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Carboxyamidotriazole Orotate and Cytotoxic Chemotherapy have a Synergistic Effect on Tumor Inhibition in Glioblastoma and Colon Xenograft Mouse Models

Research Article

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Summary

The purpose of this study was to evaluate the antitumor efficacy of carboxyamidotriazole orotate (CTO) alone or in combination with cytotoxic chemotherapy in glioblastoma and colon cancer xenograft mouse models. CTO targets VEGF and PI3K via Ca++ signaling pathways. Combination therapy with VEGF inhibitors and chemotherapy is reported to have a synergistic effect in these models. CTO was administered alone and in combination with temozolomide against U251 human glioblastoma xenografts in male, athymic NCr-*nu*/*nu* mice. CTO was administered alone and in combination with 5-fluorouracil (5-FU) and compared to 5-FU or bevacizumab given alone or in combination against HT29 human colon tumor xenografts in male, athymic NCr-*nu*/*nu* mice. Efficacy was measured by the time delay to doubling or tripling of the tumor, delay in tumor growth, and tumor weight relative to controls. The combination of CTO and chemotherapy exhibited significant antitumor effects that were more efficacious than chemotherapy alone in U251 and HT29 xenograft mouse models. CTO combined with temozolomide had synergistic activity resulting in significantly greater inhibition than temozolomide monotherapy in the GBM xenograft mouse model; however, high-dose CTO was toxic. Low-and high-dose CTO in combination with 5-FU had greater efficacy than the combination of bevacizumab and 5-FU in the colon cancer xenograft mouse model. This study supports the growing body of data reporting the efficacy of combination therapy with Calcium-VEGF-PI3K inhibitors and cytotoxic chemotherapy in GBM and colorectal cancer. Our findings establish a therapeutic approach to tumors using CTO combined with cytotoxic chemotherapy for the treatment of GBM and colon cancer.

I. Introduction

Glioblastoma multiforme (GBM) is the most aggressive malignant primary brain cancer in adults and is nearly always fatal. GBM is highly angiogenic and has genetic alterations, including PTEN, in about 80%, and PI3KCA gene mutations in about 50%, of tumors (CGAR, 2008; Kanai et al, 2011; Parsons et al, 2008; van der Heijden and Bernards, 2010). The median progression-free survival for patients treated with surgery and chemoradiation is under 7 months. Median overall survival is under 15 months. No effective therapy exists following recurrence (Reardon et al, 2008).

Temozolomide, an alkylating agent, is the current standard of care (CGAR, 2008; Jones−Bolin et al, 2006) in combination with surgical resection and irradiation (Dresemann, 2010). Although recurrent GBM has an initial response to temozolomide therapy, GBM loses sensitivity to temozolomide (Dresemann, 2010). Overall survival is not improved and tumor progression results (Jones-Bolin et al, 2006).

GBM expresses vascular endothelial growth factor (VEGF) as a pro-angiogenic factor (Reardon et al, 2008) (Brastianos and Batchelor, 2009). VEGF is involved in tumor growth rate and tumor cell migration (Jones-Bolin et al, 2006). Circulating VEGF concentration correlates with metastatic potential in various human tumors in xenografts and patients, and is linked to increased vascular density (Oliver et al, 2003).

VEGF induces nitric-oxide synthase, yielding an increase in nitric oxide. In turn, nitric oxide stimulates VEGF production and secretion (Bauer et al, 2000; Munaron, 2002). VEGF expression is also enhanced by the dysregulated signaling of the PI3K pathway resulting from PTEN mutations (Pore et al, 2003; Reardon et al, 2008). The PI3K pathway is involved in cell survival, growth, migration, and angiogenesis (Cloughesy and Mischel, 2011). PI3K is hyperactivated in nearly 90% of GBM and leads to overexpression of Akt downstream; mTOR kinase is one of its critical effectors. In addition to PI3K amplification through the PTEN mutation, the PI3KCA gene mutation, described in GBM and several other human cancers, results in increased PI3K activity (van der Heijden and Bernards, 2010).

Because GBM is highly angiogenic, several antiangiogenic agents have been evaluated as possible therapy (Ruggeri et al, 2003). Recurrent GBM has an initial response to anti-VEGF therapy and then recurs, exhibiting an altered growth pattern, including blood vessels with normal morphology, and no second wave of angiogenesis (di Tomaso et al, 2011). These results highlight the importance of pathway cross-talk and need for a rational combination therapy of mTOR/PI3K inhibition and other target signaling pathways such as calcium (Cloughesy and Mischel, 2011).

The anti-VEGF antibody bevacizumab has been approved for use as a single agent in recurrent GBM and is being tested in combination with other agents (Brastianos and Batchelor, 2009; Friedman et al, 2009). The combination of chemotherapy with agents that target VEGF has enhanced activity relative to chemotherapy alone (Friedman et al, 2009).

The Ca^{++} signaling pathway is a potential therapeutic target because it is a second messenger that activates downstream signal transduction cascades, thereby producing a constellation of effects. CA^{++}-mediated signal transduction has effects on VEGF and the PI3K pathway (Bauer et al, 2000; Oliver et al, 2003). Calcium influx is involved in VEGF/IL−8 production, is induced by VEGF and IL-8, and is required by VEGF and IL-8 for gene expression (Oliver et al, 2003).

