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THE *IN VITRO* AND *IN VIVO* EFFECTS OF TELOMERASE SUBSTRATE 6-THIO-2'-DEOXYGUANOSINE

Ilgen MENDER

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PhD THESIS

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ABSTRACT

Mender, I. The *In vitro* and *In vivo* Effects of Telomerase Substrate 6-Thio-2'-Deoxyguanosine. Hacettepe University Institute of Health Sciences, Ph.D. Thesis in Biochemistry, Ankara, 2014. Telomerase mediated telomere targeted therapy represents a new approach in cancer therapy. In this study, I report that the nucleoside analogue 6-thio-2'-deoxyguanosine (6-thio-dG) is recognized by the telomerase holoenzyme and incorporated into *de novo* synthesized telomeres to alter the structure and function of telomeres. This results in structurally and functionally modified telomeres, loss of telomeric end protective complexes, leading to telomere dysfunction. Additionally, 6-thio-dG causes progressive telomere shortening, which is independent from inhibition of telomerase activity *in vitro*. 6-thio-dG induced telomere dysfunction is observed in hTERT expressing normal human BJ fibroblast cells and cancer cells, but not in telomerase-negative BJ cells. Moreover, one week treatment with 6-thio-dG results in 80-90% cell death for the majority of the cancer cells (H2882, HCC2429, HCC827, HCC15, H2087, HCC4017, HCC515, H2009, H2073), whereas normal BJ fibroblast and human colonic epithelial (HCEC1) cells were largely unaffected. In A549 lung cancer cell based xenograft model studies, intraperitoneally 6-thio-dG treatment (2 mg/kg) caused dramatic tumor reduction as well as telomere dysfunction, superior to that observed for 6-thioguanine (2 mg/kg) treatment. These results indicate that 6-thio-dG may provide a new telomere-addressed telomerase-dependent anti-cancer approach, targeting both genomic and telomeric DNA. It was observed that some of the cancer cell lines (H1819, H1993, H1693) were resistant to 6-thio-dG compared with the sensitive cell lines. The methylation analysis showed that several genes were highly hypermethylated in resistant cell lines. In addition, in gene expression data, there were 3 different genes (FSCN1, TLE2, ALDH1A2) that were differentially expressed between resistant and sensitive cell lines. The results of this study will help in the future directions focusing on 6-thio-dG resistance.

Key Words: Telomerase, 6-thio-2'-deoxyguanosine, telomere dysfunction.

ÖZET

Mender, I. Telomeraz Substratı 6-Tiyo-2'-Deoksiguanozin'in *in vitro* ve *in vivo* etkileri. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Biyokimya Programı Doktora Tezi, Ankara, 2014. Telomeraz aracılığı ile telomerleri hedefleyen terapiler, kanserde yeni bir tedavi yaklaşımı sunmaktadır. Bu çalışmada, nükleozid analogu olan 6-tiyo-2'-deoksiguanozinin (6-tiyo-dG) telomeraz enzimi tarafından tanınıp, yeni sentezlenen telomerik yapılara eklenerek telomerlerde yapısal ve fonksiyonel bozukluğa yol açması incelenmiştir. Bu durum, telomerlerin yapısal ve fonksiyonel modifikasyonuna, telomerik uçlardaki koruyucu proteinlerin kaybına ve sonuç olarak telomerik disfonksiyona yol açmaktadır. *In vitro* şartlarda 6-tiyo-dG, telomeraz inhibisyonundan bağımsız olarak progresif telomerik kısalığa neden olmaktadır. 6-tiyo-dG, bu etkilerini telomeraz eksprese eden BJhTERT ve kanser hücreleri üzerinde gösterirken, telomeraz negatif normal BJ fibroblast hücrelerinde göstermemektedir. Akciğer kanseri hücre hatları (H2882, HCC2429, HCC827, HCC15, H2087, HCC4017, HCC515, H2009, H2073) 6-tiyo-dG ile 1 hafta inkübe edildiğinde %80-90 oranında hücre ölümü gözlenmiş, ancak normal BJ fibroblast ve kolon epitel hücrelerinin (HCEC1) etkilenmediği saptanmıştır. A549 akciğer kanseri hücrelerinin enjekte edilmesiyle oluşturulan ksenograft modellerde intraperitoneal 6-tiyo-dG uygulaması (2 mg/kg), 6-tiyoguanine (2 mg/kg) göre belirgin telomer fonksiyon bozukluğu ile birlikte etkin bir tümör küçülmesi sağlamıştır. Bu sonuçlar, 6-tiyo-dG'nin kanser tedavisinde telomerlere yönelik, telomeraz aracılı, hem genomik hem telomerik DNA'yı hedefleyen yeni bir yaklaşım olabileceğini göstermektedir. Çalışmada kullanılan bazı kanser hücre hatlarının (H1819, H1993, H1693) 6-tiyo-dG'ye dirençli olduğu saptanmıştır. Yapılan metilasyon analizinde, 6-tiyo-dG'ye direnç gösteren hücrelerde bazı genlerin yüksek seviyede metile olduğu gözlenmiştir. Bu hücrelerin gen ekspresyon profilleri karşılaştırıldığında, tedaviye dirençli ve duyarlı olan kanser hücrelerinde 3 gende (FSCN1, TLE2, ALDH1A2) farklılık olduğu tespit edilmiştir. Bu çalışmadan elde edilen veriler, hücrelerin 6-tiyo-dG'ye direnç mekanizmalarının incelenmesine yönelik yeni araştırmalarda yol gösterici olacaktır.

Anahtar Kelimeler: Telomeraz, 6-tiyo-2'-deoksiguanozin, telomer disfonksiyonu.

TABLE OF CONTENTS

ABBREVIATIONS

Ab	Antibody
ABC	ATP binding cassette
ABCB1	ATP binding cassette, sub-family B (MDR/TAP), member 1
ABCC1	ATP binding cassette, sub-family C (CFTR/MRP), member 1
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
ALDH1A2	Aldehyde dehydrogenase 1 family, member A2
ALT	Alternative lengthening of telomeres
ALT	Alanine aminotransferase
Alt-NHEJ	Alternative form of NHEJ
ASCL1	Achaete-scute homolog 1
AST	Aspartate aminotransferase
ATM	Ataxia telangiectasia mutated kinase
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related
BCRP	Breast cancer resistance protein (ABCG2)
BIBR1532	2-[(E)-3-naphthalene-2-yl-but-2-enoylamino]-benzoic acid
BRCA1/2	Breast cancer 1/2
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
CBC	Complete blood count

CDC25A	Cell division cycle 25A
Cdk1/2	Cyclin dependent kinase 1/2
Cdk4/6	Cyclin dependent kinase 4/6
cDNA	Complementary DNA
Chk1/2	Checkpoint kinase 1/2
CO_2	Carbon dioxide
CSC	Cancer stem cell
CtIP	Mammalian ortholog of ctp1
DAPI	4’, 6-diamidino-2-phenylindole
DLK1	Delta like homolog
D-loop	Displacement loop
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DSB	Double strand break
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and drug administration
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FSCN1	Fascin actin-bundling protein 1

GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal
GMPS	Guanine monophosphate synthetase
HASH1	Human achaete-scute homologue
HAT	Histone acetyltransferase
HCEC	Human colonic epithelial cell
HCT	Hematocrit
HDAC	Histone deacetylases
HGB	Hemoglobin
HPRT1	Hypoxanthine guanine phosphoribosyl transferase 1
HR	Homologous recombination
hTR/hTERC	Telomerase RNA template component
H2AX	Variant of the H2A protein family
H&E	Hematoxylin and eosin
h or hr	hour
IC50	Half maximal inhibitory concentration
IMPDH	Inosine monophosphate dehydrogenase
IP	Intraperitoneal
ITAS	Internal telomerase assay standard
kb	Kilobase

KCl	Potassium chloride
kg	Kilogram
MDC1	Mediator of DNA-damage checkpoint 1
MDR1	Multidrug resistance gene
meTIMP	S-methyl-thioinosine 5’-monophosphate
meTGMP	S-methyl-thioguanosine 5’-monophosphate
mg	Miligram
$MgCl_2$	Magnesium chloride
min	minute
miRNA	MicroRNA
MM	Milimeter
mm^3	a cubic milimetre
MRE11	Meiotic recombination 11 homolog A
MRN	MRE11-RAD50-NBS1
MRP1	Multidrug resistance protein 1
µg	Microgram
µl	Microliter
µM	Micromolar
Na-citrate	Sodium citrate
NaCl	Sodium chloride
Na_2HPO_4	Disodium phosphate

$Na_2H_2P_2O_7$	Disodium pyrophosphate
NaOH	Sodium hydroxide
NBS1	Nibrin
NHEJ	Non-homologous end joining
NP-40	Nonidet-P40
ns	Non significant
NSCLC	Non small cell lung cancer
Nu	nude
O_2	Oxygen
PARP1	Poly (ADP-ribose) polymerase 1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween 20
PCR	Polymerase chain reaction
PDX	Patient derived xenograft
PFS	Prolonged progression free survival
P-gp	P-glycoprotein
PHA	Phytohemagglutinin
PIT1	Pituitary transcription factor 1
PLT	Platelet
PNA	Peptide nucleic acid

POT1	Protection of telomeres 1
pRB	Retinoblastoma protein
pre-mRNA	Precursor mRNA
qPCR	Quantitative PCR
RAD50	DNA repair protein RAD50
Rap1	the human ortholog of the yeast repressor/activator protein 1
RBC	Red blood count
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication protein A
SA-βGal	Senescence associated betagalactosidase
SCLC	Small cell lung cancer
SCr	Serum creatinin
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	second
SP	Side population
SSB	Single strand break
SSC	Saline-sodium citrate
Telomerase	Telomere terminal transferase
TERT	Telomerase reverse transcriptase

TGMP	Thioguanosine monophosphate
TIF	Telomere dysfunction induced foci
TIMP	Thioinosine monophosphate
TIN2	TRF2 and TRF1 interacting nuclear protein 2
TLE2	Transducin-like enhancer of split 2
T-loop	Telomere loop
TPMT	Thiopurine S-methyltransferase
TPP1	Adrenocortical dysplasia homolog
TRAP	Telomere repeat amplification protocol
TRF	Terminal restriction fragment
TRF1/2	Telomeric repeat binding factor 1/2
Tric-HCl	Tris hydrochloride
V	Volt
VOD	Veno-occlusive disease
WBC	White blood count
WHO	World health organization
Wnt	Wingless
XLF	XRCC4-like factor
XO	Xanthine oxidase
XRCC1/4	X-ray repair cross complementing protein 1/4

5-aza	5-aza-2’-deoxycytidine
6-MP	6-mercaptopurine
6-TG	6-thioguanine
6-thio-dG	6-thio-2’-deoxyguanosine
6-thio-dGTP	6-thio-2’-deoxyguanosine triphosphate
6-thio-GTP	6-thio-guanosine triphosphate
53BP1	p53 binding protein 1

FIGURES

TABLES

1. INTRODUCTION

Cancer is one of the leading causes of disease in the world. According to World Health Organization (WHO), 8.2 million people died due to lung, stomach, liver, colorectal and female breast cancers in 2012 (1).

In both developed and developing countries, colorectal and lung cancers are the most common cancers, 136.830 and 224.210 people in the US will be diagnosed with colorectal and lung cancer in 2014, respectively (2). Lung cancer is divided into two different histological categories, which are small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC). SCLC presents ~25% of all bronchogenic malignancies and shows highly aggresive clinical outcomes. Metastasis are generally found in SCLC patients and combination therapy is usually chosen for treatment. While less than 5% SCLC patients survive 5 years past the initial diagnosis, 15% NSCLC patients have 5 year survival rate. However, NSCLC is less sensitive to chemotherapy than SCLC. Surgical resection is the best treatment choice for NSCLC patients (3). Therefore, prevention and early detection in addition to finding new therapeutic strategies are vitally important to prevent the high incidence and mortality risk of cancer.

Since the side effects with standard chemotherapy are highly challenging for cancer patients, targeted therapy with fewer side effects is a major focus for cancer research due to less damage to normal cells. Therefore, indefinite cell proliferation, one of the hallmarks of cancer, via activation of telomerase is a highly attractive target for cancer therapy because telomerase activity is detected in ~90% of primary human cancers (Figure 1.1), but not normal cells except proliferating progenitor and transit amplifying cells (e.g., male germline spermatocytes, activated lymphocytes, some proliferating gastrointestinal cells and skin epidermal) and embryonic stem cells (4, 5).

Although telomerase is a unique and universal target, there have been no US Food and Drug Administration (FDA) approved telomerase targeted drugs as even though this target has been studies for well over a decade. Therefore, it is important to develop new therapeutic strategies that target telomerase for anticancer therapy.

For this reason, we generated a new telomerase mediated telomere targeted agent, 6-thio-2'-deoxyguanosine (6-thio-dG), and tested its *in vitro* and *in vivo* effects on normal and cancer cells.

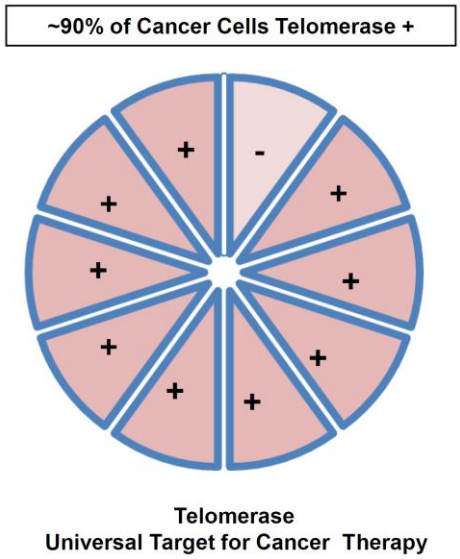


Figure 1.1. Telomerase as a universal target. While most normal cells do not have telomerase activity, ~90% of cancer cells can express telomerase. This difference between normal and cancer cells make telomerase unique and universal target for cancer therapy (Submitted to Cancer Medicine, book chapter, Mender et al.).

2. GENERAL KNOWLEDGE

2.1. Brief History on Telomere Biology

The appreciation to chromosomal ends started in the 1930's through two independent researchers. Herman Muller noted that there are unique structures at the ends of chromosomes. Muller named these ends telomeres, based on their positions on chromosomes (Telo:end, mere:part in Greek). Later, he generated various mutations by X-rays when he was working with fruit flies and realized that X-rays caused chromosome breakages and fusions (6). About the same time, Barbara McClintock observed dicentric chromosomes, chromosomes with two centromes, in maize. When dicentric chromosomes broke at mitosis, she noticed that these ends fused with any other broken ends. However, natural chromosomal ends were not involved in this process such as in embryonic cells (7).

In 1961, Leonard Hayflick discovered that cultured normal cells have limited capacity to divide, which is related to rounds of DNA replication, then they become senescent (also known as the Hayflick limit). Hayflick used phase I, II and III terminology for the growth phase of cultured cells. Phase I is the primary culture as cells from tissues adapt to the *in vitro* conditions. Phase II is the growth phase and phase III is the period when cell replication diminishes and finally stops. These observations showed that in contrary to cancer cells that have limitless capacity to divide in culture, normal cells had a limited dividing capacity (8, 9).

In the early 1970s, James Watson realized that there was a problem with replicating the ends of linear DNA because of the nature of lagging strand synthesis, not the leading strand. Since DNA polymerase can not completely replicate the 3' end of linear chromosomes, Watson called this the end replication problem (10). Olovnikov, a Russian theoretical scientist, proposed that chromosomes get shorter with each replication cycle and eventually run into essential genes, and speculated this end replication problems might be the reason for the replicative senescence, which was described by Hayflick (11, 12). When early molecular biology technologies became available, Elizabeth Blackburn was the first scientist to report tandem repeats of 6 mers (TTGGGG) at the end of chromosomes in *Tetrahymena*

thermophila (13). Human cells also consist of thousands of repeats, but this sequence is TTAGGG in mammals (14). The presence of telomeres (tandem repeats) at the ends of chromosomes was first speculated in 1938 by Hermann Muller (15) and Barbara McClintock (16). Once the sequence of telomeres was known, telomere length could be measured. Then, it was found that telomere lengths in different tissues were variable (17). These studies showed that as normal human fibroblasts divided in culture, telomeres got progressively shorter with each cell division (18).

If normal cells have telomere attrition and their growth rate is being limited as they are cultured, there had to be a different mechanism to counteract this attrition and cause unlimited growth for cancer cells. Greider and Blackburn discovered enzyme activity called telomerase (telomere terminal transferase) that elongates telomeres by adding six hexameric sequences (TTGGGG) to the ends of chromosomes in *Tetrahymena thermophila* (19). Greider also found that telomerase activity was sensitive to RNA and she co-purified RNA with telomerase and found that the RNA sequence contains 5'CAACCCCAA3' which was complementary to the Tetrahymena telomere repeat TTGGGG. She blocked this putative enzyme activity by oligonucleotides, showing that the RNA template is required for enzyme activity (20). It wasn't until the telomerase reverse transcriptase (TERT) was cloned in 1997 (21, 22), that it was shown that introduction of a single gene into telomerase silent human cells was sufficient to detect telomerase activity and this resulted in telomere elongation and greatly extended lifespan without undergoing senescence (23, 24).

In 1990s, Jerry Shay and Geron scientists showed that telomerase is present in all cancer-derived cell lines and ~90% of primary human cancers (25). Woodring Wright and Geron Scientists found that ectopic expression of telomerase in normal fibroblasts and epithelial cells bypassed the Hayflick limit, indicating that telomeres are the cellular replicometer (23). And that time, it was realized that normal cells do not have telomerase activity, while most cancer cells express telomerase activity, which is required to prevent telomere attrition and consequent replicative senescence, results in enabling proliferation and immortality of cells (25). While ~90% of cancer cells have telomerase activity, the other relatively small group of telomerase negative cancers use a non telomerase based recombination mechanism to extend their

telomere length, which is called **A**lternative **L**engthening of **T**elomeres (ALT). ALT mechanism involves intra-telomeric recombination, leading to telomere length maintenance in mostly rare cancers (26).

Following these major discoveries, pharmaceutical companies initiated large compound screen to identify compounds to inhibit telomerase in cancer cells (27).

2.2. DNA Damage Response to Endogenous and Exogenous Sources

When genomic DNA is exposed to genotoxic stresses, different types of DNA damage occurs such as base modifications, intrastrand crosslinks, interstrand crosslinks, DNA-protein crosslinks, single strand breaks (SSBs) and double strand breaks (DSBs). Double strand breaks are the most deleterious ones (28) and can be generated by reactive oxygen species produced by cellular metabolic processes and replication associated errors, which are derived from endogenous sources, as well as exogenous sources such as ionizing radiation and chemotherapeutic agents. If DSBs are not repaired, they can result in cell death. If they are accurately repaired, cells can survive with no adverse effects. If the repair occurs inaccurately, then surviving cells may gain genomic alterations that may contribute to tumor development (29). To maintain genomic integrity is important for the fate of cell. Therefore, cells that have a well coordinated network of so called DNA damage responses, can transmit signals upon damage to effector proteins leading to the induction of cellular responses to arrest the cell cycle, activate DNA repair pathways, and or cell death (28).

In response to DSBs, the MRE11-RAD50-NBS1 (MRN) complex binds to DSB sites and activates the ataxia telangiectasia mutated (ATM) kinase through autophosphorylation (30, 31). ATM along with DNA-PK phosphorylates the histone variant H2AX at serine 139, resulting in gamma-H2AX. H2AX is also phosphorylated by ATR protein kinase (ataxia telangiectasia and Rad3-related) during DNA replication associated DSB induction (32, 33). Gamma-H2AX modified chromatin is found at the DSB sites until the damage is repaired (34). There are other proteins that are retained at DSBs such as MDC1, 53BP1. The focal accumulation of gammaH2AX, 53BP1 and other factors are the markers for DSB responses (35).

DSB response involves a significant cell cycle arrest/delay checkpoints in terms of cellular proliferation, which are the early G1, the G1/S, the intra-S and the G2/M (36). Prevention of progression in G1/S phase is the most rapid cell cycle arrest and has two major pathways. These pathways start with phosphorylation of the downstream proteins by ATM (37). First, when Chk1 and Chk2 are phosphorylated, they then induce phosphorylation of the protein phosphatase CDC25A and subsequent degradation, leading to Cdk1/Cdk2 inhibition and cell cycle arrest. activation of p53 initiates transcription of the cyclin dependent kinase inhibitor p21 and triggers either cell death/apoptosis or cell cycle arrest/cellular senescence (38, 39). Physiological stresses (i.e., culture conditions) cause the upregulation of p16 (cyclin dependent kinase inhibitor), which binds specifically to CDK4 and CDK6 to inhibit the interaction with D-type cyclins, and then p16 causes pRB protein to remain in hypophosphorylated (inactive form), resulting in cell cycle arrest and eventually cellular senescence. pRB involves in heterochromatin formation, which induces cellular senescence (40).

When the DNA damage response mediator 53BP1 is activated, it contributes to DSB repair pathways via non-homologous end joining (NHEJ) (41). Double strand breaks are repaired by either NHEJ or HR (homologous recombination). NHEJ is an error-prone pathway and Ku70/Ku80 complex, DNA PK catalytic subunit (DNA-PKcs), the Artemis nuclease, XLF, XRCC4, and DNA ligase IV are involved in this pathway. In contrast, HR is an error-free repair pathway that requires non-damaged sister chromatid for recombination (42). MRN complex, CtIP, replication protein A (RPA), BRCA1, PALB2, BRCA2, and RAD51 are involved in HR. Alt-NHEJ is the alternative form of NHEJ and also involved in DSB repair that includes PARP1, XRCC1, DNAligase IIIα, polynucleotide kinase, and Flap endonuclease I (43).

2.3. Telomeres and Shelterin Complex

Telomeric DNA in eukaryotes (double stranded) are maintained by telomerase during early developments, in some proliferative stem like cells and in almost all cancer cells. Generally, the strand that constitutes the 3' end is rich in guanosine. Therefore, telomeric DNA has two strands which are called G- and

C- rich strands. Telomere length is variable in different organisms such as humans at birth (10-15 kb of the TTAGGG repeats) and mice (up to 100 kb) (44). Studies showed that even 400 bp of telomeric repeats are enough for telomeres to function properly (45-47), and in general telomere shortening to less than 1 kb by inhibiting telomerase is the needed length reduction to induce senescence in tumor cells (48). The 3' single G-stranded telomeres terminate with single stranded G-rich overhang and this overhang varies between 50-500 nt in mammalian cells. However, it is still not clear how this overhang is generated (49, 50). More recently it was shown that the single strand overhang, along with the terminal portion of the duplex TTAGGG repeats make a large duplex lariat structure that is called telomere loop (T-loop). It is believed that the single strand G-rich overhang strand invades back into the duplex telomere repeats to form a triplex structure resulting in the the overhang base pairs with displaced C-rich strand, which is called displacement loop (D-loop). These loops are the protective structures that hide telomere ends from being recognized by the DNA damage repair machinery (51).

Telomeric repeats at the end of chromosomes are associated with a 6 protein complex (TRF1, TRF2, Rap1, TIN2, TPP1, and POT1), which is called shelterin (52). They are abundant on telomeres throughout the cell cyle and they do not appear to function elsewhere in the nucleus (44). TRF1 and TRF2 (Telomeric Repeat Binding Factor 1 and 2) can bind to the duplex DNA and POT1 (Protection of Telomeres 1) can bind to the single strand DNA present at the 3' overhang. TRF1 and TRF2 can recruit with TIN2 (TRF2 and TRF1 Interacting Nuclear Protein 2), Rap1 (the human ortholog of the yeast Repressor/Activator Protein 1), TPP1 and POT1 (53, 54) (Figure 2.1).

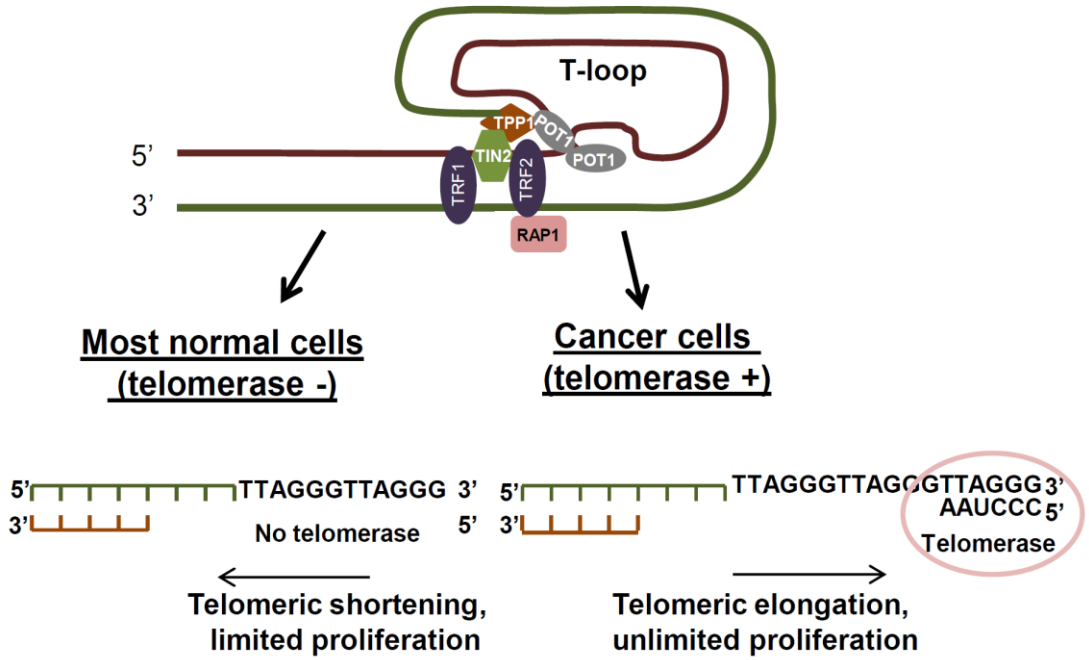


Figure 2.1. Diagram of hypothetical telomere demonstrating T-loop structure with shelterin proteins. Normal and cancer cells both have similar telomeric structure, but since normal cells generally do not express telomerase, telomere shorten progressively while telomerase expressing cancer cells can maintain their telomeres (Submitted to Cancer Medicine, book chapter, Mender et al.).

Telomere dysfunction that induces DNA damage response in mammals is associated with DNA damage response factors such as 53BP1, gammaH2AX, Rad17, ATM, and Mre11. DNA damage foci at uncapped telomeres is referred to as **T**elomere dysfunction-**I**nduced **F**oci (TIFs) (55). TIFs can be visualized by co-localization of telomeres with DNA damage response factors.

Telomere dysfunction has been implicated in aging and tumorigenesis (56-59). Telomere uncapping occurs when telomeres are criticially short in cells or telomere protective proteins are impaired (60). Under either process, telomeres become dysfunctional and activates DNA damage responses, then inducing cell cycle arrest, senescence or apoptosis and chromosome end fusions, which can be detected using immunofluorescence imaging techniques. There is no direct evidence to show if DNA damage machinery recognizes dysfunctional telomeres (55).

