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# Lack of toxicity of a STAT3 decoy oligonucleotide

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## Abstract

**Background—**STAT3 overexpression has been detected in several cancers including head and neck squamous cell carcinoma (HNSCC). Previous studies using intratumoral administration of a STAT3 decoy oligonucleotide that abrogates STAT3-mediated gene transcription in preclinical cancer models have demonstrated antitumor efficacy. This study was conducted to observe the toxicity and biologic effects of the STAT3 decoy in a non-human primate model, in anticipation of initiating a clinical trial in HNSCC patients.

**Methods—**Three study groups (two monkeys/sex/group) were administered a single intramuscular injection of low dose of STAT3 decoy (0.8 mg total dose/monkey), high dose of STAT3 decoy (3.2 mg total dose/monkey) or vehicle control (PBS alone) on day 1 and necropsies were performed on days 2 and 15 (one monkey/sex/group/day). Low and high doses of the decoy were administered in the muscle in a volume of 0.9 ml. Tissue and blood were harvested for toxicology and biologic analyses.

**Results—**Upon observation, the STAT3 decoy-treated animals exhibited behavior that was similar to the vehicle control group. Individual animal body weights remained within 1% of

pretreatment weights throughout the study. Hematological parameters were not significantly different between the control and the treatment groups. Clinical chemistry fluctuations were considered within normal limits and were not attributed to the STAT3 decoy. Assessment of complement activation breakdown product (Bb) levels demonstrated no activation of the alternative pathway of complement in any animal at any dose level. At necropsy, there were no gross or microscopic findings attributed to STAT3 decoy in any organ examined. STAT3 target gene expression at the injection site revealed decreased Bcl-$X_L$ and cyclin D1 expression levels in the animals treated with high dose of STAT3 decoy compared to the animals injected with low dose of STAT3 decoy or the vehicle as control.

**Conclusion—**Based on these findings, the no-observable-adverse-effect-level (NOAEL) was greater than 3.2 mg/kg when administered as a single dose to male and female Cynomolgus monkeys. Plans are underway to test the safety and biologic effects of intratumoral administration of the STAT3 decoy in HNSCC patients.

### Keywords

STAT3 decoy; Toxicity

## Introduction

Signal transducer and activator of transcription 3 (STAT3) is one of a group of seven transcription factors and downstream signaling proteins that are activated in response to cytokines (e.g., interleukin-6) and growth factors (e.g., epidermal growth factor). Increased STAT3 activation has been detected in a variety of human cancers, including 82–100% of prostate cancer [1, 2, 23], 70% of breast cancers [16], 54% of lung cancer [12], 71% of nasopharyngeal carcinoma [16] and in more than 95% of head and neck cancers [6, 11, 25]. Cumulative evidence suggests that dysregulation and constitutive activation of STAT3 stimulates cancer cell growth and contributes to tumor development and progression by up-regulation of STAT3 target genes including *bcl-xl*, *c-myc*, *cyclin D1*, and *VEGF*, which can enhance cell survival, proliferation and promote angiogenesis [16, 17, 19, 31].

Targeting STAT3 in preclinical models of several cancers using a variety of different approaches has demonstrated anti-cancer effects, although clinical data with any approach are lacking [6]. The interruption of persistent STAT3 activity in several different cancers by dominant-negative mutants induced apoptotic cell death [6]. Targeting STAT3 using a plasmid directed against the STAT3 translation site or with antisense oligonucleotides efficiently inhibited STAT3 activation, and stimulated apoptosis in HNSCC tumor xenografts and cell lines without a significant effect on tumor growth in vivo [11, 18,25]. Decreased STAT3 expression at both the mRNA and protein levels was demonstrated by STAT3 siRNA in human laryngeal cancer cells, with associated suppression of growth and induction of apoptosis [10]. Both in vitro and in vivo studies using HNSCC cell lines with G-rich oligodeoxynucleotides (G quartets) have shown significant inhibition of the DNA-binding activity of STAT3 [16].

Transcription factors, including STATs, have been effectively blocked by a transcription factor decoy approach, where the efficacy of a double-stranded oligonucleotide decoy has been assessed in several diseases including cancer with proven effectiveness [24, 29]. A double-stranded decoy oligonucleotide against a tanscription factor is based on the competition between the endogenous *cis*-elements within the regulatory regions of target genes. Exposure of cells, where the transcription factor is activated by the decoy mimicking the specific *cis*-elements, inhibits the binding of the transcription factor with its *cis*-elements and induction of gene expression. To evaluate the potential of a STAT3 decoy in STAT3

overexpressing tumor cells, we designed an oligonucleotide whose sequence closely resembles the response element within the promoter region of the STAT3 target gene [20]. This STAT3 decoy has been shown to decrease STAT3 promoter activity and abrogate target gene expression in HNSCC cell lines in vitro [30]. In HNSCC xenograft models, STAT3 decoy-treated tumors showed reduced STAT3 activation and decreased protein expression of target genes such as Bcl-$X_L$, cyclin D1, and VEGF [30]. The antitumor effect of the STAT3 decoy oligonucleotide has also been evaluated in other cancers such as breast and lung cancer, where the inhibition of tumor growth by the STAT3 decoy was significantly associated with the blockade of the STAT3 target gene expression [26,31]. In the absence of a small molecule inhibitor with selective activity against STAT3, the STAT3 decoy was felt to represent the most promising STAT3 inhibitor for clinical development.

In anticipation of implementing a phase 0 clinical trial in HNSCC patients to test the safety and biologic activity of the STAT3 decoy, the present study was performed to observe any local and systemic toxic effects of a single intramuscular injection (IM) of a low (0.8 mg total dose/monkey) dose and a high (3.2 mg total dose/monkey) dose of the STAT3 decoy in Cynomolgus monkeys. Nonhuman primates (NHP) are considered the animal model of choice over rodents because in NHP, oligonucleotides (ODN) elicit physiological responses similar to those observed in humans. Specifically, prolongation of clotting time, as evidenced by increased APTT and PPT and complement activation have been observed [7]. Additionally, cardiovascular changes such as increased heart rate and decreased blood pressure have been detected in NHP after ODN administration, indicating a possible concern for humans [7]. The animals were observed for 2 weeks treatment and no changes in behavior or body weight were detected. There was no activation of the complement pathway and any observed pathological abnormalities or alterations in any of the toxicological endpoints. Although cumulative evidence implicates STAT3 as a therapeutic target in cancer, the contribution of STAT3 to normal development and tissue homeostasis has raised concerns that targeting STAT3 may have undesirable consequences. Our findings indicate that direct injection of the STAT3 decoy did not cause severe acute toxicity and with appropriate safety monitoring, may be administered to patients with solid tumors.

## Materials and methods

### Test article

The STAT3 decoy (NSC-741763), which is a double stranded DNA of annealed, fully complementary, partially phosphorothioated oligonucleotides with the sense sequence being 5′ C*A*T*TTCCCGTTA*A*T*C 3′ and the antisense sequence 5′ G*A*T*TTACGGGAA* A*T*G 3′ (“*” denotes phosphorothioated sites) was manufactured at NCI-Frederick, Biopharmaceutical Developmental Program (Frederick, MD). The STAT3 decoy was formulated in phosphate buffered saline (PBS, purchased from Amresco Solon, OH), at 3.5 mg/ml stock concentration.

### Animals

The FDA required the use of non-human primates to test the safety of the STAT3 decoy. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Battelle Toxicology Laboratories. Cynomolgus monkeys, used for this study were purchased from Covance (Alice, Texas). The monkeys were quarantined for 6 weeks according to the facility standard operating procedures. Animals were housed in pairs during the quarantine and holding periods and individually during the study period. Animals were observed twice daily during the pretreatment period and on the day of dosing. Animals were observed once daily during the remainder of the study. Body weights were

recorded during the pretreatment period, one day prior to dosing and on days 2, 8 and 15 post STAT3 decoy administration.

