Tetraidothyroacetic Acid (Tetrac) and Tetrac Nanoparticles Inhibit Growth of Human Renal Cell Carcinoma Xenografts

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Abstract. *Renal cell carcinoma is the most lethal of the common urologic malignancies, with no available effective therapeutics. Tetrac (tetraiodothyroacetic acid) is a deaminated analogue of L-thyroxine (T_4) that blocks the pro-angiogenesis actions of T_4 and 3, 5, 3'-triiodo-L-thyronine as well as other growth factors at the cell surface receptor for thyroid hormone on integrin $\alpha v\beta 3$. Since this integrin is expressed on cancer cells and also on endothelial and vascular smooth cells, the possibility exists that Tetrac may act on both cell types to block the proliferative effects of thyroid hormone on tumor growth and tumor-related angiogenesis. To test this hypothesis, we determined the effect of Tetrac on tumor cell proliferation and on related angiogenesis of human renal cell carcinoma (RCC). We used two models: tumor cell implants in the chick chorioallantoic membrane (CAM) system and xenografts in nude mice. To determine the relative contribution of the nuclear versus the plasma membrane action of Tetrac, we compared the effects of unmodified Tetrac to Tetrac covalently linked to poly (lactide-co-glycolide) as a nanoparticle (Tetrac NP) that acts exclusively at the cell surface through the integrin receptor. In the CAM model, Tetrac and Tetrac NP (both at 1 μg/CAM) arrested tumor-related angiogenesis and tumor growth. In the mouse xenograft model, Tetrac and Tetrac NP promptly reduced tumor volume ($p<0.01$) when administered daily for up to 20 days. Animal weight gain was comparable in the control and treatment groups. Overall, the findings presented here provide evidence for the anti-angiogenic, and anti-tumor actions of Tetrac and Tetrac NP and suggest their potential utility in the treatment of renal cell carcinoma.*

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Renal cell carcinoma (RCC) is the most lethal of the common urologic malignancies, with approximately 40% of patients eventually dying of cancer progression (1, 2). Approximately one third of patients presented with metastatic disease, and up to 40% treated for localized disease have a recurrence (1, 2). Cytokine-based immunotherapy is considered standard treatment for metastatic RCC today (3). However, new therapies such as tumor vaccines, anti-angiogenesis agents, and small molecule inhibitors are being developed to improve efficacy in patients who are either less tolerant or resistant to systemic immunotherapy (3, 4). The aim of this investigation was to determine whether a thyroid hormone antagonist, tetraiodothyroacetic acid (Tetrac), has an antitumor effect against RCC and to define the underlying mechanism of its action. Particular emphasis was directed toward understanding whether the antitumor effects are initiated at the plasma membrane, through its interaction with the integrin $\alpha v\beta 3$ receptor (5), or at the nuclear level as a result of its potential interaction with the nuclear thyroid hormone receptor (TR). Previous studies from our laboratory have demonstrated that the thyroid hormone signal is transduced through this cell surface receptor into an angiogenic response (6, 7) in endothelial cells and vascular smooth muscle cells, as well as into a proliferative response in cancer cells that express this receptor (8, 9). The proliferative response is generated by L-thyroxine (T_4) at physiological concentrations whereas that of its analog 3, 5, 3'-triiodo-L-thyronine (T_3) is detected only at pharmacological concentrations (5). The deaminated T_4 analogue, Tetrac, was found to act as an antagonist at the integrin receptor (5) by blocking the binding of thyroid

hormones to this receptor. Previous work from our laboratory has demonstrated that, in addition to its inhibitory effects on cell proliferation, Tetrac acts also to suppress angiogenesis (10). Moreover, Tetrac was also found to inhibit tumor growth in a human breast cancer xenograft in the nude mouse (11), which suggested a dual action on endothelial and cancer cells. At the plasma membrane level, the site of action of Tetrac is located at the Arg-Gly-Asp (RGD) recognition pocket on the integrin αvβ3 (5, 12), known for its essential role in facilitating interactions of the integrin with extracellular matrix proteins and growth factors (12, 13). Evidence has also been provided that this site may be implicated in the clustering of and crosstalk between the integrin and the vascular endothelial growth factor receptor (VEGFR) (14) and basic fibroblast growth factor receptor (bFGFR) (15). For example, the pro-angiogenic activities of VEGF and bFGF are blocked by RGD peptides and by Tetrac (10).

