ELUCIDATING A ROLE FOR URACIL DNA GLYCOSYLASE (UNG)-INITIATED DNA BASE EXCISION REPAIR IN THE CELLULAR SENSITIVITY TO THE ANTIFOLATE, PEMETREXED

by

LACHELLE DAWN WEEKS

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Dissertation Advisor: Dr. Stanton L. Gerson

Department of Pathology

CASE WESTERN RESERVE UNIVERSITY

January 2014
We hereby approve the thesis/dissertation of

Lachelle Dawn Weeks

candidate for the Doctor of Philosophy degree*.

(signed) Shigemi Matsuyama (chair of the committee)

Alexandru Almasan

Ruth A. Keri

George R. Stark

Stanton L. Gerson

(date) August 23, 2013

* We also certify that written approval has been obtained for any proprietary material contained therein.
Dedication

To Clarissa Williams and Wesley Weeks.
Table of Contents

Dedication..........................................................................................................................ii
Table of Contents................................................................................................................iii
List of Tables.......................................................................................................................vi
List of Figures......................................................................................................................vii
Acknowledgements..........................................................................................................xi
List of Abbreviations.........................................................................................................xiii
Abstract.............................................................................................................................1

Chapter 1: Introduction: Uracil misincorporation and thymine-less death – old and new hypotheses on the mechanism of action of antifolates and TS inhibitors ..................................................3
  1.1 Antifolates in cancer therapy......................................................................................5
  1.2 DNA repair in human cancer development and therapy.........................................12
  1.3 Base excision repair of uracil-DNA........................................................................13
  1.4 Proposed consequences of antifolate mediated-dUTP incorporation .....................22
  1.5 Proposed clinical value for studying the role of UNG/BER in cancer chemotherapy .................................................................................................................... 34
  1.6 Summary ..................................................................................................................46

Chapter 2: Statement of objectives....................................................................................49

Chapter 3: Uracil DNA glycosylase determines human lung cancer cell sensitivity to pemetrexed.................................................................54
  3.1 Introduction ..............................................................................................................56
  3.2 Materials and Methods ..........................................................................................58
  3.3 Results ......................................................................................................................66
  3.3.1 A spectrum of UNG expression exists in human lung cancer ...............................66
  3.3.2 Loss of UNG expression increases lung cancer sensitivity to pemetrexed .............70
3.3.3 Limited uracil removal is associated with increased DNA damage in UNG deficient cells. 73

3.3.4 UNG is induced in response to acute and chronic pemetrexed exposure. 79

3.3.5 Transcriptional regulation of UNG induction in pemetrexed treated cells. 83

3.3.6 BER inhibition overrides pemetrexed resistance due to chronic exposure. 86

3.4 Discussion. 88

Chapter 4: Uracil DNA glycosylase (UNG) loss enhances DNA double strand break formation in human cancer cells exposed to pemetrexed. 97

4.1 Introduction. 99

4.2 Materials and Methods. 101

4.3 Results. 108

4.3.1 Loss of UNG expression hypersensitizes human cancer cells to pemetrexed. 108

4.3.2 Increased DNA double strand break formation in UNG−/− cells during pemetrexed exposure. 111

4.3.3 Delayed recovery from S-phase arrest in pemetrexed treated UNG−/− cells. 118

4.4 Discussion. 122

Chapter 5: Enhanced hematopoietic sensitivity in UNG−/− mice during antifolate response. 133

5.1 Introduction. 135

5.2 Materials and Methods. 137

5.3 Results. 142

5.3.1 Increased in vitro sensitivity of UNG−/− MEFs and primary bone marrow cells pemetrexed. 142

5.3.2 Increased in vivo sensitivity of UNG−/− mice to
5.3.3 Increased sensitivity to pemetrexed in UNG mutant

human lymphoblastoid cells…………………………………………………………………..150

5.4 Discussion ...........................................................................................................153

Chapter 6: Towards the development of a novel small molecule inhibitor of uracil DNA glycosylase (UNG)……………………………157

6.1 Introduction……………………………………………………………………………158

6.2 Materials and Methods……………………………………………………………..159

6.3 Results…………………………………………………………………………………161

6.3.1 Optimization and validation of HTS assay

methodology……………………………………………………………………………161

6.3.2 Determination of UNGi-2 IC_{50} using gel-based cutting assay…………………………………………………………………………………164

6.3.3 Intracellular activity of UNGi-2 in combination with pemetrexed………………………………………………………………………………169

6.4 Summary ...........................................................................................................170

Chapter 7: Summary and Conclusions.................................................................172

7.1 Summary of major findings..............................................................................172

7.2 Limitations of this research.............................................................................173

7.3 Conclusions......................................................................................................175

Chapter 8: Future Directions..............................................................................178

References Cited....................................................................................................183
# List of Tables

Table 1-1 Important antifolates and TS inhibitors........................................8
Table 1-2 Known specificities of human uracil DNA glycosylases..................19
Table 1-3 Base excision repair inhibitor strategies in various stages of
development......................................................................................................35

Table 3-1 Description of cell lines used in this study .................................65
Table 3-2 Correlation of linear regression (RQ value vs. \( \text{IC}_{50} \)).............68
Table 3-3 Multivariable regression statistics for ‘UNG+Gene’ Pairs..............69
Table 3-4 Cross-sensitivity of H1975 cells to other DNA damaging agents ......71

Table 4-1 Characteristics of \( \gamma \)-H2AX-enriched sequences.........................117
Table 4-2 Possible pathways to DSB formation and cell death in pemetrexed
treated cells....................................................................................................132

Table 5-1 Pemetrexed-induced changes in animal body weight and peripheral
blood count.........................................................................................................146
Table 5-2 Pemetrexed-induced changes in bone marrow cell number and CFU
potential.............................................................................................................149

Table 6-1 Properties of selected compounds from UNG inhibitor drug screen
efforts...............................................................................................................167
List of Figures

Figure 1-1 Important antifolates and TS inhibitors .............................................. 7
Figure 1-2 Mechanism of antifolate inhibition of nucleotide metabolism .......... 9
Figure 1-3 Schematic representation of DNA base excision repair of misincorporated uracil ................................................................. 16
Figure 1-4. Comparison of gene expression for significant BER genes in cancer cells vs. normal cells ................................................................. 17
Figure 1-5 Futile cycle hypothesis ...................................................................... 23
Figure 1-6 Deoxyribonucleotide biosynthesis ....................................................... 26
Figure 1-7 Proposed model of cell death in UNG deficient cells ......................... 32
Figure 1-8 BER inhibition strategies to improve antifolate therapy in UNG proficient cells ............................................................................. 36

Figure 3-1. UNG expression and pemetrexed sensitivity in human lung cancer .................................................................................... 67
Figure 3-2 Loss of UNG expression sensitizes lung cancer cells to pemetrexed .................................................................................. 72
Figure 3-3 Reduced uracil excision in UNG deficient cells .................................. 74
Figure 3-4 Increased DNA damage in UNG deficient cells ................................. 77
Figure 3-5 AP site detection in H1975 and H1975shUNG cells ......................... 79
Figure 3-6 Induction of UNG in response to acute pemetrexed exposure……..80
Figure 3-7 Induction of UNG in response to chronic pemetrexed exposure……82
Figure 3-8 Cross-resistance of pemetrexed resistant subline to other DNA
  damaging agents.................................................................................................83
Figure 3-9 Transcription factor binding to UNG nuclear promoter in A549 cells
during pemetrexed exposure..................................................................................85
Figure 3-10 Coordination of c-Myc expression with UNG expression and
  induction in lung cancer........................................................................................86
Figure 3-11 Coordination of UNG and TOPOIIα expression
  in human lung cancer.............................................................................................88

Figure 4-1 Loss of UNG enhances pemetrexed sensitivity in DLD1 human colon
cancer cells............................................................................................................110
Figure 4-2 Increased DNA DSB formation in UNG+/− cells treated
  with pemetrexed.....................................................................................................113
Figure 4-3 Comparison of DNA DSB repair capacity in UNG+/−
  and UNG−/− cells..................................................................................................114
Figure 4-4 γ-H2AX ChIP sequencing in UNG+/− and UNG−/− cells………………..116
Figure 4-5 Delayed recovery from cell cycle arrest in pemetrexed treated UNG−/−
cells.........................................................................................................................119
Figure 4-6 Uracil base substitutions attenuate polymerase extension efficiency in
  vitro and correspond to intracellular pemetrexed-induced

viii
replication instability .................................................................121

Figure 4-7 UNG and H2AX are over expressed in many human cancers……..130
Figure 4-8 UNG and H2AX are co-expressed in human cancer………………..131

Figure 5-1 Increased in vitro sensitivity of UNG<sup>−/−</sup> MEFs and primary bone marrow
cells to pemetrexed........................................................................144
Figure 5-2 Increased transient hematologic toxicity in pemetrexed-treated UNG<sup>−/−</sup>
mice..................................................................................................147
Figure 5-3 Increased uracil in splenocytes and bone marrow cells of pemetrexed
treated mice......................................................................................150
Figure 5-4 Increased sensitivity to pemetrexed in human lymphoblastoid cells
possessing inactivating mutations in UNG........................................152

Figure 6-1 Principle of molecular beacon substrate for HTS screening of
compounds for UNG inhibitor activity..............................................162
Figure 6-2 Optimization and validation of HTS assay..........................163
Figure 6-3 Results of HTS screening of 100 compounds for
UNG inhibitor activity........................................................................166
Figure 6-4 Comparison of UNG inhibitor activities of UNGi-2 and uracil in vitro
using gel based cutting assay..............................................................168
Figure 6-5 Intracellular cytotoxicity of UNGi-2 in combination with
Figure 6-6 Continued steps towards identification of UNG inhibitor for clinical development from lead compound library.
Firstly, I am indebted to my advisor, Stanton Gerson, for his guidance and support of this project and my career as a physician-scientist. He has been an amazing mentor – constantly challenging me to go after tough questions, think critically about data, and tell a “good story”. My skills as a scientist, writer and presenter have burgeoned as a result of his instruction and example.

I am thankful for my thesis committee: Alex Almasan, Clive Hamlin, Ruth Keri, Shigemi Matsuyama, George Stark, and Edward Stavenezer (ʼ08-ʼ10), for their direction, dedication and advice throughout the duration of this project.

Many thanks to the various collaborators on this project: Pingfu Fu, for help with statistical analysis; Anthony Berdis for help with primer extension assays (Chapter 4) and understanding implications of genomic uracil; Nooshin Sandraei, Neelesh Sharma, and Afshin Dowlati for assistance in tracking down clinical samples for analysis; Peter Scacheri and Gabriel Zentner for their assistance with ChIP Sequencing; Mike Sramkoski and the flow cytometry core facility for assistance in experimental design and execution; and all of the members of the Gerson lab past and present for trouble shooting and brainstorming ideas. I appreciate that I’ve worked in a collaborative environment where sharing of equipment, reagents, and ideas is commonplace.
I am especially grateful for my family – Mom, Dad, Jestan, Maurice, and Michael – for being an endless source of support. I cherish the love of my grandparents, which has motivated me through the completion of this work. What I wouldn’t give to hear Grandpa call me ‘Shelly Gal’ one more time. I hope to have made him proud. I am also thankful for Jerard Bennett, whose determination, positivity, and love inspire greatness.

Finally I humbly thank the Creator of all things and the Source of all knowledge for His unwavering faithfulness and for giving me the privilege, grace and strength to endure these, the best and toughest years of my life.
List of Abbreviations

5-FU 5-fluorouracil
AID activating induced deaminase
AP site apurinic/apyrimidinic/abasic site
APE AP endonuclease
BER base excision repair
CFU colony forming unit
ChIP chromatin immunoprecipitation
ChIP-Seq chromatin immunoprecipitation sequencing
CldU 5-chloro-2-deoxyuridine
CNdU 1-cyano-2-deoxuridine
Comet assay single cell gel electrophoresis assay
dATP deoxyadenine triphosphate
dCTP deoxycytidine triphosphate
dGTP deoxyguanidine triphosphate
DHFR dihydrofolate reductase
dNTP deoxyribonucleotide triphosphates
dRP deoxyribose phosphate
DSB DNA double strand breaks
dTTP deoxothymidine triphosphate
dUMP  deoxyuridine monophosphate

dUTP  deoxyuridine triphosphate

dUTPase  deoxyuridine triphosphate nucleotidohydrolase

FdUrd  5'-fluoro-2'-deoxyuridine

FPGS  folate polyglutamate synthase

GARFT  glycinamide ribonucleotide formyltransferase

HR  homologous recombination

HSC  hematopoietic stem cells

HTS  high-throughput screen

IC50  concentration inhibiting 50% of growth, survival or activity

IdU  5-iodo-2-deoxyuridine

IHC  immunohistochemistry

LCL  lymphoblastoid cell line

MBD4  methyl CpG binding domain protein 4

MEF  mouse embryonic fibroblast

MMR  mismatch repair

mRNA  messenger RNA

MTA  multi-target antifolate

MX  methoxyamine

NHEJ  non-homologous end joining

NSCLC  non-small cell lung cancer

PARP  poly(ADP-ribose) polymerase phosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PNKP</td>
<td>polynucleotide-kinase phosphatase</td>
</tr>
<tr>
<td>Polβ</td>
<td>DNA polymerase beta</td>
</tr>
<tr>
<td>RFC</td>
<td>reduced folate carrier</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNA Pol II</td>
<td>RNA polymerase 2</td>
</tr>
<tr>
<td>RNR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>RQ</td>
<td>relative quantification value</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SCLC</td>
<td>small cell lung cancer</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SMUG</td>
<td>single strand monofunctional uracil DNA glycosylase</td>
</tr>
<tr>
<td>SNP</td>
<td>small nucleotide polymorphism</td>
</tr>
<tr>
<td>SSB</td>
<td>DNA single strand breaks</td>
</tr>
<tr>
<td>TDG</td>
<td>thymine DNA glycosylase</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofolate</td>
</tr>
<tr>
<td>TIC</td>
<td>tumor initiating cells</td>
</tr>
<tr>
<td>TOPOIIα</td>
<td>topoisomerase 2 alpha</td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TYMS</td>
<td>thymidylate synthase gene</td>
</tr>
<tr>
<td>UDG</td>
<td>denotes family of uracil DNA glycosylases</td>
</tr>
<tr>
<td>Ugi</td>
<td>bacteriophage uracil DNA glycosylase inhibitor protein</td>
</tr>
<tr>
<td>UNG</td>
<td>uracil DNA glycosylase (protein encoded by gene on chr12)</td>
</tr>
<tr>
<td>UNG1</td>
<td>mitochondrial isoform of UNG gene</td>
</tr>
<tr>
<td>UNG2</td>
<td>nuclear isoform of UNG gene</td>
</tr>
<tr>
<td>uracil-DNA</td>
<td>DNA containing uracil or uracilated DNA</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>phosphorylated form of histone H2A.X</td>
</tr>
</tbody>
</table>
Elucidating a Role for Uracil DNA Glycosylase (UNG)-Initiated DNA Base Excision Repair In the Cellular Sensitivity to the Antifolate, Pemetrexed

