ORIGINAL ARTICLE

Combinatorial treatment with carboxyamidotriazole-orotate and temozolomide in sc-implanted human LOX IMVI melanoma xenografts

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Abstract

Background: Carboxyamidotriazole orotate (CTO) is the orotic acid salt of 5-amino-1(4-(4-chlorobenzoyl)-3, 5-dichlorobenzyl)-1, 2, 3-triazole-4-carboxamide (CAI). CTO possesses increased solubility as compared to CAI as the free base. The antiproliferative and antimetastatic effects of CTO are related to inhibition of receptor-operated, calcium-channel-mediated calcium influx. CTO can inhibit calcium-sensitive signal transduction in the VEGF and the PI3K pathways, inhibition of FGF-2-induced tyrosine kinase, VEGF-mediated activation of phospholipase Cy, generation of IP3 and nitric oxide synthase activation, and induction of apoptosis in imatinib mesylate resistant CML cells by downregulating bcr-abl.

Methods: Different combinations of CTO and temozolomide were first tested in female athymic NCr-*nu/nu* mice to evaluate tolerance of the combination. The tolerated combinations were then tested to evaluate the antitumor activity against subcutaneously implanted human LOX IMVI melanoma xenografts.

Results: Oral CTO at doses of 513 or 342 mg/kg/dose Q1D×14 resulted in inhibition of tumor growth ($p<0.001$ and $p=0.004$). Oral TEM at doses of 90 and 60 mg/kg/dose Q4D×3 resulted in dose-dependent inhibition of tumor growth ($p<0.001$ and $p<0.001$). Oral CTO at 513 or 342 mg/kg/dose in combination with temozolomide 90 mg/kg/dose resulted in comparable tumor inhibition to temozolomide alone. However, oral CTO at 513 mg/kg/dose in combination with temozolomide 60mg/kg/dose resulted in additive antitumor activity compared to each drug alone. Also, CTO at 342 mg/kg/dose in combination with temozolomide 60mg/kg/dose had more than additive antitumor activity and was statistically different from the group treated with temozolomide 60 mg/kg/dose alone ($p=0.001$).

Conclusions: These results suggest that this combination of previously configured therapeutic doses of temozolomide with CTO enhances the sensitivity of temozolomide and may permit use of lower temozolomide doses to obtain an optimum antitumor effect in combination therapy. This dose-dilution combinatorial strategy fulfills the need for increased tumor sensitivity and efficacy. Additionally, this strategy reduces drug resistance and toxicity associated with dose dense strategy of temozolomide treatment in this melanoma model.

Key words

Carboxyamidotriazole orotate, Melanoma, Calcium signal transduction, Anti-VEGF, Anti-PI3K

1 Introduction

Metastatic melanoma has very poor survival rates and treatment options have historically been limited. Most human melanomas feature a constitutively activated MAPK pathway through mutations in the NRAS or BRAF, a serine-threonine protein kinase, which is found in 50% to 60% of melanomas [1, 2]. Mutated V600E BRAF is constitutively active [3, 4]. High circulating levels of VEGF are associated with a poor prognosis in patients with melanoma [5]. Chemotherapy is still the standard of care [6], but response rates are low. The monoclonal antibody ipilimumab is active in patients with advanced melanoma and brain metastases [7], but is effective in only some patients [6-8]. The BRAF kinase inhibitors vemurafenib and dagrafenib (GSK2118436) are active in patients with $BRAF^{V600E}$ mutated melanoma and appear to have activity in the brain, although a small number of patients have been treated to date [9].

Maximum tolerated dose (MTD) chemotherapy given as single drug rarely cures cancer because initially responsive tumors rapidly acquire resistance after drug exposure. Dose dense strategies then aim to shrink the tumors but cause toxicities [10-11]. To minimize toxicities, metronomic regimens have been tested in which drug is administered at a lower dose than MTD but at more frequent intervals [12]. These regimens induce improved antitumor effects by a variety of mechanisms in the tumor microenvironment.

Therefore, the tumor microenvironment must be considered in the selection of cytotoxic drugs, such as determining what interferences exist or are induced in the microenvironment by chemotherapy and how these affect the tumor response to the cytotoxic drugs [13]. Presently, we tested a modification of the metronomic schedule using combinatorial varying doses of temozolomide and CTO to achieve the maximum antitumor response, and show that this response is achieved by a dose dilution strategy.

Melanoma exhibits characteristics that equip it to compensate in the face of various single-therapy approaches and curtail responses to treatment. After initial responses, inhibition of BRAF kinase [1] and VEGF [4] fail as melanoma develops resistance via treatment-induced compensatory signaling involving the MAPK or PI3K/Akt pathways [1], and rebound pERK activation and escape from BRAF inhibition [3]. Resistance to anti-VEGF therapy develops by activation of alternate proangiogenic pathways, likely in response to increased tumor tissue hypoxia. In preclinical studies, when angiogenesis is impaired, tumor mass shrinks initially, followed by enhanced tumor invasiveness and metastasis [14].

Combinatorial treatments may effectively counter drug resistance. Most successful regimens use combinatorial therapy whose drugs have different mechanisms of action [10]. Combination therapy that targets early interference by the tumor to drug therapy may enhance cytotoxic drug efficacy, thereby avoiding the need to treat with full dosages [13]. Combination therapy permits a broader range of interaction between treatment drugs and a heterogeneous tumor population [11]. Combined BRAF, PI3K and mTORC1/2 inhibition suppress compensatory signaling in melanoma cell lines [1]. Combination treatment of melanomas lacking PTEN expression with the BRAF inhibitor PLX4720 and a PI3K inhibitor enhances expression of proapoptotic BIM at the mRNA and protein level in melanoma cell lines [15]. Concurrent PI3K and mTOR targeting in melanoma show strong synergism in 23 melanoma cell lines [16].

1, 2, 3-triazole-4-carboxyamidotriazole orotate (CTO) is a novel salt form of 5-amino-1 (4 (4-chlorobenzoyl)-3, 5-dichlorobenzyl)-1, 2, 3-triazole-4-carboxamide (CAI), a cytostatic agent that has been evaluated for safety and efficacy in several clinical trials. The antiproliferative and antimetastatic effects of CTO are related to inhibition of receptor operated, calcium-channel-mediated calcium influx.