## Experimental design

Twelve monkeys (two/sex/group) were randomly assigned to two different dose groups or a vehicle control group. For injection, based on an average starting weight of 3.0 kg, the low dose (0.8 mg total dose/monkey which was equivalent to 0.267 mg/kg) and the high dose (3.2 mg total dose/monkey which was equivalent to 1.02 mg/kg) of STAT3 decoy was prepared from the stock solution. The STAT3 decoy or the vehicle control (PBS alone) was administered as a single intramuscular injection (IM) to the Cynomolgus monkeys. The monkeys were injected once with one of the two different doses of STAT3 decoy or the vehicle control on day 1 and then sacrificed on day 2 or on day 15 to determine toxicity (Table 1). Four monkeys received the low dose of STAT3 decoy (0.8 mg total dose, in 900 μl of PBS/monkey), four monkeys were injected with the high dose (3.2 mg of STAT3 decoy total dose, in 900 μl of PBS/monkey), and four monkeys were injected with an equal volume of PBS under aseptic conditions. The doses were decided based on the proposed dose for use in clinical trial and the low and high dose was used to bracket the proposed dose. The FDA concurred with our dose selection when they reviewed the pre-IND package. Clinical observation of the monkeys for evidence of toxicity was recorded twice on the day of injection and daily during the remainder of the study through day 15. Animals were weighed on day 1 prior to injection, and on days 2, 8 and 15 post injection.

## Blood collection and analysis

Blood was collected from each animal prior to injection and on day 2. Blood was also collected on days 8 and 15 from the remaining monkeys using Vacutainer CPTJ cell preparation tubes (with sodium citrate). Blood samples were collected for clinical pathology, PBMC and assessment of complement activation. Analyses of the tissues were not blinded to the treatment groups. Serum chemistries included determination of serum electrolytes, proteins and enzymes. Electrolytes examined included potassium (K), sodium (Na) and chloride (Cl). Enzymes studied included alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT) and alanine aminotransferase (ALT). Proteins and other serum chemistry parameters evaluated included total protein (TP), albumin (Alb), globulin (Glob), albumin/globulin ratio (Alb/Glob), glucose (Glu), blood urea nitrogen (Bun) and creatinine (Crea). All measurements were performed using Roche Diagnostics Hitachi 911 System. Complement activation (breakdown product Bb) was assessed in the monkeys using blood collected in presence of EDTA prior to treatment, and at 30 min, 60 min, and 24 h following injection as well as on days 4, 8, and 15 during the study period.

Hematology parameters examined included erythrocyte count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Platelet count, white blood cell count (WBC), neutrophil, total (T) lymphocytes, monocytes, eosinophils, basophils and coagulation parameters such as activated partial thromboplastin time (APTT), prothrombin time and fibrinogen were also recorded using standard procedures. RBC, HGB, HCT, MCV, MCH, MCHC, WBC, neutrophil, total (T) lymphocytes, monocytes, eosinophils, basophils and platelet counts were determined using Advia 120 Hematology Analyzer. Blood coagulation parameters such as fibrinogen (Fib), activated partial thromboplastin time (APTT) and prothrombin time (Pt) were also recorded using ACL 8000 Coagulation Systems.

Peripheral blood mononuclear cells (PBMC) were harvested from the blood collected from all animals prior to treatment, on day 2, and from surviving animals on days 8 and 15. For determination of STAT1 and STAT3 phosphorylation levels, PBMCs were fixed in 1.5% PFA/PBS for 10 min at room temperature, washed once with 2% FBS/PBS and resuspended in 100 μl of 2% FBS/PBA. The cells were stained with 20 μl of the PE conjugated isotype control, STAT1 and STAT3 antibodies (BD Bioscience, San Jose, CA) for 30 min at room temperature in the dark. The cells were then washed twice with 2% FBA/PBS and resuspended in 300 μl of 2% FBS/PBS. Single color flow cytometry was performed on a Beckman coulter flow cytometer.

For analyses of complement activation (breakdown product Bb), blood samples were collected into tubes containing EDTA and placed on wet ice immediately after collection. Blood was collected at pre-dose and at 30 and 60 min and at post-dose on day 1, 4, 8 and 15. Plasma was separated, quickly frozen and stored at −80°C until analyzed.

## Tissue analysis

Organs were harvested at the time of sacrifice (day 2 and day 15) from one male and one female monkey from each treatment group including bone (femur), bone marrow (sternum, rib, costochondral junction), brain, cecum, colon, duodenum, epididymis, esophagus, eyes, gallbladder, gonads, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes (bronchial, mandibular and mesenteric), mammary gland, pancreas, parathyroid gland, pituitary gland, prostate gland, salivary gland, sciatic nerve, skeletal muscle (thigh), skin (ventral abdomen), spinal cord (thoracolumbar), spleen, stomach (cardiac, fundic and pyloric), thymus, thyroid glands, tonsils, trachea, urinary bladder and uterus. After gross examination by a veterinary pathologist, sections of the above tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, cut approximately five microns thick, placed on slides and stained with hematoxylin and eosin (H&E). Histopathologic exam was performed by an experienced pathologist (Dr. Katherine A. B. Knostman). Microscopic parameters examined were categorized as either drug-related or non-drug related. Each lesion was listed and coded by the most specific topographic and morphologic diagnoses, severity and distribution using the Pathology Terminology Guidelines of the Toxicology Data Management system (TDMS) for the National Toxicology Program. Microscopic findings were graded semi-quantitatively according to the following scale. Minimal (Grade 1) represented the least detectable lesion; mild (Grade 2) represented an easily discernible lesion unlikely to have any biological significance; moderate (Grade 3) represented a change affecting a large area of the represented tissue that had the potential to be of some significance; and Grade 4 was reserved for lesions that approached a theoretical maximum in severity, with entire tissue involvement (i.e., hepatocellular necrosis affecting practically all hepatocytes in every section).

## Determination of STAT3 target gene expression

For determining STAT3 target gene expression, Western blot analyses were performed using lysates from the muscle tissue of animals injected with the STAT3 decoy (0.8 and 3.2 mg) and vehicle control. To extract the cell lysate, the muscle tissues were lysed with the lysis buffer (50 mM tris, 150 mM NaCl, 0.1% SDS, 1% NP40) and the protein content was quantified using the Bradford reagent. Forty micrograms of whole cell protein lysate was run on 12.5% SDS-PAGE gels and transferred onto the Trans-Blot nitrocellulose membrane (BioRad Laboratories, Hercules, CA) at 40 V using a semi-dry transfer machine (BioRad Laboratories, Hercules, CA). The membrane was blocked using 5% non-fat dry milk, 0.2% Tween-20 in 1× PBS (TBST) for 2 h. The membrane was incubated with Bcl-$X_L$ primary antibody diluted at 1:1,000 at 4°C overnight, then washed five times with TBST (5 min/wash). However, for the β-tubulin antibody, 1:5,000 dilutions were used. The membrane

was then incubated with secondary antibody for 1 h (1:3,000) at room temperature, followed by five washes in TBST. Blots were developed using ECL, according to the manufacturer's instruction (PerkinElmer Las, Waltham, MA). For RT-PCR analysis, total RNA was isolated from 30 mg frozen muscle tissue using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentration and integrity was evaluated by measuring absorbance at 260 and 280 nm. To detect cyclin D1, standard RT-PCR was done using the One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) with primers 5′CTGTGCTGCGAAGTGGAAACC3′ and 5′CAGGAAGCGGTCCAGGTAGT3′. The primers were diluted to a final concentration of 0.2 μM and incorporated into the reaction mixture. The remaining PCR reagents were diluted according to the manufacturer's protocol. Reverse transcription was done at 50°C for 30 min followed by enzyme inactivation and hot-start PCR at 95°C for 15 min. Denaturation, annealing, and extension were done at 94, 55, and 72°C, respectively, for 1 min each for a total of 35 cycles. The reaction was completed with an extension period at 72°C for 10 min. PCR products were visualized on a 1% agarose gel containing ethidium bromide. Gene expression was assessed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### Statistical analysis

This study of potential toxicity of the STAT3 decoy in Cynomolgus monkeys was not intended or designed to detect specific changes in hematology parameters or serum chemistry nor was a power analysis conducted to address the power of specific hypotheses tests. Accordingly, results can only be considered as observational without the ability to claim or disclaim that the STAT3 decoy can be considered safe. Statistical analysis was conducted to estimate rapid changes (to day 2) in paired samples from up to all 12 monkeys. The Jonckheere-Terpstra statistic was calculated as a measure of specific trend in changes to day 2 among the three groups in their natural order, i.e., from control to 0.8 mg decoy to 3.2 mg decoy. To control for inflation of type I error resulting from testing a large family of hypotheses (17 hematology parameters, 13 serum chemistries), the single step minP procedure [8] was calculated by permutation resampling. Group differences in phosphorylated STAT1 and STAT3 in peripheral blood were tested with the exact Jonckheere-Terpstra statistic, with unadjusted *P* values.