Taking this into account, it was important to determine the relative contributions of nuclear TR and the integrin αvβ3-initiated signaling pathway to the anti-tumor action of Tetrac. To differentiate between these two sites of action, we synthesized Tetrac linked to nanoparticles that, due to their size, cannot enter the cell. These particles, consisting of Tetrac bound at the outer ring hydroxyl by an ether linkage to poly (lactic-co-glycolic acid) (PLGA) (Tetrac NP), have an average size of 200 nm which represents a hindrance of their entry, and thus that of Tetrac, into the cell. Our findings indicate that Tetrac and Tetrac NP have similar effects on inhibition of cancer cell proliferation and angiogenesis both in the chick chorioallantoic membrane (CAM) and mouse tumor xenograft models, suggesting that Tetrac acts largely at the cell surface. The implication of these findings for therapy of RCC and others types of cancer is discussed.

Materials and Methods

Materials. Polyvinyl alcohol (PVA), N-(3-dimethylaminopropyl-*N*'-ethylcarbodiimide-hydrochloride (EDC) and the dialysis tubing cellulose membrane were purchased from Sigma Aldrich, St. Louis, MO, USA. Poly (d-lactide-co-glycolide) (70:30) (PLGA) was purchased from Polysciences Inc. (Warrington, PA, USA). Ethylenediamine dihydrochloride was obtained from Pierce Biotechnology, Rockford, IL, USA. Epoxy-Tetrac intermediates were custom-synthesized by Azopharma Contract Pharmaceutical Services (Miramar, FL, USA). Obtained from Biocare Medical (Concord, CA, USA) were Diva Pretreatment Solution (DV2004), aqua DePar (ADP1002), Mach 4 detection kit (M4U534), Background Sniper (BS966), rat anti-mouse CD31 detection kit (Predilute, RT517), Factor VIII (CP039, used at 1:100), Wash Buffer (TWB945) and DAB (BDB 2004). Hemoglobin standard, Drabkin's reagent and other common reagents were purchased from Sigma.

Methods. PLGA nanoparticles were synthesized by modification of a method (single emulsion solvent diffusion) originally described by Jeffer *et al*. (16) and Song *et al*. (17). Amino-functionalized PLGA nanoparticles were obtained by conjugating these PLGA nanoparticles with ethylenediamine, using carbadiimide chemistry. Finally, amino-functionalized PLGA nanoparticles were reacted with Tetrac intermediate, supplied by Azopharma Contract Pharmaceutical Services to obtain the final product, Tetrac-conjugated PLGA nanoparticles. The custom made intermediate of Tetrac composed of Tetrac conjugated to epibromohydrin through the phenolic –OH group present on it. This epoxy group reacts with the amino group (18, 19) present in the modified PLGA nanoparticles in aqueous conditions. In a typical experiment, PLGA nanoparticles were synthesized by adding 200 μl of PLGA (40 mg/ml in DMSO) to 20 ml of a 1% aqueous solution of PVA. This mixture was constantly stirred for about 12 h at room temperature to form the nanoparticles. The nanoparticle suspension was then dialyzed for 6 h using a dialysis membrane of 10-12 kDa cutoff to remove the impurities as well as the organic solvent. To 18 ml of this void PLGA nanoparticle solution were added 2 ml of PBS buffer (10×) and 500 μl of *N*-(3 dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) (180 mg/ml in 10xPBS). The mixture was stirred for about 1 h, followed by another addition of 500 μl of ethylenediamine (280 mg/ml in 10× PBS) and magnetic stirring was continued for at least 24 h. The whole solution was then dialyzed (10-12 kDa cutoff membrane) for 10-12 h to eliminate un-reacted materials. A stock solution of the custom-made expoxy activated Tetrac (Azopharma) in anhydrous DMSO (5 mg/ml) was prepared and 100 μl of this activated Tetrac solution was added to 10 ml of the above amino-functionalized PLGA nanoparticles and stirred for at least 24 h. The solution was then dialyzed for at least 12 h for purification (12 kDa cutoff membrane) and lyophilized. These lyophilized Tetrac-conjugated PLGA nanoparticles (Figure 1) were reconstituted in PBS and used for the experiments described below.