The combination of CTO and the alkylating agent temozolomide showed synergistic antitumor activity in glioblastoma xenograft mouse models. In addition, analysis of brain tissue of animals treated orally with CTO demonstrated that CTO crosses the blood-brain barrier [17]. Both low- and high-dose CTO in combination with temozolomide have synergistic activity that yields greater inhibition than temozolomide alone.

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Given that CTO in combination with temozolomide demonstrates synergistic activity that yields greater inhibition than temozolomide alone, a metronomic schedule of CTO alone, or the combination of CTO with temozolomide, offer a potentially improved approach to melanoma treatment. Different combinations of CTO and temozolomide were first tested in female athymic NCr-*nu/nu* mice to evaluate tolerance of the combination. The tolerated combinations were then tested to evaluate the antitumor activity against subcutaneously implanted human LOX IMVI melanoma xenografts. In this report, we present our evaluation of the antitumor activity of CTO when administered in combination with temozolomide against melanoma xenografts in mice, and also the anti angiogenic activity of CTO in the Matrigel plug model.

2 Materials and methods

2.1 Drug formulation

2.1.1 Reagents

CTO (MW 580.76) was synthesized by Johnson Matthey Pharma Services (Devens, MA). Avastin®, a clinical formulation of bevacizumab (25 mg/mL), was produced by Genentech Inc. (South San Francisco, CA). Temodar® (temozolomide, 20 mg/capsule) was produced by Merck & Co (Whitehouse Station, NJ). Polyethylene glycol (PEG 400, MW 400) was purchased from Aldrich Chemistry (Sigma-Aldrich Co. LLC, St. Loius, MO). Deionized water (ASTM Type II) was purchased from LabChem, Inc. (Pittsburgh, PA). Klucel (hydroxypropyl cellulose) was purchased from Aldrich. Saline (physiological saline solution, for animal use only, sterile preservative-free) was purchased from Nova-Tech, Inc. (Grand Island, NE) or Phoenix Pharmaceuticals, Inc. (St. Louis, MO). Tween 80 (T80, polysorbate 80) was purchased from Fisher Scientific (Pittsburgh, PA). BD Matrigel™ Basement Membrane Matrix (high concentration) was purchased from BD Biosciences (San Jose, CA). Recombinant human VEGF 165 (VEGF) and recombinant human FGF basic 146 aa (FGFb) were purchased from R&D Systems (Minneapolis, MN). Purified anti-mouse CD31 (PECAM-1) antibody was purchased from BD Biosciences.

2.1.2 CTO

CTO was formulated once every 5 to 7 days in 40% PEG 400 in deionized water, and the formed suspension was vortexed and sonicated briefly to achieve a homogenous suspension. For all three experiments, dosing suspensions were stored refrigerated between treatments and were warmed to room temperature and vortexed to resuspend the compound before each treatment. CTO was administered to mice by exact individual animal's body weight on each day of treatment. The injection volume was 0.1 mL/10 g body weight.

2.1.3 Temozolomide

Temozolomide was prepared on each day of treatment by emptying the contents of the capsules (assuming 100% recovery), adding several drops of T80 to the powder, and then adding 0.3% Klucel in saline to yield a concentration of 4.5 mg/mL. A portion of the resulting suspension was diluted further with 0.3% Klucel in saline to achieve a concentration of 3.0 mg/mL. Temozolomide dosing formulations were administered to mice within 30 minutes of formulation by exact individual body weight on each day of treatment. The injection volume was 0.2 mL/10 g body weight.

2.1.4 Bevacizumab

For the Matrigel plug assays, a 25.0 mg/mL solution of bevacizumab was diluted on each day of treatment with saline to yield a concentration of 1.0 mg/mL. Bevacizumab dosing solution was treated as light-sensitive. Bevacizumab was administered to mice by exact individual animal’s body weight on each day of treatment. The injection volume was 0.1 mL/10g body weight.

2.2 Tumor model

CTO/temozolomide tolerance: Animals were non-tumored.

LOX IMVI melanoma xenografts: Human LOX IMVI melanoma cells were obtained from DCTD Tumor Repository, National Cancer Institute at Frederick (Frederick, MD). Each mouse was implanted SC near the right flank with one million ($l \times 10^6$) of the LOX IMVI human melanoma cells from an *in vivo* passage using a 23 g needle. The day of tumor fragment implantation was designated as Day 0. Individual tumors of 90 animals grew to 126-198 mm^3 in size on Day 6 after tumor implantation, the day of treatment initiation. Those animals selected with tumors in the proper size range were assigned to nine treatment groups so that the mean tumor volumes in all groups on Day 6 were as close to each other as possible (mean tumor volumes were 165 or 166 mm^3; median tumor volumes ranged from 162 to 176 mm^3).

Matrigel plug assay: Non-tumored animals received Matrigel™ Basement Membrane Matrix mixed with 50 ng/mL VEGF and 50 ng/mL FGFb as antiogenic stimuli.

2.3 Animal care

Six-to-seven-week old female athymic NCr-*nu/nu* mice were purchased from Charles River Laboratories (Wilmington, MA) and acclimated in the laboratories prior to experimentation. The animals were housed in microisolator cages, 5 to 6 per cage, in a 12-hour light/dark cycle. The animals received filtered Birmingham municipal water and sterilized Teklad Global 16% protein rodent diet (2016S, Harlan Laboratories, Inc.) [Harlan-Teklad TD8656 for Matrigel plug assay] ad libitum starting on the day of arrival. Cages were changed twice weekly. The animals were observed daily and clinical signs were noted. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Southern Research Institute. Animal laboratories of Southern Research Institute are AAALAC accredited.