Carboxyamidotriazole (CAI) is an inhibitor of receptor-operated calcium channel−mediated calcium influx, and is shown to have antiproliferative and anti-invasive functions in several human cancer cell lines, including human glioblastoma cells (Fiorio Pla et al, 2008; Ge et al, 2000). By interrupting calcium mobilization as a second messenger, CAI can inhibit calcium−sensitive signal transduction pathways, including the release of arachidonic acid and its metabolites; nitric oxide release; the generation of inositol phosphates; and tyrosine phosphorylation (Ge et al, 2000; Kohn et al, 1992). CAI inhibits phosphorylation of cellular proteins STATS and CrkL, and induces apoptosis in imatinib mesylate-resistant chronic myeloid leukemia cells by down-regulating bcr−abl (Alessandro et al, 2008).

Clinical development of CAI was halted because of poor bioavailability, limited efficacy, and high toxicity. Even though oral formulations of micronized CAI achieved adequate plasma levels, CAI was bound about 99.5% to plasma proteins (Berlin et al, 1997; Desai et al, 2004; Figg et al, 1995; Hussain et al, 2003; Johnson et al, 2008; Kohn et al, 1992; Mikkelsen et al, 2007). Carboxyamidotriazole orotate (CTO) is a triazole orotate formulation of CAI. In pre-clinical studies with rats and ferrets, CTO has shown markedly improved bioavailability and reduced toxicity, respectively, compared to CAI (Grover, 2007).

Given reports that combination therapy with approved cytotoxic drugs and VEGF inhibitors or mTOR inhibitors acts synergistically on GBM and colon cancer xenograft models (Jones-Bolin et al, 2006; Sansal and Sellers, 2004), and in multiple cancer types in clinical trials (Guertin and Sabatini, 2009; Reardon et al, 2008), does CTO alone, or the combination of CTO with cytotoxic chemotherapy, offer a potentially improved approach to GBM and colon cancer treatment?

In this report we describe the antitumor efficacy of CTO when administered in combination with a chemotherapeutic agent, temozolomide, against

subcutaneously (s.c.)-implanted U251 human glioblastoma xenografts in male, athymic NCr-*nu/nu* mice.

We also describe the antitumor efficacy of CTO administered in combination with 5−fluorouracil (5-FU) versus 5−FU or bevacizumab given alone or in combination against s.c.-implanted HT29 human colon tumor xenografts in male, athymic NCr-*nu/nu* mice. Our findings identify CTO as a potent antiangiogenic small molecule with favorable pharmacokinetic and toxicokinetic profiles and multiple signaling targets, providing a strong rationale for its clinical evaluation.

II. Materials and Methods

A. Tumor model

The U251 glioblastoma model was obtained from the DCTD Tumor Repository. The HT29 colon adenocarcinoma model was obtained from the ATCC.

B. Drug formulation

For the U251 studies, 1,2,3-Carboxyamidotriazole orotate (CTO, Johnson Matthey Pharma Services, lot no. 1744-61-1) was formulated once a week at a concentration of 51.3 mg/mL in PEG 400, and the formed suspension was vortexed and sonicated in brief intervals to achieve a homogenous suspension. A portion of the resulting suspension was diluted further with 100% PEG 400 to achieve a concentration of 34.2 mg/mL. CTO dosing suspensions were stored refrigerated between injections, and were warmed to room temperature and vortexed to resuspend the compound before each treatment. Temozolomide (Schering Co.) was resuspended in several drops of polysorbate 80 and 0.3% hydroxypropylcellulose in saline to yield a temozolomide concentration of 1.7 mg/mL. Temozolomide dosing formulation was stored on wet ice and administered within 30 minutes of formulation.

For the HT29 studies, CTO (Southern Research Institute, lot no. J -84 7-51-I) at a concentration of 51.3 mg/mL was formulated once a week in 100% PEG 400. The mixture was sonicated for 15 minutes to facilitate dispersion of aggregates. A portion of the resulting suspension was diluted further with 100% PEG 400 to achieve a concentration of 34.2 mg/mL. Dosing suspensions were stored refrigerated between injections, and were warmed to room temperature and vortexed before each treatment.

A generic formulation of 5-FU, Fluorouracil Injection (American Pharmaceutical Partners, Inc.) was diluted with saline to 7.5 mg/mL, which was enough for two injections at a time. 5-FU dosing solution was treated as light-sensitive and was stored at room temperature until the next injection.

Bevacizumab (Genentech) was diluted on each day of treatment with saline to yield a concentration of 6.0 mg/mL. It was further diluted with saline to achieve a lower concentration of 4.0 mg/mL. Bevacizumab dosing solutions were treated as light-sensitive. Control group was treated with 100% PEG 400. 5-FU, CTO, bevacizumab, and the vehicle were administered to mice by exact individual animal's body weight on each day of treatment. The injection volume was 0.1 mL/10 g of body weight.

C. Animal care

For the U251 GBM xenograft studies, 5- to 6-week-old male, athymic NCr-nu/nu mice were acclimated in the laboratories for 2 weeks prior to experimentation. The animals were housed in micro-isolator cages, up to six per cage in a 12-hour light/dark cycle.