Cells and cell culture. Human renal carcinoma cells (ATCC-CR1932-786-0 cells, Lot # 4323818) were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured as instructed by the supplier, using a complete growth medium consisting of F-12K medium with 10% FBS. Cells were cultured in 5% CO_2/air atmosphere at 37˚C to sub-confluence and then treated with 0.25% (w/v) trypsin/EDTA to effect cell release from the culture vessel. After washing the cells with culture medium, cells were suspended in DMEM [free of phenol red and fetal calf serum (FCS)] and counted.

Tetrac versus Tetrac NP distribution in RCC. RCC renal cell carcinoma (RCC) were grown in RPMI-1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% FCS (Atlanta Biologicals, Lawrenceville, GA, USA). Penicillin/streptomycin (1%) was present in both culture media. The cells were trypsinized, centrifuged, and the cell pellet was re-suspended in the corresponding media. Subsequently, 500 μl of the suspension (~50,000 cells) were transferred to each well of a 4-well glass slide, Chamber slide System (Nalge Nunc International, Naperville, IL, USA) and incubated for 24 h at 37˚C with 5% CO_2 (Thermo Electron Corp., Forma Series II). The cells were treated with 20 μl of each free Tetrac tagged with Cy3 dye and PLGA-Tetrac tagged with Cy3 separately incubated (37˚C, 5% CO_2) for around 2 h. After 2 h of incubation, the plates were taken out and washed several times with PBS and then fixed in 1% formaldehyde (Sigma), and mounted with the help of Vectashield (Vector Laboratories Inc, Burlingame, CA, USA. Confocal images were