Abstract

by

LACHELLE DAWN WEEKS

Antifolates are among the oldest anti-cancer chemotherapeutic agents. Despite decades of research, precise mechanisms of antifolate-mediated cell death remain ill defined. Natural and acquired resistance limits the application and effectiveness of antifolate regimens. In an effort to identify novel molecular targets for improving antifolate efficacy in anti-cancer protocols, I have implicated uracil DNA glycosylase (UNG) initiated base excision repair (BER) as a critical determinant of cellular sensitivity to the multi-target antifolate, pemetrexed. Genomic uracil incorporation is a consequence of pemetrexed inhibition of enzymes involved in the de novo biosynthesis of thymidine nucleotides. Once incorporated into DNA, uracil is removed by UNG to initiate BER. Here, I report that UNG induction–driven in part by c-Myc transcriptional activation of the UNG promoter–contributes to pemetrexed resistance by rapidly and efficiently
removing uracil from DNA (AIM 1). Subsequently, using \textit{in vitro} and \textit{in vivo} models of UNG deficiency data show that loss of UNG expression sensitizes cells to pemetrexed through a mechanism involving uracil-DNA induced replication fork collapse, DNA double strand break formation and cell death (AIM 2). Moreover, systematic analysis of human lung cancer cells has identified UNG as a novel predictive biomarker of pemetrexed sensitivity in experimental models (AIM 3). Importantly, these data show that in the absence of functional UNG/BER, pemetrexed treatment results in the toxic accumulation of uracil (UNG loss) or the persistence of clastogenic repair intermediates (inhibition of downstream BER). These data therefore rationalize the development of existing and novel BER inhibitors for therapeutic use in combination with pemetrexed and other antifolates (AIM 4). These findings clarify the genotoxic nature of uracil misincorporation and provide clear evidence that the amount of genomic uracil and thus, a cell's capacity for UNG-initiated BER is a critical determinant of cellular sensitivity to pemetrexed. In highlighting the importance of UNG-initiated BER in the response to pemetrexed, this work provides a solid foundation for future studies, which seek to confirm a role for UNG as both a predictive biomarker and as a novel chemotherapeutic target to enhance clinical response to pemetrexed chemotherapy regimens.
Chapter 1

Introduction: Uracil misincorporation and thymine-less death – old and new hypotheses on the mechanism of action of antifolates

Antifolates are among the oldest anticancer agents in clinical use. Structurally similar to endogenous folate, these chemotherapeutic agents inhibit the activity of folate-dependent metabolic enzymes to promote cytostasis and cytotoxicity. Particularly, antifolate inhibition of dihydrofolate reductase (DHFR) and thymidylate synthetase (TS) interferes with the de novo biosynthesis of deoxythymidine triphosphate (dTTP), an essential nucleotide in DNA synthesis. Though the first antifolates, aminopterin and its derivative methotrexate, were identified in the 1940s, precise mechanisms of antifolate cytotoxicity are still being defined. Beyond nucleotide depletion, a less understood consequence of DHFR and TS inhibition is polymerase misincorporation of dUTP into DNA as a result of elevated dUTP/dTTP ratios. The genomic uracil that accumulates during antifolate exposure is a substrate for uracil DNA glycosylase (UNG), the initial enzyme in the DNA base excision repair (BER) pathway. It has been hypothesized that antifolate-induced uracil accumulation promotes futile cycles of uracil-excision, repair and reincorporation that compromise DNA integrity and
lead to cell death. However, recent data have emerged implicating uracil accumulation as a key determinant of antifolate cytotoxicity even in the absence of UNG activity, precluding futile cycles of BER as contributing factors in thymine-less death. Cancer cells possessing a high capacity for uracil removal and BER are more resistant to pemetrexed cell killing than cells with lower BER capacity. Alternatively, treatment of UNG deficient cells with antifolates results in toxic accumulation of uracil-DNA causing DNA strand breaks and cell death. Based on these data, inhibition of BER is currently in development as a clinical strategy for potentiating the cytotoxicity of the multi-target antifolate, pemetrexed. Here, I summarize the current body of knowledge regarding antifolate mechanisms of action and potential roles for UNG/BER in modulating cellular responses to antifolates. Future research in this area should include the continued evaluation of the applicability of BER modulation to enhance antifolate responses clinically and the usefulness of BER enzymes as biomarkers for clinical response to antifolates.
1.1 Antifolates in cancer therapy

Antifolates have been used in cancer therapy for more than 60 years. Drugs in this class exert their anticancer effects by inhibiting folate-dependent enzymes required for the de novo biosynthesis of DNA nucleotides. Several antifolates are currently utilized clinically, including methotrexate, 5-fluorouracil, raltitrexed, praletrexate, and pemetrexed (Figure 1-1 and Table 1-1). Three enzymes comprise the major enzymatic targets for antifolate activity: dihydrofolate reductase (DHFR), thymidylate synthetase (TS), and glycinamide ribonucleotide formyltransferase (GARFT) (Figure 1-2). The anti-metabolite, 5-fluorouracil (5-FU) also targets TS to promote cell death and has wide clinical application. Because 5-FU has been utilized widely to study the consequences of TS-inhibition, it will be discussed and compared with TS-inhibiting antifolates, where applicable.

1.1.1 Inhibitors of dihydrofolate reductase (DHFR)

The enzyme dihydrofolate reductase (DHFR) is the target of the prototypical antifolate, methotrexate and the more recent agent, pralatrexate. DHFR catalyzes the reduction of folate into its active metabolite, tetrahydrofolate (THF). This enzyme’s activity is therefore critical to the maintenance of reduced folate (THF) pools. Reduced folate, THF, is the requisite cofactor that donates carbon atoms in the enzymatic formation of thymidylate and purine nucleotides (Figure
Therefore, in cells that are in S-phase, inhibition of DHFR prevents the biosynthesis of thymidine and purine nucleotides necessary for DNA and RNA synthesis so as to lead to cell death.(1)

1.1.2 Inhibitors of thymidylate synthase (TS)

Thymidylate synthetase (TS) is the enzyme directly responsible for methylation of deoxyuridine monophosphate (dUMP) to form deoxythymidine monophosphate (dTMP). The subsequent phosphorylation of dTMP forms dTTP, an essential nucleotide in DNA synthesis. This provides the sole de novo pathway for production of thymidine deoxynucleotides (dTTPs). As alluded to above, DHFR activity is required to preserve the one carbon pool necessary for TS function. Therefore, all antifolates which specifically target DHFR will indirectly reduce TS activity via THF pool reduction.(2) Raltitrexed, a structural analog of THF directly inhibits TS and has clinical activity in advanced colon cancer and malignant pleural mesothelioma. The nucleotide analog, 5-fluorouracil (5-FU) is also a known inhibitor of TS. Within cells, 5-FU is metabolized to form the anabolite, 5-fluorodeoxyuridine monophosphate (FdUMP), which binds tightly to the dUMP-binding site of TS.

The primary effect of TS inhibition is depletion of dTTP. However, in murine tumor cells, inhibition of TS activity was also marked by decreased dATP and dGTP pool levels.(3) Together, these nucleotide pool imbalances contribute to
the overall decrease in DNA synthesis and so-called “thymine-less” death. Additionally, the corresponding elevation in dUTP levels in the context of TS inhibition promotes the incorporation of dUTP into DNA. DNA fragmentation and strand break formation that characterize thymine-less death are observed even under conditions of hypoxanthine and thymidine rescue, which partially correct nucleotide pool imbalances through salvage pathways. This feature suggests that the incorporation of dUTP into DNA is itself a source of genotoxic stress.(4)

Figure 1-1. Important antifolates and TS inhibitors. Chemical structure of folic acid is shown for comparison with that of clinically useful antifolates and TS inhibitors including the DHFR-specific inhibitors methotrexate and pralextrexate; the TS inhibitors raltitrexed and 5-fluorouracil; the GARFT inhibitor, lometrexol; and the multi-target antifolate, pemetrexed.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Approved Uses</th>
<th>Active Trials in other cancer types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pemetrexed (ALIMTA, LY231514)</td>
<td>• Malignant pleural Mesothelioma • Advanced, recurrent, metastatic NSCLC</td>
<td>• Rectal Cancer (phase I/II: NCT01397305) • Head and neck Cancer (phase I/II: NCT00573989, NCT0158049, NCT00703976) • Thyroid cancer (phase II: NCT00786552) • Ovarian (phase II: NCT01001910)</td>
</tr>
<tr>
<td>Raltitrexed (Tomudex, ZD1694, TDX)</td>
<td>• No approved uses</td>
<td>• Colorectal cancer (phase II: NCT01348412, NCT01532804) • Esophageal (phase II: NCT01732380) • Mesothelioma (phase I/II: NCT00797719)</td>
</tr>
<tr>
<td>5-Fuorouracil (Adrucil, Efudex, Fluoroplex)</td>
<td>• Locally advanced and unresectable squamous cell carcinoma of the head and neck • Pancreatic Cancer • Actinic keratosis • Basal cell carcinoma • Breast Cancer • Advanced and recurrent or treatment refractory colorectal cancer • Gastric adenocarcinoma</td>
<td>numerous post-approval studies (phase IV)</td>
</tr>
<tr>
<td>Capecitabine (Xeloda)</td>
<td>• Stage 3 colorectal cancer after surgical resection • Metastatic or treatment refractory breast cancer</td>
<td>• Pancreatic cancer (phase II/III: NCT01065870, NCT01150630) • Hepatocellular carcinoma (phase II/III: NCT01438450, NCT00522405)</td>
</tr>
<tr>
<td>Uracil-Tegafur (UFT, Uftoral)</td>
<td>• NSCLC (Japan) • Breast (Japan) • Colorectal (Japan and United Kingdom)</td>
<td>• Pancreatic (phase II, United Kingdom: NCT01050426, NCT00384800) • Hepatocellular carcinoma (Taiwan: NCT00384800)</td>
</tr>
<tr>
<td>Methotrexate (Abitrexate, Folex PFS, Mexate-AQ)</td>
<td>• Acute lymphoblastic leukemia that has spread to the CNS • Breast Cancer • Lung Cancer • Advanced and recurrent mycosis Fungoides • Advanced non-Hodgkin lymphoma • Local osteosarcoma following primary resection • Gestational trophoblastic tumors • Head and neck cancers</td>
<td>numerous post-approval studies (phase IV)</td>
</tr>
<tr>
<td>Praletrexate (Folotyn)</td>
<td>• Recurrent peripheral T cell lymphoma or refractory disease</td>
<td>• Ovarian, peritoneal (phase II: NCT01188876) • Gastro-Esophageal (phase II: NCT01178944) • Multiple Myeloma (phase I: NCT01114282)</td>
</tr>
<tr>
<td>Lometrexol (LMTX, 6R-DDATHF)</td>
<td>• No approved uses</td>
<td>Limited objective tumor responses observed with significant dose-limiting toxicity(5-8)</td>
</tr>
</tbody>
</table>
Figure 1-2. Mechanism of antifolate inhibition of nucleotide metabolism. Antifolates principally inhibit three enzymes (red): DHFR, TS and GARFT resulting in depletion of purine nucleotide pools and dTTP. Inhibition of TS also results in the accumulation of uracil in DNA, a direct consequence of increased dUTP/dTTP ratios.

1.1.3 Inhibitors of glycinamide ribonucleotide formyltransferase (GARFT)
Glycinamide ribonucleotide formyltransferase (GARFT) binds a reduced folate cofactor (THF) to catalyze several of the initial steps in the de novo synthesis of purines from activated ribose and glycine. Inhibition of GARFT therefore prevents formation of guanosine (dGMP) and adenosine (dAMP) nucleotides, in turn limiting RNA and DNA synthesis and resulting in cell death. Lometrexol was the first specific GARFT inhibitor to be identified. Clinical development of lometrexol, however, has been delayed due to extensive toxicity in humans. (8) GARFT activity is also an indirect target of DHFR inhibition, since THF molecules are necessary cofactors for GARFT function.
1.1.4 The multi-target agent, pemetrexed

The multi-target agent, pemetrexed is currently approved for use in patients with advanced or recurrent non-small cell lung cancer and malignant pleural mesothelioma. Pemetrexed is known to bind and inhibit the three major folic acid dependent enzymes, DHFR, TS and GARFT. (9-13) The primary target of pemetrexed, however, appears to be TS. (14) Once transported into cells by the reduced folate carrier (RFC), (15) pemetrexed is rapidly polyglutamated by folylpolyglutamate synthase. Polyglutamated pemetrexed is has a longer half-life and is a more potent inhibitor of TS and GARFT than the parent molecule. (9, 13) Additionally, polyglutamation rate constants are higher for pemetrexed compared to other antifolates, resulting in longer intracellular retention. (16)

Similar to the agents described above, pemetrexed exposure is characterized by decreased DNA and RNA synthesis, depletion of dTTP and consequent elevation of dUTP nucleotide pools. (10) Additionally, elevated dATP pools have also been observed, (10) presumably as a result of loss of allosteric feedback regulation of ribonucleotide reductase (RNR, see below). It is unclear the extent to which multi-enzyme targeting influences the potency of pemetrexed compared to other antifolates. However, it has been suggested that multi-target enzyme blockade may make pemetrexed the antifolate of choice in patients with acquired resistance to single enzyme-targeted antifolates. (17)
Early growth inhibition studies showed pemetrexed activity against a diverse panel of tumor cell lines(9, 11, 13, 15, 18-21) and in xenograft studies.(22) Xenograft responses were sensitive to folate levels of murine plasma(11, 21, 23) and animals maintained on low-folate diet were much more sensitive to the toxic effects of pemetrexed compared to standard diet littermates.(11, 23) Dietary folate protected animals from adverse toxicity without impairing anti-tumor effects.(21)

In humans, pemetrexed is currently approved in the treatment of advanced, recurrent or metastatic non-squamous non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma.(24-27) Combination therapy with cisplatin is first-line therapy in advanced NSCLC(24) and is single agent second-line therapy for patients with advanced NSCLC.(25) Additionally, pemetrexed maintenance therapy produces a statistically significant survival advantage over placebo.(26, 27) Numerous clinical trials evaluating the efficacy of pemetrexed in combination with other DNA damaging agents and targeted therapies have been conducted.(28-32) Pemetrexed is also being investigated for activity in other solid tumors, such as colon, pancreas, and breast cancers.(33-37)
1.2 DNA repair in human cancer development and therapy

Cellular capacity for DNA repair is central to the development and the treatment of human cancer. Genomic instability is a hallmark feature of human cancer that implies an inherent DNA repair defect in malignant cells. (38) In hereditary cancers, for example, mutations in DNA repair genes give rise to the so-called “mutator phenotype” during tumor development and progression (39-42) by increasing the spontaneous mutation rate. (43, 44) In sporadic cancers, genomic instability may arise due to mutations in oncogenes and tumor suppressors that promote de-regulation of genes related to cell growth, cell cycle and DNA repair, allowing the accumulation of additional mutations during replication stress. (45-47) Paradoxically, all cancer cells require DNA repair in order to replicate. (48) Consequently, cancer cells compensate for inherent DNA repair deficits through overuse of alternative DNA repair pathways. This “addiction” to non-defunct DNA repair pathways provides a useful target for cancer therapy. (48)

DNA damaging chemotherapy remains a major treatment modality for human malignancy. DNA damaging agents work by producing excessive DNA damage in the form of bulky DNA adducts (alkylating agents and cross-linking agents), aberrant nucleotide utilization (fluoropyrimidines), and nucleobase depletion (anti-metabolites and antifolates). The efficacy of DNA damaging chemotherapy is highly influenced by the cellular capacity for DNA repair. For example, the
removal of base lesions by DNA base excision repair (BER) dampens the cytotoxicity of chemotherapeutics such as alkylating agents (49-56) and anti-metabolites.(57-59)

Targeting non-defunct DNA repair pathways in cancers with mutated DNA repair genes leads to synthetic lethality. For two genes or pathways to have a synthetic lethal effect on a cell, mutations or inactivation of two genes must lead to cell death whereas mutation in any one of the genes does not.(60) The concept of synthetic lethality as it relates to DNA repair in cancer cells has recently been reviewed.(48, 61) A classic example of synthetic lethality is the observation that HR-defective cells are specifically sensitive to PARP inhibitors.(62-72) Inhibition of PARP in this milieu combines ineffective single strand break (SSB) repair, which in turn increases replication-associated double strand breaks (DSB) resulting in cell death.(60, 61) Based on the central role of DNA repair pathways to the development and the treatment of human cancer, targeting DNA repair to enhance cancer therapy is considered one of the most innovative advances in cancer drug development over the last decade.

1.3 Base excision repair of uracil-DNA

DNA base excision repair (BER) is an evolutionary conserved pathway that removes the preponderance of endogenous DNA lesions as well as DNA
damage arising during exposure to chemotherapeutics. BER gene expression is elevated in cells with high turnover rates, including gut mucosa, bone marrow, and malignant cells suggesting a critical role for BER in the maintenance of genomic integrity in highly replicative cells. It follows that BER has been identified as an important DNA repair pathway in tumor cells and has emerged as a target for enhancing cancer therapy. Table 1-3 summarizes the various strategies that have been considered for targeting the BER pathway to enhance chemotherapeutic efficacy.