2.4 Drug treatment

CTO/temozolomide tolerance: The experiment consisted of five groups of five mice per group. One group received CTO only at a dose of 1026 mg/kg/dose once a day for fifteen consecutive days (Q1D×15, Days 1-15). Four groups received combination treatment with CTO and temozolomide, both administered by oral gavage (PO). Beginning on Day 1, combination treatment groups received CTO at 513 and 342 mg/kg/dose once a day for 14 consecutive days (Q1D×14, Days 1-14), and temozolomide at 90 and 60 mg/kg/dose once every 4 days for a total of three injections (Q4D×3, Days 1, 5, and 9) in the following combinations: CTO 513 mg/kg/dose with temozolomide 90 mg/kg/dose; CTO 342 mg/kg/dose with temozolomide 90 mg/kg/dose; CTO 513 mg/kg/dose with temozolomide 60 mg/kg/dose; and CTO 342 mg/kg/dose with temozolomide 60 mg/kg/dose. On the days when both compounds were administered, CTO was administered first, followed immediately by the administration of temozolomide.

LOX IMXI melanoma xenografts: The experiment consisted of nine groups of 10 mice per group. All treatments were administered starting on Day 6. CTO (or its vehicle) was administered PO on a Q1D×14 schedule (Days 6-19). Temozolomide (or its vehicle) was administered PO on a Q4D×3 schedule (Days 6, 10, and 14). The control group received the combination of CTO vehicle and temozolomide vehicle. Two treatment groups received CTO at 513 or 342 mg/kg/dose. Two treatment groups received temozolomide at 90 or 60 mg/kg/dose. The four combination treatment groups received CTO 513 mg/kg/dose with temozolomide 90 mg/kg/dose; CTO 342 mg/kg/dose with temozolomide 90 mg/kg/dose; CTO 513 mg/kg/dose with temozolomide 60 mg/kg/dose; and CTO 342 mg/kg/dose with temozolomide 60 mg/kg/dose. On the days when both compounds were given, CTO was given first, followed immediately by the administration of temozolomide.

Matrigel plug assay: The experiment consisted of five groups of six mice per group. All treatments were administered starting on Day 0. The control group received CTO vehicle administered PO once a day for ten consecutive days (Q1D×10, Days 0-9).Three treatment groups received CTO administered PO at 513, 342, and 228 mg/kg/dose on a

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Q1D×10 schedule. The fourth treatment group received bevacizumab intravenously (IV) at 10 mg/kg/injection on a Q4D×3 schedule (Days 0, 4, and 8).

2.5 Parameters evaluated

CTO/temozolomide tolerance: Days of deaths and a 22-day survival were evaluated. Group mean body weights on each day of data collection, and change in mean body weight on each day of data collection relative to the mean body weight on Day 1 (in grams and as a percent), were calculated.

LOX IMVI melanoma xenografts: The number of nonspecific deaths, number of complete tumor regressions, number of tumor-free survivors on Day 35, and the median time for the tumors to reach four tumor mass doublings were determined. The median time to reach four tumor mass doublings in each of the treated groups (T) and in the control group (C) was used in the calculation of the overall delays in the growth of the median tumors (T - C, days). Median tumor volume in the treatment groups (T) was compared with the median tumor volume in the control group (T/C × 100%) on Day 19 (the last day of treatment with CTO and the last day of data collection when mice in the control group were still alive) to further evaluate the antitumor efficacy of the combination treatments.

Matrigel plug assay: The number of CD31-positive blood vessels in one entire section of each Matrigel plug was counted under the microscope. The average number of micro vessels for each mouse group was calculated.

2.6 Plasma drug levels

CAI levels in plasma were measured using animals from the CTO/temozolomide tolerance and LOX IMVI melanoma xenograft experiments.

2.6.1 Blood collection

CTO/temozolomide tolerance: Five mice treated with CTO at a dose of 1026 mg/kg/dose was bled on Day 15, 4h after the last treatment with CTO. Blood was collected from each mouse by retro-orbital puncture under CO_2/O_2 anesthesia and plasma was separated, frozen on dry ice, and stored at below -70℃ until analysis. After bleeding, each mouse was euthanized.

LOX IMVI melanoma xenografts: Five mice in the groups treated with CTO, CTO plus temozolomide, and the vehicles were bled on Day 19, 4 hours after the last treatment with CTO. Blood was collected from animals given CTO only at 513 and 342 mg/kg/dose; CTO at 513 and 342 mg/kg/dose, each in combination with 90 mg/kg/dose of temozolomide; and CTO at 513 and 342 mg/kg/dose, each in combination with 60 mg/kg/dose of temozolomide. Approximately 300 μL of blood was collected from each mouse by submandibular route (without anesthesia, survival bleeding) and plasma was removed, frozen on dry ice, and stored at below -70℃ until analysis.

2.6.2 Statistical analysis

There was no statistical analysis done in the CTO/temozolomide tolerance experiment. In the LOX IMVI melanoma xenograft study, the individual animal's time to reach four tumor mass doublings was used as the endpoint in a life tables analysis (Kaplan-Meier survival analysis followed by a log-rank test). The life tables analysis allows for the comparison of the growth data between the groups using the animals whose tumors did not reach the evaluation point, by censoring them. The individual animal's tumor volume on Day 19 was used as the endpoint in a Student's t-test (or Mann-Whitney rank sum test) in order to statistically compare the growth data between the groups. A nonparametric test was used when the data set did not the pass the normality or equal variance test. In the Matrigel plug assay, the number of blood vessels between the groups was compared by t-test or Mann-Whitney rank sum test (used when the data set did not pass the equal variance test) using SigmaStat 3.5 software. Measured CAI plasma levels were compared using a one tailed t-test and one-way ANOVA. SigmaStat version 3.5 or GraphPad version 4.03 were used for statistical analysis. The difference between the groups was considered significant if the *P* value was less than or equal to 0.05.

