Development of a Novel STAT3 Inhibitor for the Treatment and Chemoprevention of Lung Cancer

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Christian V Nzineku Njatcha

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Advisor: Jill Siegfried PhD

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Abstract

The limited successful treatment options and the development of resistance to therapy continues to make lung cancer the leading cause of cancer death in men and women in the United States and accounts for greater than 15% of all deaths from cancer. Because of its high mortality rate, designing and developing novel strategies to successfully circumvent intrinsic and acquired resistance represents a major challenge in lessening the burden of lung cancer as a disease. With the overwhelming majority (approximately 85%) of lung cancer diagnoses represented by patients who are former or current smokers, the opportunity to develop chemopreventive strategies requires preclinical evaluation of candidate agents to address the lack of strategies for lung cancer prevention. Following tobacco exposure, numerous mechanistic events such as DNA adduct formation, permanent gene mutation, and transformation, the development of lung carcinogenesis during these early events is also accompanied by carcinogen-induced immune suppression.

Identification of the epidermal growth factor receptor (EGFR) sensitizing mutations (namely L858R and exon 19 in-frame deletion) in NSCLC is currently used in the clinic to select patients who would respond to EGFR-directed targeted therapy but only 10% of the patient population harbor those mutations. In spite of the initial benefits observed in patients receiving EGFR tyrosine kinase inhibitors as first or second line therapy, resistance develops in more than 50% of patients as a result of a secondary T790M mutation in EGFR rendering therapy
ineffective. On the other hand, NSCLC patients without those sensitizing genetic alterations can't benefit from targeted therapy and a sub-population of those patients harbor K-RAS mutations, especially in smokers, but unfortunately, K-RAS is currently an undruggable target. Previous analysis of NSCLC patient-derived and mouse xenograft tissue reveals the presence of signal transducer and activator of transcription 3 (STAT3) as a critical signaling node, and its constitutive activation is independent of oncogene mutation status.

STAT3 is a key transcriptional activator in the initiation and pathogenesis of diverse human cancers. Its role in lung cancer can be ascribed to the functional interplay between suppressing the immune system, and induction of a gene machinery in the tumor cells that translate to critical biological events such as proliferation, survival, and anti-apoptosis. Despite understanding the biology and function of STAT3 in oncogenic signaling pathways, its lack of enzymatic activity as a transcription factor has made STAT3 elusive and “undruggable” which has particularly limited STAT3 inhibitors to research purposes. Most STAT3 inhibitors either exhibit low potency, inadequate membrane penetrance or poor stability requiring higher drug concentrations to achieve therapeutic benefits, which can cause adverse effects. Efforts to develop STAT3 therapeutics for lung cancer is been pursued from different angles but none is currently FDA-approved and early results from clinical trials is not very promising. It is therefore imperative that we continue to assess candidate STAT3 inhibitors in preclinical models of lung cancer that could be viable for clinical studies. To develop a novel approach to treat and potentially prevent lung cancer
development, preclinical studies were undertaken to evaluate a double-stranded oligonucleotide molecule which mimics the STAT3 response element within the promoters region of STAT3 target genes thereby acting as a “STAT3 decoy” (S3D). S3D containing hexa-ethylene glycol molecules as linkers can be ligated to form a nuclease-resistant and more stable circular STAT3 “decoy” (CS3D). To evaluate CS3D in preclinical lung cancer models, we selected NSCLC cells that are intrinsically resistant to EGFR-targeted therapy (201T), acquire resistance to EGFR-targeted therapy (H1975), and a K-RAS-induced carcinogenic model to determine its therapeutic potential and assess its chemopreventive properties.

**Major Results**

The findings associated with CS3D were compared to an inactive circularized oligonucleotide (CS3M) harboring a single nucleotide mutation rendering it unable to bind to STAT3. We observed a greater than 90% uptake of fluorescent-labelled CS3D and CS3M by NSCLC cell lines and also detectable 48 hours post-injection via systemic delivery in various organ site such as the lung, spleen, and liver. CS3D was determined to inhibit proliferation by 50%, induce apoptosis, decrease independent-anchorage growth by 70%, and suppress c-Myc and Bcl-XL gene expression. Surprisingly, CS3D increased p-STAT3 ubiquitination thereby enhancing its degradation. This effect is accompanied by a decrease in nuclear and cytoplasmic p-STAT3 pools, suggesting a CS3D-induced degradation process.
To support the preliminary in vitro findings, CS3D demonstrated a robust antitumor effect measured as a 96.5% reduction in xenograft models inherently resistant (201T) to EGFR targeted therapy and 81.7% in drug-induced resistant models (H1975). CS3D promoted an anti-proliferative phenotype measured as a decrease in Ki67 index, increase in apoptosis, and increase in lymphocyte infiltration suggesting an enhanced immune function, with no systemic toxicity observed through the course of the studies. Assessment of CS3D in a carcinogen-induced lung cancer model that induces K-RAS mutations, showed that CS3D causes a decrease in preneoplasia, tumor size, and number of tumors by 30%, 50%, and 40% respectively, highlighting the chemopreventive properties of CS3D. Early histological analysis revealed a lung microenvironment that is less primed for tumor initiation or progression because markers of angiogenesis, and cell cycle progression such as VEGF, and MYC respectively, were downregulated. In addition to key factors in survival (VEGF and MYC), markers of chronic inflammation such as NF-kB were also suppressed at 8 weeks post CS3D administration except for COX-2 which was elevated. These effects were accompanied by an increase in macrophage infiltration. However, at a later timepoint, 20 weeks post-treatment, we were able to detect higher levels of IL-6 which might point to a rescuing signaling feedback loop that could possibly lead to development of resistance to CS3D. Flow cytometry analysis to define the immune cell profile revealed that CS3D favored an antitumor immune response over an immunosuppressive TME by increasing the number of lung M1
macrophages while decreasing M2 macrophages and MDSCs. Ratio of M1 to M2 was 3:1 with CS3D and 1:3 with the control construct (CS3M).

Despite the robust anti-tumor effect observed in the xenograft and chemopreventive model, CS3D also activates feedback loops by increasing IL-6, HER and FGFR receptor ligand secretion. Production of those ligands in turn activates corresponding receptors that leads to the phosphorylation of MAPK in an autocrine fashion. In combination with EGFR and FGFR inhibitors, CS3D produces anti-proliferative effects that are synergistic as a measure of proliferation, migration, and anchorage-independent growth.

**Conclusion and Significance**

Confocal analysis indicates that the “STAT3 decoy” (CS3D) can successfully be incorporated into NSCLC cells and exhibit stability via systemic delivery, conferring a strong translational potential. CS3D effectively alters STAT3 function by suppressing its transcriptional activity and causing its subsequent degradation, demonstrating the ability of CS3D to act as a molecular sink for STAT3 dimers. This provides a unique feature compared to other STAT3 inhibitors which are designed to target the STAT3 phosphorylation step. The ability of unphosphorylated dimers to be transcriptional active renders phospho-STAT3 inhibitors ineffective compared to decoy oligonucleotides like CS3D. Comparison of CS3D to an oligonucleotide that differs by a single base-pair (CS3M), which showed no therapeutic effects, was imperative to confirm CS3D selectivity against STAT3.
The CS3D-induced antitumor effects in EGFR resistant diseases (inherent and acquired) as well as in the setting of KRAS mutation provides a novel treatment modality for many molecular types of NSCLC and has great potential to transcend the therapeutic constraints associated with current targeted therapies such EGFR inhibitors. Evidence of CS3D exhibiting chemopreventive properties in a carcinogen-induced lung cancer model positive for K-RAS mutations, shows that CS3D inhibits STAT3-mediated tumor formation and progression and further shows that its anti-tumor activity is broad against tumors of different mutation status. CS3D also promote an immune permissive microenvironment by increasing the proportion of M1 macrophages (known to promote an antitumor immune response) present in the lung tumor microenvironment. Most importantly, no major systemic toxicity was associated with CS3D, which was given for up to 8 weeks in mice, supporting its translational potential as an alternative treatment strategy for NSCLC.

Taken together, these findings have already contributed to the implementation of an IND-directed pharmacology and toxicology study needed to initiate a phase I clinical trial of CS3D. The clinical usefulness of CS3D in NSCLC could also have therapeutic implications for a broad range of heterogeneous malignancies in addition to NSCLC, that are also STAT3 dependent to prevent cancer progression and possibly prevent or treat resistance that develops to other targeted therapies.
# Table of Contents

1. List of figures .................................................................................................................. x
2. Background .................................................................................................................... 1
2. An “undruggable” transcription factor, STAT3 ......................................................... 7
3. Phenotypic changes associated with STAT3 inhibition .............................................. 22
4. Exploring a novel approach to treat and prevent lung cancer ................................. 30
5. Focus of research ........................................................................................................ 32
6. Materials and methods ................................................................................................. 33
7. Chapter I: Targeting STAT3 using a novel approach demonstrate a robust 
antitumor effect ........................................................................................................... 49
8. Chapter II: The cyclic STAT3 “decoy” exhibit chemopreventive properties ............. 74
9. Chapter III: STAT3 inhibition promotes “bypass” signaling mechanisms ............. 94
10. Discussion .................................................................................................................. 108
11. References ................................................................................................................ 119
List of Figures

Background Figures:

Figure 1. The six functional domains of STAT3 and important phosphorylation sites

Figure 2. Structures of STAT3α and STAT3β isoforms

Figure 3. Meta-analysis studies of STAT3 overexpression in NSCLC

Figure 4. Ligation of a cyclic STAT3 decoy (CS3D)

Figures in Chapter I

Figure 5. Ligation reaction and quantification analysis

Figure 6. Fluorescent detection of CS3D via confocal microscopy

Figure 7. In vivo detection of fluorescein-labelled CS3D

Figure 8. CS3D specificity by target genes analysis

Figure 9. Effects of the CS3D on cell viability

Figure 10. CS3D mediates growth inhibition in NSCLC

Figure 11. CS3D downregulates STAT3 target genes critical for cell cycle progression and survival

Figure 12. CS3D induces STAT3 degradation via ubiquitination
Figure 13. CS3D suppresses NSCLC xenograft tumor growth.................64

Figure 14. CS3D suppresses c-Myc expression and activates cleaved caspase 3.................................................................66

Figure 15. CS3D alters tumor cellularity.............................................................67

Figure 16. Short-term CS3D-treatment decreases proliferation (by Ki-67 index measurement) and suppresses p-STAT3 levels.............................................69

Figures in Chapter II

Figure 17. NNK-induced lung carcinogenesis model.................................77

Figure 18. Quantification of preneoplasia and adenomas in response to CS3D.................................................................79

Figure 19. Tumor burden quantification in response to CS3D.................80

Figure 20. CS3D impairs NNK-induced lung cancer growth......................81

Figure 21. Proportion of tumors bearing K-RAS mutations post-CS3D treatment.................................................................83

Figure 22. CS3D downregulates STAT3 and NF-kB expression...............84

Figure 23. CS3D disrupts the protumorigenic phenotype.........................86

Figure 24. STAT3 inhibition by CS3D alters the lung tumor microenvironment.................................................................90
Figures in Chapter III

Figure 25. CS3D activates compensatory signaling mechanisms…………………..97

Figure 26. NSCLC cell lines secret IL-6 in response to CS3D…………………..99

Figure 27. Cross-signaling activation of HER-family of receptors in response to IL-6………………………………………………………………………………100

Figure 28. Model of CS3D-mediated autocrine loop…………………..101

Figure 29. Effects of tyrosine kinase inhibitors on cell viability……………103

Figure 30. Dual effects of STAT3 and FGFR inhibition…………………..105
1. Background

1.1. Lung Cancer Prevalence and Risk Factors

Lung cancer remains the leading cause of cancer deaths for both men and women in the United States and worldwide, with an estimated 234,030 new cases of lung cancer and estimated 154,050 deaths from the disease in 2018 [1]. Despite major research efforts, it remains the most aggressive cancer type with a dismal overall survival, as only 18.6% of all patients are expected to be alive 5 years after diagnosis. Lung cancer also accounts for 13.5% of all new cancer cases and a staggering 25.3% of all cancer deaths raising the question on the effectiveness of current prevention and treatment strategies [2]. A recent report as of 2018 suggests lung cancer incidence rates continue to decline as fast in men as in women, reflecting historical differences in tobacco uptake, and cessation with individual cigarette smoking the most common risk factor for lung carcinoma; other risks factors includes passive smoke inhalation, residential radon, occupational exposures, infection and genetic susceptibility [3].

Advances in lung carcinoma prevention and treatment have been slow in contrast to the steady increase in survival observed for most cancer types because lung cancer is typically diagnosed at a distant stage which is due to the limitation of screening methodology and biomarkers of early detection [2]. The use of tobacco cigarettes is the single greatest risk factor in the development of lung cancer, with up to 90% of lung cancers attributed to smoking [3]. Other behavioral risk factors include the association between marijuana smoking and
initiation of tobacco use in young people; and despite the lack of short- or long-term safety data on electronic nicotine delivery systems (ENDS), the molecular histological changes and inflammatory milieu produced by ENDS as well as marijuana consumption, mirrors the premalignant events that facilitates lung carcinogenesis [3]. Exposure to other culprits and risk factors includes environmental factors such as radon, asbestos, and polluted air quality. Studies on second hand smoking as an environmental pollutant has shown that nonsmoking spouses of smokers have a 20–30% increased risk for developing lung cancer [4]. Damage to the lung from infection has also been implicated to lung carcinogenesis. For example, HIV-infected individuals with immunosuppression contributing to disease progression, these individuals have 2.5 fold higher risk of succumbing to lung cancer [5, 6, 7].

Even though lung cancer is often intimately linked to tobacco smoking and inflammation, not all tobacco users develop lung cancer, reinforcing genetic susceptibility to lung malignancy. Genome wide association studies (GWAS) have identify genomic signatures in the form of specific chromosomal regions, locus mutations, epigenetic changes, and signaling driven mechanisms such as the ErbB protein family (EGFR/HER1-4) that confers susceptibility to lung cancer independent of smoking status [8, 9, 10]. The proportion of lung cancer diagnoses in nonsmokers continues to rise as the 7th leading cause of cancer deaths. It accounts for 25% of all lung cancer cases and predominantly affecting women [11, 12, and 13]. Estrogen for example plays a biological role in lung cancer progression with preclinical models demonstrating the therapeutic value
of antiestrogens [14, 15, and 16]. Globally, this demonstrate a marked gender bias underlining the role for gender-dependent hormones in patients with lung cancer.

The risk factors and the genetic predisposition to lung carcinoma in smokers and never smokers respectively, indicates diseases of different molecular characteristics. Lung cancers consist of four major types of lung cancer and multiple minor or rare forms. For clinico-pathological reasons they are often divided into the broad categories of small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) [13]. SCLCs express a range of neuroendocrine markers and transcription factors that play important roles in neuroendocrine differentiation arising from the proximal (central) airway. NSCLC can be divided into three major histologic subtypes: squamous-cell carcinoma (originating from the bronchioles as Clara cells expressing CC10), adenocarcinoma (cell of origin differentiated as alveolar Type II cells secreting surfactant), and large-cell lung cancer [17]. Because large cell carcinomas might represent poorly or undifferentiated forms of the other types of cancers, it is a vaguely defined entity, and criteria for its diagnosis vary widely. Classification based on the tumor histology, molecular testing results, and stages provides healthcare professionals such as oncologists a platform to tailor personalized treatment.
1.2. Lung Cancer Staging and Remedies

Decades of research from understanding lung cancer incidence, risk factors and etiology, better stratifying tumor histology and advances in genetic profiling based on superior molecular testing approaches, has contributed to better understanding of the disease and the development of advanced therapeutic strategies. This has also led to the advent of personalized medicine primarily in lung cancer amongst many other types of cancer. Unfortunately, clinical manifestation of this disease is often detected at the most aggressive and late stage. In patients considered at high risk for developing lung cancer, the only screening modality for early detection that has been shown to alter mortality is low-dose helical CT scanning while other screening methodologies such as chest radiography and sputum cytology have failed to impact the mortality rates [18].

The most common symptoms at presentation are worsening cough or chest pain. Other presenting symptoms include the following: Hemoptysis, malaise, weight loss, dyspnea, and hoarseness. Patients with stage I, II, or III non-small cell lung cancer (NSCLC) are generally treated with curative intent using surgery, chemotherapy, radiation therapy (RT), or a combined-modality approach. Immunotherapy may be part of the treatment strategy for some with unresectable stage III disease. Systemic therapy is generally indicated for patients who present with advanced disease, including those who present with metastases (stage IV) or recur following initial definitive treatment [19]. Dependent on performance status (1 – 3 score), patients receive chemotherapy
but are often subjected to serious adverse effects and radiotherapy is mostly effective in controlling local Stage I NSCLC (20). The limitation to these treatment modalities has led to the development of targeted therapy and personalized medicine.

The search for biomarkers of drug response in NSCLC therapy has become important in helping select patients that would benefit from a selected treatment. Currently tested biomarkers in NSCLC includes the classical EGFR mutations that dictates clinical response to tyrosine kinase inhibitors (TKIs) such as Erlotinib, Gefitinib, and Afatinib which are used after first-line chemotherapy. Continuous EGFR-directed therapy often selects for tumors positive for EGFR T790M mutations and Osimertinib is used to treat these patients. Other standard treatment options for progressive stage IV, relapsed, and recurrent NSCLC includes: anaplastic lymphoma kinase (ALK)-directed TKIs (Crizotinib after first-line chemotherapy), ALK-directed TKI after prior ALK TKI therapy (Ceritinib, Alectinib, Brigatinib), ROS1-directed therapy (Crizotinib), and BRAF V600E and MEK inhibitors (for patients with BRAF V600E mutations) are treated with Dabrafenib and trametinib. More recently, patients with elevated levels of PD-L1 tumor expression responded favorably to immune checkpoint blockade in several randomized clinical trials which led to the approval of three monoclonal antibodies which includes Nivolumab, Pembrolizumab, and Atezolizumab as second-line immunotherapy [22].
Targeted therapy to date has obviously had a major impact on the patient overall survival but majority of the patient population lack those biomarkers of drug response or those who are positive for those biomarkers, tend to develop resistance to the selected therapy. It is thus imperative that we continue to identify new targets, explore novel approaches, and implement treatment regimens that would address the problem of intrinsic and acquired resistance in lung cancer therapy. In addition to kinase dependence (EGFR, ALK, ROS1, BRAF, and MEK), NSCLC cells are also highly dependent on transcription factors whose role are to mediate biological events such as proliferation and survival.