2.4. Telomerase and Its Regulation

Telomerase is a ribonucleoprotein enzyme that includes the catalytic core subunit with **TE**lomerase **R**everse **T**ranscriptase activity (hTERT), **TE**lomerase **R**NA **C**omponent (hTERC/hTR also known as the telomerase RNA template component) and other less characterized protein components. Telomerase can add new telomeric repeats to the end of chromosomes by using RNA (hTERC/hTR) as a template to bind to the 3' telomeric overhang end structure and hTERT for catalytic enzyme (reverse transcriptase) activity to maintain telomere lengths (61) (Figure 2.2).

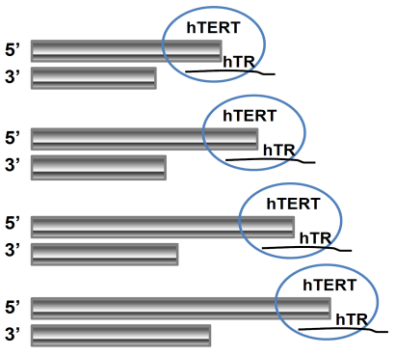


Figure 2.2. Telomere Elongation by Telomerase. hTERT: telomerase catalytic subunit, hTR: telomerase RNA subunit (Submitted to Cancer Medicine, book chapter, Mender et al.).

While cancer cells have telomerase activity at variable levels and almost all of them have short telomeres, somatic cells repress their telomerase activity during development except a small subset of proliferating stem like progenitor cells (25, 62-64). Normal cells experience telomere shortening with each cell division until a few shortened telomere signal a DNA damage response and cells enter replicative senescence. However, cancer cells that lack of cell cycle checkpoints (i.e., p53, p16) escape replicative senescence and continue to divide (called extended lifespan or crisis). During this extended lifespan phase, telomeres continue to shorten until there are so many shortened telomeres that crisis occurs. Crisis is the second growth arrest that cause chromosome end fusion, resulting in chromosome bridge-breakage-fusion

cycles. The cells that continue to divide eventually enter to the crisis, which generally results in apoptosis. Senescence and crisis are the two independent mechanisms that are important in protecting most large, long-lived species from the early occurence of cancer. Rarely, cells can acquire telomerase activity, which leads to an escape from crisis, and a hallmark of this event is that cells no longer progressively shorten their telomeres but maintain generally very short telomere lengths and become immortal (65). Importantly, immortalization is one of the hallmarks of cancer. Therefore, strict regulation of telomerase is highly important for fate of cell.

While we still know very little about the regulation of telomerase, there is mounting evidence that enzyme activity can be controlled at the transcriptional, posttranscriptional (alternative splicing) and epigenetic levels. While almost all cells have high levels of the template RNA (hTR) component, irrespective of having telomerase activity, hTERT can be detected at very low levels in telomerase positive cells with longer telomeres such as stem cells, progenitor cells and even in cancer cells (66). However, both components are necessary for telomerase to be activated (67).

Alternative splicing is one mechanism that is proposed to activate telomerase in cancer or repress telomerase in normal cells. Precursor mRNA (pre-mRNA) is generated by transcription of TERT DNA, then the generated pre-mRNA can be processed by excluding introns (noncoding sequences) and joining exons (coding sequences) in order to code protein sequence. During RNA splicing, exons might be included or excluded from the final mRNA to create multifunctional proteins, a process called alternative splicing (67). Misregulation of alternative splicing of telomerase and many other genes is a hallmark of almost all cancers. Therefore, understanding hTERT alternative splicing can also help to develop new strategies for anticancer therapies.

A recent study speculated that low abundance transcripts that are regulated by alternative splicing need more specific mechanisms for fine-tuning regulation such as hTERT. While hTERT coding sequence is conserved among species, some of the intronic elements that regulate hTERT splicing are only conserved among Old World

primates (68), suggesting that more specialized regulatory mechanisms for low abundance transcripts may be necessary for proper splicing (67).

Full length of hTERT that has 16 exons is the only transcript has reverse transcribed activity. Other identified alternative spliced forms do not have telomerase activity (69, 70). The major alternative spliced forms of hTERT are minus alpha, minus beta or minus alpha beta. The minus alpha form is a dominant-negative form, which does not have reverse transcriptase activity (70, 71) but overexpression of this spliced form causes inhibition of telomerase activity in telomerase positive cells, which can lead to senescence or apoptosis (71). The minus beta splicing creates a frame shift by skipping TERT exon 7 and 8 leading to a stop codon in exon 10, and is often the major hTERT spliced form in cancer cells (68). Although this form has a stop codon and does not have telomerase activity, it was shown that it can be translated to protein and overexpression of this form can be advantage for growth of breast cancer cells (72). A recent study showed that hTERT alternative splicing is regulated by variable number tandem repeats (VNTR), far from exon/intron junctions, that may use RNA:RNA pairing to regulate hTERT splicing (73).

Telomerase activity is detected in early fetal development but is lost during human gestations (between 12-18 weeks) due to a dramatic shift from full length hTERT to other isoforms (mostly minus beta) (74). These studies showed that alternative splicing of hTERT can be an important mechanism for telomerase regulation.

There have been different approaches to better understand telomerase regulation. Breakpoints of DNA rearrangement in TERT promoter region causes increased TERT expression in some cancers (75), and also mutations upstream of the TERT promoter can lead to telomerase activation by changing transcriptional regulatory sites (76). Different studies showed that the mutations in TERT promoter (77) and gene amplification (78, 79) is also related with transcriptional activation of hTERT.

2.5. Telomere and Telomerase Targeted Therapies

Considering the link of telomerase activity in tumor development and progression, researchers have focused on developing telomerase targeted strategies for anticancer therapy. Ideally, telomerase based anticancer strategies could have toxic effect on cancer cells with no or less toxicity on normal cells (25, 80, 81). Most tumor cells that express telomerase activity can accelerate their proliferation rate upon oncogenic stimuli. This may partially explain why tumor cells have shorter telomeres than healthy tissues. Because as their proliferation rate increases, they will continue to divide and eventually their telomere length will decrease (81-85). Normal progenitor cells and stem cells have relatively long telomeres, undergoing less divisions. This should make them more resistance to the therapy compared to cancer cells (65). Therefore, the expression of telomerase in cancer cells versus normal cells is the fundamental rationale for telomerase based cancer therapy. The most promising telomerase based strategies reviewed in Table 2.1.

Table 2.1. Telomerase based therapeutic approaches (Submitted to Cancer Medicine, book chapter, Mender et al.).

Therapeutic approach	Inhibitors
G-quadruplex stabilizers	BRACO19, RHPS4, Telomestatin, porphyrin, TMPyP4, CX-3543/quarfloxin, AS1411/ Cytarabine/Ara-C
Immunotherapy	GV1001, VX-001,GRNVAC1/2
Gene therapy	Telomelysin, Ad-hTERT-NTR/CB1954, hTERTp-HRP/IAA
Telomere and telomerase associated proteins	Geldanamycin (GA), Curcumin
T-oligo approach	PARP inhibitors
Small molecule inhibitors	BIBR1532
Antisense oligonucleotides (ASO)	GRN163L

Very few small molecule inhibitors have been found to inhibit telomerase activity in cancer cells. BIBR1532 (2-((E)-3-naphthalene-2-yl-but-2-enoylamino)-benzoic acid) is the one canditate small molecule that is non-competitive inhibitor of both hTERT and hTERC (86). Although *in vitro* and *in vivo* effects of BIBR1532 has been shown (i.e.; telomere shortening, inhibition of cell proliferation, cellular senescence, delayed tumor growth with pretreatment) (48, 87), it was not effective enough to enter into clinical trials (88, 89). After many years of screening new drugs a promising oligonucleotide, GRN163L (Imetelstat, Geron Corporation) was tested, GRN163 was the first generation of this oligonucleotide that is a 13 mer that is complementary to the hTR template region and thus is a competitive telomerase inhibitors (not a typical antisense approach). Although GRN163 showed good telomerase inhibition *in vitro*, its potential was reduced due to rapid uptake in cells (90, 91). Then researchers modified GRN163 by adding a lipid carrier molecule (covalently bound lipophilic palmitoyl (C16) group to the 5'-thio-phosphate) to increase its uptake into cells (91). GRN163L, competitive enzyme inhibitor, is a 13 mer N3'-P5'-thio-phosphoramidate oligonucleotide and shows highly potent telomerase inhibition as a direct hTR template antagonist (91, 92) (Figure 2.3).

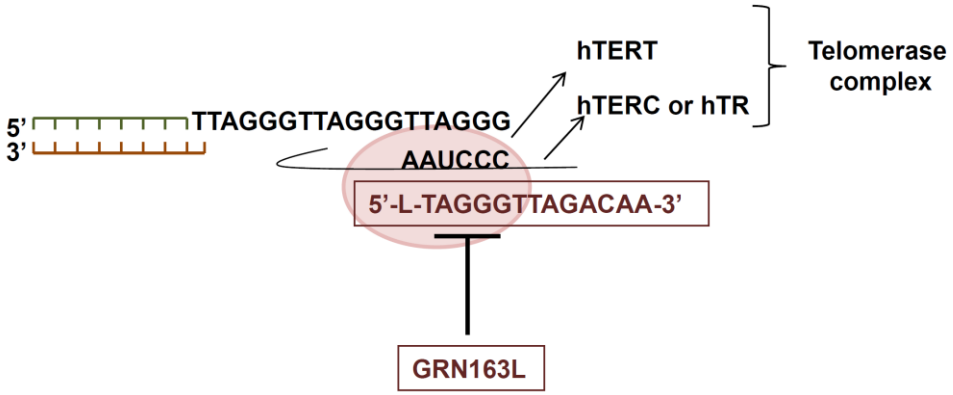


Figure 2.3. Targeting telomerase with GRN163L (Imetelstat). GRN163L is a 13 mer oligonucleotide that has a lipid moiety at 5' end to increase the efficiency of GRN163L uptake in to the cell. This oligonucleotide directly binds to the telomerase hTERC or hTR subunit with high specificity and affinity as a telomerase RNA template antagonist resulting in telomerase enzyme inhibition (hTERT: telomerase catalytic subunit, hTERC or hTR: telomerase RNA subunit).

After showing promising results in preclinical studies of different cancer cell lines by inhibiting telomerase activity and resulting progressive telomere shortening (92-101), GRN163L progressed into 17 different clinical trials (10 phase 1, and 6 phase 2 trials) (Table 2.2). However, it has not progressed in most clinical trials due to hematological side effects and liver function abnormalities besides other adverse events. When GRN163L causes hematological toxicity in patients, these patients have to go off trial until their platelet numbers come back to normal levels, resulting transient telomerase reactivation and telomere re-elongation during the drug holiday period. The other potential challenge for telomerase inhibitors, such as GRN163L, is the "lag period". The lag period is the time between the beginning of therapy and the therapeutic response. Telomere attrition is required to induce senescence or apoptosis with telomerase inhibitors, but the lag period of cancer cells is variable. Therefore, if a tumor has heterogeneous telomere length, there could be a long treatment period and this might result in increased side effects and costs. Yet, if they are used following conventional therapy to kill the surviving cells, it might reduce the chance of cancer recurrence (102). A clinical trial for this approach was completed for advanced non-small cell lung cancer (NSCLC) (clinicaltrials.gov: NCT01137968). Eligible stage IV or recurrent locally advanced NSCLC patients were randomly divided to different groups that are GRN163L with/without bevacizumab for day 1 of each 21 day cycle or observation versus standard of care alone (patients who had received bevacizumab before randomization were continued with bevacizumab maintenance or had no followup maintenance treatment). This study showed a trend in prolonged progression free survival (PFS) in the patients with the shortest quartile of telomere at the initiation of the trial in the GRN163L arm (103). Experience from these type of trials can facilitate our understanding how we can treat cancer patients to take a better advantage of anti-telomerase therapies. GRN163L clinical trials are reviewed in Table 2.2.

Table 2.2. Clinical trials of GRN163L (Submitted to Cancer Medicine, book chapter, Mender et al.).

ClinicalTrials.gov Identifier/phase	Condition	Drug Intervention	Status
NCT01568632/phase 1	Refractory or Recurrent Solid tumors/Lymphoma	GRN163L	Withdrawn
NCT01273090/phase 1	Refractory or Recurrent Solid tumors/Lymphoma	GRN163L	Completed
NCT01243073/phase 2	Essential Thrombocythemia/Polycythemia Vera (ET/PV)	GRN163L, standard of care	Suspended
NCT00594126/phase 1	Refractory or Relapsed Multiple Myeloma	GRN163L	Completed
NCT01916187/phase 1	Neuroblastoma	GRN163L, 13-cis retinoic acid	Withdrawn
NCT00732056/phase 1	Recurrent or Metastatic Breast Cancer	GRN163L, paclitaxel, bevacizumab	Completed
NCT00310895/phase 1	Refractory or Relapsed Solid Tumor Malignancies	GRN163L	Completed
NCT00718601/phase 1	Multiple Myeloma	GRN163L, bortezomib, dexamethasone	Completed
NCT01242930/phase 2	Multiple Myeloma	GRN163L, standard of care	Active, not recruiting
NCT00510445/phase 1	Advanced or Metastatic Non-Small Cell Lung Cancer	GRN163L, paclitaxel, carboplatin	Completed
NCT01265927/phase 1	Her2+ Breast cancer	GRN163L, trastuzumab	Active, not recruiting
NCT00124189/phase 1	Chronic Lymphoproliferative Disease (CLD)	GRN163L	Completed
NCT02011126/phase 2	Relapsed or Refractory Solid Tumors	GRN163L	Withdrawn
NCT01137968/phase 2	Non-small Cell Lung Cancer	GRN163L, bevacizumab	Completed
NCT01731951/Not provided	Primary or Secondary Myelofibrosis	GRN163L	Active, not recruiting
NCT01256762/phase 2	Recurrent or Metastatic Breast Cancer	GRN163L, paclitaxel, w/wo bevacizumab	Completed
NCT01836549/phase 2	Recurrent or Refractory Brain Tumors	GRN163L	Recruiting

Guanosines can assemble by themselves in the presence of guanine tandem repeats and generate G-quadruplex structures by monovalent cations (i.e.; potassium). G-quadruplexes can be found in telomeres, oncogene promoter sequences and other regions of genome (104). G-quadruplex structures are the another target to design small molecule ligands for telomerase-based therapies. G-quadruplex ligands stabilize G-quadruplex structure by preventing G-quadruplex from unwinding and opening T-loops, which makes telomerase indirectly a target and can result in telomerase inhibition. Also, G-quadruplex ligands may cause telomere uncapping by leading to dissociation of telomeric proteins (80, 105). BRACO-19, RHPS4 and telomestatin have been the most commonly studied G-quadruplex ligands to date. They inhibit telomerase activity by activating DNA damage responses (106). However, the specificity of G-quadruplex stabilizers for telomerase is very limited (107) and they may affect quadruplex structures on normal cells as well as cancer cells (Figure 2.4.A). Thus, this approach to telomerase inhibition has also not progressed.

An immunotherapy approach was designed to induce CD8+ cytotoxic T-lymphocyte responses for hTERT antigens in tumors. Telomere associated antigens can produce immune responses resulting in lysis of telomerase expressing tumor cells (108-110). Multiple approaches for immunotherapy have been successfully tried *in vitro* and *in vivo*, some of vaccines have been moved into clinical trials such as GV1001 (peptide vaccine), GRNVAC1 (dendritic cell priming *ex vivo*). Clinical trials have involved in solid tumors (breast, prostate, renal, lung, pancreatic cancer and melonama) and hematological tumors (acute myeloid leukaemia) (80, 111). One of the problems is that the immune system can be compromised in many patients due to standard of care therapy, this may cause weak TERT immunological responses (80). Also, since most chemotherapeutic agents are immunosuppressive, the combination of chemotherapeutic drugs with vaccines might limit the utilize of vaccines with other chemotherapeutic drugs (112) (Figure 2.4.B).

Gene therapy is a well established technique by delivering therapeutic DNA into the cancer cells via oncolytic viral vectors or suicide genes. While often encouraging, there are several challenges for gene therapy approaches as well. These

concerns are about whether delivery of gene therapy to cancer cells throughout the body can be achieved efficiently or not and also immunological responses to vector systems can limit dosing schedules (80) (Figure 2.4.C). Mutant hTERC (dominant-negative approach) is the other gene therapy approach in preclinical studies. Wild type telomeres contain DNA sequences for binding of telomere associated proteins (i.e.; POT1, TRF1, TRF2) (113, 114). Mutant DNA that is synthesized from mutant hTERC disrupts the binding of telomere associated proteins (115-117) and this results in telomere uncapping. Although *in vitro* and *in vivo* effects of mutant hTERC therapy approach via lentiviral vectors has been succesfully reported (116, 117), it is still a gene therapy approach that needs to be more efficient to target the vast majority of cancer cells.

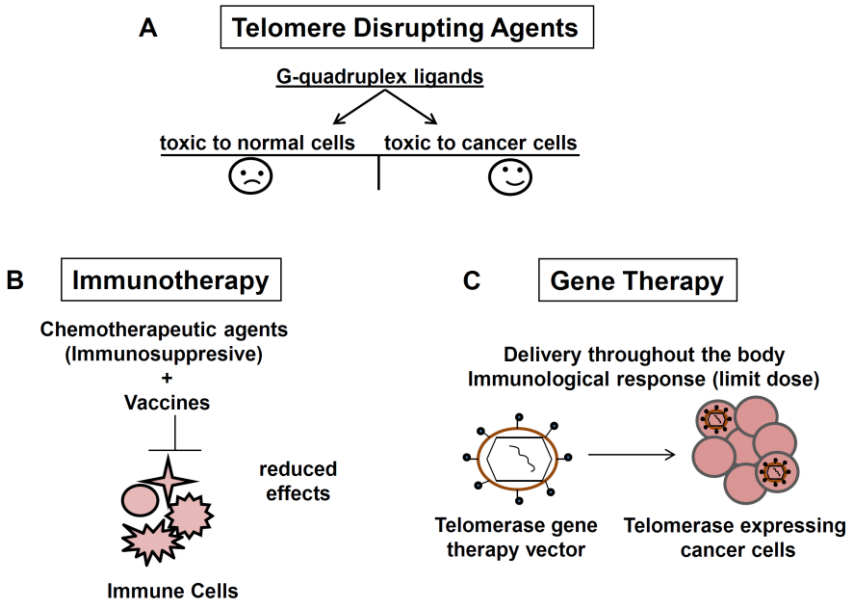


Figure 2.4. Disadvantages of different telomerase based therapy strategies. A) G-quadruplex structures can be found at the other region of chromosomes besides telomeres, thus they can be toxic normal cells as well as cancer cells. B) One of the important disadvantages of immunotherapy is the immunosuppresive effects of chemotherapeutic agents when they are used together as an adjuvant therapy. C) Delivery of gene therapy into the cancer cells throughout the body is challenging for cancer patients and oral therapy is not generally available for efficient delivery (Submitted to Cancer Medicine, book chapter, Mender et al.).

2.6. The Role of Telomeres on Cellular and Organismal Aging and Cancer

Studies showed that having short telomeres in mice (with knockout of mTERT) can eventually lead to some aspect of aging observed in humans. When telomerase activity and expression of p53 pathway were increased at the same time, they found that these mice extended their life span by ~40% (118). A different study showed that the mice with critical short telomeres had many hallmark of advance age immunological problems, wound healing problems, and stem cell regenerative problems such as errosive dermatitis and failure of liver regeneration. However, when telomerase was reactivated in these mice with experimentally shortened telomeres, animals became fertile (119), more directly showing that telomeres play a role in aging process. In addition, individual critically short telomeres trigger cellular responses to the telomere dysfunction (120). Each human cell has 92 telomeres, and each telomere has its own unique length and the lowest percentiles of telomere length have a higher risk for aging related disease. Therefore, it is important to understand aging and cancer process so that we can increase the years of healthy life by diminishing age associated diseases and controlling unlimited proliferation for prevention of cancer (121).

Cellular senescence is one of the tumor suppressor mechanisms to counteract the development of cancer. Cellular senecence is the proliferative arrest that forces cells to exit the cell cycle permanently (122). There are several nuclear biomarkers for senescence such as telomere dysfunction induced DNA damage foci, TIFs (123, 124), altered gene expression (upregulation of p16, $p21^{Waf1}$/Cip1 and p53) (125-129), evidence of chromosomal instability but only in combination with other alterations in important cell cycle check point genes (130), an increased DNA damaged response (123), and changes in chromatin structure [the development of senescence-associated heterochromatin foci (SAHF)] (131-133). Different studies showed in mouse models that high levels of oncogene expression can cause benign tumors in which cells display cellular senescence features such as high levels of certain heterochromatin proteins and senescence-associated-beta galactosidase (SA-βGal) activity. Disruption of senescence pathways, for example by inactivating p53 tumor suppresor, promotes

malignant cancer progression, indicating that cellular senescence supresses cancer development in humans and mice (61).

When double strand breaks (DSBs) are generated by genotoxic stresses such as DNA replication stress, ionizing radiation, endonucleases, or ROS, most DBSs are repaired. In contrast, breaks that occur on or near telomeres (in the absence of telomerase activity) can not be repaired for months, maybe for years, both *in vitro* and *in vivo* (134, 135). Persistent telomeric damage foci are also generated by ionizing radiation in the presence of endogenous and ectopically expressed telomerase, suggesting that even telomerase is unable to prevent formation of telomeric damage under some ionizing radiation conditions (136). However, telomerase can suppress the formation of telomeric damage under the DNA damage stress in somatic human cells (136). Dysfunctional telomeres that are generated by progressive telomere erosion, DNA replication stress, telomeric DSB formation, or other genotoxic events induce cellular senescence in normal cells, which are called **T**elomere **D**ysfunction-**I**nduced **S**enescence (TDIS). This telomere dysfunction induced senescence is a tumor suppressing mechanism. Telomeres or telomere associated proteins can inhibit the DNA damage repair machinery under some conditions (61). It might seem that permanency of damage on telomeres is disadvantageous, however, as DSB machinery tries to repair damage, it will cause mutation at the breakage site. Therefore, it provides that dysfunctional telomeres induce senescence prematurely, accumulate in our tissues as we age. This is potentially a beneficial mechanism to the organism to prevent accumulation of mutations and protect organism from uncontrolled proliferation and invasive tumors early in life (61).

2.7. The Thiopurines

Azathioprine, 6-thioguanine and 6-mercaptopurine are the thiopurines that are used as an anti-inflammatory, anticancer and immunosuppresive drugs. Although they have been in clinical trials for well over a half century, only 6-mercaptopurine and azathioprine were approved by FDA (137). However, it has been known that prolonged treatment with thiopurines is associated with high risk of various cancers

such as non-Hodgkin lymphoma, skin squamous cell carcinoma. Therefore, it is advisable to follow up with patients for any signs of therapy related cancers (138).

Thiopurines are prodrugs that need to be converted to metabolically active compounds (139). 6-thioguanine is the final active metabolite of all the thiopurines. The first step of this metabolic pathway is the removal of the nitroimidazole group off azathioprine in a non-enzymatic reaction involving glutathione. This step occurs in erythrocytes which release the active metabolite 6-mercaptopurine. 6-mercaptopurine and 6-thioguanine then enters the purine salvage pathway in cells.

Hypoxanthine-guanine phosphoribosyl transferase 1 (HPRT1) is one of the important enzymes in purine biosynthesis, which catalyzes the addition of ribose-5-phosphate to 6-mercaptopurine and 6-thioguanine to generate thioinosine monophosphate (TIMP) and thioguanosine monophosphate (TGMP), respectively. TIMP and TGMP are the precursors for incorporation of 6-thioguanine into RNA or DNA. Additional reactions with deoxynucleoside kinases and reductases are required to convert thioguanine nucleotides to 6-thio-GTP and 6-thio-dGTP, which are substrates for incorporation of 6-thioguanine into RNA and DNA (138).

The degradation of 6-mercaptopurine is catalysed by xanthine oxidase, also both 6-mercaptopurine and 6-thioguanine are inactivated by thiopurine S-methyltransferase (TPMT) (140) (Figure 2.5).

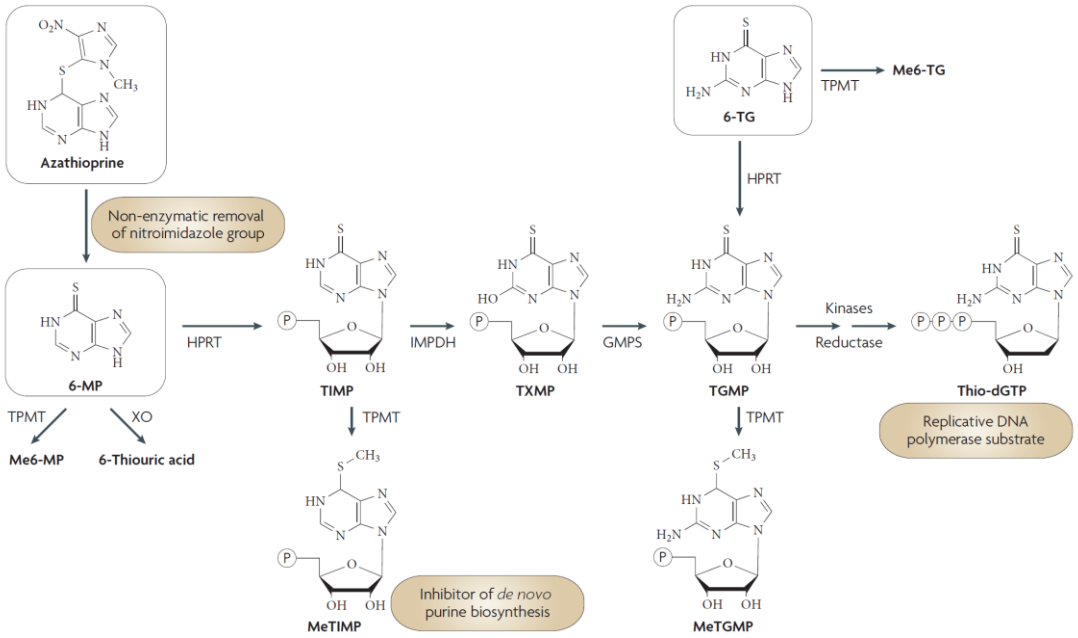


Figure 2.5. Shematic illustration of thiopurine metabolism. Azathioprine is converted to 6-mercaptopurine (6-MP) by non-enzymatic activation. 6-mercaptopurine and 6-thioguanine (6-TG) are converted to nucleoside monophosphates (TIMP and TGMP, respectively) by hypoxanthine–guanine phosphoribosyl transferase enzyme (HPRT). These nucleoside monophosphates are eventually converted to 6-thio-dGTP by deoxynucleoside kinases and reductases to be incorporated to DNA. In catabolic reactions, thiopurine *S*-methyltransferase (TPMT) is the catabolic enzyme for 6-mercaptopurine and 6-thioguanine, which catalyzes TIMP and TGMP to meTIMP (inhibitor of de novo purine biosynthesis) and meTGMP, respectively. In addition, TPMT and xanthine oxidase (XO) catalyze 6-mercaptopurine to 6-thiouric acid. TIMP that escapes from catabolism can be further metabolized to TGMP and eventually 6-thio-dGTP by inosine monophosphate dehydrogenase (IMPDH) and guanine monophosphate synthetase (GMPS) with additional kinases and reductase. (138).