1.3.1 Overview of the BER pathway.

There are two major sources of genomic uracil: hydrolytic deamination of cytosine and misincorporation of dUTP during DNA replication. Spontaneous hydrolytic deamination of cytosine to uracil forms a U:G mismatch that generates C:G→T:A transition mutations upon subsequent rounds of DNA replication. Misincorporation of dUTP by DNA polymerases during DNA replication, and genomic uracil accumulation (uracil-DNA) is a consequence of antifolate inhibition of the enzymes TS and DHFR. DNA base excision repair (BER) is the major pathway responsible for the rapid and efficient repair of DNA base lesions including uracil-DNA (Figure 1-3). Briefly, the BER pathway begins with uracil DNA glycosylase excision of uracil to generate abasic (AP) sites. AP sites are subsequently processed by AP Endonuclease (APE), which hydrolyzes phosphodiester bonds at the 5’ end of the AP site yielding a DNA single strand.
break. This processing step also generates a 3’hydroxyl group, which primes the DNA for re-synthesis and a 5’-dRP residue, which blocks premature DNA ligation. In a rate-limiting reaction, the dRP lyase domain of DNA polymerase β (Polβ) removes the 5’dRP moiety allowing either the short- or long-patch pathways to accomplish further repair. Short-patch BER is reserved for single nucleotide base lesions. In short patch BER, Polβ, completes gap filling by adding a single nucleotide to the DNA strand. To complete the repair process, the DNA-ligase III-XRCC1 heterodimeric complex then rejoins DNA ends. Long-patch BER is signaled by the oxidation or reduction of the sugar residue of the AP site, which occurs in the context of large flaps of damaged bases (2-10 nucleotide). Polβ initiates gap filling by adding the first nucleotide to the DNA strand and DNA polymerase δ or ε add subsequent nucleotides until the gap is filled. Flap endonuclease 1 (FEN1) removes the flap of damaged or aberrant nucleotides and DNA ligase I completes the repair. PCNA also participates in BER by activating BER enzymes and facilitating the formation of DNA repair complexes.

Several BER enzymes including UNG, APE1, Polβ and PARP are significantly elevated in certain human cancers (Figure 1-4) and their increased activity in human cancer may provide protection against antifolate induced DNA base damage. In addition, these enzymes represent significant bottleneck points in the
BER pathway that govern BER initiation and the resolution of significant clastogenic BER intermediates.

Figure 1-3. Schematic representation of DNA base excision repair of misincorporated uracil. Briefly, uracil is excised by uracil DNA glycosylase to generate an AP site. AP sites are processed by APE endonuclease I (APE1) to generate a DNA strand break. PARP-1 is localized to the site of DNA damage to facilitate single strand break repair. DNA polymerase removes the 5’dRP moiety and initiates re-synthesis by adding the first nucleotide to the single strand gap. Gap filling is followed by ligation by the DNA ligase-XRCC1 heterodimer. Longer patches of damaged bases are excised by flap endonuclease (FEN1) prior to DNA ligation (not shown).
Figure 1-4. Comparison of gene expression for significant BER genes in cancer cells vs. normal cells. These data are a composite of published microarray studies retrieved from the Oncomine database (Oncomine™, Compendia Biosciences). Analysis of gene expression for UNG, APE1, Polβ, PARP1 and PARP2 are shown. Red boxes indicate studies with significantly higher expression in cancer cells compared to normal cells for a given cancer type while blue boxes indicate significantly lower expression in cancer vs. normal. The numbers within the boxes indicates the number of independent studies with the observed trend. The threshold fold change is 2 (all data are log2 median centered) and p value significance was set at $1 \times 10^{-4}$. 

![Gene Expression Table]

**Note:** This table illustrates the comparison of gene expression for significant BER genes across various cancer types, including UNG, APE1, Polβ, PARP1, and PARP2. The data are derived from published microarray studies and are filtered through the Oncomine database. Red boxes denote studies with significantly higher expression in cancer cells compared to normal cells, while blue boxes indicate lower expression. The numbers within the boxes represent the number of independent studies supporting the observed trend. The analysis uses a threshold fold change of 2 for all data, with a p value significance set at $1 \times 10^{-4}$. 

**Legend:**
- **Low expression:** Decrease in expression compared to normal cells.
- **High expression:** Increase in expression compared to normal cells.
- **Significant Unique Analyses:** Numbers indicate the specific studies meeting the criteria for significant expression changes.
1.3.2 Uracil DNA glycosylases

The base excision repair of uracil DNA begins with hydrolytic cleavage of uracil bases from the DNA phosphodiester backbone by uracil DNA glycosylases. This initiating step of BER generates an AP site, a clastogenic lesion that will result in cell death when left unrepaired.(78) In humans, there are four distinct glycosylases responsible for the excision of uracil in different contexts (Table 1-2). Conventionally, the abbreviation, UDG denotes the enzymatic activity of glycosylases with uracil specificity. The abbreviation, UNG, refers to the uracil DNA glycosylase encoded by the UNG gene on chromosome 12. From here on, the discussion will follow this convention. The UNG gene encodes mitochondrial (UNG1) and nuclear (UNG2) isoforms of the major uracil DNA glycosylase. UNG2 has a noted role in resolving uracil that is misincorporated during DNA replication and comprises the bulk of uracil excision activity.(77) In contrast, SMUG1 plays a minor role in excising single strand uracil and has a slightly broader specificity for damaged pyrimidines. Additionally, a role for SMUG1 in preferentially excising deaminated cytosine residues has been proposed.(79) SMUG1 has also been observed to excise 5-FU from DNA.(80) TDG and MBD4 excise mismatched uracil and damaged thymidines from double-stranded DNA. MBD4 has a specific role in CpG contexts and also engages in crosstalk with mismatch repair (MMR) complexes through MLH1 interactions.(81)
There is evidence to suggest that in the context of chemotherapy-induced DNA base lesions, glycosylase expression must be maintained within certain homeostatic bounds for cell survival. In studies of methyl-purine glycosylase (MPG), for example, both induction (82) and complete loss (83) of glycosylase expression have been noted to drive sensitivity to alkylation-induced base damage. A similar concept of glycosylase homeostasis may also be applicable to uracil DNA glycosylases. Overexpression of UNG has been shown to increase cytostasis during raltitrexed exposure (84) and we report herein that deficiency in UNG potentiates pemetrexed cytotoxicity in in vitro and in vivo models (chapters 3-5). However, a complete understanding of cellular sensitivity to glycosylase perturbations remains muddled by an incomplete knowledge of threshold tolerances for the various types of base damage targeted by BER.

<table>
<thead>
<tr>
<th>Glycosylase</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG 1/2</td>
<td>U:A, U:G, Flu</td>
</tr>
<tr>
<td>SMUG 1</td>
<td>U:F, U:A, OH-MeU, 5-FU</td>
</tr>
<tr>
<td>TDG</td>
<td>U:G, T:G, ethenoC</td>
</tr>
<tr>
<td>MBD4</td>
<td>U:G, T:G in CpG domains</td>
</tr>
</tbody>
</table>

U = uracil, OH-Meo = hydroxy methyl uracil, T = thymine, G = guanine, ethenoC = ethenocytosine, Flu = fludarabine
1.3.3 AP Endonuclease

AP site end processing is essential to facilitate downstream re-synthesis. Following glycosylase removal of uracil, AP Endonuclease (APE) acts to create a nick or single strand break in the phosphodiester backbone of the AP site. APE, via its phosphodiesterase function, participates in the end processing at the 5’ end of the single strand break to generate a 3’-hydroxyl (3’OH) end that primes for DNA polymerase extension and a 5’dRP moiety that blocks premature ligation. This end processing is promoted by polynucleotide-kinase phosphatase (PNKP). AP sites, when left unrepaired, can act as position-specific endogenous topoisomerase poisons by stimulating covalent topoisomerase-DNA complexes, inhibiting DNA replication and generating permanent double strand breaks.(85-88) Like the AP site, the 5’dRP moiety is a clastogenic intermediate, which can trigger NAD+/ATP depletion and necrotic cell death when unrepaired.(89)

1.3.4 DNA polymerase beta (Polβ)

In both short- and long-patch BER, DNA polymerase beta (Polβ) is responsible for the template-directed nucleotide transfer reaction that adds the first nucleotide to the nick formed by APE.(90, 91) Polβ is a non-replicative polymerase, the activity of which appears to be limited to the BER pathway.(91-93) It is distinguished from replicative polymerases by lack of proofreading capability and high DNA replication infidelity.(94, 95) Because Polβ is error prone, its expression is tightly regulated with levels typically maintained consistently low.
throughout the cell cycle. (96, 97) It has been suggested that perturbations of Polβ expression exceeding homeostatic boundaries or expression of certain genetic variants of Polβ results in more frequent DNA synthesis errors and contributes to mutator phenotypes in human cancer. (98-103)

In addition to DNA synthesis, Polβ is comprised of a dRP lyase domain that is responsible for the removal of the 5’dRP moiety created during APE/PNKP mediated end processing of AP site. (89) While the DNA synthesis activity of Polβ can be complemented by several additional polymerases and is not rate determining, the dRP lyase activity is both essential and rate limiting for BER. (104, 105) In the absence of Polβ expression, or in cells specifically lacking the dRP lyase domain of Polβ, persistence of the dRP moiety leads to cross-linking of repair enzymes to DNA lesions, strand breaks and cell death. (106) In this way, Polβ deficiency results in hypersensitivity to base lesions formed by genotoxic agents.

1.3.5 Poly (ADP-ribose)-polymerase (PARP)

Upon detection of DNA nicks, Poly (ADP-ribose)-polymerase (PARP)s -1 and 2 bind damaged DNA and rapidly stimulate the NAD⁺ dependent poly (ADP-ribosyl)ation of multiple proteins including itself. (107) Upon poly(ADP-ribosyl)ation, PARP loses affinity for DNA and is released from its binding site permitting access of repair proteins. (108, 109) Binding and activation of PARP
also serves to sequester DNA repair enzymes to the site of damage and single
strand breaks (SSBs), thus facilitating the formation of functional repair foci.(110)
Therefore, while PARP does not directly function to repair DNA base lesions or
BER intermediates, PARP activity often referred to as a critical BER enzyme.
Deficiency in PARP results in increased accumulation of single strand break
intermediates in response to base damage by genotoxic agents.(110, 111)

1.4 Proposed consequences of antifolate mediated-dUTP incorporation

1.4.1 Futile cycles of BER

Though an exact mechanism of thymine-less death has not been fully elucidated,
a dominant hypothesis has suggested that futile cycles of UDG-initiated BER
contribute to TS inhibitor cytotoxicity.(4, 112) The basis of this hypothesis is that
when TS inhibition increases dUTP/dTTP pool ratios, and the abundance of
dUTP overwhelms dUTPase (an enzyme that degrades dUTP to dUMP to
prevent dUTP utilization in DNA synthesis). DNA polymerases, having equal
affinities for dUTP and dTTP, will incorporate dUTP opposite dATP.
Misincorporation of dUTP then triggers UDG/BER to remove uracil. However,
because dUTP/dTTP ratios remain high, dUTP is reinserted during repair. This
futile cycle of excision, repair and reinsertion is hypothesized to lead to the
accumulation of BER intermediates that are converted to DNA double strand
breaks during replication resulting in cell death (Figure 1-5).(113)
Figure 1-5. Futile cycle hypothesis. Elevated dUTP:dTTP ratios during antifolate exposure results in DNA polymerase misincorporation of uracil instead of thymine during both replication and repair. UDG removes the uracil but re-synthesis in the milieu of elevated dUTP levels results in reinsertion of uracil leading to a futile cycle of DNA repair. This is hypothesized to result in the relative accumulation of BER intermediates, which weaken the DNA strand and contribute to chromosome fragmentation and cell death.

Whether futile cycling of BER is the predominant mechanism of antifolate-induced thymine-less death remains controversial. Several early reports failed to detect dUTP or uracil in cells treated with antifolates. (114, 115) Failed detection of uracil, however, could have simply been a consequence of rapid and efficient uracil DNA glycosylase excision. Indeed we have recently provided evidence of robust uracil accumulation in UNG−/− cells after treatment with the antifolate pemetrexed or 5-FU, where levels remain similar to baseline in UNG+/+ cells (58). Other studies have identified a strong correlation between elevations of intracellular/intragenomic dUTP and the DNA fragmentations and strand breaks characteristic of antifolate toxicity. (4, 116-118) DNA strand breaks do not have to arise from futile cycles of BER. Using double stranded circular plasmids researchers illustrated that UNG directed excision of uracil residues positioned...
within 12 bases on opposing DNA strands was sufficient to cause double strand break formation. (119) This *in vitro* series of experiments conceivably recapitulates a situation wherein uracil accumulates in both the template and coding strands and therefore would imply that several rounds of replication must occur before uracil and UNG-mediated toxicity becomes apparent.

Still, while it has been assumed by some that antifolate-induced DNA fragmentation and strand break formation is BER-mediated, little to no direct evidence of BER involvement in strand break formation exists. In one study favoring the futile-cycle hypothesis, researchers observed FdUrd resistance in cells when UNG was prematurely degraded in S-phase or under conditions of siRNA directed UNG knockdown. (120) However a contrasting result in UNG−/− MEFs indicated no differential sensitivity to 5-FU or FdUrd. (121) Furthermore, only a modest growth inhibition phenotype was reported for A549 cells over-expressing UNG. (84) This result is in striking contrast to the increased cytotoxicity one would expect with enhanced UNG-directed futile cycles.

Understanding of the impact of UNG/BER and genomic uracil on antifolate and TS-inhibitor sensitivity is further complicated by observations of apparent uracil toxicity in *E. Coli* (122) and *S. Cerevisiae*. (123) In *E. Coli* it has been reported that between 93% and 96% of the thymidine in DNA is replaced by uracil in dUTPase deficient strains. (122) Moreover, in both *E. Coli* (122) and yeast (124), dUTPase
mutants are not viable, presumably due to high levels of dUTP incorporation and the accumulation of uracil-DNA. The inviability of *S. Cerevisiae* deficient in both dUTPase and UNG has suggested that uracil-DNA is itself toxic, even in the absence of UNG-initiated BER cycles. (124) In mammalian cells, dUTP incorporation and uracil accumulation have also been identified as critical determinants of cytotoxicity of the antifolate, pemetrexed. (58) Therefore, it appears that futile cycling of BER, if it occurs, is not necessary for antifolate-induced cytotoxicity.

In short, comprehension of the contributions of futile cycles of BER to antifolate toxicity is limited by the paucity of direct experimental measurement of this phenomenon and relatively few model systems. Further evaluation in this area is warranted to directly link BER excision of uracil to antifolate toxicity and explicitly identify the source of DNA strand breaks in UNG proficient systems.