Signal transducer and activator of transcription 3 (STAT3) is a highly phosphorylated protein in NSCLC with distinct role in tumor initiation and growth, metastasis, immune evasion, and drug resistance. This form of activation is only transient in normal cells making it an attractive therapeutic target. However, STAT3 as a transcription factor is a highly “undruggable” target that has eluded therapy for a long-time creating a challenging opportunity to design, develop, and test STAT3 inhibitors in the context of treatment and chemoprevention. Several STAT3 inhibitors have failed at different phases of drug development due to limitations highlighted as anti-STAT3 targeting modalities.
2. An “Undruggable” Transcription Factor, STAT3

2.1. Structural Biology of STAT3 and Isoforms

Signal transducer and activator of transcription (STAT) is a family of cytoplasmic transcription factors (TFs) that includes STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 are responsible for signaling events initiated by multiple receptors and non-receptor associated kinases. Amongst these TFs, STAT3 undergoes post-translational modifications which dictates its functional activities, and regulation of several biological processes such as proliferation, differentiation, apoptosis, angiogenesis and immuno-inflammatory [22]. STAT3 is characterized by six main structural motifs: Amino-terminal domain (NTD), coiled-coil domain, DNA-binding domain, linker domain (LD), Src Homology 2 (SH2) domain and the transactivation domain (TAD). The TAD domain is a highly conserved domain containing the tyrosine residue (Y705) and STAT3 phosphorylation at Y705 reinforces protein-protein interactions [23, 24].

![Diagram showing the six functional domains of STAT3 and important phosphorylation sites.](image)

**Figure 1.** The six functional domains of STAT3 and important phosphorylation sites (Levy et al., 2002, Lim et al., 2006, Droescher et al., 2012).
The dimerization interface is formed by the SH2 domains of STAT3 monomers containing Arginine 609 with positive NH3 amino group favoring the interaction with negatively charged phosphate, stabilizing p-Tyr705 binding [25, 26]. STAT3 is primarily activated through Y705 and the widespread protumorigenic function of STAT3 is attributable to its phosphorylated form (p-STAT3) and this form is often activated by kinases such as the epidermal growth factor receptor (EGFR). STAT3 is overexpressed in 65% of NSCLC [27] and meta-analysis studies shows that STAT3 expression is associated with a worse 3-year overall survival (OS) in NSCLC [28]. Recent evidence however suggests that the STAT3 NTD domain might facilitate the non-canonical activation of STAT3 through the formation of unphosphorylated dimers (USTAT3) and this mode of activation, should limit therapeutic strategies that target phosphorylation [29 – 33]. Other STAT3 inhibitors are primarily designed to inhibit SH2 domains to prevent p-STAT3 monomers from dimerizing and subsequently localizing to the nuclear compartment but they lack potency which accounts for the treatment failure in NSCLC [33 – 35].

Other STAT3 post-translational modifications also exist which are critical for its function. For example, mitochondrial translocation of STAT3 is triggered by phosphorylation at serine727 (S727) amongst several modifications such as acetylation, and methylation contributing to tumorigenesis [36 – 41]. Subsequently, many STAT3 inhibitors have been screened to target STAT3 activation but none has yet to result in an FDA-approved STAT3-targeting drug which might be attributable to STAT3 various post-translational modifications
contributing to treatment failure in various cancers including NSCLC. In NSCLC, STAT3 upregulates genes important for cancer cell proliferation (MYC), preventing apoptosis (Bcl-XL), and angiogenesis (VEGF) and the DBD is critical for the ability of STAT3 to bind gamma interferon activation site (GAS) elements in the DNA promoter regions upon dimerization and translocation. Multiple efforts are currently been explored to inhibit the DBD of STAT3, and we have shown that targeting the DBD of STAT3 using an oligonucleotide-based approach suppresses the expression of MYC in tumor cells [42].

There are four different STAT3 isoforms, STAT3α (92 kDa), STAT3β (83 kDa), STAT3γ (72 kDa) and STAT3δ (64 kDa). These isoforms are derived from alternative splicing and proteolytic processing. STAT3α and STAT3β are both detected in NSCLC biopsies in contrast to STAT3γ and STAT3δ isoforms that are not expressed [43]. Alternative splicing of exon 23 in mouse and human transcript via a highly conserved acceptor site generates STAT3α and a truncated version, STAT3β. Structurally, STAT3β lacks the C-terminal transactivation domain, but instead carries a unique seven amino acid tail [44] and STAT3γ and STAT3δ isoforms are generated as a result of proteolytic cleavage [45, 46]. STAT3α and STAT3β are able to form homo- and heterodimers upon growth factor or cytokine stimulation and both isoforms are activated by phosphorylation (Y705) in NSCLC [43]. Despite the slight differences in dimer stability, localization, and nuclear retention, they retain a common denominator that is the DNA binding ability [47 – 54]. Thus, designing
STAT3 inhibitors to target the DBD should not discriminate between STAT3α and STAT3β for NSCLC therapy.

Figure 2. Structures of STAT3α and STAT3β Isoforms (Schütz et al., 2015, Shao et al., 2001).

With respect to primary tumor, lymph node metastases, and distant metastases, biopsies obtained from patients with advanced stage NSCLC displayed both isoforms (STAT3α and STAT3β) constitutively phosphorylated, suggesting both isoforms might contribute to pro-oncogenic effects in NSCLC.

2.2. STAT3 Activation, Localization, and Function

STAT3 is a latent cytoplasmic transcription factor that was initially described by two independent group as an acute phase response factor in 1994 [55, 56]. In its inactive state, STAT3 is localized in the cytoplasm and once phosphorylated on Y705 and S727, STAT3 monomers dimerize to form homodimers or heterodimers by reciprocal SH2 phosphotyrosine interaction. This leads to STAT3 localization to the nucleus (Y705) followed by DNA binding activity to
regulate transcription [57]. STAT3 (S727) also translocate to the mitochondria where its non-canonical activity involves gene regulation and cellular metabolism [58]. Bromberg et al. initially engineered a mutant (STAT3-C) and constitutively active form of STAT3 that can spontaneously dimerize [59], and when over-expressed in mice alveolar type II epithelial cells, it promotes chronic inflammation and causes lung bronchoalveolar adenocarcinoma [60]. STAT3 mutations are rare in solid tumors and none have been reported in NSCLC but there is a significant body of evidence that points to endogenous activator of STAT3 that are up-regulated, overexpressed, or mutated in lung cancer and they all promote aberrant STAT3 activation. Canonical activation of STAT3 is mediated through the IL-6R/JAK pathway [57] and IL-6, a key upstream activator of STAT3, was reportedly detected in 75 patients with lung cancer driving a poor prognosis in NSCLC [61]. STAT3 can also be activated in a non-JAK dependent manner [62 – 64].

Receptor and non-receptor tyrosine kinases (NRTKs) such as EGFR or Src respectively, are significantly active during lung cancer development and they promote a malignant phenotype in part, via STAT3 activation [62 – 64]. Enhanced EGFR kinase activity through classical mutations (L885R, T790M, & exon 19 deletion) also amplifies STAT3 activation which contributes to proliferation, and survival of NSCLC [65, 66]. Other RTKs (VEGFR, PDGFR) and NRTKs (MAPK, PKCδ, and mTOR) have been reported to catalyze the phosphorylation of STAT3 [57, 67]. STAT3 phosphorylation via RTKs and non-RTKs can therefore be occur independent of the canonical IL-6/JAK receptor
complex activation. The dependence of multiple signaling pathway on STAT3 in NSCLC, illustrate that STAT3 is a critical common denominator with widespread functions that should reinforce the need to continuously focus on developing novel STAT3 therapeutics. Upon activation, STAT3 nuclear translocation is dependent on the ability of Ran GTPase to bind to importin-β1 which is necessary for STAT3 to traffic from the cytoplasm to the nucleus to exert its function [68]. Once imported to the nucleus, STAT3 functions as a transcriptional regulator of genes involved in cell cycle progression (such as Myc, Pim-1, and cyclin D1), angiogenesis (such as VEGF and bFGF), and anti-apoptotic genes (such as Bcl-XL, and Fas) in NSCLC [200]. Binding of STAT3 to the respective gene promoter regions creates a transcriptional profile uniquely favorable for NSCLC development.

To support the anti-apoptotic function of STAT3, lung carcinoma cell lines deprived of serum displayed an increase in STAT3 activation and prolonged survival [69]. Complex analysis of STAT3 in redox homeostasis also suggest that IL-6-induced STAT3 activation promotes ROS detoxification which impairs apoptosis [70, 71]. STAT3 also confers resistance to apoptosis by modulating Ca2+ release because increased cytosolic Ca2+ influx induces apoptosis [72, 73]. With knowledge of STAT3 central role in protecting cancer cells from apoptosis, researchers have developed and tested STAT3 SH2 domain inhibitors that could induce apoptosis by inhibiting STAT3 phosphorylation and dimerization. In NSCLC cell lines, SH2 domain inhibitors often fail to demonstrate a robust effect in preclinical mouse models or produce therapeutic effects in
clinical trials. Such failure is associated to the therapeutic strategy employed to block STAT3 activity which doesn’t always account for the non-canonical activation of STAT3. A non-canonical function of STAT3 involves the activated but unphosphorylated STAT3 (USTAT3) dimers which adds a new dimension to the array of functions that could be executed by USTAT3. USTAT3 influences cell migration, inhibit autophagy, and can be protective against cell death which are all tumorigenic phenotypes in lung cancer through its DNA binding activities [74 – 75]. These novel function of STAT3 makes the DNA-binding domain of STAT3 the most targetable region for NSCLC therapy amongst all its domain because it is void of any mutations and doesn’t undergo any post-translational modification.

Under normal conditions, STAT3 is tightly controlled by a variety of endogenous protein regulators such as protein tyrosine phosphatases (PTPs), protein inhibitor of activated STAT (PIAS), and suppressors of cytokine signaling (SOCS 1 – 7). The short duration of activation in normal cells makes it an attractive target for human malignancies and targeting STAT3 in normal bronchial epithelial cells, we show limited toxicity [42]. In NSCLC, those negative STAT3 regulators are inactivated and down-regulated leading to sustained STAT3 signaling in tumor cells driving proliferation, survival, invasion, angiogenesis, and metastasis [67]. These endogenous inhibitors are often down-regulated in certain lung cancer cell lines especially PIAS3 and SOCS3 that negatively regulates STAT3 [76]. It is plausible that in blocking STAT3, the oncogenic process shifts from a STAT3-dependent process to an independent
one that allows PIAS3 and SOCS3 to further mediate STAT3 proteasomal degradation. We have shown that STAT3 inhibition in NSCLC can result in proteasomal degradation via ubiquitination and further work would require elucidating the role of PIAS3 and SOCS3 in that process [43].

2.3. **STAT3 in Cancer – Friend or Foe?**

Early studies to study the physiological role of STAT3 suggested a diversity in its biological function using a STAT3 conditional knockout mouse model to define unique phenotypes [77]. Those phenotypes explain the distinct tissue-specific role of STAT3 (either oncogenic or tumor suppressive) using various models of the lungs, brain, skin, prostate and mammary gland [78]. Evidence to suggest STAT3 could be involved in cellular transformation was initially described by Bromberg and coworkers and further work to strengthen those observations showed that STAT3 activation by Src also contributes to cellular transformation [59]. The paradigm that has longtime defined the critical role of STAT3 in tumor development is continuously been supported by a growing body of work identifying its novel tumor-promoting functions in mitochondria metabolism, drug resistance, epigenetic regulation, cancer stem cells, and pre-metastatic niches. In lung cancer in particular, elevated STAT3 expression in tumor tissue correlates with poor survival. A subgroup meta-analysis study using tumor types as a stratifying criteria showed that STAT3 expression was associated with worse 3-year overall survival (OS) of lung cancer.
(Odd Ratio (OR) = 2.22, 95% CI = 1.31 to 8.89, P = 0.003) as illustrated in Figure 3 below from Wu et., al [28].

**Figure 3. Meta-analysis studies of STAT3 overexpression in NSCLC.**

We can infer from this meta-analysis studies that STAT3 is associated with a malignant phenotype in NSCLC and effective pharmacological inhibition of STAT3 should be favorable for therapeutic purposes. Certain attempts to restrain STAT3 activity in NSCLC preclinical models are limited because the molecule either exhibit low potency, poor membrane penetrance, or is easily degradable. In addition to this barrier, STAT3 multifaceted functions in NSCLC also contributes to drug resistance. A hallmark feature of cancer progression is the ability of tumor cells to escape immune surveillance and lung cancer cells depend on STAT3 signaling to evade anti-tumor immunity. The seminal work by Wang and colleagues demonstrated that STAT3 signaling played a significant role in regulating innate and adaptive immune response in the tumor microenvironment (TME) [79]. In a carcinogen-induced lung tumorigenesis model, silencing STAT3 increased NK-cell mediated toxicity [80]. Targeting
STAT3 can also restore NSCLC cells susceptibility to cytotoxic T lymphocyte-mediated killing [81]. Restraining STAT3 function in the TME can also alter the function of other immune cell infiltrates such as macrophages and myeloid-derived suppressor cells to help sustain an antitumor immune response which can be highly model-dependent [82]. A classical trait of cancer cells is their ability to metastasize to secondary sites. Blocking STAT3 activation in an orthotopic mouse model of lung cancer, significantly impaired lymph node and brain metastasis [83].

This plethora of evidence thus solidifies the role of STAT3 as an oncogene in NSCLC making STAT3 an optimal target for therapy. However, over the last decade, a possible tumor-suppressive role of STAT3 has emerged as a protective mechanism in various cancers including lung carcinoma. Most studies describing this unique role has identified a STAT3 anti-tumor role in the context of a specific genetic background. In a K-RAS-mutated inducible lung cancer model, genetic ablation of STAT3 in the airways correlated with decreased survival in the mouse model. Low STAT3 mRNA expression levels in lung adenocarcinoma patients with a smoking history also correlated with a decrease in patient survival suggesting a tumor-suppressive function of STAT3, and these effects were found to be specific to KRAS mutant lung cancer [84]. The NF-κB/IL8 axis, which was activated in the genetic absence of STAT3, was identified as the survival mechanism enabling an aggressive phenotype in the STAT3 knockout mouse model [84]. This work by Casanova group however, was later challenged by another group that was able to show that in a K-RAS-mutant
model, IL6 blockade by pharmacological inhibition produced an antitumor phenotype and reprogrammed the lung TME to limit lung cancer development emphasizing that pharmacological targeting of the IL-6/STAT3 axis should produce a favorable response as opposed to genetic ablation, that requires the TME network to promote an aggressive and pro-tumorigenic phenotype [85]. Pharmacological inhibition of STAT3 might not necessarily reprogram the function of NF-kB to promote an aggressive phenotype, because STAT3 protein would still be available to bind NF-kB in the cytoplasm [85]. Other studies have elucidated the tumor suppressive role of STAT3 in various carcinoma such as prostate, glioblastoma, liver, thyroid, head and neck cancer, and breast cancer. These tumors are either characterized by Pten-deficient, p19\textsuperscript{ARF} loss of function, STAT3 knockdown-induced metabolic switch, and other biochemical defects [86 – 93]. Pencik and colleagues surprisingly identified STAT3 deletion mutations in prostate cancer providing an insight on how tumor cells might inactivate the tumor suppressive function of STAT3.

The body of work elucidating the anti-tumorigenic role of STAT3 highlights the complexity of STAT3 in cancer development and could challenge the therapeutic benefit of targeting the STAT3 axis, but also highly suggests stratifying patients based on genetic background, and possibly STAT3 mutation status if those findings can be confirmed in other types of cancer such as lung cancer. Nonetheless, STAGE IV specimens resected from patients with NSCLC have high STAT3 expression. The patient-derived xenografts as well as mouse-derived xenografts from NSCLC are characterized by a complex TME,
dependence on aerobic glycolysis, highly vascularized, populated by drug resistant cells and cancer stem cells, and possess invasive properties, and STAT3 is at the center of maintaining an aggressive phenotype that supports NSCLC malignancy.

2.4. Role of STAT3 in the Tumor Microenvironment

Tumorigenesis is a highly dynamic process that depends on the numerous biological process coordinated by the TME. This complex environment is composed of cellular and noncellular components which consist of malignant cells, tumor stroma (infiltrating immune cells, cancer-associated fibroblasts (CAFs), cancer stem cells, lymphatic and angiogenic vascular networks and the extracellular matrix (ECM)) [94]. Cancer cells control the TME by recruiting stromal cells and subverting their functions to their own advantage. Crosstalk amongst the different cell type in the TME is essentially responsible for the establishment and maintenance of the pro-oncogenic niche, primarily initiated by cancer cells and relying on cell-cell contacts as well as on a complex network of released cytokines, chemokines and growth factors [94]. Tumor cells also instruct stromal cells to acquire pro-tumorigenic features and prevent immune cells from mounting an effective immune response. ECM remodeling, angiogenesis, epithelial to mesenchymal transition (EMT), and chemo resistance are pro-tumorigenic changes that can provide a conducive milieu for tumor growth and STAT3 is central to all the processes occurring in the TME.
The interplay between tumor cells and various immune cell types such as Natural killer (NK) cells, dendritic cells (DC), and macrophages, myeloid-derived suppressor cells (MDSCs), B cells, CD4+ and CD8+ is increasingly recognized to play a decisive role throughout multiple stages of carcinogenesis [95]. STAT3 suppresses immune function by inhibiting the expression of proinflammatory cytokines and chemokines necessary for tumor-specific T-cell responses [79]. STAT3 as a culprit seems to tilt the balance from immunosurveillance to favor tumor-promoting inflammation.

To demonstrate that STAT3 plays a pivotal role in lung cancer progression by shifting anti-tumor immunity towards a pro-inflammatory TME in a urethane-induced lung cancer model, targeted deletion of STAT3 in the myeloid compartment decreased the number of pro-tumorigenic cells that includes MDSCs, M2 macrophages and Tregs and reversed lung tumorigenesis by increasing CD4+ and CD8+ T cells [96]. Caetano and colleagues were also able to demonstrate that blockade of the IL-6/STAT3 pathway impaired tumor progression by decreasing the number of regulatory T cells (Tregs) and MDSC cells in the TME and showed an inverse correlation between STAT3 mRNA levels and overall survival from 150 patients with lung adenocarcinomas challenging the idea that all patients with K-RAS mutations would not benefit from STAT3 targeted therapy [85].