6-thio-dGTP that is generated from 6-thioguanine and 6-mercaptopurine is a good substrate for DNA polymerases (141, 142). 6-thioguanine can accumulate in DNA and high levels of DNA substitution can be toxic or partially mutagenic, which is dependent on active purine salvage. Thiopurine shows delayed toxic effect, which

can partially be explained with the requirement of passage through S-phase of the cell cycle (138). 6-thioguanine (more reactive than canonical DNA bases) is incorporated into the template DNA, rather than the daughter DNA, therefore DNA polymerases can not find the precise daughter-strand partner for the aberrant base. This results in potentially lethal DNA lesions (143). Increased levels of spontaneous mutations by 6-thioguanine related DNA damage is due to uncorrected replication errors. It is also reported that DNA that has 6-thioguanine substitution are photochemically active and generate reactive oxygen species (ROS), which can eventually cause damage to DNA, protein and other macromolecules (138).

2.8. The Importance of Liver and Kidney Function on Drug Toxicity

Assessment of organ function (i.e.; liver, kidney) is an important aspect in selecting the appropriate drug and its doses for cancer patient after assessment of tumor histology. The possibility of organ abnormalities following treatment can be due to drug regime rather than progressive disease (144). An appropriate strategy for effective and safe chemotherapy dosing is poorly defined in cancer. Cancer patients have different pharmacokinetics/pharmacodynamics, and many agents have narrow therapeutic windows (e.g. differences between toxicity to normal versus cancer cells). Chemotherapy dosing decisions are often made base on limiting toxicity in cancer (144). Therefore, selecting an appropriate chemotherapy and its dosing can be a challenge for clinicians and their patients.

The liver is a vital organ that has functions in multiprocesses such as metabolism, biosynthesis, excretion, secretion and detoxification. The liver is an oxygen dependent tissue since the processes in liver are energy required. The liver is a regenerative organ that can regrow from even massive cellular losses. However, cellular loss above certain threshold will prevent tissue regeneration and lead to hepatic failure and death (145). Several chemotherapy agents cause liver injury, which can lead to necrosis, steatosis, fibrosis, cholestasis, and vascular injury (146). This can be determined by serum liver biochemistry or histology. Serum liver biochemical tests are generally an indicator of liver inflammation or damage, rather than liver function (147). Several studies showed the relation between abnormal serum biochemical tests and toxic effects of chemotherapeutic drugs. For instance,

the toxicity of antimetabolites such as 6-mercaptopurine, 6-thioguanine have been shown in preclinical and clinical trials. When 6-mercaptopurine dose exceed 2 mg/kg daily dose in adults, it causes either hepatocellular or cholestatic liver disease and a hepatocellular injury (148, 149). It has been shown that 14 of 40 patients treated with 6-mercaptopurine developed aspartate aminotransferase (AST) or alanine aminotransferase (ALT) values above 150 U/L (150). 6-thioguanine, which is another thiopurine metabolite causes the production of hepatic veno-occlusive disease (VOD) and in a single case of peliosis hepatitis as well as jaundice (144, 151-155).

The kidney is the essential organ that functions in many important processes such as excretion of waste products of metabolism, reabsorption of vital nutrients, acid-base homeostasis, osmolality regulation, blood pressure regulation, hormone secretion. Also the importance of kidney on drug toxicity is due to most drugs are eliminated by the kidneys and therefore dose adjustments are needed in patients with renal insufficiency (156). The amount of damage in the kidneys is dependent on the type of chemotherapy, patient age, and underlying disease. Most potent chemotherapeutics have been shown to have nephrotoxic effects. These effects can lead to acute (frequently reversible) or chronic kidney disease. Acute toxicity can progress to chronic toxicity, which develops chronic tubulointerstitial nephritis, papillary necrosis, or prolonged proteinuria with some chemotherapeutics (157). Impaired renal function such as excretion and metabolism might cause systemic toxic effect such as bone marrow suppression (158, 159). Therefore, it is important to know commonly used chemotherapeutics that cause renal dysfunction and also available preventive strategies to decrease the risk of renal disease in cancer patients under treatment (160).

2.9. Tumor Resistance to Therapy

There have been different mechanisms to explain tumor resistance to chemotherapy or radiotherapy. Tumor that arises from one single clonogenic cells accumulates multiple mutations and genetically diverse clones are found in the same tumor (161-166). This diversity in intratumor cells exerts distinct treatment sensitivity and different clones in the same tumor may have different resistance

mechanisms (167, 168). In addition, the cells in the same clone have different fundamental and phenotypic differences that might be explained by the stem cell model of cancer development. Recent studies showed that some cancer cells have a hierarchical organization, which is similar to normal tissue. For instance, tumorigenic cancer stem cells (CSCs) differentiate into non-tumorigenic progenies (168). Cancer stem cells represent a different subpopulation that can be identified and isolated from the tumor tissues by using putative cancer stem cell markers. Cancer stem cells, which are different than bulk tumor cells, have self renewal capacity and long term repopulation potential. These features of cancer stem cells can facilitate the initiation and maintainence of tumorigenesis (169, 170). Cancer stem cells can be either stable or transient cell populations in human malignant neoplasms (170). Studies have shown that although CSC and their non tumorigenic progenies in the same clone have same genotype, they display different epigenetic profiles, which cause alteration of the signaling pathways (171-174). In addition, different studies have shown that the number of molecular mechanisms contribute to resistance of certain CSCs to conventional therapy (168, 169), but this property can not be generalized since some CSCs are sensitive to conventional therapy and some are not. However, cancer can potentially arise from a single CSC, therefore to target all CSCs might be required for efficient therapy and durable responses in patients (175, 176).

Cancer stem cells can be detected in human tumors and cell cultures by specific cell surface proteins such as CD133, CD44, CD24, α2β1 integrin and others (169). In addition, cancer stem cells can be determined by biochemical activity of the marker proteins. For instance, the aldefluor assay is based on enzymatic activity of aldehyde dehydrogenase (ALDH) in putative tumor initiating populations. Also identification of CSCs is possible by the capability to efflux lipophilic fuorescent dye Hoeschst 33342 efflux. The cells that are negative for the dye staining have a tail like shape called side population (SP) (177). This phenotype is correlated with upregulation of ATP-Binding Cassette (ABC) transporter superfamily proteins. ABC transporters are known to participate in multidrug resistance functions in various human cancers (178-180). The ABC transporter family has 49 proteins; P-glycoprotein (P-gp, MDR1, ABCB1), multidrug resistance protein 1 (MRP1, ABCC1), and breast cancer resistance protein (BCRP, ABCG2) have been

commonly investigated for drug resistance mechanisms (181, 182). Increased expression of these proteins in some type of cancers results in ATP-dependent efflux of cytotoxic drugs from cells. This leads to the inability to maintain within the cancer cells a toxic drug concentration (183). Population of cancer stem cells in different tumors have high expression of ABC transporters, leading to drug resistance (184, 185).

Aldehyde dehydrogenase activity is one of the common features of cancer stem cells. The proteins of aldehyde dehydrogenase family are enzymes that catalyze oxidation of intracellular aldehydes to carboxylic acids and also contribute the synthesis of retinoic acid and neurotransmitter gamma-amino butyric acid (GABA), which are also important for normal and cancer stem cells maintenance and differentiation (186, 187). ALDH1A1 is the most studied isoform of ALDH protein family (188). Studies showed that increased level of ALDH1A1 is the marker for poor clinical outcome in breast and prostate cancer patients (189-191). It is also known that ALDH1A2 is involved in cell proliferation, colony formation and drug resistance (192) .

Epigenetic changes are the heritable changes in gene expression that occur without any alterations in the DNA sequence including DNA methylation, histone modification, and posttranslational gene regulation by microRNAs (miRNAs) (193, 194). DNA methylation is a major epigenetic factor that influences gene activities and plays an important role in cancer and chemotherapy drug resistance. DNA methylation is catalyzed by DNA methyltransferases that transfer methyl group from S-adenosyl methionine (SAM) to the fifth carbon of a cytosine residue to generate methylated cytosine (195). Cytosines are followed immediately by a guanine, it is so-called CpG dinucleotides. Most genome are lack of CpG dinucleotides, and those that are present in the genome are generally methylated. In contrast, CpG islands that are comparatively rich in CpG nucleotides are almost methylation free. These CpG islands are frequently located in the promoters of human genes and methylated cytosines in the CpG islands are generally associated with transcriptional inactivation of gene such as tumor supressor gene like p53. Aberration in DNA methylation is correlated with cancer development and it has been shown that methylation of CpG

islands plays an important role on transcriptional repression of tumor suppresor genes (196). The second epigenetic mechanism is the histone modification. Histones are the major components of chromatin and can undergo multiple post-translational modifications such as acetylation by histone acetyltransferases (HATs) and deacetylation by histone deacetylases (HDACs). Acetylated form of histone proteins causes less-condensed packaging of genes in chromatin that might lead to the re-expression of silenced tumor suppressor genes. It is known that micro-RNAs (miRNAs) are endogenous small non-coding RNAs that negatively regulate gene expression. Previous studies showed the crucial role of miRNAs in regulating gene expression and the relation between differential expression of miRNAs with drug resistance in cancer (197).

Genetic alterations in signaling pathways have also effect on drug response. In most cases, mutated oncogenes start the signals in pathways leading to upregulation of survival, drug resistance or downregulation of cell death responses. For example, Wnt/β-catenin and Notch pathways are one of the signaling pathways that are important for cancer progression. Transducin-like enhancer of split 2 (TLE2) is a mammalian homologue of the Drosophila transcriptional repressor groucho, which plays a role in Wnt/β-catenin and Notch pathway. It was shown that increased Wnt signals and nuclear β-catenin results in elevated Cyclin D1 and cellular proliferation in Wnt/β-catenin pathway, additionally increased TLE2 affected tumor progression. In Notch pathway, Notch3 repressed HASH1/ASCL1, resulting in reduced DLK1 and inhibited cellular differentiation, PIT1 and growth hormone expression. However, how increased TLE2 levels affect tumor progression requires further investigation (198). It is also known that Wnt and Notch signaling pathways participate in the regulation of drug resistance in several cancers (199, 200).

2.10. Purpose and Significance

Telomerase is an highly attractive and almost universal target for cancer therapy, yet there are few telomerase based therapies in clinical trials. Since telomerase inhibition based therapies depend on initial telomere length of cancer cells, long treatment period is necessary to induce senescence or apoptosis by progressive telomere shortening in the presence of the inhibitors. Therefore, this long

treatment period (lag phase) may lead to increased toxicities (side effects) and require high costs. Standard telomerase inhibitors have not been effective in long-term clinical trials due to the increased toxicities and the long lag period. In this study, we first aimed to generate a nucleoside analog to reduce this long lag period treatment period and hopefully with minimal side effects. Secondly, we aimed that this nucleoside analog should be recognized by telomerase, then incorporated into the telomeres, upcapping them immediately and resulting in telomere induced dysfunctional foci and cell death. Importantly, we sought to identify a nucleoside that was more specifically toxic to telomerase expressing cancer cells compared to normal cells. We hypothesized that this nucleoside analogue would alter the telomeric structure and function, cause telomeric DNA damage due to telomere uncapping and reduce the treatment phase, leading massive and fast cancer cell death by being converted to nucleoside triphosphate. The biochemical conversion of this nucleoside analogue (6-thio-dG) to 6-thio-dGTP is shown in Figure 2.6. The overarching focus of this study was to identify such a nucleoside, to test it both *in vitro* and in xenograft mouse experiments and finally to determine the side effects in wild type mice.

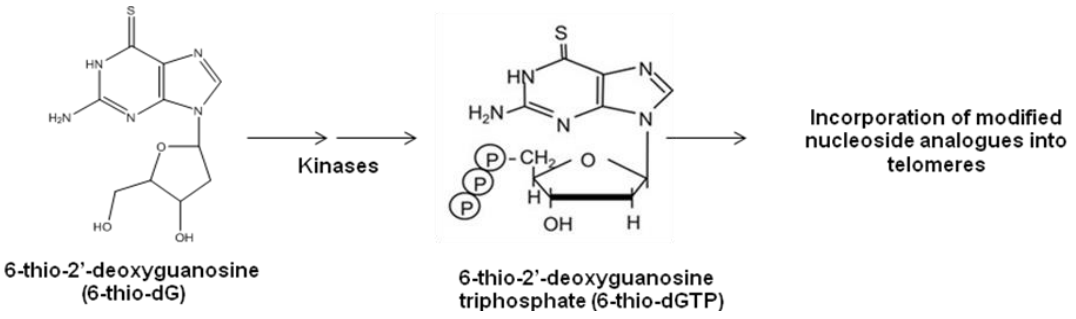


Figure 2.6. The biochemical conversion reaction of 6-thio-dG to 6-thio-dGTP to be incorporated into telomeres by telomerase.

3. METHODS

3.1. Cell lines

The panel of non-small lung cancer cell lines (H2882, HCC2429, HCC827, HCC15, H2087, HCC4017, HCC515, H2009) were provided by John D. Minna at the University of Texas Southwestern Medical Center. HCT116 human colon, A549 human non-small cell lung cancer (NSCLC), the panel of additional non-small cell lung cancer cell lines (H2882, HCC2429, HCC827, HCC15, H2087, HCC4017, HCC515, H2009) and BJ human fibroblasts were grown in a Medium X (DMEM:199, 4:1, Hyclone, Logan, UT) supplemented with 10% cosmic calf serum (Hyclone) without antibiotic. HCEC1 cells were cultured in medium consisting of mediumX (DMEM:199, 4:1), 2% cosmic calf serum, insulin, and gentamycin. BJ and HCEC1 cells were cultured at 37˚C in low oxygen (2-5%) to prevent damaging cells in culture (three gas mixture; 2% oxygen, 5% CO_2 and 93% nitrogen) (201). BJ cells were immortalized by transfection of a retroviral hTERT-TK-hygromycin cassette. Successful hTERT-hygromycin expression was confirmed in clones by testing for hygromycin resistance and the presence of telomerase activity.

3.2. Drug preparation

6-thio-dG (Metkinen Oy, Kuopio, Finland) and 6-thioguanine (Sigma, St Louis, MO) was dissolved in DMSO/water (1:2) to prepare 50 mM or 10 mM stock solutions, which were kept frozen at –20˚C. Once *in vitro* experiments were conducted, a 1 mM final concentration was prepared in serum free medium and added at varying amounts for cell treatments. For mouse *in vivo* studies, drugs were prepared in 5% DMSO solution.

3.3. Cell Viability Assay

HCT116 (0.5×10^3 cells/well), A549 (0.6×10^3 cells/well), and H2882, HCC2429, HCC827, HCC15, H2087, HCC4017, HCC515, H2009 (1.5×10^3 cells/well), BJ and HCEC1 cells (2×10^3 cells/well) were plated in growth media on 96 well plate. Cells were incubated for 1 week and treated with varying concentrations of 6-thio-dG and 6-thioguanine or DMSO every three days. The 96

well plates were analyzed according manufacturer's directions for the CellTiterGlo Luminescent Cell Viability Assay (Promega, Madison, WI) to obtain IC_{50} values, The IC_{50} is defined as the concentration of drug at which 50% of the cells are inhibited by the drug. Sigmoidal dose-response curves (GraphPad Prism, La Jolla, CA) were used to calculate IC_{50} values.

3.4. Long-term cell culture studies

For long-term cellular experiments, HCT116 (1,000 cells/cm^2) and BJ (10,000 cells/cm^2) cells were treated with 6-thio-dG (1, 3, 10 μM) containing medium every three days. The cells were counted and replated every week for 10-12 weeks. Additionally, HCT116 cells (1,000 cells/cm^2) were fed with 6-thioguanine (1, 3, 10 μM) every three days, the cells were counted each week, collected $1x10^6$ cells for TRF (Telomere Restriction Fragment) analysis and replated.

3.5. Telomerase activity assay (TRAP, Telomere Repeat Amplification Protocol)

General Information and Principle

TRAP is a standard method to determine telomerase activity. It is developed and referred to as the TRAP assay by Kim et al. (25). TRAP assay includes three steps that are extension, amplification, and detection of telomerase products (Figure 3.1). In the extension step, telomeric repeats are added to the telomeric substrate, which is a non telomeric oligonucleotide, by telomerase. In amplification step, the extension products are amplified by the polymerase chain reaction (PCR) by using specific primers and in detection step, mostly the presence or absence of telomerase is analyzed by electrophoresis. The extraction of telomerase from cell or tissue is very important step since the amount of telomerase affects the quantitative determination of telomerase activity (202). However, the standard TRAP assay has the ability to detect telomerase activity even in a single cell. But it is worth to note that the accuracy of detection of telomerase activity depends on amplification step and the primers used (202). Although this is an advantageous assay, there are some disadvantages due to the annealing problem of primers. The extended product by PCR utilizes TS specific primers that can produce primer-primer interactions and these dimers might generate false positive results. Therefore, there have been

different primers generated to eliminate primer interaction (203-205). The products that are generated by telomease are visualized as a ladder of bands and each band represents 6 bp or telomeric repeats. Using fluorescent moieties in primers improves the annealing and amplification efficiency (202). PCR amplification is sensitive to most inhibitors that may inhibit Taq polymerase activity such as heme containing compounds. Therefore, internal telomerase assay standard (ITAS) helps to detect of these inhibitors in samples and serves to normalize telomerase activity (206).

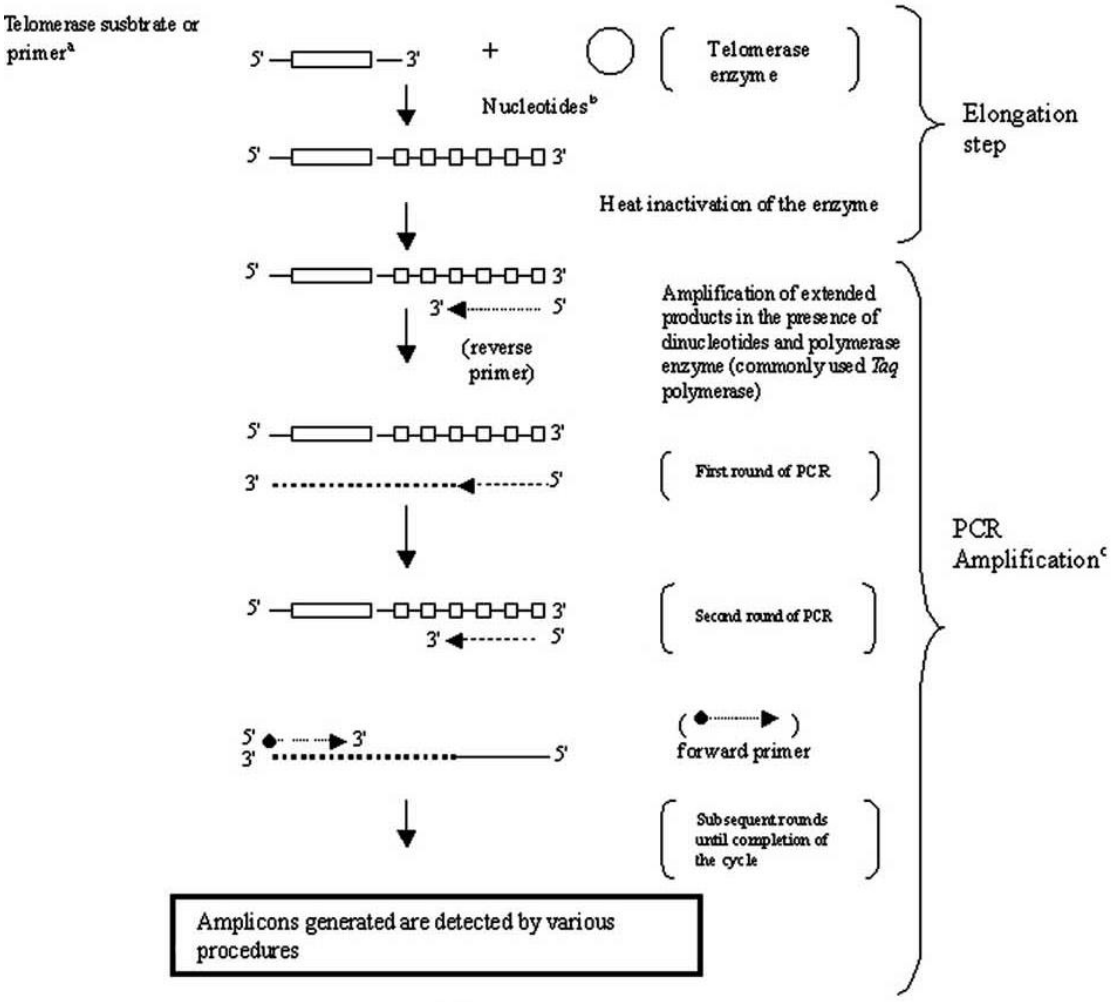


Figure 3.1. The elongation and amplification steps of Telomere Repeat Amplification Protocol (TRAP) (202).

Procedure

Telomerase activity was measured by the TRAP (Telomeric Repeat Amplification Protocol) assay as mentioned above (62). Briefly, HCT116 cells were treated with 1 or 10 μM 6-thio-dG for 1 week. $1x10^5$ cells were collected and lysed with ice-cold NP-40 lysis buffer (10 mM Tris–HCl pH 8.0, 1.0 mM $MgCl_2$, 1 mM EDTA, 1% NP-40, 0.25 mM sodium deoxycholate, 10% glycerol, 150 mM NaCl, 5 mM β-mercaptoethanol) for 30 min. One microliter cellular lysate was used for each reaction. Hela cells were used as a positive control and lysis buffer was used as a negative control. Samples were prepared and then the telomerase extension products were amplified using PCR (95˚C for 5 min to inactivate telomerase, then 95˚C for 30 sec, 52˚C for 30 sec, 72˚C for 30 sec; 24 cycles). Samples were run on a 10% nondenaturating acrylamide gel and visualized using a Typhoon PhosphorImager scanner system (Molecular Dynamics, GE Healthcare, Piscataway, NJ) that is capable of reading Cy5 fluorescence.

3.6. Telomere length assay (TRF, Terminal Restriction Fragment)

General Information and Principle

Telomere restriction fragment analysis, which is based on southern blot technique, can be used to obtain average telomere length of cell population. This method measures canonical and noncanonical telomere components and also provides telomere length distribution in each DNA. The principle of this analysis lies in the relative lack of restriction sites in telomeric (TTAGGG repeats) and subtelomeric (adjacent to telomeric repeats) regions. Genomic DNA digestion by restriction endonucleases cut within chromosomal DNA, not telomeric repeats. HinfI and RsaI is typically used in TRF analysis rather than HphI and MnlI because while HphI/MnlI cuts DNA within noncanonical subtelomeric region, HinfI/RsaI leaves this region intact, resulting in ~1 kb longer than mean telomere length (207).

Procedure

$1x10^6$ cells were collected and washed with PBS. DNA was isolated using the manufacturer's instructions (Qiagen, Valencia, CA). 2.5 μg DNA was digested with

six different restriction enzymes (HhaI, HinfI, MspI, HaeIII, RsaI, AluI) (New England Bio, Ipswich, MA) and incubated at 37°C overnight. Digested DNA was separated on a 0.7% agarose gel overnight at 70 V. The terminal restriction fragment (TRF) gel was placed in denaturating solution for 20 min (0.5 M NaOH, 1.5 M NaCl, pH 13.2) and dried on Whatman 3MM paper under vacuum for 3 hr at 56°C. The gel was then placed for 15 min in neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) and then probed with a radiolabeled telomeric probe (C-rich) for 16 hr at 42°C in 5x SSC buffer, 5xDenhardt's solution, 10 mmol/L Na_2HPO_4, and 1 mmol/L $Na_2H_2P_20_7$. The gel was washed once with 2x SSC, 0.1% SDS, twice with 0.5x SSC, 0.1% SDS and then twice with 0.5x SSC, 1% SDS at room temperature for 15 min. Gels were exposed to a PhosphorImager screen overnight and analyzed using a Typhoon PhosphorImager scanner system (Molecular Dynamics). ImageQuant and graphpad prism were used to determine telomere length of cells.

3.7. 6-thio-dG and GRN163L Treatment for Cell Killing Effect and Telomere Restriction Fragment Analysis

HCT116 cells were grown without any treatment, with only 3 μM GRN163L treatment, 3 days pretreatment with 3 μM GRN163L, and 3 μM GRN163L+1 μM 6-thio-dG treatment at the same time. Drugs were added every three days for 1 week. At the end of treatment, cells were countedand compared with each treatment design.

HCT116 cells ($5x10^4$ per 10 cm dish) were treated with 10 μM 6-thio-dG and/or 3 μM GRN163L every three days for 11-14 weeks. Also, HCT116 cells treated with 10 μM 6-thio-dG for 12 weeks were allowed to grow without any treatment for additional 2-4 weeks or combination of 10 μM 6-thio-dG and 3 μM GRN163L for additional 2-4 weeks. Each week cells were counted, collected for TRF (Telomere restriction fragment) analysis and replated.

3.8. Telomere dysfunction Induced Foci (TIF) assay

The TIF assay is based on the co-localization detection of DNA damage by an antibody against DNA damage markers, such as gamma-H2AX, and telomeres using an antibody against the telomeric protein TRF2. Briefly, HCT116 cells were plated in 4-well chamber slides and after the cells attached to the surface, either 3 μM

6-thio-dG or 3 μM 6-thioguanine was added to the medium at different time points (0, 30 min, 12 hr, 24 hr, 48 hr, 72 hr). BJ and BJ hTERT cells were plated in 4-well chamber slides and after the cells attached to the surface, either 10 Mm 6-thio-dG or 10 μM 6-thioguanine was added to the medium for 48 hr. Slides were rinsed once with PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Then, cells were washed twice with PBS and permeabilized in 0.5% Nonidet-P40 in PBS, blocked with 0.5% Bovine Serum Albumin (BSA) and 0.2% fish gelatin in PBS for 30 min. Gamma-H_2AX (mouse) (Millipore, Billerica, MA) was diluted 1:1000 and TRF2 (rabbit) (Abcam, Cambridge, MA) was diluted 1:200 in blocking solution and this primary Ab mixture was incubated on cells for 2 hr. Following three washes with PBST (1x PBS in 0.1 % Triton) and 3 washes with PBS, cells were incubated with Alexaflour 488 conjugated goat anti rabbit (1:500) (Invitrogen, Grand Island, NY) and Alexaflour 568 conjugated goat anti mouse (1:500) (Invitrogen) for 40 min, then washed six times with PBS. After drying, the slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were captured with Deltavision wide-field microscope using the 60X objective, then deconvoluted using Autoquant X3. TIFs were quantified using Imaris software.