For the HT29 colon cancer xenograft studies, 5-week-old, male athymic NCr-nu/nu mice were acclimated in the laboratories for 10 days prior to experimentation. The animals were housed in a pathogen-free barrier facility in micro-isolator cages, five per cage in a 12-hour light/dark cycle.

The animals in both sets of experiments were purchased from the NCI-Frederick Animal Production Area (Frederick, MD), and received filtered Birmingham municipal water and sterilizable rodent diet (Harlan-Teklad TD8656) ad libitum. 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.

D. Drug treatment

For the U251 studies, each mouse was implanted s.c. near the right flank with an approximate 30-40 mg fragment of U251 human glioblastoma xenograft from an in vivo passage using a 13-gauge trocar needle. The day of tumor fragment implantation was designated as Day 0. Individual tumors grew to 88-198 mg in weight (88-198 mm3 in size) on the day of treatment initiation, Day 13 after tumor fragment implantation. Those animals selected with tumors in the proper size range were assigned to groups so that the mean tumor weights in all groups on the first day of treatment were as close to each other as possible (mean tumor weights being 135 or 136 mg, median tumor weights ranging from 120 to 153 mg).

The injection on Day 14 in groups receiving CTO 513 mg/kg/dose and 342 mg/kg/dose was timed for each animal. Three mice in each group were euthanized 6 hours after the CTO treatment and the other three mice per group were euthanized 8 hours after the treatment. Whole brain of each animal was collected, flash frozen in liquid nitrogen, and stored at below -70°C until evaluated for the CAI concentration. Brain of three untreated, tumored mice was also collected.

In earlier studies, PEG 400 given daily caused bloating, so a dosing schedule comprising two rounds of dosing was implemented. In the combination groups, temozolomide was administered first, followed immediately by the administration of CTO in the same group order.

Drugs and dosages: Temozolomide vehicle and CTO vehicle were both administered by oral gavage (p.o.) once daily for five consecutive days for two rounds, starting on Day 13 (Days 13-17) and then again on Day 27 (Days 27-31); CTO was administered p.o. at doses of 513 and 342 mg/kg/injection once daily for five consecutive days for two rounds, starting on Day 13 (Days 13-17) and then again on Day 27 (Days 27-31); temozolomide was administered p.o. at a dose of 17 mg/kg/injection once daily for five consecutive days for two rounds, starting on Day 13 (Days 13-17) and then again on Day 27 (Days 27-31); and treatment groups (n = 10/group) received CTO 513 mg/kg and 342 mg/kg alone; temozolomide

17 mg/kg alone; and temozolomide in combination with CTO at the dosages described above.

Two groups of animals received a single oral treatment of CTO at a dose of 513 mg/kg or 342 mg/kg on Day 14. Both test compounds and their vehicles were administered to mice by exact individual animal's body weight on each day of treatment, with an injection volume of 0.1 mL/10 g body weight. These animals were euthanized on Day 14 for tissue collection. The study was terminated on Day 76 after tumor fragment implantation. Any animal whose tumor reached 4,000 mg in weight was euthanized prior to scheduled day of study termination for humane reasons.

For the HT29 studies, each mouse was implanted s.c. near the right flank with an approximate 30-40 mg fragment of HT29 human colon tumor from an in vivo passage using a 13-gauge trocar needle. The day of tumor implantation was designated as Day 0. Tumors were allowed to reach 75-198 mg in weight (75-198 mm3 in size) before the start of treatment. A sufficient number of mice were implanted so that tumors in a weight range as narrow as possible were selected for the trial on the first day of treatment (Day 13 after tumor implantation). Those animals selected with tumors in the proper size range were assigned to the various treatment groups. The median tumor weights on the first day of treatment ranged from 135 mg to 153 mg and the mean tumor weights ranged from 145 mg to 147 mg.

Drugs and dosage were as follows: CTO vehicle + 100% PEG 400, p.o. daily x 14 days (Days 13-26); CTO 513 mg/kg and CTO 342 mg/kg p.o. daily for 14 days (Days 13-26); 5-FU 75 mg/kg/dose intraperitoneal (i.p.) injections every 4 days on Days 13, 17, 21, and 25; bevacizumab 60 mg/kg/dose and 40 mg/kg/dose intravenously (i.v.) every 4 days on Days 13, 17, 21, and 25. Treatment groups (n = 10/group) received CTO 513 mg/kg and 342 mg/kg alone; bevacizumab 60 mg/kg and 40 mg/kg alone; 5-FU 75 mg/kg alone; and 5-FU in combination with CTO at the dosages described above, and in combination with bevacizumab at the dosages described above.

On the days when both 5-FU and CTO or 5-FU and bevacizumab were administered, 5-FU was administered first to all four combination groups, followed immediately by the administration of CTO or bevacizumab. Animals in the control group were treated p.o. with the CTO vehicle, 100% PEG 400, administered on a once-daily x 14 schedule. Except for one animal, all dead and two moribund animals in the p.o. treated groups were necropsied to check for signs of possible gavage-related trauma.

