

Anti-tumor effects of nitrosylcobalamin against spontaneous tumors in dogs

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Summary Purpose: Given the limited options available to treat canine cancers, the use of companion animals for evaluating new drugs may identify better therapies for veterinary and human oncology. The anti-tumor effects of nitrosylcobalamin (NO-Cbl), an apoptosis-inducing, vitamin B12-based carrier of nitric oxide (NO), was evaluated in four dogs with spontaneous cancer. *Experimental Design:* (1) A 13 year-old female spayed Giant Schnauzer with inoperable thyroid carcinoma and hypercalcemia. (2) A 6 year-old male neutered Golden Retriever with a malignant peripheral nerve sheath tumor (MPNST). (3) A ten yr-old neutered male Bichon Frise with apocrine gland anal sac adenocarcinoma (AGACA). (4) A 7 year-old female spayed Labrador mix with spinal meningioma following partial surgical resection. Tumor regression was

measured by physical exam and verified using ultrasound (case 1) and MRI (case 2–4). Serum chemistries and hematologic parameters were monitored throughout the studies. *Results:* (1) The Giant Schnauzer demonstrated a 77% reduction in tumor volume after ten weeks of daily NO-Cbl treatment. (2) The Golden Retriever demonstrated a 53% reduction in tumor volume after 15 months of daily NO-Cbl therapy. (3) The Bichon Frise demonstrated a 43% regression of the primary tumor and a 90% regression of an iliac lymph node measured by MRI after 15 months of treatment. After 61 months, the dog currently has stable disease, normal liver enzymes, CBC analysis, and no evidence of toxicity. (4) The Labrador demonstrated complete regression of the residual tumor after 6 months of treatment. *Conclusion:* We have shown previously that NO-Cbl is endocytosed by malignant cells, resulting in intra-tumoral NO release. In this study, we have shown that daily long-term use of NO-Cbl induced responses in all dogs without any signs of toxicity. The use of NO-Cbl capitalizes on the tumor-specific properties of the vitamin B12 receptor and represents a promising anti-cancer therapy.

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Abbreviations

AGACA	Apocrine gland anal sac adenocarcinoma
Apo2L/TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
MPNST	Malignant peripheral nerve sheath tumor
SM	Spinal meningioma
TCII	Transcobalamin II

Introduction

Current treatment of cancer in dogs involves the sole use or combination of surgery, irradiation, chemotherapy, biological therapy, immunotherapy, and anti-cancer vaccines. Although combinations of these therapies have been used to increase efficacy and decrease toxic side effects, the clinical response of dogs with advanced solid tumors is far from ideal.

We designed nitrosylcobalamin (NO-Cbl), a vitamin B12 analogue with nitric oxide as an axial ligand, to function as a biologic “Trojan Horse” that uses receptor-mediated Cbl uptake to target NO-Cbl to cancer cells and cause apoptosis [1] (Fig. 1). Radiolabeled vitamin B12 conjugates that bind TCII R have been used clinically in the detection of cancer [2, 3].

We have previously demonstrated the anti-tumor activity of NO-Cbl as a single agent or in combination with biological therapies such as Interferon- β (IFN- β)(1) and Tumor Necrosis Factor-related Apoptosis-inducing Ligand (Apo2L/TRAIL) in xenograft models [4]. We have combined NO-Cbl with a variety of chemotherapeutic agents to achieve synergistic antiproliferative effects due to inhibition of survival signaling mediated by two key regulators of survival, NF- κ B or AKT activation *in vitro* [5]. We have shown that NO-Cbl sensitized Apo2L/TRAIL-resistant cells to Apo2L/TRAIL-mediated cell death [4]. NO-Cbl mediated apoptosis was induced in part via activation of the Apo2L/TRAIL receptor DR4 by S-nitrosylation [6]. DR4-mediated apoptosis is important since it is a tumor-specific response as functional DR4 is expressed on the surface of tumors but not on normal tissues such as liver or kidney [7, 8].

The NCI Developmental Therapeutics Program independently tested NO-Cbl in a human tumor 60-cell line screen. In general, colon, ovarian, and breast carcinomas as well as CNS tumors were most responsive to the antigrowth effects of NO-Cbl [1]. Based on these findings, we hypothesized NO-Cbl would be effective in treating a variety of cancers such as MPNST, thyroid carcinoma, AGACA and spinal meningioma.