3 Procedures for parameter evaluations

3.1 Microvessel analysis

Matrigel plug assay: The Matrigel plug assay was adapted from Hotchkiss et al. [18] (2002) and optimized. Matrigel™ Basement Membrane Matrix was mixed with 50 ng/mL VEGF and 50 ng/mL FGFb, as angiogenic stimuli, and kept at 4℃. Each mouse was implanted SC bilaterally (in the right and left flanks) with 0.5 mL of Matrigel using a 23-gauge needle. The injection was done rapidly to ensure that the entire content was delivered in one plug. The day of implantation was designated as Day 0. Formed plugs from each mouse were collected on Day 10 and fixed in 10% neutral buffered formalin. After formalin fixation, Matrigel plugs were embedded in paraffin and a 4 μm thickness slide was prepared from each paraffin block. Only intact plugs of not less than 4 mm in diameter were analyzed. Sections collected around the center part of each plug were processed with antigen retrieval and then immunostained with specific antibody against CD31 and counterstained with H&E.

3.2 Tumor Measurements and Body Weights

CTO/temozolomide tolerance: Animals were checked and mortality was recorded once daily. The animals were weighed twice a week starting on Day 1.

LOX IMVI melanoma xenografts: The SC tumors were measured and the animals were weighed two times a week starting on the first day of treatment. Tumor volume was determined by caliper measurements (mm) and using the formula for an ellipsoid sphere: $LxW^2/2 = mm^3$, where L and W refer to the larger and smaller perpendicular dimensions collected at each measurement. Limit of detection was 4×4 mm.

3.3 Study Duration

CTO/temozolomide tolerance: Mice treated with CTO at a dose of 1026 mg/kg/dose were euthanized after blood collection on Day 15. The experiment was terminated on Day 22.

LOX IMVI melanoma xenografts: The experiment was terminated on Day 35. Any moribund animal or any animal whose tumor ulcerated or reached 4,000 mm^3 in volume was euthanized prior to the scheduled day of termination for humane reasons.

Matrigel plug assay: The experiment was terminated on Day 10.

4 Results

4.1 CTO / temozolomide tolerance

The tolerance of female athymic NCr-*nu/nu* mice to the combination treatment of CTO and temozolomide was evaluated. The combination treatment of CTO at 513 or 342 mg/kg/dose and temozolomide at 90 or 60 mg/kg/dose was tolerated without deaths at all doses tested. Animals in all four groups gained weight after the end of the treatment. Thus, the maximum tolerated dose (MTD, defined as the dose which does not result in death or produces no more than 20% mean body weight loss) of the combination treatment of CTO, when administered PO on a Q1D×14 schedule, plus temozolomide, when administered PO on a Q4D×3 schedule to nontumored female athymic NCr-*nu/nu* mice, was above 513 mg/kg/dose for CTO and 90 mg/kg/dose for temozolomide in this experiment (Table 1).

Treatment with CTO administered PO on a Q1D×15 schedule to nontumored female athymic NCr-*nu/nu* mice at a dose of 1026 mg/kg/dose was also tolerated without deaths. Thus, the MTD was above 1026 mg/kg/dose in this experiment (Table 1).

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Table 1. Mean Gross Body Weight Percent Change CTO Tolerance Experiments

Compound	**Day**						
Mg/kg/dose	**1**	**4**	**8**	**11**	**15**	**18**	**22**
CTO 513							
Temozolomide 90							
Mean gross body weight (g)	21.9	20.6	20.8	21.2	22.6	23.6	24.3
Mean gross body weight change (g)		-1.3	-1.1	-0.7	+0.7	+1.7	+2.4
Mean gross body weight change (%)		-6	-5	-3	3	8	11
CTO 513							
Temozolomide 60							
Mean gross body weight (g)	23.7	22.3	22.8	23.0	25.2	26.3	26.9
Mean gross body weight change (g)		-1.4	-0.9	-0.7	+1.5	+2.6	+3.2
Mean gross body weight change (%)		-6	-4	-3	6	11	14
CTO 342							
Temozolomide 90							
Mean gross body weight (g)	22.4	22.0	21.4	21.9	23.4	24.1	25.1
Mean gross body weight change (g)		-0.4	-1.0	-0.5	+1.0	+1.7	+2.7
Mean gross body weight change (%)		-2	-4	-2	4	8	12
CTO 342							
Temozolomide 60							
Mean gross body weight (g)	24.4	23.0	23.7	24.4	25.3	25.9	26.5
Mean gross body weight change (g)		-1.4	-0.7	0.0	+0.9	+1.5	+2.1
Mean gross body weight change (%)		-6	-3	0	4	6	9
CTO 1026							
Mean gross body weight (g)	25.7	24.8	25.6	25.5	26.1		
Mean gross body weight change (g)		-0.9	-0.1	-0.2	+0.4		
Mean gross body weight change (%)		-4	0	-1	2		

4.2 LOX IMVI melanoma xenografts

The effects of administering CTO alone, temozolomide alone, and CTO combined with temozolomide on tumor regression, number of tumor-free survivors, and median time to four doublings were evaluated using LOX IMVI melanoma xenograft model in NCr *nu/nu* female mice. A summary of the LOX IMVI melanoma xenograft experimental results, including maximum mean body weight loss, median days to four doublings, median tumor growth delay, and median T/C, are presented in Table 2.

Control, vehicle-treated LOX IMVI melanoma xenografts grew progressively in all 10 animals, reaching 4,800 mm^3 in volume on Day 19.

In the CTO 513 mg/kg/dose group, one animal died on Day 20. Growth of the tumors in the group administered CTO at 513 mg/kg was found to be statistically different from the growth of the tumors in the control group, when individual animal's times to reach four tumor mass doublings were compared (P<0.001). Tumor volumes on Day 19 in the 513 mg/kg CTO treated group were statistically different from the tumor volumes in the vehicles treated control group (P<0.010).

In the CTO 342 mg/kg/dose group, growth of the tumors was found to be statistically different from the growth of the tumors in the control group, when individual animal's times to reach four tumor mass doublings were compared (P=0.004). Tumor volumes on Day 19 in the 342 mg/kg CTO-treated group were statistically different from the tumor volumes in the vehicles-treated control group (P=0.010).