On Day 41, the day of study termination, five animals from the group treated with the vehicle, CTO at a dosage of 513 mg/kg/dose, and bevacizumab at a dosage of 60 mg/kg/dose were necropsied. All mice received postmortem examination of the gastrointestinal (GI) tract and macroscopic observations, if any, were recorded, including the color of the tissues. Stomach, duodenum, jejunum, ileum, cecum, colon, rectum, and mesenteric lymph node were collected. Any moribund animal was euthanized prior to the scheduled day of study termination.

E. Parameters evaluated

For the U251 studies, the numbers of nonspecific deaths, complete tumor regressions, tumor-free survivors on Day 76, and median time to reach three tumor mass doublings were determined. The median time to reach three 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). Median tumor weight in the treatment groups (T) was compared with the median tumor weight in the control group (T/C x 100%) on Day 34 (3 days after the last treatment) and on Day 76 (the day of study termination) to further evaluate the antitumor efficacy of the test compounds. Results are summarized in Table 1.

For the HT29 studies, the numbers of nonspecific deaths, partial and complete tumor regressions, tumor-free survivors, and the individual animal's time to reach two tumor mass doublings were determined. The individual animal's time to reach two tumor mass doublings was used in the calculation of the overall delay in the growth of the median tumor (T-C). Median tumor weight in the treatment groups (T) was compared with the median tumor weight in the control group (T/C x 100%) on Day 27 (1 day after the last treatment with CTO and 2 days after the last treatment with 5-FU and bevacizumab) and on Day 41 (the day of study termination) to further evaluate the antitumor efficacy of the test compounds.

F. Statistical analysis

For the U251 studies, the individual animal's time to reach three tumor mass doublings was used as the endpoint in a life tables analysis (survival analysis followed by a log-rank test). The difference between the groups was considered to be significant if the P value was equal to or less than 0.05. 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.

For the HT29 studies, the individual animal's time to reach two tumor mass doublings and the individual animal's tumor weight on Day 41 were used as the endpoint in Student t-test (or the Mann-Whitney rank sum test) in order to compare statistically the growth data between groups. A nonparametric test was used in place of the t-test when the data set did not pass the normality or equal variance test.

III. Results

The effects of the administration of CTO alone, temozolomide alone, and CTO in combination with temozolomide on tumor regression, number of tumor-free survivors, and median time to three tumor mass doublings were evaluated using U251 GBM xenografts in NCr-*nu*/*nu* male mice. A summary of the U251 GBM xenograft experimental results, including median days to three doublings, growth delay, and median T/C, is presented in **Table 1**.

Treatment Compound	Dose mg/kg/inj	Route	No. of Animals	Non-specific Deaths	Regressions (100%)	Tumor-free on Day 76	Median Days to 3 Doublings	Growth Delay (T-C)[a]	Median T/C (%) Day 34	Median T/C (%) Day 76
Temozolomide vehicle	0	PO	10	0	0	0	21.1			
CTO vehicle	0	PO	10	0	0	0				
CTO	513	PO	10	0	0	0	31.9	10.8	61	88
CTO	342	PO	10	0	0	0	22.3	1.2	104	94
Temozolomide	17	PO	10	1	0	0	49.5	28.4	13	56
Temozolomide	17	PO	10	0	0	0	>63	>41.9	10	33
CTO	513	PO	10	0	0	0				
Temozolomide	17	PO	10	0	0	0	59.9	38.8	11	41
CTO	342	PO	10	0	0	0				
CTO	513	PO	6	0	0	0	N/A	N/A		
CTO	342	PO	6	0	0	0	N/A	N/A		

Table 1. Summary of U251 CNS Xenograft Results. Response of SC U251 GBM Tumor to Treatment with CTO or CTO in Combination with Temozolomide.
a growth delay calculations are based on median days to 3 doublings.

Control, vehicle-treated U251 human glioblastoma xenografts grew progressively in all 10 animals, reaching 1,017 mg in weight on Day 34 and 3,612 mg in weight on Day 76. CTO 342 mg/kg monotherapy had no significant inhibitory effect on tumor growth, measured by median time to three tumor mass doublings, relative to vehicle-treated mice (P=0.615).

Conversely, CTO 513 mg/kg monotherapy had a statistically significant inhibitory effect on tumor growth relative to vehicle-treated mice (P=0.034). Median tumor delay relative to vehicle-treated mice is shown in **Figure 1a**.