During angiogenesis, STAT3 phosphorylation is only expressed in endothelial cells (ECs) from mouse and human tumors, but undetectable in normal mouse organs suggesting a tumor-dependent pathway for angiogenesis.
[97]. STAT3 also mediates myeloid cell-dependent tumor angiogenesis. MDSCs and macrophages isolated from mouse tumors are able to induce angiogenesis in an in vitro tube formation assay via STAT3 activation by inducing VEGF and bFGF [98]. This implies that STAT3 inhibition should impair angiogenesis by blocking critical factors like VEGF. A network is also established between CAFs and cancer cells via the IL-6/STAT3 pathway priming the TME for enhanced tumor growth [99 – 103]. This implies that STAT3 inhibition should impair angiogenesis and derail the function of CAFs in the TME. The broad ability of STAT3 to participate in tumor cell proliferation, and educate immune cells in the TME help propagate signals that generate a powerful multilayered network prompting immune evasion and cancer progression. Another layer of complexity in the TME is deregulated metabolism. That adds an extra element to tumor cell proliferation and cancer-related inflammation. STAT3 is key feature in the metabolic regulation of NSCLC cells, the TME, and its components.

2.5. STAT3 and Metabolism

Dependence on aerobic glycolysis and reduced mitochondrial activity is a hallmark feature of tumor cells. The central role of energy metabolism in tumor progression might raise the question on why and how do proliferating cells switch to a less efficient metabolic pathway? One possible explanation is that rapidly proliferating cancer cells have metabolic requirements that extend beyond ATP production and there are other critical components that dictate the metabolic fate of cancer cells. Interestingly, research over the last decade has uncovered novel
STAT3 functions in regulating cellular metabolism in both canonical and non-canonical ways.

The activated form of STAT3 (S727) present in the mitochondria appears to function as a hub in shifting cancer cells metabolic needs from oxidative phosphorylation to aerobic glycolysis [104]. Mitochondrial STAT3 (S727) seems to be required for Ras-induced transformation and loss of STAT3 causes a 50% reduction in cellular ATP levels [104]. STAT3 can also induce a metabolic switch towards aerobic glycolysis by decreasing reactive oxygen species (ROS) production while inhibiting apoptosis [105]. Effectively targeting STAT3 in NSCLC should diminish ROS production. STAT3-dependent glycolytic metabolism is not only utilized by tumor cells, but also plays a gatekeeper role in immune cell metabolism which has major implications in cancer development [106 – 112]. The remarkable role of STAT3 in regulating tumor cell proliferation while priming immune cells to promote a pro-tumorigenic phenotype makes STAT3 a unique target to enhance antitumor immune response in NSCLC.
3. Phenotypic changes associated with STAT3 inhibition

3.1. Targeting STAT3 to Enhance Antitumor Immunity

Based on the role of STAT3 in mediating tumor cell survival and impairing immune cell function, targeting STAT3 should unleash an antitumor immune response. STAT3-dependent active immune-evasive mechanisms are currently been explored for NSCLC therapy. Despite the recent success associated with immune checkpoint blockade in NSCLC amongst many other forms of cancer, only a subset of NSCLC patients is able to benefit from immunotherapy. The response rate in the majority of NSCLC patients with innate or acquired resistance does not exceed 30% and 45% in the first and second line setting respectively [113 – 116]. STAT3 assist in tumor immune evasion by upregulating program-death ligand 1 (PD-L1), a transmembrane protein expressed in many tumors. The correlation between PD-L1 expression and poor clinical outcome is well established lung cancers [117, 118]. PD-L1 expression in NSCLC tumors bind to the PD-1 receptor inhibiting CD8-positive T cell activation [119]. In EGFR-mutant NSCLC, PD-L1 expression is highly dependent on STAT3 activation and STAT3 knockdown downregulates PD-L1 expression [120]. Evidently suppressing PD-L1 expression by blocking STAT3 enhances the effect of EGFR inhibitors in naïve and drug resistant models of NSCLC [121]. We can infer that hematopoietic cells and nonhematopoietic expressing PD-L1 might alter their function as a result of STAT3 blockade boosting anti-tumor immunity [119].

STAT3 activation in DCs, M2 macrophages, Tregs, and MDSCS can also help cancer cells evade immune surveillance. Two independent groups showed
that STAT3 knockout in lung cancer models, reprograms cells in the myeloid compartment to enhance innate and adaptive immunosurveillance [85, 96]. This suggest STAT3 signaling contributes significantly to the ability of tumor cells to evade antitumor immunity in lung cancer. Finding potent and selective STAT3 inhibitors to enhance antitumor immunity would require designing inhibitors that can also impede STAT3 function in tumor immunosurveillance. Most STAT3 inhibitors are somewhat ineffective because STAT3 is largely implicated in mediating innate or acquired resistance in NSCLC.

3.2. STAT3 Mediated Drug Resistance in NSCLC

Regardless of the treatment regimen implemented for NSCLC therapy, evidence suggest that feedback activation of STAT3 plays a prominent role in mediating drug resistance to a broad spectrum of targeted therapies, radiation, and chemotherapies. Multiple studies have shown that feedback activation of STAT3 as a compensatory signal is triggered from the blockade of cancer-addicted signaling pathways or the use of chemotherapy contributes to drug resistance following primary therapy. STAT3-mediated compensatory mechanisms thus allow cells to respond dynamically and adapt to drug-induced perturbation thereby impeding the therapeutic efficacy of various targeting modalities. Numerous studies demonstrate that cancer cells depend on receptor or non-receptor tyrosine kinase (RTK), and downstream molecules to drive signaling.
Targeted inhibition of one RTK can be circumvented via the activation of a secondary RTK as a detour mechanism which should allow neoplastic cells to maintain a parallel and adaptive response independent of RTK amplification or gain of function mutations [122]. STAT3 signaling happens to have emerged as one of the most critical axis in mediating resistance to targeted therapy [123]. Targeting EGFR in NSCLC using erlotinib (a tyrosine kinase inhibitor), directly increases STAT3 activation in EGFR mutant cell lines but does not induce STAT3 activation in EGFR wild-type cells [124]. STAT3 has been reported to play a major role in de novo resistance to irreversible EGFR-TKIs (such as afatinib) in NSCLC cells with the classical T790M secondary mutation [125]. In response to afatinib, histological analysis of NSCLC tumors showed an increased in p-STAT3 that was potentiated by autocrine and paracrine production of IL-6 [125].

Thus, IL-6 derived from cells in the TME contribute to drug resistance via STAT3 activation modulates IL-6 production leading to NF-kB activation [126]. NF-kB activation in turn ignites a feed-forward loop which is notable in lung cancers with K-RAS mutations and mostly associated with a cancer-prone inflammatory microenvironment observed in lung cancer patients with a smoking history [127]. This malicious feed-forward loop displayed in lung carcinogenesis puts STAT3 at the center of drug resistance providing an opportunity to assess STAT3 inhibitors in the context of lung cancer chemoprevention and therapeutic purpose to ameliorate treatment failure in NSCLC. Finding potent and selective
STAT3 inhibitors should be designed to alleviate innate and acquired resistance in NSCLC.

3.3. Anti-STAT3 Targeting Modalities

The unique functions of STAT3 supporting its pivotal role in tumorigenesis have gain significant attention over the last decade and subsequently, STAT3 inhibitors have been developed to target distinct STAT3 structural domain thereby disrupting several hallmarks of cancer progression [128]. STAT3 as a transcription factor (TF) has been considered highly “undruggable” and the lack of FDA-approved direct STAT3 inhibitors underscore the challenges faced in successfully developing a beneficial approach. STAT3 role in tumor onset and progression can either be impeded by direct STAT3 inhibitors or upstream (cell surface receptor) inhibitors. The limitations with direct STAT3 inhibitors is that they might exhibit low membrane penetrance, poor stability and low potency requiring a high concentration which can cause significant toxicity. On the other hand, upstream kinases can be nonspecific and resistance is highly associated with indirect interventions [128]. However, TFs like STAT3 are still biologically attractive for therapeutic intervention because they happen to be either overexpressed or constitutively activated in 70% of solid tumors but transiently activated in normal cells and dispensable for normal cell growth [128, 129]. The present modalities aim at either disrupting phosphorylation, dimerization, nuclear translocation, and/or DNA binding activity of STAT3 [128].
Amongst direct STAT3 inhibitors that have successfully reached clinical trials for NSCLC therapy, OPB-51602, a STAT3 SH2 domain binder, demonstrated a partial response in 2 out of 37 patients with NSCLC highlighting the limitation of STAT3 inhibitors aimed at only targeting STAT3 via phosphorylation, to disrupt dimerization and translocation. NSCLC patients in this study also experienced drug-induced pneumonitis and peripheral neuropathy [130]. Other compounds such as STATTIC and WP1066 with similar mode of actions like OPB-51602 have failed to reach clinical trials and they only serve as research tools. The novel role of unphosphorylated STAT3 dimers in oncogenesis may limit the ability of STAT3 SH2 domain inhibitors and kinase inhibitors [131].

Another approach to disrupt the function of STAT3 is to target STAT3 mRNA with an antisense oligodeoxynucleotide (OND) that would suppress its expression. AZD9150 is an antisense OND that demonstrated reduction in tumor burden in preclinical lung cancer models but human clinical trials by AstraZeneca revealed major toxicities via induction of thrombocytopenia [130, 131]. An additional but unique strategy to directly target STAT3 in NSCLC involves competitively inhibiting the ability of STAT3 dimers to bind to STAT3 response element within the promoter region of STAT3 target genes such as c-MYC, Cyclin D1 and Bcl-XL. The prototype of this molecule was designed as a STAT3 “decoy” by our collaborator Dr. Grandis and this approach has shown promising preclinical results in lung and head-and-neck squamous cell carcinoma (HNSCC). In a phase 0 clinical trial, the first generation of a double-stranded
oligonucleotide corresponding to the STAT3 response element within the c-FOS promoter element, was injected intratumourally in patients with HNSCC, and suppressed STAT3 target genes such as Cyclin D1 and Bcl-XL [133]. This approach did not reveal any apparent toxicity in preclinical studies which provides a major opportunity to be assessed in various cancers including NSCLC [134]. It is however established that only NSCLC patients harboring specific genetic alteration benefit from first line targeted therapy which is represented by less than twenty percent of patients suffering from the disease and such observation is also associated with immunotherapy [135, 136].

Targeting upstream kinase receptors (UKIs) such as JAK, EGFR, and FGFR have led to STAT3 activation via secondary compensatory signals that inevitably leads to NSCLC progression [137] and receptor inhibition of certain upstream kinases in normal cells might also lead to toxicity. For example, Janus kinase (JAK) inhibitors showed unexpected neurologic toxicities, such as Wernicke’s encephalopathy, in late clinical studies [138]. In summary, even though STAT3 is still an elusive target in clinical settings, focusing our attention on assessing direct STAT3 inhibitors such as oligonucleotide-based drugs in preclinical settings is necessary to identify candidate compounds for clinical evaluation.
3.4. Oligonucleotide-Based Designs and Delivery for Cancer Therapy

The initial discovery of antisense and siRNA oligonucleotides (OND) has led to the development of OND-based therapeutics for a spectrum of diseases including cancer. Several ONDs have been widely evaluated for cancer therapy but they can cause off-target effects. Some of those effects are mediated by their interaction with the immune system or components within the blood or intracellular compartment. Rapid clearance by the kidney is also associated with OND-based therapies limiting their potency [139]. Oligonucleotide-based designs have utilized distinct chemical modifications (such as phosphorothioates, peptide nucleic acids (PNA), and locked nucleic acid (LNA) chemistry) to increase the stability and delivery of antisense and siRNA molecules [139]. However, the most common problem with OND-based therapeutics is the delivery system. Some delivery system that are widely used either for research purposes or clinically includes antibody conjugated, lipid and gold nanoparticle, and PEGylated conjugated ONDs [140].

The first and most recent OND to selectively target STAT3 to be evaluated in a clinical setting was developed by the laboratory of Jennifer Grandis [133, 134, 141 – 143]. The first-generation of the parent molecule was designed as a linear double-stranded OND consisting of 15 base pairs in length with free end. This OND sequence is congruent with the STAT3-response element in the c-fos promoter region and acts as a synthetic STAT3 decoy (S3D), competitively inhibiting STAT3 by acting as a functional sink preventing STAT3 from manipulating gene expression. S3D exhibited antitumor phenotypes in HNSCC
with no observable adverse effects in mice [141 – 143]. In an effort to develop a S3D more resistant to degradation by nucleases and, thereby, amenable to systemic delivery, hexaethylene glycol spacers were covalently inserted between complementary sequences of the double-stranded linear S3D which can be ligated to create the second-generation cyclic STAT3 decoy (CS3D) (Figure 4). CS3D as its linear predecessor, S3D, is non-toxic and highly active in HNSCC [133, 134]. Compared to S3D with 2.5 h of half-life, CS3D also demonstrate greater stability in human serum with an average half-life of 7.9 h in human serum indicating CS3D might exhibit greater stability for intravenous delivery [144]. All evidence thus points to CS3D as a viable therapeutic agent for a subset of cancers such as NSCLC that is STAT3 dependent [28].

**Figure 4. Ligation of a cyclic STAT3 decoy (CS3D).** Ligation reaction of S3M and S3D to CS3M and CS3D respectively as the cyclic form. Schematic representation of CS3M and CS3D enzymatic ligation using T4 DNA ligase following self-annealment to generate the cyclic forms.
Broad molecular profiling of NSCLC for targeted therapy has led to improvements in the progression-free survival of patients but the majority of NSCLC patients lack the biomarkers of drug response that are standards in the clinic [135]. By classification, patients without EGFR mutations or other known targetable mutations, and those positive for K-RAS mutations are left to depend on traditional and less efficacious cytotoxic agents. This provides a niche to evaluate CS3D as a therapeutic agent in NSCLC resistant to classical targeted therapies and assess its potential chemopreventive properties.

4. EXPLORING A NOVEL APPROACH TO TREAT AND PREVENT LUNG CANCER

The advent of precision medicine based on a patient’s genetic profile has transformed the landscape of treatment strategies shifting from more conventional chemotherapy and radiotherapy towards a more precise targeted approach in NSCLC. This involves the use of first, second, and third generation kinase inhibitors to target proteins with enzymatic activity. Other strategies in the realm of targeted approaches also include immunotherapy to enhance the antitumor response [19, 145, and 146]. These strategies have demonstrated clinical success in NSCLC but only in minor subset of the patient population. To initiate the development of a different treatment strategy for NSCLC, we explored a novel state-of-the-art oligonucleotide approach to target STAT3 which serves as a common denominator for hallmark processes such as proliferation, angiogenesis, resisting cell death, and evading the immune system. Critical modification from the 1\textsuperscript{st} generation S3D to the 2\textsuperscript{nd} generation CS3D took
advantage of hexa-ethylene glycol to primarily allow for the hybridization of the double-stranded form, and this simple but yet unique modification might also contribute to its biological activity.

Oligonucleotide-based therapies for cancer treatment have been evaluated in preclinical models in the past, but no PEGylated double-stranded oligonucleotide to target STAT3 in NSCLC has been assessed in the past. Modification of aptamers by addition of polyethylene glycol (PEG) does alter the pharmacokinetics by improving the stability and the size increase avoids rapid renal elimination [147]. Biologically, PEG modifications have high affinity for cell-derived vesicles such as exosomes and numerous studies have illustrated that PEGylating compounds can enrich for exosomes and also enhance cellular membrane penetrance [148, 149]. With a PEGylated-CS3D, we plan to effectively inhibit STAT3 function during tumor progression and maintenance providing the first evidence for treatment and chemoprevention of NSCLC using a PEGylated-CS3D STAT3 inhibitor.
5. FOCUS OF RESEARCH

It is our overall hypothesis that targeting STAT3 using CS3D in NSCLC that is EGFR wild type, EGFR mutant or K-RAS mutant, would produce an antitumor phenotype that might transcend therapeutic constraints associated with intrinsic or acquired drug resistance in NSCLC. This hypothesis was tested in three studies:

I. Determine the ability of the CS3D compared to CS3M to produce antitumor effects in intrinsically EGFR TKI resistant (EGFR wild-type) and acquired EGFR TKI resistant mutant EGFR NSCLC.

II. Examine the chemopreventive properties of CS3D in a carcinogen–induced lung cancer model by assessing CS3D’s ability to impair tumorigenesis in an NNK-induced lung cancer mouse model, determine the percent of tumors positive for K-RAS mutations, analyze STAT3-target gene expression, and characterize the immune cell profile which might make it less conducive for aggressive tumor formation.

III. Investigate CS3D-mediated compensatory signaling mechanisms that could be promoting resistance to STAT3 inhibition in NSCLC.
6. MATERIALS AND METHODS

Chemical Reagents

The single-stranded ONDs (S3M and S3D) were synthesized by Integrated DNA Technologies (Coralville, IA) and reconstituted in normal saline at 1mM stock solutions. The Cell Titer 96 Aqueous One Solution (MTS) Reagent containing 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was obtained from Promega. Tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was purchased from TRC (ON, Canada).

Ligation of S3M and S3D

S3M and S3D were purified by HPLC according to the manufacturer’s reports (IDT). The sense and antisense strands were modified by two hexa-ethylene glycol linkers attached to both strands and circularized upon enzymatic ligation of the 3’ and 5’ ends of the annealed oligonucleotide. Using 15µL of either S3D or S3M, 3µL T4 DNA ligase (400,000 U/mL; New England BioLabs, M0202S) and 2µL 10X T4 DNA ligase reaction buffer (New England BioLabs, B0202S), we performed an overnight ligation at room temperature to produce the CS3D and the inactive circularized mutant (CS3M) (Figure 4). Ligation efficiency was analyzed on a 15% Tris-Borate-EDTA (TBE) urea/polyacrylamide gels after a 5 min incubation at 60°C with DNA loading buffer. The ONDs were detected by
incubating gels in 1X SYBR Gold solution (Thermo Fisher Scientific, Waltham, MA) for 45 minutes in the dark, followed by exposure to ultraviolet light and image detection using the Universal Hood II Gel Doc System (BIO-RAD, Hercules, CA). Quantification was done using Image 1.X software to assess ligation efficiency [144].