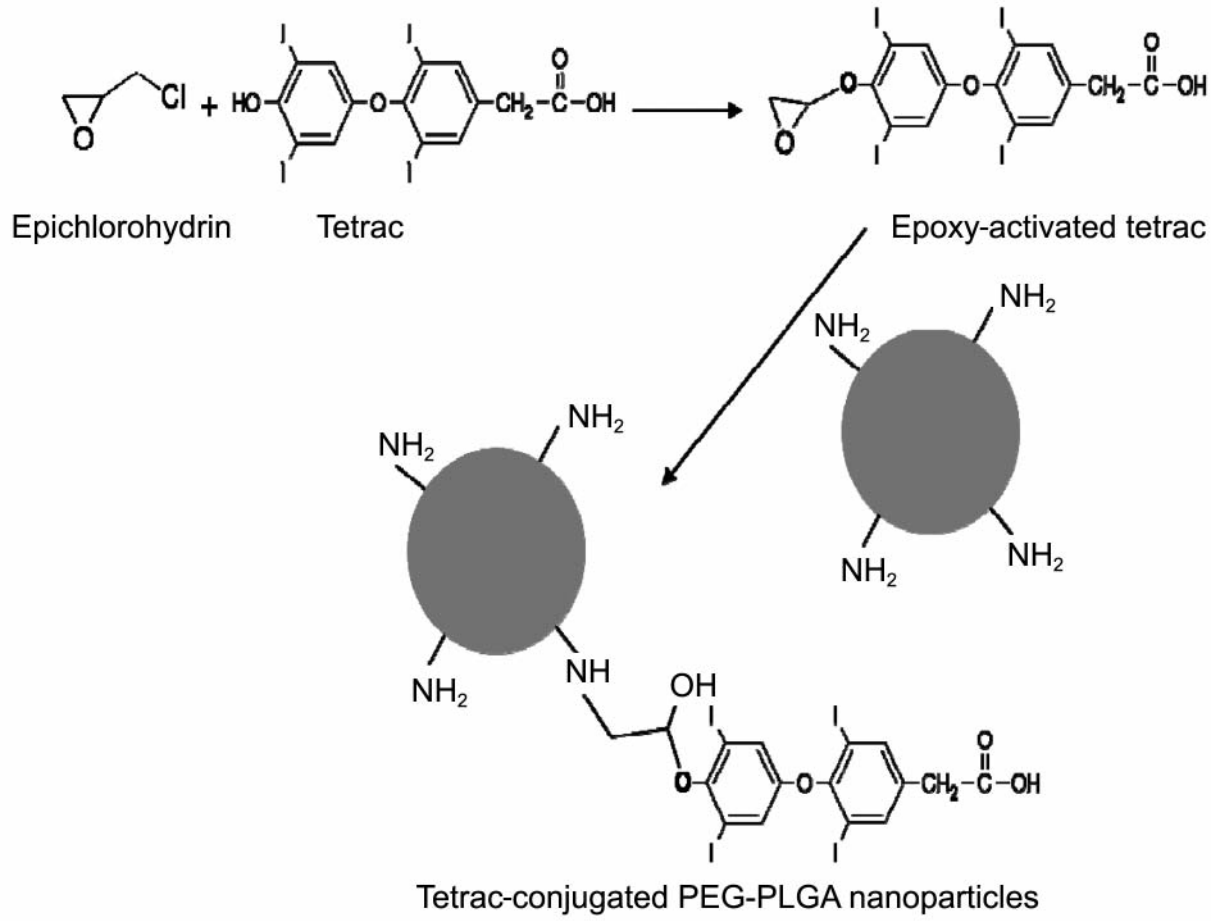

Figure 1. *Description of the structure of the nanoparticles used.*

taken using a Leica TCS SP5 Confocal with a 63x (NA=1.3 glycerol immersion) objective. A 405nm laser was used for excitation, and emission was detected between 565 nm and 688 nm. Figure 2 is a representative confocal image illustrating the cellular distribution of Cy3-Tetrac (A) *versus* Cy3-Tetrac NP (B) in renal cell carcinoma.

Tumor growth in the CAM cancer implant model. The effect of Tetrac *versus* Tetrac nanoparticles at 1.0 μg/CAM on tumor angiogenesis and tumor growth of 1×10^6 renal carcinoma cells implanted in matrigel in 7-day old chick eggs was determined 8 d after implant (20). Matrigel® (BD Bioscience, San Jose, CA, USA) was thawed overnight at 4˚C and placed on ice. The RCC cells in exponential growth phase were harvested using 0.25% trypsin–EDTA washed and suspended in medium. Only suspensions of single cells with a viability exceeding 95% were used. Approximately 1×10^6 cells in 30 μl of medium were mixed with the same volume (30 μl) of Matrigel® and implanted on the chorioallantoic membrane. Results are presented as mean tumor weight (mg) per treatment group and tumor hemoglobin (mg/dl)±SEM, n=8 per group.

Cell implantation in nude mice. Female NCr nude homozygous mice aged 5-6 weeks and body weights of 20 g were purchased from Taconic Farms (Hudson, NY, USA). Animals were maintained under specific pathogen-free conditions and housed 4 animals per cage, under controlled conditions of temperature (20-24° C) and humidity (60-70%) and a 12 h light/dark cycle. Water and food were provided *ad libitum*. Xenograft experiments were carried out in the animal facility of the Veterans Affairs (VA) Medical Center, Albany, NY, and the experimental protocol was approved by the VA-IACUC. Mice were allowed to acclimatize for 5 d prior to the start of treatments. Subcutaneous implant of 1×10^7 renal cell carcinoma cells was carried out in the flank regions of each mouse.

Treatment of animals with unmodified or nanoparticle Tetrac. Tumors were measured daily by calipers and tumor volume was calculated according to a standard formula ($W\times L^2/2$), where W=width and L=length. Tumor volume measurements were made after the implant every other day for the first 4 weeks after inoculation. Tumor volumes were measurable 3 d after implant and the treatment was started on day 10. Mice with tumors of 350-450 mm^3 proximal to the injection site were randomized into 3 groups (n=8): control, Tetrac (1 mg/kg) group and nanoparticle Tetrac (1 mg/kg) group. Drugs were administered intraperitoneally (*i.p.*) every 2 d for 32 d. Mice were weighed every other day and tumor size was measured every other day until the end of the experiment. Animals were then sacrificed in a CO_2 chamber and tumor masses were collected and weighed.