## Results

### STAT3 decoy administration did not alter animal behavior or body weight

Non-human primate models are required for oligonucleotide toxicology studies. All animals were observed for evidence of toxicity daily and weighed pretreatment and on days 2, 8 and 15 after the treatment. Treated monkeys exhibited behavior throughout the study that was similar to that observed in the control group monkeys. Individual animal body weights are presented by group in Fig. 1. Body weight changes between measurement days were small (<0.2 kg) and similar among the three groups.

### STAT3 decoy administration did not alter serum chemistries or hematologic indices

Serum electrolytes (Na, K, Cl), serum enzymes (ALP, AST, GGT, ALP), total (T) protein, albumin (Alb), globulin (Glob), albumin/globulin ratio (Alb/Glob), glucose (Glu), blood urea nitrogen (Bun) and creatinine (Crea) were also examined prior to treatment and on days 2, 8 and 15. Comparison of serum chemistries between the treatment groups and the control group demonstrated no significant difference due to STAT3 decoy treatment at any dose level. The statistical evaluations demonstrating no significant difference in the serum chemistries of animals in the two STAT3 decoy groups (0.8 and 3.2 mg) and the vehicle control group at day 2 are presented in Table 2. No significant difference in any of the serum chemistry parameters was observed between the control and the treatment groups on pre and

post-STAT3 decoy treatment (Fig. 2a). Erythrocyte (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, white blood cell count (WBC), neutrophil, total (T) lymphocytes, monocytes, eosinophils, basophils and coagulation parameters such as activated partial thromboplastin time (APTT), prothrombin time and fibrinogen were determined prior to treatment and on days 2, 8 and 15. Due to the extensive blood collections throughout the study, and the smaller size of one of the animals, blood collection on day 15 was difficult from that animal in the group injected with low dose of STAT3 decoy (0.8 mg). As a result, the sample for that animal on day 15 was insufficient to obtain a hematology profile. There were no salient changes due to STAT3 dose with the possible exception of APTT (Fig. 2b). After accounting for inflation of type I error resulting from the testing of 17 hypotheses, the minimum adjusted *P*value was 0.337 and none of the dose-dependent changes could be considered statistically significant (Table 3).

## Intramuscular STAT3 decoy administration effects on pSTAT3 or pSTAT1 expression in PBMC

We previously reported that the STAT3 decoy abrogates both STAT3 and STAT1-mediated signaling in HNSCC cells, likely due to the high sequence homology at the DNA binding domain [21]. To determine if the STAT3 decoy had an effect on STAT3 or STAT1 activation in peripheral blood, flow cytometry was performed to assess phosphorylated STAT3 or STAT1 levels in PBMC. PBMC harvested from each animal blood sample at pre-dose, day 2, and on days 8 and 15 from the monkeys remaining after sacrifice, on day 2 were stained for pSTAT1 and pSTAT3 levels. Although results were only available for eight monkeys with paired flow cytometry data prior to treatment and on day 2, a decrease in pSTAT3 mean fluorescence index (MFI) was observed for the high dose of STAT3 decoy (exact two tailed $P = 0.0124$). No dose effect was seen for pSTAT1 MFI ($P = 0.6107$) (Fig. 3).

## Complement levels remained unaffected by STAT3 decoy administration

Activation of the alternative pathway of complement by the phosphorothioate backbone of oligonucleotides has been reported in primates [9]. These effects are not sequence specific and have been attributed to the non-specific binding and inactivation of inhibitory factors, such as complement factor H [13]. Complement activation (breakdown product Bb) was assessed in the monkeys prior to treatment with STAT3 decoy and at 30 min, 60 min and 24 h post-dose to capture early signs of inflammation as well as on days 4, 8 15 post-treatment to assess recovery from effects of complement activation if any. No activation of the alternative pathway of complement occurred in any animal at any dose at any time point (Fig. 4).

## STAT3 decoy administration did not result in organ damage

A complete necropsy was performed on each animal at the time of sacrifice. One male and one female monkey from each dose group were euthanized on day 2 (approximately 24 h post-dosing). The remaining two animals per group were euthanized on day 15. The only gross findings observed were three nodules and a cystic structure in the liver of one monkey euthanized on day 2 that had received the low dose of STAT3 decoy (0.8 mg). Microscopically, these lesions were consistent with chronic abscesses and pyogranulomas with abundant eosinophil infiltration and hemosiderosis (data not shown). While no parasites were seen, the lesions were highly suggestive of parasitic migration and were thought to be unrelated to treatment with the STAT3 decoy. Histopathologic studies on the following organs were performed after being examined and fixed in 10% neutral buffered formalin: bone (femur), bone marrow (sternum, rib, costochondral junction), brain, cecum,

colon, duodenum, epididymis, esophagus, eyes, gallbladder, gonads, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes (bronchial, mandibular and mesenteric), mammary gland, pancreas, parathyroid gland, pituitary gland, prostate gland, salivary gland, sciatic nerve, skeletal muscle (thigh), skin (ventral abdomen), spinal cord (thoracolumbar), spleen, stomach (cardiac, fundic and pyloric), thymus, thyroid glands, tonsils, trachea, urinary bladder and uterus. No histopathological lesions were reported upon microscopic observation of the aorta, mammary gland, spleen, tonsil, bone marrow, duodenum, gall bladder, lung bronchi, ovary, stomach, trachea, esophagus, ileum, lung, mandibular lymph nodes, thymus, jejunum, mesenteric lymph node and spinal cord in any animal at any dose levels. Mild or minimal mononuclear cell infiltrate, minimal inflammation or minimal cytoplasmic vacuolization was observed in several organs including brain, liver, heart, kidney, pituitary gland, salivary gland, urinary bladder or pancreas in some STAT3 decoy-injected animals as well as the untreated control animals. All changes were typical background lesions in Cynomolgus macaques and were interpreted to be unrelated to STAT3 decoy administration (data not shown).

### STAT3 decoy abrogated target gene expression at the injection site

We previously reported that intratumoral administration of the STAT3 decoy in a HNSCC xenograft model decreased STAT3 target gene expression including Bcl-xl and cyclin D1 [30]. Despite the relatively large size of the primate muscle (compared with murine xenograft tumor), tissue at the injection site was processed to assess STAT3 target gene expression. Immunoblotting for Bcl-$X_L$ was performed with the cell lysate extracted from the muscle tissue (Fig. 5a). Treatment with both low and high dose of STAT3 decoy decreased Bcl-$X_L$ level compared to the control animals. RT-PCR analyses for cyclin D1 in the RNA samples isolated from the muscle tissue of monkeys injected with low and high dose of STAT3 decoy showed a dose related decrease in cyclin D1 expression (Fig. 5b).

## Discussion

Increased activation of STAT3 has been reported in a wide variety of human cancer cell lines and primary human tumors compared with levels in corresponding normal tissue [11] [31] [6] [1]. The oncogenic role of STAT3 was demonstrated by the ability of a constitutively-active mutant to transform fibroblasts in vitro and initiate tumor formation in vivo [3]. Observations that elevated levels of total and/or tyrosine phosphoryalated STAT3 in the tumor are associated with decreased survival in cancer patients suggests that STAT3 may serve as a therapeutic target [19]. To date, no small molecule with selective activity against STAT3 has been identified. We developed a transcription factor decoy approach that specifically inhibits STAT3-mediated DNA binding with antitumor effects reported in preclinical models of several cancers including HNSCC [20] [30] [31] [4, 26]. In anticipation of implementing a phase 0 clinical trial, the present study was carried out to determine the toxicity of the STAT3 decoy in a non-human primate model.

