg/kg, 300 mg/kg and 500 mg/kg, respectively, whereas gemcitabine showed 61% TGI at the 120 mg dose (Figure 2D). Interestingly, RX-3117 showed intermittent efficacy in MiaPaca2 pancreatic cancer and was ineffective in another pancreatic tumor BxPC3, in which gemcitabine was ineffective as well (Figure 2E and F). All TGIs of all xenografts of all treatments are summarized in Table I. All xenograft models in which the anti-tumor effect of RX-3117 was examined did not show any significant body weight changes.

Oral treatment of RX-3117 showed anti-tumor effects even in gemcitabine-insensitive tumor models. Since RX-3117 demonstrated efficacy in various xenograft models, the anti-tumor effect of RX-3117 was examined in several models, which are experimentally known to be insensitive to gemcitabine. In two lung carcinoma xenografts models, H460 non-small cell lung carcinoma (NSCLC) and H69 small cell lung carcinoma (SCLC), in which gemcitabine had intermittent efficacy, RX-3117 showed a potent antitumor effect (Figure 3A and B). In the CaSki human cervical cancer and Colo 205 human colorectal cancer xenografts gemcitabine was completely ineffective but RX-3117 had a potent antitumor effect (Figure 3C and D). The anti-tumor activities of RX-3117 and gemcitabine in gemcitabine-insensitive tumor models are compared in Figure 3E.

Correlation between UCK enzyme activity and the efficacy of RX-3117 in several models. Although RX-3117 showed potent anti-tumor effects in a variety of tumor types, the BxPC3 pancreatic cancer xenograft displayed a poor response to RX-3117. Since pre-clinical studies in cell lines demonstrated that RX-3117 is activated by phosphorylation by the enzyme UCK (11), we measured the UCK activity in Colo 205, H460 and BxPC3. The Colo 205 and H460 models, which had excellent responses to RX-3117, also showed high UCK enzyme activity,

