Selective Inhibition of NF-kappa-B with NBD Peptide Reduces Tumor-Induced Wasting in a Murine Model of Cancer Cachexia In vivo

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Abstract
Cancer cachexia is a severe wasting syndrome characterized by the progressive loss of lean body mass and systemic inflammation, which is seen in as many as 80% of patients with advanced malignancy. It accounts for an estimated 20-30% of all cancer-related deaths. The mechanism by which cancer induces skeletal muscle atrophy in cachexia involves tumor-derived cytokines, including TNFα, IL-1, and IL-6. Upon interaction with their unique receptors on skeletal muscle, these cytokines activate NF-kappaB, a transcription factor crucial for atrophy-related sarcomere proteolysis to occur. The significance of NF-κB is highlighted in studies demonstrating that genetic inhibition of NF-κB ameliorates cancer-induced muscle loss in vivo. In the present study, we evaluate a selective NF-kappaB inhibitor (NBD peptide) which targets the IkappaB complex to prevent cancer-induced skeletal muscle atrophy in an established mouse model (C26 adenocarcinoma). We identified for the first time that NBD peptide can directly inhibit tumor-induced NF-kappaB activation in skeletal muscle, resulting in a decrease loss of lean muscle. We also identified that NBD peptide reduces the expression of the tumor induced ubiquitin ligases MuRF-1 and MAFbx/Atrogin-1 necessary for atrophy. These findings highlight that NBD peptide may be a potential selective therapeutic agent for the treatment of cancer cachexia.

Keywords: Cachexia; NF-kappa-B; Muscle atrophy; Cancer; NBD peptide

Abbreviations: FM: Fat Mass; IκB: Inhibitory κB; IKK: IκB Kinase; LBM: Lean Body Mass; MuRF1: Muscle Ring Finger-1; MAFbx: Muscle Atrophy F-Box also called Atrogin-1; NBD: NEMO Binding Domain; NEMO: NF-κB Essential Modulator; NF-κB: Nuclear Factor-κB

Introduction
Cancer cachexia is a severe wasting syndrome characterized by the progressive loss of skeletal muscle and an increase in systemic inflammation. This highly debilitating condition is also associated with weight loss, depletion of fat stores, anemia, insulin resistance, and alterations in carbohydrate, lipid, and protein metabolism [7,30]. Cachexia is seen in as many as 80% of patients with advanced malignancy and, rather than tumor burden, accounts for an estimated 20 to 30% of all cancer related deaths [30,37,65]. Tumor-induced wasting also lowers patient response to chemotherapy, corresponding to a poorer prognosis of different ways. Both genetic or pharmacologic inhibition of NF-κB prevents muscle mass loss, improves strength, and increases muscle regeneration [2,9,16,28,31,36,48,57,70,75]. These numerous studies implicate NF-κB as having much potential as a therapeutic target in cancer-induced muscle loss.

Recent studies have reported novel therapeutic interventions which specifically target acute increases in NF-κB, while not affecting the necessary basal NF-κB activity in cells, including NBD peptide. These interventions target the activation of the IκB kinase (IKK) complex that...
phosphorylates NF-xB during its activation. In the present study, we test a competitive inhibitor of the IKK complex (NBD peptide) for its utility in preventing cancer-induced skeletal muscle atrophy for the first time using an established mouse model of cancer cachexia.

Materials and Methods

Cell lines

The transplantable C26 adenocarcinoma cells were maintained as monolayers in culture dishes at 37°C with 5% CO₂ in a culture medium consisting of Roswell Park Memorial Institute (RPMI) 1640 (Gibco; Rockville, MD) supplemented with 5% fetal bovine (Hyclone; Logan, UT) and 1% penicillin/streptomycin (Gibco; Rockville, MD). Prior to injection, cells were harvested at approximately 80% confluence, washed, and resuspended in phosphate-buffered saline (PBS) at a concentration of 1 x 10⁶ cells per milliliter.