### 1.4.2 Impact of dUTP/dTTP pool imbalance on nucleotide homeostasis

Ribonucleotide reductase (RNR) is an enzyme responsible for maintaining balanced nucleotide pool levels through the synthesis of deoxyribonucleotides (dNTP) from ADP, GDP, CDP and UDP. Because of allosteric feedback regulation of RNR, inhibition of dTMP biosynthesis causes decreased dGTP and dTTP levels, while dCTP, dATP and dUTP levels increased (Figure 1-6). (125) Excess thymidine corrects this imbalance by shifting the equilibrium in the
Figure 1-6. Deoxyribonucleotide biosynthesis. Biosynthesis of deoxyribonucleotides is regulated through ribonucleotide reductase (RNR), an allosteric enzyme with an active site (A) and a specificity site (S). dNTP synthesis is regulated by binding of other dNTPs to (A) or (S) to allosterically inhibit or activate RNR towards additional dNTP production. Allosteric activators (up arrows) and inhibitors (down arrows) are shown corresponding the allosteric binding site for RNR for each dNTP. dATP binding at (S) stimulates CDP and UDP reduction, while dATP binding at (A) inhibits RNR reduction of all dNTPs. dTTP binding at (S) stimulates dGTP formation and inhibits dCTP, dUTP and dTTP formation. dGTP binding at (S) stimulates dATP inhibits dCTP, dUTP, dTTP and dGTP production.
opposite direction raising dGTP and dTTP levels and lowering dCTP, dATP and dUTP pools. Low levels of any dTTP may therefore trigger additional nucleotide pool imbalances and inhibit DNA replication. The elevation of dCTP by dTTP may be particularly problematic in the replication of CGG repeats. (126) It has further been suggested that aberrantly high dCTP levels may render AP site repair error prone, leading to increased mutagenesis in cells exposed to base damaging chemotherapeutics. (125, 127, 128) As a testament to the potential importance of the nucleotide pool aberrations induced by low dTTP, Sanchez et al recently reported evidence of increased sensitivity to elevated dCTP:dTTP ratios. (129) Replication fork stall, fork collapse and genome instability were observed in cells with inactivating mutations in deoxycytidylylate deaminase, an enzyme responsible for catalyzing the deamination of dCMP to form dUMP, a precursor in dTMP biosynthesis. (129) Additionally, synergistic enhancement of pemetrexed efficacy has been observed with the RNR inhibitor, gemcitabine, suggesting that RNR-mediated nucleotide pool maintenance is a critical determinant of cellular response to pemetrexed. (130) Antifolate and TS-inhibitor cell killing could then be directly related to the numerous nucleotide pool aberrations that likely occur in the context of dTTP depletion.

1.4.3 Direct toxicity of uracil-DNA

Recently, as an alternative to BER mediated futile cycling, replication stress caused by uracil-DNA accumulation has been proposed as a mechanism of
antifolate toxicity (Figure 1-7). (58, 59) It is well accepted that U:G mispairs are mutagenic, generating C:G→T:A transition mutations during both replication and transcription. (131-134) In contrast, several observations of equivalent in vitro polymerase extension efficiencies for synthetic oligonucleotides containing single uracil substitutions (135) have erroneously lead researchers to believe that U:A (uracil-DNA) is a completely innocuous lesion.

Studies in yeast and E. Coli provided the first hints of uracil-DNA toxicity. In S. Cerevisiae and E. Coli lacking dUTPase expression, uracil accumulation, impaired growth and decreased viability are marked by failed DNA synthesis. (122, 124) In human cancer cells, overexpression of dUTPase, which limits dUTP incorporation and genomic uracil loads, lead to resistance to thymidine deprivation. (136, 137) Elevated expression of dUTPase was also observed to be associated with fluoropyrimidine resistance in colon cancer patients. (138) Small interfering RNA knockdown of dUTPase expression resulted in increased dUTP misincorporation and sensitization of human cancer cells to TS inhibition. (139) Together, these data clearly indicate that the incorporation of dUTP into DNA is an essential feature of TS inhibitor and antifolate-mediated cell death.

That uracil-DNA were a toxic lesion which promotes cell death in a manner independent of UNG-BER futile cycling would imply that limiting BER activity by
blocking UNG expression and activity may enhance uracil-DNA accumulation and sensitize cells to fluoropyrimidine TS inhibitors and antifolates. Consistent with this supposition, we have shown that loss of expression of UNG sensitizes human lung (59) and colon cancer (58) cells to pemetrexed (Chapters 3 & 4). Additionally, pemetrexed treatment results in a greater degree of bone marrow suppression in UNG deficient mice than in wild type littermates (Chapter 5).

Because of our observations of enhanced DNA double strand breaks in UNG deficient cancer cell models, we have hypothesized that UNG deficient cancer cells succumb to antifolate-mediated cell death via a mechanism involving uracil accumulation, DNA double strand break formation and disassembly of DNA replication fork components. (58) The ability to partially rescue sensitivity to antifolates in UNG deficient cells using thymidine suggests that TS-inhibitor induced cell death is mediated by the incorporation of uracil. Furthermore, the lack of uracil excision precludes futile cycles of BER in this model of thymine-less cell death. Thus, we have proposed that the accumulation of genomic uracil is intrinsically genotoxic and directly contributes to DNA double strand break formation via collapse of DNA replication forks and ultimately cell death (Figure 1-7).

Structural differences in uracil-DNA may explain cellular sensitivity to this lesion. Polymerase catalyzed dUTP incorporation, produces a DNA macromolecule with an inherently different electron stacking structure than thymine containing
DNA. (140) Uracil-DNA lacks thymine 5’methyl groups that many DNA regulatory proteins rely upon for sequence recognition and binding. (141, 142) Through van der Waals interactions with amino acids, these thymine methyl groups provide specific contact points for DNA binding proteins. (142) For example, it has been suggested that uracil in DNA may impede transcription factor binding to target sequences (141, 143) which could potentially have multiple effects on cell signaling, metabolism and survival. Following this logic, high uracil content could also be an impediment to the association of the replicate with DNA. In the studies that follow, we will show that pemetrexed treatment in UNG−/− cells results in dissociation of PCNA, a replisome component from S-phase gated cells (Chapters 3&4). Additionally, the in vitro efficiency of polymerase nucleotide transfer is decreased when uracil-containing oligonucleotides are used as template DNA or when the reaction mixture contains excess dUTP (Chapter 4).

The presence of uracil in DNA may also provoke the activity of nucleases and topoisomerase leading to DNA degradation. For example, in E. Coli, uracil containing DNA stimulates the activity of endonuclease V (endoV) which cleaves the phosphodiester bond 3’ to uracil in an Mg2+ dependent manner to generate a 3’OH and 5’P termini. (144) Recently, a human homolog of endoV has been identified. (145) While human endoV appears to lack endonuclease activity against uracil-DNA, (145) other human endonucleases such as NEIL1 (endoVIII) and hNTH (endoIII) have been shown to incise dihydrouracil containing
Additionally, it has also been suggested that uracil located immediately 3' prime to topoisomerase cleavage sites may stimulate topoisomerase cleavage activity, suggesting that like AP-sites, uracil-DNA may also function as a positional topoisomerase poison. High uracil burden therefore may increase DNA fragmentation by endogenous nuclease and topoisomerase activity, leading to cell death.

In earlier reports, TS inhibition by raltitrexed, 5-FU, and FdUrd in mammalian cell models of UNG deficiency evinced robust uracil accumulation and decreased proliferation compared to wild type cells. However, no differential cytotoxicity was observed in three independent investigations. In contrast, siRNA directed knockdown of UNG in prostate cancer cell lines, even without TS inhibition, was capable of increasing DNA damage, decreasing cell proliferation, and increasing expression of pro-apoptotic proteins. In this model, UNG deficient cells were also more sensitive hydrogen peroxide and doxorubicin exposure, suggesting loss of UNG expression may alter sensitivity to genotoxic stress and cell fate decisions.
Figure 1-7. Proposed model of cell death in UNG deficient cells. In UNG deficient cells, uracil accumulates as a result of misincorporation of dUTP opposite dATP during DNA replication. This accumulation of genomic uracil has been observed to stall DNA replication forks and result in the formation of DNA double strand breaks upon replication fork collapse.
It is incumbent upon us to address the difficulty in application of data derived from one TS inhibitor to other TS inhibitors/antifolates. Particularly challenging is the knowledge that 5-FU and FdUrd, though inhibitors of TS, are different from raltitrexed and pemetrexed in that they engage in suicide inhibition via dUTP analogous metabolites (FdUTPs). FdUTP, is also a substrate for DNA polymerase nucleotide transfer and is incorporated into DNA. SMUG1, TDG, and UNG have each been identified as glycosylases capable of FdUTP excision, yet, whether futile cycles of FdUTP excision, repair and reincorporation occur has not been evaluated. Thus, it must be cautioned that the extent to which studies using 5-FU and FdUrd can be generalized among all antifolates or TS inhibitors is unclear. The differences in the study outcomes described may indeed be a reflection of vastly different model systems. Different cell lines may have distinctive threshold tolerances for uracil, and there may also be considerable variation in the degree of uracil accumulation induced by each anti-metabolite. Therefore, the extent to which uracil-DNA is a mediator of antifolate toxicity may likely depend on the model system (anti-metabolite + cell line combination) under investigation.
1.5 Proposed clinical value for studying UNG/BER in human cancer

1.5.1 Targeting BER to improve antifolate efficacy

From studies of UNG deficiency, it appears that misincorporated uracil is a determinant of antifolate cytotoxicity. Our findings have suggested that uracil may stall DNA replication and subsequent collapse of stalled replication forks can generate DNA strand breaks and trigger apoptosis. (58, 59) In this model, the efficient base excision repair of uracil-DNA is actually a protective mechanism that prevents uracil-mediated toxicity. Therefore, while enzymes in the BER pathway have not been typically referenced in discussions of antifolate resistance, induction of BER pathway activity in response to antifolates limits the cytotoxic potential of these agents. This knowledge has motivated rational evaluation of BER inhibition as a strategy to improve antifolate therapy.

BER proteins have been studied as therapeutic targets to sensitize cells to a variety of genotoxic agents, including alkylating agents. In principal, BER inhibition aims to prevent the excision of potentially toxic base lesions or increase the persistence of clastogenic repair intermediates. For example, AP sites and the DNA strand breaks generated following glycosylase excision and APE activity, respectively, are potent topoisomerase II poisons and contribute to cell death when unrepaired. (51, 78, 153) Such observations have lead to the development of BER inhibition strategies, such as targeting the excision of
damaged bases by DNA glycosylases, AP site repair by APE, DNA re-synthesis by DNA polymerase beta, and PARP-1 mediated resolution of single strand break BER intermediates (Figure 1-8). Several small molecule inhibitors of BER are in various stages of development for use in combination with chemotherapeutics in the treatment of human cancers (Table 1-3). Here, we summarize the present knowledge of the utility of BER inhibition for potentiating antifolate efficacy while drawing on studies that have effectively used BER inhibition to enhance the cytotoxicity of other genotoxic agents.

| Table 1-3 Base excision repair inhibitor strategies in various stages of development |
|---------------------------------|------------|-----------------|-----------------|-----------------|
| Agent (company) | BER Enzyme | Combination Chemotherapy | Phase of development | References |
| Small molecule inhibitors | APE | MMS | Pre-clinical | (154) |
| Methoxyamine TRC102 (Tracon) | APE | Temozolomide Pemetrexed | Phase I/II | http://www.traconpharma.com/content/pipeline_overview.html (28) |
| Small molecule inhibitors | Polβ | Temozolomide | Preclinical | (49, 50) |
| Lithocolic Acid | Polβ | Temozolomide | Preclinical | (55) |
| ABT-888 (Abbott) | PARP | Temozolomide | Phase III | http://clinicaltrials.gov/show/NCT01026493 (154) |
| INO-1001 (Inotek) | PARP | Temozolomide | Phase I/II | http://clinicaltrials.gov/show/NCT00272415 (155) |
1.5.1.1 Targeting uracil DNA glycosylase

Uracil excision by DNA glycosylases is the initial rate-limiting step in the UNG/BER reaction. (157) Models implicating direct toxicity of uracil-DNA in antifolate-mediated cell killing imply that decreased UNG expression or activity
results in persistence of uracil-DNA allowing it to function as a genotoxic stressor (Figure 1-7). As supporting evidence for this hypothesis we have shown that accumulation of uracil-DNA coincides with replication instability and contributes to pemetrexed hypersensitivity in UNG-deficient cancer cells(58, 59) These data suggest that uracil-DNA is a toxic base lesion, the effects of which are normally limited by proficient BER.(58) Others have also illustrated increased intolerance to genotoxic stress in human prostate cancer (LNCaP) cells.(149)

Specific intracellular targeting of UNG with bacteriophage-derived and small molecule inhibitors is of interest. The 9.5 kDa PBS2 bacteriophage protein based inhibitor of UNG, Ugi, forms a tight inhibitory complex that mimics the UNG:DNA transition state.(158-160) Expression of this protein in human cells has not generated strong phenotypes. In U251 glioma cells, expression of Ugi increased the spontaneous mutation frequency by 3-fold, without any appreciable difference in growth rate or cell cycle distribution.(159) Mitochondrial expression of Ugi was not mutagenic.(161) Hek293 cells expressing Ugi were not differentially sensitive to 5-FU treatment.(147)

The complexity of protein transduction as a mode of enzyme inhibition lies in the inability to accurately quantify and titrate inhibitor levels delivered in situ. For this reason, small molecule inhibitors are preferable. Nucleic acid based transition state mimics have been illustrated to inhibit both bacterial and viral UNG, albeit
with generally low specificity and efficiency at physiologic pH. (162, 163) The nucleotide analog, 1-cyano-2'-deoxyuridine (CNdU) has been identified as an inhibitor of UNG that may also be incorporated into DNA by replicative polymerases. (164)

Based on our data, we have initiated the identification of a novel class of small molecule inhibitors of UNG. We are currently characterizing compounds within this library for intracellular toxicity, efficacy of UNG inhibition and potentiation of TS inhibitor toxicity (Chapter 6 and 8). At this time, we believe the impact of such inhibitory compounds exists for pemetrexed. As discussed above, however, the reported impact of UNG loss varies depending on the agent investigated. For example, in our hands UNG−/− MEFs, are more sensitive to pemetrexed exposure. However, Andersen et al. report no differential sensitivity of UNG−/− MEFs to 5-FU. (121) This disparity suggests that perhaps UNG targeting may be an efficacious strategy only when used in combination with certain TS-inhibitors/antifolates. It is possible that different TS inhibitors produce varying levels of genomic uracil and have varying degrees of dependence upon uracil misincorporation for cellular toxicity. The redundancy of proteins with uracil excision activity (Table 1-3) is another important consideration that we address in this work with pemetrexed studies. UNG has been reported to be the major glycosylase involved in the excision of U:A lesions arising during replication, (75, 77) and we have not observed any compensatory induction of TDG, SMUG1, or
MBD4 activity during pemetrexed exposure in UNG deficient cells (Chapter 3).(59) This would suggest that other glycosylases do not play a significant role in uracil excision in this particular context. We cannot however, exclude the possibility of backup glycosylase involvement with other TS-inhibitors and antifolates.