3.9. Quantitative PCR (qPCR)

HCT116 cells were pelleted and total RNA was isolated according to manufacturer's instructions (Qiagen). cDNA was done by reverse transcryptase and qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) with the following cycling parameters: 95°C for 30 sec and 40 cycles of 95°C for 10 sec, 60°C for 24 sec, and 60°C for 1 sec. Samples were run in triplicate, and data were normalized to untreated control. GAPDH was used as a housekeeping gene.

3.10. Colony formation assay

HCT116 cells were treated with 10 μM 6-thio-dG for 1 week and 10 weeks. Then treated cells were plated at low density (100-300 cells per 10 cm^2 dish) and incubated for 10 days without giving any treatment. Cells were then washed with PBS, fixed and stained with 6% glutaraldeyde plus 0.5% crystal violet, incubated at room temperature for 30 min, washed and the number of colonies was counted.

3.11. Isolation and Culture of Primary Human Lymphocyte

Antecubital vein blood was drawn into a 10 ml EDTA Vaccutainer. One part blood was mixed with three parts 1xPBS and layered over Ficoll (Ficoll-Paque, Amersham-Pharmacia) and centrifuged at 300xg for 30 min at room temperature. Mononuclear cells were collected, washed with 1xPBS and centrifuged at 300xg for 10 min at room temperature. Cells were resuspended in 10 ml of 20% FBS in 1640 RPMI Glutamax media (Life Technologies, Invitrogen, Carlsbad, CA, USA) supplemented with penicilin and streptomycin, then counted. 6-thio-dG (1, 3 and 10 μM) was added ~5 hr after cells were plated into two different 6 well plates and one of them was stimulated with PHA (phytohemagglutinin, 500 ng/ml, Sigma-Aldrich) for the indicated period of time prior to cell counts.

3.12. Drug toxicity animal experiments

All procedures and experiments involving mice were approved by The University of Texas Southwestern Institutional Animal Care and Use Committee and conducted as per institutional guidelines. Wildtype female 129S2 mice were used to determine a 6-thio-dG drug toxicity curve. Mice were randomly divided to control, 6-thio-dG and 6-thioguanine treatment groups. Animals were injected intraperitoneally every day at a dose 5 mg/kg or 1.67 mg/kg in 100 μL DMSO/PBS mixture per mouse. Animals were weighed and observed every day for 25 days (n=3).

3.13. Histology and serum analysis

General Information About Liver Function Tests

Conventional serum liver biochemical tests show current status of liver functions; Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT) are the enzymes that are commonly used for liver tests.

AST catalyses a reversible reaction, which participates in transfering an amino group from aspartic acid to α-ketoglutaric acid resulting in formation of

oxaloacetic acid and glutamic acid. Likewise, ALT transfers an amino group from alanine to α-ketoglutaric acid resulting in formation of pyruvic acid and glutamic acid (Figure 3.2). All transaminases require the coenzyme pyridoxal phosphate (PLP), which is converted into pyridoxamine in the first reaction, when an aminoacid is converted to α-ketoacid. ALT and AST are intracellular enzymes that are found in the cytoplasm including mitochondria (147, 208). However, when hepatocytes are damaged, ALT and/or AST are released from cells into the serum. While AST is found in other tissues such as skeletal muscle, heart muscle, kidney, ALT is more specific to hepatocytes (147).

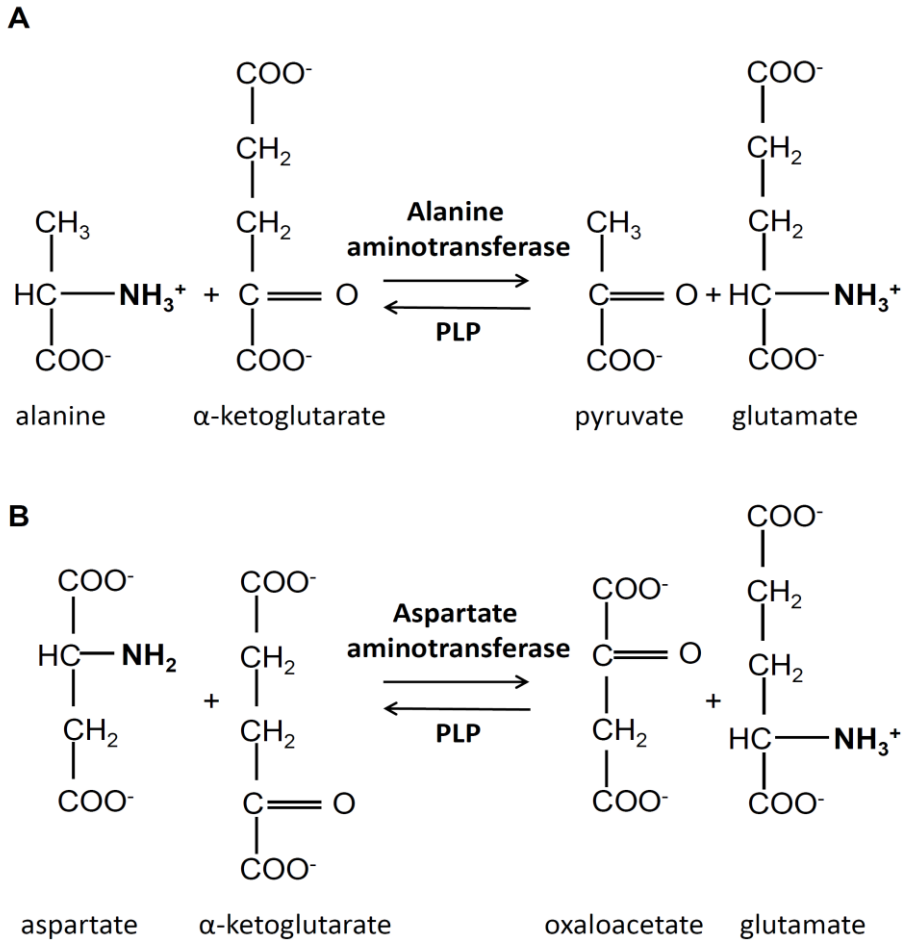


Figure 3.2. Reactions of transaminases. Alanine aminotransferase catalyses the transfer of amino group from L-alanine to α-ketoglutarate. This irreversible reaction produces pyruvate and L-glutamate. Likewise, aspartate aminotransferase catalyses the transfer of amino group from L-aspartate to α-ketoglutarate, then produces oxaloacetate and L-glutamate.

General Information About Kidney Function Tests

There are two serum biomarkers commonly used to detect kidney toxicity in preclinical and clinial studies as well as in routine clinical care. These are serum creatinine and blood urea nitrogen (BUN) (209). Creatinine is excreted by the kidneys. Creatinine is produced by creatine, phosphocreatine and adenosine triphosphate (ATP). Creatine is synthesized from the methylation of glycocyamine by S-adenosyl methionine in liver, then phosphorylated while it is being transported through the blood to the other organs by creatine kinase. While creatine kinase produces phosphocreatinine, creatinine can spontaneously occur (210). Creatinine reacts with picric acid in an alkaine environment and produces a colored compound. The color change that occured in 520/800 nm is directly proportional to the creatinine concentration (211).

Urea is produced by liver in the urea cycle following protein digestion. Urea cycle consists of five reactions: two mitochondrial and three cytosolic. Urea cycle converts two amino groups (one from ammonium, the other from aspartate) and one carbon atom (from bicarbonate) to the excretion product urea. Ornithine is the carrier of these nitrogen and carbon atoms. Urea nitrogen is an other indication marker for kidney function as well as creatinine in blood. Urea is hydrolized to ammonia and carbondioxide by urease. Ammonia then reacts with 2-oxoglutarate and NADH by glutamate dehydrogenase. This reaction produces glutamate and NAD^+. The decrease of NADH absorbance is correlated with the urea concentration (211).

Additionally, histological examination is another way to detect the kidney injury in preclinical animal studies (212, 213).

Procedure

Wildtype female 129S2 mice were used for histology and serum analysis. Mice were randomly divided to control, 6-thio-dG and 6-thioguanine treatment groups. Animals were injected intraperitoneally every two days at a dose 2 mg/kg in 100 μL DMSO/PBS mixture per mouse for 14 days and sacrificed two days after last injection. After euthanasia by CO_2, some organs were examined and fixed in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin, sectioned at 5 μM thickness, stained with hematoxylin and eosin. The following tissues were examined: livers, kidneys, spleen and colon.

For hematology analysis, whole blood was collected via cardiac drawl in Sarstedt 100 μl K3E EDTA tubes and immediately analyzed for red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), white blood cell count (WBC), platelets (PLT), lymphocytes, monocytes, neutrophils, basophils using automated IDEXX ProCyte DX Hematology analyzer (IDEXX Laboratories, Inc., Westbrook, ME, USA) in which laser flow cytometry is used to perform the analysis of white blood cell types and laminar flow impedance for the count of red blood cells.

The liver function tests (AST, ALT) and the kidney function tests (creatinine, BUN) were performed at the same time on a Vitros 250 Analyzer (Ortho Clinical Diagnostics, Johnson & Johnson Co, Rochester, NY) which uses dry slide technology based on reflectance spectrophotometry. The colorimetric/rate slides used in the analyzer are composed of spreading layer, reagent layer, indicator layer and support layer. As the drop of the sample (plasma or serum) moves down these layers, a series of chemical reactions occur. The spreading layer uniformly distributes the sample over the slide area and filters out large molecules such as proteins, lipids and hemoglobin that interfere with the assay. The reagent layer contains enzymes, buffers and catalysts necessary for the chemical reactions. The indicator layer contains a dye or indicator to produce a colored complex. The support layer is made of clear plastic which allows light to pass through it, so the colored complex can be measured. The color development in the slide is proportional to the analyte concentration.

3.14. Establishment of xenograft models

A subcutaneous xenograft mouse model of the human A549 NSCLC was used to evaluate the effects of 6-thio-dG and 6-thioguanine treatment *in vivo*. Athymic NCR nu/nu female mice (~6 weeks old) were used (National Cancer Institute, Bethesda, MD). A total of 2.5 x 10^6 A549 cells were inoculated subcutaneously into the left and right dorsal flanks of the nude mice in 100 μL phosphate buffered saline (PBS). When tumors reached ~40mm^3 average volume, mice were randomly divided as control, 6-thio-dG and 6-thioguanine treatment groups (3 animals in each group). Animals were injected intraperitoneally every two days for 17 days at a dose of 2 mg/kg in 100 μL DMSO/PBS mixture per mouse. In addition, different animals were injected intratumorally every day for 16 days at a dose of 2.5 mg/kg in 50 μL DMSO/PBS mixture per mouse. Tumor size was measured by calipers and recorded either every day or every two days. Tumor volumes were calculated by taking length to be the longest diameter across the tumor and width to be the corresponding perpendicular diameter, using the following formula: (length x $width^2$) mm^2 x 0.5. No animal died during the experimental period. The animals were sacrificed by CO_2 inhalation 24 hr following the last dose of treatment. The tumors were resected, fixed with 10% neutral buffered formalin and paraffin-embedded for sectioning and immunohistochemistry staining.

3.15. Ki67 proliferation assay

Tissues were processed, paraffin-embedded, and cut in to 5-μm sections. Sections were deparaffinized, rehydrated, and then antigens retrieved with citrate buffer (10 mmol/L sodium citrate, pH 6.0; 0.05% Tween 20) in microwave medium power (20 min). Endogenous peroxidase, biotin, and proteins were sequentially blocked with solutions of 3% hydrogen peroxide (Sigma-Adrich), the Avidin/Biotin Blocking Kit (Vector Laboratories), and 10% bovine serum albumin (Vector Laboratories). Ki67 primary antibody (1:1000) were diluted in 1× PBS and then sections were incubated 30 min at room temperature. After washing with PBS, secondary antibody and ABC reagent were applied using the VECTASTAIN ABC Kit (Vector Laboratories) following the manufacturer's protocol. Tissue sections were then incubated with ImmPACT DAB peroxidase substrate (Vector

Laboratories), counterstained with hematoxylin, and then dried overnight before mounting coverslip. Images were captured with Axiovision software v4.6.3 on Axioskop 2 plus microscope mounted with AxioCamHR color camera (Carl Zeiss Microscopy) using Plan-APOCHROM 20x, 40x and 63x objectives.

3.16. ImmunoFISH

Tissue sections were processed for TIF analysis by fluorescence in situ hybridization (FISH) as described previously (136). Briefly, to deparaffinize 5 μM tissue sections, xylene (2x5 min), 100% ethanol (2x2 min), 95% ethanol (1x2 min), 75% ethanol (1x2 min), 50% ethanol (1x2 min) were sequentially used and then washed with tap water (2x3 min). Deparaffinized tissue sections were incubated in sodium citrate buffer (10 mM Na-citrate, 0.05 % Tween 20, pH 6.0) at microwave (medium power) for 20 min to retrieve antigens. After tissue sections cooled down, they were rinsed with 1 x PBS for 5 min and then dehydrated in 95% ethanol for 3 min. After air-drying, denaturation was conducted with hybridization buffer containing FITC-conjugated telomere sequence $(TTAGGG)_3$-specific peptide nucleic acid (PNA) probe (PNA Bio, Thousand Oaks, CA), 70% formamide, 12 mM Tris-HCl pH=8.0, 0.5 mM KCl, 1mM $MgCl_2$, 0.08% Triton X-100 and 0.25% BSA for 5 min at 80°C, followed by 2 hr incubation in the same buffer at 37°C. Slides were washed sequentially with 70% formamide (Ambion, Life Technologies, Grand Island, NY) / 0.6 x SSC (Invitrogen) (3 x 15 min), 2 x SSC (1 x 15 min), PBS (1 x 5 min), PBST (PBS + 0.1% Tween 20; 1 x 5 min) and incubated with blocking buffer (4% BSA in PBST) for 30 min. Sections were incubated with primary polyclonal anti-53BP1 antibody (1:500) (Novus Bio, Littleton, CO) in blocking buffer at room temperature for 1 hr. Following 2 x 5 min washes with PBST, tissue sections incubated with Alexaflour 568 conjugated goat anti Rabbit (1:500) (Invitrogen) in blocking buffer at room temperature for 1 hr. Sections were washed sequentially with PBST (3 x 5 min) and PBS (1 x 5 min). The slides were mounted with Vectashield mounting medium with DAPI. Images were captured with a Deltavision wide-field microscope using a 100X objective, then deconvoluted using Autoquant X3. TIFs were quantified using Imaris software.

3.17. Immunohistochemical analysis

Formalin-fixed, paraffin-embedded tissue was used for immunohistochemical analyses. Sections were cut to 5 μm, deparaffinized and stained with haematoxylin I and eosin Y (Fisher Scientific, Pittsburgh, PA).

3.18. Statistical analysis

The observed datas were presented as mean values ± Standard Deviation (SD). SDs were showed at above to make graphs more clear. Comparisons of different groups for statistical significance were analyzed using a two-tailed, unpaired Student t test. *P* value of 0.05 or less was considered significant. Statistically analyses were performed using GraphPad Prism software version 6.01.

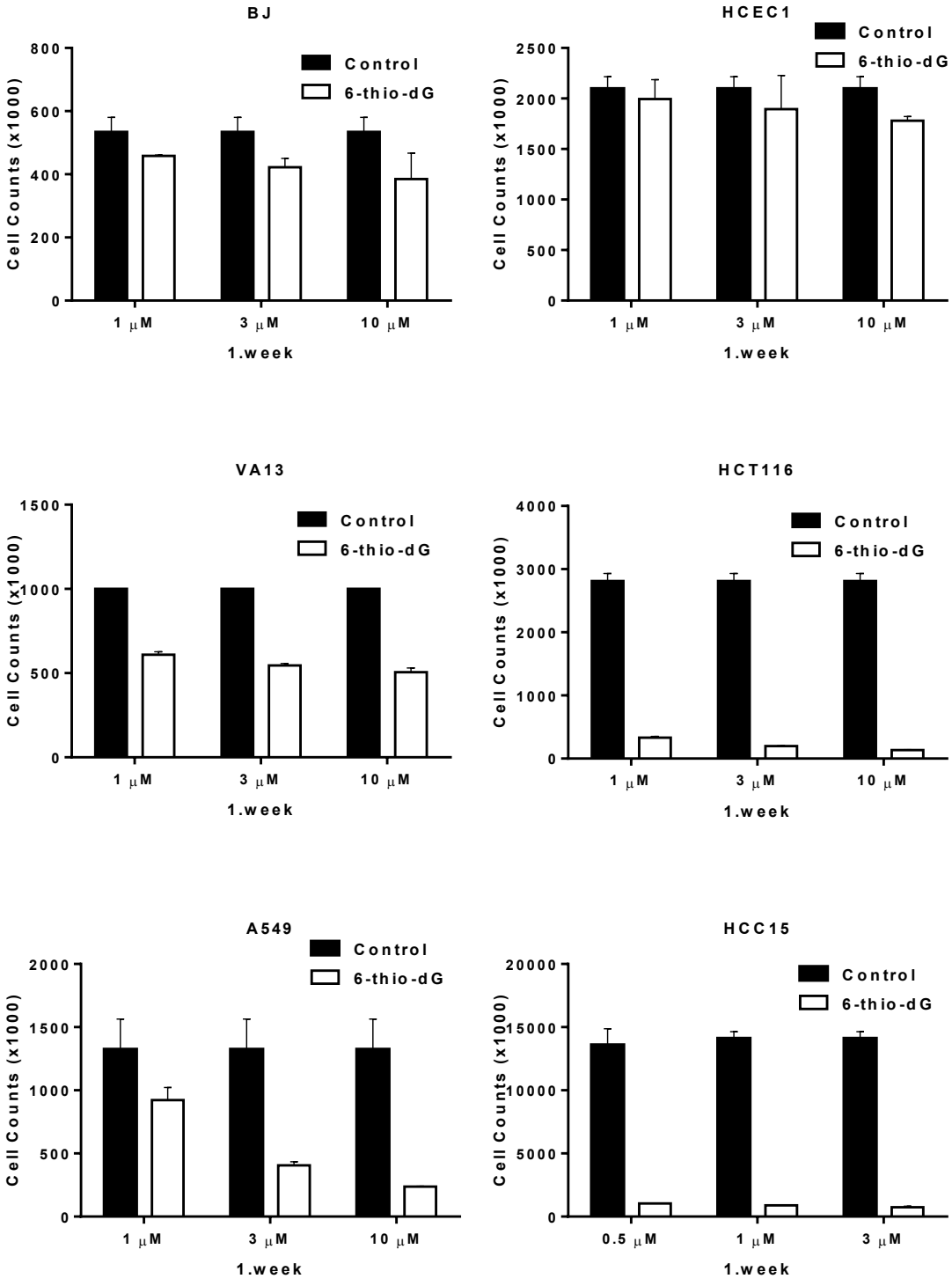
4. RESULTS

4.1. Induction of Telomere Dysfunction Mediated by the Telomerase Substrate Precursor 6-Thio-2'-Deoxyguanosine

(Ilgen Mender, Sergei Gryaznov, Zeliha G. Dikmen, Woodring E. Wright, Jerry W. Shay) (Cancer Discovery, 2014, in press)

Human BJ normal fibroblasts, normal diploid immortalized human colonic epithelial cells (HCEC1), colon cancer (HCT116), VA13 (ALT-telomerase negative) and the panel of non small cell lung cancer (H2882, HCC2429, HCC827, HCC15, H2087, HCC4017, HCC515, H2009) cell lines were tested for *in vitro* effects of 6-thio-dG and 6-thioguanine (0.5-10 μM) every three days for a one week period. At the end of one week treatment period, 6-thio-dG and 6-thioguanine treatment resulted in the death of the vast majority of HCT116, A549, H2882, HCC2429, HCC827, HCC15, H2087, HCC4017, HCC515 and H2009 cancer cell lines, whereas human BJ fibroblasts, HCEC1 and VA13 ALT cells were not significantly sensitive. The growth inhibition results of BJ fibroblasts, HCEC1, VA13, HCT116, A549 and HCC15 cells following one week 6-thio-dG treatment and the morphology of BJ, HCEC1, HCT116, A549 and HCC15 cells following one week 6-thio-dG and 6-thioguanine treatment are shown in Figure 4.1A, and B, respectively.

A


BJ
Control
6-thio-dG
Cell Counts (x1000)
800
600
400
200
0
1 μM
3 μM
10 μM
1.week
HCEC1
Control
6-thio-dG
Cell Counts (x1000)
2500
2000
1500
1000
500
0
1 μM
3 μM
10 μM
1.week
VA13
Control
6-thio-dG
Cell Counts (x1000)
1500
1000
500
0
1 μM
3 μM
10 μM
1.week
HCT116
Control
6-thio-dG
Cell Counts (x1000)
4000
3000
2000
1000
0
1 μM
3 μM
10 μM
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A549
Control
6-thio-dG
Cell Counts (x1000)
2000
1500
1000
500
0
1 μM
3 μM
10 μM
1.week
HCC15
Control
6-thio-dG
Cell Counts (x1000)
20000
15000
10000
5000
0
0.5 μM
1 μM
3 μM
1.week

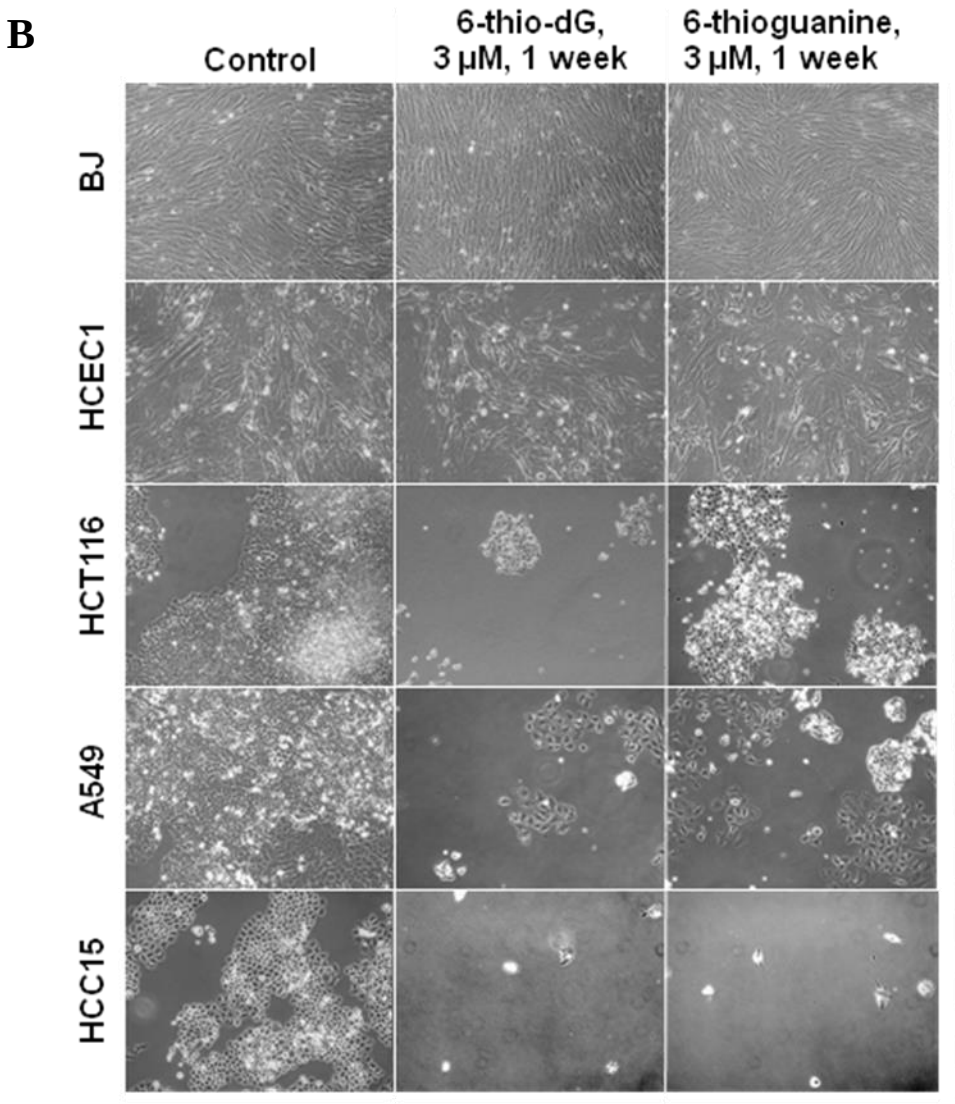
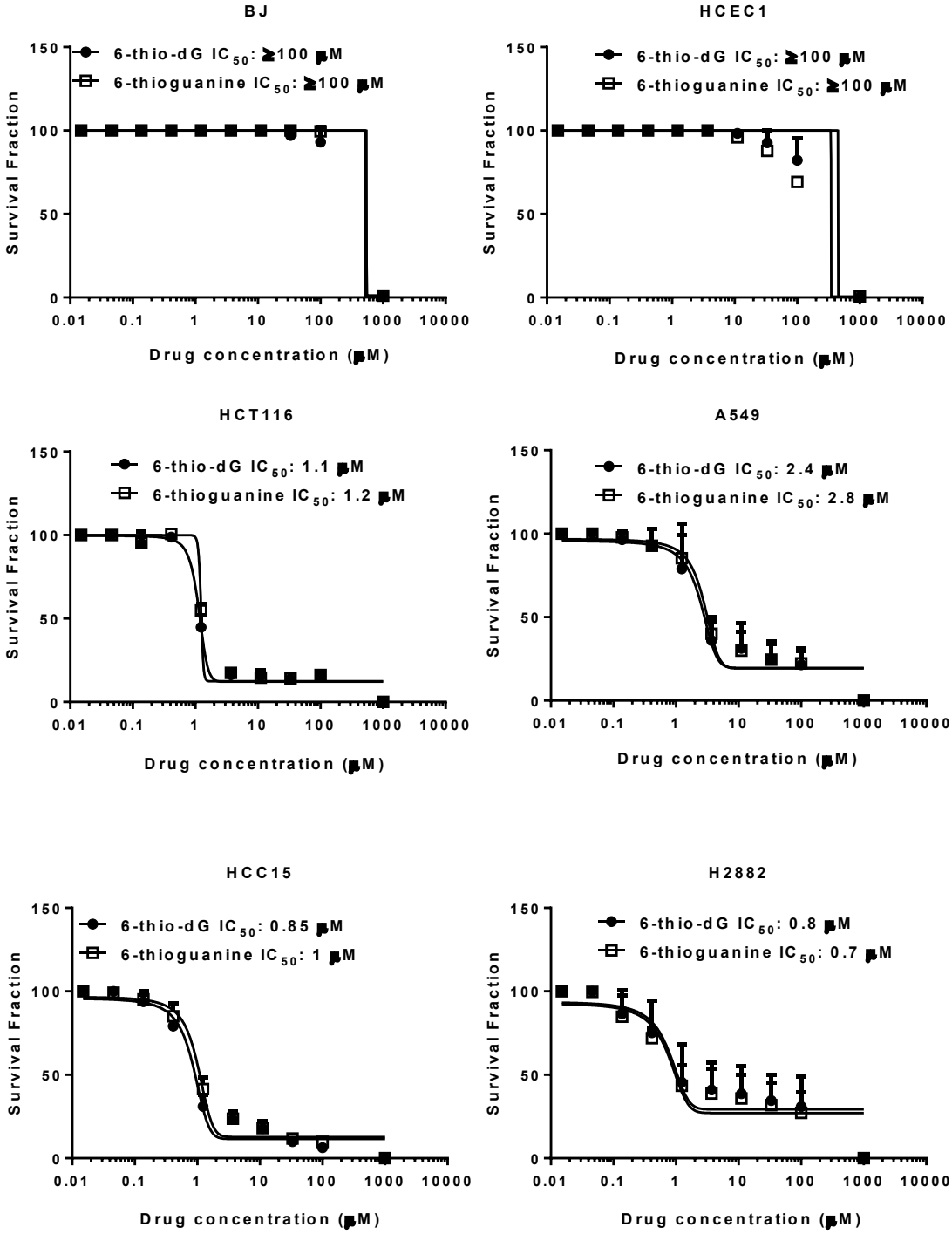


Figure 4.1. The growth inhibition kinetics of normal human BJ fibroblasts, colonic epithelial cells (HCEC1), VA13 (ALT-telomerase negative), HCT116 colon, A549 and HCC15 NSCLCs. A) The cell counts of BJ, HCEC1, VA13, HCT116, A549 and HCC15 cells were treated with 0.5-10 µM 6-thio-dG for 1 week. B) The morphology of BJ, HCEC1, HCT116, A549 and HCC15 cells were treated with 3 µM 6-thio-dG and 3 µM 6-thioguanine for 1 week. SDs are from two independent experiments.