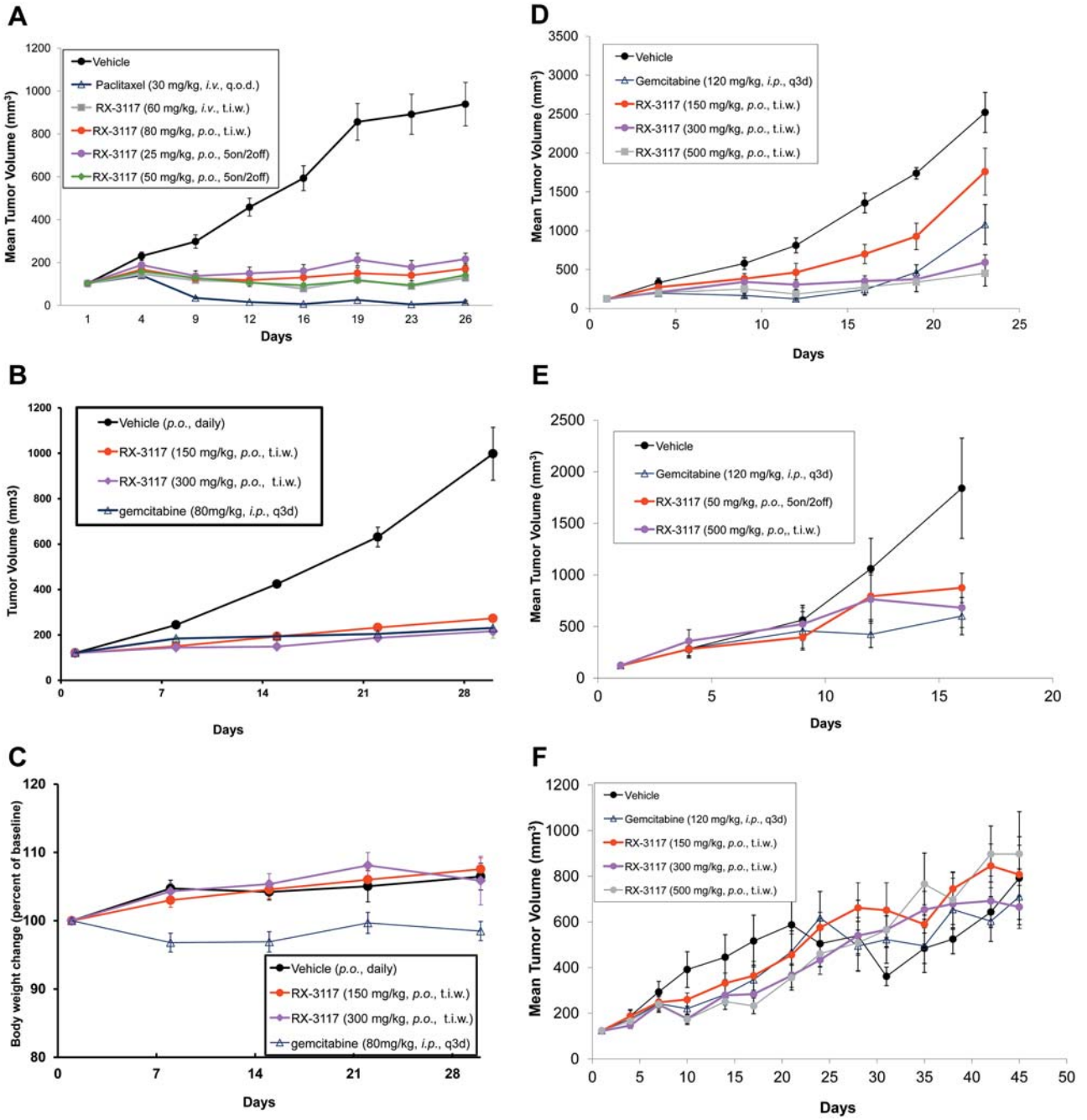

Figure 2. *Anti-tumor effects of RX-3117 in various xenograft models. The cancer cell lines were implanted subcutaneously into female immunodeficient nude mice. When the tumors reached target sizes, mice were sorted into several groups (n=10) on day 1 of the study and treated with various regimens of RX-3117 or gemcitabine. Tumors and body weights were measured regularly until the study was terminated. Error bars indicate SEM. HCT116 (A), HT29 (B), Caki-1 (D), MiaPaca2 (E) and BxPC3 (F) tumor growth was compared in various treatments of RX-3117, including vehicle control. In HT29 (C) tumor growth model, body weight was compared to each group.*

whereas the BxPC-3 model with little efficacy after treatment with RX-3117 displayed low UCK activity, possibly showing a correlation between the efficacy of RX-3117 and UCK levels in xenograft models (Figure 4).

Anti-tumor activity in primary tumor models builds further support for the potential efficacy and use of RX-3117. In order to extend the results established in cell line xenograft models and to test efficacy in a potentially more relevant