Animals and tumor implantation

Sixty-five male BALB/c mice aged 43 to 63 days old (weight 22 to 24 grams) were obtained from the Charles River Laboratories (Wilmington, MA). Mice were evenly and randomly divided into groups: healthy control mice, tumor-bearing mice, and tumor-bearing plus NBD peptide (80 µg, 200 µg, or 500 µg). Mice were housed five animals per cage in a temperature controlled room on a 12 hour light-dark cycle. All animals had access to unlimited food and water. Mice were allowed to acclimate to their new environment for approximately 3 days prior to beginning the study.

On day 0, the mice in tumor-bearing groups were injected subcutaneously in the right flank with 100 µL (approximately 500,000 cells) of C26 adenocarcinoma cells. Body weight, tumor volume, and food consumption were measured every other day from inoculation to completion of the study. Beginning on day 6, untreated tumor-bearing mice received daily single intraperitoneal injections of sterile DMSO while treated tumor-bearing mice received injections of NBD peptide at 80 µg, 200 µg, or 500 µg. On day 17 when tumor-bearing mice had a significant tumor burden and clinical signs of cachexia were apparent, all animals underwent body composition analysis using magnetic resonance imaging (MRI). A final total body weight measurement was also obtained. Tumors were resected, measured, and weighed and total carcass weight (total body weight - tumor weight) was calculated. Hind limbs were weighed separately. Quadriceps and gastrocnemius muscles, and tumors were excised from each animal immediately after sacrifice, snap frozen in liquid nitrogen and stored at -80°C. Liver and kidneys were removed and placed in 10% neutral phosphate-buffered formalin for fixation at 4°C.

Body composition analysis

Prior to tumor injection, all animals underwent body composition analysis using magnetic resonance imaging (Lunar PIXIms densitometer, GE Medical Systems). Animals were gently placed in a tube designed for mice < 40 g and placed in the scanner. All scans were done using the full body mode. Lean body mass (LBM), fat mass (FM), and water weight were measured in each animal. Tumors were found to have the same density as LBM and were subtracted out after weighing the tumors in tumor-bearing animals.

Western blot analysis

For Western blot analysis, gastrocnemius muscle samples were pulverized, extracted, and solubilized using T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) then centrifuged for 10 minutes at 13,000 x g at 4°C. The protein-containing supernatant was stored at -80°C. After determination of the protein concentration for each sample using Bradford reagent (Bio-Rad), 40µg of total quadriceps or gastrocnemius muscle protein extracts were separated using NuPAGE Novex 4% to 12% bis-Tris gels (Invitrogen). Proteins were transferred to nitrocellulose membranes using I-Blot Gel Transfer (Invitrogen); The membranes were blocked with 5% dry milk protein (Carnation, Wilkes-Barre, PA), diluted in 1X tris-buffered saline with 0.1% Tween-20 (TBS-T) and incubated with antibodies of interest diluted in 5% bovine serum albumin (BSA) diluted in 1X TBS-T or 5% milk. After washing with TBS-T and labeling with secondary antibodies, light emission after addition of electrochemiluminescence (ECL) detection reagent (GE Healthcare UK Limited, Buckinghamshire, UK) was captured onto X-ray film. Bound antibodies were stripped from membranes by incubation in stripping buffer (Thermo Scientific, Rockford IL). Stripped blots were washed and re-probed with other antibodies of interest. To control for equal loading, all blots were stained with anti-tubulin antibody since the more commonly used actin may be cleaved in these experimental models of muscle wasting.

Antibodies

Antibodies directed against p65 (1:500), phosphorylated p65 (p-
p65)(1:500) were purchased from Cell Signaling Technology (Danvers, MA). β-tubulin antibodies (Santa Cruz Biotechnology, 1:25,000) were used to normalize loading.

Cross-sectional muscle samples

Hind limbs were harvested from approximately three animals per group at day 17 and placed in 10% formalin buffered in phosphate (Electron Microscopy Sciences, Hatfield, PA) for approximately 48 hours. Tissue samples were then washed in water and placed in 70% ethanol. Tissues were sliced in five micrometer sections, placed on slides, and stained with hematoxylin and eosin (H&E) stain using standard techniques.

Drug toxicity

Liver and kidney specimens were harvested and immediately placed in 10% neutral phosphate-buffered formalin for fixation at 4°C. After approximately 24 hours, blocks were rinsed with water and placed in 70% ethanol at room temperature. All samples were embedded in paraffin, sliced in 5 micrometer sections and placed on slides. Tissues


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were stained with H&E staining and evaluated by an independent, blinded pathologist for evidence of drug toxicity.