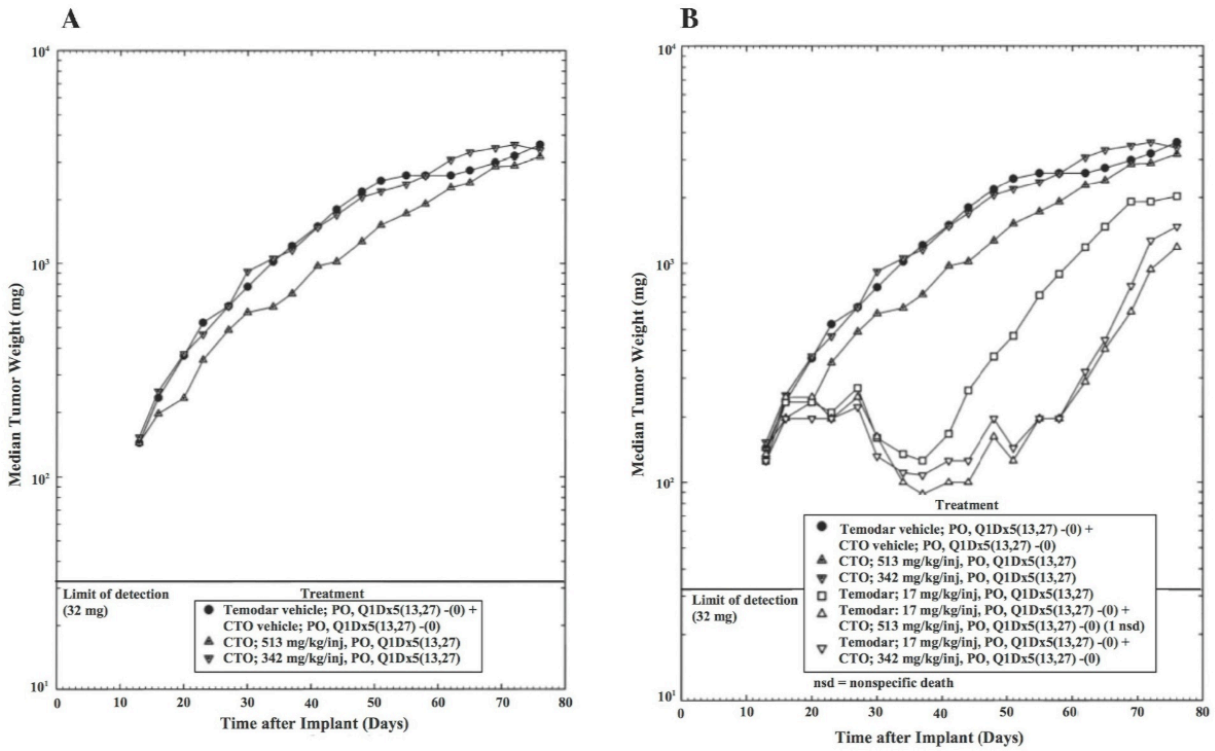

Figure 1a: Response of s.c. U251 Human CNS Tumor to Treatment with CTO
Figure 1b: Response of s.c. U251 Human CNS Tumor to Combination Treatment with Temodar (Temozolomide) and CTO

Temozolomide 17 mg/kg monotherapy had a statistically significant inhibitory effect on tumor growth relative to vehicle-treated mice (P=0.004). Median tumor delay relative to vehicle-treated mice is shown in **Figure 1b**.

The combination of temozolomide and CTO 342 mg/kg also had a statistically significant inhibitory effect on tumor growth relative to vehicle-treated mice (P<0.001). Median tumor delay relative to vehicle-treated mice is shown in **Figure 1b**. This combination therapy was not statistically different from the temozolomide monotherapy group (P=0.182). However, the antitumor activity of the combination was greater than additive compared to the antitumor activity of each compound alone (**Figure 1b**).

The combination of temozolomide with CTO 513 mg/kg also had a statistically significant inhibitory effect on tumor growth. In this combination group, six of nine tumors did not reach three mass doublings (P<0.001). Median tumor delay relative to vehicle-treated mice is shown in **Figure 1b**. Tumor growth in this combination group was also statistically significant (P<0.001) compared with the growth of the tumors in the group treated with CTO at a dose of 513 mg/kg alone, and statistically significant (P=0.028) compared to the growth of the tumors in the group treated with temozolomide at a dose of 17 mg/kg alone (**Figure 1b**).

Treatment with temozolomide and combination treatment with temozolomide and CTO 342 mg/kg was tolerated without deaths. Combination treatment with temozolomide and CTO 513 mg/kg resulted in one non-specific death.The CAI concentration in mouse brain after a single oral treatment with CTO at a dose of 513 mg/kg or 342 mg/kg 6 hours and 8 hours after the treatment is presented in **Table 2**.

The effects of the administration of CTO alone, 5-FU alone, bevacizumab alone; CTO in combination with 5-FU; and 5-FU in combination with bevacizumab on tumor regression, number of tumor-free survivors, and median time to two tumor mass doublings were evaluated using HT29 colon cancer xenografts in NCr-*nu*/*nu* mice. A summary of the HT29 colon cancer xenograft experimental results, including median days to two doublings, growth delay, and median T/C, is presented in **Table 3**.