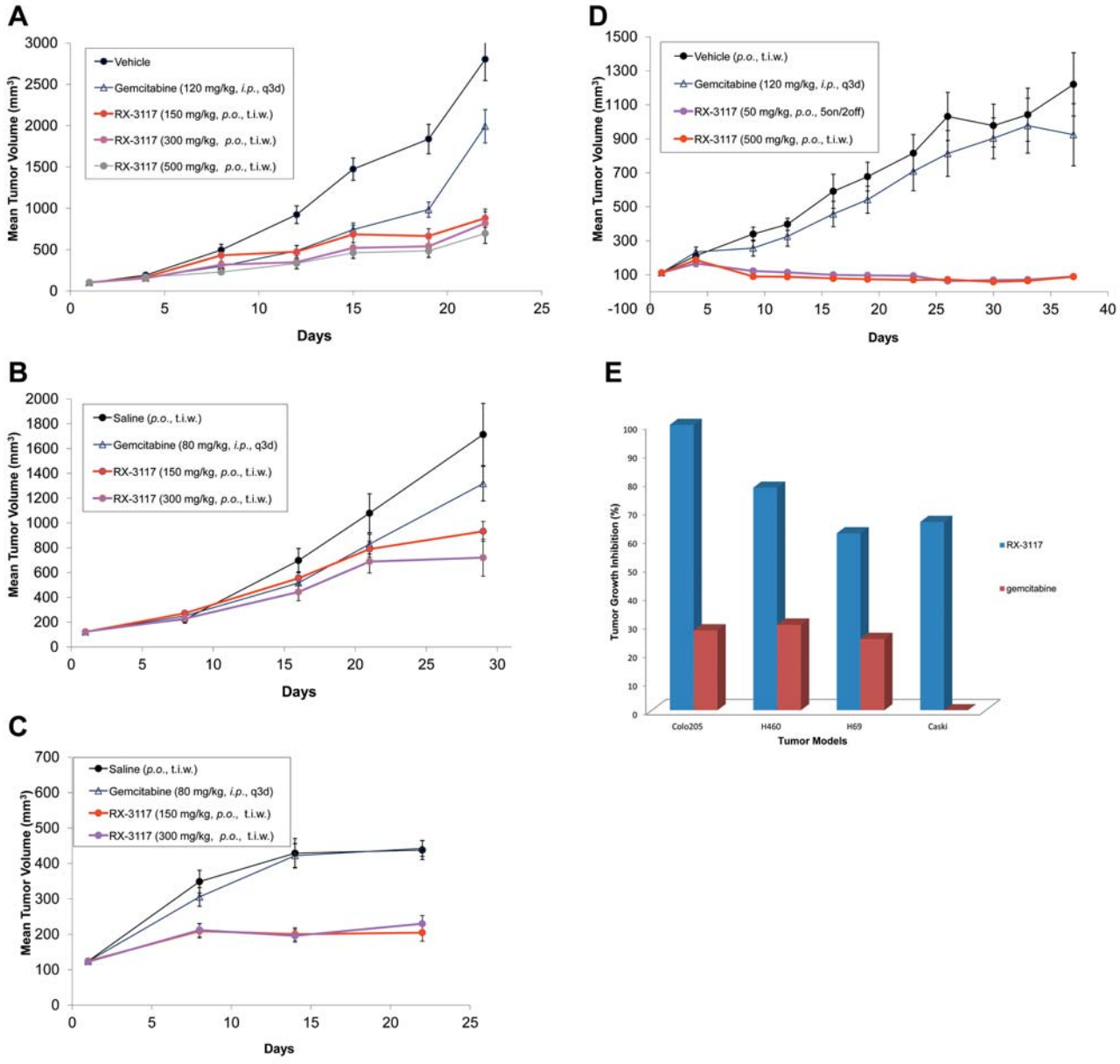

Figure 3. *Efficacy of RX-3117 in subcutaneous human xenograft models that are insensitive to gemcitabine. Cancer cells were inoculated subcutaneously into athymic nude mice. When the tumors reached 80-130 mm³, mice were sorted into several groups on day 1 of the study, when dosing was initiated. Tumor volume was monitored over time. Values represent mean±SEM. H460 (A), H69 (B), CaSki (C) and Colo 205 (D) tumor growth was compared for various treatments of RX-3117, including vehicle control and gemcitabine. (E) Tumor growth inhibition (TGI) was compared following treatment with RX-3117 and gemcitabine in gemcitabine-insensitive models.*

clinical system, RX-3117 was evaluated in a single primary low passage human pancreatic TumorGraft™. CTG-0298 was selected based upon resistance to gemcitabine, as well as high UCK activity (Figure 5A). Treatment with RX-3117 resulted in dose responsive tumor growth inhibition (TGI 76%) and was also shown to be superior to the standard-of-care agent gemcitabine (TGI 38%) (Figure 5B).

Discussion

This study demonstrated that oral administration of the novel cytidine analog RX-3117 is highly effective in various xenograft models. Most interestingly, RX-3117 was effective in Colo 205, H460, H69 and CaSki xenograft models, which are experimentally known to be insensitive to gemcitabine.