Materials and methods

Synthesis of nitrosylcobalamin

Nitrosylcobalamin was synthesized as previously described [9]. Hydroxocobalamin (vitamin B12a) acetate (Hebei Huarong Pharmaceutical Co, Hebei Province, China) was dissolved in dichloromethane (OmniSolv, EMD Chemicals, Gibbstown, NJ) and exposed to CP grade NO gas (Praxair, Wickliff, OH) at 150 psi. The reaction proceeds in a closed system within a high-pressure stainless steel reactor (Parr Instrument Co, Moline, IL). The system was purged daily and evacuated prior to NO exposure. The NO gas was scrubbed prior to entering the system using a stainless steel cylinder (Midwest Process Controls, Bay Village, OH) containing NaOH pellets. The reaction progress was monitored on a daily basis utilizing HPLC and UV/Vis. The solid NO-Cbl product was collected following rotary evaporation of the solvent and stored under argon at -80°C prior to use. NO-Cbl was sterile filtered, aseptically bottled [prepared at 40 mg/mL in THAM (Tromethamine, 300 mM, pH adjusted to 7.9 using 6 M HCl, Abbott Labs,

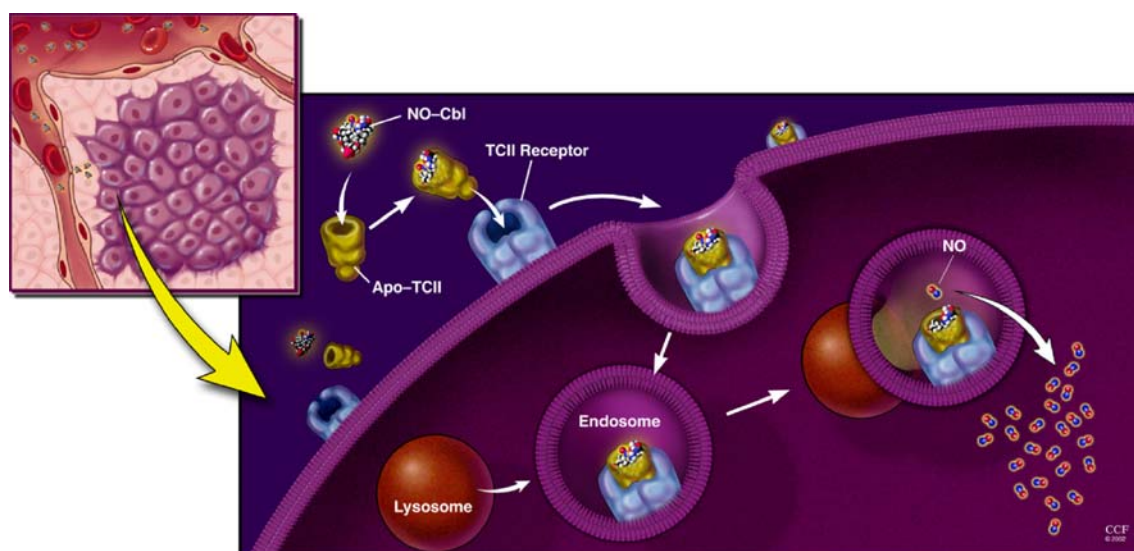


Fig. 1 NO-Cbl uptake. NO-Cbl binds the carrier protein apo-TCII in the plasma and then binds the plasma membrane bound TCII receptor. The receptor is internalized and fuses with a lysosome, releasing NO into the cytosol

North Chicago, IL): Cleveland Clinic, Sterile Solutions Pharmacy, Cleveland, OH] and administered subcutaneously (s.c.). NO-Cbl is stable under these conditions as verified by chemiluminescence headspace-gas analysis [9].