**Total RNA isolation/real time PCR determination of mRNA expression**

Frozen quadriceps muscle tissue samples taken immediately after euthanasia were thawed in Trizol reagent (Sigma Chemical). Total RNA was extracted and purified using the ABI PRISM 6100 Nucleic Acid PrepStation according to the manufacturer’s protocols (Applied Biosystems, Foster City, CA). Quantitative mRNA expression was determined using a two step reaction. cDNA was made using the High Capacity cDNA Archive kit (Applied Biosystems). Real time PCR (RT-PCR) was amplified on an ABI Prism 7900 HT Sequence Detection system using cDNA and TaqMan probe sets in TaqMan® Universal PCR Master Mix. The TaqMan probes used in these studies included MuRF1 (Mm01188690_m1), MAfbx/Atrogin-1/Fbxo32 (Mm00499518_m1), and 18S (Hs99999901_s1) (Applied Biosystems). Data are depicted as mean fold change (MFC) versus the 18S expression (non-tumor-bearing animals). Three replicate plates and averages were calculated for each treatment group.

**Statistical analysis**

Results are presented as the mean ± standard error throughout the manuscript. The significance of observed differences between groups when greater than 2 groups are present was determined using a one-way analysis of variance (ANOVA) with a repeated measures analysis (using Student’s t-test) when the F ratio reached significance [14,69]. Analyses were performed using SPSS (SPSS, Inc., Chicago, IL) and basic statistics on Microsoft Excel 2007 (Microsoft, Seattle, WA). Statistical significance was defined as p<0.05.

**Results**

C26 adenocarcinoma cells produce tumor-induced wasting in BALB/c mice

The C26 adenocarcinoma murine model of cancer cachexia was chosen as a model because it has been extensively characterized over the past 30 years to test chemotherapeutics in vivo [8]. C26 adenocarcinoma cells were placed in the right flank of BALB/c mice to induce cancer cachexia. Palpable tumors were identified by day 6 after inoculation in experimental mice. Weight loss was detected on approximately day 8, and on day 17, carcass weights of untreated tumor-bearing mice were 89% that of healthy controls (p <0.001) (Figure 1A). Untreated tumor-bearing mice lost an average of 4% of their starting body weight, while control mice gained approximately 10% (Figure 1B). Using MRI for body composition analysis, untreated tumor-bearing mice lost more lean body mass (20.51 ± 0.44g vs. 19.39 ± 0.18g, p=0.02) and fat mass (2.64 ± 0.11g vs. 1.71 ± 0.27g, p=0.001) than control mice (Figure 1C), despite equivalent food intake among all groups (Figure 1D).

**Figure 1:** NBD peptide treatment is associated with a reduction in tumor-induced wasting in an experimental model of cancer cachexia. (A) The effects of NBD peptide (injected daily for 11 days) on carcass weight (g). (B) Mean final % weight loss of each of the five groups. (C) Using MRI for body composition analysis, NBD peptide was associated with a dose response increase in lean body mass and fat mass when compared to untreated tumor-bearing mice on day 17. (D) There were no significant differences in average daily food intake for each of the five groups. * p < 0.05. ** p < 0.001.
Nemo-binding domain peptide (NBD) peptides inhibit skeletal muscle NF-κB and reduce tumor-induced weight loss

Tumor-bearing mice treated with 80 µg, 200 µg, and 500 µg of NBD peptide gained 1%, 4%, and 6% of their starting body weight, respectively, compared to untreated tumor-bearing mice who lost 4% (p<0.05) (Figure 1A, 1B). Using MRI body composition analysis, tumor-bearing mice treated with NBD peptide had a dose dependent increase in lean body mass (LBM) and fat mass (FM) when compared to untreated tumor-bearing mice (Figure 1C). LBM was 19.39±0.18 g for untreated animals compared to 19.85±0.44 g, 20.71±0.51 g, and 21.08±0.53 g for increasing doses of NBD peptide. FM also increased in animals treated with NBD peptide 1.63±0.17 g, 1.81±0.21 g, and 2.20±0.17 g compared to 1.71±0.27 g for untreated tumor-bearing animals. Significant protection against the loss of lean body mass was reached at NBD levels of 200 µg (p=0.03) and 500 µg (p=0.03) and at 500 µg for fat mass (p=0.04). Food intake did not significantly differ across control, untreated or treated tumor-bearing groups (Figure 1D), suggesting that NBD effects were independent of the amount of food that was ingested by each group.