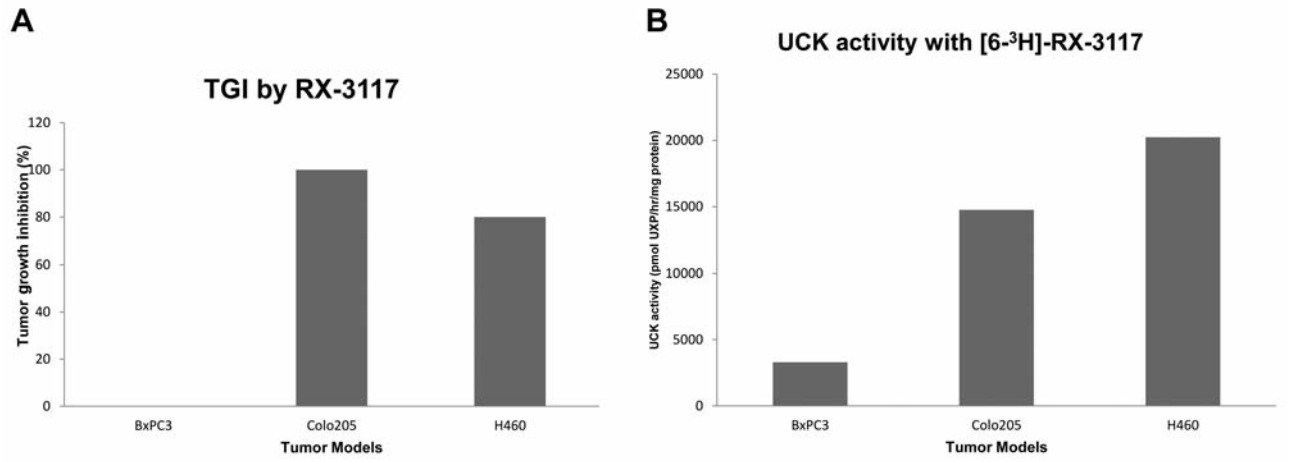

Figure 4. *Relationship between the UCK activity and the efficacy of RX-3117. (A) Tumor growth inhibitions (TGIs) by RX-3117 in BxPC3, Colo 205 and H460 xenograft models were compared. (B) UCK activity, using [6-^{3}H]-RX-3117, was measured in BxPC3, Colo 205 and H460 tumors as described in the Materials and Methods section.*

Table I. *Summary of anti-tumor activities of all treatments, and optimization of dosing and scheduling of RX-3117 (All treatments were well-tolerated without any significant body weight loss and there were no treatment-related deaths).*

Drug	Dose (mg/kg)	Schedule	Route	Xenograft (TGI*****%)								
				HCT116	HT29	Caki-1	MiaPaca2	BxPC3	H460	H69	CaSKi	Colo205
Gemcitabine	80	q3d*x4	*i.p.*		88%					25% (q3dx10)	0% (q3dx10)	
Gemcitabine	120	q3dx4	*i.p.*			61%	56%	0%	30%			27%
Paclitaxel	30	q.o.d**.x5	*i.v.*	regression								
RX-3117	60	t.i.w.***x3	*i.v.*	96%								
	80	t.i.w. x3	*p.o.*	91%								
	150	t.i.w. x3	*p.o.*		83%	31%		0%	71%	49%	74%	
	300	t.i.w. x3	*p.o.*		89%	81%		0%	78%	62%	66%	
	500	t.i.w. x3	*p.o.*			87%	68%	0%	78%			100%
	25	5on/2off****x3	*p.o.*	87%								
	50	5on/2off x3	*p.o.*	96%			67%					100%

*q3d means every three days; **q.o.d. means every other day; ***ti.w. means three times a week; ****5on/2off means once daily dosing for five days separated by two days of no dosing. *****The TGI was calculated at the last day of each tumor growth graph.