Pharmacokinetics

For pharmacokinetic analysis, two dogs (one male, one female: Hodgins Kennels, Howell, MI) received a bolus of NO-Cbl (40 mg/kg, s.c.). Blood samples (5 mL) were withdrawn at various time points using gold top BD Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Tubes were immediately centrifuged and separated serum was sent to Texas A&M, GI lab, Department of Small Animal Medicine and Surgery (College Station, TX) and analyzed for vitamin B12 using an automated chemiluminescent immunoassay system (Immulite 2000, Siemens, Malvern, PA). Institutional Animal Care and Use Committee at the Cleveland Clinic Foundation approved all procedures for animal experimentation. Pharmacokinetic analysis was performed using Kinetica version 4.4.1 (Thermo Electron Corporation, Waltham, MA). Area under the curve (AUC) was calculated using the linear trapezoidal rule.

Serum chemistry, hematology, imaging, NO-Cbl administration

Informed consent was obtained from the dog's owners prior to blood draws, imaging and drug administration. In all cases NO-Cbl was administered daily by the dog owner under the direction and supervision of a licensed veterinarian. Case 1: Blood work was processed at The Ohio State University, College of Veterinary Medicine (Columbus, Ohio). Ultrasound and x-ray were performed there as well. NO-Cbl was administered at an initial dose of 28 mg/kg which was escalated to 35 mg/kg 8 weeks later (Dose escalation was performed to assess toxicity and did not correlate to response). Case 2: Blood work was processed at the Gastrointestinal Lab, Texas A & M University (College Station, TX) and Marshfield Clinic Laboratories (Cleveland, Ohio). Contrast enhanced MRI was performed at PetsDx Veterinary Imaging (Pittsburgh, PA) with radiographic assessment at PetRays Veterinary Radiology Consultants (Spring, TX). NO-Cbl was administered at 20 mg/kg throughout the study. Case 3: Blood work was processed at Angell Animal Medical Center and at Tufts University School of Veterinary Medicine. Magnetic resonance imaging was performed at Tufts using T1, T2 and fat-suppressed pulse sequences. T1 and fat-suppressed sequences were obtained following the intravenous injection of 1.7 mL of gadolinium contrast agent. NO-Cbl was initially administered at 28 mg/kg s.c.

b.i.d., which was increased to 42 mg/kg t.i.d. at week 12, and later increased to 56 mg/kg t.i.d. at week 20. Dose escalation was performed to assess toxicity and did not correlate to response. Carboplatin (300 mg/m² : 150 mg, i.v.) was administered for 6 cycles alternating every two weeks (during week 42–56). Case 4: Blood work and MRI were performed at Carolina Veterinary Specialists Medical Center (Charlotte, NC).

Results

Pharmacokinetics and canine dosage determination

Pharmacokinetic analysis was performed in two dogs, one male and female. Peak serum levels (C_{max}) of 11,674 and 5,842.5 pg/mL were achieved following single dose bolus subcutaneous (s.c.) administration (40 mg/kg) in the male and female dog respectively. The area under the curve (AUC_{tot}) was 81,360 and 52,026 h × pg/mL with an average elimination half-life ($T_{1/2\beta}$) of approximately 4.8 and 5.7 h, in the male and female dog respectively, similar to the $T_{1/2\beta}$ of an i.v. bolus of 70 mg/kg ($T_{1/2\beta}$ =7.36 h) and 140 mg/kg ($T_{1/2\beta}$ =6.00 h) of hydroxocobalamin in dogs [10].

Case 1

A 13 year old female spayed Giant Schnauzer was diagnosed with inoperable thyroid carcinoma and hypercalcemia. Initial treatment of the thyroid carcinoma consisted of intra-lesional ethanol injection which resulted in a qualitative decrease in tumor vascularization and marginal reduction in tumor size upon one-month follow-up although calcium levels remained unchanged from pre-treatment levels (week 4). NO-Cbl was administered 10 months later (week 40) at an initial dose of 28 mg/kg which was escalated to 35 mg/kg at 8 weeks later (week 48). Tumor volume was assessed by ultrasound at ten weeks after NO-Cbl administration (week 50) resulting in a 77% reduction in tumor volume compared to baseline (Fig. 2). Prior to NO-Cbl treatment ionized calcium levels (maximum value 6.32 mg/dL) were elevated. During NO-Cbl treatment, calcium decreased and remained within normal limits (5.0–6.1 mg/dL). Blood chemistries including BUN were normal during NO-Cbl administration. The dog was taken off study due to progression of severe arthritis (unrelated to NO-Cbl therapy) that was diagnosed more than one year prior to initiation of NO-Cbl treatment.