The hind limbs of the mice were weighed immediately after euthanasia to determine differences in muscle mass. Tumor-bearing mice had significant hind limb atrophy when compared to non tumor-bearing controls (1.27±0.01 g vs. 1.06±0.02 g; p<0.001) (Figure 2). Hind limb weight was increased in NBD peptide treated animals: 1.14±0.03 g, 1.26±0.01 g, and 1.22±0.03 g in animals treated with 80 µg, 200 µg, and 500 µg of NBD peptide, respectively (p=0.23, p=0.004, p<0.001). Treatment with NBD peptide at the mid and high doses showed an almost complete reversal of hind limb wasting comparable to control animals with a hind limb weight of 1.27±0.01 g. Visual differences in hind limb wasting can be appreciated between control, treated, and untreated mice in gross hind limb and H&E stained transverse sections of the lower leg muscles (Figure 2B, 2C).

To determine if NBD peptide affected the C26 tumor in vivo, we investigated how NBD affected tumor volume. The groups given daily treatment with 200 µg and 500 µg NBD had a reduction in final tumor volume compared to untreated tumor-bearing mice (p=0.02, p=0.03) (Supplemental Figure 1). However, significant tumor burden was still present in all tumor-bearing animals. The final weight of the isolated tumors at day 17 according to treatment was: 1) Tumor only 1.28±0.14 g; 2) NBD 80 µg 0.86±0.13 g; 3) NBD 200 µg 0.67±0.12 g; and 4) NBD 500 µg 0.70±0.13 g.

Phosphorylated p65 down regulated with NBD peptide

To confirm that NBD inhibited NF-κB activity in our model, we

**Figure 2:** NBD peptide shows an almost complete reversal of hind limb wasting. (A) Hind limb weights in control, untreated tumor, and NBD peptide treated mice at day 17. (B) Visual differences in hind limb wasting can be appreciated between control, untreated, and treated mice in gross hind limb and (C) H&E stained transverse sections of the lower leg muscles. (Top to bottom: control, untreated tumor-bearing, tumor-bearing mice treated with 200µg, and 500µg of NBD peptide). ** p < 0.001.

**Figure 3:** Phosphorylated p65 is down-regulated with NF-κB inhibition using NBD peptide. Gastrocnemius muscles taken from control, tumor-bearing, and NBD peptide treated tumor-bearing animals were homogenized and examined by immunoblot probing for p-p65, p65, and tubulin as a loading control.
investigated the amount of active NF-κB (p-p65) in a representative skeletal muscle, the gastrocnemius. Gastrocnemius was harvested from control, DMSO-injected tumor-bearing and NBD peptide treated tumor-bearing BALB/c mice after 17 days and were homogenized and examined by immunoblots probing for p-p65, p65, and tubulin as a loading control. We identified increased NF-κB activity (i.e. increased p-p65 compared to total p65) in cachectic muscles compared to controls, which was reversed with NF-κB inhibition using NBD peptide (Figure 3).

### Reduced induction of muscle E3 ubiquitin ligases with NF-κB inhibition

In multiple models of skeletal muscle atrophy, muscle specific ubiquitin ligases MAFbx/Atrogin-1 and MuRF1 have been demonstrated to be upregulated and mediate the induced atrophy [13]. Furthermore, they are increased in response to chronic NF-κB activation [16]. Therefore, we quantitatively determined MuRF1 and MAFbx/Atrogin-1 mRNA expression in quadriceps from each animal from all the groups investigated. As expected, we identified that tumors significantly increased MuRF1 and MAFbx/Atrogin-1 levels relative to non-tumor-bearing control animals approximately 6.7- and 6.1-fold, respectively (Figure 4). NBD treatment of tumor-challenged mice significantly decreased expression of both MuRF1 and MAFbx/Atrogin-1, suggesting that NBD-inhibition of NF-κB acted to inhibited skeletal muscle atrophy by down-regulating the ubiquitin ligases previously shown to be pivotal in mediating atrophy.