STAT3 has been shown to play an essential role in early development as demonstrated by the early embryonic lethality of STAT3 deletion [27]. The function of STAT3 has also been assessed in normal adult tissues where STAT3 has been implicated in stimulation of proliferation in B lymphocytes, activation of growth arrest in monocytes, and induction of an acute-phase response in hepatoma cells [22]. Thus, attempts to block STAT3 activation for cancer therapy have to be tested for their effects on normal cellular and tissue homeostasis.

Several molecular strategies have been reported to down-regulate STAT3 protein and/or decrease STAT3 phosphorylation. These include peptide aptamers that bind the STAT3 DNA-binding site or the SH2 domain and surrounding region, phosphotyrosyl

peptidomimetics, G-quartet oligonucleotides, siRNA or antisense strategies (summarized in [19]). A STAT3-inhibiting peptide conjugated to an ErbB2 peptide mimetic and linked to an HIV TAT domain inhibited breast cancer xenograft growth in vivo [28]. G-quartet oligonucleotides targeting STAT3 have also been described with high STAT3-selective activity [16].

Synthetic double stranded DNA with a high affinity for a target transcription factor may be introduced into target cells as a “decoy” to bind the transcription factor and inhibit target gene expression [27]. The DNA decoy closely corresponds to the response element within the promoter region of specific genes. By administering a sufficient concentration of decoy, the authentic interaction between a transcription factor and its response element is diminished due to the saturation of the transcription factor binding site, with subsequent modulation of gene expression [15]. This approach is particularly attractive for several reasons: (1) the potential drug targets (transcription factors) are readily identifiable; (2) the synthesis of the sequence-specific decoy is relatively simple; and (3) knowledge of the exact molecular structure of the target transcription factor is unnecessary.

The only transcription factor decoy to undergo clinical evaluation to date was designed to competitively inhibit the transcription factor E2F, a critical regulator of the cell cycle, for the treatment of intimal hyperplasia following vascular bypass procedures. This compound, edifoligide, completed phase III testing and while it failed to induce patency in vascular bypass grafts compared with placebo, no dose-limiting side effects were reported [14]. In the clinical trials of this transcription factor decoy, vascular grafts were bathed in the decoy in a pressurization chamber where the drug was infused through the lumen to fill the chamber and immerse the graft. This process was performed in the operating room prior to anastamosis of the graft in the patient. In a series of preclinical and clinical studies, local delivery of the decoy resulted in undetectable plasma levels of the drug and no toxic effects were observed with local delivery. Systemic administration of antisense oligonucleotides has been associated with complement activation, prolonged APTT, increased transaminases, thrombocytopenia, and splenomegaly in animal models [5]. In the present study, intramuscular administration of the STAT3 decoy was chosen, as this most closely reflects the planned intratumoral delivery method for the human trial. Using this approach, we found no evidence of local or consistent systemic abnormalities using a lower or higher dose of the STAT3 decoy in monkeys.

A single intramuscular injection in Cynomolgus monkeys did not result in the death of any animal or demonstrate evidence of systemic or organ-specific toxicity. The two different doses administered resulted in only mild or minimal cellular mononuclear cells infiltration of some organs. In one animal, injected with the low dose of STAT3 decoy (0.8 mg), a cystic structure of the liver associated with three nodules reflecting chronic abscesses with profuse eosinophil infiltration was observed which was felt to be unrelated to treatment with the STAT3 decoy. Even though the primate muscle was substantially larger than our previous murine xenograft model, the clinical grade STAT3 decoy abrogated STAT3 promoter activity and gene expression in HNSCC cell lines and decreased expression of STAT3 target genes at the site of injection (data not shown; Fig. 5). The high dose of STAT3 decoy, 3.2 mg was associated with a reduction in pSTAT3 in peripheral blood. These results support the biologic potency of the material injected.

To date, in vitro studies have failed to demonstrate any toxicity of the STAT3 decoy in normal mucosal epithelial cells after being treated with the STAT3 decoy and there are no reports of any toxicity study using STAT3 targeting agent in any in vivo model [20]. This is the first in vivo study to demonstrate lack of toxicity of the STAT3 decoy upon targeting STAT3 in normal muscle tissue of Cynomolgus monkeys. Given the small number of

animals used in these toxicology studies, we are not able to conclude that the STAT3 decoy is absolutely safe for human testing. The planned phase 0 trial will utilize a single intratumoral injection, performed in the intraoperative setting in conjunction with planned resection of the tumor, which is likely to be well tolerated. In the trial, patients will be closely monitored to detect evidence of local or systemic toxicity resulting from intratumoral STAT3 decoy treatment. If the decoy results in decreased STAT3 target gene expression in the resected HNSCC tumor, a phase I trial will be considered. Current efforts are directed towards stabilizing the decoy via chemical modification so that it can be delivered systemically.