Case 2

A 6 year-old male neutered Golden Retriever presented with right foreleg lameness and pain during cervical range of

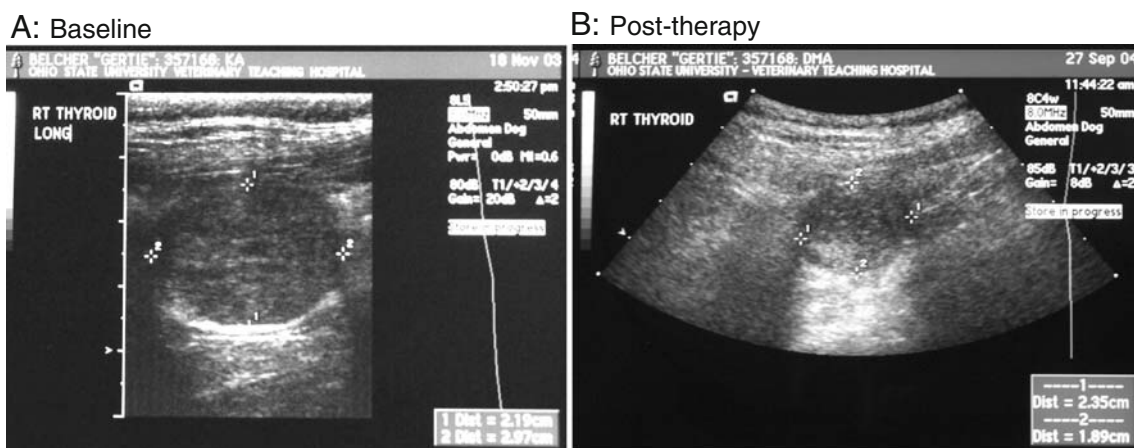


Fig. 2 Case 1—(Thyroid carcinoma): Quantitation of tumor volume by ultrasound. Ultrasound of the thyroid tumor indicates a 77% reduction in tumor volume compared to baseline images

motion. These signs are consistent with malignant peripheral nerve sheath tumor (MPNST); 41/50 dogs exhibited unilateral foreleg lameness and muscle atrophy [11]. MR imaging confirmed a malignant peripheral nerve sheath tumor in the right brachial plexus (C7-T1) that invaded into the spinal canal. Surgical reduction and radiation therapy were declined by the owners. NO-Cbl treatment was started 4 weeks after diagnosis at 20 mg/kg s.c., b.i.d.. After 9 months of therapy, invasion of the mass into the spinal canal was no longer evident on MR. The tumor decreased in volume by 39% compared to pre-treatment. The decrease in tumor size was evident in all dimensions and the contrast enhancement pattern suggested that regions of the tumor had become less vascular. After 15 months of daily NO-Cbl treatment the tumor had shrunk by 53% compared to pre-treatment (Fig. 3).

Case 3

A 10 year old male neutered Bichon Frise presented with apocrine gland anal sac adenocarcinoma (AGACA) confirmed by biopsy. Enlargement of the iliac lymph node was observed one month later. Initial therapy consisted of three cycles of mitoxantrone (weeks 1–4 post-diagnosis) followed by palliative radiation therapy (24 Gy in three 8 Gy fractions, beginning week 5) and 5 cycles of adriamycin (beginning week 12) NO-Cbl therapy began 28 weeks after diagnosis. Over the course of the study, the dose of NO-Cbl was escalated from 28 mg/kg to 42 mg/kg at 12 weeks and increased again to 56 mg/kg at 20 weeks (post-NO-Cbl therapy).