There are several mechanisms to explain gemcitabine resistance (2, 4, 9). First, a decrease in expression levels of hENT1 can cause resistance to gemcitabine. Since most of gemcitabine uptake is mediated by hENT1, a correlation between the sensitivity to nucleoside analogues has been reported, including gemcitabine and the expression of hENT1 both *in vitro* and in the clinical setting (14-18). hENT expression in these xenografts was relatively low, except in H69 tumors. Second, gemcitabine resistance can be explained by dCK deficiency (19-21). Gemcitabine is a prodrug that requires intracellular phosphorylation, which is mediated by dCK; a deficiency was correlated with gemcitabine resistance (22). The most frequently described form of acquired resistance to gemcitabine *in vitro* was dCK deficiency (2, 9, 20). In the clinic, the correlation between dCK expression and efficacy is not consistent with positive and negative associations in pancreatic adenocarcinoma (2, 23, 24). The role of the inactivation enzyme for gemcitabine, cytidine deaminase (CDA), does not seem to be important for intrinsic resistance to gemcitabine; only acquired resistance of gemcitabine may be related to high CDA expression (25-27). Another important determinant of gemcitabine's efficacy is the expression of RR, which was found to be associated with a poor response to gemcitabine in non-small cell lung cancer and with acquired resistance in several model systems (2, 28). In the model systems tested by us, H69 has a relatively high RR expression, explaining its insensitivity to gemcitabine despite having a relatively high dCK and hENT expression. RX-3117 is activated by UCK instead of dCK. It is not inactivated by CDA since it is a very poor substrate compared