CAI Concentration in Brain After Single Oral Treatment with CTO		
	6h (animals 1, 2, 3)	**8h (animals 4, 5, 6)**
CTO 513 mg/kg/inj	11,883 ± 1,291 ng/g	15,167 ± 2,372 ng/g
CTO 342 mg/kg/inj	12,483 ng/g ± 1,020 ng/g	10,950 ng/g ± 1,704 ng/g

Treatment			No. of Animals	Non-specific Deaths	Regressions	Tumor-free Survival	Median Days to 2 Doublings	Growth Delay (T-C)[a]	Median T/C (%) Day 27	Median T/C (%) Day 41
Agent	**Dose mg/kg**	**Route**								
Control	0	PO	10	0	0	0	13.2	—	—	—
5-FU	75	IP	10	0	0	0	20.0	6.8	77	77
CTO	513	PO	10	0	0	0	20.3	7.1	62	61
CTO	342	PO	10	1	0	0	20.6	7.4	61	62
5-FU	75	IP	10	5	0	0	22.5	9.3	48	46 (TOXIC)
CTO	513	PO								
5-FU	75	IP	10	2	0	0	23.3	10.1	63	62
CTO	342	PO								
Bevacizumab	60	IV	10	0	0	0	18.7	5.5	84	66
Bevacizumab	40	IV	10	0	0	0	18.4	5.2	81	69
5-FU	75	IP	10	0	0	0	21.3	8.1	71	66
Bevacizumab	60	IV								
5-FU	75	IP	10	0	0	0	22.1	8.9	74	62
Bevacizumab	40	IV								

a. Growth delay calculations are based on median days to 2 doublings.

Table 2: CAI Concentration in Mouse Brain

Table 3: Summary of HT29 Xenograft Results. Response of SC HT29 Colon Tumor to Treatment with 5-FU in Combination with CTO or Bevacizumab

Control, vehicle-treated HT29 human colon tumor xenografts grew progressively in all 10 animals reaching 600 and 1,405 mg in weight on Days 27 and 41, respectively. Growth of the tumors in the vehicle-treated animals was associated with a maximum average body weight loss of 3% (0.8 g). In this study, CTO was prepared in 100% PEG 400 and administered daily. However, this regiment caused PEG-400 related toxicity and, therefore, later studies used an intermittent administration of CTO and/or 40% PEG 400 diluted with deionized water.

CTO monotherapy had a statistically significant inhibitory effect on tumor growth for the 513 mg/kg and 342 mg/kg doses relative to vehicle-treated mice, respectively (P<0.0001 for both CTO doses) and tumor weights relative to vehicle-treated mice on Day 41 (P=0.001 and P=0.005 for 513 and 342 mg/kg doses, respectively). Antitumor activity was not dose dependent. 5-FU monotherapy treatment had a statistically significant inhibitory effect on tumor growth relative to vehicle-treated mice (P=0.0046). However, the difference in tumor weights on Day 41 between the two groups was not statistically significant (P=0.182).

Bevacizumab monotherapy had statistically significant inhibitory effects on tumor growth when tumor weights on Day 41 were compared to vehicle-treated mice (P=0.0024 and P=0.0286 for 60 and 40 mg/kg/dose, respectively). However, only the 60 mg/kg/dose treatment with bevacizumab had a statistically significant inhibitory effect
on tumor growth relative to vehicle-treated mice when individual animal's times to reach two tumor mass doublings were compared (P=0.0028). Bevacizumab monotherapy treatment resulted in measurable antitumor activity, producing median tumor growth delays at dosages of 60 and 40 mg/kg/dose, respectively, relative to vehicle-treated mice.

The combination treatment of 5-FU at a dosage of 75 mg/kg/dose plus CTO 513 mg/kg/dose yielded a median time to doubling of 22.5 days. However, the combination was toxic, resulting in death of four of 10 animals. One more animal was euthanized due to being moribund. The treatment was associated with a maximum average body weight loss of 18%. The combination treatment of 5-FU at a dosage of 75 mg/kg/dose plus CTO at a dosage of 342 mg/kg/dose resulted in death of two of 10 animals and was associated with a maximum average body weight loss of 14%. Because MTD is defined as no deaths or no more than 20% average body weight loss during and within 14 days of the end of the treatment in this experiment, both combination treatments were in excess of the MTD.

Hence, additional studies are needed to determine a non-toxic combination of 5-FU and CTO. The combination of 5-FU with CTO 342 mg/kg/dose had a statistically significant inhibitory effect on tumor growth when the median time to reach two tumor mass doublings and tumor weights on Day 41 were compared to vehicle-treated mice (P=0.0001 and P=0.001, respectively). However, growth of the tumors in the combination group was not statistically different from the growth of the tumors in the group treated with CTO alone at the corresponding dosage (P=0.001 versus P=0.005). The difference in tumor growth in the combination group was statistically significant compared to the tumor growth in the 5-FU monotherapy-treated group (P=0.001 versus P=0.182). Response of the HT29 colon tumor xenografts to the combination treatment of 5-FU with CTO is graphically presented in **Figure 2**.