Intrinsic toxicity of UNG inhibitors is another important consideration. Human cancers have characteristically high UNG expression suggesting a baseline reliance upon or addiction to UNG-directed BER. High basal UNG expression is similarly observed in normal human cells with high turnover rates, such as marrow, intestinal epithelial cells, and thymocytes.(165) Enhanced marrow suppression observed in UNG<sup>−/−</sup> mice treated with pemetrexed (Chapter 5) suggests that myelosuppression, an already established impediment to antifolate chemotherapy, may be enhanced with UNG inhibition. An encouraging finding is the fact that UNG levels are even more elevated in malignant cells compared to normal cells so it is possible that higher UNG levels in malignant cells combined with preferential uptake of folic acid analogs will provide a relative “sink” for the cytotoxic effects of combined drug regimens. Despite these anticipated challenges, the development of inhibitors to UNG is a critical measure toward a more complete understanding of the role of UNG activity and the role of uracil misincorporation in the mechanism of action of antifolates.
1.5.1.2 Targeting AP endonuclease 1 (APE1)

The AP site, generated following glycosylase excision of a damaged base, is a clastogenic lesion that can trigger cell death when unrepaired. Under normal circumstances, the AP site is rapidly and efficiently targeted by AP endonuclease I (APE1), generating a single strand break to facilitate re-synthesis and completion of BER. The expression of APE1 may have prognostic and predictive significance in human cancer. APE1 is over-expressed in several human tumors(166-170) and the increased expression of APE1 has been associated with resistance to radiotherapy,(168) chemotherapy(171) and reduced overall survival.(171) Direct enzymatic targeting of APE1 has been pursued in various preclinical studies as a means to sensitize cells to genotoxic agents. Homozygous deletion of APE1 in the mouse leads to embryonic lethality,(172, 173) and APE1 heterozygous mice are hypersensitive to oxidative stress.(174) In human cells, antisense oligonucleotide or RNA interference approaches to APE1 down-regulation have been successful in sensitizing human cells to alkylating agents and oxidating agents due to the accumulation of unrepaired AP sites.(54, 175-177) Small molecule inhibitors of APE have recently been developed as a more facile means of targeting the enzyme and sensitizing to a variety of DNA base-targeting compounds.(178)

Methoxyamine has been developed as an active inhibitor of BER to overcome BER conferred drug resistance.(51) MX binds irreversibly to the aldehydic
oxygen of the sugar moiety at the AP site forming an MX-bound AP site that is refractory to the repair activity of APE.\(^{(179, 180)}\) In this way, MX enhances the therapeutic efficacy of several DNA damaging chemotherapeutics\(^{(51-53, 56, 57, 78, 181)}\) and is currently being evaluated in multiple clinical trials (as TRC102, Tracon Pharmaceuticals, Inc). Because uracil-DNA lesions inhibit DNA replication and contribute to collapse of DNA replication forks, the excision of uracil by DNA serves to blunt antifolate cytotoxicity.\(^{(58)}\) This limitation of pemetrexed cytotoxicity can be successfully overridden with methoxyamine, which when stably complexed at UNG generated AP sites, promotes topoII\(\alpha\) dependent cleavage and double strand break formation.\(^{(58)}\) The lethal combination of methoxyamine and pemetrexed is selective towards cancer cells, which have higher expression of UNG and topoII\(\alpha\) than normal cells (Chapter 3). The pemetrexed and methoxyamine combination has successfully completed phase I clinical trial testing and phase II and randomized clinical trials are planned.\(^{(28)}\)

### 1.5.1.3 Targeting DNA polymerase beta (Pol\(\beta\))

AP site processing generates a DNA single-strand break modified with a 5’dRP terminator. The bi-functional DNA repair polymerase, DNA polymerase beta (Pol\(\beta\)) participates in nucleotide gap filling and removes the dRP moiety. The latter function, dRP lyase activity, is rate limiting and considered essential for maintaining cell survival following DNA damage. Therefore, inhibition of Pol\(\beta\)
enhances the cytotoxicity of several DNA damaging therapeutics. (89-91) It has been suggested that the cell death in Polβ deficient cells is regulated by poly(ADP-ribose) synthesis and NAD⁺/ATP depletion, a process requiring PARP functionality. (182) The concept of Polβ targeting has been successfully explored with alkylating agents, which generate DNA base lesions repaired by BER. (49, 50) Cellular sensitivity to genotoxic agents had been increased through Polβ depletion and with inhibitors of Polβ (49, 50) and a role for polymerase beta in cellular recovery from TS inhibitors has been suggested. (183)

Many cancer cells possess mutations in other DNA repair pathway proteins. To compensate the inherent deficit in DNA repair, cancer cells are said to be addicted to remaining intact DNA repair pathways. (48) Loss of function mutations in DNA base excision repair enzymes are rarely described in human cancer. To the contrary, malignant cells typically have elevated expression of BER genes compared to non-malignant cells of the same tissue type. Thus, cancer cell addiction to BER is plausible. Both mismatch repair (MMR) and DNA double strand break repair genes are mutated in human cancers. It has been suggested that MMR may have repair activity towards several antifolate-relevant base lesions, including 5-FU, (184) uracil, (185) and 8-oxoguanine, (186) a lesion formed by antifolate induced oxidative stress. Targeted deletion in Polβ in MMR deficient cells results in enhanced sensitivity to methotrexate, suggesting BER inhibition is selectively lethal in cells already deficient in MMR. (187) Additionally, Inhibition of
Polβ is also more effective in potentiating the cytotoxicity of genotoxic agents when DNA double strand break repair is compromised. (55) Based on these data, the enhanced sensitivity to antifolate-induced base lesions conferred by targeting BER may be particularly lethal in cells possessing mutations in other DNA repair pathways.

1.5.1.4 Targeting Poly(ADP)-Ribose Polymerase

The poly(ADP)-ribose polymerase (PARP) family of enzymes play a critical role in maintenance of genomic stability as a part of the base excision repair pathway and single strand break repair. PARP-1 and PARP-2 are DNA damage sensing molecules that quickly bind to sites of DNA damage and poly(ADP)-ribosylate a wide range of proteins involved in DNA damage response and repair. The addition of PAR to DNA repair enzymes recruits them to the sites of DNA damage to facilitate DNA repair. In BER, PARP interacts with, modifies and recruits proteins such as the scaffolding protein XRCC1 (110) to facilitate the formation of repair foci. PARP-1 is over expressed in a variety of human cancers (Figure 1-4) and expression of PARP has been associated with prognosis in solid tumors including breast cancer. (188) PARP-1 deficient animals and cells are sensitive to base damage caused by alkylating agents. (111)

The combination of PARP loss or inhibition with deficiency in DNA repair capacity has been targeted as a synthetically lethal combination that is particularly useful.
in certain human cancers. Inhibition of PARP interferes with single strand break repair. (111) Unrepaired SSBs degenerate to double strand breaks (DSBs). In BRCA-(69-72), PTEN-(65-68) and Rad51-(62-64) deficient cancer, which have reduced capacity for DNA DSB repair, inhibition of PARP is a synthetic lethal anti-cancer strategy. (48, 61)

1.5.2 BER Enzymes as predictive biomarkers for antifolate chemotherapy

1.5.2.1 Previously investigated predictive biomarkers of TS-inhibitors
Antifolate resistance has been associated primarily with up-regulation of the expression of the enzymatic targets TS(189-193) and DHFR(194), decreased expression of the reduced folate carrier (RFC)(195) or decreased expression of folate polyglutamate synthase (FPGS). However, pathway specific immunohistochemistry (IHC) has failed to predict clinical response in some studies. (196) Of note, inactivation of p53, a common genotype of many malignant cells, appears to increase resistance to pemetrexed and the TS inhibitor raltitrexed, irrespective of TS levels. (197) Therefore, particularly in the context of advanced and recurrent disease, it is prudent to identify additional predictive markers for antifolate therapy.

1.5.2.2 Predictive value of UNG and BER enzymes
In recent years, DNA repair enzymes have emerged as promising predictive and prognostic markers in cancer. Proficient BER in cancer cells results in enhanced ability to repair DNA damage caused by chemotherapeutic agents leading to resistance. Treatment with antifolates, like pemetrexed, causes uracil accumulation that is repaired by UNG and the BER pathway. Published microarray data reveals that certain cancer types have increased expression of UNG compared to normal tissues (Figure 1-4). Histological subtypes of cancer that are resistant to antifolate based chemotherapy, such as squamous cell lung cancer, express UNG at higher levels compared to antifolate sensitive histological subtypes (Chapter 3). We have observed a positive correlation between pemetrexed sensitivity and cellular expression of UNG (Chapter 3). These findings suggest that UNG-initiated BER in human cancer could potentially dampen the cytotoxic effects of antifolate-induced uracil accumulation and limit therapeutic indices of these drugs. Supporting this hypothesis are data that show that tumor overexpression of dUTPase, an additional enzyme involved in limiting genomic uracil levels, contributes to resistance to the TS inhibitor 5-FU.(138) Based on these data we are motivated to investigate UNG expression and pemetrexed response in clinical samples to determine the utility of UNG capacity as a molecular predictor of pemetrexed response (Chapter 8).

In addition to absolute gene expression levels, single nucleotide polymorphisms (SNPs) that alter the activity of uracil DNA glycosylases have been identified.
Future studies evaluating the frequency of these SNPs in human cancers that are intended to receive TS-inhibitor based chemotherapy and their association with drug response may be warranted.(198, 199)

1.6 Summary

Uracil incorporation into DNA triggers DNA base excision repair to limit DNA uracil content. Though futile cycles of uracil excision – reinsertion – and repair have been suggested, limited data supporting this hypothesis have been generated. In contrast, in studies using the multi-target antifolate pemetrexed, a clear role for uracil removal via BER in antifolate resistance has been established (Chapter 3).(58, 59) This body of work will describe data that show that targeting of BER through knockdown of UNG expression (Chapters 3 & 4), small molecule inhibition of UNG (Chapter 6), and inhibition of AP site processing by methoxyamine (Chapter 3) may be effective strategies for sensitizing cancer cells to pemetrexed. These reports are consistent with findings that BER inhibition potentiates cytotoxicity of DNA base damage-inducing chemotherapeutics. Moreover, these data show that the dominant hypothesis of thymine-less death (futile cycles of BER) cannot be a significant contributor to double strand break formation in pemetrexed-treated cells. In lieu of the traditional hypothesis for thymine-less death, the data that follow support a novel hypothesis wherein
genomic uracil accumulation is intrinsically cytotoxic leading to enhanced antifolate cytotoxicity in UNG/BER deficient cells (Figure 1-7).

Still, while recent data imply uracil-DNA is a toxic lesion, a threshold tolerance for uracil accumulation in DNA has yet to be defined. Without knowledge of a cell’s capacity for uracil tolerance, it is difficult to discern discrepancies in reported sensitivities of UNG deficient systems to certain TS inhibitors/antifolates. It is possible that pemetrexed is a more potent inhibitor of dTTP biosynthesis that leads to a greater uracil burden than other agents that have been studied. This is an area that warrants direct investigation in future studies.

The genetic background of cells utilized in sensitivity studies is another important consideration. We have observed more significant sensitization to pemetrexed in UNG deficient cancer cells compared to non-malignant UNG deficient MEFs and human lymphoblastoid cell lines from patients bearing germline inactivating mutations in UNG. Additionally, a synthetic lethal combination of BER inhibition and methotrexate treatment has been observed in cells with a MMR deficient background. Given the likelihood of a delicate balance in the activity of BER enzymes,(200, 201) changes in BER enzyme expression or activity are expected to have profound effects on DNA maintenance. This effect is amplified in cancer cells, which rely upon BER for survival. As such, cancer cells are thought to be preferentially sensitive to DNA base lesions during BER inhibition.
It remains clear, however, that targeting BER is a potentially efficacious strategy for overcoming pemetrexed resistance in the clinical setting. Studies to evaluate the clinical potential of BER inhibitors in combination with pemetrexed show considerable promise.(28, 58, 59) Moving forward, it is conceivable that pemetrexed tumor response, or adverse events may be predicted by the expression levels of UNG or other BER enzymes in cancer cells or bone marrow.
Chapter 2

Statement of Objectives

Tumor sensitivity to chemotherapy is influenced by several factors, including recognition and repair of DNA damage caused by anti-cancer agents. The anti-folate pemetrexed causes misincorporation of dUTP and genomic uracil accumulation through inhibition of the enzymes thymidylate synthetase (TS) and dihydrofolate reductase (DHFR), which are involved in thymidine biosynthesis. Base excision repair (BER), initiated by uracil DNA glycosylase (UNG), actively recognizes and removes misincorporated uracil from the genome. The limited viability of UNG deficient strains of *E. Coli* suggests that excessive genomic uracil is not well tolerated. However, limited and conflicting data in mammalian cells has muddled the understanding of the impact of UNG levels on response to chemotherapy-induced uracil accumulation. The present study elucidates the impact of UNG initiated BER on cellular sensitivity to pemetrexed. This work was governed by the hypothesis that **UNG expression will determine pemetrexed response in human cancer cells such that cells having low UNG expression and activity will be more sensitive to pemetrexed-mediated genotoxic uracil accumulation.** This hypothesis was addressed with the following specific aims:
AIM 1: To identify the intracellular mechanisms regulating increases in UNG expression following pemetrexed treatment. UNG is the primary uracil DNA glycosylase involved in the excision of uracil arising as a result of dUTP misincorporation during replication. (116) Treatment with fluoropyrimidine TS inhibitors and antifolates causes genomic uracil accumulation. Thus, it follows that treatment with the multi-target antifolate pemetrexed would cause induction of UNG/BER. In untreated cells, UNG levels are cell cycle regulated at the G1/S boundary. (202, 203) Antifolate treatment, however, is known to cause cell cycle arrest in S-phase and induction of DNA damage markers. Therefore, I have tested the hypothesis that UNG protein and mRNA are induced in response to pemetrexed treatment as a result of regulation by transcription factors related to DNA damage recognition and signaling. To address this hypothesis subaims were pursued to: (i) confirm that UNG expression increases following pemetrexed treatment occur at the transcriptional level (ii) identify transcription factors regulating UNG promoter activity after pemetrexed treatment; and (iii) validate that the observed induction and transcriptional regulation of UNG were specific to conditions of uracil incorporation (Chapter 3).

AIM 2: To determine the impact of variations in UNG expression on cellular sensitivity to pemetrexed. Uracil misincorporation and accumulation has been identified as a source of genotoxic stress in E. Coli and S. Cerevisiae lacking expression of the dUTPase enzyme. In human cells, dUTPase overexpression
confers resistance to TS inhibition. However, studies with cells that have either increased(84) or decreased(77) UNG expression have only yielded modest phenotypic changes such as decrease proliferation without differences in cell death. The lack of a strong phenotype in response to alterations in UNG expression has made the impact of uracil accumulation in mammalian cells difficult to discern. Here, I have tested the hypothesis that **UNG expression determines the cellular sensitivity to pemetrexed.** I employed the following UNG deficient systems to complete this work: UNG deficient systems including UNG−/− DLD1 colon cancer cells, siRNA and shRNA targeted UNG−/− lung cancer cells, UNG−/− mouse embryonic fibroblasts, human UNGmut/mut lymphoblastoid cell lines, and UNG+/+ mice. Subaims specifically were designed to: (i) test whether UNG deficiency corresponded with decreased survival and increased evidence of DNA damage after pemetrexed treatment in vitro (Chapter 3); (ii) determine if UNG deficiency in live mice corresponded to increased pemetrexed toxicity in vivo (Chapters 3-5); (iii) identify a mechanism of cell death in UNG−/+ cells treated with pemetrexed (Chapter 4) and (iv) confirm that the mechanism of cell death in UNG deficient cells treated with pemetrexed is reliant upon uracil-DNA accumulation (Chapter 4).

**AIM3: Evaluate the ability of UNG expression levels to predict sensitivity to pemetrexed treatment in human non-small cell lung cancer (NSCLC).** I tested the hypothesis that **the level of UNG expression would predict the relative**
sensitivity of NSCLC cells to pemetrexed. Activities in this aim included: (i) an evaluation of UNG expression and pemetrexed sensitivity in a panel of non-small cell lung cancer cell lines; (ii) evaluation of cell lines for sensitivity to pemetrexed measured by DNA damage response, induction of BER proteins, and sensitivity to pemetrexed-mediated cell killing; (iii) a statistical correlation between UNG expression and pemetrexed response (Chapter 3) and (iv) a preliminary investigation of UNG expression in primary human cancer tissue (Chapters 3 and 6).