**Cell Lines and Culture Conditions**

The WT EGFR (201T) cell line was previously derived in our laboratory from a patient with lung adenocarcinoma (150). H1975 cells with the EGFR-sensitizing point mutation L858R and the acquired EGFRi resistance mutation T790M were purchased from the American Type Culture Collection (ATCC). 201T and H1975 were cultured in BME and RPMI, respectively, containing 10% heat-inactivated FBS and 1x penicillin/streptomycin (Thermo Fisher) and 1xGlutaMax (Life Technologies). A549 and H3255 cells were also purchased from the ATCC and cultured in BME and RPMI, respectively. Human bronchial epithelial cells were purchased from the ATCC, and primary normal lung fibroblasts were derived from human lung tissue (151). Cell lines were authenticated by short tandem repeat DNA profiling and used within 6 months of testing. Frozen cell stocks were passaged a maximum of 15 times, and cells were mycoplasma-free. Cells were grown to 70-80% confluence in full serum media prior to experimental setup.
## Characteristics of Cell Lines by Mutation Status

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tumor Type</th>
<th>EGFR Status</th>
<th>K-Ras Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>201T</td>
<td>Adenocarcinoma</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>H3255</td>
<td>Adenocarcinoma</td>
<td>Mutant (L858R substitution in exon 21)</td>
<td>Wild-type</td>
</tr>
<tr>
<td>H1975</td>
<td>Adenocarcinoma</td>
<td>Mutant (L858R mutation and T790M insertion in exon 20)</td>
<td>Wild-type</td>
</tr>
<tr>
<td>A549</td>
<td>Adenocarcinoma</td>
<td></td>
<td>G12D</td>
</tr>
</tbody>
</table>

**Liposome-Mediated Transfections**

Transfections were performed using Lipofectamine 2000 (Life technologies) in opti-MEM media (Life Technologies) as follows: Cells were plated at 75% to 80% confluency and exposed to transfection media containing the respective circularized ONDs for 24 hours at 37°C. After transfection, cell
recovery was initiated in media containing 10% heat-inactivated FBS unless otherwise noted under different experimental designs.

**Confocal microscopy**

Fluorescently-labeled CS3M and CS3D (with iFluor) were produced using enzymatic ligation as described above and used to assess uptake efficiency. After transfection with fluorescein-tagged CS3D, 201T and H1975 cells were fixed in 4% paraformaldehyde, and prolong antifade reagent (CST) was applied directly to the slides. Fixed cell imaging was performed using a Nikon Eclipse Ti Confocal Microscope System. Images were captured and analyzed using the imaging software NIS Elements and Image J. Fluorescence was detected at 488 nm. Images were taken and processed at 40X and 60X.

**MTS assays and programmed cell death analysis**

Dose–response experiments assessing metabolically active viable cells were performed by transfecting cells with increasing concentrations of CS3D or CS3M, followed by performance of MTS assays. After 72 hours, 5 mg/mL of MTS reagent was added in the plates and incubated at 37°C for 20 minutes. The plates were then read at 490nm in a Synergy microplate reader (BioTek) using Gen5 2.05 software. Data obtained following treatment with C3SD or CS3M were normalized to treatment with lipofectamine alone. IC50s determined from these experiments were used to perform independent assays to confirm the effects of CS3D relative to CS3M. To assess induction of apoptosis, cells were transfected
with 100 nmol/L of CS3M or CS3D. After 24 hours, cells were resuspended in Annexin V–binding buffer, incubated with 5 mL of propidium iodide (BD Biosciences), and subjected to flow cytometric analysis, using a BD FACS Canto II Flow Cytometer.

**Anchorage-Independent Growth Assay**

Following transfection as above, cells transfected with either CS3D or CS3M were detached from the plate using Trypsin-EDTA (Life Technologies) and seeded at a density of 2 x 10⁴ cells/well (in a 6-well plate) in soft agar to determine anchorage-independent growth. Sea Plaque Agarose (Lonza) was used to prepare 0.8% base agarose layer and a 0.48% upper agarose layer containing the transfected cells. Note that 1 mL of growth media was added into each well and incubated at growth conditions of 37°C with 5% CO₂. Media were replaced every 3 to 4 days, and colony formation was monitored. After 15 to 20 days, media were aspirated, and wells were incubated with crystal violet in 10% formalin for 1 hour. Using a dissecting scope, colonies were photographed in a 6-well plate (with 4 quadrants/well) using ImageJ software analysis to automatically count colonies, using 35 pixels as a cutoff.
Protein Analysis

Immunoblotting

Cells were seeded at 0.5 x 10^6 cells/well in 6-well plates in BME/RPMI containing 10% FBS and transfected with 0.1 mmol/L CS3D/CS3M for 24 hours. After transfection, cell lysates were extracted, and protein concentrations quantified using DC assay reagents. Whole-cell lysates (20 mg/sample) were electrophoresed on 7.5% SDS–polyacrylamide gels for 1 hour and transferred onto Trans-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) for 1 hour at 100 V. The membranes were blocked using 5% nonfat dry milk and 0.1% Tween 20 in 1x phosphate-buffered saline (TBST) for 1 hour. Membranes were incubated in primary antibody (in 1% milk in 1x TBS-Tween) at 4°C overnight and washed 3 times with TBST (15 min/wash) followed by incubation with secondary horseradish peroxidase-conjugated IgG antibody for 1 hour (1:2,000) at room temperature (RT), and three washes in TBST. Blots were developed using a superenhanced chemiluminescence substrate according to the manufacturer’s protocol (ThermoScientific). To quantify the results, ImageJ 1.X software (National Institute of Health, Bethesda, MD) was used to assess changes in protein expression patterns.

Immunoprecipitation

After transfection with either 100 nmol/L CS3D or CS3M, H1975 were lysed in immunoprecipitation (IP) lysis buffer (Thermo Scientific) supplemented
with protease inhibitor cocktail (Roche) and phosphatase inhibitors (NaF and Na3VO4). IPs were performed using pSTAT3 antibody at 1:500 and Protein A Magnetic Beads (Thermo Scientific) at 4°C. Immunoprecipitated lysates were electrophoresed on 7.5% SDS-polyacrylamide gels followed by exposure to ubiquitin antibody (1:1,000) at 4°C overnight and the appropriate secondary antibody while following the immunoblotting procedure as above.

**Primary Antibodies**

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Species: Dilution</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>Phospho-STAT3</td>
<td>Rabbit; 1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>(Y705)</td>
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<td></td>
</tr>
<tr>
<td>STAT3</td>
<td>Rabbit; 1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>Rabbit; 1:500</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Histone 3</td>
<td>Rabbit; 1:500</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>c-Myc</td>
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<td>Cell Signaling</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Rabbit; 1:1000</td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>

**Quantitative Real Time-PCR analysis**

After transfection, cells were treated with 10 ng/mL EGF for 1.5 hour to activate STAT3. Trizol (Invitrogen) was then used to extract total RNA. One microgram of total RNA was reverse transcribed using a cDNA synthesis kit (Quanta Biosciences) using a T100 Thermal Cycler (BioRad). Real-time qPCR was performed using a SYBR Green Super Mix kit on a CFX connect Real-Time
System (BioRad). Gene-specific primers for STAT3 target genes were used to assess mRNA levels normalized to GAPDH mRNA levels as internal control, and the ratio of normalized mRNA to the control conditions was determined using the comparative DCT method for analysis. The primers used for real-time qPCR are as follows:

**Primers**

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bcl-xL</strong></td>
<td>5’- ATGCAGGTATTGGTGAGT CG-3’</td>
<td>5’- CTGCTGCATTGTTCCCATA G-3’</td>
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<tr>
<td><strong>GAPDH</strong></td>
<td>5’-GGA GCG AGA TCC CTC CAA AAT-3’</td>
<td>5’-GGC TGT TGT CAT ACT TCT CAT GG-3’</td>
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<td><strong>IL6</strong></td>
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<td>5’-CCA TCT TTG GAA GGT TCA GGT TG-3’</td>
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<tr>
<td><strong>c-MYC</strong></td>
<td>5’- CCGCATCCACGAAACTTT G-3’</td>
<td>5’- GGGTGTTGTAAGTTCCAGT GCAA-3’</td>
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<td>5’- TCCAGGGCTTCATCATAT-3’</td>
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**In Vivo Lung Cancer Xenograft Model**

All studies were approved by the University of Minnesota Institutional Animal Care and Use Committee and were carried out in accordance with institutional guidelines for animal care. Female Foxn1 nu (nude) mice (4–6 weeks old) were injected with 1X10^6 cells (201T, A549, H1975, and H3255) in the flanks. After 3 weeks, animals with palpable tumors (200 mm3) were randomized into two treatment groups with 10 tumors/group. Daily tail vein injections (5 days per week) of the CS3D or mutant STAT3 control (CS3M; 100 µg in 200 mL per injection) was delivered for 2 to 3 weeks. Tumor measurements were made with calipers every 2 days. Volumes were calculated from the formula \( V = \frac{\text{Length} \times \text{Width}^2}{2} \). Animals were sacrificed according to Institutional Animal Care and Use guidelines.
Committee guidelines. Tumors were later harvested for RNA, immunoblotting, and immunohistochemical analysis.

**Carcinogen-induced Lung Carcinogenesis Model**

All animal experiments were approved by Institutional Animal Care and Use guidelines. FVB mice used for experiments were sex, weight, and age matched. Tumors were induced by intraperitoneal injections of 3mg/kg of the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) per body weight; two injections of NNK were done weekly for 4 weeks followed by 1 week of wash out period after NNK administration. The mice were then administered daily injections of CS3D or mutant STAT3 control (CS3M; 100 µg in 200 mL per injection) via tail vein injections for 8 weeks (every 2 days). At 1, 8, and 20 weeks removed from the end of treatment, lungs were harvested and fixed in phosphate-buffered formalin, transferred within 2 days to 70% alcohol, and evaluated under a dissecting microscope for the number of tumors and tumor size. Tumors on the lung surface were counted under a dissecting microscope by at least two blinded readers. Tumor sizes were measured using the LAS V4.12 Leica program. Lungs were further used for histological analysis of tumors, immunohistochemistry, tissue isolation via laser capture microdissection, and DNA sequencing analysis.

**Tumor Histology and Immunohistochemistry**
From the *in vivo* xenograft model, tumors were excised for immunohistochemical (IHC) staining; 5-mm sections were cut from formalin-fixed paraffin-embedded tissue blocks, deparaffinized, and rehydrated using successive washes of xylene followed by ethanol. Antigen retrieval was performed in a microwave oven (for 20 minutes) in an unmasking solution containing sodium citrate buffer followed by peroxidase blocking in 3% hydrogen peroxide. The sections were incubated in ABC blocking buffer (Vector laboratories Inc.) for 1 hour at RT. Sections were then stained with hematoxylin and eosin (H&E) or subjected to sequential incubations with different primary antibodies and peroxidase-conjugated goat anti-rabbit secondary antibodies. Sections were developed with DAB and counterstained with hematoxylin. Bright field microscopy was performed using Leica DM 4000 B LED microscope, and images were captured at 20X and 40X magnification using LASv4.7 software.

IHC analysis was done as a blinded study with 100 images from each treatment group graded as low, moderate, or high. Staining was graded as low (<30% positive cells per field, scored as 1), moderate (30%–60% positive cells per field, scored as 2), or high (>60% positive cells per field, scored as 3).

For fixed lung samples, they were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). H & E-stained lung sections from three predetermined depths were evaluated in a blinded manner to assess tumor burden categorized in reference to the criteria of the Mouse Models of Human Cancers Consortium [152]. For IHC analysis of fixed lung tissue, we followed the same IHC protocol used to analyze tumor xenograft tissue with the exception of
specific and different primary antibodies and peroxidase-conjugated goat anti-rabbit secondary antibodies.

<table>
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**Laser Capture Microdissection (LCM)**

Lung tissue samples were cut at 5 – 10 μM and mounted on special Leica foiled slides. Sections were deparaffinised with xylene, rehydrated through a gradient series of alcohol, washed in water, stained with hematoxylin and counterstain with eosin followed by dehydration. Within 30 minutes of air drying, the slides were mounted on the LCM stage of an LMD6500 laser dissection.
microscope and desired regions of the same tumor were microdissected into individually labelled cap of 200-µL PCR tubes using a laser directed guide provided by the manufacturer (Leica Microsystems, Exton, PA). This procedure was repeated for individual lung tumors identified from animals either treated with CS3M or CS3D.

**DNA K-RAS Sequencing Analysis**

Formalin-fixed paraffin embedded (FFPE) tissue isolated by LCM were used to isolate genomic DNA. DNA extracts were isolated using an AllPrep DNA/RNA FFPE kit purchased from Qiagen (Cat. No. 80234) and a PCR purification kit (Cat. No. 28104) was used to purify DNA and eluted DNA was stored at -20°C. To amplify exon 1 that contains codon 12/13, 50ng of DNA sample was used for the first PCR amplification reaction in a final 25µL volume containing 1X GoTaq Green Master Mix (12.5µL) (Promega), .2µM each of the pair of primers (forward: 5′-GACATGTTCTAATTAGTTG-3'; reverse: 5′-AGGCGCTTTGCCTACGGCA-3′) [153], and nuclease-free water to 25µL. The mixture was heated at 95°C for 9 minutes then subjected to 40 cycles (94°C/1 min, 53°C/1 min, 72°C/1 min). For the 2nd round PCR, 1µL of each of the first round PCR products was then diluted into a final 25µL reaction mixture containing the same buffer composition as the 1st reaction with a different set of primers (forward: 5′-GCCGCTTGACAGCCGCGCCGCCCCTGTGCCCCCGCCCCGCCCCGCCCCGCCCCGCCCCCG.45
CGCCCTATTGTAAGGCCTGCTGAAAAT-3'; reverse: 5' - AGGCGCTCTTGCCTACGGCA-3') [153]. The 2nd reaction was heated at 95°C for 9 min then subjected to 25 PCR cycles (94°C/1 min, 60°C/1 min, 72°C/1 min). The PCR products containing the 126 bp exon 1 fragment were then sequenced (Sanger) and analyzed for the G12D substitution mutation using ExPASy Translate tool.

**Flow Cytometry Analysis**

Cells were stained with fluorescently labeled antibodies using CD45, CD11b, F4/80, Ly6C, Ly6G, I-A/I-E markers on ice for 30 minutes [154]. Cells were analyzed and gated on an LSRFortessa machine. Data were further analyzed by FlowJo.

**Enzyme-Linked Immunosorbent Assay**

NSCLC cell lines were plated at 85 – 95% confluency and transfected with either CS3D or CS3M. Conditioned medium (CM) was collected at 24 hr post-transfection and samples were centrifuged at 1500 rpm for five minutes at 4°C to concentration ligands of interest. Protein lysates were collected for protein concentration normalization as described in the protein analysis methodology section. Ligands were measured using commercially available ELISA kits from R&D systems. IL-6 (add catalogue number) and AREG (add catalogue number)
detection kits were used to measure their concentration in CM. Each assay was performed three times for each sample.

**Combination Index analysis**

Based on Chou-Talalay method of combination analysis, we determined the combination index (CI) between two drugs. These analysis were performed based on the Median-Effect Equation (MEE) developed by Chou-Talalay [180]. Using Compusyn (designed based on the MEE), we calculated the Combination Index (CI) to determine the synergistic effect of 2 or more drugs by assessing the fraction of affected cells. CI<1 were classified as synergistic, equal to 1 (CI=1) was additive, and CI>1 was determined to be antagonistic. The cytotoxic effects of single treatments or combined therapies was determined on several cell lines and entered into the Compusyn program to generate a CI report using a non-constant ratio between the various treatment groups.

**Statistical Analysis**

For statistical analyses, data were reported as mean ± SD or SEM. To assess significance between different treatment groups, a Student t test (two tailed) was used to determine significance with P values at least < 0.05 categorized as statistically significant. To analyze IHC scoring data, the Chi-Squared test was used to compare the frequency of scores of 1, 2, or 3 in
treatment groups. Data represent biological triplicates with n=4 per group per treatment group for all in vitro experiments.
7. Chapter I: Targeting STAT3 using a novel approach demonstrate a robust antitumor effect

7.1 Introduction

Lung cancer remains the leading cause of cancer-related mortality in the United States, which is largely attributed to the dismal survival of stage IV disease [1]. In non–small cell lung carcinoma (NSCLC), accounting for 85% of lung cancer cases [2, 3], patients are stratified based on genetic alterations in the tumor to choose targeted therapy. Patients with activating somatic EGFR mutations initially respond to EGFR tyrosine kinase inhibitors (TKI), but eventually develop acquired resistance through new mutations such as the EGFR T790M substitution, accounting for about 50% of EGFR-mutant patients who develop EGFR TKI resistance. The majority of patients without EGFR mutation, including those who lack other "drivers," are intrinsically resistant to EGFR TKIs [155].

In this study, we tested the ability of a novel therapeutic, CS3D, to inhibit the growth of lung tumors with the T790M mutation or with intrinsic resistance to EGFR TKIs, by targeting STAT3. STAT3 is a central node downstream of individual oncogenic tyrosine kinase "drivers," which could be a therapeutic niche for both intrinsic and acquired resistance to multiple TKIs. STAT3 is often activated during in vitro induction of resistance to erlotinib, as the result of feedback upregulation of other kinases, suggesting that increased STAT3 activity could commonly occur in response to TKIs [124]. During lung cancer
progression, STAT3 is constitutively active in approximately 65% of all NSCLC cases and its primary function is to upregulate the expression of important target genes necessary for cellular proliferation and survival [156].

As a point of convergence for many dysregulated signaling pathways, STAT3 mediates adaptive mechanisms of resistance to molecular targeted therapy in NSCLC, such as induction of epithelial-to mesenchymal transition by IL6 in response to erlotinib [157, 158]. Because STAT3 plays a critical role in malignant cell transformation, but is not essential for normal cell growth [129], it is potentially a prime therapeutic target for NSCLC treatment. Efforts to apply STAT3 inhibition in the clinical setting have been limited due to a paucity of potent and selective inhibitors [128, 159]. The ability of nonphosphorylated STAT3 to function as an active dimer [74, 75] also may limit the ability of kinase inhibitors to completely block STAT3.