Prior to NO-Cbl therapy, calcium levels were elevated (max 13.1 mg/dL). During NO-Cbl treatment, calcium decreased to within the normal range (9.7–11.4 mg/dL). Initially liver enzymes, especially ALT, were elevated 2-fold and decreased with NO-Cbl treatment. After 46 weeks of NO-Cbl therapy, combination treatment with

carboplatin was begun. Combination therapy resulted in a 2.5 fold increase in liver enzyme levels. Co-administration of NO-Cbl and carboplatin resulted in mild anemia; the RBC count was $4.37 \times 10^6/\mu\text{L}$, (range: $5.8\text{--}8.9 \times 10^6/\mu\text{L}$) accompanied by mild leukocytosis, the WBC counts rose to $19.5 \times 10^3/\mu\text{L}$ (range $6.0\text{--}14.3 \times 10^3/\mu\text{L}$). Myelosuppression was not observed. Serum creatinine values were within the normal range throughout the study, although BUN levels were consistently elevated (>3 times normal). During the first three years of therapy, creatinine remained within normal limits (0.4–1.5 mg/dL). However, in the past three months the canine has developed mild renal azotemia (max. 2.4 mg/dL), cause unknown, as a renal biopsy has not been performed. Vitamin B12 levels were randomly monitored and were consistently greater than 1200 pg/mL throughout the duration of the study (normal: 249–733 pg/mL).

CT scans were used to assess tumor volume prior to treatment. However, due to the invasive nature of the AGACA, the tumor margins could not be clearly visualized with CT. Therefore, MRI was subsequently utilized. The first MRI was obtained after 7 weeks of NO-Cbl treatment. After 20 weeks of NO-Cbl treatment the primary perianal mass was unchanged and the volume of the iliac lymph node decreased by 46%. At 28 weeks the size of the primary tumor had not changed and the metastatic lesion decreased by an additional 30%.

We decided to add carboplatin to the treatment regimen since the combination of NO-Cbl and carboplatin demonstrated synergistic antitumor effects *in vitro* [5]. The dog was given six cycles of carboplatin (week 42–56). MRI at 50 weeks indicated a reduction in the primary by 45% and an additional reduction in the metastatic lesion by 75%. NO-Cbl was continued following carboplatin treatment. At 62 weeks, there was an overall reduction of 43% and 90% in the primary tumor and the iliac lymph node, respectively,