Table 2. Response of SC LOX IMVI Melanoma Tumor to Treatment with CTO or CTO in Combination with Temozolomide

Treatment			No. of Animals	Maximum Mean Body Weight Loss	Median Days to 4 Doublings	Growth Delay (T-C)[a]	Median T/C (%) Day 19
Compound	**Dose mg/kg/dose**	**Route**					
Control							
CTO vehicle	0	PO	10		8.7	N/A	N/A
Temozolomide vehicle	0	PO					
Monotherapy							
CTO	513	PO	10	4%	11.8	3.1	65
CTO	342	PO	10	2%	10.3	1.6	70
Temozolomide	90	PO	10	7%	17.2	8.5	36
Temozolomide	60	PO	10	7%	12.3	3.6	57
Combination therapy							
CTO	513	PO	10	16%	20.2	11.5	27
Temozolomide	90	PO	10				
CTO	342	PO	10	15%	16.9	8.2	35
Temozolomide	90	PO	10				
CTO	513	PO	10	8%	15.5	6.8	47
Temozolomide	60	PO	10				
CTO	342	PO	10	10%	19.4	10.7	39
Temozolomide	60	PO	10				

The observed CTO antitumor activity was not dose dependent when individual animal's times to reach four tumor mass doublings were compared (513 mg/kg versus 342 mg/kg: P=0.556) and when individual animal's tumor volumes on Day 19 were compared (513 mg/kg versus 342 mg/kg: P=0.204). Response of the SC-implanted human LOX IMVI melanoma xenografts to the treatment with CTO is presented graphically in Figure 1.

In the temozolomide 90 mg/kg/dose group, one animal died on Day 24. Growth of the tumors in the 90 mg/kg/dose group was found to be statistically different from the growth of the tumors in the control group, when individual animal's times to reach four tumor mass doublings were compared (P<0.001).Tumor volumes on Day 19 in the 90-mg/kg group were statistically different from the tumor volumes in the vehicles treated control group (P<0.001).

In the temozolomide at 60 mg/kg/dose group, growth of the tumors was found to be statistically different from the growth of the tumors in the control group, when individual animal's times to reach four tumor mass doublings were compared (P<0.001).Tumor volumes on Day 19 in the 60 mg/kg group were statistically different from the tumor volumes in the vehicles-treated control group (P<0.001).

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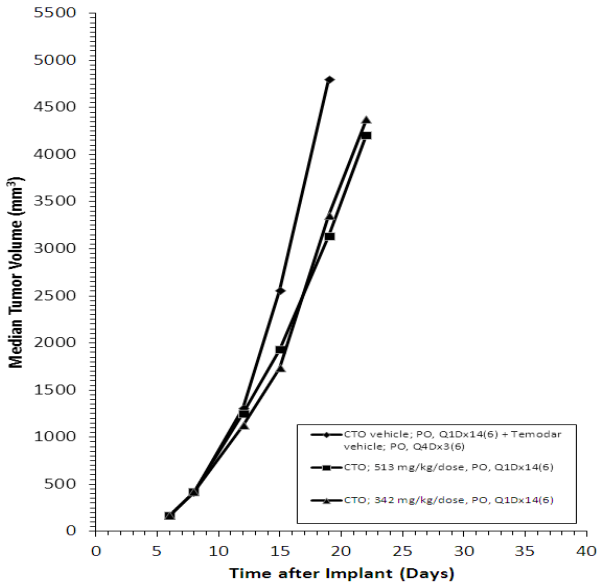

Figure 1. Response of SC Implanted LOX IMVI Human Melanoma to Treatment with CTO

The observed temozolomide antitumor activity was dose dependent when individual animal's times to reach four tumor mass doublings were compared (90 mg/kg versus 60 mg/kg: $P<0.001$) and when individual animal's tumor volumes on Day 19 were compared (90 mg/kg versus 60 mg/kg: $P=0.004$). Response of the SC-implanted human LOX IMVI melanoma xenografts to the treatment with temozolomide is presented graphically in Figure 2.

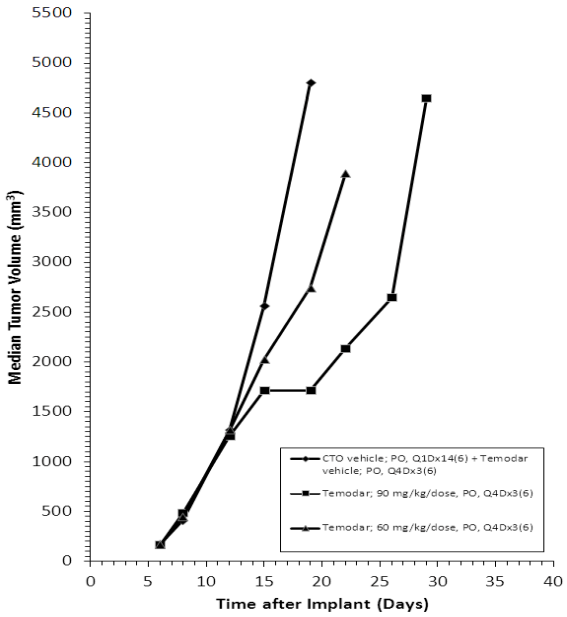

Figure 2. Response of SC Implanted LOX IMVI Human Melanoma to Treatment with Temozolomide

In the group administered CTO 513 mg/kg/dose in combination with temozolomide 90 mg/kg/dose, one animal died on Day 22. Growth of the tumors in this combination treatment group was found to be statistically different from the growth of the tumors in the control group, when individual animal's times to reach four tumor mass doublings were compared (control vehicle versus CTO 513 mg/kg with temozolomide 90 mg/kg: $P<0.001$). However, tumor growth was not different from that in the group administered temozolomide at 90 mg/kg/dose alone ($P=0.383$). Tumor volumes on Day 19

in this combination group were statistically different from the tumor volumes in the vehicles treated control group (control vehicle versus CTO 513 mg/kg with temozolomide 90 mg/kg: $P<0.001$) and from the tumor volumes in the group treated with temozolomide 90 mg/kg/dose alone (temozolomide 90 mg/kg versus CTO 513 mg/kg with temozolomide 90 mg/kg: $P=0.021$).