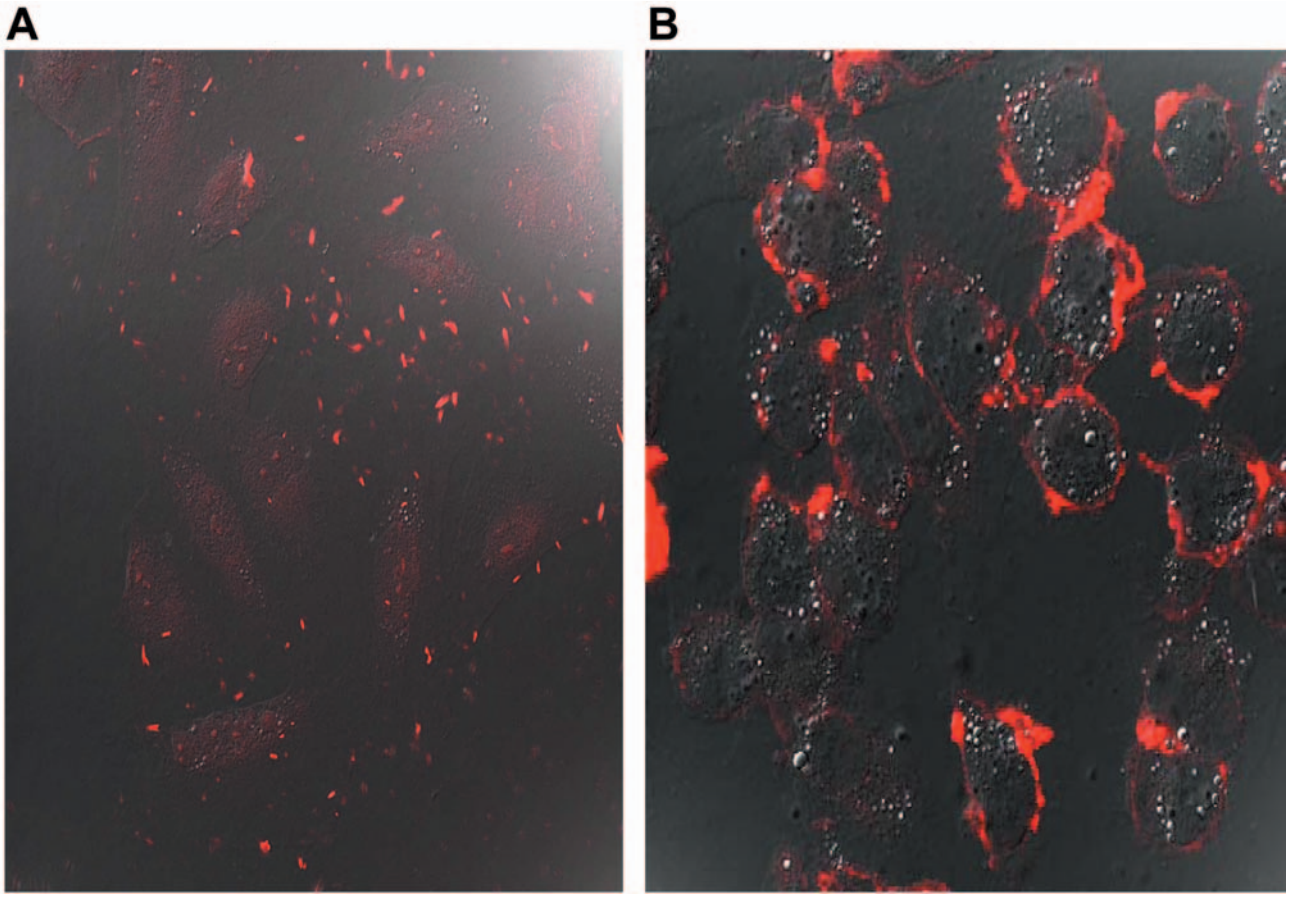

Figure 2. *Representative confocal images illustrating the differences in distribution between Tetrac (A) and Tetrac NP (B) in human renal carcinoma cells. Cy3-labeled Tetrac NP (PLGA-Tetrac nanoparticles) is on the cell surface (B), whereas Cy3-labeled Tetrac is distributted homogenously in the cytoplasm and over the nucleus (A).*

Statistical analysis. Statistical analysis was performed by one-way ANOVA, using Statview software (Adept Scientific, Acton, MA, USA) and by comparing the mean±SD from each experimental group with its respective control group. Statistical significance was defined as $p<0.05$.

Results

Tetrac versus Tetrac NP distribution in renal cell carcinoma. In order to evaluate the preferential binding of Tetrac NP to the plasma membrane, we used nanoparticles labeled with Cy3. The localization of these particles was compared to that of Tetrac labeled with Cy3. The cellular localization of these two derivatives was then determined by fluorescence microscopy. As shown in Figure 2, Tetrac-labeled Cy3 was present in the cytoplasm as well as in the nucleus, whereas Tetrac NP was present only on the plasma membrane. This confirms the predictions made earlier and suggest, that putative biological effects of this formulation would be exclusively mediated through the plasma membrane.