AIM 4: Evaluate the ability of inhibitors of UNG-BER activity to sensitize cells to pemetrexed. The identification of uracil-DNA as a source of genotoxic stress in pemetrexed treated cells suggested that limiting UNG excision of uracil-DNA would enhance the potency of pemetrexed cell killing. Moreover, because BER generates clastogenic intermediates, inhibition of BER will result in accumulation of intermediates and promote cytotoxicity. For this reason I have tested the hypothesis that inhibition of BER excision of uracil or repair of clastogenic intermediates will result in potentiation of pemetrexed cell killing. Strategies investigated include: (i) inhibition of AP site repair by methoxyamine (Chapter 3) and (ii) direct inhibition of UNG with novel UNG inhibitors (Chapter 6).
Together, these studies have identified UNG-BER as a critical determinant of pemetrexed cytotoxicity in \textit{in vitro} and \textit{in vivo} models. Based on these data, future studies to understand the clinical value of UNG/BER expression and activity as a predictive biomarker for pemetrexed and other antifolates. Additionally, uracil accumulation has been identified as a source of replication stress, capable of inducing replication fork collapse and double strand break formation – a novel, uracil-mediated mechanism of action for antifolates. The inhibition of critical bottleneck points in the base excision repair of uracil-DNA has been proposed with \textit{in vitro} preliminary data indicating a capacity to significantly enhance pemetrexed antitumor efficacy. These novel findings warrant the rational development of clinically active inhibitors of UNG-BER to enhance pemetrexed efficacy.
Uracil DNA Glycosylase Determines Human Lung Cancer Cell Sensitivity to Pemetrexed

reproduced with permission from

Uracil misincorporation into DNA is a consequence of pemetrexed inhibition of thymidylate synthase. The base excision repair (BER) enzyme, uracil DNA glycosylase (UNG) is the major glycosylase responsible for removal of misincorporated uracil. We previously illustrated hypersensitivity to pemetrexed in UNG⁻/⁻ human colon cancer cells. Here, we examined the relationship between UNG expression and pemetrexed sensitivity in human lung cancer. We observed a spectrum of UNG expression in human lung cancer cells. Higher levels of UNG are associated with pemetrexed resistance and are present in cell lines derived from pemetrexed-resistant histological subtypes (small cell and squamous cell carcinoma). Acute pemetrexed exposure induces UNG protein and mRNA, consistent with up-regulation of uracil-DNA repair machinery. Chronic exposure
of H1299 adenocarcinoma cells to increasing pemetrexed concentrations established drug-resistant sublines. Significant induction of UNG protein confirmed up-regulation of BER as a feature of acquired pemetrexed resistance. Co-treatment with the BER inhibitor, methoxyamine (MX) overrides pemetrexed resistance in chronically exposed cells, underscoring the utility of BER directed therapeutics to offset acquired drug resistance. Expression of UNG-directed siRNA and shRNA enhanced sensitivity in A549 and H1975 cells, and in drug-resistant sublines, confirming that UNG up-regulation is protective. In human lung cancer, UNG deficiency is associated with pemetrexed-induced retention of uracil in DNA that destabilizes DNA replication forks resulting in DNA double strand breaks and cell death. Thus, in experimental models, UNG is a critical mediator of pemetrexed sensitivity that warrants evaluation to determine clinical value.
3.1 Introduction

Lung cancer remains a leading cause of mortality worldwide. Greater than 80% of lung cancer cases are non-small cell lung cancer (NSCLC) and of newly diagnosed patients, 40% have advanced disease.(204) Chemotherapy is a cornerstone therapy for patients with advanced and recurrent lung malignancy.(205)

Pemetrexed, a multi-target antifolate, has proven clinical activity against non-squamous non-small cell lung cancer (NSCLC). It is currently approved as single agent second-line therapy for patients with advanced NSCLC(25) and as first-line therapy in combination with cisplatin for patients with advanced NSCLC.(24) Pemetrexed maintenance therapy results in statistically significant progression-free and overall survival compared to placebo.(26) Still, despite initial responses, patients receiving pemetrexed-based chemotherapy ultimately progress highlighting a need for novel strategies to enhance pemetrexed efficacy.

Pemetrexed inhibits several key enzymes in folate dependent metabolism including thymidylate synthetase (TYMS), dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyl transferase (GARFT) causing decreased nucleotide synthesis.(13) Importantly, pemetrexed metabolites are most active
against TYMS, the primary target for pemetrexed cytotoxicity. (206) Inhibition of TYMS limits reductive methylation of deoxyuridine nucleotides (dUMP) to form deoxythymidine nucleotides (dTMP). As a result, aberrantly large pools of dUMP accumulate, are phosphorylated to dUTP, and are utilized in DNA synthesis in place of dTTP. (207) The misincorporated genomic uracil that results is a substrate for uracil DNA glycosylase (UNG)-initiated base excision repair (BER). (25) Though uracil accumulation is a well documented sequelae of cellular exposure to pemetrexed and other TYMS inhibitors, (113) the impact of uracil misincorporation on genomic stability and cellular survival remains undefined.

Pemetrexed resistance has been evaluated in lung and other cancer cell types. (191, 195, 208, 209) In NSCLC cell lines, elevated expression of TYMS, DHFR and GARFT corresponds to pemetrexed resistance. (189, 192, 209) UNG activity, however, is seldom cited as a mechanism of antifolate resistance, despite the apparent toxicity of uracil-DNA in some human (139, 149) and non-mammalian model systems. (122, 124)

Recently, we reported that DLD1 human colon cancer cells lacking UNG are hypersensitive to pemetrexed-induced uracil accumulation resulting in cell cycle arrest, DNA double strand break (DSB) formation, and apoptosis. (58) Since pemetrexed is primarily used in the treatment of lung cancer and is limited by a response rate of 30-40% with no long-term sustained responses, we evaluated
the relationship between UNG expression and pemetrexed response in human lung cancer cell lines. Gene expression data in cell lines and primary human lung tumor tissue samples suggest a spectrum of UNG expression in lung cancer specimen that is significantly correlated with pemetrexed response. Based on evidence of DNA replication fork instability in the context of deficient uracil excision, we propose a novel role for misincorporated uracil as a genotoxic lesion that contributes to antifolate-induced DSB formation and cell death. Induction of UNG in response to acute and chronic pemetrexed exposure also suggests UNG activity limits pemetrexed cytotoxicity. Differential UNG expression among lung cancer histological subtypes motivates the investigation of UNG as a clinical predictive marker for pemetrexed response. The correlation between UNG expression and pemetrexed sensitivity in experimental models justifies targeting UNG to enhance pemetrexed anti-cancer activity NSCLC.

3.2 Materials and Methods:

3.2.1 Cell lines and reagents. Pemetrexed was purchased from LC Laboratories. Thymidine, 5-Fluorouracil, Cisplatin, Methoxyamine-HCL and Raltitrexed were purchased from Sigma Aldrich. Temozolomide was purchased from O-Chem, Inc (Des Plaines, IL). All cell lines were obtained from ATCC and expanded upon delivery into numerous vials of low passage cells for cryopreservation. Cells were passaged for no longer than 3 months. Cell line
characterization by ATCC is conducted through short tandem repeat (STR) typing. Re-authentication was not conducted. Adherent cells were maintained in complete DMEM (10% FBS, 2 mM L-Glutamine) and suspension cells were maintained in complete RPMI-1640 (10% FBS, 2 mM L-glutamine) at 37°C in a 5% CO₂ incubator.

3.2.2. Cell cycle analysis. Propidium iodine (PI) staining of methanol fixed cells was for cell cycle determinations. Where indicated, FITC labeled PCNA antibody (PCNA-FITC, Abcam) was added for PCNA detection. Uni-parameter (PI-only) and dual-parameter (PI + PCNA-FITC) analysis was performed on a Coulter flow cytometer (EPICS-XL-MCL). Cell cycle histograms (PI) and PCNA dot plots (PCNA-FITC) were de-convoluted from ≥ 20,000 events using FlowJo software.

3.2.3 Western blot. Protein extracts (25 µg) were resolved by SDS-PAGE and transferred to PVDF membrane (Millipore). Non-specific binding sites were blocked in 5% milk in PBST (1X PBS + 0.1% Tween-20). Incubation with primary antibody at 4°C in 5% BSA/PBS was followed by incubation with HRP-conjugated secondary antibody in 2.5% milk in PBST. Proteins were visualized with ECL reagent (Amersham Corp). Chromatin-bound proteins were extracted from formaldehyde (1%) cross-linked cells using Pierce Chromatin Prep Module (Thermo Pierce). Antibody sources: UNG-23936 (39 kDa band, nuclear UNG) and PCNA (Abcam); Tubulin (Calbiochem); γH2AX and Histone-H3 (Millipore);
Cleaved PARP (Cell Signaling); and p-chk1, chk1, cdc2, and p-cdc2 (Santa Cruz).

### 3.2.4 UNG activity assay.
UNG activity was measured using a 40-mer oligodeoxynucleotide duplex:

5’-[HEX] GTAAAACGACGGCCAGTGUCTTCGAGCTCGGTACCCGGGG (top)

3’-CATTTTGCTGCACGAGCATGGGCCCC[Cy5] (bottom).

In fluorescent images, the top and bottom strands appear green and red, respectively. Oligonucleotide duplexes were incubated with either purified enzymes (1 unit) or whole cell extract (2.5 μg) at 37°C for 30 minutes. The reaction was heat-killed at 95°C, reaction products were resolved by electrophoresis on denaturing 20% polyacrylamide gels, and visualized with a Typhoon 9200 fluorescence imager (Amersham Bioscience, Piscataway, NJ, USA). UNG activity (percentage of cutting) was defined as the fluorescence density of the cut band (20-mer) relative to the sum of the fluorescence intensity of the cut (20-mer) and uncut (40-mer) bands using ImageQuant software (Amersham BioScience).

### 3.2.5 Abasic (AP) site detection.
Following drug treatment cellular DNA extracts were labeled with a biotinylated aldehyde reactive probe (ARP) for chemiluminescent AP site detection as previously described.(210) For UNG deficient cells, an additional incubation at 37°C with recombinant UNG (1U UNG
/100 μg DNA) liberated genomic uracil prior to ARP labeling. Quantitative densitometry was performed using Image J software.

**3.2.6 Neutral comet assay.** Treated cells were processed for comet tail formation under neutral comet assay conditions according to the manufacturers instructions for cell lysis and single cell electrophoresis (Trevigen). Tail lengths were recorded for at least 50 comets on two separate slides (~100 cells per treatment) using Image J software.

**3.2.7 Colony survival assay.** For cells in suspension, colony survival was determined by crystal violet staining of colonies formed after 10-day exposure of 5x10³ cells to pemetrexed in soft agar. For adherent cells, colony survival was determined by methylene blue staining of colonies formed after 10-day exposure of 100 cells to pemetrexed in 6-well culture dishes. Only colonies containing ≥50 cells were counted. Data points represent percent of colonies relative to untreated control averaged over 3 experiments.

**3.2.8 Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR).** Lung cancer cDNA microarray was purchased from Origene. For cell lines, total RNA was extracted from cells using RNAqueous-4PCR kit (Ambion, Austin, TX). Random hexamers (Invitrogen) were used to PCR amplify cDNA from 1μg of RNA extract. TaqMAN MGB probes (FAMTM dye labeled, Applied Biosystems)
for nuclear UNG (UNG2), SMUG1, MBD4, TDG, TYMS and Polβ amplified cDNA using 40 cycles of PCR in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Target quantification was achieved after normalization to β-actin amplification as an endogenous control and is presented as relative quantification (RQ) values or log-mean RQ values.

3.2.9 siRNA and shRNA transfection. UNG directed shRNA and siRNA plasmids were purchased from Origene. Transfection was carried out according to manufacturer specifications. Stably transfected clones (shRNA) were selected with puromycin with subsequent expansion of a well-isolated colony of cells.

3.2.10 Pemetrexed-resistant cell lines. H1299 cells were exposed to step-wise increasing concentrations of pemetrexed over a period of 4 months. UNG expression was monitored in the bulk population at 2, 8, 12 and 16 weeks prior to administration of the induction dosage and at 24 weeks in a population of bulk chronically exposed cells that had been without pemetrexed for 8 weeks. H1299 cells capable of growth in 50nM pemetrexed selection pressure were subcloned by limiting dilution in 96 well plates. Independent sublines were designated H1299/PR-1 and H1299/PR-2.

3.2.11 Xenografts in NODSCID Mice. Tumor cells in early passage (5x10⁶) were injected into bilateral flanks of female NODSCID mice (6 weeks old). When
tumor volumes reached 100 mm³, mice were divided into control (n=4) and treatment (n=6) groups. Mice bearing tumors were treated with pemetrexed (150 mg/kg) or 100μl sterile PBS (control) by daily intraperitoneal injection (IP) for 5 consecutive days. Tumor measurements were taken every 2 days and response was quantified by tumor volume.

### 3.2.12 Chromatin Immunoprecipitation (ChIP) assays

After drug treatment cells were cross-linked in 1% formaldehyde solution. DNA cross-linking was quenched by the addition of 125mM glycine. Cell lysates were prepared using standard protocol in the presence of protease inhibitors and sonicated to obtain DNA fragments of 200-500 base pairs. A 10 μl aliquot of cell lysate was maintained as an input control. Protein A Dynabeads (Dynal Invitrogen Corp., Carlsbad, CA), 50 μl per sample, are pre-incubated with antibody overnight at 4°C. A non-antibody linked Dynabead sample is prepared as a no-antibody control. After washing with BSA/PBS, Dynabeads are incubated with fragmented cell lysate for 4 hours at 4°C with continuous gentle agitation. After washing in lysis buffer, NaCl rich washing buffer, and Tris-HCL-EDTA samples are eluted from with 2 separate additions of 30uL elution buffer for 10 minutes at 65°C. 15 minute incubation at 95°C reversed crosslinking and immunobloting was carried out according to western blot protocols. Primers for amplification of immunoprecipitated chromatin fragments were designed as described.(202) Primers flanking the nuclear promoter: npUpper (5’-
CCCGAGCTCTTGAGATGCCTCGGATTACAGTG-3') and npLower
(5’CCGCTCGACTCCTGGAGCTGAGGAGGCAG-3’) amplified a 603bp DNA
fragment. HPLC purified primers were obtained from Eurofins MWG Operon
(Huntsville, AL). PCR products were resolved by agarose gel electrophoresis
using ethidium bromide staining for visualization.

3.2.13 Pooled lung cancer microarray dataset analysis. Microarray data (log-
median expression values) were retrieved from the Oncomine website
(http://www.oncomine.org) and normalized to set log-median expression of non-
malignant samples to 1.

3.2.14 Statistical analysis. Results are presented as the mean ± SEM.
Significance, assigned for p-values < 0.05, was determined by unpaired 2-tailed
student's t-test with standard software (GraphPad Prism, San Diego, CA, USA).
Correlations of gene expression with pemetrexed IC_{50} were estimated using
Pearson correlation coefficient. Expression of expression UNG and other genes
of interest was compared using analysis of variance (ANOVA) followed by
Tukey's pair-wise comparison procedure. The effects of multiple genes on drug
IC_{50} were estimated using multivariable regression models, i.e. IC_{50} = intercept +
coefficient1(gene1) + coefficient2(gene2) + ε.
<table>
<thead>
<tr>
<th>Name</th>
<th>Histological Subtype (Lung)</th>
<th>Tumor suppressor/Oncogene status</th>
<th>Doubling time (h)</th>
<th>Pemetrexed IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI-38</td>
<td>Normal fibroblast</td>
<td>wt</td>
<td>24</td>
<td>NP</td>
</tr>
<tr>
<td>IMR-90</td>
<td>Normal fibroblast</td>
<td>wt</td>
<td>23</td>
<td>NP</td>
</tr>
<tr>
<td>H1299</td>
<td>Adenocarcinoma</td>
<td>del</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>*H1299-PR1</td>
<td>Adenocarcinoma</td>
<td>del</td>
<td>26</td>
<td>468</td>
</tr>
<tr>
<td>H460</td>
<td>Large Cell</td>
<td>wt</td>
<td>23</td>
<td>153</td>
</tr>
<tr>
<td>A549</td>
<td>Adenocarcinoma</td>
<td>wt</td>
<td>22</td>
<td>416</td>
</tr>
<tr>
<td>H1838</td>
<td>Adenocarcinoma</td>
<td>mut</td>
<td>26</td>
<td>1402</td>
</tr>
<tr>
<td>H1975</td>
<td>Adenocarcinoma</td>
<td>wt</td>
<td>20</td>
<td>777</td>
</tr>
<tr>
<td>*H1975 shUNG</td>
<td>Adenocarcinoma</td>
<td>wt</td>
<td>22</td>
<td>131</td>
</tr>
<tr>
<td>HCC827</td>
<td>Adenocarcinoma</td>
<td>wt</td>
<td>25</td>
<td>1023</td>
</tr>
<tr>
<td>Calu-1</td>
<td>Squamous Cell</td>
<td>del</td>
<td>30</td>
<td>1064</td>
</tr>
<tr>
<td>H69</td>
<td>Small Cell</td>
<td>mut</td>
<td>65</td>
<td>1733</td>
</tr>
</tbody>
</table>

Del = gene deletion, mut = mutated gene, wt = wild type gene expression, NP= not performed
*Cell lines were subcloned as a part of this study.
3.3 Results

3.3.1 A spectrum of UNG expression exists in human lung cancer. Our previous observation of profound pemetrexed sensitivity in UNG<sup>−/−</sup> colon cancer cells(58) prompted us to investigate the value of UNG as a mechanistic and predictive marker for pemetrexed response in human lung cancer. To do this, we evaluated UNG expression and pemetrexed sensitivity (IC<sub>50</sub>) in a panel of 8 lung cancer cell lines and two non-malignant lung cell lines. Details of the cell lines used in this study are summarized in Table 3-1.