To address this, we utilized a decoy approach by circularizing (through ligation) a double-stranded oligonucleotide containing a 15 base-pair sequence corresponding to the STAT3 response element [141]. The circular molecule was produced through inclusion of two hexaethyleneglycol spacers that provide flexibility and upon ligation become a thermally stable cyclic double-stranded oligonucleotide [133]. The cyclic STAT3 decoy (CS3D) was compared with a mutant inactive control (CS3M) molecule that differs from the active compound by a single base pair and lacks binding to the STAT3 protein [133, 141], allowing an assessment of the specificity of CS3D to block STAT3's functions. The STAT3 decoy was previously shown to decrease luciferase activity in cells expressing a
luciferase reporter gene controlled by the STAT3 consensus sequence; CS3D competitively bound to pSTAT3 protein in comparison to CS3M, which showed no affinity for pSTAT3 protein [141]. The decoy approach allows STAT3 dimers to preferentially interact with the oligonucleotide, acting as a molecular sink to competitively inhibit binding of the dimer to the promoters of STAT3 target genes [133, 141]. This approach demonstrated efficacy in head and neck squamous cell carcinoma models (HNSCC; refs. 134, 160), and the ligated circularized decoy exhibits greater potency when injected in vivo compared with earlier linear versions [133]. Here, we examined the antitumor effects of CS3D in NSCLC in comparison to an inactive decoy mimetic harboring a single mutation (CS3M) providing an imperative assessment of CS3D specificity to block STAT3's functions in NSCLC. The data presented in this chapter have been published by Njatcha and colleagues in Molecular Cancer Therapeutics [42].
7.2 RESULTS

Ligation of S3M and S3D

The oligonucleotides (S3D and S3M) are synthesized as single-stranded molecules. To ensure a ligation reaction occurred, we performed an overnight enzymatic ligation using T4 DNA ligase following self-annealment to generate the cyclic version of CS3D and CS3M (Figure 5A). Ligation efficiency was analyzed on a 15% TBE gel, and the cyclic form migrated faster than the unligated strands. Extrapolating from the band intensity, ligation efficiency ranges between 85% and 95%, showing that amount of the cyclic forms predominates over the linear forms (Figure 5B).

![Figure 5B](image-url)
Fluorescently-labelled Oligonucleotide Uptake

To determine whether CS3D and CS3M were equally taken up by NSCLC cells, both molecules were tagged with a fluorescein dye (iFluorT) to assess transfection efficiency. Twenty-four hours after transfection with the iFluorT-labeled oligonucleotides at a concentration of 25 nmol/L, NSCLC cell lines showed intracellular location of the cyclic molecules (CS3D or CS3M), demonstrating that integrating hexaethyleneglycol linkers into the oligonucleotide, or circularizing the DNA, still allowed intracellular uptake. More importantly, altering the sequence of the CS3D by a single base pair to generate the inactive STAT3 mutant (CS3M) did not affect its uptake, demonstrating that the cytotoxicity of CS3D was not due to preferential uptake compared with CS3M (Figure 6A). The efficiency of transfection was >90% for both CS3M and CS3D and confocal microscopy illustrates that the cyclic forms are mostly concentrated.
within endosomes suggesting that CS3M/CS3D cellular uptake is partly mediated by endocytosis.

Further confocal microscopy showed clear fluorescent signal in both the nucleus and the cytoplasm of lung cancer cells, with cytoplasmic signal predominating (Figure 6B). This suggests that CS3D can interact with its target STAT3 dimers in both compartments.
To evaluate the systemic stability of CS3D, we implanted H1975 characterized by EGFR mutations into the flanks of nude mice to examine whether it can be detectable at the flanked tumors and also organs such as the lung, liver, and spleen. Once the tumors established, mice were administered a single dose of 5mg/kg of CS3D constituting a 1:1 ratio of unlabeled “decoy” to the labelled “decoy”. Frozen tumor sections were evaluated for green-fluorescent staining at various timepoints (1hr, 24hrs, 48hrs, 72hrs, 96hrs, and 120hrs). Fluorescein-labelled CS3D was detectable at the tumor site after 1 hour, 24 hours and 48 hours but undetectable beyond that (72hrs, 96hrs, and 120hrs) (Figure 7). This suggest that CS3D potentially exhibit resistance to nuclease degradation for 48 hours with sustainable biological activity after a single injection. Imaging analysis of the liver also shows that CS3D is detectable after 1 hour compared to a no fluorescence control (data not shown).
Specificity of CS3D on Gene Regulation

It has been demonstrated that STAT3 transcriptional activity overlaps with other transcription factors (TFs) such as STAT1 and NF-kB in gene regulation. Since STAT1 and NF-kB have been documented to regulate gene expression in NSCLC, we assessed the ability of CS3D to specifically regulate STAT3-target genes without modulating STAT1 and NF-kB target genes. Examination of STAT1 target genes (IFIT1 and IRF7) [173, 174] and NF-kB target genes (IL1b and IL8) [84] that are predominantly regulated by these TFs showed no significant decrease in mRNA expression level by qRT-PCR when comparing CS3D- with CS3M-treated cells (Figure 8).
Wild-type and Mutant EGFR NSCLC cells exhibit similar sensitivity to CS3D

To determine effect of CS3D on cell viability, we transfected increasing concentrations of CS3D, and after 72 hours, MTS assays were used to assess cell viability. Data were normalized to cells treated with lipofectamine alone. The inactive mutant control (CS3M) showed no cytotoxicity (Figure 9A), and the percentage of cytotoxicity induced by CS3D allowed calculation of the IC50s as described in Materials and Methods. The IC50 values for CS3D in both the WT (201T) and mutant (H1975) EGFR cell lines were approximately 0.3 mmol/L (Figure 9A). Other cell lines with sensitivity to CS3D is the EGFR TKI sensitive cell line H3225 (Figure 9B). Normal lung fibroblasts and primary bronchial
epithelial cells had IC50s that were 100-fold higher (300 mmol/L) than tumor cells (data not shown). Images captured 24 hours post-transfection illustrates that CS3D-mediated cell death is robust and is indiscriminate of mutation status (Figure 9B).

![Graph showing cell viability](image-url)

**Figure 9. Effects of the CS3D on cell viability.** A, NSCLC cell lines (201T and H1975) were transfected with CS3D or CS3M at concentrations ranging from 0 nmol/L to 600 nmol/L. Using MTS assays, cell viabilities was assessed 72 hours later. Three independent experiments were performed, using 24-well plates and 4 wells/concentration.

**Figure 9B.** Effects of the CS3D on cell viability independent of EGFR genetic alterations. NSCLC cell lines with or without EGFR mutations (201T, H1975, H3255) were transfected with .3µM of either CS3M or CS3D and images were captured 24 hours later.
CS3D Inhibits Anchorage Independent Growth of NSCLC and Induces Apoptosis

We next examined the effect of CS3D on the ability of NSCLC cells to grow in an anchorage-independent manner. 201T and H1975 cells were transfected with either CS3D or CS3M (300 nmol/L), then seeded in soft agar. Compared with CS3M, CS3D produced a significant decrease in colony formation. The number of colonies formed was quantified by using a size cutoff (with greater than 35 pixels counted as a colony). A single transfection with CS3D significantly ($P < 0.051$) disrupted anchorage-independent growth of the NSCLC cells by 70% (at 15 days in H1975 cells) and 50% (at 20 days in 201T cells, $P < 0.053$) compared with CS3M (Figure 10A). Flow cytometric analysis of Annexin V/propidium iodide–stained cells also demonstrated that transfection with CS3D significantly increased apoptosis in comparison with CS3M. CS3D treatment (100 nmol/L) caused a 2-fold increase ($P < 0.05$) in the number of cells undergoing apoptosis as compared with CS3M at 24 hours (Figure 10B).
CS3D Suppresses the Expression of c-Myc in Response to EGF

c-Myc is a STAT3 target gene that is known to be activated by EGF treatment. To investigate the effects of STAT3 blockade on c-Myc expression, cells were first treated with 10 ng/mL EGF for 1.5 hours (deemed the optimal time to observe increased c-Myc RNA), 24 hours after transfection of the cyclic oligonucleotides. RT-qPCR was used to assess differences in mRNA expression in STAT3 decoy–treated versus mutant decoy–treated cells (Figure 11A and B). A single transfection of CS3D caused a 50% inhibition in mRNA level of c-Myc in 201T cells (Figure 11A) and a 25% to 30% inhibition in H1975 cells (Figure 11B).
Immunoblotting analysis also showed a 31% reduction in c-Myc protein expression post-CS3D transfection compared with either CS3M or control (lipofectamine alone) in 201T cells. Similarly, in H1975 cells, a 40% reduction in c-Myc protein expression was found (Figure 11C). H3225 cells and A549 cells also showed similar decrease in c-Myc expression. Examination of another STAT3 target gene (for example, Bcl-xL as shown in Figure 11A & B) also responded with decreased mRNA and/or protein expression across the four cell lines, but not as consistently as c-Myc.

**Figure 11. CS3D downregulates STAT3 target genes critical for cell cycle progression and survival.** Downregulation of c-Myc and Bcl-xL mRNA expression by CS3D. Twenty-four hours after transfection, cells were treated with EGF (10 ng/mL) for 1.5 hours, and mRNA was harvested from wild-type (A) and mutant EGFR T790M (B) NSCLC cell lines, and mRNA expression was assessed by RT-qPCR. Relative mRNA expression was normalized to GAPDH mRNA levels as an internal control.
CS3D induces STAT3 Ubiquitination

Immunoblotting analysis of cell extracts showed that after stimulation with IL6, a potent inducer of p-STAT3, there was a 35% reduction in both nuclear pSTAT3 and nuclear total STAT3 after transfection of CS3D compared with CS3M or control (lipofectamine alone; Figure 12). In the cytoplasm, the levels of pSTAT3 (but not total STAT3) were also reduced by CS3D, suggesting that the selective binding of pSTAT3 to CS3D might alter its stability and/or its ability to shuttle to the nucleus. (Figure 12A). To test whether CS3D could increase ubiquitination of p-STAT3 protein, an IP was carried out for p-STAT3, followed by immunoblotting for ubiquitin. After 24 hours, CS3D increased the ratio of p-STAT3 that was ubiquitinated relative to amount of STAT3 present (input), compared with CS3M (0.95 vs. 0.74; Figure 12B). The input of total cell lysate also showed less STAT3 after CS3D treatment, confirming the cell compartment results in Figure 12A.
Systemic Administration of CS3D inhibits NSCLC tumor growth

Based on the in vitro effects of CS3D, we next evaluated antitumor effects in mice harboring established NSCLC xenografts (10 tumors/group). Mice were given daily intravenous injections (tail vein) of CS3D or CS3M (5 mg/kg/d), 5 days per week, and tumor growth was monitored for 14 to 20 days. This method of delivery and dose were previously shown to be efficacious in head and neck cancer models [133, 134]. CS3D caused a significant and robust tumor growth inhibition in 201T- and H1975-derived xenografts compared with treatment with
STAT3 Blockade in Vivo by CS3D Downregulates c-Myc and Promotes Cell Death

To determine how CS3D blocks NSCLC growth, we performed Western blot, qRT-PCR, and IHC analysis to determine expression of c-Myc in residual tumors. These analyses identified a substantial downregulation of c-Myc protein (Figure 18A) and mRNA levels (Figure 14B) in individual CS3D-treated tumors relative to CS3M-treated tumors in both 201T and H1975 xenografts. Some
variability in protein levels among individual xenografts was observed (Figure 18A); the densitometry units for c-Myc protein after normalizing for GAPDH levels ranged from 0.11 to 0.16 units for CS3D-treated xenografts and 0.25 to 0.46 units for CS3M-treated tumors in H1975. Similarly, in 201T-derived tumors, these measurements ranged from 0.015 to 0.51 units for CS3D and 0.28 to 0.97 units for CS3M (Figure 14C). Even with this variability, the effect across the groups in both cell lines was significant ($P < 0.05$ for H1975 and $P < 0.042$ for 201T xenografts), with a mean decrease of 65% and 70%, respectively. For mRNA levels in tumor lysates, CS3D produced a 60% to 70% reduction of c-Myc gene expression relative to GAPDH compared with CS3M (Figure 14B). IHC analysis of tumors harvested after the last day of treatment also revealed a substantial increase in the levels of CC3 (observed predominantly in tumor cells) in response to CS3D. Sections of the H1975 xenografts in response to CS3M showed 10% of fields scoring for high level of CC3, whereas in CS3D-treated xenografts, there was an increase to 83% of fields in the high scoring tumor sections ($P < 0.05$; Figure 14D). Some CC3 staining was localized in nuclei, where caspase-3 is known to be active.
Figure 14. CS3D suppresses c-Myc expression and activates cleaved caspase 3.  

A, c-Myc is suppressed after CS3D treatment in vivo. At the end of the treatments, tumors were harvested, whole-cell lysates were prepared, and RNA extracted for target gene analysis by Western blotting and RT-qPCR. GAPDH protein was used as internal loading control for immunoblotting. CS3D-treated tumors (C4, C5, and C6) from separate animals show a decrease in c-Myc expression level relative to the CS3M-treated group (C1, C2, and C3) in 201T. Immunoblotting analysis of H1975 residual tumors treated with CS3D (C8–C8) also showed reduction in c-Myc expression levels relative to CS3M (C1–C4). The expression of c-Myc was significantly reduced in response to CS3D, relative to CS3M, in both wild-type (201T, $P < 0.042$) and mutant EGFR (H1975, $P < 0.05$)-derived tumors ($P < 0.05$). Data are shown as mean±SEM.  

B, c-Myc mRNA expression levels in 201T and H1975: CS3D suppresses c-Myc gene expression levels in 201T and H1975: CS3D suppresses c-Myc gene expression levels in 201T- and H1975-derived tumors. Data are presented as mean±SEM. * $P < 0.05$ compared with the mutant control group (CS3M).  

C, Densitometry quantification of c-Myc protein expression levels in 201T and H1975: CS3D suppresses c-Myc gene expression levels in 201T- and H1975-derived tumors. The expression of c-Myc was significantly reduced in response to CS3D, relative to CS3M, in both wild-type (201T, $P < 0.042$) and mutant EGFR (H1975, $P < 0.05$)-derived tumors. D, Representative sections of H1975 tumors staining positive for cleaved caspase 3 (CC3). CS3D significantly increases the number of cancer cells staining positive in the tumor microenvironment relative to CS3M ($P < 0.05$). Data shown are mean ± SEM between two groups (CS3M and CS3D).
Long-Term CS3D Treatment Significantly Alters Tumor Cellularity

H&E staining of sectioned tumors harvested after treatment showed that the CS3D-treated tumors were composed of large areas of debris and infiltration with stroma and lymphocytes, whereas the CS3M tumors had a high tumor cellularity (Figure 15). The animals showed no significant loss of body weight or decreased activity, and histologic examination of the lungs, liver, and spleen showed no signs of toxicity with CS3D.

Figure 15. CS3D alters tumor cellularity. Representative sections of 201T and H1975 tumors sectioned in-depth to analyzed tumor morphology. 201T- and H1975-derived xenografts administered daily intravenous injections of either CS3D or CS3M were harvested at the end of treatment (day 14), and changes in tumor architecture was analyzed. CS3D significantly changes the tumor microenvironment relative to CS3M in 201T and H1975-derived xenografts.
Short-term CS3D Treatment Promotes an Antitumor Phenotype Mediated by Reduction in Nuclear p-STAT3 and Ki-67 expression

Because tumors treated for several weeks with CS3D were largely composed of debris (Figure 15), we examined xenografts after only 5 daily tail vein injections to determine what cellular changes were induced prior to collapse of the intracellular tumor structure. We observed few histologic changes in CS3D-treated tumors after only 5 days (Figure 16). IHC staining, however, revealed a significant decrease in the proliferative capacity of NSCLC, shown by Ki-67 staining in response to CS3D treatment ($P < 0.001$) compared with CS3M-treated tumors after 5 daily injections (Figure 16A). We observed a shift from 41% of cells scored as high-grade staining with CS3M treatment to 13% with high-grade staining in the presence of CS3D, with corresponding increases in moderate- and low-grade staining in the STAT3 decoy–treated group (Figure 16A).

Expression levels of p-STAT3 protein (which was mainly observed localized to the nucleus) were likewise significantly suppressed by the STAT3 decoy ($P < 0.0001$) after 5 days of treatment in vivo compared with mutant decoy (Figure 16B). Frequency of high-grade staining for p-STAT3 differed between CS3D and CS3M treatment groups (23% in CS3D-treated and 51% in CS3M-treated, with corresponding increase in low-grade staining in presence of the active decoy; Figure 16B). The p-STAT3 result suggests that multiple doses of the decoy promoted the reduction in p-STAT3 levels in vivo. At this early time
point, no cellular debris was observed (Figure 16A and B), suggesting that effects on STAT3 and proliferation occur prior to these histologic changes observed in the long-term treatment analysis. We also observed no change in NF-κB nuclear protein with the active decoy, suggesting that the decoy does not alter ... κB movement to the nucleus (data not shown).

Given that K-RAS mutations are also prevalent in patients with NSCLC, evaluating CS3D in that context should give us an insight into the effects of CS3D in NSCLC cells with K-RAS mutations. Preliminary results showed that CS3D can impair the proliferative capacity of A549 cells with a G12S activating
mutation. Because STAT3 is known to form a complex with NF-kB, A549 cells transfected with CS3D to determine if the STAT3-NF-kB complex is still present as a result of CS3D-mediated mechanism. CS3D did not alter the STAT3-NF-kB in A549 cells and other NSCLC cell lines (H1975 and 201T) with wild-type K-RAS (data not shown). After 5 days of treatment with CS3D, residual tumors from mice bearing A549 xenograft showed no difference in NF-kB expression by IHC scoring and western blot analysis (data not shown). This indicates that tumors harboring K-RAS mutations treated with CS3D doesn't disrupt the STAT3-NF-kB complex but we speculate that CS3D could modulate the expression of NF-kB as a target gene. It is important to note that no toxicity was associated with CS3D in monitoring the mice body weights and histological assessment of organs such as the liver and spleen did not indicate and form of toxicity.
7.3 CONCLUSION AND DISCUSSION

These findings illustrate that CS3D with hexaethyleneglycol linkers the cyclic form (CS3D) does have biological activity in vivo when given via systemic delivery. Verifying the presence of CS3D by confocal microscopy in NSCLC cell lines and tumor-derived xenograft from \textit{in vivo} analysis suggest CS3D exhibits strong translational potential for NSCLC therapy. CS3D specificity to STAT3 by gene regulation analysis confirmed that target genes predominantly regulated by other critical TFs such as STAT1 or NF-\kappa B were unaffected by CS3D. Additional analysis showing that STAT3 target genes (namely c-Myc and Bcl-xL) are suppressed in response to CS3D in comparison to the inactive construct (CS3M) further demonstrates the specificity of CS3D. In a critical experiment, we identified a CS3D-mediated mechanism that leads to STAT3 ubiquitination, leading to enhanced degradation in the setting of IL-6 stimulation.