4.1.1. The Effects of 6-thio-dG and 6-thioguanine on Cell Viability

To investigate if there is any difference between 6-thio-dG and 6-thioguanine on cell viability, BJ, HCEC1, HCT116, A549, H2882, HCC2429, HCC827, HCC15, H2087, HCC4017, HCC515 and H2009 cells were seeded into 96 well plates. Following cell attachment, they were treated with 10 different concentrations of 6-thio-dG and 6-thioguanine (1:3 serial dilutions) every three days. Following one week treatment, the cell viability was determined by using CellTiterGlo luminescence assay. The results showed that 6-thio-dG (IC50 values: 0.7-2.9 μM) and 6-thioguanine (IC50 values: 0.7-3.5 μM) have similar toxic effects on cancer cells that were tested. Importantly, both BJ and HCEC1 normal cells were mostly resistant to 6-thio-dG and 6-thioguanine treatment in cell viability assays (Figure 4.2).


BJ
6-thio-dG IC50: ≥100 μM
6-thioguanine IC50: ≥100 μM
HCEC1
6-thio-dG IC50: ≥100 μM
6-thioguanine IC50: ≥100 μM
HCT116
6-thio-dG IC50: 1.1 μM
6-thioguanine IC50: 1.2 μM
A549
6-thio-dG IC50: 2.4 μM
6-thioguanine IC50: 2.8 μM
HCC15
6-thio-dG IC50: 0.85 μM
6-thioguanine IC50: 1 μM
H2882
6-thio-dG IC50: 0.8 μM
6-thioguanine IC50: 0.7 μM
Survival Fraction
Drug concentration (μM)
0
50
100
150
0.01
0.1
1
10
100
1000
10000

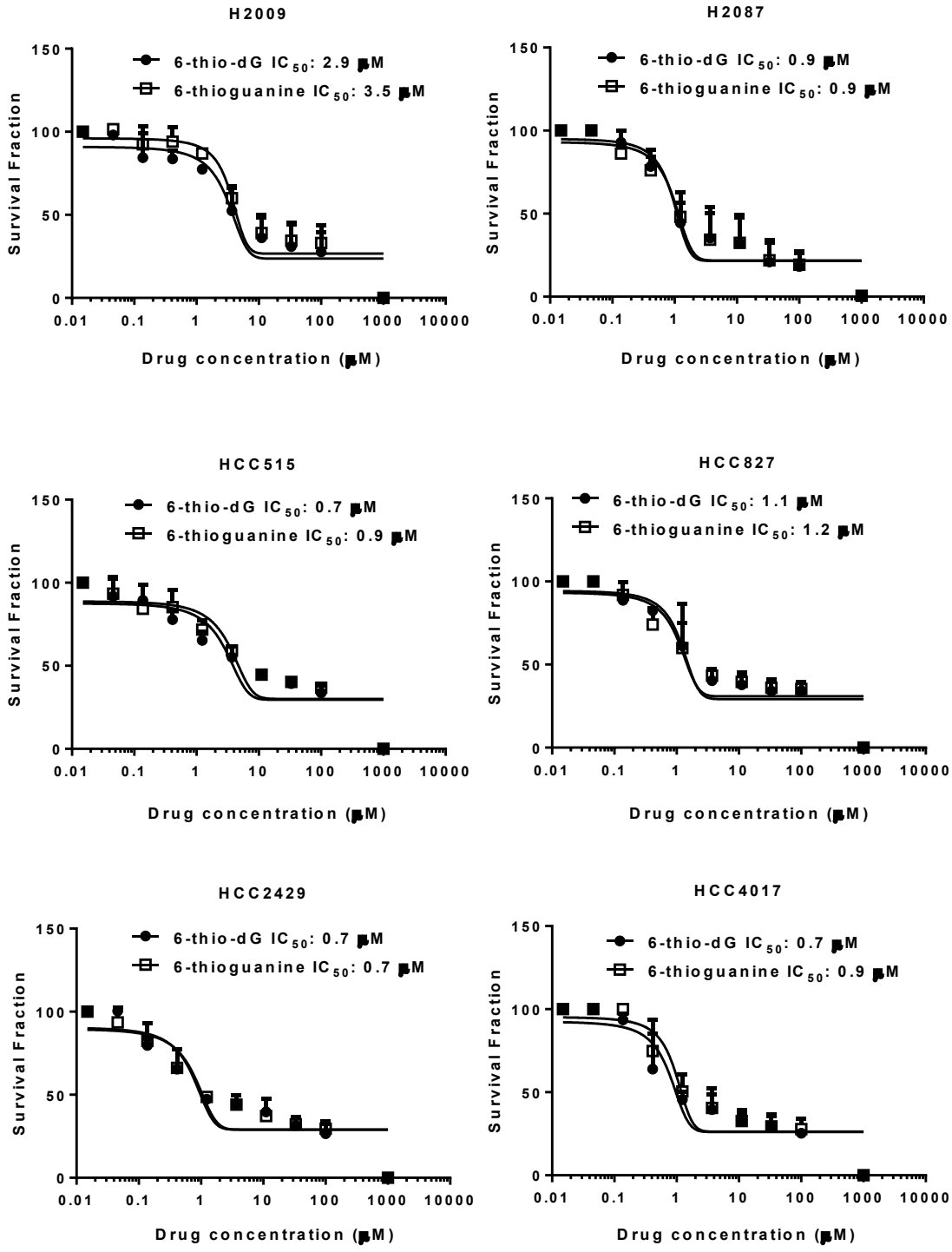


Figure 4.2. IC50 values of BJ, HCEC1, HCT116, A549, HCC15, H2882, H2009, H2087, HCC515, HCC827, HCC2429 and HCC4017 following one week treatment with 6-thio-dG and 6-thioguanine. All samples were analyzed in triplicate and SDs are from two independent experiments.

Since these tumor cells lines had different telomere lengths, the cell killing effects of 6-thio-dG and 6-thioguanine were mostly independent from initial telomere length (Table 4.1).

Table 4.1. Average telomere lengths of normal versus cancer cell lines and IC50 values of these cell lines following one week treatment with 6-thio-dG and 6-thioguanine.

Cell Type	Telomere Length (kb)	6-thio-dG (μM)	6-thioguanine (μM)
BJ	6±0.35	≥100	≥100
HCEC1	7.5±0.5	≥100	≥100
HCT116	7.7±0.66	1.1	1.2
A549	5.1±2.85	2.4	2.8
H2882	3.4±0.08	0.8	0.7
HCC2429	2±0.1	0.7	0.7
HCC827	1.6±0.5	1.1	1.2
HCC15	3.1±0.36	0.85	1.0
H2087	2.4±0.004	0.9	0.9
HCC4017	3.4±0.58	0.7	0.9
HCC515	3±0.39	0.7	0.9
H2009	8.4±0.54	2.9	3.5

4.1.2. Telomere Dysfunction Induced Foci (TIF) Induced by 6-thio-dG, but not 6-thioguanine

To understand whether 6-thio-dG or 6-thioguanine causes telomeric damage on cancer cell, HCT116 cells were seeded on chamber slides and treated with 3 μM 6-thio-dG and 3 μM 6-thioguanine following cell attachment at variable time points (30 min, 3 hr, 12 hr, 24 hr, 48 hr and 72 hr). After gamma-H2AX and TRF2 co-localization staining was conducted, images were captured by fluorescence microscopy and analyzed by Imaris Software. Cells that had 4 or more than 4 TIFs were accepted as damaged telomeres in interphase. The results showed that 6-thio-dG treatment induced TIFs starting at 12 hr post drug treatment. At 72 hr post 6-thio-dG treatment resulted in a 7.8 fold increase in telomeric DNA damage compared to 6-thioguanine (Figure 4.3A and B). Since telomeres constitute only ~1/6000th of total genomic DNA, any TIFs above a background threshold are likely to be significant. When general DNA damage was analyzed, it was found that 6-thio-dG causes modest general DNA damage as well as telomeric damage compared to 6-thioguanine which only caused general DNA damage (Figure 4.3C). To understand better if these results are dependent on telomerase, we generated normal BJ cells overexpressing hTERT that have detectable telomerase activity. BJ and BJhTERT cells were treated with 10 μM 6-thio-dG or 10 μm 6-thioguanine for 48 hr, stained with gamma-H2AX and TRF2 and co-localization analysis was conducted (Figure 4.4A). We observed that 6-thio-dG and 6-thioguanine did not induce telomere dysfunction (TIFs) in telomerase silent BJ cells following 48 hr treatment (Figure 4.4A and B), whereas 6-thio-dG, but not 6-thioguanine, caused significant increased of telomere dysfunction in hTERT telomerase expressing BJ cells (BJ hTERT+) compared to controls (Figure 4.4A and C). This increase in co-localization was 3.3 % (TIF≥5), 4.2 % (TIF≥6) or 5.7% (TIF≥7) of cells treated with 6-thio-dG. These results indicate that 6-thio-dG is rapidly converted to 6-thio-dGTP, recognized by telomerase and incorporated into telomeres in telomerase expressing cells, while 6-thioguanine is not.

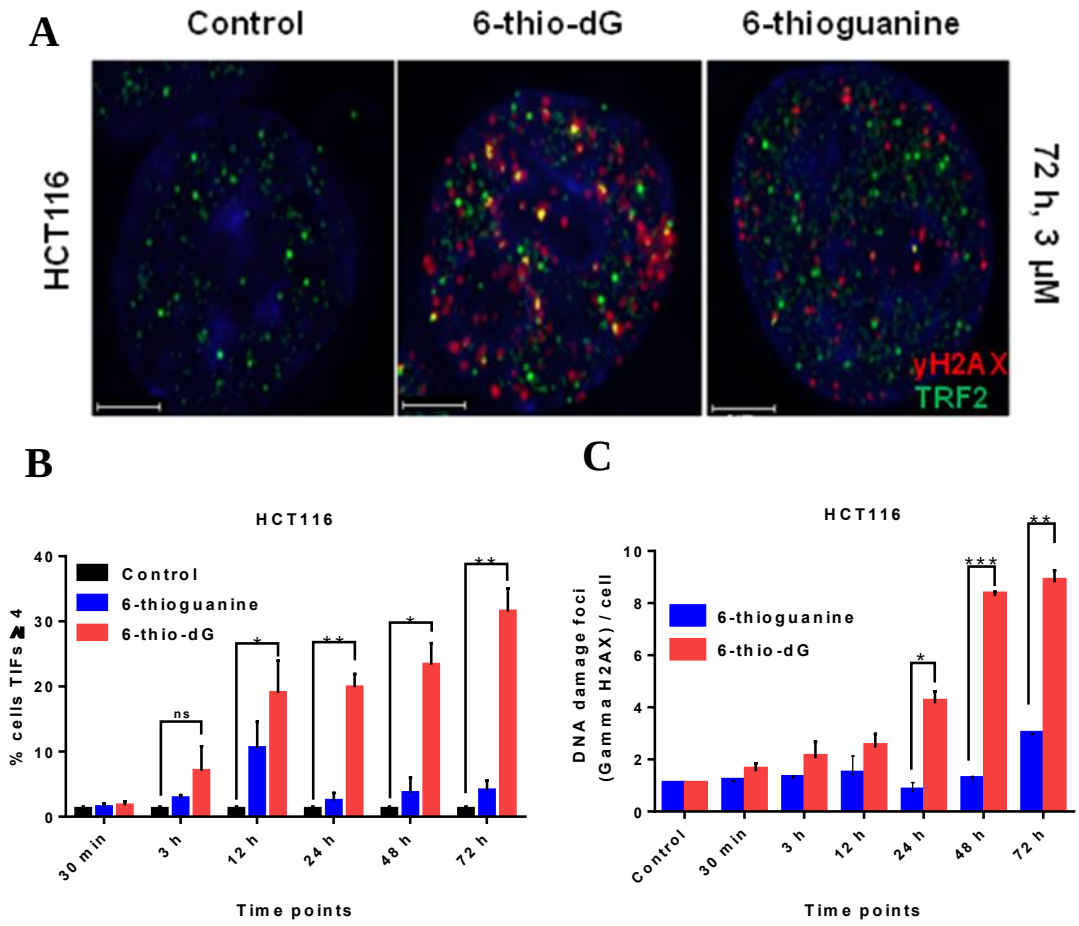


Figure 4.3. Colocalization of gamma-H2AX and TRF2 exerts DNA damage on telomeres. A) Representative image of HCT116 cells treated with 3 μM 6-thio-dG and 3 μM 6-thioguanine or without any treatment. 6-thio-dG treatment induces telomeric damage (Red: dots: gamma-H2AX, green dots: TRF2, yellow dots: TIF or telomeric damage). B) Graphs show the TIF index of 6-thio-dG (3 μM), 6-thioguanine (3 μM) treatment or control. The cell that has more or more than 4 TIFs (gamma-H2AX and TRF2 colocalization) as scored TIF positive. The percentage of TIF positive cells following 6-thio-dG treatment showed gradually TIF induction at variable time points in HCT116 cells compared to control or 6-thioguanine treated cells (n>55, SDs from two independent experiments, **P*<0.05, ***P*=0.006 (control:6-thio-dG), ns: non-significant differences in the unpaired Student *t* test). C) Relatively DNA damage per foci. HCT116 cells were treated with 3 μM 6-thio-dG and 3 μM 6-thioguanine (n>55, SDs from two independent experiments, **P*=0.0141, ***P*=0.003, ****P*=0.0005 (6-thio-dG:6-thioguanine), ns: non-significant differences in the unpaired Student *t* test).

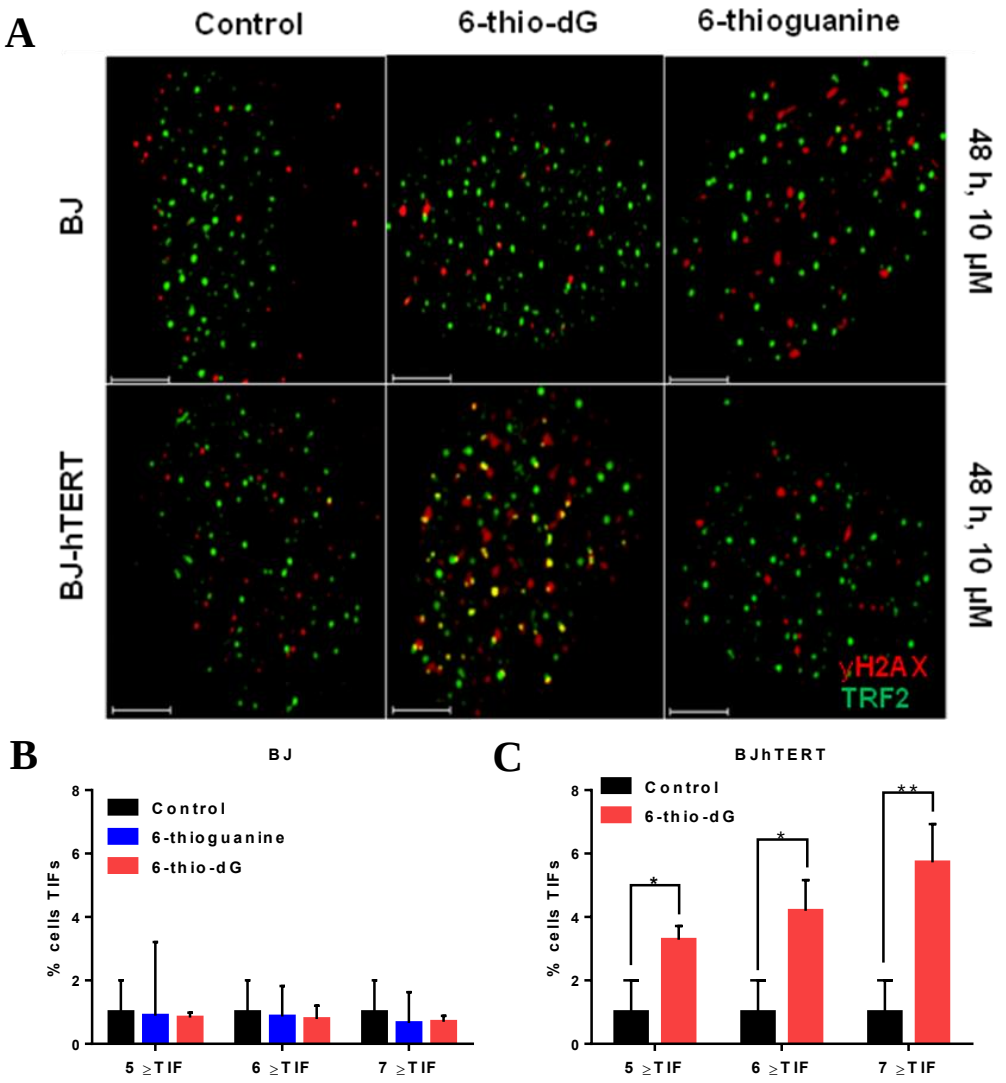


Figure 4.4. Binding of gamma-H2AX on damaged telomeres. A) Representative image of BJ and BJ hTERT cells following with 6-thio-dG (10 μM) or 6-thioguanine (10 μM) treatment for 48 hr and also comparison with control. Merged images show either gamma-H2AX (red) and TRF2 (green) seperately or colocalized (yellow). B) Shown the percentage of TIF positive cells (5≥TIF, 6≥TIF, 7≥TIF) following 6-thio-dG (10 μM) and 6-thioguanine (10 μM) treatment for 48 hr in BJ and, C) BJ hTERT cells. 6-thio-dG induced telomeric colocalization in BJhTERT cells, but not in BJ cells. 6-thioguanine did not cause significant increase of telomeric colocalization in BJ and BJhTERT cells (n=85 for control, n=83 for 6-thio-dG and n=81 for 6-thioguanine treated BJ and BJhTERT cells, SDs are from two independent experiments for BJ and three independent experiments for BJhTERT cells). *P<0.02, **P=0.064 (control:6-thio-dG) in the unpaired Student t test.

4.1.3. 6-thio-dG, but not 6-thioguanine, Induces Progressive Telomere Shortening via Independent Mechanism from Telomerase Inhibition in Cancer Cells

In order to investigate whether 6-thio-dG or 6-thioguanine causes telomere shortening as well as acute TIF induction, HCT116 and BJ cells were treated with two different concentrations of 6-thio-dG (1 and 10 μM) and 6-thioguanine (1 and 10 μM) every three days for up to 12 weeks. Telomere lengths of HCT116 and BJ cells were evaluated using the TRF (Telomere Restriction Fragment) assay. While 6-thio-dG starts to shorten telomere lenghts of surviving HCT116 cells even after 1 week treatment (Figure 4.5A), 6-thioguanine did not cause any changes in telomere length of HCT116 cells (Figure 4.5B). In addition, following 12 weeks treatment, 6-thio-dG leads to even more dramatic telomere shortening in HCT116 cells that are not immediately killed (Figure 4.5A). This indicates that 6-thio-dG participates in biochemical and metabolic processes to a lesser extent and is more rapidly converted to 6-thio-dGTP to be incorporated into telomeres compared to 6-thioguanine. In addition, 6-thio-dG (Figure 4.5C) and 6-thioguanine (data not shown) treatment for up to 10 weeks did not cause any additional telomere shortening on telomerase negative BJ cells. In order to investigate the effects of 6-thio-dG on telomerase activity, HCT116 cells were treated with 1-10 μM 6-thio-dG for 1 week and as expected there was no telomerase inhibition on HCT116 cells (Figure 4.6). These results show that 6-thio-dG causes progressive telomere shortening as well as acute telomere dysfunction by being recognized by telomerase and incorporated into telomeres via independent mechanism from telomerase inhibition.

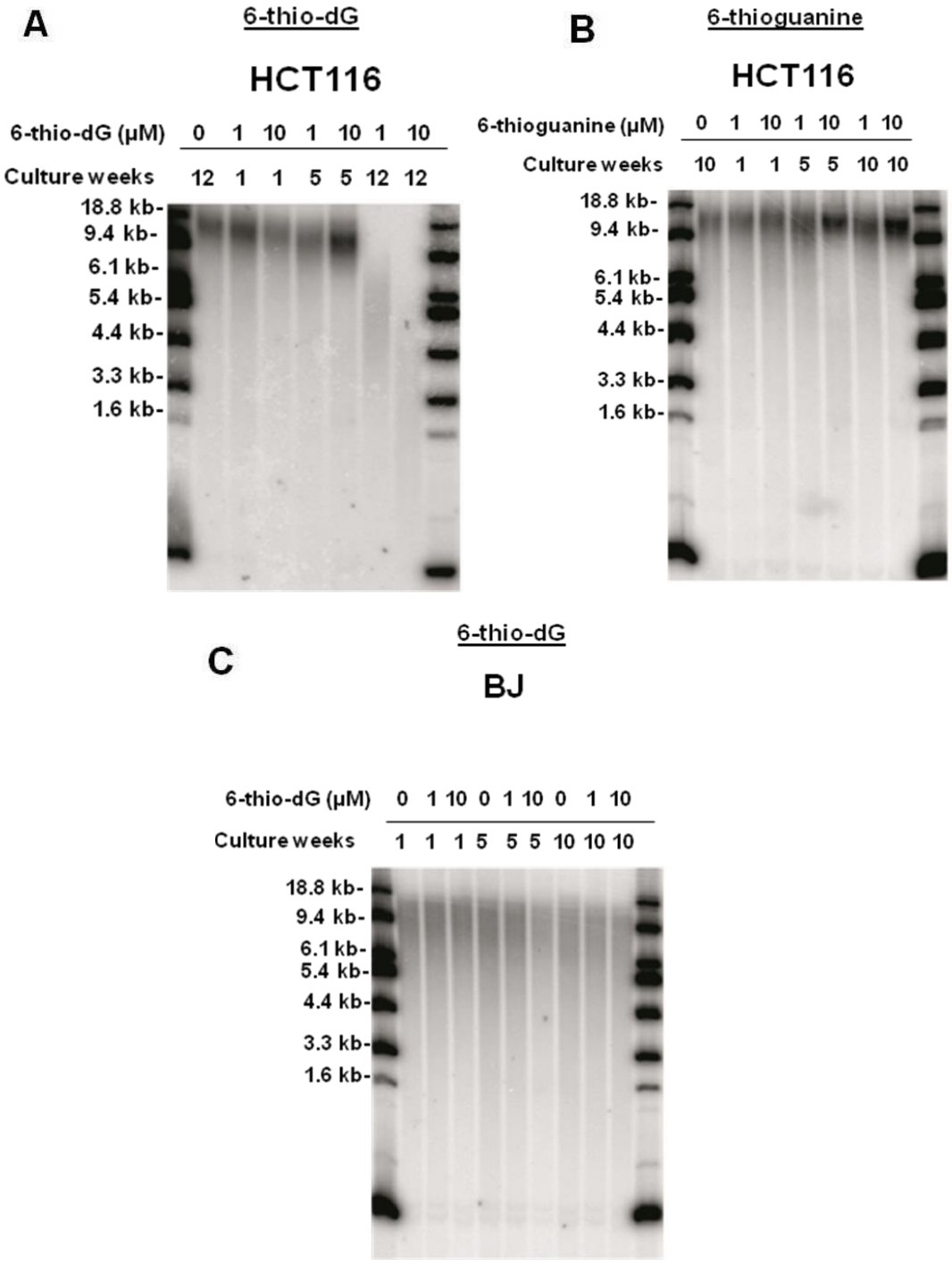


Figure 4.5. Telomere shortening analysis by TRF (Terminal Restriction Fragment) assay A) HCT116 cells treated with 6-thio-dG B) HCT116 cells treated with 6-thioguanine C) BJ cells were treated with 6-thio-dG (1-10 μM) every three days for 1-12 weeks. Cells were collected in indicated numbers of weeks for TRF. TRF lenghts are expressed as kilobase pairs (kb) (Control: untreated).

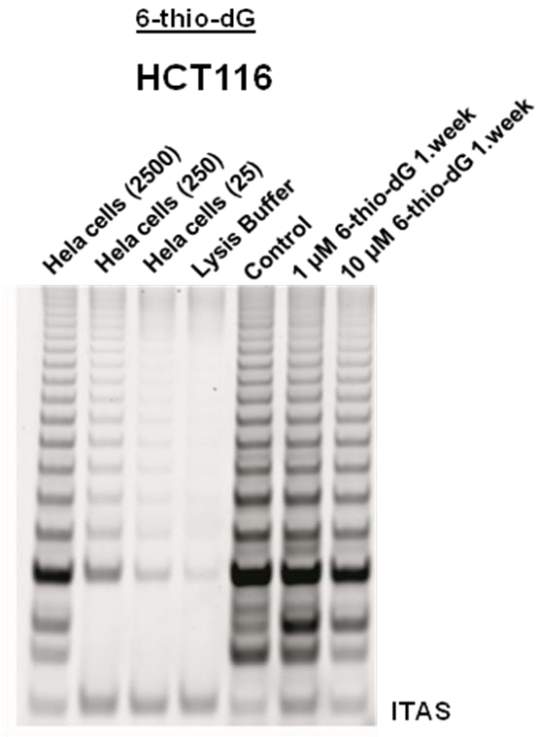


Figure 4.6. Telomerase activity analysis by TRAP (Telomeric Repeat Amplification Protocol). HCT116 cells were treated with 1-10 μM 6-thio-dG every three days for 1-10 weeks. Cells were collected following one week treatment for TRAP assay. Serial dilutions of Hela cells and lysis buffer were used as a positive and negative control, respectively (Control: untreated).