UNG protein (Figure 3-1a) and transcript (Figure 3-1b) levels were significantly higher in lung cancer cell lines compared to non-malignant lung epithelial cells (WI38 and IMR90). Additionally, lung cancer cell lines derived from small cell (H69) and squamous cell (Calu-1) carcinoma, known to be clinically unresponsive to pemetrexed,(211-213) had higher levels of UNG compared to adenocarcinoma and large cell carcinoma cell lines (Figure 3-1a-b). Pemetrexed IC<sub>50</sub> was determined from colony survival experiments (Figure 3-1c). Plotting UNG levels (protein band density, Figure 3-1d) or UNG mRNA levels (relative quantification (RQ) value, Figure 3-1e) against pemetrexed IC<sub>50</sub> for each cell line indicated that UNG expression was well correlated with pemetrexed IC<sub>50</sub> (protein – Pearson r = 0.79, p = 0.021; mRNA – Pearson r = 0.71, p = 0.047).
Figure 3-1. UNG expression and pemetrexed sensitivity in human lung cancer. (a) Western blot for UNG protein levels in 8 lung cancer cell lines and 2 lung fibroblast lines (WI38 and IMR90). (b) Mean RQ-values from RT-PCR determination of relative UNG expression. (c) 10-day colony survival assay for lung cancer cell lines treated with 0-800 nM pemetrexed. (d) Graph of UNG protein band density plotted against pemetrexed IC50. (e) Graph of UNG RQ value and pemetrexed IC50. (f) RT-PCR for UNG expression was performed on lung cancer cDNA microarray (Origene). The log-ratio UNG expression was calculated and is represented for each sample as a box and whisker plot. OncomineTM (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization of data from 3 published microarray data sets(27-29). Data were normalized so that non-malignant samples had a median log-ratio of UNG (g) or TYMS (h) expression values of 1. For f & g, p<0.05 for all pairs except small cell and squamous cell carcinoma. For h, p<0.05 for squamous vs. adenocarcinoma and squamous vs. normal only (ANOVA, Tukey’s procedure).
Table 3-2. Correlation of Linear Regression (RQ value vs. IC50)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pearson r</th>
<th>95% CI</th>
<th>p value</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG</td>
<td>0.7128</td>
<td>0.01618 to 0.9436</td>
<td>0.0472</td>
<td>0.51</td>
</tr>
<tr>
<td>SMUG1</td>
<td>0.3376</td>
<td>-0.4818 to 0.8420</td>
<td>0.4134</td>
<td>0.11</td>
</tr>
<tr>
<td>TDG</td>
<td>0.4075</td>
<td>-0.4171 to 0.8641</td>
<td>0.3163</td>
<td>0.17</td>
</tr>
<tr>
<td>MBD4</td>
<td>-0.1707</td>
<td>-0.7815 to 0.6071</td>
<td>0.6862</td>
<td>0.029</td>
</tr>
<tr>
<td>Polβ</td>
<td>0.057</td>
<td>-0.6747 to 0.7325</td>
<td>0.893</td>
<td>0.003</td>
</tr>
<tr>
<td>TYMS</td>
<td>0.406</td>
<td>-0.4184 to 0.8637</td>
<td>0.318</td>
<td>0.170</td>
</tr>
<tr>
<td>TOPOIIα</td>
<td>0.436</td>
<td>-0.3883 to 0.8725</td>
<td>0.281</td>
<td>0.190</td>
</tr>
</tbody>
</table>

Pemetrexed IC_{50} was not significantly correlated with mRNA expression of other BER genes, including other glycosylases with uracil excision activity (SMUG1, TDG, and MBD4), DNA polymerase β (Polβ) or the pemetrexed target gene, TYMS (Table 3-2). UNG mRNA levels were still marginally predictive of the pemetrexed IC_{50} (p<0.1) when paired with other pathway specific genes in multivariable regression analysis (Table 3-3). Overall, these data illustrate a spectrum of UNG expression in human lung cancer cell lines that is positively correlated with pemetrexed response.
Table 3-3. Multivariable Regression Statistics for ‘UNG+Gene’ Pairs

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>&quot;R^2&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG</td>
<td>0.079</td>
<td>0.06</td>
<td>0.59</td>
</tr>
<tr>
<td>SMUG1</td>
<td>-0.299</td>
<td>0.358</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>&quot;R^2&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG</td>
<td>0.136</td>
<td>0.012</td>
<td>0.79</td>
</tr>
<tr>
<td>TDG</td>
<td>-0.456</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>&quot;R^2&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG</td>
<td>0.058</td>
<td>0.044</td>
<td>0.6</td>
</tr>
<tr>
<td>MBD4</td>
<td>-0.575</td>
<td>0.327</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>&quot;R^2&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG</td>
<td>0.058</td>
<td>0.054</td>
<td>0.56</td>
</tr>
<tr>
<td>Polβ</td>
<td>0.321</td>
<td>0.48</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>&quot;R^2&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG</td>
<td>0.049</td>
<td>0.084</td>
<td>0.58</td>
</tr>
<tr>
<td>TOPOIIα</td>
<td>0.064</td>
<td>0.399</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>&quot;R^2&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG</td>
<td>0.075</td>
<td>0.088</td>
<td>0.56</td>
</tr>
<tr>
<td>TYMS</td>
<td>-0.095</td>
<td>0.477</td>
<td></td>
</tr>
</tbody>
</table>

*Pairs of genes contribution to drug IC50 was estimated using multivariable regression models, i.e. IC50 = intercept + coefficient1 * gene1 + coefficient2 * gene2 + ε. R^2 shown is for multivariable regression model. R^2 for UNG alone is 0.51.

Measurement of UNG expression in primary human lung cancer tissue cDNA microarrays evinced significant variation in UNG expression among histological subtypes (Figure 3-1f). Similar to cell lines, UNG was elevated in lung cancer compared to non-malignant tissue cDNAs. Additionally, cDNA from small cell and squamous cell carcinoma had significantly higher UNG expression compared to adenocarcinoma (p<0.0001, Tukey’s procedure). Pooled analysis of published microarray data (214-218) corroborated our findings (Figure 3-1g). These data
also showed that UNG expression was correlated with higher grade adenocarcinoma (214) and reduced 1-year survival (218). While clinical response data are unavailable for these samples, higher UNG levels in lung cancer histological subtypes that are reportedly pemetrexed resistant (211-213) suggest a clinically relevant correlation between UNG expression and pemetrexed response. Thymidylate synthetase (TYMS), which has been associated with pemetrexed resistance, was also differentially expressed among the various lung cancer subtypes in the datasets analyzed (214-218). Squamous but not small cell carcinoma TYMS expression was significantly higher than adenocarcinoma (Figure 3-1h). We were unable to locate tissue sets from recent pemetrexed clinical trials in lung cancer to interrogate for UNG expression. We have initiated prospective collection of lung cancer tissue samples from patients receiving pemetrexed to directly evaluate the relationship between UNG expression and pemetrexed response.

3.3.2 Loss of UNG expression increases lung cancer sensitivity to pemetrexed. We validated the correlation between UNG expression and pemetrexed response through direct targeting of UNG expression with siRNA and shRNA. For this analysis we used adenocarcinoma cell lines, A549 and H1975, which have moderate and high baseline UNG expression, respectively. Targeting of A549 cells with UNG directed siRNA resulted in 70% reduction of UNG protein expression (Figure 3-2a-b). A549 siRNA cells were 7-fold more
sensitive to pemetrexed compared to parental cells (A549 siUNG IC$_{50}$ = 60.54 nM; A549 parental IC$_{50}$ = 416.1 nM, p<0.0001, Figure 3-2c). Similarly, stable transfection of H1975 cells with UNG directed shRNA resulted in 50-60% knockdown of UNG protein expression (Figure 3-2d-e). In colony survival assays (Figure 3-2f), shRNA targeted H1975 clones were > 5-fold more sensitive to pemetrexed than parental cells (IC$_{50}$ values: H1975 = 776.6 nM; H1975 66 shUNG = 130.9 nM; H1975 67 shUNG = 146.2 nM, p<0.0001). UNG deficient H1975 cells displayed some cross sensitivity to raltitrexed and 5-fluorouracil but not temozolomide or cisplatin suggesting the effects of UNG loss are specific to TYMS inhibitors (Table 3-4).

| Table 3-4. Cross-sensitivity of H1975 cells to other DNA damaging agents |
|-----------------------------|--------------------------|--------------------------|----------------|----------|
| DNA damaging agent          | IC$_{50}$ H1975 (parental) | IC$_{50}$ H1975 (shUNG)  | P-value       | FS       |
| Pemetrexed                  | 770.1 (374.8–1582) nM     | 140.3 (107.1–183.9) nM   | P<0.001       | 5.48     |
| Pemetrexed + 3mM MX         | 115.0 (84.8–270.5) nM     | 98.52 (70.46–137.3) nM   | P=0.6598      | 1.16     |
| Raltitrexed                 | 217.3 (116.8–404.5) nM    | 84.41 (55.75–127.7) nM   | P=0.0082      | 2.57     |
| 5-Fluorouracil              | 808.2 (586.7–1113) nM     | 514.2 (351.3–752.6) nM   | P=0.0347      | 1.58     |
| Cisplatin                   | 1.940 (0.5473–6.879) μM   | 1.648 (0.2946–8.134) μM  | P=0.7454      | 1.18     |
| Temozolomide                | 273.9 (187.4 – 400.2) μM  | 263.7 (135.4–513.8) μM   | P=0.8770      | 1.04     |

Data are presented as mean IC$_{50}$ (95% confidence interval) of 3 independent experiments
FS – fold sensitivity of shUNG cells to indicated agent (IC50 parental/IC50 shUNG)
Figure 3-2. Loss of UNG expression sensitizes lung cancer cells to pemetrexed. (a-c) UNG directed (siUNG) and non-specific (NS) control siRNAs (Origene) were transfected into A549 cells according to manufacturer’s protocol. (a) 36-hours-post transfection, puromycin selected cells were harvested for western blot analysis of UNG expression. (b) Protein band densities from 3 independent transfections were determined by image J, normalized to WT expression levels and analyzed using GraphPad Prism software (*p<0.005 for all pairs except WT vs. NS). (c) Sensitivity to pemetrexed was analyzed by colony forming assay. (d-f) UNG directed shUNG clones (Origene) were transfected into H1975 cells and stably transfected cells were selected by puromycin. UNG knockdown was confirmed by western blot (d) and protein band density (e) was calculated using ImageJ software. (f) Colony survival was used to determine sensitivity to pemetrexed. Data points for colony survival represent the mean ± SEM of 3 independent experiments, *p<0.001. (g) H1975 and H1975 shUNG (h) cells were xenografted onto NOD-SCID mice. When tumor volume reached 100mm3 mice were treated with IP injections of PBS (control) or pemetrexed (150mg/kg) for 5 days. Tumor volume was measured to determine therapeutic effect. Data points represent mean ± SEM tumor volume for 4-6 mice bearing 2 tumors each, *p<0.0001.
To determine the impact of UNG expression on pemetrexed sensitivity \textit{in vivo}, H1975 and H1975 66 shUNG cells were xenografted subcutaneously into NODSCID mice. Treatment with 5 daily consecutive intraperitoneal (IP) injections of pemetrexed (150mg/kg) evinced a tumor quadrupling time of 9.68± 0.31 days in H1975 wt tumors compared to 13.67±0.28 days for H1975 shUNG tumors. At day 20, pemetrexed treated H1975 66 shUNG tumors were 55.6% smaller than untreated controls while H1975 wild type tumors were only 37.8% smaller than untreated controls, p<0.001 (Figure 3-2g). These data indicate a significant increase in the anti-tumor effect of pemetrexed on tumors with lower levels of UNG expression, \textit{in vivo}.

\textbf{3.3.3 Limited uracil removal is associated with increased DNA damage in UNG deficient cells.} UNG is the major glycosylase responsible for removing uracil that is misincorporated during DNA replication.(77) To relate pemetrexed sensitivity in UNG deficient cells to the retention of uracil in DNA, we first evaluated uracil excision capacity of UNG knockdown cells. UNG knockdown did not alter expression of other glycosylases capable of uracil excision (Figure 3-3a). Protein extracts from untreated H1975 66 shUNG cells had diminished capacity to excise uracil from a synthetic oligonucleotide duplex containing a single uracil residue (Figure 3-3b-c). In pemetrexed-exposed cells, AP site detection was used as a surrogate measure of accumulated uracil. H1975 parental and H1975 66shUNG cells were treated with pemetrexed for 0-48 hours.
Figure 3-3. Reduced uracil excision in UNG deficient cells. (a) Western blot of SMUG1, TDG and MBD4 in H1975 parental cells and H1975 cells expressing shUNG clones 66 and 67. (b) In vitro UNG activity assay. A fluorescently labeled oligonucleotide duplex containing U:A mispair were incubated for 30 minutes at 37°C with 2.5μg of whole cell extract from H1975 parental cells and H1975 cells stably expressing one of the shUNG clones (66 and 67). Reactions with purified recombinant enzyme were used as controls. Reaction products were resolved by electrophoresis in a denaturing 20% polyacrylamide gel. In fluorescent images, the green band represents the Hex-labeled oligonucleotide containing a U base and the red band represents the Cy5-labeled complementary strand. Yellow produced by overlay of the fluorescent signals. (c) Percentage of cutting was calculated as the density of the 20-mer band divided by the sum of the density of the 20-mer and 40-mer bands. (d) H1975 and H1975shUNG (66) Cells were treated with pemetrexed as indicated and DNA extracts were incubated with ARP reagent to determine AP sites relative to untreated control. (e) DNA extract of H1975 shUNG was reacted in vitro with purified recombinant UNG and ARP was used to detect AP sites. Data represent mean ± SEM of three independent experiments, *p<0.05.
Following treatment, DNA extracts from treated cells were labeled with chemiluminescent aldehyde reactive probe (ARP) that binds glycosylase-generated AP sites. Compared to control cells, H1975 shUNG cells had decreased AP site detection, p<0.05 (Figure 3-3d). Lack of AP site formation following pemetrexed exposure suggests decreased uracil excision and accumulation of uracil bases in DNA.(58) To verify that uracil was retained in the DNA of cells with low UNG expression, we incubated DNA extract of pemetrexed-treated H1975 shUNG cells with recombinant UNG enzyme before labeling with ARP to detect AP sites. This in vitro uracil excision reaction resulted in the chemiluminescent detection of AP sites thereby confirming the persistence of uracil in H1975 shUNG DNA (Figure 3-3e).

To determine the mechanisms responsible for enhanced pemetrexed sensitivity in UNG deficient cells, we compared cell cycle progression and expression of DNA damage response proteins in pemetrexed treated UNG competent and UNG knockdown cells. Stable knockdown of UNG (H1975 66 shUNG cells) conferred increased sensitivity to pemetrexed-mediated accumulation of early S-phase and sub-G1 cells (Figure 3-4a), despite similar doubling times (Table 3-1). S-phase accumulation was accompanied by induction of phospho-cdc2, phospho-chk1, and cyclin B1 (Figure 3-4b). H1975 66 shUNG cells also had increased DNA DSB formation, as indicated by increased levels of γ-H2AX (Figure 3-4b) and significantly increased comet tail lengths (p<0.0001) in neutral
assay conditions (Figure 3-4c). H1975 66 shUNG cells were also more sensitive to pemetrexed-induced apoptosis as indicated by increased levels of cleaved poly-ADP ribose polymerase (PARP) (Figure 3-4b). These data are consistent with the DNA damage response observed in DLD1 UNG<sup>-/-</sup> cells treated with pemetrexed(58) and support our prior conclusion that cells lacking UNG are more sensitive to pemetrexed induced cell cycle arrest, DNA DSB formation and apoptosis.