Effect of Tetrac and Tetrac nanoparticles on tumor angiogenesis and tumor growth in the CAM model. RCC cells were implanted at 1×10^6 in the CAM model (n=8 per group) and the effect of Tetrac and Tetrac nanoparticles (Tetrac NP) at 1 μg/CAM on tumor-related angiogenesis and on tumor growth were determined 8 d after implant in 7 days old chick eggs. Treatment with Tetrac and Tetrac NP resulted in significant inhibition ($p<0.01$) of tumor-mediated angiogenesis (Figure 3A). Additionally, both Tetrac and Tetrac NP resulted in effective inhibition ($p<0.01$) of tumor growth (Figure 3B). There was no significant difference between the effects of Tetrac alone *versus* that of Tetrac linked to nanoparticles, providing a first indication that the antitumor action of this hormone antagonist is initiated at the plasma membrane.

Response of tumor implants to Tetrac administration. Daily treatment of xenografted animals with Tetrac (2.0 mg/kg; *i.p.*) or Tetrac nanoparticles (1.86 mg/kg; *i.p.*) resulted in immediate suppression in tumor volume (Figure 4A).

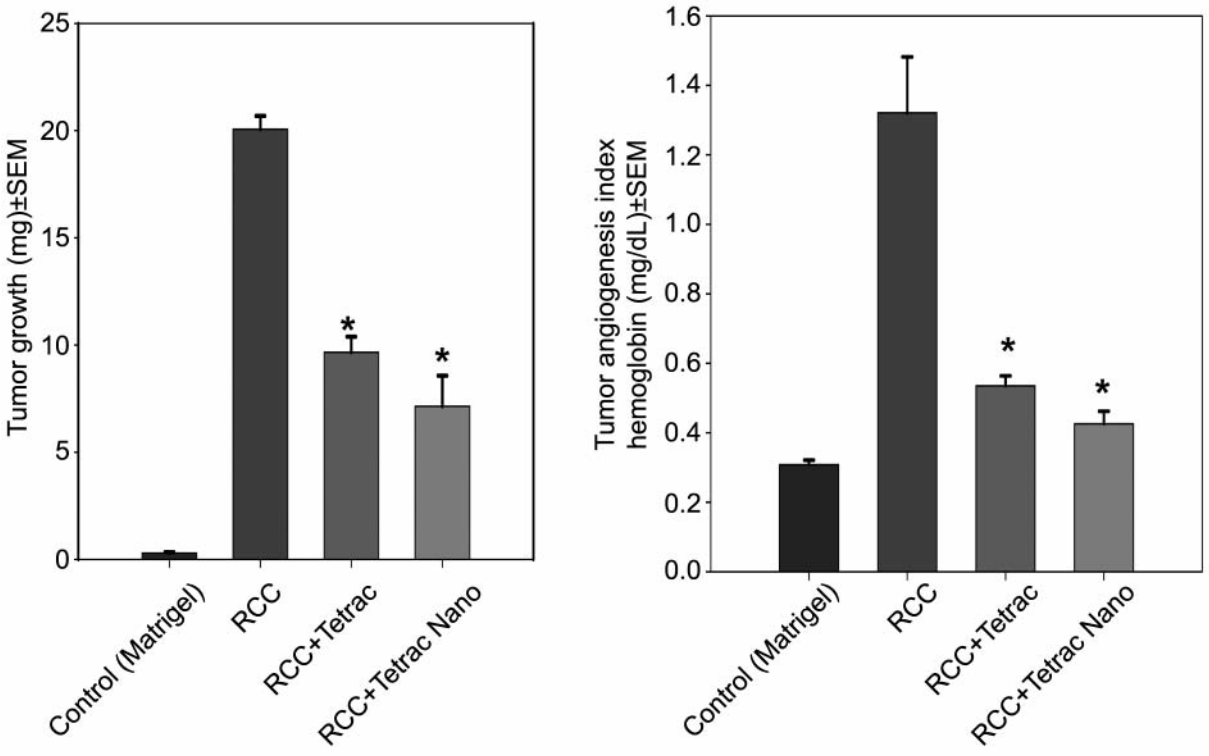

Figure 3. *Effect of Tetrac and Tetrac nanoparticles at 1 μg/CAM Tetrac equivalents on renal cell carcinoma-mediated angiogenesis (A) and tumor growth (B) in the CAM cancer cell implant model. Data represent mean±SEM, n=8 per group, *p<0.01.*