### NBD peptide treatment showed no evidence of drug induced toxicity at the levels used in this study

Recent studies have reported the use of NBD peptide therapy for the treatment of colitis and arthritis in mouse models without any evidence of toxicity [22,61]. Similarly, in the present study, we did not identify any toxicity as a result of treatment of NBD peptide, including any lethality or acute and chronic effects on behavior after daily injection of NBD peptide up to 500 µg per mouse. At the end of the study, we carefully looked for morphological evidence of toxicity and did not find any. No hepatotoxicity or nephrotoxicity across all doses of NBD peptide delivery were found, as confirmed by a blinded, independent pathologist and shown in Figure 5.

### Discussion

The C26 adenocarcinoma murine model of cancer-induced cachexia was created in 1975 as a model to test biological and chemotherapeutics in vivo [19]. Since then, nearly 190 manuscripts have been written using the C26 adenocarcinoma cell line, which delineate the natural history of cachexia and its underlying mechanisms [8]. When placed in mice, the C26 adenocarcinoma cell line form tumors which secrete pro-inflammatory cytokines. IL-6 was initially identified as a prominent player in C26-induced cachexia using anti-IL-6 antibodies, but later studies also identified TNFα and IL-1 [32,76], paralleling human cancer cachexia [17]. While all these cytokines interact with their own unique receptors on striated muscle, each receptor converges on the activation...
of the transcription factor NF-kB to induce skeletal muscle atrophy. NFkB induces proteolysis via the ubiquitin proteasome system, including increases in the ubiquitin ligases MuRF1 and MAFbx/Atrogin-1, which specifically interact with sarcomere proteins and mediate their degradation directly [16,67].

The activation of the Inhibitor κ B Kinase complex (IKK) is central to NF-kB activation. Phosphorylation of IkB proteins by the IKK complex is a critical regulatory step in proinflammatory signaling pathways leading to NF-kB activation [41]. The IKK complex contains two catalytic subunits (IKK-α and IKK-β) and a regulatory component, NF-kB essential modulator (NEMO or IKK-γ) [34]. The IKK complex phosphorylates the IkBa, which is then targeted by the ubiquitin proteasome system for degradation, allowing NF-kB to translocate to the nucleus. The NBD peptide used in this study potently inhibits stress-induced NF-kB activation without affecting basal NF-kB activity [45], by competitively inhibiting the IKK complex from forming. Specifically, the NBD peptide prevents the IKKγamma (NEMO) from forming its interaction with IKKβ by competitive inhibition [45]. This mechanism is distinct from other NF-kB inhibitor strategies, such as proteasome inhibition, because it doesn’t affect the necessary basal proteasome activity vital to protein quality control [47,50,71,72]. This peptide has been used successfully in diminishing inflammatory responses in several animal models of disease, including inflammatory bowel disease [2,18,21,36,55].

In the present study, we demonstrate for the first time that selective inhibition of NF-kB using a cell permeable peptide to block the NEMO domain of the IkB complex is able to inhibit skeletal muscle atrophy. We also identified that NBD peptide inhibits the C26 tumor-induced increases in the ubiquitin ligases MuRF1 and MAFbx/Atrogin-1. MuRF1 and MAFbx/Atrogin-1 expression were investigated because of their upregulation during skeletal muscle atrophy, and their necessary role in mediating skeletal muscle atrophy in different experimental models [13]. Recent studies have implicated that NF-kB regulates both MuRF1 and MAFbx/Atrogin-1 [16,33], making it plausible that NF-kB inhibition is preventing the up-regulation of these ubiquitin ligases fundamental in skeletal muscle atrophy [13]. However, MuRF1 and MAFbx/Atrogin-1 have been reported to be regulated by the FOXO transcription factors as well as NF-kB [54,58,68]. Additional studies will be needed to determine the role of MuRF1 and MAFbx/Atrogin-1 in cancer cachexia to delineate this relationship between MuRF1 and MAFbx/Atrogin-1 expression regulation and their actual role in the skeletal muscle atrophy process.