4.1.4. 6-thio-dG does not Lead to Signficant Weight Loss or Hematological Toxicities in Wild Type Mice

To determine effective and less toxic drug concentration for *in vivo* experiments, we used 129S9 wild type mice. We randomly separated the mice into different groups as controls, 6-thio-dG and 6-thioguanine at different concentrations were injected intraperitonealy daily. We initiated the experiments with 1.67 and 5 mg/kg drug concentrations because it was reported that higher than 3 mg/kg 6-thioguanine has toxic effects on mice (214). 6-thio-dG, 6-thioguanine and DMSO controls were injected intraperitoneally every day for 25 days. Three mice treated with 5 mg/kg 6-thioguanine died following 17 days of treatment (Figure 4.7A). Three mice treated with 1.67 mg/kg 6-thioguanine did not gain weight and started to lose weight during the 25 days treatment period (Figure 4.7B). In contrast to

6-thioguanine, 5 mg/kg 6-thio-dG treatment did not lead to mice death and the mice weight was stable during the 25 days of treatment (Figure 4.7A). 6-thio-dG did not cause weight loss as well as any mice death at 1.67 mg/kg (Figure 4.7B). Based on these results, we initiated mice xenograft experiments with ~2 mg/kg drug concentration.

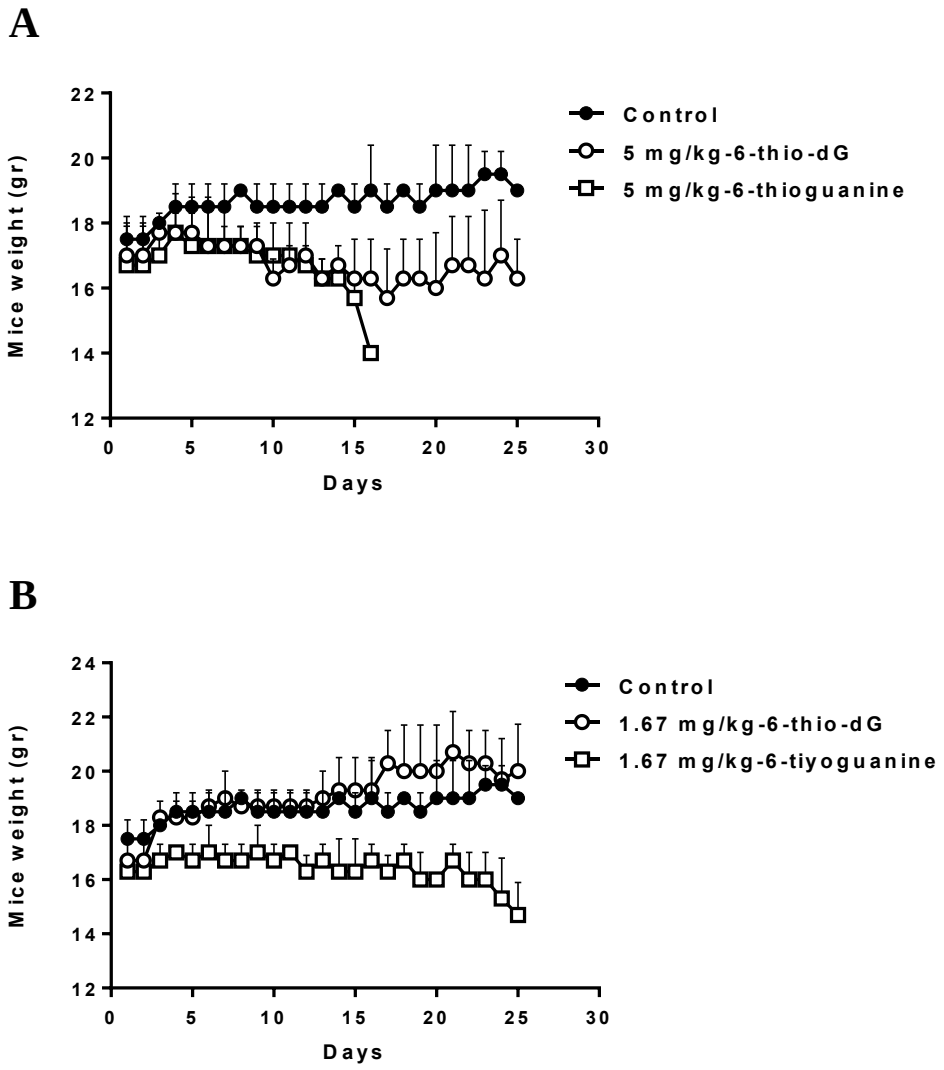


Figure 4.7. Wild type mice weight during 6-thio-dG and 6-thioguanine treatment with different doses. A) 5 mg/kg 6-thio-dG, 5 mg/kg 6-thioguanine or DMSO control, B) 1.67 mg/kg 6-thio-dG, 1.67 mg/kg 6-thioguanine or DMSO control were injected intraperitoneally to wild type mice and mice were observed and scaled for 25 days, daily.

In addition to weight loss, we wanted to investigate if there were any hematological toxicities in mice treated with 6-thio-dG. 129S2 wild type were injected every other day with 2 mg/kg 6-thio-dG, 2mg/kg 6-thioguanine or DMSO control for 14 days. Two days after the last injection, mice were sacrificed and blood was collected for complete blood count (CBC) analysis. Red blood cell (RBC), white blood cell counts (WBC), hemoglobin (HGB), lymphocytes, hematocrit (HCT), monocytes, neutrophils, and basophils were evaluated. 6-thio-dG and 6-thioguanine did not cause hematological toxic effects except minor neutropenia, although the neutrophil difference between 6-thio-dG or 6-thioguanine versus control was not significant (Figure 4.8).

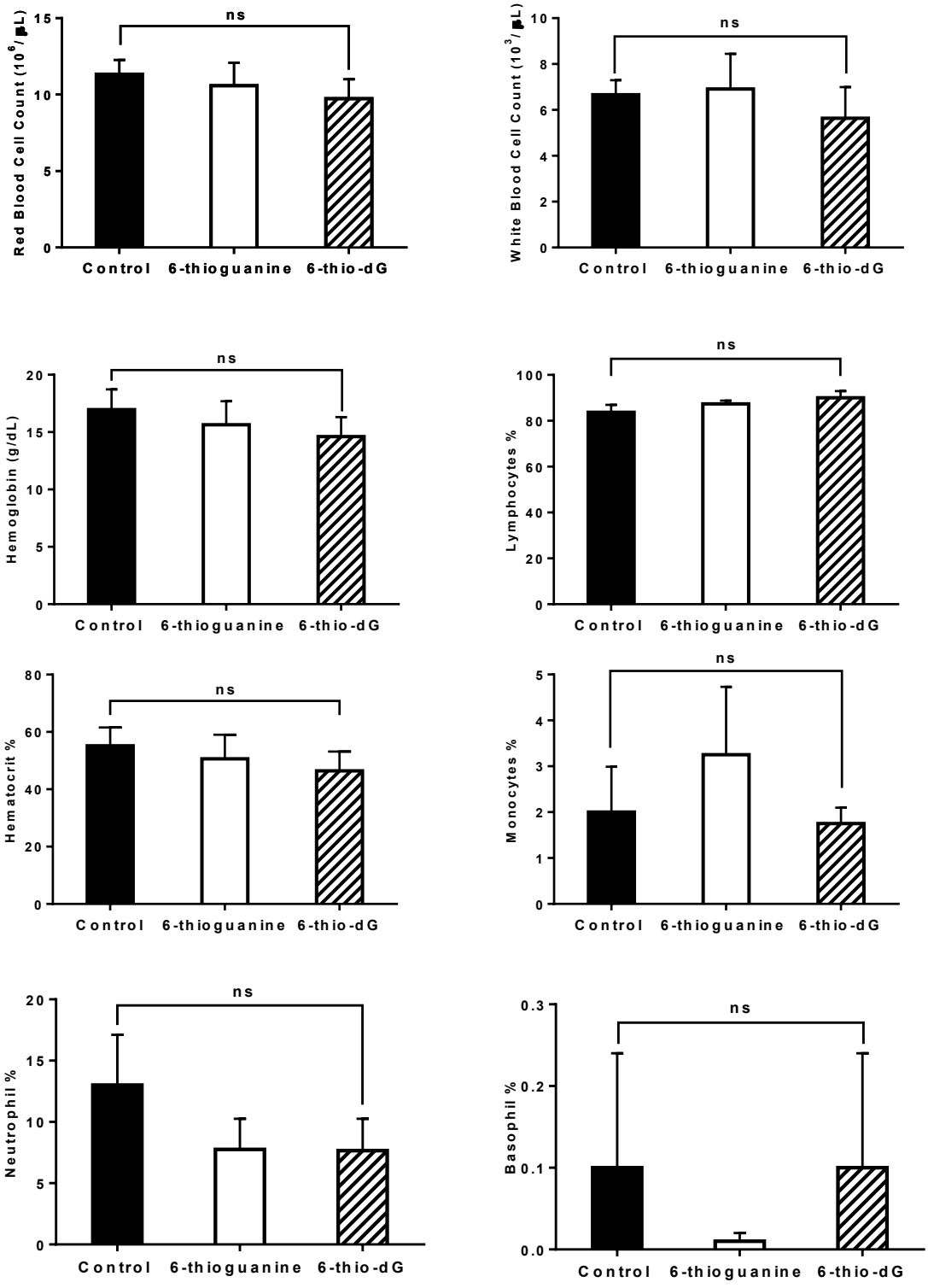


Figure 4.8. Complete blood count following 6-thio-dG (2 mg/kg) and 6-thioguanine (2 mg/kg) treatment in wild type mice. 6-thio-dG and 6-thioguanine were injected intraperitoneally and every other days for 14 days (ns: non-significant).

4.1.5. 6-thio-dG does not Cause Significant Liver, Kidney and Gastrointestinal Tract Side Effects

In order to determine if 6-thio-dG caused any organ toxicities such as liver, kidney, gastrointestinal (GI) tract, 129S2 wild type mice were intraperitoneally injected with 6-thio-dG (2 mg/kg), 6-thioguanine (2 mg/kg) and DMSO control every two days for 14 days. Two days after the last injection, mice were sacrificed and blood was collected for liver and kidney function tests. In addition, organs were resected, fixed, and prepared for H&E staining. Liver functions tested by aspartate amino transferase (AST), alanine aminotransferase (ALT) levels. Kidney function tests included creatinine and blood urea nitrogen (BUN) levels. The results showed that 2 mg/kg 6-thio-dG did not cause any significant toxic effect on liver and kidney functions over a two week treatment period. While 2 mg/kg 6-thioguanine (already approved compound) did not cause increased of ALT, creatinine and BUN levels, it did result in a slight increase in AST levels, but this increase was statistically non significant (Figure 4.9A and B). Additionally, we evaluated the histopatology of the liver, kidney, spleen and colon with 6-thio-dG treatment and we did not observe any histopathological alterations with 6-thio-dG treatment compared to controls (Figure 4.9C). We did observe some liver necrosis in the 6-thioguanine treated animals (data not shown).

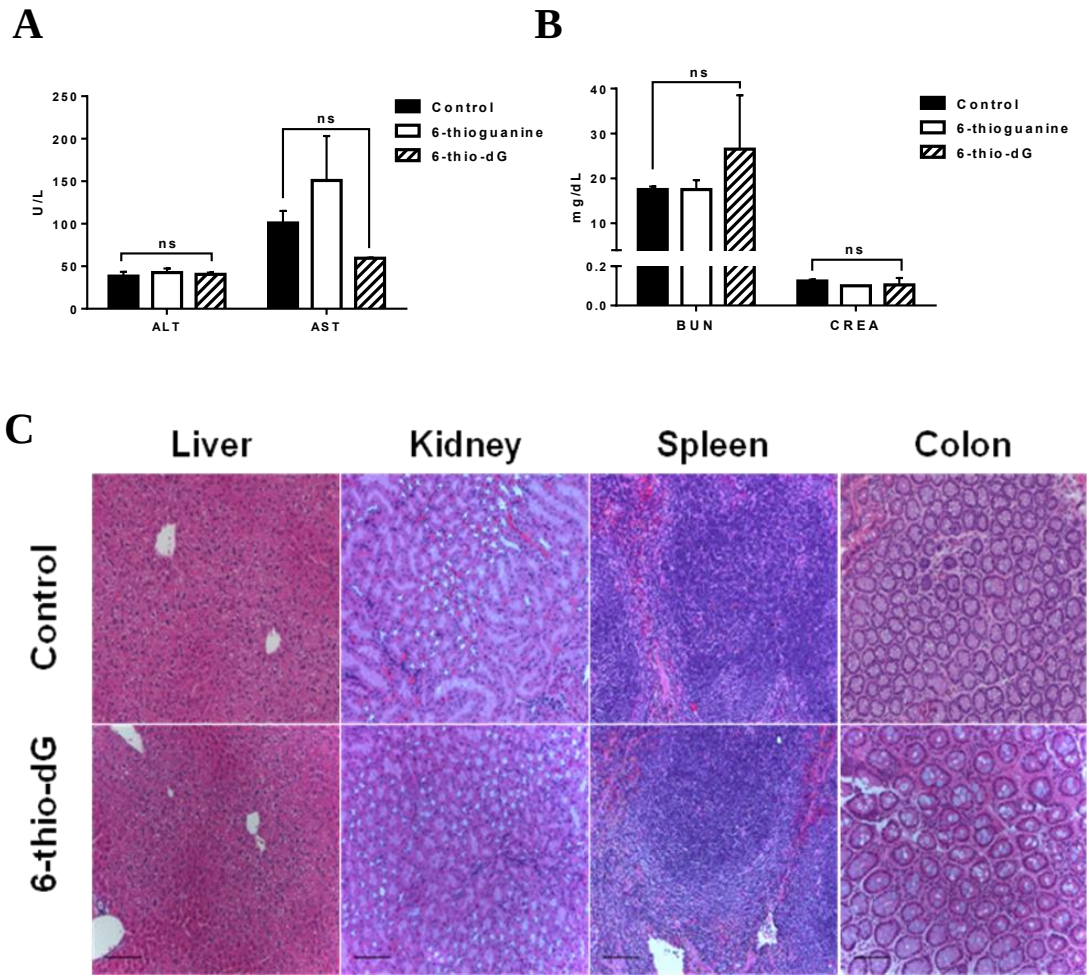
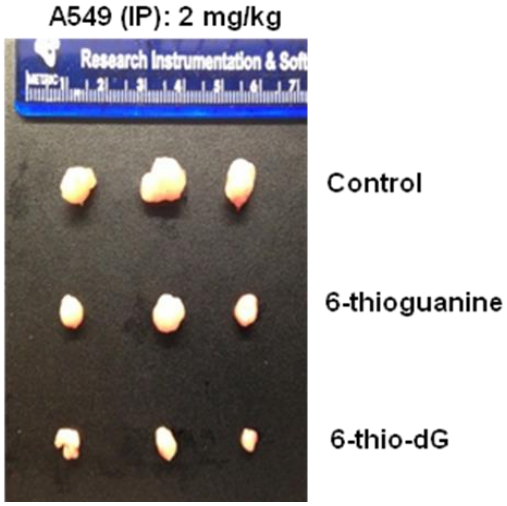


Figure 4.9. Liver, kidney function tests and histopatological evaluation of liver, kidney, spleen and colon. A) ALT (Alanine transminase) and AST (Aspartate transaminase) levels for liver function. B) CREA (Creatinin) and urea nitrogen (BUN) levels for kidney function following 6-thio-dG (2 mg/kg) and 6-thioguanine (2 mg/kg) treatment for 14 days (every other day, i.p.). C) The images show the liver, kidney, spleen and colon histologies with H&E staining (Control: DMSO/PBS). Magnifications 20X, scale bars: 100 μM.

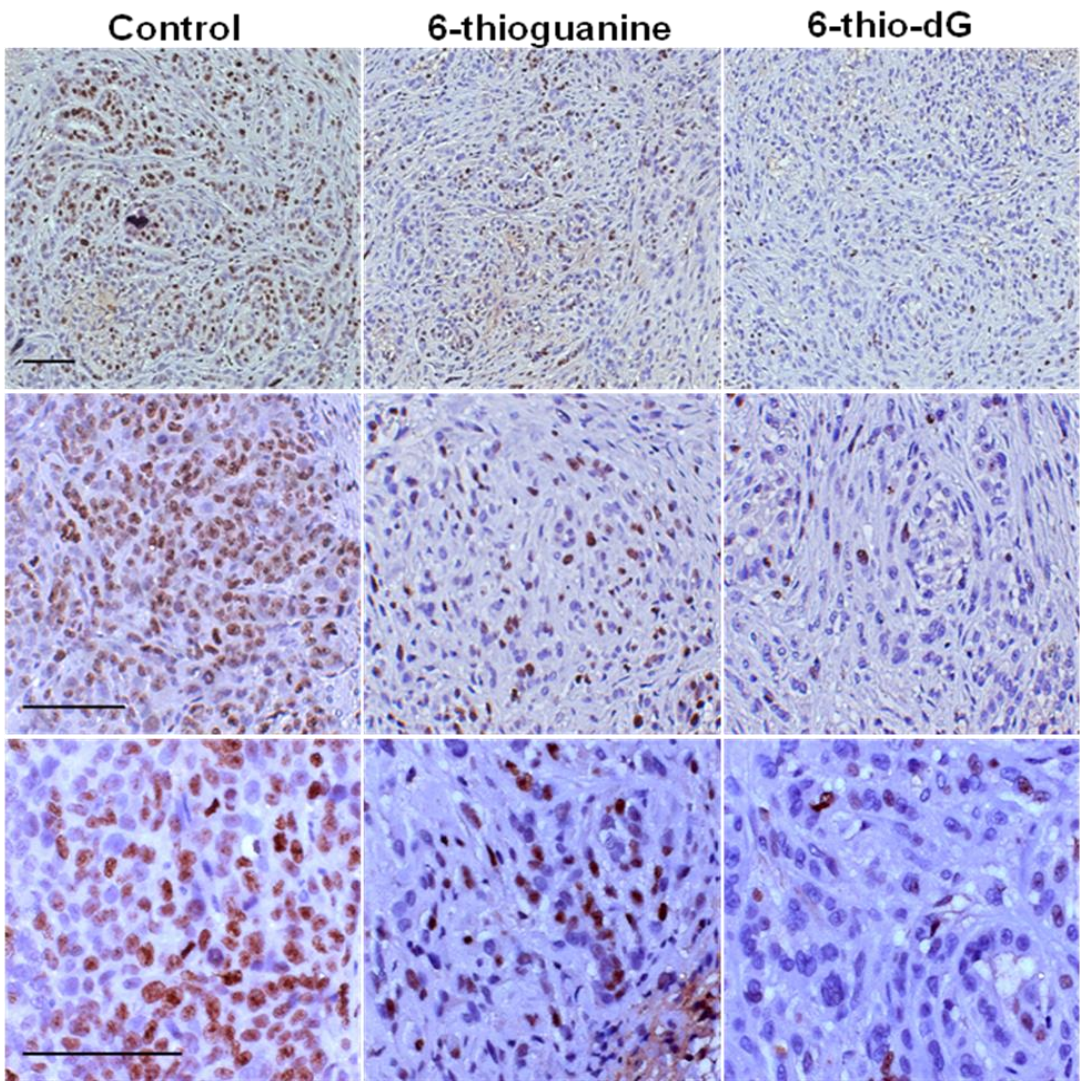
4.1.6. 6-thio-dG Reduces Tumor Growth Rate *In Vivo*

In order to investigate the anticancer effects of 6-thio-dG on tumor growth compared to 6-thioguanine and control, we used A549 NSCLC derived xenograft murine models. 6-thiodG and 6-thioguanine injections were initiated when the xenografted tumor size reached 50-100 mm^3. 6-thio-dG and 6-thioguanine at a dose of 2 mg/kg were intraperitoneally injected into athymic nude mice every other day for 17 days. At the end of treatment, 6-thio-dG caused a decreased of tumor growth rate compared to 6-thioguanine and control (Figure 4.10A). In addition, Ki67 staining, which is a proliferation marker, showed that 6-thio-dG treatment reduced Ki67 levels in A549-derived tumor xenografts compared to 6-thioguanine treatment and controls (Figure 4.10B). These results document *in vivo* differences between 6-thio-dG and 6-thioguanine on tumor growth rates and suggests 6-thio-dG is more effective compared to 6-thioguanine. To test whether intratumoral injection caused any additional therapeutic improvement on tumor growth compared to intraperitoneal injection, 6-thio-dG and 6-thioguanine were intratumorally injected every day at a dose of 2.5 mg/kg into the nude mice after xenograft establishment. 6-thio-dG treatment with intratumoral injection showed even more dramatic reduction on growth rate compare to 6-thioguanine and control (Figure 4.10C). In addition, 6-thio-dG treatment exerted highly vascularized tumors with infiltrating inflammatory cells, which were not observed with 6-thioguanine injections. Thus, the residual small tumors following 6-thio-dG treatment underrepresent the true effectiveness *in vivo* (Figure 4.10D). It is certain that additional, and longer treatments will be required to determine if tumors can be completely eliminated.

A


A549 (IP): 2 mg/kg
Control
6-thioguanine
6-thio-dG

B


Ki67
Control
6-thioguanine
6-thio-dG

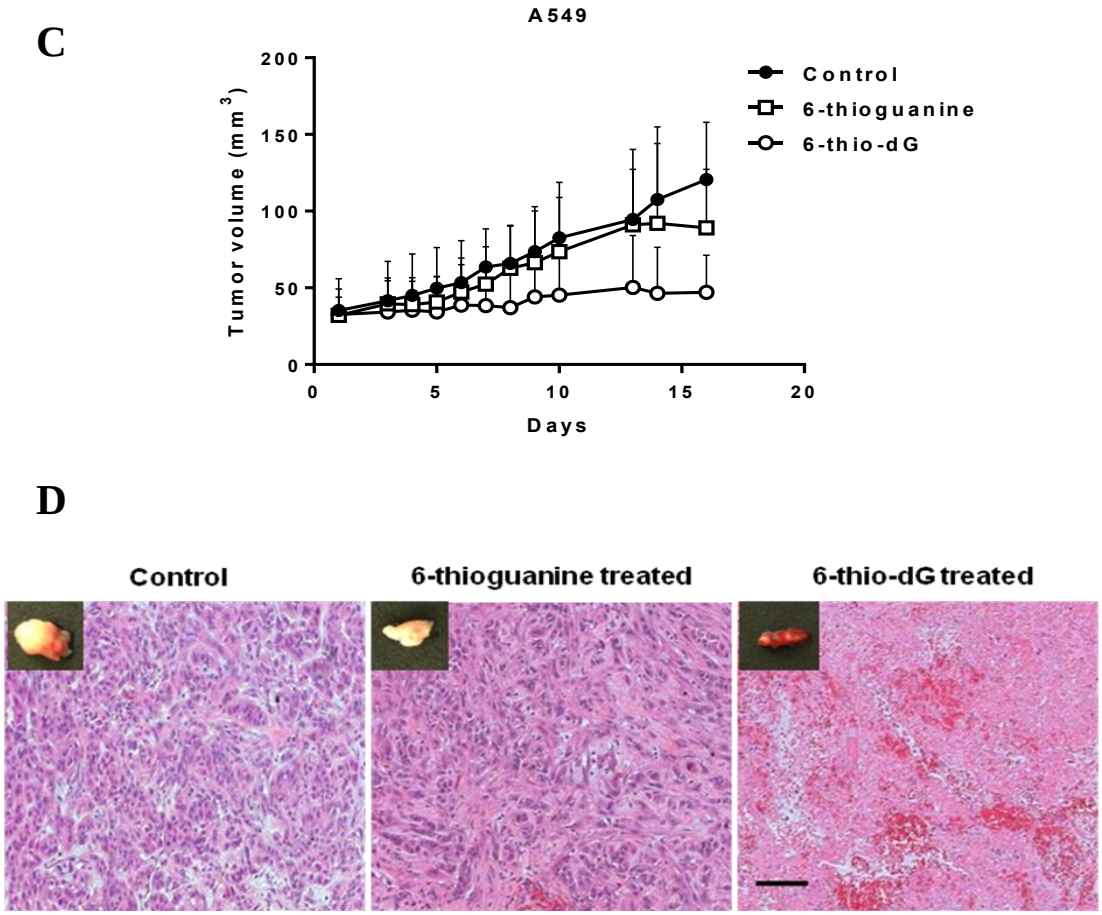
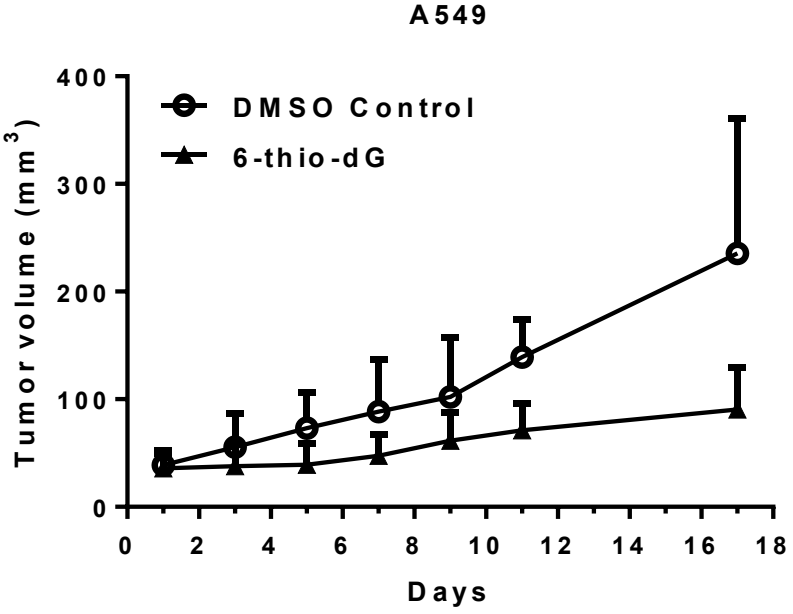


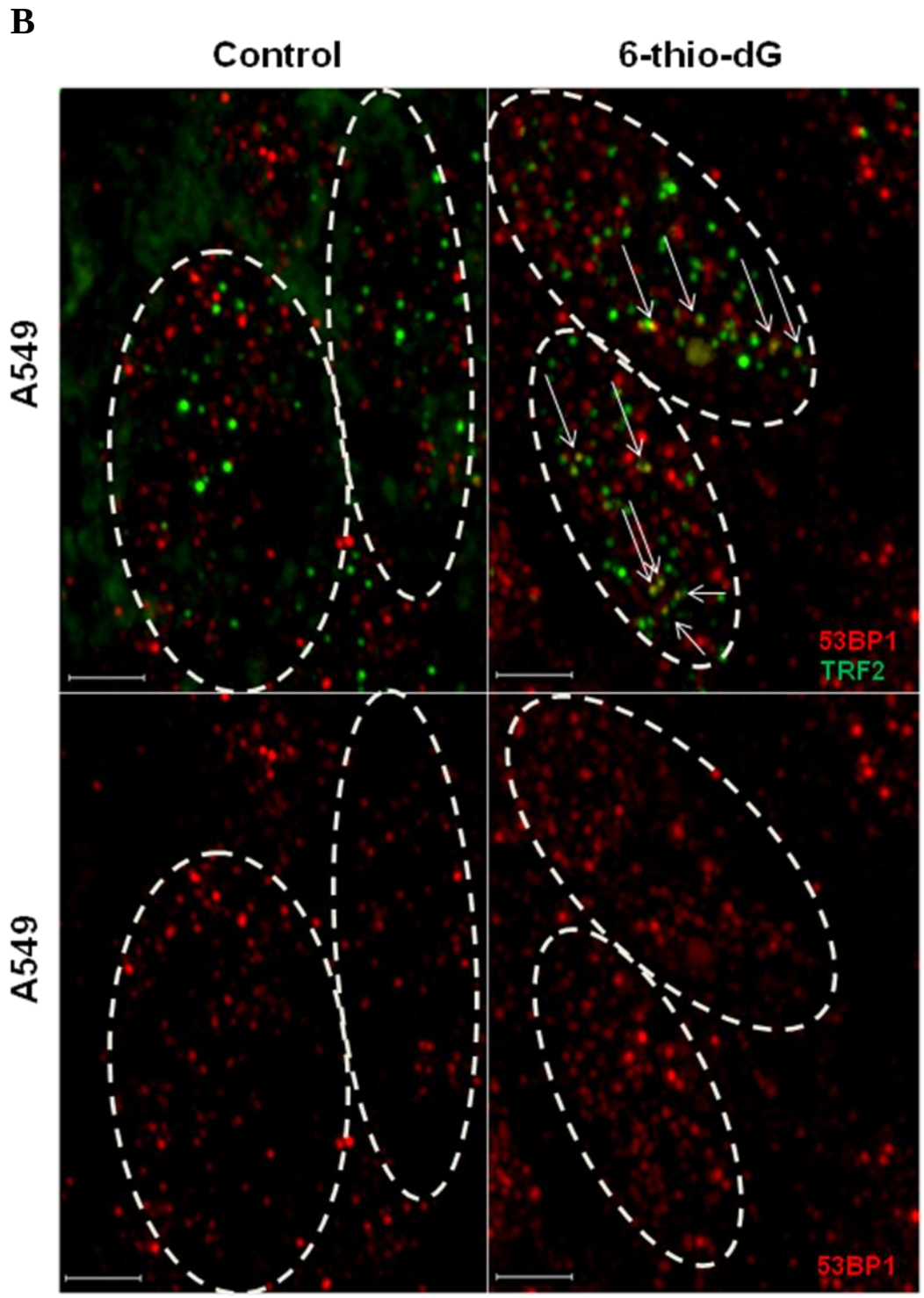
Figure 4.10. Intraperitoneally and intratumoral injection with 6-thio-dG and 6-thioguanine. A) Representative images form isolated tumors. 2 mg/kg 6-thio-dG and 2 mg/kg 6-thioguanine intraperitoneally (IP) injected to nude mice every other day for 17 days. B) Representative images show Ki67 proliferation level in 6-thio-dG (2 mg/kg) treated A549 derived tumor compared to 6-thioguanine (2 mg/kg) and control. Magnifications 20X, 40X, 60X, scale bars: 100 μM. C) The tumor volumes (mm^3) are shown following 6-thio-dG treatment (2.5 mg/kg) compared to 6-thoguanine (2.5 mg/kg) and control. Treatments were done as intratumoral injections, every day for 17 days. Tumor volumes were calculated using the following formula: (length x $width^2$) mm^2 x 0.5. D) The images show the histology of A549-derived tumors with hematoxylin and eosin staining following 6-thio-dG, 6-thioguanine and DMSO control treatment with intratumoral injection at a dose of 2.5 mg/kg, every day. Magnification 10X, scale bar: 100 μM.