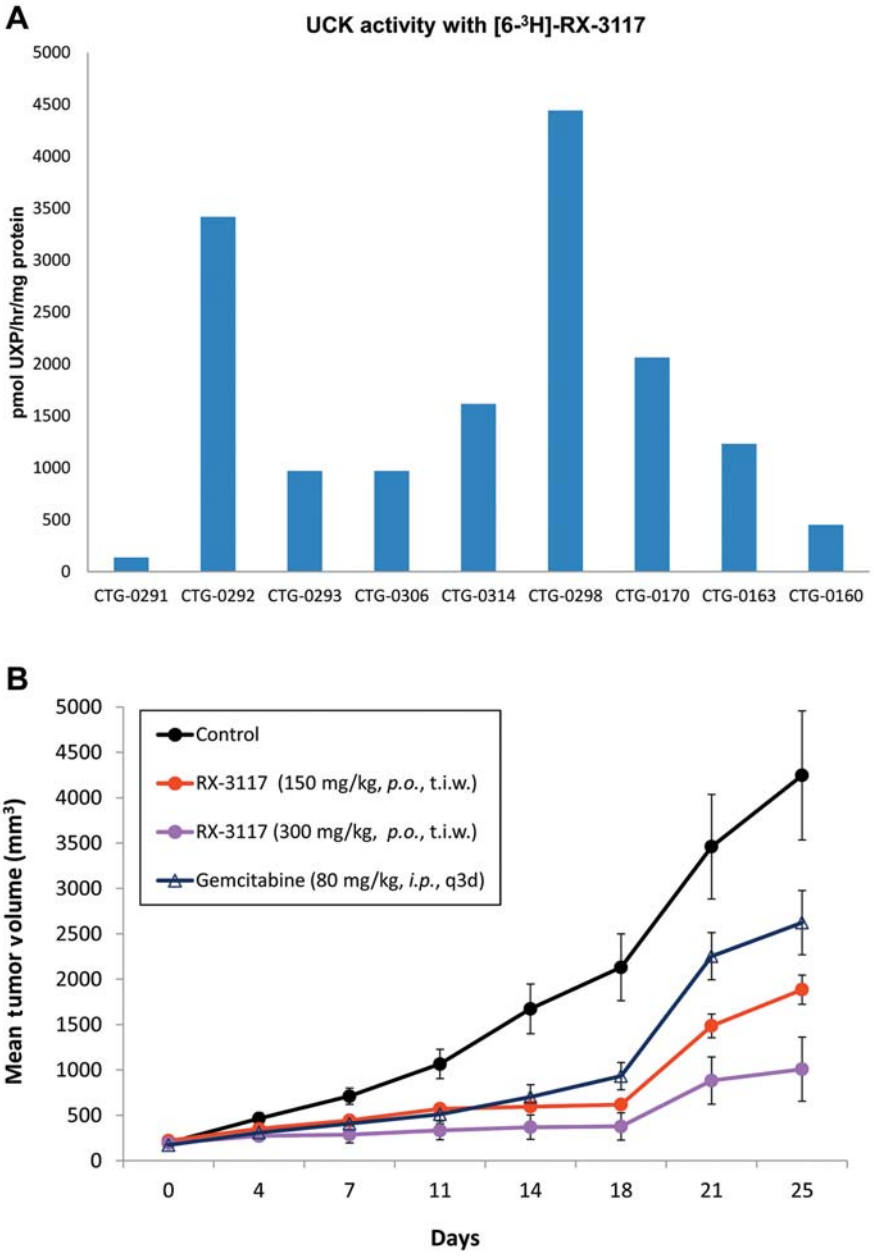

Figure 5. *Anti-tumor effect of RX-3117 in a primary low-passage human pancreatic TumorGraft™ , CTG-0298. (A) UCK activity, using [6-3H]-RX-3117, was measured in nine primary low-passage human pancreatic or NSCLC TumorGraft™ . (B) Tumor fragments from animals carrying the CTG-0298 model were implanted into nude mice and studies were initiated at a mean tumor volume of approximately 164 mm^3. When the tumors reached 80-130 mm^3, mice were sorted into 4 groups (n=9) and treated with vehicle (p.o., t.i.w ×3), gemcitabine (80 mg/kg, i.p., q3d ×4), RX-3117 (150 mg/kg, p.o., t.i.w ×3) or RX-3117 (300 mg/kg, p.o., t.i.w ×3). Values represent mean±SEM.*

to gemcitabine (11), providing a potential explanation why RX-3117 showed anti-tumor effects even in gemcitabine-insensitive xenograft models. In addition, RX-3117 seems to have a high UCK activity in tumors that are sensitive to the drug (Figure 4). BxPC3 is known to be resistant to gemcitabine due to a low activity of dCK (22).

By showing a dose-dependent response in primary human pancreatic TumorGraft™ CTG-0298, which has also a relatively high UCK activity, RX-3117 demonstrated its therapeutic potential in a clinical setting. However, each individual Champions TumorGraft™ is a model of an individual patient and evaluating a compound in a single model

may not yield results that are indicative of the whole target population. As such, this study may not be predictive for all pancreatic cancer patients. The antitumor activity seen in this study may be model-specific and is not broad enough to predict activity in pancreatic cancer patients in general. Therefore, more patient-derived xenograft models representing different tumor types should be tested for both gemcitabine resistance and favorable UCK activity. This will provide more insight into the potential clinical efficacy of RX-3117.

UCK is required for the intracellular activation of certain pyrimidine nucleoside analogues to cytotoxic nucleotides (29-32). There are two UCK family members, UCK1 and UCK2, sharing 68.8% identity at the amino acid level (33). Their distinct roles in activating pyrimidine nucleoside analogues and sensitivity of cancer cells to these analogues were investigated in several studies (29-31), which demonstrated that UCK2 expression level and activity, but not UCK1, are correlated with the sensitivity of cancer cells to cytidine analogs. Our unpublished data also show that *UCK2* mRNA levels were matched with UCK activity. In addition, the existence of single-nucleotide polymorphisms in the *UCK2* gene has been reported (34). Two SNPs were detected in exon 4 and three SNPs were found in the promoter region of *UCK2*. Further study of these SNPs is required to confirm the role of *UCK2* polymorphism in the expression patterns of *UCK2* mRNA. All these data suggest that UCK2 may be a useful marker to predict chemosensitivity to antitumor uridine and cytidine analogs, including RX-3117.

This study successfully established the potent anti-tumor effects of orally administered RX-3117 in several models while demonstrating the therapeutic potential of RX-3117 in gemcitabine-resistant cancer patients.

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Conflicts of Interest

The Authors have no conflicts of interest.

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