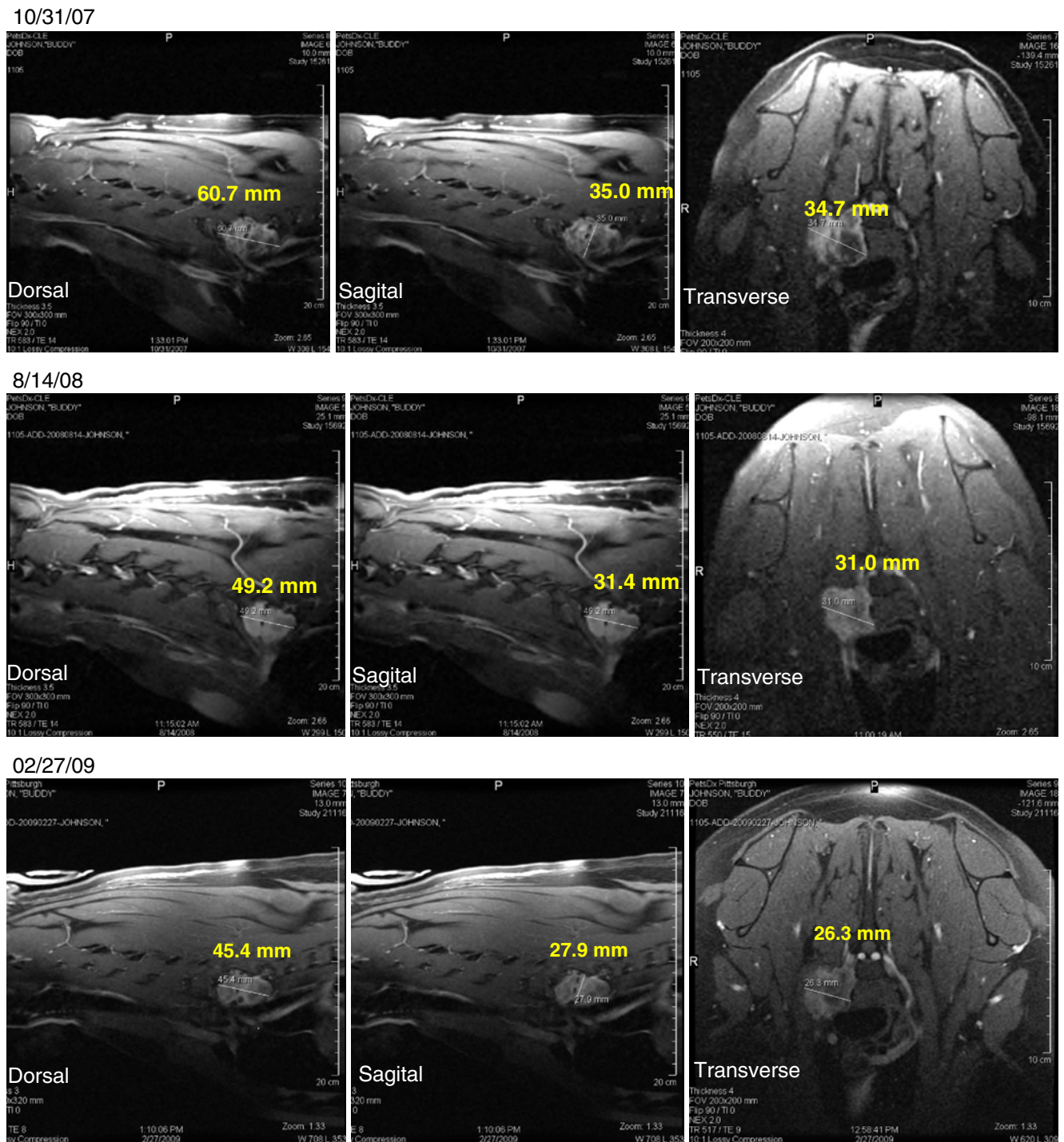


Fig. 3 Case 2—(MPNST): Quantitation of tumor volume by MRI. Contrast enhanced sequences depict dorsal, sagittal, and transverse views comparing baseline (10/31/07) to images at 9 month follow-up

as compared to baseline MRI (Fig. 4). MRIs were discontinued due to sensitivity to the anesthesia. NO-Cbl treatment was discontinued after 41 months upon diagnosis of stable disease as assessed by physical exam. The dog has been off-study for 18 months without evidence of disease progression.

(8/14/08). Composite views depict a 39% reduction in tumor volume compared to baseline. (2/27/09) follow-up scans depict a 53% decrease in tumor volume compared to pre-treatment

Case 4

A 7 year-old female spayed Labrador mix presented with spinal meningioma following partial surgical resection. Prior to NO-Cbl therapy there was an irregular increased T2 signal noted in the spinal canal at the level of T3

Fig. 4 Case 3–(AGACA): Quantitation of tumor volume by MRI. A) Tumor volume index compared to weeks of treatment (week 7– week 62). The primary tumor demonstrated an overall reduction of 43% compared to baseline. The iliac lymph node was reduced in volume by 90% compared to baseline. B) T1 and fat-suppressed sequences were obtained following the intravenous injection of 1.7 mL of gadolinium contrast agent. MR images depict transverse and sagittal views of primary tumor at baseline (7 weeks) compared to transverse and sagittal views of primary tumor at 62 weeks