4.1.7. 6-thio-dG Induces Telomere Dysfunction as well as Tumor Growth Rate Reduction on A549 NSCLC Derived Tumor

To test if this reduction of tumor growth rate (Figure 4.11A) correlated with telomere dysfunction, A549-derived tumor treated with 6-thio-dG (2 mg/kg) for 17 days were analyzed for telomere dysfunction induced foci (TIFs), as determined by the presence of 53BP1 and telomeric probe co-localization. When the cells were analyzed for TIFs ≥4 and TIFs ≥5, 6-thio-dG caused significant ~2 fold increases in telomere dysfunction *in vivo* (Figure 4.11B and C). Interestingly, in contrast to *in vitro* HCT116 general DNA damage response, there was no significant changes in general DNA damage between control and 6-thio-dG (2 mg/kg) treatment *in vivo* (Figure 4.11B and D). These results show that 6-thio-dG alters telomere structure and function and the reduction of tumor growth rate correlates with both general DNA damage and specific telomere dysfunction *in vivo*.

A




B
Control
6-thio-dG
A549
A549
53BP1
TRF2
53BP1

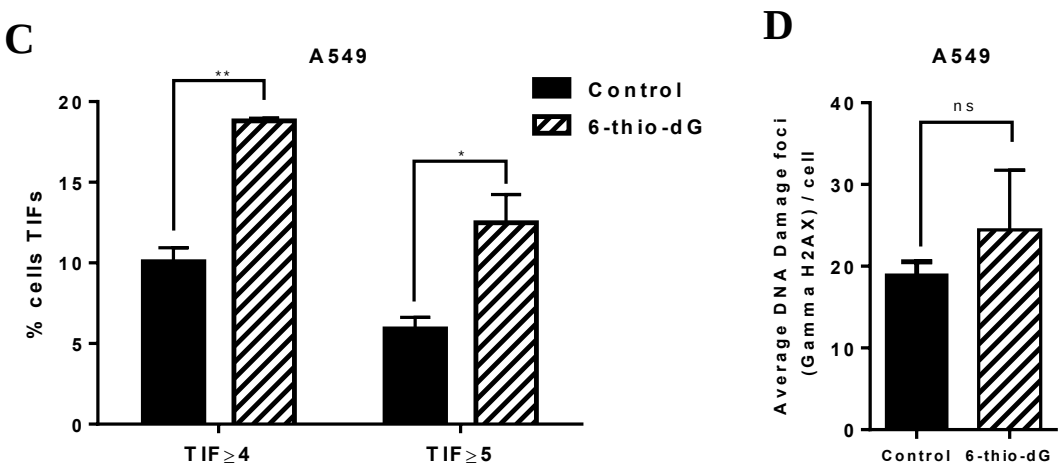


Figure 4.11. Correlation of tumor growth reduction and telomere dysfunction in A549-derived tumor. A) Tumor volume (mm^3) of A549 derived tumor following with 2 mg/kg 6-thio-treatment compared to control (intraperitoneally, every other day for 17 days). B) Representative image of binding of 53BP1 on uncapped telomeres following with 6-thio-dG treatment (2 mg/kg). Images were obtained by Deltavision and then deconvoluted by Autoquant X3. Red dots: DNA damage (53BP1), green dots: telomeres and yellow dots: TIFs (DNA damage on telomeres) in merged images. Scale bars: 3 μM. C) Percentage of TIF positive cells in A549 derived tumor treated with 6-thio-dG. Cells with ≥4 or ≥5 53BP1 foci colocalizing with telomere was scored as TIF positive by Imaris software (n>150 for control, n>200 for 6-thio-dG treatment, SDs from two independent experiments, $^{**}P$=0.0051, $^{*}P$<0.05 (control:6-thio-dG) in the unpaired Student *t* test. D) DNA damage foci per cell. A549 derived tumor cells treated with 6-thio-dG (2 mg/kg) (n>150 for control, n>200 for 6-thio-dG treatment, SDs from two independent experiments, ns: non-significant in the unpaired Student *t* test (Control:6-thio-dG).

4.2. Possible Resistance Mechanisms of 6-thio-dG Treatment

While it is promising that while 6-thio-dG kills the vast majority of cancer cell lines and it does not have major toxic effect on normal cells (BJ and HCEC1), the question of drug resistance must be addressed. In this study when HCT116 cells were treated with 6-thio-dG for long-term treatment, they eventually developed resistance to 6-thio-dG treatment (Figure 4.12A and B). This indicates that following the death of vast majority of HCT116 cells, there are still some cells that survive, gain the benefits of drug resistance and proliferate.

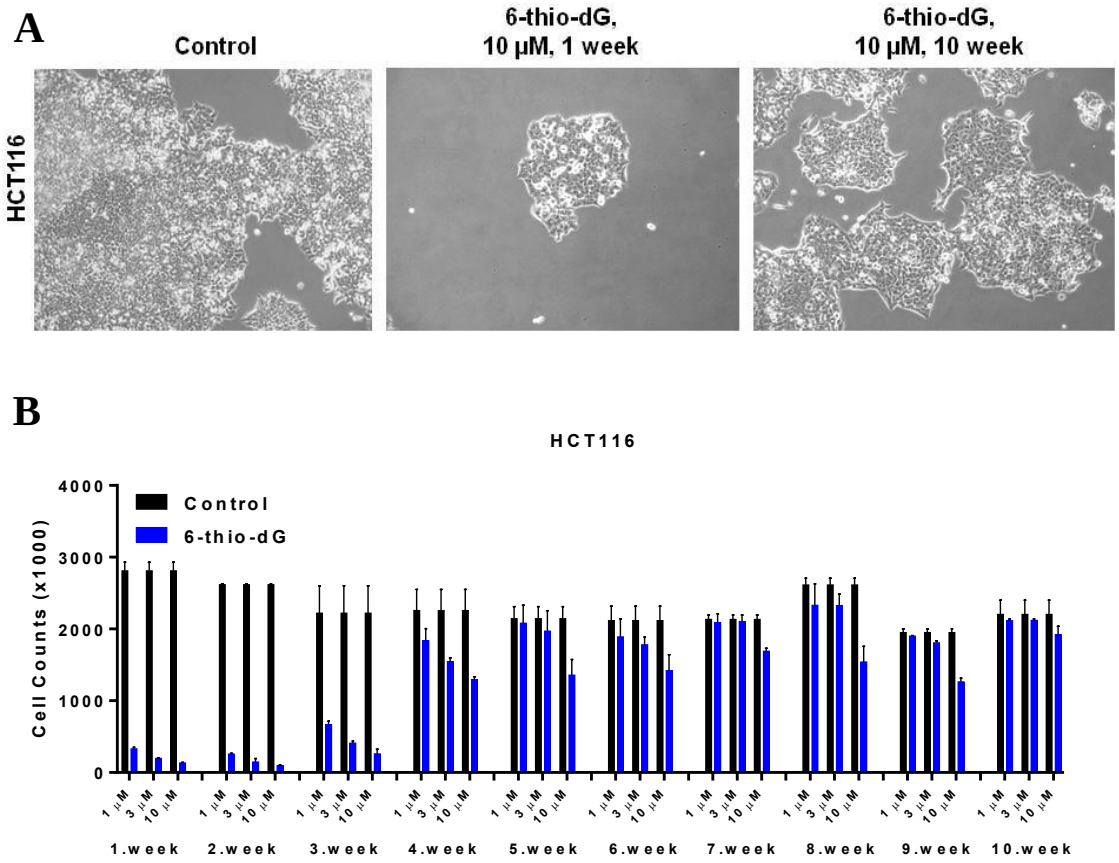


Figure 4.12. The development of 6-thio-dG resistance in HCT116 cells. A) The morhology of HCT116 cells treated with 10 µM 6-thio-dG for 1 and 10 weeks. B) The cell counts of HCT116 cells with 1, 3 and 10 µM 6-thio-dG treated every three days for 1-10 weeks. While HCT116 cells are highly sensitive to 6-thio-dG treatment for 1-2 weeks, they start to develop resistance to 6-thio-dG with over 3 weeks treatments.

We next sought to determine if 6-thio-dG resistance in HCT116 cells can be explained by cancer stem cell properties and/or increased levels of ATP-Binding Cassette (ABC) transporter proteins, that are known to be important for multidrug resistance functions in various human cancers. ALDH1A1 (aldehyde dehydrogenase 1A1) and ABCG2 are the most commonly studied markers for cancer stem cell and ABC transporter functions, respectively. Therefore, we compared the ALDH1A1 and ABCG2 mRNA levels of the populations that were treated with 10 μM 6-thio-dG for 1 and 8 weeks by using qPCR. We found that while the ALDH1A1 and ABCG2 mRNA levels of HCT116 cells treated with 6-thio-dG for 1 week are decreased compared to control, 6-thio-dG treated HCT116 cells for 8 weeks had similar or more ALDH1A1 (Figure 4.13A) and ABCG2 (Figure 4.13B) mRNA expression levels compared to controls and 1 week 6-thio-dG treatment. These results indicate that the surviving cells in the population following 6-thio-dG treatment shows increases cancer stem cell biomarkers and also 6-thio-dG drug levels are insufficient to be toxic due to development of increased drug efflux capability of cells.

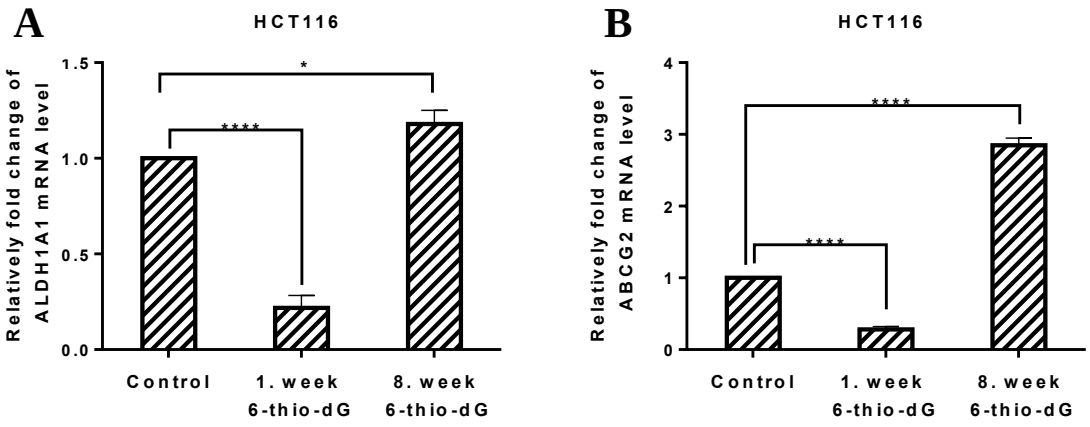


Figure 4.13. The mRNA levels of ALDH1A1 and ABCG2 in HCT116 cells following 6-thio-dG treatment. A, B) HCT116 cells treated with 10 μM 6-thio-dG every three days for 1 and 8 weeks. A) ALDH1A1 and B) ABCG2 mRNA levels were significantly decreased with 1 week and increased with 8 weeks 6-thio-dG compared to control (*P<0.05, ****P<0.0001 in the unpaired student t test).

To examine the difference of clonogenic potential of HCT116 cells treated with 10 μM 6-thio-dG for 1 week and 12 weeks, colony formation assays were done. Treated HCT116 cells were plated at low density and colonies were allowed to form for 10 days in the absence of 6-thio-dG. HCT116 cells treated with 6-thio-dG showed decreased ability to form colonies following 12 weeks of treatment, while untreated cells were still able to form colonies. Interestingly, 12 weeks treated HCT116 cells had higher capability to form colonies compared to 1 week treated cells, yet the difference was not statistically significant (Figure 4.14). However, at 1 week of treatment the cells may still have accumulated damage explaining the poor colony formation. The explanation of why colony formation is significantly reduced at 12 weeks is less clear since at this time point the cells have increased drug efflux capability. Overall, these results indicate that long-term treated HCT116 cells develop resistance to 6-thio-dG by multiple mechanisms.

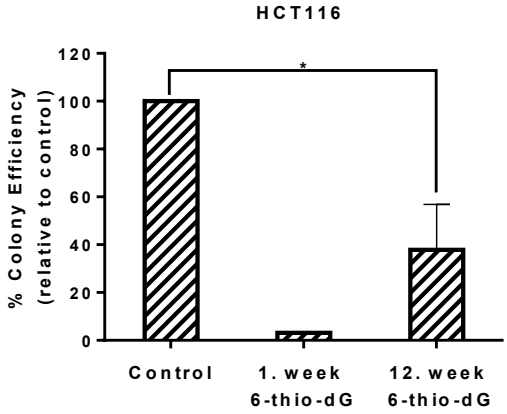


Figure 4.14. The colony formation efficiency of HCT116 cells following 10 μM 6-thio-dG treatment with different time points. 1 week and 12 treated HCT116 cells with 6-thio-dG were allowed to form colonies for 10 days without any treatment. Counted colonies in 1 and 12 week treated groups following 10 days no treatment were compared and colony formation efficiency of HCT116 cells were increased in 12 week treatment compared to 1 week 6-thio-dG treatment (*P<0.05 in the unpaired student t test).

4.2.1 HCT116 Cells are not Sensitive to 6-thio-dG with GRN163L Pretreatment

In order to test the effects of combined therapy with GRN163L and 6-thio-dG, we initiated experiments with two different designs. First, HCT116 cells were treated with 3 μM GRN163L and 1 μM 6-thio-dG at the same time every three days for 1 week. We observed that HCT116 cells were partially sensitive to combined therapy compared to control and only GRN163L treatment. It is important to note that HCT116 cells are more sensitive to 6-thio-dG treatment compared to combined therapy of GRN163L and 6-thio-dG that were added at the same time. In the second series of experiments, HCT116 cells were pretreated with 3 μM GRN163L for 3 days and then GRN163L (3 μM) and 6-thio-dG (1 μM) were added together to the HCT116 cells. Pretreatment with GRN163L inhibited the 6-thio-dG toxic effect on HCT116 cells following 1 week treatment. The partial response in the first experimental design can be explained by the earlier effect of 6-thio-dG on HCT116 cells. Thus, 6-thio-dG induced initial telomere dysfunction by 12 hr treatment and initiated cell death in some of the HCT116 cells in the population by 72 hr until telomerase activity is inhibited by GRN163L. After GRN163L inhibits telomerase activity, the toxic effect of 6-thio-dG on HCT116 was partially lost. This would be totally expected since the mode of action of 6-thio-dG for induction of telomere dysfunction depends on active telomerase activity. In second series of experiments since telomerase activity is inhibited with pretreatment of GRN163L, 6-thio-dG can not be incorporated into telomeres without active telomerase. It was not expected to see any toxicity with only GRN163L following 1 week treatment because inducing senescence or apoptosis with GRN163L on cancer cells would require many weeks of progressive telomere shortening, which can be achieved with long-term treatment (Figure 4.15A and B). Overall, these experiments provide a further proof of principal of the mode of action of 6-thio-dG.

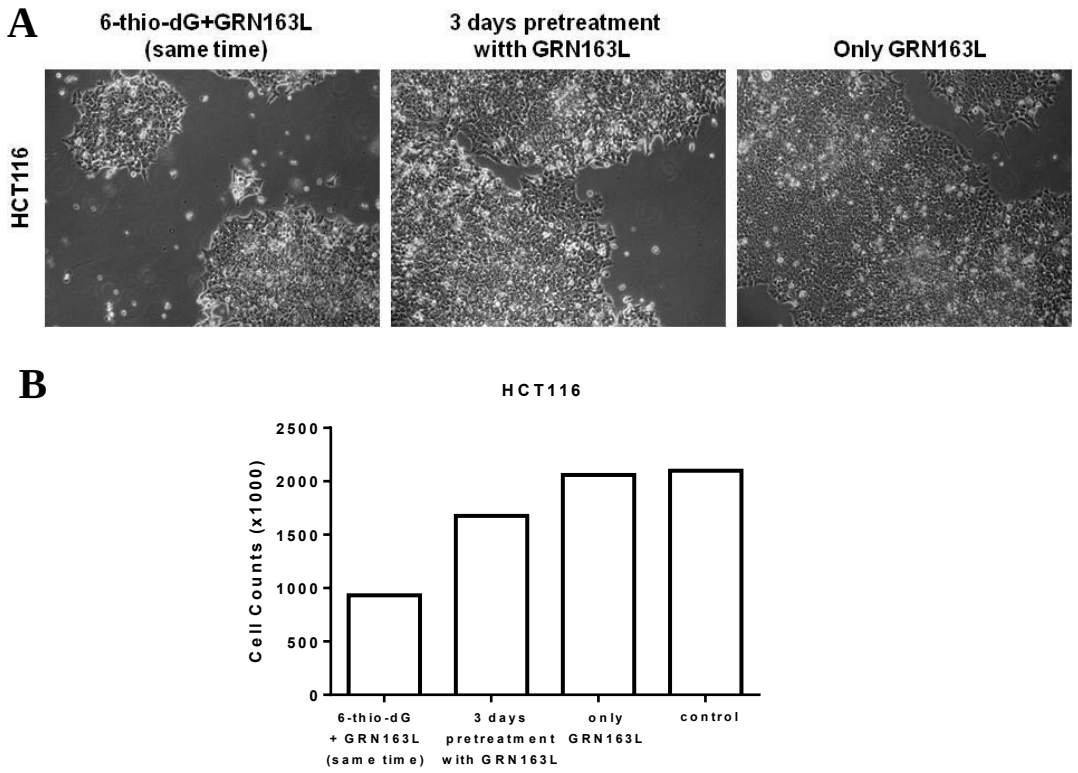


Figure 4.15. Two different experimental designs of combination therapy with GRN163L and 6-thio-dG. A) The morphology and B) The cell counts of HCT116 cells without any treatment, with only 3 μM GRN163L treatment, 3 days pretreatment with 3 μM GRN163L, and 3 μM GRN163L+1 μM 6-thio-dG treatment at the same time.

We tested if combination therapy of 6-thio-dG and GRN163L treatment induces additional telomere shortening compared to 6-thio-dG alone or GRN163L alone. The time when GRN163L is added to the culture medium is important (pretreatment or at the same time). Therefore, if we pretreated HCT116 cells with GRN163L and then co-treated with 6-thio-dG, we would expect not to see any additional telomere shortening with 6-thio-dG due to telomerase inhibition activity of GRN163L. However, combination therapy with GRN163L and 6-thio-dG that were added at the same time would be predicted to induce telomere shortening on cancer cells due to telomerase inhibition effect, which is only due to the effect of GRN163L and not telomere dysfunction effects of 6-thio-dG. While 12 week treatment with 6-thio-dG resulted in progressive telomere shortening, 2 or 4 week off 6-thio-dG

following 12 week treatment did not show any further telomere shortening or re-elongation (Figure 4.16). This suggest the surviving population have reset their telomere length. Under these conditions adding GRN163L as a single agent after 12 weeks of 6-thio-dG treatment results in additional progressive telomere shortening (Figure 4.16). This indicates that HCT116 cells that have already short telomeres following only 6-thio-dG are sensitive to telomerase inhibition by GRN163L, but this sensitivity is not because of the effect of 6-thio-dG. This also suggests a possible combinatorial approached to effective durable remissions.

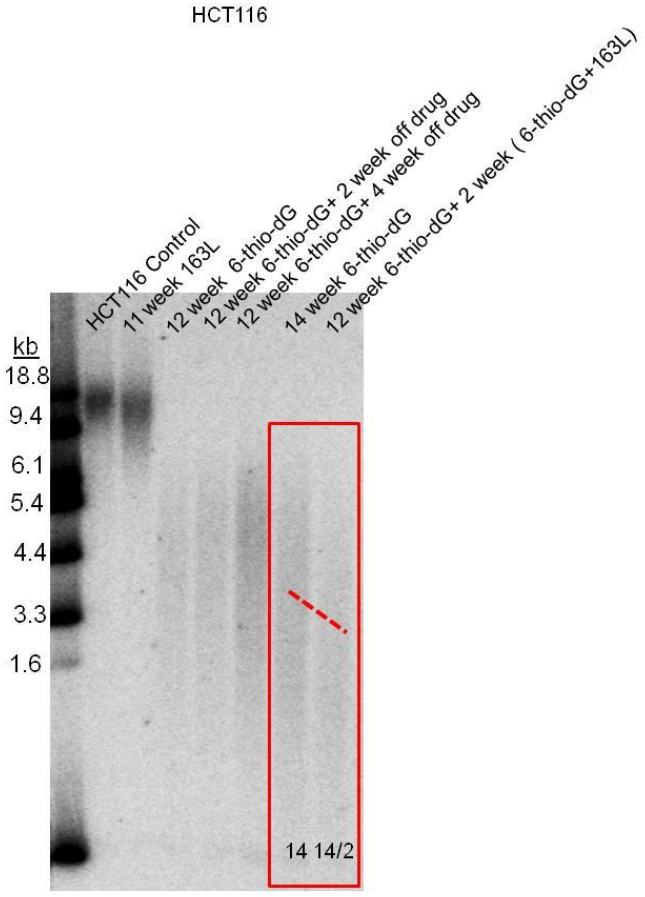


Figure 4.16. Telomere Restriction Fragment analysis in HCT116 cells treated with only GRN163L, only 6-thio-dG, or combination of 6-thio-dG and GRN163L. HCT116 cells were treated with 3 μM GRN163L or 10 μM 6-thio-dG for 11-14 weeks. After treating with 10 μM 6-thio-dG for 12 weeks, the cells were treated with combination of 10 μM 6-thio-dG and 3 μM GRN163L for 2-4 weeks or without any treatment for 2-4 weeks.

4.2.2. H1819, H1993 and H1693 NSCLCs are resistant to 6-thio-dG

6-thio-dG was toxic for 10 NSCLCs (A549, H2882, HCC2429, HCC827, HCC15, H2087, HCC4017, HCC515, H2009, H2073), whereas H1819, H1993 and H1693 NSCLCs were not sensitive to 6-thio-dG. H1819/H1693 and H2073/H1993 are the isogenous cell lines. Each cell line of one pair are from same patient and have different telomere lengths (Table 4.2). H1693 cell line was established prior to chemotherapy from a patient with IIIB adenocarcinoma lung cancer. Residual primary lung tumor was treated with cisplatin, etoposide, radiotherapy and then H1819 was established. Likewise, another patient who had stage IIIA adenocarcinoma lung cancer was biopsied at a regional lymph node. H1993 was established prior to chemotherapy. H2073 was established from residual primary lung tumor after cisplatin and etoposide treatment. H2073 is the chemo-resistant primary lung tumor with shorter telomeres compared to H1819, H1993 and H1693 cells with long telomeres. Since the methylation patterns and gene expression profiles of these NSCLCs are known, we compared and contrasted the aberrant methylation and gene expression profiles of sensitive and resistance NCSLCs. Figure 4.17 shows complete data for 13 NSCLCs sorted using a clustering algorithm. Since cell lines have heterogenous population, there is hypomethylated or hypermethylated forms of all genes. When we compared the sensitive and resistant cell lines, some genes were hypermethlated in both resistant cell lines, but not sensitive cell lines. And interestingly, H2073/H1993 matched pair of NSCLCs that is both sensitive and resistant to 6-thio-dG treatment had different methylation pattern (Figure 4.17). This cluster demonstrates that methylation profiles of some genes can be important for the cell lines that are resistant to 6-thio-dG.