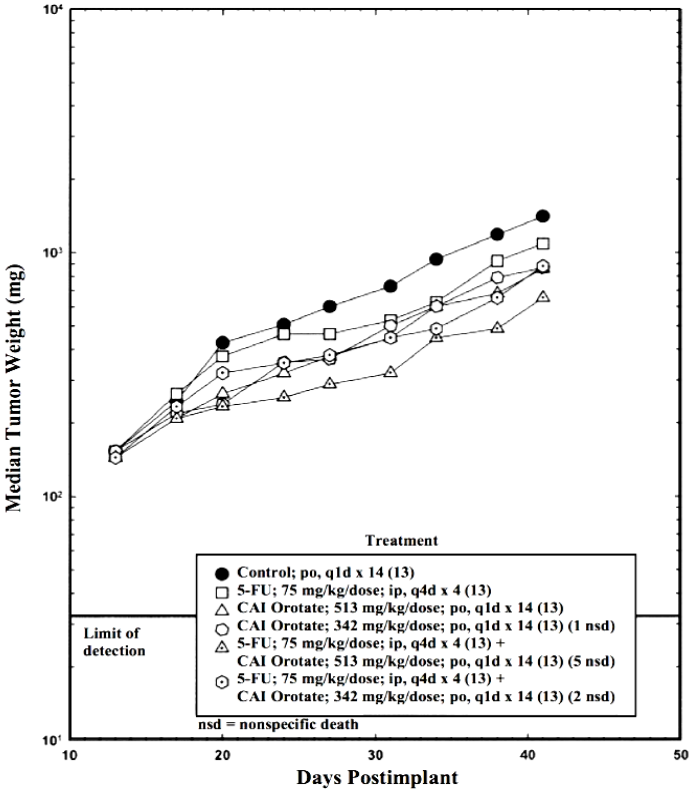

Figure 2: Response of s.c. HT29 Colon Tumor to Combination Treatment with 5-FU and CAI Orotate (CTO)

The combination of 5-FU with bevacizumab at dosages of 60 or 40 mg/kg/dose had a statistically significant inhibitory effect on tumor growth for the 60 mg/kg and 40 mg/kg doses, respectively, relative to vehicle-treated mice (P=0.0036 and P=0.0029 for the 60 and 40 mg/kg dose, respectively), and tumor weights relative to vehicle-treated mice on Day 41 (P=0.0017 and P=0.0035 for the 60 and 40 mg/kg dose, respectively). However, growth of the tumors in the combination groups was not statistically different from the growth of the tumors in the group treated with 5-FU alone or bevacizumab alone at corresponding dosages. Response of the HT29 colon tumor xenografts to the combination treatment of 5- FU with bevacizumab is graphically presented in **Figure 3a**.

5-FU at 75 mg/kg/dose, CTO at 513 mg/kg/dose, bevacizumab at 60 mg/kg/dose, and 5-FU plus bevacizumab combination therapy at both bevacizumab doses were tolerated without deaths. CTO 342 mg/kg monotherapy resulted in one death. Bevacizumab 40 mg/kg resulted in one death that was not treatment-related. 5-FU plus CTO 513 mg/kg was toxic and resulted in five deaths, while the combination with CTO 342 mg/kg resulted in two deaths; both combinations were in excess of the MTD in this experiment. The toxicity was partly due to the use of 100% PEG 400 and daily administration of CTO, which caused loss in appetite and body weight loss even in the control group.

Figure 3b shows a comparison of 5-FU at 75 mg/kg/dose alone or in combination with CTO at 513 mg/kg/dose or bevacizumab at 60 mg/kg/dose. The combination of 5-FU with CTO 513 mg/kg/dose resulted in inhibition of tumor burden compared with 5-FU and bevacizumab.