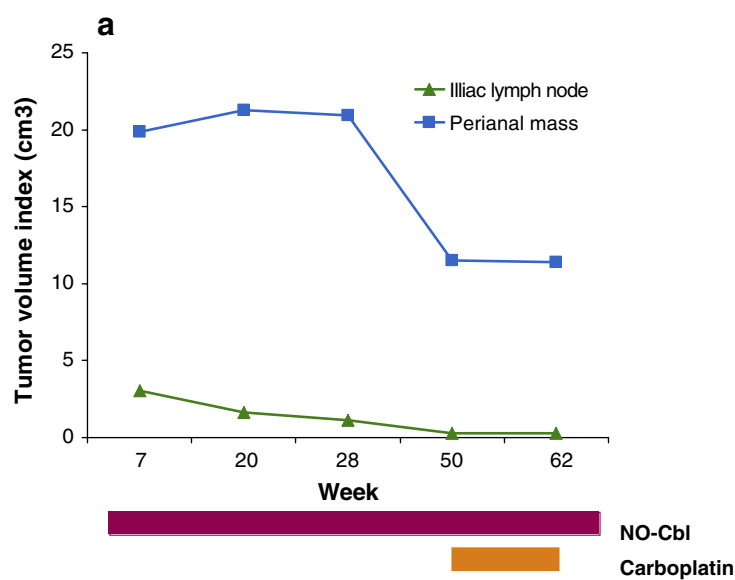




Fig. 5 Case 4—(Spinal meningioma): Quantitation of tumor volume by MRI. A) T3 region, dorsal view of tumor at baseline prior to partial surgical resection. B) T3 region, dorsal view of tumor after surgical resection. C) T3 region, dorsal view after 6 months of NO-Cbl treatment

consistent with the area of the previously removed tumor mass. A right sided laminectomy is noted at the level of T3-4. There was a focal, contrast enhancing mass effect noted in the spinal cord at this level which measured $6.5 \times 6.5 \times 9$ mm and was eccentrically located towards the right. There is also contrast enhancement noted of the soft tissues in the region of the laminectomy and extending out the skin surface consistent with reactive tissue from the surgery. This contrast enhancing tissue extends into the spinal canal adjacent to the spinal cord and can't be separated from residual tumor. Prior to surgery, the mass measured $7.5 \times 7.7 \times 18$ mm.

After 6 months of daily NO-Cbl treatment (20 mg/kg, s.c. b.i.d.) the previously seen contrast enhancing mass in the spinal cord at the level of T3 is barely visible (Fig. 5). Minimal contrast enhancement is noted. The spinal cord is deranged in shape at the level of the previous mass effect but no progression of mass is visible. It is not possible to accurately measure any mass effect as it is not visible. The spinal cord is slightly heterogeneous in the area of the previous mass but the intense signal change is not visible. The contrast enhancement of the paraspinal tissues in this region has resolved (from the previous surgery). Blood values were within normal ranges over the course of NO-Cbl treatment.

Discussion

Little progress has been made in the treatment of advanced human or canine cancers. The most common treatment options for dogs include surgery, radiation therapy and a limited arsenal of chemotherapeutic agents.

Treatment options for thyroid carcinoma include resection, radiation therapy or radioactive iodine and a few chemotherapeutic agents [12]. Surgical resection alone

demonstrated a median survival of 20.5 months in a study of 20 dogs without evidence of metastasis [13]. A study of 39 dogs with nonresectable thyroid tumors treated with iodine 131 demonstrated a median survival time of 28 months (tumor restricted to within the neck) and 12 months for dogs with distant metastasis [14]. A similar case study in 65 dogs evaluated the effectiveness of ^{131}I as an adjuvant to therapy with a median survival of 34 months compared to 30 months using ^{131}I as a single agent [15]. External beam radiation has also been effective with a median survival of 24.5 months [16, 17]. Cisplatin [18, 19], Actinomycin D [20], mitoxantrone [21], and doxorubicin [22] have been utilized in the treatment of thyroid carcinoma with some success as demonstrated in a study of 9 dogs with a response rate of 40% following chemotherapy [23].

The preferred treatment of malignant peripheral nerve sheath tumors (MPNST), schwannomas or “soft tissue sarcomas” is typically surgical resection although adjuvant radiation therapy has shown some benefit [24]. Survival times of non-treated cases ranged from 5 to 21 months [24]. A study of 12 dogs with MPNST indicated a post-operative median survival of 180 days [25]. Local recurrence is common [26]. A study involving 11 dogs with recurrent tumor following resection resulted in the death of all dogs studied within 2 years of the last surgical resection [27].

Treatment options for AGACA include surgery, radiation, and chemotherapy. AGACA is highly aggressive and frequently recurs after resection, with a median time of recurrence of 10 months [28, 29]. A review of seven studies of AGACA ($n=179$) suggests that the mean survival for dogs receiving palliative care (7 dogs were treated with piroxicam and the other 3 were treated with IV fluids, prednisone and furosemide for hypercalcemia) was 8.7 months compared to surgery (21.3 months), chemotherapy (7.9 months), surgery + chemotherapy (17.4 months), and the combined treatment of

surgery, chemotherapy and radiation (31.8 months) [29–35]. A survival advantage of 4.8 months was observed for dogs that underwent surgery as part of their treatment [32]. Some studies have shown that hypercalcemia and metastatic disease were associated with poor prognosis [33] whereas others did not [29]. Chemotherapy induced myelosuppression and GI-related toxicities in 37% of cases [32].