Anchorage-independent growth has been shown to be a STAT3-dependent process [59]. We found that after a single transfection, CS3D was very effective in suppressing anchorage-independent growth. It is important to note that in vitro transfections were done using a lipid based approach compared to in vivo studies which did not require any carrier molecule. This therefore suggest that because we inject CS3D as a naked DNA for systemic delivery, there is a possibility that CS3D binds to either proteins like albumin in circulation or is packaged in vesicles like exosomes rendering CS3D resistant to nuclease
degradation. Biochemical experiments would be needed to identify how CS3D as a naked DNA, is specifically packaged and delivered to tumor site.

In a series of subsequent experiments, CS3D was determined to decrease cellular proliferation, and induce apoptosis, and NSCLC cell line sensitivity to CS3D was independent of EGFR mutation status which is known to dictate clinical response to EGFRi. These findings have huge implications for CS3D to be utilized in the setting of NSCLC treatment as a therapeutic strategy to overcome resistance associated with current FDA-approved EGFRi therapies. These early experiments demonstrated that CS3D could be effective in the context of inherent or acquired resistance and could represent a novel treatment modality for NSCLC diseases resistant to FDA-approved EGFR targeted therapies.

To support the early evidence for CS3D therapeutic potential, CS3D demonstrates robust antitumor effects in vivo accompanied by decrease in p-STAT3 nuclear pool, suppression of STAT3-target genes, increase in apoptosis, and altered tumor architecture characterized by large areas of debris and increase in lymphocytes infiltration corroborating the in vitro results. Most importantly, no systemic toxicity is associated with CS3D. The use of liposomes for in vitro experiments compared to tail-vein injections of “naked” DNA molecules (such as CS3D) suggest that CS3D maybe packaged into biological vesicles such as exosomes during systemic circulation contributing to enhanced stability and the robust antitumor effects in vivo. Imaging analysis shows that
fluorescein-labelled CS3D is detectable at an early timepoint (1 hour) after injection and accumulates at the tumor site after 48 hours. These results indicates that CS3D might concentrate at the tumor site after 48 hours suggesting that single injections could be scheduled every 48 hours to achieve therapeutic benefits. In summary, CS3D exhibits great therapeutic potential and these studies represent the first step towards assessing its translational use in NSCLC.
8. Chapter II: The Cyclic STAT3 “decoy” exhibit chemopreventive properties

8.1 Introduction

More than 85% of all lung cancer cases are associated with tobacco exposure [2, 3] and carcinogens found in tobacco products have been well documented to facilitate tumor initiation and promote lung carcinogenesis [161]. Statistically, the risk of developing lung cancer due to exposure to tobacco carcinogens makes it the leading cause of preventable cancer-related mortality in the United States [161]. Pathologically, the sequential events that lead to lung carcinogenesis are defined by preneoplastic lesions that are strong indicators of tobacco exposure and offers a unique opportunity for chemoprevention [162]. Preneoplasias identified as thickening of the epithelial airways, are histological changes that eventually leads to tumor progression and subsequent formation [162]. Clinical evidence from NSCLC patients exposed to carcinogens via cigarette smoking points to genetic abnormalities which results in the activation of oncogenes such as KRAS [163], and may also activate signaling molecules, all contributing to chronic inflammation within the lung paramount to cancer initiation and progression [164].

The nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), found in tobacco smoke, is sufficient to induce KRAS mutations among many other mutations such as p53 mutations required for lung carcinogenesis in mice. Susceptible strains to NNK-induced lung carcinogenesis includes A/J or FVB/N
mice [164]. The NNK-induced lung cancer murine model is widely used to mirror the genetic and molecular alterations found in human lung adenocarcinomas due to cigarette smoking. In addition to KRAS-driven signaling in NSCLC, STAT3 also plays a critical role in cellular transformation. STAT3 was first identified as an oncogene by Bromberg and colleagues as a requirement for epithelial cell transformation [59]. Following this seminal work, researchers have mainly focused on targeting STAT3 for treatment purposes, without really addressing the opportunity to impair STAT3’s function as a cancer preventable strategy.

It was later reported that STAT3 activation plays a critical role in chemical and UV-induced carcinogenesis [165]. Consistent with these observations, STAT3 deficient mice are resistant to tumor development. Caetano et al., recently observed a substantial STAT3 increase in a KRAS-induced lung cancer model, with an increase in STAT3 mRNA expression indicating that the IL-6/STAT3 axis is critical for tumors harboring KRAS mutations and tumor burden formation and growth dependence [85]. This provides a unique opportunity to assess whether disrupting its function can translate to chemopreventive measure in an NNK-induced lung cancer model. In the present study, we sought to determine the chemopreventive property of a STAT3 “decoy” (CS3D) which mimics the response element within STAT3 target genes.

To assess CS3D as chemopreventive strategy, mice exposed to NNK as described in materials and methods, were treated with CS3D following NNK administration. The effects of CS3D were compared to an inactive mutant
oligonucleotide (CS3M) that is unable to bind to STAT3 dimers due to a single base-pair mutation within the CS3M construct. We hypothesized that CS3D should bind to STAT3, alter gene expression patterns, impair tumor formation, tumor growth (measured in size), and should reprogram the TME from an immunosuppressive phenotype towards a more active anti-tumor immune response.
8.2 RESULTS

CS3D Delays Preneoplasia Lesion Formation

Using an NNK-induced mouse model, we investigated the effects of STAT3 inhibition on preneoplasia (defined as the abnormal thickening of the airways epithelium) formation and on progression to tumors. Following NNK exposure, FVB/N mice were administered 5mg/kg of CS3D three times a week, via tail-vein injections, for a period of 8 weeks, and lungs were collected at various timepoints removed from the end of treatment after 1 week, 8 and 20 weeks as depicted in our timeline (Figure 17).

**Figure 17. NNK-induced lung carcinogenesis model.** Evaluation of CS3D chemopreventive effects compared to a mutant control construct (CS3M) in an NNK-induced tumor model parallel to an ex-smoker status. 3mg/kg of NNK was administered intraperitoneally 2 days per week followed by 1 week washphase, 8 weeks of CS3D or CS3M (5mg/kg) treatment (3 times/week), and lungs were collected at various timepoints for different endpoint analysis as depicted in the timeline.
Cohorts of mice treated with CS3D were compared to CS3M-treated control group and no body weight changes were observed or organ toxicity (by histological analysis of the liver and spleen) was detectable at the end of treatment. Histopathological analysis of the lungs from CS3D treated mice revealed approximately 15 fewer number of preneoplasias on average per animal as compared to CS3M at week 1 post-CS3D administration (Figure 18A). Tumor burden was also assessed at the week 1 timepoint and consistent with reduction in preneoplasia, we observed a decrease in the average number of tumors (by a count of 3 less tumors from the CS3D-treated animal) (Figure 18B). Preneoplasias and tumor burden were assessed by histological analysis providing evidence for the antitumorigenic property of CS3D on tumor onset after NNK exposure (Figure 17).

To determine if the effects of CS3D is sustainable in a long-term experimental model, we analyzed FFPE sections from lung tissue harvested at the 8 weeks timepoint and we counted a lower number of adenomas from the CS3D group compared to the CS3M control group. But predictably, the CS3D-treated group showed a higher count of preneoplasia compared to CS3M, which was mostly characterized by fully blown adenomas with limited amount of preneoplasias to score.
Figure 18. Quantification of preneoplasia and adenomas in response to CS3D. Histological analysis of H&E sections of lungs average number of preneoplasia (defined as the abnormal thickening of the airways epithelium) and adenomas (benign tumors starting to form from the epithelial tissue which are advance stages from preneoplasias) at (A) week 1 post CS3D, and (B) week 8 post CS3D administration. Representative photomicrographs were captured at 40X (scale bar for 200µM) magnification and quantitative analysis of adenomas were processed at respective timepoints for each treatment group. Five slides were analyzed per animal (n=5) with 15 slides apart from one another to integrate an in-depth analysis of preneoplasia and adenomas. (Data represent means ± SEM****P<0.001)
STAT3 Blockade Decreases NNK-induced Lung Carcinogenesis

With evidence that CS3D can delay preneoplasia formation, and impair tumor formation (identified as adenomas) at early timepoints (week 1 and 8), we wanted to assess tumor burden formation at a later timepoint of 20 weeks post-eight weeks of CS3D administration. As predicted, the average number of tumors per animal was significantly reduced by approximately 40% in CS3D-treated animals (Figure 19) as compare to CS3M control cohort. These data suggest targeting preneoplasia and the transition towards adenomas by STAT3 inhibition with CS3D suppresses tumor formation in an NNK-induced lung cancer model.

**Figure 19. Tumor burden quantification in response to CS3D.** Mice susceptible to NNK-induced lung tumorigenesis were treated with either CS3M or CS3D. A, Lung appearance and representative tumors are indicated by arrows. B, Number of tumors per animal was counted and averaged per treatment group. Data are the mean ± SD (n = 10 *P<.05)
STAT3 Inhibition Impairs NNK-induced Lung Cancer Growth

After illustrating that mice treated with CS3D are more resistant to NNK-induced lung preneoplasia and tumor burden formation, we wanted to determine whether CS3D would impair the growth rate of tumors in this NNK model well-characterized by K-RAS mutations and as a result, highly dependent on K-RAS mediated signaling. Tumor size measurement determined using the LAS V4.12 Leica program illustrates that CS3D causes 49.8% tumor growth inhibition (Figure 20) and this effect might be independent of K-RAS mutations known to be key oncogenic alterations in NNK-induced lung carcinogenesis.

*Figure 20. CS3D impairs NNK-induced lung cancer growth. Mice susceptible to NNK-induced lung tumorigenesis were treated with either CS3M or CS3D. A, Tumor size were measured by the proportion of area covered on lungs using LAS V4.12 Leica program. Data are the mean ± SD (n = 10 *P<.05)
CS3D Effects on Tumor Burden are Sustained Independent of K-RAS Mutations

The potential tumor suppressive role of knocking out STAT3 in a K-RAS mutant lung cancer model was recently shown, suggesting that there could be a select group of patients with K-RAS mutations that are less likely to benefit from STAT3-targeted therapy [84]. This evidence was strictly generated in a STAT3 knockout mouse model and we aimed to show that pharmacological targeting of STAT3 with CS3D should impair the growth of K-RAS driven tumors. To evaluate the percent of tumors positive for K-RAS mutations, we isolated samples from individual tumors by laser capture microdissection. We proceeded to sequence exon 1 of the K-RAS gene to determine the presence G12D missense genetic mutation which is detectable in greater than 30% of all lung adenocarcinoma [160]. This is the only KRAS mutation known to be produced by NNK in mice. After Sanger sequencing analysis, we found that there was no significant difference in the proportion of tumors positive for K-RAS mutation between CS3D and CS3M control group with greater than 10 tumors per group (Figure 21). These sequencing results demonstrate that pharmacological inhibition of STAT3 potentially impairs the growth of tumors harboring K-RAS mutations producing an antitumor phenotype contradicting the observations made in a STAT3 (genetic) knockout model as described by Grabner and colleagues [84].
**STAT3-induced Degradation by CS3D Alters NF-kB Activation**

The selective survival of K-RAS driven tumors in a STAT3 knockout system as described by Grabner and colleagues identified an NF-kB escape signaling mechanism. NF-kB promotes the survival of mutant K-RAS clones by promoting an immunosuppressive phenotype that involves blood vessel formation and macrophage infiltration [84]. With knowledge of this potential drug-resistant mechanism, we wanted to assess the effects of CS3D on NF-kB-mediated signaling. As previously reported in the xenograft studies, CS3D causes STAT3 degradation and following that observation, we were able to confirm that CS3D also causes STAT3 reduction 20 weeks post-treatment in the NNK model (Figure 22A). The sustainable CS3D-mediated effect is also accompanied by a decrease in p-NF-kB activation (Figure 22B) indicating that

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**Figure 21. Proportion of tumors bearing K-RAS mutations post-CS3D treatment.** Tumors from mice treated with CS3D after NNK exposure are similarly susceptible to K-RAS mutations as the control group (CS3M) with no significant increase in K-RAS positive tumors. Sanger sequencing analysis of K-RAS mutations at codon 12 of exon 1 to detect G12D missense mutation. (n = 10)
CS3D can effectively interrupt STAT3 signaling while simultaneously impairing NF-kB signaling. We also observed a decrease in total NF-kB protein (data not shown).

**Figure 22. CS3D downregulates STAT3 and NF-κB expression.** A, CS3D treatment suppresses p-STAT3 and (B) subsequently causes a decrease in p-NF-κB. FFPE lung tissue were analyzed by IHC staining for p-STAT3 and p-NF-κB. Representative IHC staining for p-STAT3 and p-NF-κB. Quantification analysis indicates the percent of cells staining positive as low (less than 30% cells per field), moderate (30-60% of cells per field), and high (greater than 60% per field) defined by the brown intensity. Images represent adenomas scoring positive as high grade tumors. See Methods section for details on scoring assessment. Data are the mean ± SD (n > 10 *p < 0.05).
CS3D Disrupts the Pro-tumorigenic Phenotype Associated with Lung Cancer Development

STAT3 as a TF has been widely implicated in several oncogenic events that defines the hallmarks of cancer development. Angiogenesis, cell cycle progression, and chronic inflammation are all STAT3 dependent processes that respectively regulate pro-survival factors such as VEGF, MYC and IL-6. Biopsies from smokers at high risk of lung cancer shows that preneoplastic changes in the lungs consist of capillary blood vessels and angiogenic protrusions which helps establish a pattern of microvascularization [166]. Contrary to smokers, biopsies from nonsmokers lack those vascularized lesions suggesting that VEGF-dependent microvascularization at early stage of lung carcinogenesis is a clinical feature that can be targeted to prevent tumor formation. VEGF activation in the endothelial compartment is known to be partly regulated by STAT3 [97]. Enhanced vascularization in preneoplastic lesions suggest targeting STAT3 might disrupt the NNK-induced pro-tumorigenic phenotype. IHC analysis of preneoplasia at 8 weeks and 20 weeks caused a decrease in VEGF staining (Figure 23A). CS3D also suppresses CD31 expression (data not shown) suggesting that CS3D does impair the degree of angiogenesis from premalignant stage to tumor formation.
In addition to vascularization, chronic inflammation is now recognized to be important in the pathogenesis of lung cancer. Given that patients with lung cancer have elevated levels of IL-6 and known to mediate inflammatory signals via canonical activation of STAT3, it was critical to evaluate the short and long term effects of CS3D on this pathway using an immunocompetent NNK model. Because IL-6 is known to activate STAT3, which in turn regulates IL-6, this sustains a feed-forward loop which supports a pro-inflammatory environment.
conducive for tumor formation [166]. We proceeded to evaluate IL-6 expression in response to CS3D and results shows that IL-6 levels are upregulated at the early timepoint (week 8) and the later timepoint (20 weeks), indicating a potential rescue mechanism (Figure 23B).

Figure 23. B, CS3D treatment upregulates IL-6 at respective timepoints (8 and 20 weeks). FFPE lung tissue were analyzed by IHC staining for IL-6. Stainings are quantified as low, moderate, and high. Quantification analysis indicates the percent of cells staining positive as low (less than 30% cells per field), moderate (30-60% of cells per field), and high (greater than 60% per field) defined by the brown intensity. Images represent preneoplasias scoring positive as high grade tumors with respect to the control (CS3M) group See Methods section for details on scoring assessment. Data are the mean ± SD (n > 10 *p < 0.05).

A defined immunoinflammatory mechanism in lung cancer development amongst many other is the role of IL-6 in inducing COX-2 during chronic inflammation
In addition to IL-6 upregulation, STAT3 inhibition by CS3D also mediated an increase in COX-2 expression (P<.0001) (Figure 23C).

We previously demonstrated that MYC, critical for cell cycle progression, is a STAT3 target gene because CS3D is able to suppress its expression independent of EGFR mutation status. In this NNK model, we can also show that MYC expression is also suppressed (data not shown) post-CS3D treatment.

Figure 23. C, CS3D treatment upregulates COX-2. FFPE lung tissue were analyzed by IHC staining for COX-2. Staining is quantified as low, moderate, and high. Quantification analysis indicates the percent of cells staining positive as low (less than 30% cells per field), moderate (30-60% of cells per field), and high (greater than 60% per field) defined by the brown intensity. Images represent preneoplasias scoring positive as high grade tumors with respect to the control (CS3M) group. See Methods section for details on scoring assessment. Data are the mean ± SD (n > 10 *p < 0.0001).
(week 1). Altogether, CS3D suppresses STAT3 activity thereby favoring a tumor microenvironment (TME) less proliferative, more apoptotic, and less angiogenic phenotype. An increase in IL-6 and COX-2 expression suggest these markers could represent biomarkers of CS3D response.