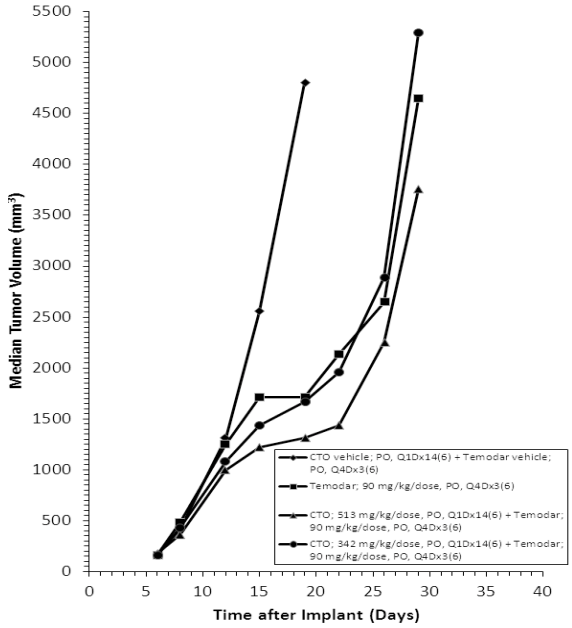

Figure 3. Response of SC Implanted LOX IMVI Human Melanoma to Treatment with CTO in Combination with Temozolomide (90 mg/kg/dose)

In the group administered CTO 342 mg/kg/dose in combination with temozolomide 90 mg/kg/dose, three animals died (with deaths occurring on Days 19, 25, and 28). Growth of the tumors in this combination treatment group was found to be statistically different from those in the control group, when individual animal's times to reach four tumor mass doublings were compared (control vehicle versus CTO 342 mg/kg plus temozolomide 90 mg/kg: $P<0.001$). However, tumor growth was not different from the growth of the tumors in the group treated with temozolomide 90 mg/kg/dose alone ($P=0.890$). Tumor volumes on Day 19 in this combination group were statistically different from those in the vehicles-treated control group (control vehicle versus CTO 342 mg/kg plus temozolomide 90 mg/kg: $P<0.001$). However, tumor volumes were not different from those in the group treated with temozolomide 90 mg/kg/dose alone (temozolomide 90 mg/kg versus CTO 342 mg/kg plus temozolomide 90 mg/kg: $P=0.211$). Response of the SC-implanted human LOX IMVI melanoma xenografts to the treatment with CTO in combination with temozolomide 90 mg/kg/dose is presented graphically in Figure 3.

In the group administered CTO 513 mg/kg/dose in combination with temozolomide 60 mg/kg/dose, one animal died on Day 25. Growth of the tumors in this combination treatment group was found to be statistically different from the those in the control group, when individual animal's times to reach four tumor mass doublings were compared (control vehicle versus CTO 513 mg/kg with temozolomide 60 mg/kg: $P<0.001$); and from the growth of the tumors in the group treated with temozolomide 60 mg/kg/dose alone (temozolomide 60 mg/kg versus CTO 513 mg/kg plus temozolomide 60 mg/kg: $P=0.001$).Tumor volumes on Day 19 in this combination group were statistically different from those in the vehicles-treated control group (control vehicle versus CTO 513 mg/kg with temozolomide 60 mg/kg: $P<0.001$) and from the tumor volumes in the group treated with temozolomide 60 mg/kg/dose alone (temozolomide 60 mg/kg versus CTO 513 mg/kg with temozolomide 60 mg/kg: $P=0.017$). The antitumor activity of the combination treatment was additive compared to the antitumor activity produced by the administration of each compound alone: a median tumor growth delay

in the CTO 513 mg/kg/dose group was 3.1 days; that of the temozolomide 60 mg/kg/dose group, 3.6 days. In comparison, the median tumor growth delay in the combination group administered CTO 513 mg/kg/dose plus temozolomide 60 mg/kg/dose was 6.8 days.

In the group administered CTO 342 mg/kg/dose in combination with temozolomide 60 mg/kg/dose, one animal died on Day 19. Growth of the tumors in this combination treatment group was found to be statistically different from those in the control group, when individual animal's times to reach four tumor mass doublings were compared (control vehicle versus CTO 342 mg/kg plus temozolomide 60 mg/kg: $P<0.001$); and from the growth of the tumors in the group treated with temozolomide 60 mg/kg/dose alone (temozolomide 60 mg/kg versus CTO 342 mg/kg plus temozolomide 60 mg/kg: $P<0.001$).Tumor volumes on Day 19 in this combination group were statistically different from those in the vehicles-treated control group (control vehicle versus CTO 342 mg/kg plus temozolomide 60 mg/kg: $P<0.001$) and from the tumor volumes in the group treated with temozolomide 60 mg/kg/dose alone (temozolomide 60 mg/kg versus CTO 342 mg/kg plus temozolomide 60 mg/kg: $P=0.001$). The antitumor activity of the combination treatment was more than additive compared to the antitumor activity produced by administration of each compound alone: a median tumor growth delay in the CTO 342 mg/kg/dose group was 1.6 days; that of the temozolomide 60 mg/kg/dose group, 3.6 days. In comparison, the median tumor growth delay in the combination group administered CTO 342 mg/kg/dose plus temozolomide 60 mg/kg/dose was 10.7 days. Response of the SC-implanted human LOX IMVI melanoma xenografts to the treatment with CTO in combination with temozolomide 60 mg/kg/dose is presented graphically in Figure 4.