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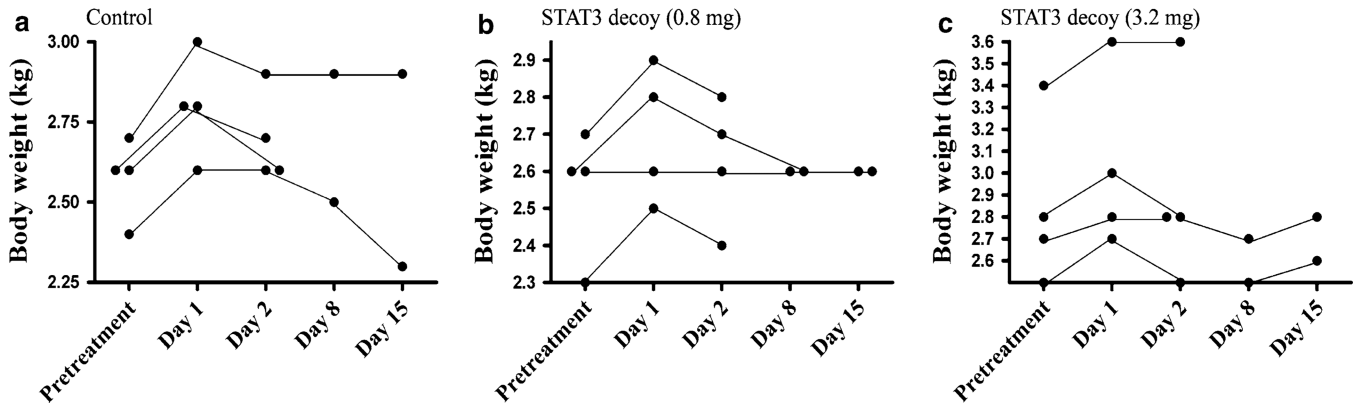
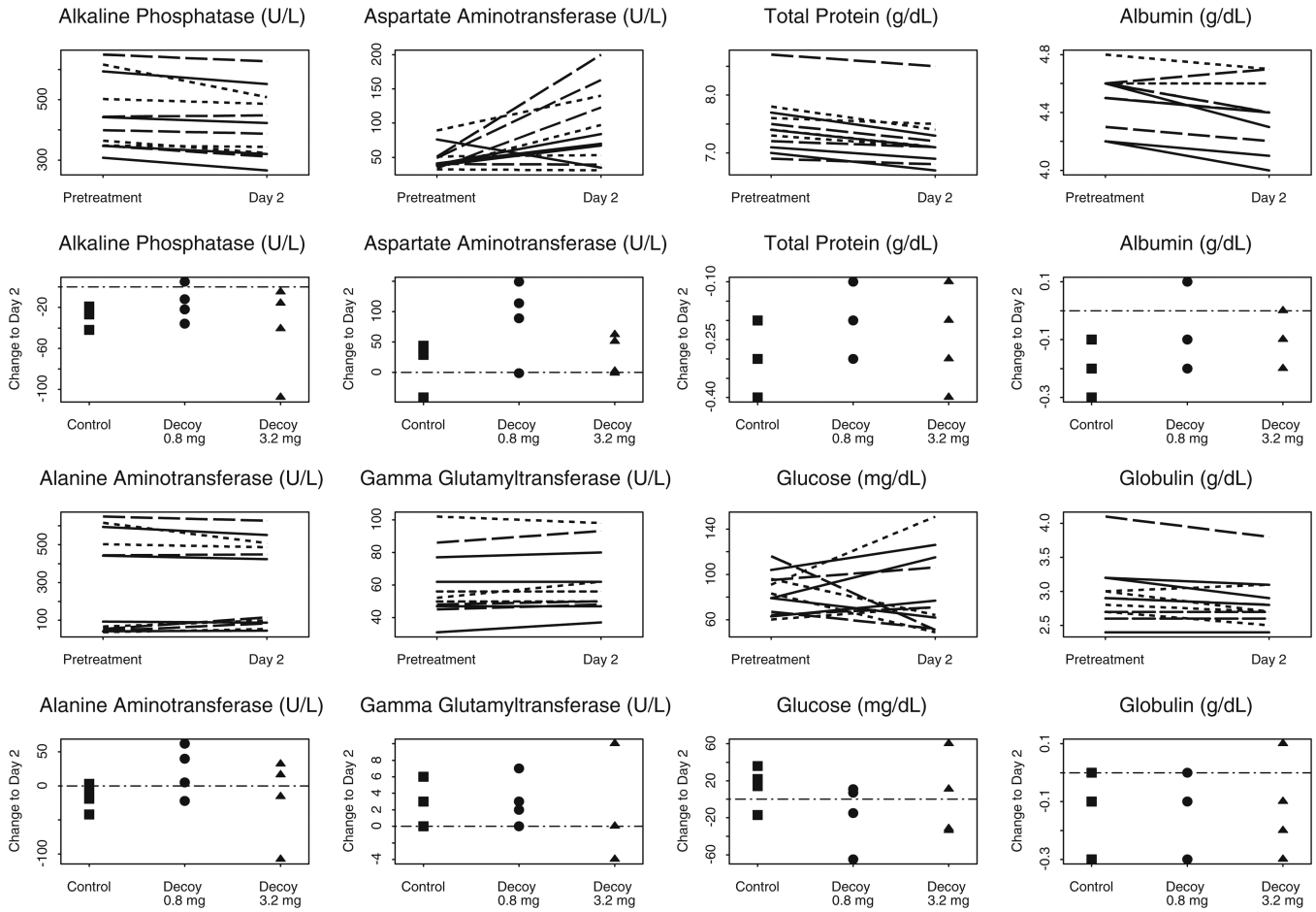
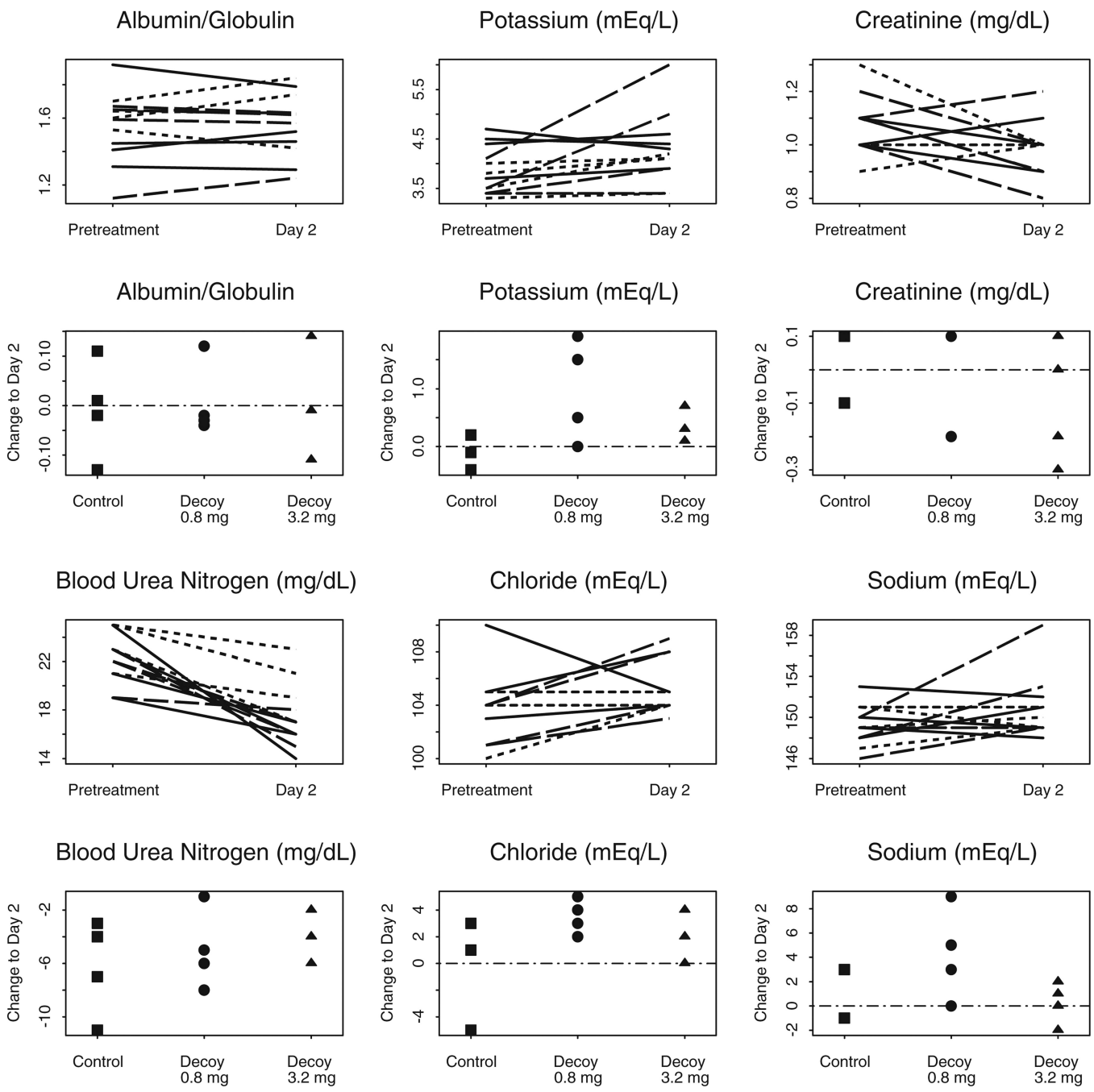
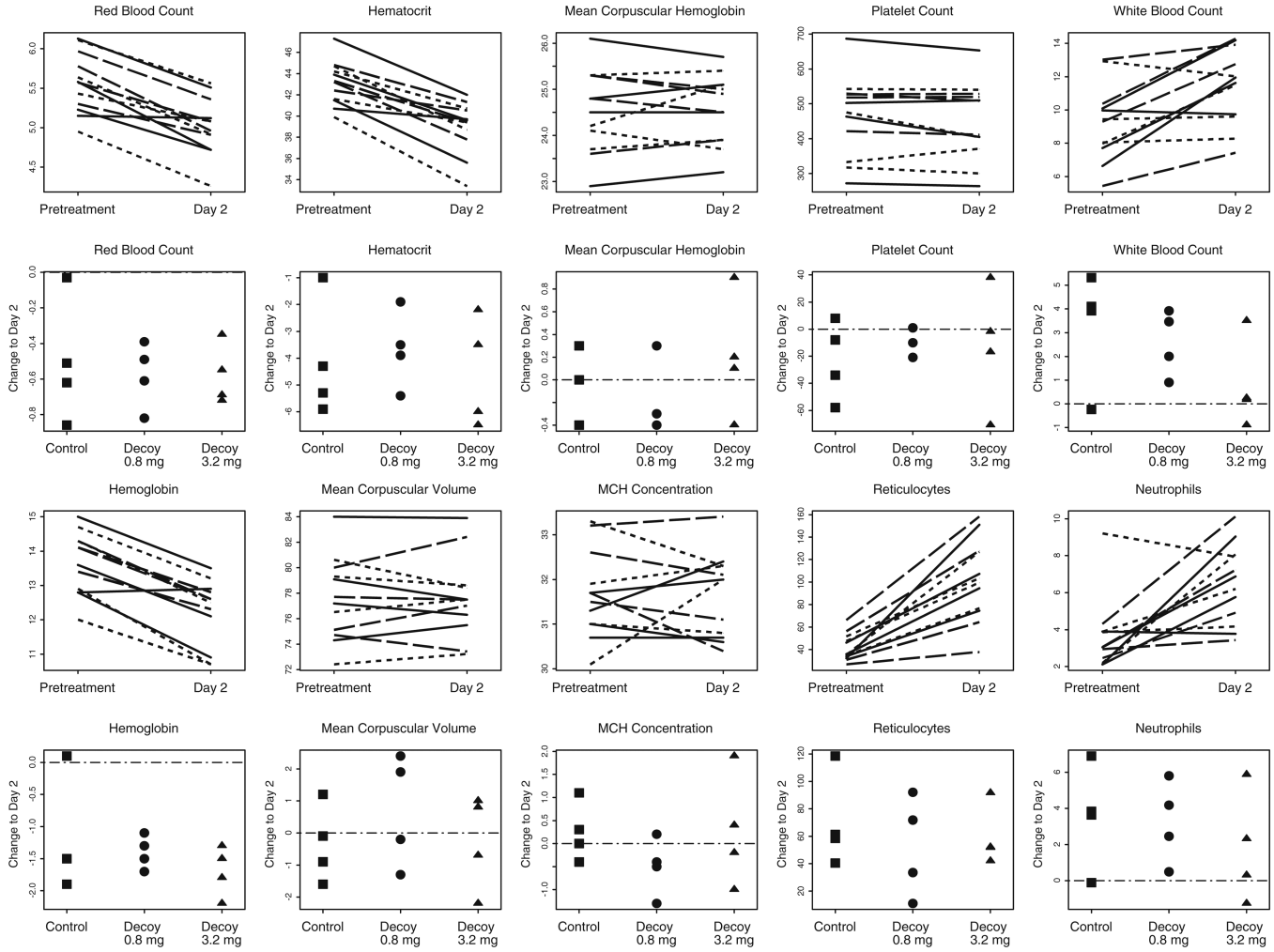