**Pharmacological Inhibition of STAT3 by CS3D Shifts the Immunosuppressive Phenotype towards an Antitumor Immune Response**

To this point, we have generated evidence clearly demonstrating that STAT3 functions to sustain lung cancer proliferation, induce angiogenesis, and resisting cell death but its pleiotropic effects extend beyond those. STAT3 also plays a central role in immune evasion by promoting inflammation [79]. STAT3 specifically orchestrate immunosuppression by promoting M2 macrophage and myeloid-derived suppressor cells (MDSCs) activity while suppressing M1 macrophage function [168]. Thus STAT3 inhibition by CS3D should favor an antitumor immune response by enhancing M1 macrophage activity. Flow cytometry analysis revealed that CS3D promotes an antitumor response by increasing the proportion of M1 macrophages identified as an increase in F4/80+, Ly6C-, and MHCII+ macrophages but a reduction in the proportion of macrophages staining positive for F4/80+, Ly6C+, and MHCII+ indicated a decrease in M2 macrophages. Further assessment of MDSCs cells positive for CD11b, and Ly6G markers indicated that CS3D also caused a reduction in the relative proportion of MDSCs present within the lung TME.
Figure 23. STAT3 inhibition by CS3D alters the lung tumor microenvironment. Representative flow cytometry data and percentage of (live/CD45+) activated M1 and M2 macrophage population, and MDSCs after 4 weeks of NNK exposure followed by 1 week of CS3D administration.
8.3 CONCLUSION AND DISCUSSION

Lung cancer is often diagnosed in former smokers as a result of exposure to carcinogens like NNK. From the time of smoking cessation to advance stages of lung cancer, there is window for intervention to prevent the transition from premalignant growth to full tumor onset. Using a carcinogen-induced mouse model characterized by signal driven mechanisms involving essential factors such as STAT3, we addressed the role of STAT3 by pharmacologically targeting STAT3 in an NNK mouse model known to induce K-RAS activating mutations. The “decoy” STAT3 inhibitor used for these studies demonstrates efficacy in significantly delaying tumor formation and growth. Contrary to a previous observation which suggested that disruption of STAT3 via genetic knockout promotes KRAS-induced lung tumorigenesis [84], intervention by this novel approach confirms that pharmacological inhibition of STAT3 produces an antitumor phenotype, without selecting for KRAS positive tumors. With evidence that CS3D can promote an antitumor phenotype by growth inhibition in tumors bearing K-RAS mutations and similar observations in wild-type derived tumors (201T and H1975), comparing mutant and wild-type tumors in response to CS3D would provide a great insight into the similarities and differences in terms of tumor biology.

Interestingly, work by one group shows that genetic ablation of IL-6 can increase tumor initiation [169] while another group demonstrated that an anti-IL-6 antibody-based approach does not promote tumor initiation, but rather inhibits K-RAS induced tumorigenesis [85]. This evidence thus corroborates the results
observed in our model. The differences observed between pharmacological inhibition of STAT3 and genetic ablation is attributable to the distinct timing and cell-specific effects of mutant K-RAS induction during pulmonary carcinogenesis. Epithelial cell-specific STAT3 knock-out might promote an aggressive phenotype involving NF-kB and IL8 that might suggest STAT3 has “tumor suppressive” functions, but that phenotype is not recapitulated with antitumor agent like CS3D. This suggests the STAT3-knockout models studied in the context of K-RAS mutations provide a model to identify potential rescuing mechanisms required to compensate for STAT3 inhibition via a pharmacological approach. Once identified, those drug resistant signaling pathways could be targeted with selective inhibitors and maybe combined with CS3D to determine if synergy exist in K-RAS-induced lung cancer models.

After generating evidence illustrating the ability of CS3D to significantly impair lung tumorigenesis in an NNK-induced lung cancer model, determining the percentage of residual tumors positive for K-RAS mutation would inform us on the selectivity of CS3D with respect to K-RAS mutant clones. Analysis of K-RAS G12D activating mutation in residual tumors seems to suggest that CS3D does not enhance K-RAS oncogenic activation because no significant differences were observed compared to mice treated with the mutant control construct (CS3M). These findings support the hypothesis that K-RAS mutant clones utilize STAT3 function for survival as opposed to the tumor suppressive functions described in the literature in a context dependent manner. In line with STAT3 tumor suppressive function, STAT3 can sequester NF-kB in a complex depriving the
cancer cells of NF-kB-mediated signaling critical for a pronounced tumor angiogenesis, and myeloid cells infiltration necessary to enhance lung cancer development [84]. Here, we found that CS3D degrades STAT3 without eliminating it completely and significantly reduces NF-kB activation, disrupting downstream signaling. These effects culminate in a less proliferative, less angiogenic, and ultimately, a less pro-tumorigenic environment, thus impairing lung cancer development. CS3D also induces a phenotypic switch by recruiting immune cells with antitumor properties such as M1 macrophages while suppressing the immunosuppressive phenotype.

Collectively, our data provide the first evidence that CS3D has chemopreventive properties in lung cancer without promoting K-RAS-driven tumors, and modulates the TME in the process resulting in malignant growth impairment.
9. STAT3 inhibition promotes “bypass” signaling mechanisms

9.1 Introduction

NSCLC is a set of heterogeneous diseases with genetic alterations mostly manifesting as mutations. Frequently occurring mutations in adenocarcinoma are mostly identified as either EGFR OR K-RAS mutations [145]. These driver mutations maintain constitutive signaling which highly converges on STAT3 as a central node. Data generated by our laboratory at this point clearly demonstrates that the STAT3 “decoy” inhibitor impairs lung cancer development in NSCLC harboring EGFR or K-RAS mutations (Figure 13, 19, and 20). However, the complex cascade of signaling in NSCLC also constitute cross-signaling via the MAPK and PI3K-AKT pathways. Research by multiple groups has shown that STAT3 inhibition in several type of cancers produces a drug-resistant phenotype dependent on “bypass” activating signaling mechanisms may involve MAPK and AKT downstream signaling [145].

Activation of redundant secondary pathways are regulated by RTKs such as the HER (Human Epidermal Receptor) family of receptors. This family of receptors comprises of EGFR, HER2, HER3, and HER4 [170]. These receptors can be activated by ligand dependent and independent mechanisms (such as EGFR activating mutations). In NSCLC without activating mutations in the HER family of receptors, ligand-dependent signaling is the primary route of activation and their structural features creates a complex signaling cascade caused by receptor dimerization. The diversity in their biology is also paralleled by a corresponding subset of ligands such as epidermal growth factor (EGF),
amphiregulin (AREG), and neuregulin (NRG1) with preferential binding for a distinct receptor over another [170]. Within the TME, cellular communication dependent on growth factor and cytokine secretion, have been demonstrated to foster paracrine signaling in NSCLC [171]. IL-6, a cytokine associated with inflammation in NSCLC, is secreted by tumor and immune cells. IL-6 binds to the IL-6 receptor and in a non-canonical fashion, the IL-6 receptor complex can form a complex with the EGFR receptor prolonging downstream signaling [172]. Targeting STAT3, a critical downstream effector molecule, should rewire the cellular circuit to activate secondary pathways via compensatory mechanisms involving pathways such as the EGFR/IL-6 cross-signaling cascade. Another receptor activated in a ligand-dependent fashion is the fibroblast growth factor receptor (FGFR) and FGFR plays a critical role in tumor development and maintenance. We hypothesize that STAT3 inhibition in NSCLC using CS3D should induce IL-6, EGFR and FGFR ligands production as compensatory signals to overcome STAT3 blockade. We predicted that the corresponding EGFR and FGFR receptors could be activated by their corresponding ligands or, activated via an unknown IL-6-mediated mechanism. By targeting STAT3, while simultaneously inhibiting those secondary rescuing signals, we might enhance the effects of the STAT3 “decoy” (CS3D) in a combinatorial approach.
9.2 RESULTS

CS3D Activates “Bypass Signaling” Mechanisms in Wild-type EGFR-Derived tumors

We have previously demonstrated that targeted inhibition of STAT3 using the STAT3 “decoy” inhibitor (CS3D) results in significant tumor growth arrest (Figure 13, 19, & 20). Given the pleotropic functions of STAT3 in tumor cell and stromal cells in the TME, impaired STAT3 function should evoke a compensatory response that promotes cancer cell survival. Thus cancer cells can become highly dependent on “bypass signaling” pathways as a coping mechanism to evade drug therapy. To address whether CS3D evokes those “coping” mechanisms, nude mice bearing tumors derived from EGFR wild-type cell lines were treated with either CS3D or CS3M for 5 days, weekly for 3 weeks.

Evaluating residual tumor by western blot analysis revealed an increase in HER3 phosphorylation (Figure 25A). Because HER3 lacks a kinase domain [170], HER3 phosphorylation suggest HER3 is forming a complex with one of the HER family members (either EGFR, HER2, or HER4) to maintain proliferative signaling. In response to CS3D, we also observed an increase in IL-6 expression accompanied by JAK2 phosphorylation (Figure 25A). Cytokine array analysis also showed that HER family ligands such as EGF and AREG was detectable at higher levels in response to CS3D (Figure 25B).

On the one hand, CS3D triggers compensatory signaling mechanisms but it also simultaneously activates key components in the apoptotic machinery.

Another transcription factor in the STAT family of protein with unique and
opposite functions associated with STAT3 is STAT1. STAT1 activation has been linked to induction of apoptosis and STAT1-induced apoptosis is in part mediated by Caspase 3 [173]. STAT1 activation has also been implicated in interferon-alpha-mediated cell death [174]. Interestingly, STAT3 inhibition by CS3D was also determined to activate STAT1 with subsequent cleaved caspase 3 activation (Figure 25A). Increase in interferon-gamma was also detected in 201T residual tumors via cytokine array analysis (Figure 25B) which might be linked to STAT1 activation in response to STAT3 inhibition as previously described [175].
STAT3 Inhibition by CS3D Promotes IL-6 Secretion by Cancer Cells

STAT3 is canonically activated via the IL-6/JAK axis but analysis from tumor-derived xenografts showed an increase in IL-6 expression in response to CS3D treatment (Figure 25A). In the TME, IL-6 secretion by tumor or stromal cells maintains autocrine and paracrine loop that supports a malignant phenotype [176]. Due to the pleiotropic functions of IL-6 that may involve an autocrine loop in tumor cells, we predicted that STAT3 inhibition in cancer cells is contributing to the high levels of IL-6 detectable in the tumors. NSCLC cell lines (H3255, H1975, and 201T) transfected with the STAT3 decoy (CS3D) secreted higher levels of IL-6 compared to tumors treated with Control (CS3M).

Figure 25. B, Secretion of HER ligands in response to CS3D treatment. At the end of the treatments, tumors were harvested and cytokine array analysis was utilized to assess AREG, EGF, and IFN-gamma expression. Positive and negative protein was used as internal loading control for cytokine profiling. Lysates from separate CS3D-treated tumors were pulled together and compared to a CS3M-treated group from 201T xenografts. The difference in cytokine or growth factor expression by immunoblotting from duplicate experiments between the CS3D- and CS3M-treated groups was significant for AREG (P=.016), EGF (P=.011), and IFN-gamma (P=.02).
6 in comparison to CS3M (Figure 26). Other cytokines detectable in response to CS3D were IL-8, FGF2, and NRG1 (data not shown) but the cytokine profiles in response to CS3D varied across cell lines. The observed CS3D-mediated cytokines and growth factors secretion suggest that despite the robust anti-proliferative effects associated with CS3D (Figure 9B), it also triggers compensatory mechanisms which cancer cells can utilize to evade drug therapy.

**Wild-type EGFR NSCLC Activates Growth Factor and Cytokine Receptors in Response to IL-6 Stimulation**

With evidence that CS3D can induce STAT3 degradation via ubiquitination (Figure 12), and subsequent STAT3 downregulation in tissue-derived tumors (Figure 16B & 22A), the IL-6/STAT3 axis is significantly impaired. Therefore autocrine secretion of IL-6 in response to STAT3 inhibition, might gain diverse function by activating compensatory signaling mechanisms. Wang and colleagues have shown in an elegant fashion that IL-6 can bind to the IL-6 receptor and prolong EGFR activation [172] with subsequent downstream effects.
effects. We show that in response to IL-6 stimulation, various receptors (such as EGFR, HER3, HER4, c-Met, and FGFR) are phosphorylated within 5 minutes in EGFR wild-type cell lines (201T) sustaining downstream signaling. However, no changes were observed in the mutant EGFR cell line (H1975) due to high basal levels of receptor activation (Figure 27A).

CS3D-mediated IL-6 release combined with IL-6 mediated phosphorylation events (Figure 26 and 27), led us to hypothesize that conditioned media from CS3D-transfected cells should activate signaling receptors (identified in Figure 27A) in naïve “untransfected” cancer cells promoting an autocrine feedback loop. To determine whether IL-6 secretion from CS3D-transfected cancer cells can initiate an autocrine loop via phosphorylation events, protein lysates from untransfected cells primed with conditioned media (CM) from CS3D-transfected cells was collected and we performed western blot analysis. The data shows that EGFR, FGFR, HER3, and MAPK are phosphorylated within 5 minutes of CM-exposure identifying an autocrine feedback loop that might serve as a rescuing mechanism (Figure 27B).

**Figure 27. Cross-signaling activation of HER-family of receptors in response to IL-6 secretion.** **A,** Increase in receptor phosphorylation in response to IL-6 in 201T compared to H1975. Cells are treated with recombinant IL-6 for 5 minutes. **B,** Untransfected cells are serum starved and primed with conditioned media from transfected cells. For western blot analysis lysates are collected from wild-type (201T) and mutant EGFR T790M (H1975) NSCLC cell lines.
Targeted Kinase Inhibition in NSCLC Produces Anti-Proliferative an Effect Independent of EGFR Mutations

The advent of molecular profiling continues to dictate how we design and test novel therapeutic regimens in preclinical models. Overexpression of a target gene or activating mutations can help determine whether a patient with a known genetic profile might respond to targeted therapy. More than 80% of NSCLC patients without the known genetic alterations namely EGFR mutations, cannot benefit from targeted therapy and are left to be treated with the less efficacious
and toxic approach such as chemotherapy [145]. *K-RAS* mutations in NSCLC are also detectable in approximately 30% of the patient population but *K-RAS* mutations are “undruggable” [163]. Lack or presence of these prognostic markers in tumors, suggest that there is a need to develop a therapeutic intervention independent of detectable mutations. The lack of mutations in STAT3 in NSCLC based on notable evidence, suggest STAT3 is a prime target for intervention and functions as a common denominator in tumors harboring EGFR or *K-RAS* mutations.

Altogether we have generated evidence that STAT3 inhibition by a STAT3 “decoy” (CS3D) approach is effective independent of EGFR or *K-RAS* mutation status in NSCLC. We have however demonstrated that STAT3 blockade leads to activation of “bypass” signaling mechanisms. We are therefore suggesting that HERs and FGFR could be directly activated via the IL6 receptor complex in addition to secretion of corresponding ligands (as depicted in Figure 28). This therefore provides an opportunity to test CS3D in combination with inhibitors aimed at kinases activated as a result of STAT3 inhibition.

Based on western blot analysis, IL-6 induces the activation of receptor tyrosine kinases such as HER family of receptors, FGFR, and c-Met (Figure 27). These results led to a drug screen with the goal of identifying potent kinase inhibitors in NSCLC cell lines that are efficacious independent of mutation status. Other inhibitors included in these studies were selected based on the cytokine profile analysis performed on the tumor-derived xenografts. Cell viability was assessed in response to drugs aimed at corresponding targets as listed in the
material and method section following the MTS assays. One notable observation is the response rate to the FGFR inhibitor (LY2874455) with maximum effect in diverse NSCLC cell lines independent of their EGFR mutation status (Figure 29). For the drug screen, we used different inhibitors aimed at various target identified in the table below. Afatinib is a drug designed to target mutation in EGFR such as T790M compared to dacomitinib that binds to cysteine residues in the catalytic domains of the HER receptors. LY2874455 is a pan-inhibitor of FGFRs.

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**Figure 29. Effects of tyrosine kinase inhibitors on cell viability.** NSCLC cell lines (H3255, H1975, 201T, and HCC827) were treated with increasing concentrations of TKIs ranging from 0 nmol/L to 5000 nmol/L. Using MTS assays, cell viabilities was assessed 48 hours later. Three independent experiments were performed, using 96-well plates and 8 wells/concentration.
Dual Inhibition of STAT3 and FGFR Produces Synergistic Effects in Wild-type and Mutant EGFR NSCLC

STAT3 has been implicated in two distinct signaling pathways, one by binding to the c-Myc promoter region, a critical regulator of cell cycle progression from the G1 to the S phase. Secondly, STAT3 mediates anti-apoptotic signals by regulating the expression of genes such as Pim and Bcl-XL critical for survival [177]. On the other hand, aberrant FGFR signaling drives tumor cell proliferation and angiogenesis. FGFRs are also associated with an increase in chromosomal instability via mislocalization of FGFR fusion proteins to the mitotic spindles [178]. FGFR1 amplification and FGFR3-TACC3 fusion are known to occur in patients with NSCLC largely contributing to chromosomal instability mediated by the FGFR family of proteins [179]. The various functions of FGFR suggest NSCLC cells are highly dependent on FGFRs and identifies FGFRs as unique targets for therapeutic purposes.

The distinct functions of STAT3 and FGFR at different phases of the cell cycle might independently contribute to NSCLC cell survival. Altogether, our data strongly suggest that combining the STAT3 “decoy” (CS3D) plus the FGFR inhibitor (LY2874455) should produce an enhanced effect. To determine the effect of CS3D plus LY2874455 on cell viability, we transfected .3µM of CS3D, and increasing concentration of LY2874455 and after 48 hours, MTS assays were used to assess cell viability. Data were normalized to cells treated with lipofectamine alone plus ethanol. .3µM concentration was determined to be the
IC50 of CS3D compared to an inactive mutant control (CS3M) with no cytotoxicity (Figure 9A).

Using the Chou-Talalay Method [180], we determined synergy by assessing combination index (CI) values. CI<1 implies synergy, CI=1 produces an additive effect, and CI>1 is antagonistic [180]. The combination of CS3D plus LY2874455 was determined to be synergistic in H3255, H1975, and 201T (Figure 30). These in vitro results suggest that CS3D or LY2874455 alone, respectively might target independent events required for cell cycle progression.

![Figure 30. Dual effects of STAT3 and FGFR inhibition.](image)

**Figure 30. Dual effects of STAT3 and FGFR inhibition.** Combination index (CI) for treatment in H3255, H1975 and 201T cells, respectively. The CI was determined using the Compusyn software to evaluate the interaction of CS3D and LY2874455. A solid horizontal line marks CI=1. The CI offers definition for additive (CI=1), synergism (CI<1), and antagonism (CI>1) in drug combination. The data are the mean values from three independent experiments.
9.3 CONCLUSION AND DISCUSSION

Despite the robust antitumor effect associated with CS3D due to STAT3 targeted inhibition, we understand that NSCLC is a heterogeneous and complex disease that can be challenging to treat. Therefore depriving NSCLC cells of STAT3 transcriptional functions is critical for their fate and as a result, prompts cancer cells to depend on alternate signaling mechanisms for survival. In these studies, we focused on identifying the feedback loops primarily activated as a result of STAT3 inhibition. Oncogene addiction in NSCLC is known to be associated with activating mutations such as EGFR mutations that might sustain constitutive signaling. To identify alternate “bypass” signaling mechanisms, we utilized a wild-type EGFR cell line (201T) with minimal constitutive signaling.