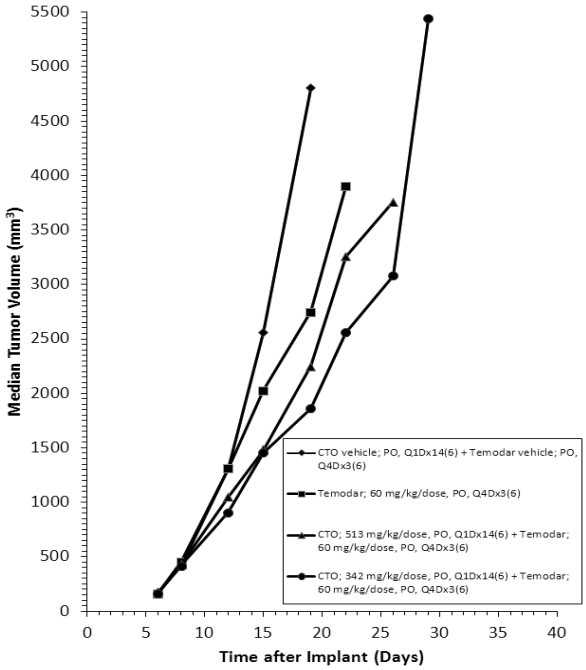

Figure 4. Response of SC Implanted LOX IMVI Human Melanoma to Treatment with CTO in Combination with Temozolomide (60 mg/kg/dose)

4.3 Matrigel plug assay

Oral treatment of Matrigel plug-bearing mice with CTO at a dose of 513 mg/kg/dose for 10 consecutive days resulted in statistically significant reduction in new blood vessel formation, producing a 44% inhibition as assessed by the Matrigel plug assay. The CTO treatment at doses of 342 and 228 mg/kg/dose showed only 4% and 5% inhibition, respectively. Bevacizumab treatment, when administered IV on a Q4D×3 schedule at a dose of 10 mg/kg/injection, resulted in a statistically significant 50% reduction in new blood vessel formation (Figure 5).

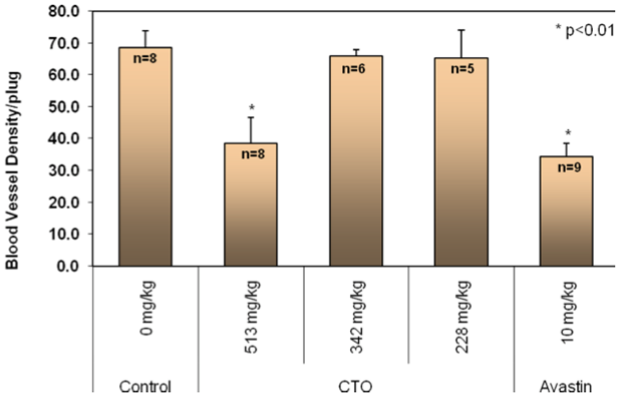

Figure 5. Blood Vessels Formation within Matrigel Plugs in Mice Receiving Different Treatments (Average number of blood vessels + SE)

4.4 Plasma drug levels

Table 3. CAI Plasma Levels for CTO/tolerance Experiment

Sample	Final Concentration (ng/mL)
Average of 5 samples	11702
Standard deviation	2693

Table 4. CAI Plasma Levels for LOX IMVI Melanoma Xenograft Experiment

Treatment*	Final Concentration (ng/mL)
CTO vehicle + temozolomide vehicle	No peak
CTO 513	
Average	10320
Standard deviation	1527
CTO 342	
Average	6385
Standard deviation	1731
CTO 513 + temozolomide 90	
Average	11760
Standard deviation	2977
CTO 342 + temozolomide 90	
Average	10530
Standard deviation	2703
CTO 513 + temozolomide 60	
Average	9770
Standard deviation	1512
CTO 342 + temozolomide 60	
Average	8610
Standard deviation	2275

* Doses are in mg/kg/dose.

The CAI levels measured in plasma of non-tumored mice collected 4 hours after the last, fifteenth daily treatment with CTO at a dose of 1026 mg/kg/dose ranged from 8,810 ng/mL to 15,000 ng/mL. The average plasma drug level of the five samples was 11,702 ng/mL with a standard deviation of 2,693 ng/mL (Table 3).

The CAI levels in plasma samples from the LOX IMVI melanoma xenograft study are shown in Table 4. For the 513 mg/kg/dose group, no statistical differences in the mean values were found between the groups administered CTO only (10,320 ng/mL), CTO with 60 mg/kg of temozolomide (9,770 ng/mL), and CTO with 90 mg/kg of temozolomide (11,760 ng/mL).

For the 342 mg/kg/dose group, no statistical difference between the mean values was found in the groups administered CTO only (6,385 ng/mL) and CTO and 60 mg temozolomide (8,610 ng/mL). There was, however, a statistical difference between the mean values of the groups administered CTO only (6,385 ng/mL) and CTO and 90 mg temozolomide (10,530 ng/mL).

Table 5. Summary of Statistical Comparison of Plasma Levels for LOX IMVI Melanoma Xenograft Experiment

Groups Compared	Statistically Significant
CTO 513* vs. CTO 342[1]	Yes
CTO 513 vs. CTO 513 + temozolomide 90[1]	No
CTO 513 vs. CTO 513 + temozolomide 60[1]	No
CTO 342 vs. CTO 342 + temozolomide 90[1]	Yes
CTO 342 vs. CTO 342 + temozolomide 60[1]	No
CTO 513 vs. CTO 513 + temozolomide 90 vs. CTO 513 + temozolomide 60[2]	No
CTO 342 vs. CTO 342 + temozolomide 90 vs. CTO 342 + temozolomide 60[2]	Yes

* Doses are in mg/kg/dose.

1 One-tailed T test with 95% confidence interval

2 One-way ANOVA with 95% confidence interval

Further statistical analysis using one-way ANOVA showed a statistical difference between the mean values for the groups administered CTO 342 mg/kg/dose, CTO 342 mg/kg/dose and temozolomide 90 mg/kg/dose, and CTO 342 mg/kg/dose and temozolomide 60 mg/kg/dose. A statistical difference was not seen for the groups administered CTO 513 mg/kg/dose, CTO 513 mg/kg/dose and temozolomide 90 mg/kg/dose, and CTO 513 mg/kg/dose and temozolomide 60 mg/kg/dose. A summary of statistical comparisons is shown in Table 5.

5 Discussion

Our experiments show that a metronomic schedule of CTO alone or the combination of CTO with temozolomide may offer a potentially improved approach to melanoma treatment. Oral administration of CTO 513 or 342 mg/kg/dose for 14 consecutive days resulted in a measurable inhibition of the growth of the human LOX IMVI melanoma xenografts when implanted SC in female athymic NCr-*nu/nu* mice. The observed antitumor activity of CTO was not dose dependent. Oral administration of temozolomide 90 and 60 mg/kg/dose once every four days for a total of three treatments resulted in a measurable, dose-dependent inhibition of the growth of human LOX IMVI melanoma xenografts.