In order to investigate the effect of 6-thio-dG following 5-aza-2'-deoxycytidine pretreatment (500 nM), which is an inhibitor of DNA methylation, we seeded resistant NSCLCs in 96-well plates, and treated the cells with different concentrations of 6-thio-dG every three days. Following one week of treatment (using different doses with serial dilutions, 1000-0.03 μM), the cell viability was determined by the celltiterglo luminescent cell viability assay. We observed that 6-thio-dG inhibited cell viability in a dose dependent manner after

pretreatment with 5-aza in H1993 NSCLCs, but neither H1819 nor H1693, showing that the resistance mechanism of H1993 NCSLC. The reason for this increased sensitivity or resistance to 6-thio-dG may be due to epigenetic variations in some important genes (Table 4.2).

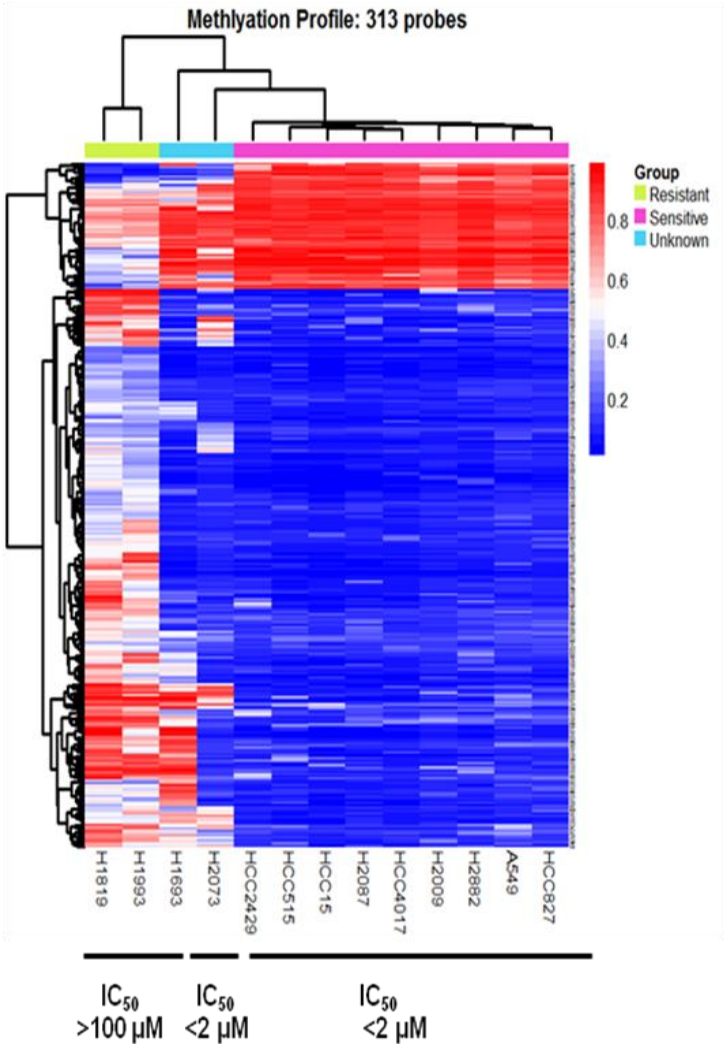


Figure 4.17. Clustering analysis of DNA methylation profiles for 13 NSCLCs. Sensitive and resistance NSCLCs were clustered and seperated by different colors (purple: sensitive, green: resistant). Generally, neighboring clusters have similar methylation patterns. The tone differences between red and blue shows the range between hypermethylation to hypomethylation (This heatmap was created with the help of Kimberly Batten).

The average telomere lengths and IC_{50} values of two pairs of cell lines for 6-thio-dG (with/without 5-aza pretreatment) are summarized in Table 4.2.

Table 4.2. The average telomere lenghts and IC50 values of H1819/H1693 and H1993/H2073 isogenic cells with 6-thio-dG treatment with/without 5-Aza pretreatment.

Cell Lines	Average Telomere Length (kb)	6-thio-dG (μM)	5-Aza pretreatment (500 nM)
H1819	8	Resistance (IC50>100 μM)	Resistance (IC50>100 μM)
H1693	6.2	Resistance (IC50>100 μM)	Resistance (IC50>100 μM)
H1993	10	Resistance (IC50>100 μM)	Sensitive (IC50~4.1 μM)
H2073	3	Sensitive (IC50~0.7 μM)	-

In order to compare gene expression profiles of sensitive and resistance cell lines (11 NSCLCs), they were clustered by using algorithm. We identified 3 different genes that are differentially expressed in sensitive and resistant cell lines. H1819 and H1993 resistant cell lines showed upregulation of ALDH1A2, TLE2 and downregulation of FSCN1 gene expression (Figure 4.18).

As it was shown in previous studies (192, 199, 200), resistant cell lines had overexpressed ALDH1A2 and TLE2 gene expression levels in our study as well, showing the relation of upregulation of ALDH1A2 and TLE2 gene expression (might be related with Wnt/β-catenin and notch pathways) with drug resistance. FSCN1 is a fascin actin-bundling protein 1, which is an absent in most normal eptihelia but expressed in many human carcinomas. A number of previous studies have shown that fascin-1 is correlated with poor prognosis (215-217). It is unclear that while upregulation of fascin-1 represents poor prognosis of cancer, why in our gene expression results showed down regulation of fascin-1 in resistant cell lines compared to sensitive cell lines and also there are no studies that showed the relation of down regulation of FSCN1 and drug resistance. It needs to be further investigated.

These alterations of gene expression and DNA methylation in drug resistance cancer might be related to mechanisms of drug resistance and maybe useful as biomarkers of cancer drug sensitivity.

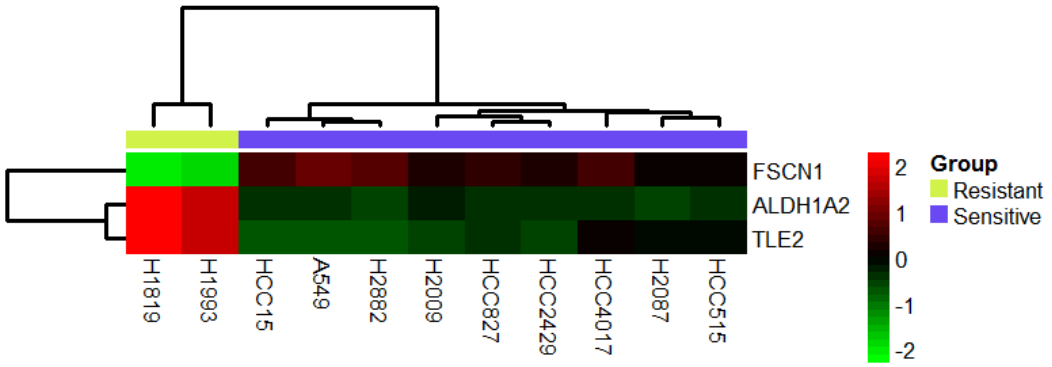


Figure 4.18. Hierarchical clustering of the gene expression profile in sensitive and resistance NSCLCs to 6-thio-dG treatment. 3 different genes that are down or upregulated were identified. Relative expression levels of these genes are color coded (This heatmap was created with the help of Kimberly Batten).

4.2.3. Telomerase positive peripheral blood mononuclear cells (PBMC) are partially sensitive to 6-thio-dG

Since normal lymphocytes express a low but measurable telomerase activity, we next tested the effects of 6-thio-dG on normal resting lymphocytes (telomerase silent) versus proliferating lymphocytes (telomerase positive). To evaluate this, we treated peripheral blood mononuclear cells (PBMC) with 1, 3, and 10 μM 6-thio-dG together with/without phytohemagglutinin (PHA), which is a mitogen lymphocte activator, for 24 hr, 72 hr and 120 hr. We observed that 120 hr is required for maximal proliferation and telomerase activity in PBMCs following PHA stimulation. Telomerase positive proliferating cells were sensitive to 6-thio-dG whereas 6-thio-dG did not have any toxic effects on normal resting lymphocytes, showing that when the proliferating rate of PBMCs was accelerated, 6-thio-dG can easily be recognized by telomerase and cause cell death (Figure 4.19). This suggests that long-term treatment with 6-thio-dG could have some hematological toxicities,

therefore dosing regimens of 6-thio-dG may be required to reduce these predicted side effects.

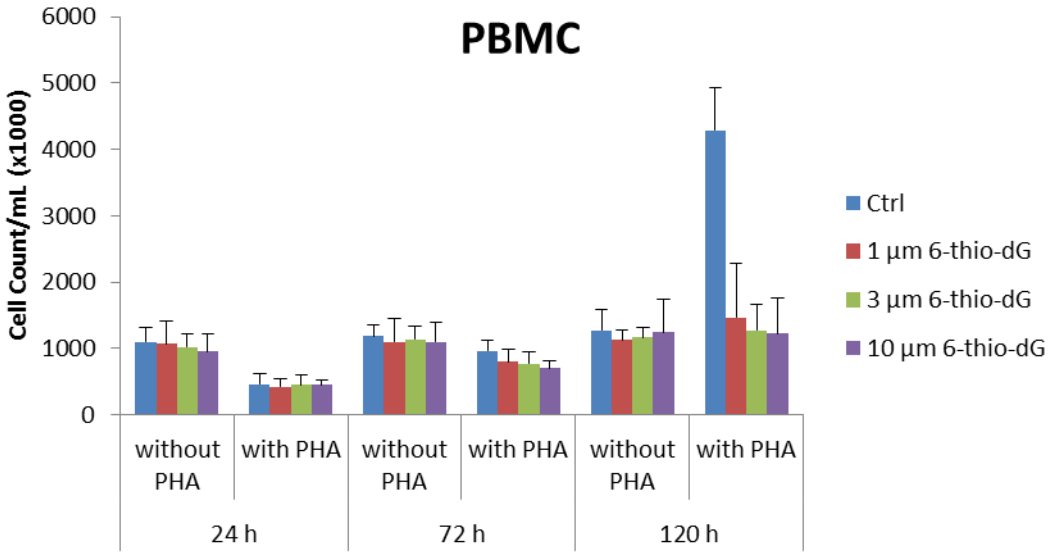


Figure 4.19. The cell counts of peripheral blood mononuclear cells (PBMCs) following 6-thio-dG treatment in the presence/absence phytohemagglutinin (PHA), which is a telomerase stimulator.

5. DISCUSSION

Telomerase remains a potent and attractive target for cancer therapy. However, while most previous telomerase targeted therapies showed some promising results in preclinical and early clinical studies, there are still concerns about side effects and drug resistance that leads to cancer recurrence and metastasis. GRN163L (Imetelstat) is the potent telomerase inhibitor that entered into clinical trials over the last several years. One of the main limitations of GRN163L is the variable telomere lengths of tumor cells that patients have. Therefore, initial telomere length variability and a potential long "lag period" (the time between the initiation of treatment and therapeutic effect) are the main limitations of this and other telomerase inhibition based therapies (Figure 5.1) in additional to hematologic and hepatic toxicities. A combination therapy with GRN163L and standard of care or traditional chemotherapy following surgical resection might have been the most effective way to target telomerase positive tumors. Unfortunately, combining GRN163L with other chemotherapeutics in human clinical trials have mostly been terminated primarily due to toxicities in patients (218, 219). In addition, because of the drug administration cycle, GRN163L was administered on days 1 and 8 of 21 day cycle, it is likely that telomerase can not be inhibited sufficiently, resulting telomere re-elongation or maintenance of telomeres. Due to the hematologic toxicities most patients had to be taken on protocol and this likely results in re-elongation of telomeres. Therefore, it is important to find appropriate drug dose, administration cycle with combination therapy to increase therapeutic efficacy with fewer side effects.

In this study, we aimed to identify a nucleoside analogue that is toxic for cancer cells, but less so far for normal cells, and one that is recognized and then incorporated into telomeres by telomerase. We predicted that the heterocyclic guanine base modified nucleoside analogue 6-thio-2’-deoxyguanosine would make an immediate change in the telomere structure and function, causing immediate telomeric damage due to telomere uncapping and thus lead to a significantly reduced lag phase. Our goal was to identify a telomerase mediated telomere uncapping small molecule that would lead to massive and fast cancer cell death independent from the initial tumor telomere length (Figure 5.1). In contrast to other telomerase inhibitors,

6-thio-2'-deoxyguanosine is not a direct telomerase inhibitor *per se*, but a substrate of telomerase to be incorporated into telomeres following being converted to 6-thio-dGTP.

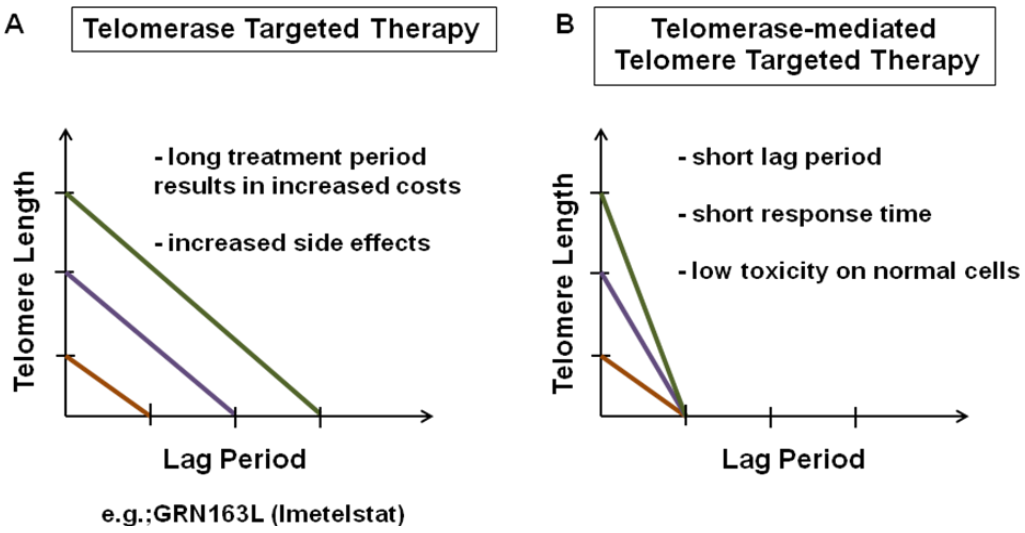


Figure 5.1. Two different approaches for telomerase based therapies. A) Variable telomere length of cancer cells are important for telomerase inhibition based therapies. For example, GRN163L (Imetelstat) treatment depends on initial tumor cell telomere length. Therefore, the treatment period will be longer for the cells that have longer telomeres compared to cells with shorter telomeres to induce senescence or apoptosis. As a conclusion, any long term treatment to target telomerase may cause side effects, high costs, and potentially re-elongation of telomeres to maintain telomere stability. B) In contrast to telomerase inhibition based therapies, telomerase mediated telomere targeting therapies such as 6-thio-dG or MT-hTERC gene therapy are independent from initial telomere length, thus they do not need long treatment periods. Therefore it is expected to have short response time to reduce tumor burden with less side effects, which are advantages of these approaches. However, although the MT-hTERC approach has the known limitations of all gene therapy approaches, the 6-thio-dG nucleoside analogue will be a new approach by excluding the limitations of GRN163L and gene therapy approaches such as MT-hTERC. And also 6-thio-dG should be oral available and have the advantage that all small molecules have.

Purine analogues such as 6-thioguanine and 6-mercaptopurine are chemotherapeutic agents that can be converted to thioguanine nucleotides. 6-thioguanine is one of the thiopurine drugs that are used in the treatment of acute leukaemia. 6-thioguanine is synthesized by substitution of oxygen by sulphur at carbon 6 of guanine. It was shown that 6-thioguanine inhibits purine utilisation and was initially shown to be effective against rodent tumors and then in children with acute lymphoblastic leukaemia. 6-thioguanine is the prodrug that is metabolized and incorporated into DNA and RNA as thioguanine nucleotides to exert its cytotoxic effect (220). 6-thioguanine has various bioavailability characteristics ranging from 14 to 46% (221) and a plasma half life is 90 min (222). 6-mercaptopurine is the other thiopurine which is also metabolised to 6-thio-dGTP, but 6-mercaptopurine participates in more biochemical processes to be converted to 6-thio-dGTP compared to 6-thioguanine. It was suggested that since 6-thioguanine is metabolised to thioguanine nucleotide more directly than 6-mercaptopurine, 6-thioguanine might be the drug to choice instead of 6-mercaptopurine in children with acute lymphoblastic leukaemia who had failed 6-mercaptopurine treatment (220). The idea was since 6-thioguanine participated in fewer biochemical and metabolic processes, the efficiency and perhaps toxicity in patients would be different from 6-mercaptopurine. In previous studies, 6-thioguanine and 6-mercaptopurine treatment was compared in patients and thioguanine nucleotide concentration was higher in patients on 6-thioguanine compared to 6-mercaptopurine. There was no difference in anaemia or neutropenia in 6-thioguanine and 6-mercaptopurine treated group except increased thrombocytopenia was seen in 6-thioguanine treated group (223-225). It was also shown that 6-thioguanine was associated with sinusiodal obstructive syndrome, hypertension and veno-occlusive disease (VOD). Since toxicities of 6-thioguanine was significant in trials, the use of 6-thioguanine has largely been stopped (220).

In this study, the nucleoside analogue, 6-thio-2'deoxyguanosine shows

- Both general DNA and telomere specific damage but only in cells that express telomerase *in vitro* and *in vivo*.
- Progressive telomere shortening in cancer cells that are not immediately killed by 6-thio-dG.
- Less toxicity compared to approved 6-thioguanine.
- Reduction of tumor growth rate.

We found that 6-thio-dG that is rapidly converted to 6-thio-dGTP by kinases and is recognized by telomerase and incorporated into telomeres. This guanine-based modified nucleoside incorporation causes increased telomere dyfunction induced foci in HCT116 and telomerase expressing BJ cells (BJhTERT), but not in telomerase negative BJ cells, indicating that the effect of 6-thio-dG is mostly telomerase dependent (which was not observed with 6-thioguanine in HCT116, BJhTERT and BJ cells). In addition, 6-thio-dG also caused increased general DNA damage in HCT116 cells. We also found that while 6-thio-dG treatment causes progressive telomere shortening in rare surviving telomerase positive cancer cells, 6-thioguanine did not show telomere shortening on cancer cells, showing that 6-thio-dG has a bifunctional role that targets both genomic and telomeric DNA *in vitro* as well as antimetabolite functions. Interestingly, the effect of 6-thio-dG on telomeres was not dependent on inhibition of telomerase activity, indicating that 6-thio-dG is not a direct telomerase inhibitor, but telomerase is a mediator for its action. Importantly, while 6-thio-dG kills the majority of cancer cells, it does not appear to have major toxic effects on normal telomerase silent cells, which provide a therapeutic and selective window of opportunity for cancer treatment. 6-thioguanine also showed similar toxic effect on cancer cells besides no short-term major toxic effect on normal cells. In contrast to the *in vitro* studies, *in vivo* studies showed more efficiency and less toxic effects of 6-thio-dG (lack of weight loss, no changes hematological except minor neutropenia, liver or kidney functions) compared to 6-thioguanine. We believe that these toxicity differences might be because of the

differences in structure of 6-thio-dG (a nucleoside) and 6-thioguanine (a heterocyclic base). And also while 6-thioguanine participates in multiple biochemical processes to be converted to thiopurine nucletiode including purine salvage, 6-thio-dG participated in biochemical processes to a lesser extent, which was also shown in comparison between 6-thioguanine and 6-mercaptopurine treatment. The differences in biodistribution, relative solubility (6-thio-dG is more hydrophilic than 6-thioguanine), plasma protein binding and major organ accumulation may also be related with observed toxicity differences *in vivo.*

It has been demonstrated that telomere dysfunction induces senescence or apoptosis and can limit tumor growth rate in animal models (226, 227). Our results also showed that 6-thio-dG significantly induced telomere dysfunction in A549-derived tumor cells compared to control, suggesting that 6-thio-dG can be incorporated into telomeres (~1/6000 of the genome) during replication and causes tumor growth reduction.

Telomerase positive stem cells are a potential concern going forward with 6-thio-dG, but stem cells often grow slower compared to cancer cells, and also cancer cells have relatively shorter telomeres (thus cancer cells may be more susceptible to anticancer agents compared to normal cells). However, we showed that increased telomerase activity caused sensitivity to 6-thio-dG in transient telomerase positive lymphocytes compared to resting lymphocytes (telomerase silent), showing that while there is likely to be some effects on transient amplifying telomerase positive normal transit amplfying cells, quiescent stem cells (which as telomerase negative) are unlikely to be affected by 6-thio-dG. Going forward, other potential side effects will be tested in broader toxicology studies. As a conclusion, these novel findings in this dissertation research provide encouraging support to further develop new telomerase-based telomere targeted therapy that primarily target telomerase positive cancer cells.

Cancer relapse is often a predicted outcome with single (monotherapy) anti-cancer therapy. In our study, we observed that when we started to treat HCT116 colon cancer cells with 6-thio-dG, there was massive cell death. However, there were still a small percentage of cells that appeared to survive and become resistant to

6-thio-dG. Eventually, after several weeks of treatment these cells started to grow and became a resistant population of cells. There are several drug resistance mechanisms to explain why these cells gained the ability to grow when continuously treated with 6-thio-dG. ABC transporter proteins and cancer stem cell markers as well as epigenetic biomarkers are commonly investigated for drug resistance. We showed that resistant populations had higher levels of ABCG2 (ABC transporter family member) and ALDH1A2 (aldehyde dehydrogenase, cancer stem cell marker) compared to sensitive populations. In addititon, colony formation was higher in the resistant cell population compared to sensitive cells. However, when compared to control cells, the resistant cell population had less colony formation, a finding suggesting that additional factors are reducing the ability of these resistent cells to form colonies. Almost all lung cancer cell lines tested were sensitive to 6-thio-dG except H1819, H1993 and H1693 cells. H1819/H1693 and H2073/H1993 were isogenic cell lines from the same patient and each had different telomere lengths. H1693 was established prior to chemotherapy from patient with lung adenocarcinoma at regional lymph node. H1819 was established from residual primary lung tumor following treatment with cisplatin, etoposide, and radiotherapy. Likewise, H1993 was established prior to chemotherapy from a patient with adenocarcinoma lung cancer. H2073 was established from a residual primary lung tumor following cisplatin and etoposide treatment. Interestingly, while 6-thio-dG causes less toxic effects on H1819, H1693 and H1993 cells, H2073 were more sensitive.

To attempt to dissect the mechanism for the differential sensitivity to 6-thio-dG, methylation patterns were examined. A differential methlylation pattern between sensitive and resistant cell lines was observed. While resistant cell lines were hypermethylated in some genes, sensitive cell lines were hypomethylated in the same genes. In addition, 5-aza (demethylation agent) treatment induced H1993 sensitivity to 6-thio-dG, but not H1819 or H1693 cells, indicating that the resistance mechanism of H1993 can be correlated with hypermethylation status but not in the other two cell lines. Gene expression profiles showed 3 different genes between resistant (H1819, H1993) and sensitive cell lines, which are ALDH1A2, TLE2 and FSCN1. ALDH1A2 is a cancer stem cell marker, which is upregulated in drug

resistance. Therefore, it is possible that resistant cells also have cancer stem cell properties such as self renewal that play a role in the drug resistance mechanism. TLE2 is correlated with Wnt/β-catenin and notch signaling pathways, which have important roles in cell proliferation and also drug resistance. Thus, these methylation and gene expression results show that resistant cells have several properties to become resistant compared to sensitive cells. There are no published studies that show a correlation between FSCN1 (Fascin-actin bundling protein 1) and drug resistance, therefore it is unclear why FSCN1 is downregulated in resistant cancer cells. Thus, FSCN1 should be further investigated.

Since GRN163L inhibits telomerase activity, it is expected that adding GRN163L together with 6-thio-dG treatment would inhibit the effect of 6-thio-dG on telomeres due to inability of using telomerase as a mediator of telomere uncapping. We showed that GRN163L treatment that was added before 6-thio-dG did not cause any toxic effect on HCT116 cells, predicting that by inhibiting telomerase activity with GRN163L would not lead to telomere upcapping with 6-thio-dG treatment. In one series of experiment we showed additional telomere shortening and partially toxic effect on HCT116 cells following with GRN163L after initial effects of 6-thio-dG. This indicated that additional telomere shortening effect with GRN163L in cancer cells that have already short telomeres following only 6-thio-dG treatment may cause increased sensitivity to GRN163L. However, this sensitivity would not be because of the effect of 6-thio-dG. This also suggests a possible combinatorial approach to sensitize the surviving cancer cells following short term treatment with 6-thio-dG.

In future experiments, additional toxicity studies with long term 6-thio-dG treatment are needed. In addition, chosing appropriate double therapy (carboplatin, paclitaxel or cisplatin) and dose scheduling with combination therapy *in vivo* is needed. We have established patient derived xenografts (PDX) by implanting of primary tumor directly from patients into immunodeficient mouse. PDXs provide partial solutions to the challenge that researchers face in cancer drug discovery such as the problems with translating tumor responses to humans. Therefore, in progress are experiment testing the effect of 6-thio-dG with/without combination therapy in PDXs and also oral gavage of 6-thio-dG with proper drug concentration will be

tested to mimic the therapeutic efficiency of oral 6-thio-dG and minimize the risk of adverse effects. In addition, the effect of oral 6-thio-dG on telomere dysfunction will be investigated in PDX. Another direction to be pursued is treating with 6-thio-dG and then in the maintenance setting examining the sensititivity of surviving cells to radiation therapy. It may be that cancer cells with somewhat dysfunctional telomeres may be more sensitive to radiation therapy.

We are also planning to further investigate the resistance mechanism of cancer cells. HCT116 colon and/or A549 lung cancer cells will further be tested for colony formation capability after 6-thio-dG treatment *in vitro* to understand if this resistance of cancer cells are rare or common events and also what causes the resistance? In addition, to show more efficiency (maybe tumor shrinkage) and eliminate drug resistance with single agent, we are going to use combination therapy with 6-thio-dG and cisplatin/paclittaxel (conventional therapies for lung cancer) in H1819, H1993 or H1693 resistant cell lines *in vitro* and *in vivo*.

Telomerase negative ALT (Alternative Lengthening of Telomeres) cell lines will be tested for telomere dysfunction following 6-thio-dG treatment to see if 6-thio-dG causes damage on telomeres in ALT cell lines as well as telomerase positive cancer cells. This is somewhat unexpected since ALT cells do not express telomerase. However, during telomere recombination in ALT cells it is possible that 6-thio-dGTP could be incorporated into ALT telomeres.

Further understanding of resistance mechanisms to eliminate and find an appropriate dose and dose scheduling with multimodal (chemotherapy, radiation therapy, and possibly other telomerse directed therapies) will provide a path forward to treat patients with less side effects and perhaps more therapeutic and durable responses.

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