We show that secondary pathways are activated in residual tumors derived from 201T xenografts post-CS3D treatment. These findings suggest that the second wave of signaling cascade initiated due to STAT3 inhibition might promote a drug-resistant phenotype. We identified growth factors (AREG, and EGF) and cytokines (IL-6) to be upregulate in response to CS3D. These observations were substantiated by activation of corresponding signaling cascade via receptor phosphorylation. From tumor xenograft studies to in vitro analysis, we demonstrate that IL-6 and AREG are secreted from cancer cells transfected with CS3D. Wild-type EGFR cell lines such as 201T without certain beneficial EGFR or K-RAS mutations might rely on growth factors and cytokines to sustain cell signaling. In this work, we show that 201T cells stimulated with
recombinant IL-6 mediates trans-phosphorylation of receptors such as EGFR, HER3, c-Met, and FGFR.

Further experiments confirmed that conditioned media from transfected cells containing IL-6 also trans-phosphorylate tyrosine kinase receptors. Altogether, these data suggest that CS3D elicit an IL-6 autocrine mediated loop that leads to activation of receptors (such as EGFR, HER3, c-Met, and FGFR) which are critical for NSCLC progression. In addition to IL-6, growth factors such as AREG and EGF also secreted in response to CS3D might enhance the phosphorylation of their corresponding kinase receptors which includes the HER family of proteins.

With knowledge of these autocrine loops, we screened compounds to target the various receptors activated via IL-6-mediated phosphorylation. Dose response experiments revealed that FGFR inhibition (using LY2874455) produced the most pronounced effects in all the NSCLC cell lines. Because STAT3 or FGFR functions at different stages of the cell cycle, the combined effect of CS3D plus LY2874455 produced a combination index (CI) value <1 in all the NSCLC cell lines demonstrating synergy. We have also continuously evaluated the STAT3 “decoy” (CS3D) in combination with an EGFR and HER2 inhibitor, Afatinib, and early preliminary results suggest an additive effect that is independent of EGFR mutation status. In summary, this work has culminated to identifying a secondary pathway that could be targeted in combination with CS3D. Future experiments will therefore entail assessing the therapeutic potential of these dual therapies in in vivo models.
10. DISCUSSION

STAT3 is a critical mediator of tumor progression in NSCLC. As a transcription factor that regulates the expression of multiple target genes, STAT3 relays signals through multiple tyrosine kinases, including EGFR, IL6R, and SRC. The key role of STAT3 in mediating proliferation and suppressing apoptosis in NSCLC makes it a prime target for therapeutic intervention [156]. Prior STAT3 inhibitors have failed in clinical development due to factors including nonspecificity, low potency, and minimal cell membrane penetrance, limiting their efficacy [128, 159]. We examined a novel therapeutic that targets STAT3, a second-generation cyclic, double-stranded decoy oligonucleotide that exhibits a long half-life, high potency, and limited toxicity [133]. The oligonucleotide decoy was previously found to specifically and competitively bind activated STAT3 protein via its similarity to the STAT3 response element located in the promoter region of the c-fos gene [141], and to reduce expression of STAT3 target genes in head and neck cancer models [133, 134, 141].

Here, we demonstrate that the STAT3 decoy produces a robust antitumor effect in NSCLC models known to be resistant to FDA-approved EGFRi therapies. We found that the decoy decreased the viability of NSCLC cells and induced apoptosis, and sensitivity was independent of EGFR mutation status. These effects were specific compared with a mutant version of the double-stranded decoy that is unable to recognize STAT3 protein [133, 134, 141], and were demonstrable after a single transfection. Normal bronchial epithelial cells transfected with CS3D did not show any toxicity at the IC50 determined to be
toxic in NSCLC cell lines indicating that CS3D only exhibit anti-proliferative properties in cancer cell lines characterized by a constitutively active pSTAT3 dimers. In vivo studies illustrated that daily i.v. injection of the active decoy had strong antitumor effects in mouse NSCLC xenografts, accompanied by inhibition of c-Myc protein expression and destruction of the cellular integrity of tumor cells within the xenografts. When tumors were analyzed after only 5 daily treatments, nuclear p-STAT3 protein was greatly reduced and the proliferative state of tumor cells was greatly decreased. Because the phosphorylation step that activates STAT3 is not affected by STAT3 decoy [141], this result suggests one effect of the decoy is to reduce accumulation of p-STAT3 in the nucleus. In cell culture, we observed a decrease in the nuclear pool of STAT3. The cytoplasmic pool of p-STAT3 was reduced as well, with an increase in ubiquitination of p-STAT3 protein following CS3D treatment, which could cause increased degradation of STAT3 dimers when bound to the active decoy. A CS3D–pSTAT3 dimer complex may trigger a ubiquitination process that reduces the amount of active STAT3 available for signaling. Further analysis to gain mechanistic insight into how and why pSTAT3 is degraded should inform us on the CS3D effectively causes ubiquitination and facilitate the development of oligonucleotide molecules with similar properties.

STAT3 is activated by modifications through phosphorylation at tyrosine and serine residues that allow dimerization [57]. Despite alternative splicing generating STAT3α and STAT3β isoforms in NSCLC, both isoforms retain their DNA binding domain [43]. This implies that in a homodimer or heterodimer
complex of either isoform would effectively bind to CS3D. Independent of phosphorylation state, STAT3 nuclear localization is dependent on the coiled-coil domain region interacting with a specific import carrier importin-α3 [181]. We speculate that CS3D unlike other STAT3 inhibitors that are mostly designed to target the SH2 domain, should impair STAT3 nuclear localization and that subsequently accompanied by STAT3 degradation. We also predict that CS3D may also disrupt the ability of STAT3 to localize to the mitochondria. The mode of activation and kinetics of STAT3 therefore limits small-molecule inhibitors of STAT3 designed to only target the phosphorylation SH2 domain site.

By targeting the DNA binding domain of STAT3, CS3D disrupts the function of STAT3 dimers (phosphorylated or unphosphorylated), without requiring the interruption of phosphorylation. Potentially, this strategy could be more effective, because it focuses on the active dimeric moieties. We showed that CS3D downregulates the expression of the STAT3 target gene c-Myc in NSCLC cell lines in culture and in residual tumors after systemic administration. The transcriptional activity of STAT3 overlaps with other transcription factors such as STAT1 and NF-κB, but genes controlled predominantly by STAT1 or NF-κB such as IRF7 or IL8 respectively, were unaffected by CS3D. Although c-Myc is a direct STAT3 target gene, it is possible that indirect effects on c-Myc mRNA or protein expression could also be occurring in response to the STAT3 decoy, which we were unable to detect. The decoy effects were also specific because the mutant control oligonucleotide was unable to induce antitumor effects. The cellular uptake of fluorescently labeled CS3D and CS3M also did not differ,
supporting the specificity of the decoy that is designed to bind with selectivity to dimeric STAT3 protein.

Imaging analysis showed that the localization of CS3D mostly stained positively in the cytoplasm, although some was present in the nucleus, suggesting the main effect is to bind to pSTAT3 dimers in the cytoplasm. This also suggest there is a possibility that molecular cargo such as CS3D localization to the nucleus could be mediated by endosomal trafficking. CS3D mechanism of action also suggest that it might traffic to the nuclear compartment in a complex with pSTAT3 dimers impairing its function or directly packaged into endosomes and released in the nuclear compartment. The development of oligonucleotide-based therapies have traditionally been limited because of their poor stability and cleared rapidly [139]. It was thus imperative that we evaluate the stability of CS3D and we demonstrated that the fluorescein-labelled CS3D is detectable at various timepoints after a single tail-vein injection including 1hr, 24hr and further accumulates after 48hours. The uptake of CS3D by NSCLC cell lines after a single transfection and its detection in a time-dependent manner illustrates that CS3D confers a great amount of translational potential.

It has been reported that the constitutively active mutant form of STAT3, STAT3-C, which is dimerized by cysteine-cysteine residues, mediates epithelial cell transformation by promoting anchorage-independent growth [59], suggesting that this phenotype is especially dependent on STAT3 signaling. We found that after a single transfection, CS3D was very effective in suppressing anchorage-
independent growth. The inhibition of c-Myc expression in cultured cells and in xenografts also was a strong indicator of CS3D activity, suggesting that the ability to suppress this STAT3 target gene may be critical for its antitumor mechanism in NSCLC.

As a transcription factor, STAT3 has been generally considered an “undruggable” target in NSCLC amongst many other malignancies. The antitumor effects of CS3D were comparable in both EGFR wild-type and mutant NSCLC. These findings have huge implications for the potential of the STAT3 “decoy” to be utilized as a therapeutic for patients who develop resistance to EGFR therapy and those without the EGFR sensitizing mutations. A proportion of NSCLC patients without those EGFR sensitizing mutations who have been exposed to tobacco carcinogens harbor K-RAS mutations. K-RAS mutations often associated with smoking and to date, K-RAS is considered to be an “undruggable” target [163]. K-RAS mutations amongst many others, are mostly detectable in patients with a smoking history. Genetic alterations such as K-RAS mutations eventually leads to tumor formation but key components such as STAT3 can also be activated via carcinogen-induced mechanisms [164]. Prior to tumor formation, premalignant events are mostly identified as preneoplasia characterized by STAT3 expression.

STAT3’s role during tumor initiation and progression has been well documented and evidence suggests STAT3 can also be activated as a result of carcinogen exposure found in tobacco smoke [164, 165]. Exposure to tobacco
carcinogens such as NNK leads to mutations in genes like K-RAS in premalignant lesions, which progress to adenocarcinoma [161, 162]. Early events in carcinogenesis are also characterized by inflammation. Central to these processes, STAT3 activation has pleiotropic pro-tumor effects on proliferation, angiogenesis, and immune evasion [166]. Using an NNK-induced lung cancer model, we demonstrated that CS3D delays preneoplasia lesion formation and impairs lung cancer growth and development. These findings provide an opportunity for an intervention that might transcend the therapeutic constraints associated with the challenges clinicians face in designing therapies for patients harboring K-RAS mutations. Evidence from another group has also demonstrated that neutralizing IL-6, a strong inducer of STAT3, can also impair lung cancer development [85] supporting the usefulness of pharmacologically targeting STAT3 in the context of K-RAS mutations.

In stark contrast to our findings and the results generated by Caetano and colleagues [85], another group have showed that STAT3 ablation instead contributed to an aggressive lung cancer phenotype indicating a “tumor suppressive” function of STAT3. It is important to point out that this was a “genetic” approach compared to a pharmacology strategy that we utilized to block STAT3 activity, which does not result in a complete knockdown. Therefore pharmacological intervention using CS3D demonstrates that there is a therapeutic niche for STAT3 targeted therapy in lung cancer patients harboring K-RAS, challenging the idea that those patients will not benefit from such therapy [84]. NNK exposure in mouse models is known to induce G12D K-RAS
mutations, and after assessing individual residual tumors in response to CS3D, we find that there is no significant difference between CS3D compared to mice treated with the mutant control construct (CS3M) in terms of the percentage of tumors for G12D K-RAS mutations. These results suggest that STAT3 blockade by CS3D does not enhance K-RAS oncogenic activation. Our data also suggest that STAT3 inhibition by CS3D shows that STAT3 is required for survival rather than creating tumor suppressive TME in K-RAS-induced lung cancer.

In line with STAT3 tumor suppressive function, STAT3 sequesters NF-κB and impairs NF-κB-mediated signaling that primarily contributes to tumor angiogenesis and cancer progression via stromal cell infiltration [84]. Based on this evidence, CS3D mechanism of action is unique because it is designed to only disrupt the p-STAT3 dimers and not the p-STAT3-NF-κB complexes through genetically ablation of STAT3 thus promoting NF-κB protumorigenic functions. In addition to STAT3 degradation, CS3D significantly suppressed NF-κB activation, disrupting downstream signaling. CS3D is also determined to suppress VEGF expression creating a TME less angiogenic and subsequently a less pro-tumorigenic. However, we noted an increase in IL-6 and COX-2 expression as a result of STAT3 blockade as compensatory signals primarily utilized by cancer cells as rescuing mechanisms. These markers (IL-6, COX-2, primarily activated by PGE$_2$) could also be used as biomarkers of CS3D response. Future studies assessing the amount of IL-6 or PGE2 detectable in serum could be used to predict responsiveness to CS3D treatment. Evidence from our laboratory have also shown that NSAIDs such as Aspirin designed to target COX-2 also exhibit
preventive effects in NSCLC [182]. Therefore combined targeting of STAT3 and COX-2 or IL-6 could show enhanced antitumor effects.

In addition to maintaining tumor cell proliferation, STAT3 is also known to promote an immunosuppressive phenotype by promoting M2 macrophage and myeloid-derived suppressor cells (MDSCs) activity while suppressing M1 macrophage function [168]. In a series of experiments, we demonstrate that targeting STAT3 orchestrate an immune permissive TME by increasing the proportion of M1 macrophages present in the lung TME while reducing the number of M2 macrophages and MDSCs infiltrating the lung TME. Altogether this data suggest CS3D tilts the balance to favor an antitumor immune response. The chemopreventive properties of CS3D observed in the K-RAS-driven model might be enhanced by a “bystander” effect mainly associated with the shift from an immunosuppressive phenotype towards an antitumor immune response. Given that immunotherapy is approved as first line therapy for NSCLC and CS3D exhibit immunomodulatory function, screening CS3D in combination with an immune checkpoint neutralizer might provide an insight into that interaction within the immunogenic milieu within the TME.

Despite the remarkable effects observed that clearly demonstrates that CS3D exhibit chemopreventive properties and can impair lung can growth and development by attenuating STAT3 signaling, we also identified secondary signaling mechanisms activated as a result of STAT3 inhibition. Residual tumor analysis from the xenograft studies and NNK-induced lung cancer model also revealed that IL-6 overexpression is detectable post-CS3D treatment. This
findings however creates the possibility that IL-6 is either secreted from cancer cells or stromal cells and regardless of the cell type, IL-6 is involved in autocrine and paracrine functions in the TME. We demonstrate that targeting STAT3 leads to IL-6 transcriptional upregulation by an unknown molecule in NSCLC cell lines.

IL-6 is a key upstream activator of STAT3 therefore substantial increase in IL-6 levels as a result STAT3 blockade raises the possibility that cancer cells might depend on IL-6 signaling kinetics for survival via mechanisms independent of STAT3 activity. As referenced previously, prolonged activation of EGFR is in part mediated by the IL-6 receptor complex in response to IL-6 stimulation [171, 172]. We therefore predicted that cancer cells deprived of STAT3 critical transcriptional activity might revert to the IL-6 axis point to initiate survival mechanisms which might contribute to drug resistance. We hypothesize that other receptors such as FGFR, c-MET, EGFR, and other HER family of receptors will be phosphorylated following IL-6 addition and this cross-talk was confirmed within 5 minutes of stimulation. We further confirmed an autocrine loop involving the IL-6 receptor complex. We collected conditioned-media containing IL-6 from transfected cells, primed serum-starved and “untransfected” cells and confirmed the activation of FGFR, c-MET and the HER family of receptors at the 5 minute timepoint suggesting that a potential cross-talk activation might exist.

Parallel with these observations, we also determined that STAT3 inhibition also causes an increase in EGFR and FGFR ligands such as AREG, EGF, and FGF2 highlighting the possibility of a cross-talk amongst receptors at the membrane level. Co-immunoprecipitation assays will be needed to determine
whether receptor complexes composed of homodimers or heterodimers are initiated as a result of growth factor or cytokine secretion. Immunofluorescent analysis will further confirm complex formation initiated in response to STAT3 blockade. In addition to receptor activation, the autocrine secretion of growth factor and cytokines in response to STAT3 targeted inhibition, shows that we have identified compensatory mechanisms that are associated with the STAT3 “decoy”. Using a drug screen, we showed that targeted inhibition of the receptor kinases (FGFR, c-MET, and HERs) decreases cell viability but the FGFR inhibitor (LY2874455) produced the most pronounced effect on across different NSCLC cell lines. FGFR is an integral component in lung cancer growth and development and the potential cross-talk activation of FGFR via IL-6, a canonical STAT3 activator, suggest that LY2874455 might enhance the effects of CS3D. We discovered that when we combined LY2874455 and CS3D, we often observed synergy in terms of their anti-proliferative effects compared to single treatment alone.

Based on this body of work, we have to perform more experiments to fully determine the impact of these cross-talk mechanisms and determine which complexes are formed as a result of STAT3 inhibition. We predict that evaluating the combined effect of CS3D and various compounds either targeting FGFR or EGFR in animal models should further inform us on whether this approach is a viable therapeutic regimen. Further studies to inform us on why synergy might exist and how this intervention impedes the activity of key players whose functions are independently regulated by either FGFR, EGFR or STAT3 during
cell cycle progression. Because CS3D only caused a static effect in terms of
tumor growth development, it would be intriguing to determine whether targeting
FGFR or EGFR in combination with CS3D can lead to tumor regression. Further
studies should also evaluate the impact of CS3D alone or in combination with
other agents on tumor metastasis. Once we confirm that CS3D alone or in
combination with other agents like the FGFR inhibitor (LY2874455) can cause
tumor regression or impede metastasis, it would be imperative to do a robust
assessment on the long-term survival of animals bearing lung tumors. Confirming
the therapeutic value of these different combination regimens primarily designed
to enhance CS3D antitumor properties could be useful in the future for NSCLC
treatment.

However, our findings to date support the ability of a systemically
administered, double-stranded, cyclic oligonucleotide decoy to attenuate STAT3
signaling in a lung cancer model, leading to tumor growth arrest in EGFRi
resistant and K-RAS mutant models known to evade therapy. The effects in
NSCLC support the idea that a circularized double-stranded oligonucleotide
targeting STAT3 as single therapy has promise as a viable therapeutic agent.
The STAT3 “decoy” (CS3D) showed no sign of toxicity in immunosuppressed and
immunocompetent animals and efforts are underway to complete the IND-
directed pharmacology and toxicology studies needed to initiate a phase I clinical
trial of CS3D.
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