While the combination treatment of CTO at both doses tested with temozolomide 90 mg/kg/dose did not result in an increased antitumor activity compared to the antitumor activity produced by the administration of temozolomide alone at 90 mg/kg/dose, the combination treatment of CTO at both doses tested with temozolomide 60 mg/kg/dose resulted in an increased antitumor activity compared to the antitumor activity produced by the administration of temozolomide alone at 60 mg/kg/dose. While the antitumor activity of the combination treatment of CTO at 513 mg/kg/dose plus temozolomide at 60 mg/kg/dose was additive, the antitumor activity of the combination treatment of CTO at 342 mg/kg/dose plus temozolomide at 60 mg/kg/dose was more than additive. These results demonstrate that a dose-dilution strategy of using lower doses of temozolomide produced the maximum antitumor activity without more frequent intervals as in metronomic chemotherapy. This outcome is contrary to the established practice of dose dense strategies currently used in treating cancer patients [10, 11]. In combination therapy, nonoverlapping patterns of normal organ toxicity allow both drugs to be used at full dosages for optimal efficacy [10]; however, our results reveal the potential to achieve maximum treatment effects and lower toxicities through a dose-dilution strategy.

There was a dose-related difference with regard to CAI plasma levels between the two groups treated with CTO in the absence of temozolomide. A statistically significant difference was observed in the CAI mean plasma levels of samples from the CTO 342 mg/kg/dose groups as a function of temozolomide dosing. However, the observed difference was not statistically significant for the CTO 513 mg/kg/dose groups as a function of temozolomide. Mean CAI plasma levels in the groups administered 342 mg/kg/dose administered with 90 mg/kg of temozolomide were not statistically different from the mean levels of the groups administered 513 mg/kg dose with or without temozolomide. Despite having comparable CAI levels in these groups, there were differences in the response of SC LOX IMVI tumor growth as a function of temozolomide. In a comparison of the group administered 513 mg/kg CTO with 90 mg/kg of temozolomide versus 90 mg/kg temozolomide alone, the median days to 4 doublings increased by 3 days and the T/C value decreased by 9%. In a comparison of the group administered 342 mg/kg CTO with 90 mg temozolomide versus the 513 mg/kg CTO with 90 mg/kg of temozolomide, the median days to 4 doublings increased by 3.3 days and the T/C value decreased by 8%. This result demonstrates some degree of synergistic activity of the dual mode therapy at comparable mean CAI plasma levels.

Plasma samples from non-tumored mice dosed with 1026 mg/kg/dose once a day for 15 days collected 4 hours after dosing on day 15, had measured levels of CAI ranging from 8,810 ng/mL to 15,000 ng/mL. The average plasma drug level of the five samples was 11,702 ng/mL with a standard deviation of 2,693 ng/mL.

Statistical analysis of plasma levels from tumored mice in the IMVI xenograft experiment showed a statistical difference between mean plasma levels of CAI (342 mg/kg/dose) as a function of administration of temozolomide at 0, 60 or 90 mg/kg/dose Q4D×3. No statistical difference was found in the 513 mg/kg/dose groups as a function of temozolomide dosing. The observed difference in mean plasma levels is not likely due to metabolic drug-drug interaction, as temozolomide is converted to its active form via hydrolysis at physiological pH rather than by CYP450-mediated enzymatic biotransformation.

Enhanced expression of the P-gp transport protein (MDR-1) can increase drug efflux and affect drug sensitivity. Inhibition of P-gp has been used as a strategy to reverse drug resistance [19]. However, inhibition may decrease systemic cytotoxic chemotherapy elimination and increase toxicity and adverse events [20]. Initial clinical success with first- and

second-generation inhibitors has been limited by unacceptable toxicity and unpredictable pharmacokinetic interactions. Third-generation P-gp inhibitors have been developed that have greater specificity and do not have PK effects on co-administered cytotoxic drugs [21].

A possible explanation for the reported differences in mean CAI plasma levels could involve P-gp efflux transporters. Because CAI has previously been shown to be a substrate for P-gp, if temozolomide were to act as weak P-gp efflux inhibitor, an increase in absorption of CAI in a dose-related manner could be observed. One variant in the MDR1 genotype is associated with the survival of patients with glioblastoma who are treated with temozolomide. This MDR variant may alter the affinity of P-gp for temozolomide [22]. If temozolomide were indeed a weak P-gp efflux inhibitor, its contribution would not be as readily observed at higher doses of CTO, where absorption by passive diffusion would dominate.

Administration of CTO at a dose of 513 mg/kg/dose for 10 consecutive days resulted in a statistically significant reduction in new blood vessel formation. The main cause of visual loss in AMD is the development of choroid neovascularization. This antiangiogenic activity of CTO could potentially limit the development of neovascularization in complications of AMD [23].

CTO is a small molecule with favorable pharmacokinetic and toxicokinetic profiles and multiple signaling targets. CTO is an inhibitor of calcium-mediated signal transduction, thereby inhibiting the PI3K and VEGF pathways, leading to inhibition of angiogenesis, thus providing a strong rationale for its clinical evaluation for the treatment of melanoma. CAI (the active component of CTO) has been shown to inhibit VEGF-induced vascular endothelial cell proliferation. In nonclinical studies, CTO showed improved bioavailability and diminished toxicity, suggesting that higher plasma concentrations can be achieved with lower doses, thereby avoiding the toxicity observed with CAI. Given its significant inhibitory effect at low doses in combination with temozolomide, this dose dilution strategy, versus a dose-dense strategy, shows potential in the treatment of melanoma.

6 Conclusion

These results suggest that CTO enhances the sensitivity of temozolomide and may permit use of lower doses of temozolomide to obtain an optimum antitumor effect in combination therapy, thus reducing toxicity of high temozolomide doses in this melanoma model.

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Conflicting interests

Rashida A. Karmali, shareholder of Tactical Therapeutics, Inc.

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