Repeated administration of these two treatments exhibited sustained inhibition of tumor growth up to 20 d (Figure 4A). At the end of the study, tumor weight was directly measured in the untreated, Tetrac and Tetrac NP groups. The results indicate that both treatments resulted in 30-40% ($p<0.05$) inhibition of tumor mass after 8 d of discontinuation as compared to control (Figure 4B). The similarity of the treatment effects of the two agents strengthens the notion that Tetrac exerts anti-proliferative and anti-angiogenic effects at the plasma membrane through its specific interaction with integrin αvβ3 (10-12). There was no effect of these treatments on animal body weight (Figure 4C) as one index of the lack of toxicity of the agents.

Discussion

Although renal cell carcinoma (RCC) accounts for only 3% of adult malignancies, it has been increasing in incidence by 2-4% per year for the past three decades (1, 2). Cigarette smoking, obesity and end-stage renal disease apparently are important risk factors for RCC (1, 2, 21). Genetic syndromes such as von Hippel-Lindau disease are also associated with an increased incidence of RCC (22-25). Localized disease is usually treated with surgical resection and complete resection of primary tumors can result in long-term survival. Removal of the primary renal tumor in patients with unresectable disseminated disease has also been shown to improve survival in selected good performance status patients who receive systemic immunotherapy. While chemotherapy has been relatively ineffective in the treatment of renal cell carcinoma, biologic therapy with interleukin-2 or interferon may lead to responses in a minority of patients, with occasional long-term survivors. Recently, promising results have been reported with allogeneic stem cell transplantation, using a non-myeloablative conditioning regimen (26). Therapy for metastatic renal cell carcinoma nonetheless remains inadequate.

We show here that unmodified Tetrac and nanoparticle Tetrac (Tetrac NP) are effective inhibitors of the growth of human renal carcinoma xenograft in the nude mouse. Administered parenterally, the agents promptly reduced tumor volume and given every other day for 20 d significantly reduced tumor size.

As mentioned above, the nanoparticle does not gain access to the cell interior and thus the Tetrac ether-bonded to the PLGA particle *via* the outer ring hydroxyl can act only at the integrin receptor – where it is exclusively an antagonist – and not at the nuclear receptor for thyroid hormone (TR). Unmodified Tetrac does gain access to the cell and can interact with TR where it is a low potency agonist