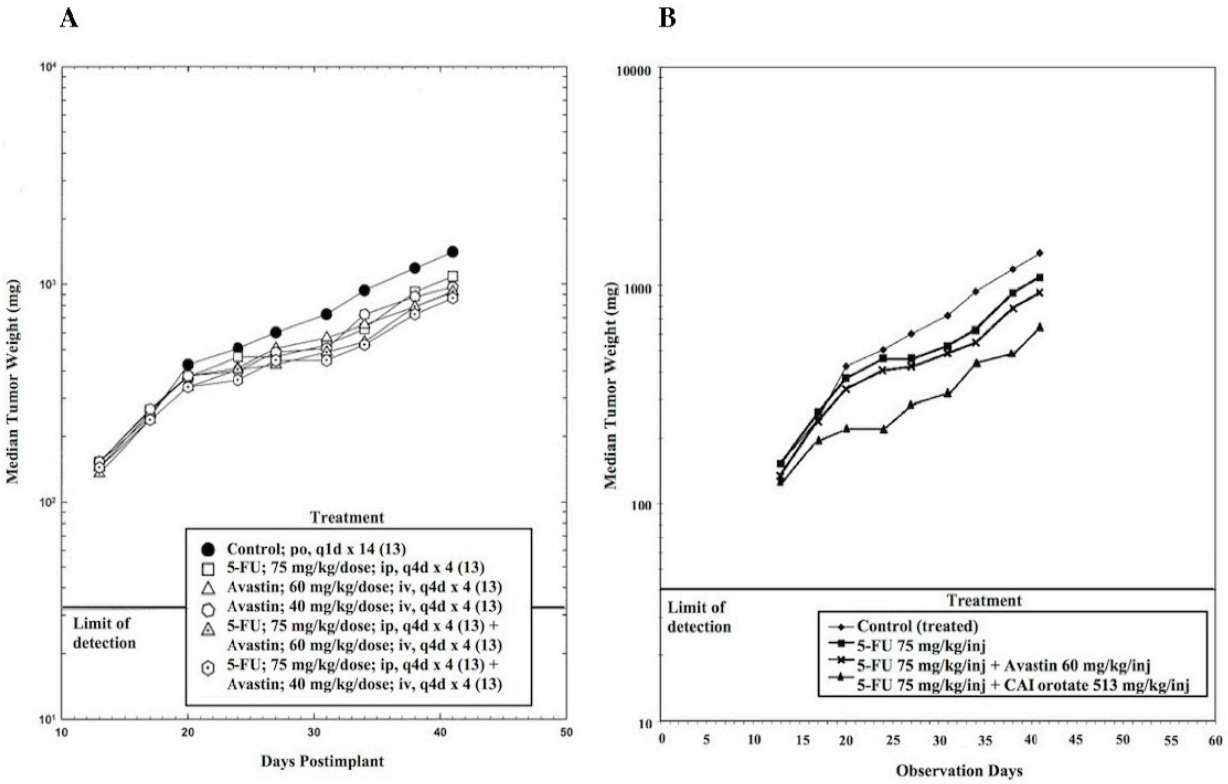

Figure 3a: Response of s.c. HT29 Colon Cancer Tumor to Combination Treatment with 5-FU and Avastin (Bevacizumab)

Figure 3b: Response of s.c. HT29 Colon Cancer Tumor to 5-FU alone or in Combination Treatment with 5-FU and CTO or 5-FU and Avastin (Bevacizumab)

IV. Discussion

Does CTO alone, or the combination of CTO with cytotoxic chemotherapy offer a potentially improved therapeutic approach to GBM and colon cancer treatment? To answer this question, we tested CTO alone and in combination with chemotherapeutic agents against human glioblastoma xenografts and human colon tumor xenografts in athymic NCr-*nu*/*nu* mice. Our results indicate that CTO in combination with chemotherapeutic agents offers a potentially improved approach to GBM and colon cancer treatment.

In these studies, efficacy was determined by measuring the time delay to doubling or tripling of the tumor, delay in tumor growth, and tumor weight relative to vehicle controls. We have found that CTO exhibits significant antitumor effects when administered in combination with cytotoxic chemotherapy in two mouse xenograft tumor models; and that the combination is more efficacious than chemotherapy alone.

No drug-associated toxicity was observed in the administration of CTO monotherapy, or in combination therapy with temozolomide in our GBM xenograft model.

In contrast, combination therapy with 5-FU and both doses of CTO was toxic in our HT29 colon cancer xenograft model, and further studies are needed to determine a non-toxic combination.

In the GBM xenograft model, temozolomide monotherapy shows inhibitory activity. High-dose CTO monotherapy has some inhibitory activity but is less active than temozolomide; however, both low- and high-dose CTO in combination with temozolomide have synergistic activity that yields significantly greater inhibition than temozolomide alone. Particularly striking was the combination of CTO 513 mg/kg with temozolomide in the GBM xenograft model, in which six of nine tumors did not reach three mass doublings. Our U251 GBM xenograft studies also demonstrate that CAI is found in mouse brain after treatment with CTO.

In the HT29 colon cancer xenograft model, CTO and bevacizumab have equivalent efficacy. Low- and high-dose CTO in combination with 5-FU show improved efficacy compared to combination therapy with 5-FU and bevacizumab, although a non-toxic high-dose CTO needs to be determined.

Our results add to the growing body of data supporting the efficacy of combination therapy with VEGF inhibitors and cytotoxic chemotherapy. Treatment with the combination of temozolomide and CEP-7055, a VEGFR inhibitor, improved the median survival of nude mice bearing GBM xenografts compared to temozolomide alone (Jones-Bolin et al, 2006). Treatment with bevacizumab and irinotecan in patients with recurring GBM was well tolerated, with notable antitumor activity of single-agent bevacizumab and its combination with irinotecan (Friedman et al, 2009). The combination of bevacizumab and irinotecan for the treatment of grade 3 malignant glioma was found to be an active regimen with acceptable toxicity (Desjardins et al, 2008). In a study of patients with colorectal cancer treated with a chemotherapy cocktail (irinotecan, bolus fluorouracil, and leucovorin [IFL]) with and without bevacizumab, the addition of bevacizumab resulted in improved survival (Hurwitz et al, 2004).

CAI inhibits calcium influx and calcium release from intracellular stores (Faehling et al, 2002) and has been tested in patients with solid cancers in Phase 1-3 trials. However, NCI stopped the trials for failure to demonstrate efficacy, or because CAI exhibited poor bioavailability, severe toxicity, and tolerability issues that prevented optimum dosing. Clinical studies using micronized CAI in PEG-400 improved bioavailability (Alessandro et al, 2008; Bauer et al, 2000; Kohn et al, 2001; Yasui et al, 1997), but dose-limiting toxicities persisted (Berlin et al, 1997).

The hydrophobic properties of CAI make oral absorption variable and low. The addition of the orotate improves dissolution, increases oral bioavailability, and speeds absorption into the bloodstream, leading to higher plasma concentrations. CTO and CAI have a similar half-life profile. Importantly, CTO has a safer toxicity profile than CAI in rats, dogs, and humans, thus enabling the use of CTO as adjunctive therapy with cytotoxic agents (Grover, 2007).

Given its significant inhibitory effect on tumor growth in combination with temozolomide, CTO shows potential in the treatment of human GBM. A Phase 1 trial is currently underway to evaluate CTO monotherapy and CTO in combination with temozolomide in patients with GBM.

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