**Fig. 1.**

Apparent lack of effect of STAT3 decoy on body weight. Body weight measurements of the monkeys injected with PBS as the vehicle control (**a**), low dose (0.8 mg) of STAT3 decoy (**b**) and high dose (3.2 mg) of STAT3 decoy (**c**) were determined during the pretreatment period, on day 1 before treatment and on days 2 8 and 15 after STAT3 decoy treatment. No statistically significant difference in body weight was observed between the treatment groups at each time point. *Each line* represents an individual animal







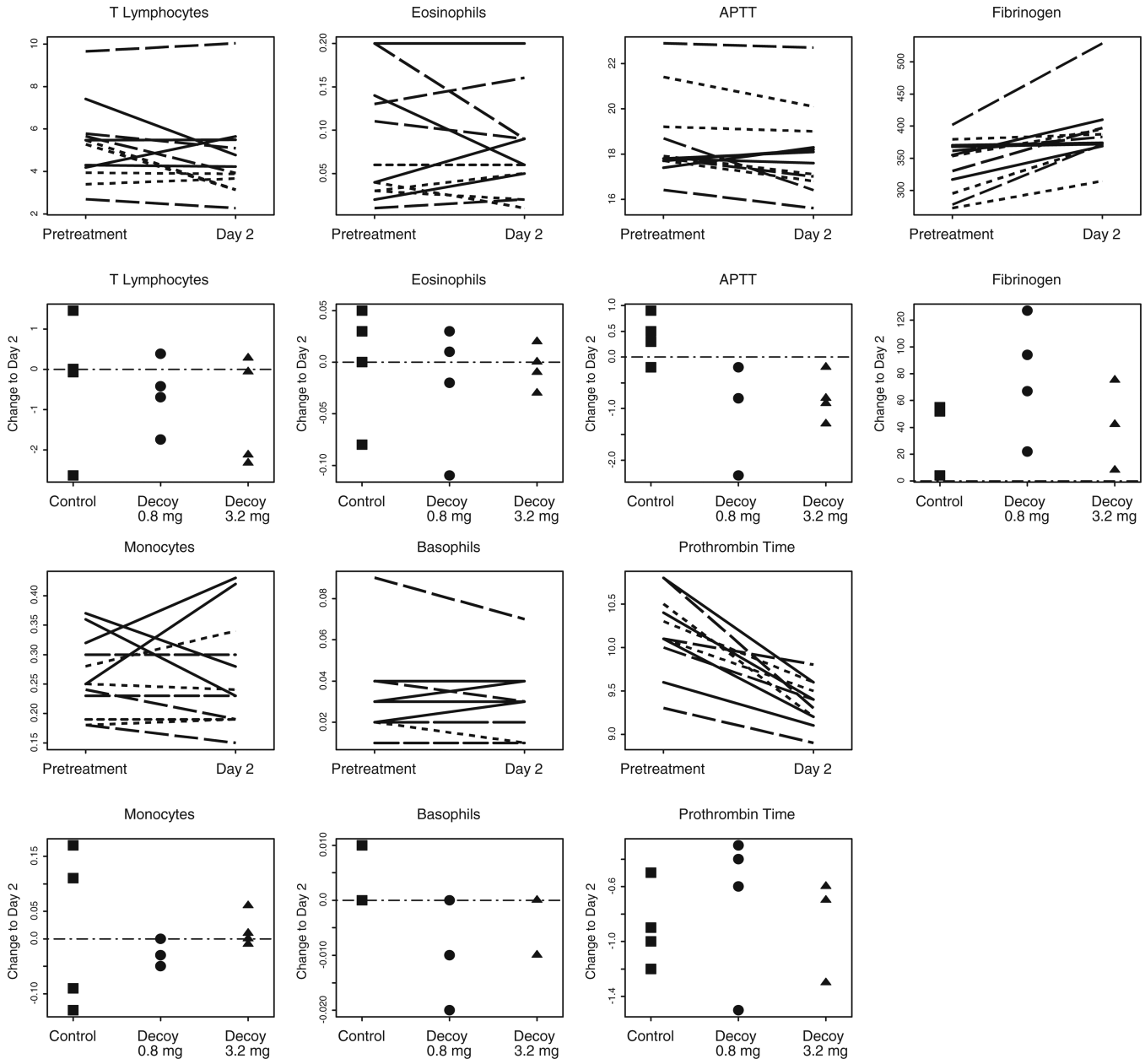



**Fig. 2.**

STAT3 decoy administration may not affect serum chemistry or hematological parameters in monkeys. **a** Distribution of serum chemistries and **b** hematological indices in the blood of monkeys injected with the STAT3 decoy. Four monkeys were injected with PBS as control. Four monkeys were injected with the low dose of STAT3 decoy (0.8 mg) and four monkeys were injected with the high dose of STAT3 decoy (3.2 mg) and blood was collected from each animal at the times indicated. Data for each constituent are shown in pairs (*top* to *bottom*). The *top graph* is the day 2 profile for each monkey, as a *line* connecting each pretreatment to day 2. The *lower graph* is the magnitude of the change to day 2 by group. Line codes are *solid line* for control, *small dashes* for 0.8 mg STAT3 decoy and *big dashes* for 3.2 mg STAT3 decoy. The *lower graph* of each pair may include a *dashed line* to depict zero change