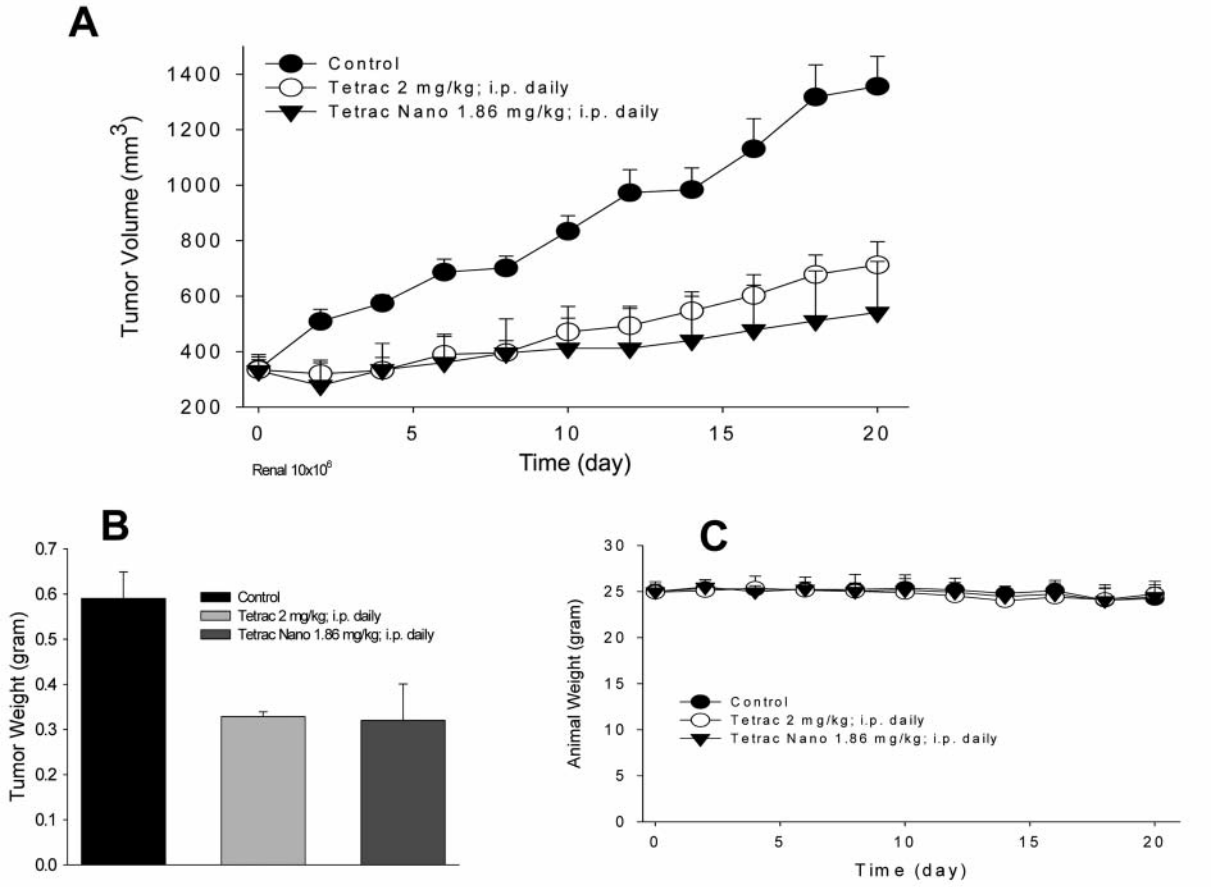

Figure 4. *A, Effect of Tetrac and Tetrac nanoparticles treatment on tumor xenograft volume overtime for up to 20 days. Data represent mean tumor volume (mm^3)±SD, n=8 per group, *p<0.05. B, Effect on tumor xenograft mass at the end of study on day 20. C, Effect on animal weight.*

(thyromimetic) (12). Thus, the possibility existed that the antitumor effect of Tetrac could be initiated either at the plasma membrane or at the nucleus. Our findings revealed that since Tetrac and Tetrac NP have similar action on angiogenesis and tumor growth, the antitumor effects of Tetrac are initiated at the plasma membrane and at the receptor for thyroid hormone analogues, such as Tetrac, on αvβ3 integrin.

Regarding the dual effect of Tetrac on angiogenesis and tumor growth, anti-angiogenic agents clinically directed at a specific vascular growth factor have come to be regarded as adjuncts to standard chemotherapy, rather than as primary anticancer modalities (4, 27). We might conclude that the effectiveness of Tetrac against renal cell carcinoma secondarily involves anti-angiogenesis. On the other hand, Tetrac blocks the effects of more than one vascular growth factor, *e.g.* VEGF and bFGF, and thus anti-angiogenesis in the present studies of Tetrac may be more important than the adjunctive effect obtained clinically with agents that target a single specific vascular growth factor or vascular growth factor receptor (10). The *in vivo* data (Figure 4) also indicate that the action of Tetrac on renal carcinoma tumor volume had two components: a relatively acute effect that was apparent within 2 days of the initiation of Tetrac treatment (Figure 4A) and a progressive effect for up to day 20. We have ascribed conventional pro-apoptotic activity to Tetrac (9) that would account for the progressive decrease in tumor volume that occurred over 20 days of treatment. Additionally, recent studies showed propylthiouracil-induced hypothyroidism reduces xenograft tumor growth in athymic nude mice (28), suggesting that lowering the pro-angiogenesis (7, 8) and anti-apoptotic effects (9) of endogenous thyroid hormone would limit tumor growth. These data using the thyroid antagonist Tetrac are in agreement with data reported on the suppression of thyroid hormone levels or induction of hypothyroidism on tumor growth in nude mice (28).

In conclusion, results from this study provide evidence for the efficacy of both Tetrac and Tetrac NP in inhibiting angiogenesis and RCC tumor growth without any noticeable toxic effect. These encouraging findings suggest that Tetrac can be used alone for the treatment of RCC and perhaps

other types of cancer. More importantly, we show that nanoparticle formulations of Tetrac are safe and thus, may provide an alternative approach for delivering this hormone antagonist, particularly to optimize its pharmacokinetics and pharmacodynamics. Further investigation of these treatments in clinical trials is therefore warranted.

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