Meningioma and peripheral nerve sheath tumors are the most common intradural-extramedullary tumors in dogs [36]. Spinal meningioma accounts for 14% of all CNS meningioma. Spinal meningioma (SM) has a predilection for the cervical spinal cord (40–77%) but can affect the thoracic region (0–32%). Prognosis after complete surgical removal is guarded but good [37–39]. Surgical resection is the standard of care for SM. Radiation is recommended as adjuvant therapy for incompletely resected tumors and may improve survival.

In all cases, NO-Cbl was well tolerated without apparent toxicity. The response in case 1 was remarkable considering the duration of treatment was only 10 weeks. The dog was taken off study due to severe arthritis unrelated to NO-Cbl therapy.

In case 2, surgical resection was not an option due to the location of the tumor. Prior to NO-Cbl therapy, nerve involvement in the right forelimb had progressed to tarsal laxity and pronounced muscle atrophy. After 9 months of NO-Cbl therapy, the tumor disappeared in the spinal canal with a 39% reduction in overall tumor volume. Physical exam revealed decreased atrophy and mobility of the right foreleg. The dog currently remains on study.

In case 3, prior to the administration of NO-Cbl, treatments included mitoxantrone (3 cycles), doxorubicin (5 cycles) and palliative radiation therapy. However, the tumor continued to progress and hypercalcemia persisted. After two weeks of NO-Cbl treatment, calcium levels returned to normal. This may be important, as treatment outcome has been shown in some cases to be negatively affected by hypercalcemia. Daily administration of NO-Cbl over a period of 3 years caused minimal toxicity. Although BUN levels were elevated, this may have been secondary to dehydration since this has not been observed in other dogs. Since initiation of NO-Cbl the dog has survived 61 months, in comparison to the longest documented case of AGACA in the literature (42 months) following combined surgery and chemotherapy.

In case 4, NO-Cbl was used as an adjuvant subsequent to incomplete surgical resection. The tumor completely regressed after 6 months of daily NO-Cbl therapy.

Given the limited options available to treat canine cancers, the use of companion animals (pets) for evaluating new drugs may be a superior alternative, given the failure rate of mouse xenografts in predicting drug efficacy in humans [40–42]. Companion animals with spontaneously

occurring malignancies may be more biologically relevant in that they are exposed to similar etiologic factors as humans [43–45]. In 2003, the NCI initiated the Comparative Oncology Program (COP) including a Comparative Oncology Trials Consortium, which standardizes treatment protocols for integrated drug discovery and development [46, 47]. The hope is that human clinical trials designed using canine clinical data will have higher response rates than studies based on xenograft data, and may identify better therapies for veterinary oncology. NO-Cbl based therapies may represent new treatment options for combating animal carcinomas.

Statement of Translational Relevance Currently, the NCI's Comparative Oncology Program (COP) is evaluating the anti-tumor efficacy of chemotherapeutic agents in dogs with spontaneous occurring tumors. The impetus for this program stemmed from the high discordance of chemotherapeutic agent response rates observed in xenograft studies to the frequently low rates observed with the same agent in clinical studies. Our work is innovative because it involves the use of “biological Trojan horse technology” to target cancer. Since the 1950's researchers have tried in vain to synthesize cobalamin analogs as anti-tumor agents, utilizing the vitamin B12 receptor (transcobalamin II receptor, TC II-R) or methionine synthase as a target. We synthesized a vitamin B12 based compound that delivers nitric oxide to cells. We have demonstrated previously that cancer cells which overexpress the vitamin B12 receptor (TCII-R) are very sensitive to nitrosylcobalamin (NO-Cbl). Our case studies demonstrate anti-tumor efficacy with limited toxicity to normal tissues. NO-Cbl sensitizes multidrug-resistant cancer cells to the antitumor effects of several different cytotoxic drugs and may therefore be valuable when utilized in combination regimens.

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