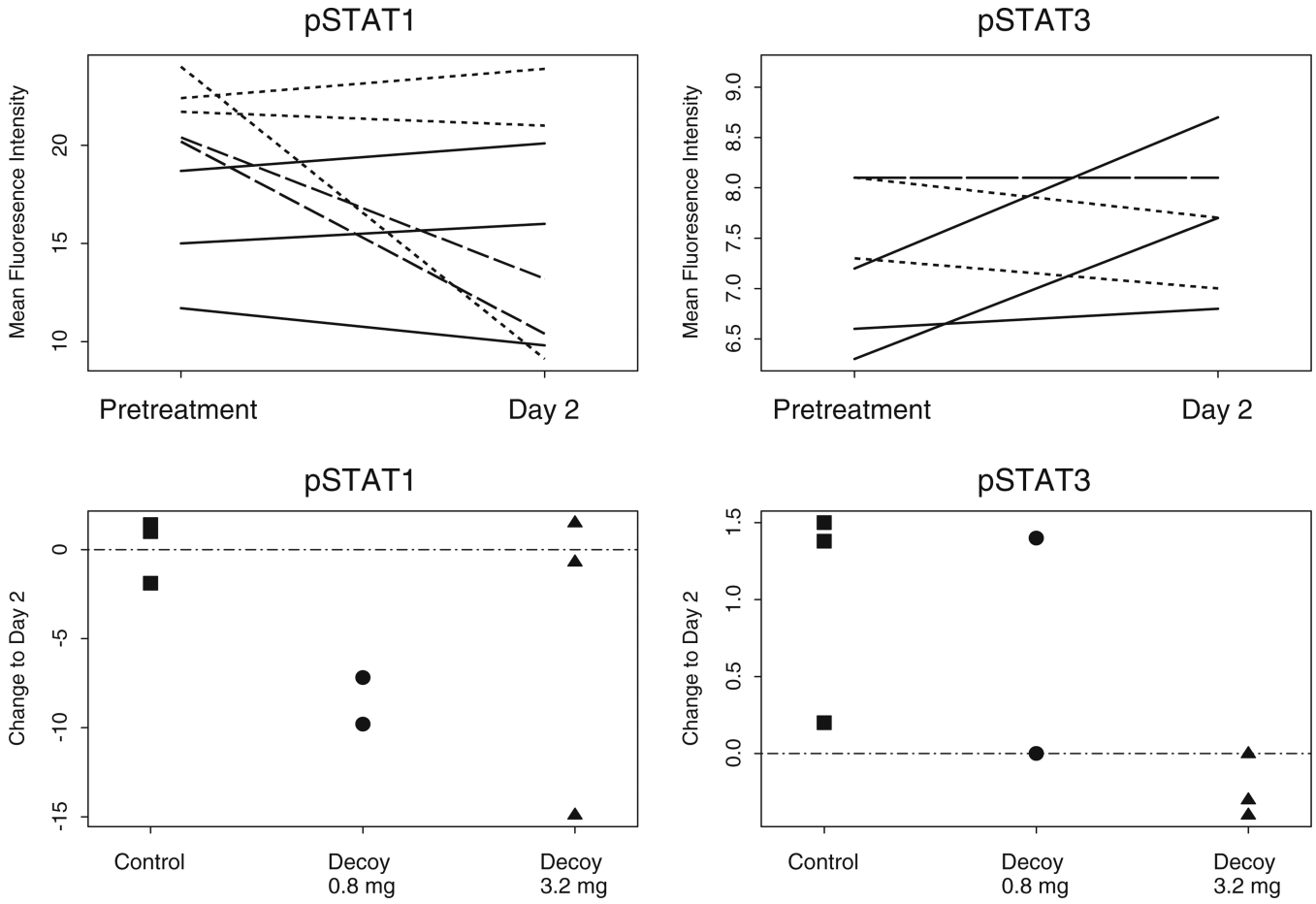



**Fig. 3.**

High-dose STAT3 decoy may reduce pSTAT3 levels in PBMCs of monkeys. PBMCs from the monkeys injected with vehicle control, low (0.8 mg) or high dose (3.2 mg) of STAT3 decoy were fixed, washed and stained with phosphoSTAT1 (*pSTAT1*) and phosphoSTAT3 (*pSTAT3*) antibodies. Single color flow cytometry was performed on a Beckman coulter flow cytometer. The *upper panels* demonstrate the mean fluorescence intensity (MFI) from *pSTAT1* (*left panel*) and *pSTAT3* (*right panel*) from PBMCs of each animal administered the STAT3 decoy. The *lower panels* demonstrate the change in *pSTAT1* (*left panel*) and *pSTAT3* (*right panel*) MFI in PBMC from animals administered with vehicle control, low dose 0.8 mg STAT3 decoy or high dose 3.2 mg STAT3 decoy

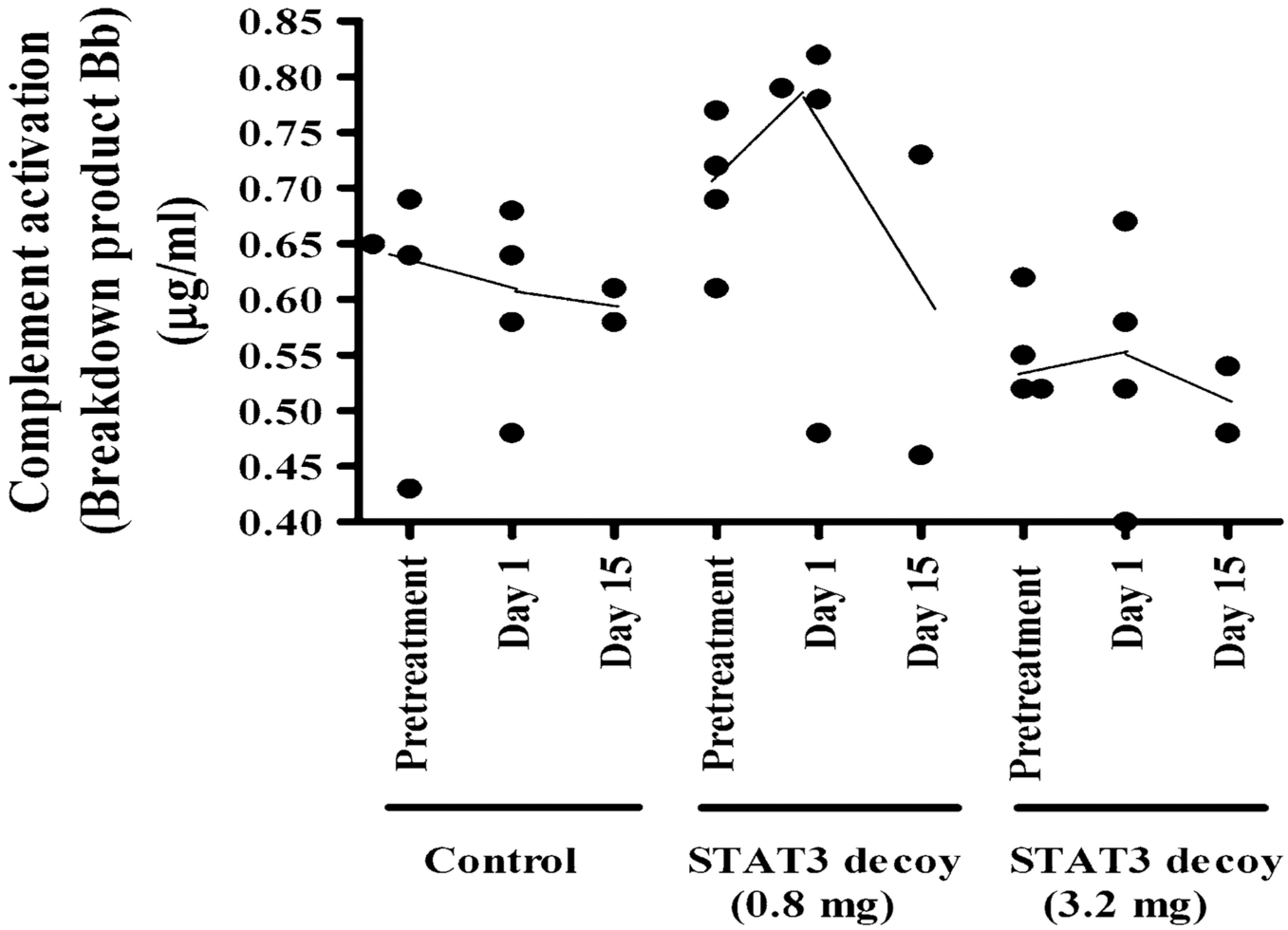



**Fig. 4.**

STAT3 decoy administration does not induce complement activation. Complement activation (breakdown product Bb) in the blood of monkeys injected with vehicle control, low (0.8 mg) or high dose (3.2 mg) of STAT3 decoy determined prior to treatment and after 24 h. Group medians are connected with a *solid line*. No significant difference in complement activation was observed between the control group and the low and high dose of STAT3 decoy injected groups