In the present study, both the 200 and 300 µg of NBD inhibited both the C26 tumor growth AND prevented lean body mass and fat mass loss, skeletal muscle NF-kB signaling, and MuRF1/Atrogin-1 expression. While this indicates the NBD may have clinical utility in vivo in a clinically relevant model, it does not allow the differentiation of the 2 possible mechanisms by which it may be protective. The protection against skeletal muscle injury may either be direct, due to the cytokine mediated NF-kB inhibition at the muscle and adipocyte itself. However, it is possible that the protective effects of NBD may be indirect, due to its inhibition of the tumor growth itself. This is an important point to make because the dysregulation of NF-kB signaling is a major driver of cancer [53] and its inhibition has been shown experimentally to prevent oncogenic initiation and progression [10]. However, the magnitude between the tumor size inhibition (~30%, Suplemental Figure 1) and the skeletal muscle protection (~5%, Figure 2) are mismatched, suggesting that inhibition at the skeletal muscle may be prominent, although both mechanisms may be in play in the model tested.

There are few therapies currently available to treat cancer cachexia. They include mainly appetite stimulants and anabolic agents such as prednisolone [15,51] and progesterone [12,42,43,77] and gherelin, a ligand which stimulates growth hormone release [44,59]. Anecdotally, the use of α-3 omega fatty acids (eicosapentaenoic acid) [52] and thalidomide (potent anti-TNF activity) have been reported [48]. Experimentally, glucocorticoids, aspirin [63], indomethacin [35,79], methotrexate [60], and celecoxib [23], all have been shown to have some efficacy, which are believed to be effective, in part, due to their ability to inhibit NF-kB activity [10,32,73,78]. However, these agents are neither potent nor selective for the NF-kB pathway. In addition, they have many off-target side effects. In the present study, using a specific inhibitor of the IkB kinase complex, we did not find evidence of toxicity to the liver or kidney across all three doses of NBD peptide. These results parallel those of Jimi et al. [39], who treated daily with NBD peptide for several weeks in a murine model of rheumatoid arthritis.

The discordance in absolute carcass weight and the total weight determined by quantitative magnetic resonance (QMR) in Figure 1A and Figure 1C has been previously identified and is largely due to the QMR methodology. Until recently, Dual X-Ray Absorptiometry (DEXA) scans have been largely utilized to determine body composition. However, recent publications comparing QMR to DEXA in mice highlight some of the advantages and caveats of using QMR [64]. QMR is more precise than DEXA in determining body composition and could be performed in non-anesthetized mice [64]. This is the reason that we chose QMR for our current studies. In fact, the precision of QMR in these studies allowed us to determine that the higher two doses of NBD peptide could prevent both the lean muscle and fat mass loss induced by tumor (Figure 1C). However, while QMR more precise, it’s estimation of lean mass is underestimated because, in part, it does not accurately measure water bound in tissues. In fact, rodent studies have found a very high correlation between QMR and carcass weights. QMR was found to overestimate fat mass and underestimate lean mass and total water [40]. Despite these absolute differences in QMR values and carcass weights, the measure of fat, lean, and water were highly correlated with the chemical analyses of these (r²>0.99) [40].

Although it is generally believed that treating cachexia will not cure the underlying neoplastic process, reversal of wasting may improve treatment response, prolong survival, and improve quality of life. Of note, an attenuation of tumor growth at the highest doses of NBD peptide was seen in our experiment. However, this cannot be the sole reason for the reversal of the wasting as beneficial effects on cachexia were seen at the lowest dose where tumor growth was not significantly different than untreated tumor-bearing animals. In addition, there was still a significant tumor burden present in animals treated at the highest doses.

Our findings suggest that inhibiting NF-kB by targeting the IkB Kinase complex is able to ameliorate tumor-induced muscle wasting and significantly reduces weight loss through inhibition of NF-kB activity and decreased expression of muscle atrophy genes. These pre-clinical studies highlight the therapeutic potential of the NBD peptide therapy, which may prove to be a potent and safe therapeutic agent in cancer cachexia.

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