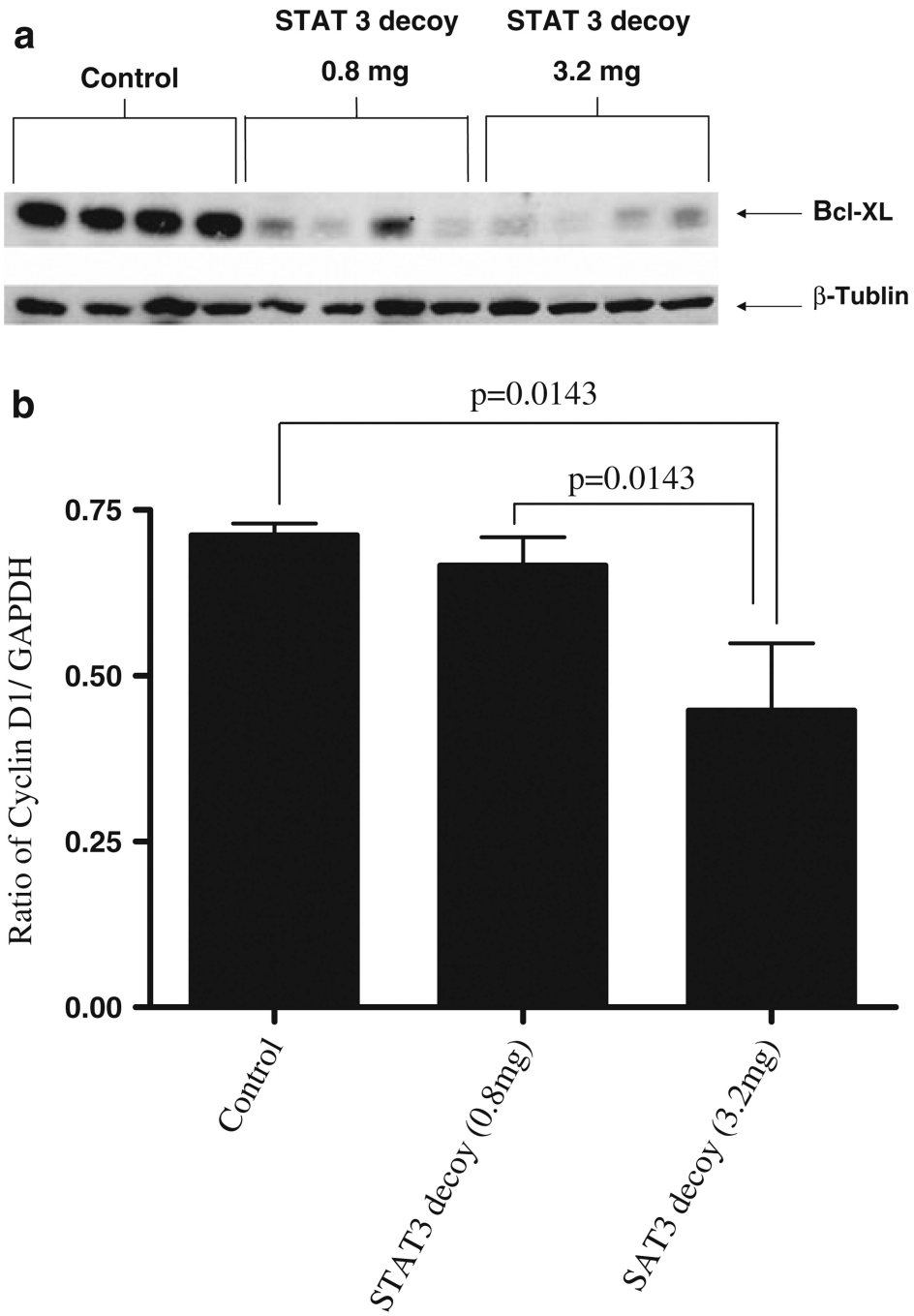



**Fig. 5.**

STAT3 decoy modulates target gene expressed at the injection site. **a** Whole cell lysates were prepared from the muscle tissue surrounding injection sites in control animals and animals treated with 0.8 mg or 3.2 mg STAT3 decoy. Proteins (40 μg /lane) were resolved on a 12.5% SDS/PAGE gel and subjected to immunoblotting with anti-Bcl-$X_L$ antibody. The blot was stripped and reprobed with anti-β-tubulin to assess protein loading. The experiment was performed twice, with similar results each time. **b** RNA was extracted from the muscle tissue of monkey injected with low (0.8 mg) and high dose (3.2 mg) of STAT3 decoy and PBS as the vehicle control and RT-PCR was performed to determine cyclin D1

level. Expression of cyclin D1 is relative to GAPDH level. The *bar graph* represents cumulative results from four samples per treatment group

**Table 1**

Animal grouping for toxicity studies

| Group | Dosage[a] (mg/dose) | Dose concentration (mg/mL) | Dosage volume[a] (mL) | Number of monkeys at study start[b] | Number of monkeys necropsied | |
|---|---|---|---|---|---|---|
| | | | | | Day2[c] | Day 15[c] |
| Control | 0 | 0 | 0.9 | 4 | 2 | 2 |
| STAT3 decoy (low dose) | 0.8 | 0.9 | 0.9 | 4 | 2 | 2 |
| STAT3 decoy (high dose) | 3.2 | 3.5 | 0.9 | 4 | 2 | 2 |

[a]Flat dose, i.e., mass and volume administered will be independent of body weight

[b]Equal number of males and females

[c]Following treatment

## Table 2

Jonckheere-Terpstra 2-tailed test for equality between the three groups, vehicle control group, 0.8 mg and 3.2 mg STAT3 decoy administered groups

| Variable | Asymptotic test statistic | Raw *P* | Adjusted *P* |
|---|---|---|---|
| Alkaline phosphatase | 0.5855 | 0.5582 | 1 |
| Alanine aminotransferase | 0.5855 | 0.5582 | 1 |
| Aspartate aminotransferase | 0.366 | 0.7144 | 1 |
| GG | 0.5123 | 0.6084 | 1 |
| Total protein | 0.6587 | 0.5101 | 1 |
| Glucose | 0.9515 | 0.3413 | 0.994 |
| Albumin | 0.9515 | 0.3413 | 0.994 |
| Globulin | 0.366 | 0.7144 | 1 |
| Albumin/globulin | 0.8051 | 0.4208 | 0.998 |
| Blood urea nitrogen | 1.1711 | 0.2416 | 0.954 |
| Potassium | 1.0247 | 0.3055 | 0.981 |
| Chloride | 0.366 | 0.7144 | 1 |
| Creatinine | 1.0979 | 0.2723 | 0.974 |
| Sodium | 0.2928 | 0.7697 | 1 |

**Table 3**

Jonckheere-Terpstra 2-tailed test for equality between the three groups, vehicle control group, 0.8 mg and 3.2 mg STAT3 decoy administered groups

| Variable | Asymptotic test statistic | Raw *P* | Adjusted *P* |
|---|---|---|---|
| RBC | 0.0000 | 1.0000 | 1.000 |
| HGB | 0.8051 | 0.4208 | 0.999 |
| HCT | 0.3660 | 0.7144 | 1.000 |
| MCV | 0.0000 | 1.0000 | 1.000 |
| MCH | 0.0732 | 0.9417 | 1.000 |
| MCHC | 0.2196 | 0.8262 | 1.000 |
| PLT | 0.2928 | 0.7697 | 1.000 |
| RET | 0.4392 | 0.6605 | 1.000 |
| WBC | 2.0494 | 0.0404 | 0.422 |
| NEUT | 2.0494 | 0.3798 | 0.998 |
| TLYM | 0.8783 | 0.4642 | 1.000 |
| MONO | 0.7319 | 0.4642 | 1.000 |
| EOS | 0.8051 | 0.4208 | 0.999 |
| BASO | 1.4639 | 0.1432 | 0.867 |
| APTT | 2.1226 | 0.0338 | 0.337 |
| PTT | 0.1464 | 0.8836 | 1.000 |
| FIB | 0.5855 | 0.5582 | 1.000 |