Prolonged S-phase arrest is known to result in DNA DSB formation due to the collapse of stalled replication forks.(219, 220) We evaluated the stability of the DNA replication fork in pemetrexed treated lung cancer cells with native and reduced levels of UNG protein. Nucleotide incorporation experiments using CldU and IdU indicate decreased post-pemetrexed treatment nucleotide incorporation in cells deficient in UNG.(58) However, CldU and IdU compete with dU for incorporation sites, making these data difficult to interpret and prompting us to employ alternative measures of replication stability. Using bi-parametric flow cytometry, we measured the dissociation of the replication fork processivity factor, PCNA at the single cell level in H1975 cells expressing UNG directed shRNA. Similar assays have been utilized to detect replication fork disassembly following etoposide and hydroxyurea exposure.(221, 222) PCNA dissociation was determined by the percentage of cells in S phase having low PCNA (red box, Figure 3-4d). In wild type cells, less than 1% of cells in treated and untreated
Figure 3-4. Increased DNA damage in UNG deficient cells. (a) Comparison of cell cycle progression before and after pemetrexed treatment between H1975 cells and H1975 shUNG cells determined by PI-mediated flow cytometry. Cell cycle gates were resolved using Flow Jo software. (b) Protein alterations in cell cycle checkpoint, DNA damage and apoptosis proteins detected by western blot of whole cell extracts from pemetrexed treated and untreated H1975 and H1975 shUNG cells. (c) Neutral comet assay in pemetrexed treated and untreated H1975 and H1975 shUNG cells. Representative comet images are shown on the left and comet tail lengths of 100 cells per treatment are quantified in the right panel, *p<0.0001. (d) PCNA and PI bi-parametric flow cytometry in H1975 and H1975shUNG cells treated with 25 nM pemetrexed for 6 and 24 h. (e) Western blot of PCNA in chromatin extract from H1975 and H1975 shUNG cells before and after pemetrexed treatment. (f) Model of pemetrexed inhibition of primary target, TS to block dTMP formation. Supplemental thymidine supports salvage pathway formation of dTMP to bypass this effect. (g) Colony survival experiment in H1975 and H1975 shUNG cells treated with 0-400 nM pemetrexed alone or in combination with supplemental thymidine (10 μM). (h) Western blot from whole cell extracts of H1975shUNG cells treated with 100 nM pemetrexed (P) alone or in combination with 10 μM supplemental thymidine (T).
samples had low PCNA staining in S phase compared to 3.88 ± 0.93% and 7.81 ± 1.19%, H1975 shUNG cells treated with 25 nM pemetrexed for 6 and 24 hours, respectively, p<0.001 (Figure 3-4d). As a complimentary experiment, we examined the expression of chromatin-bound PCNA after pemetrexed treatment by western blot of chromatin cellular extracts. Pemetrexed treatment resulted in reduced expression of chromatin-bound PCNA in shUNG cells (Figure 3-4e). Such dispersal of PCNA and other replication fork components from chromatin is indicative of collapsing replication forks(222) and our data implicate pemetrexed-mediated replication fork instability and subsequent fork collapse in the mechanism of DSB formation and cell death observed in pemetrexed-treated UNG deficient cells.

Lastly, to link the accumulation and retention of genomic uracil with pemetrexed cytotoxicity, we used supplemental thymidine to promote salvage pathway production of dTMPs (Figure 3-4f). In H1975 cells treated with varying concentrations of pemetrexed alone or in the presence of 10 μM supplemental thymidine, the addition of thymidine rescued pemetrexed sensitivity in H1975 shUNG cells (Figure 3-4g). Supplemental thymidine also significantly decreased pemetrexed-mediated induction of γ-H2AX (Figure 3-4h). Importantly, AP site detection in H1975 parental cells and in H1975 shUNG DNA extracts incubated in vitro with purified UNG was also limited by the addition of thymidine (Figure 3-5). Reduced UNG excision (fewer AP sites) suggests supplemental thymidine dampens genomic uracil misincorporation. These data support the hypothesis that pemetrexed-induced cell cycle arrest and DSB formation are consequences of uracil misincorporation.
Figure 3-5. AP site detection in H1975 and H1975shUNG (66) cells. Quantification of chemiluminescent ARP-based AP site detection in DNA extracts of H1975 and shUNG cells treated with pemetrexed and supplemental thymidine (a) and extracts of H1975 66shUNG cells treated with pemetrexed and supplemental thymidine with and without in vitro incubation with purified UNG (b) as in figure 3(d-e).

3.3.4 UNG is induced in response to acute and chronic pemetrexed exposure. Resistance to anti-cancer agents that induce DNA damage has long been associated with up-regulation of DNA repair genes. Pemetrexed resistance in cells with high UNG expression led us to hypothesize that UNG, by limiting uracil-DNA, promotes survival of pemetrexed exposed cells. We assessed the impact of acute and chronic pemetrexed exposure on lung cancer cell expression of UNG. Acute pemetrexed exposure in the pemetrexed-sensitive adenocarcinoma cell line, H460 revealed time- and dose-dependent induction of UNG protein and transcript (Figure 3-6a-d). Supplemental thymidine dampened the UNG induction response, linking the observation of UNG induction to TYMS inhibition and consequent uracil accumulation (Figure 3-6e).
Figure 3-6. Induction of UNG in response to acute pemetrexed exposure. Time dependent changes in UNG protein (a) and mRNA (b) expression in H460 cells treated with 200 nM pemetrexed. Dose-dependent changes in UNG protein (c) and mRNA (d) expression in H460 cells treated with 0-200 nM pemetrexed for 48 h. (e) UNG expression in H460 cells treated with 200 nM pemetrexed and 10 μM thymidine alone or in combination for 6, 24 and 48 h.

To investigate whether chronic pemetrexed exposure would select for cells with elevated UNG expression, we established pemetrexed resistant sublines. We chose to induce resistance in H1299 cells (adenocarcinoma), the most pemetrexed sensitive cell line in our panel, which also expressed low levels of UNG. Sequential exposure of H1299 parental cells to increasing concentrations of pemetrexed over a 16-week period resulted in chronically elevated UNG protein expression that persisted for 8 weeks when pemetrexed was withdrawn (Figure 3-7a). Clonogenic sublines, PR-1 and PR-2, were established and colony
survival revealed 25-fold and 71-fold relative resistance to pemetrexed compared to parental cells, p<0.0001 (Figure 3-7b). Western blot analysis confirmed induction of UNG in PR1 and PR2 (Figure 3-7c). UNG activity was also enhanced, as indicated by UNG cutting assay (Figure 3-7e) and increased AP site detection in DNA extracts from pemetrexed treated pemetrexed resistant sublines (Figure 3-7f). PR-1 and PR-2 cells displayed cross-resistance to the TYMS inhibitors raltitrexed and 5-fluorouracil but were not resistant to other DNA damaging chemotherapeutics such as cisplatin or temozolomide that are not known to induce DNA repair through UNG initiated-BER (Figure 3-8).

Transfection of UNG-directed siRNA into PR-1 cells restored pemetrexed sensitivity (Fig 3-7g), indicating that UNG expression contributes significantly to the development of acquired pemetrexed resistance. The acute and chronic induction of UNG protein in lung cancer cells exposed to pemetrexed suggests that UNG activity is a pro-survival response to pemetrexed induced uracil incorporation into DNA and the resulting DNA damage.
Figure 3-7. Induction of UNG in response to chronic pemetrexed exposure. Exposure of H1299 cells to sequentially increasing concentrations of pemetrexed over a period of 16 weeks was conducted to establish pemetrexed resistant cells. (a) UNG expression was monitored in bulk cells prior to induction with 10, 30, 40, and 50 nM pemetrexed at 2, 8, 12 and 16 weeks respectively as well as in cells cultured for 8 additional weeks in pemetrexed-free media. Pemetrexed-resistant H1299 sublines (PR-1 and PR-2) were established by chronic exposure of H1299 parental cells to sequentially increasing concentrations of pemetrexed. (b) Clonogenic survival assay illustrates the difference in sensitivity between the resistant sublines (PR-1 and PR-2) and H1299 parental cells. (c) Representative western blot of UNG levels in H1299 cells (WT) and resistant sublines (PR-1 and PR-2). (d) Representative UNG activity assay in H1299 cells and resistant sublines. (e) Quantification of percent cutting from UNG activity assay, ** and * p<0.05. (f) H1299 pemetrexed-resistant sublines were treated with pemetrexed as indicated and DNA extracts were reacted with ARP reagent for AP site detection. (g) H1299 PR-1 cells expressing non specific (siNS) or UNG directed (siUNG) siRNAs were selected with puromycin and colony survival experiments determined pemetrexed IC50, *p<0.05.
3.3.5 Transcriptional regulation of UNG induction in pemetrexed-treated cells. Time and dose-dependent induction of UNG transcript and protein in lung cancer cells prompted an investigation of transcriptional regulators of UNG during pemetrexed exposure. Pemetrexed-mediated protein level induction was extirpated by pre-treatment with the transcriptional inhibitor, actinomycin D (Figure 3-9a), suggesting transcription level regulation of UNG expression.

Regulation of UNG in untreated cells is reported to be cell cycle regulated at the G1/S boundary by E2F.(165, 202, 203) We used chromatin immunoprecipitation
(ChIP) to identify transcription factors that potentially regulate UNG expression in the context of pemetrexed treatment. The nuclear promoter region of UNG is depicted in figure 3-9b with arrow indicating putative transcription factor binding sites. UNG nuclear promoter activity was suggested by the binding of RNA polymerase II (RNA Pol II) to the nuclear promoter (Figure 3-9c-d). In H460 cells, pemetrexed treatment causes an increase in E2F-4 protein while E2F-1 protein peaked only at 12 and 24-hour exposure to pemetrexed and declined with longer treatment times. ChIP analysis indicated that E2F-1 and E2F-4 both bind the UNG nuclear promoter in untreated cells, but dissociate at 12-hours of pemetrexed exposures (Figure 3-9c-d). These findings preclude these E2F transcription factors as activators of transcription in this context, but do not rule out the possibility of E2F de-repression of the UNG promoter.

The transcription factors c-Myc and Sp-1 have been associated with DNA damage response signaling(223, 224) and have been identified as positive regulators of the UNG nuclear promoter in untreated cells.(165) Western blot revealed both c-MYC and Sp-1 protein levels were enhanced by pemetrexed treatment (Figure 3-9c). Accordingly, c-MYC and Sp-1 associated with the UNG nuclear promoter in cells treated with 200uM pemetrexed at 12 hours, but not in untreated samples (Figure 3-9d), suggesting these transcription factors may activate the nuclear promoter in the context of pemetrexed induced DNA damage.
Figure 3-9. Transcription factor binding to UNG nuclear promoter in A549 cells during pemetrexed exposure. (a) A549 cells were pre-treated with actinomycin D (8μM solution) or vehicle (0.1% DMSO in PBS) for 30 minutes and subsequently exposed to 200nM pemetrexed for 24 hours. Western blots of whole cell extracts were performed to determine UNG expression in each sample. (b) Schematic of UNG promoter indicating location of putative transcription factor binding sites. Primers flanking a 603 bp region of PA and a proximal segment of Exon1A were used to amplify segments of DNA that were bound to transcription factors bound to the nuclear promoter in ChIP assays. (c) Input westerns were performed for RNA POLII, c-MYC, SP-1, AP-1(c-Jun), E2F-4 and E2F-1 to determine total relative expression of proteins within each sample. (d) PCR amplification products of chromatin immunoprecipitated DNA were resolved by agarose gel electrophoresis (2% gel) and visualized using ethidium bromide.

Mining of published microarray data revealed that up-regulation of c-Myc is associated with increased UNG expression (Figure 3-10a). (225) Hence we further evaluated the relationship between c-Myc and UNG induction through siRNA inhibition of c-Myc expression. Reduced c-Myc levels were achieved by siRNA targeting in A549 cells and confirmed by western blot (Figure 3-10b). A549 cells expressing lower c-Myc expression lacked a pemetrexed-mediated...
UNG induction response (Figure 3-10b) pointing to a role for c-Myc in activating the UNG nuclear promoter in the context of antifolate-induced DNA damage.

Figure 3-10. Coordination of c-Myc expression with UNG expression and induction in lung cancer. (a) Published microarray data mined from Oncomine (216) indicates UNG and c-Myc are co-expressed in pemetrexed resistant human lung cancer subtypes (small cell and squamous cell carcinoma. (b) In A549 human adenocarcinoma lung cancer cell lines, transient knockdown of c-Myc abrogated UNG induction response to pemetrexed exposure (200nM, 24h) and reduced baseline levels of UNG.

3.3.6 BER inhibition overrides pemetrexed resistance due to chronic exposure. Increased BER gene expression and activity prompted us to investigate BER inhibition as a means to re-sensitize chronically exposed cells to pemetrexed. The BER inhibitor methoxyamine (MX) covalently binds the aldehyde of glycosylase-formed AP sites and blocks downstream BER. This agent is now in clinical trials. Recent data has documented in-human tolerance
and potential efficacy when combined with pemetrexed. (28) Because MX is well tolerated at 3mM in cell culture and does not impact cellular sensitivity to non-AP site producing chemotherapeutics, we have surmised that the potentiation of cytotoxicity is due primarily to MX interaction with AP sites versus non-specific interactions with other intracellular aldehydes. MX-bound AP sites are substrates for topoisomerase IIα (TOPOIIα) cleavage and DNA DSB formation. Cells with elevated TOPOIIα are more sensitive to MX potentiation of DNA damaging agent cytotoxicity. (78) Interestingly, TOPOIIα expression is elevated in pemetrexed-resistant histological subtypes of lung cancer primary tissues compared to adenocarcinoma, (216-218) and we observed up-regulation of TOPOIIα in both pemetrexed resistant H1299 subclones compared to parental cells (Figure 3-11a). Additionally, published microarray data also indicates that UNG levels and TOPOIIα levels trend coincidently higher in cancer cells vs. normal cells (Figure 3-11b). Potentiation of pemetrexed cytotoxicity by MX is attenuated in UNG deficient cells (ref 32 and Table 3-3) suggesting that MX effects are critically linked to UNG expression and activity. In colony survival experiments, 3mM MX co-treatment restored cellular sensitivity to pemetrexed in PR-1 cells (Figure 3-11c) further suggesting that pemetrexed response is critically linked to uracil excision by UNG, and in doing so highlighting the utility of BER blockade to override acquired pemetrexed resistance.
Figure 3-11. Coordination of UNG and TOPOIIα expression in human lung cancer. (a) Published microarray data was retrieved from Oncomine™ (216) and illustrates that UNG expression and TOPOIIα genes are coincidently elevated in pemetrexed resistant cancer subtypes (small and squamous cell carcinoma). (b) Results of qRT-PCR indicating elevation of UNG and TOPOIIα gene expression in H1299 cells chronically exposed to pemetrexed (PR1 and PR2). The coincident induction of UNG and TOPOIIα in cells that acquire resistance to pemetrexed due to chronic exposure motivates the utilization of methoxyamine as a re-sensitization strategy since methoxyamine mechanism of action relies upon both glycosylase excision and topoisomerase targeting of the resulting abasic site. (c) Colony survival data indicating that targeting BER with methoxyamine re-sensitizes pemetrexed resistant cells (PR-1) to pemetrexed.

3.4 Discussion:

The present study reveals a significant role for UNG directed BER as a determinant of pemetrexed sensitivity in lung cancer. We observe a spectrum of
UNG expression in human lung cancer that is well correlated with pemetrexed IC$_{50}$ in cell lines and trends higher in pemetrexed-resistant small and squamous cell lung cancer subtypes. UNG has been identified as a prognostic marker in NSCLC.(226) Among lung adenocarcinoma tissues, relationships between elevated high UNG expression and both decreased survival (215, 218) and advanced disease stage(214) were noted. While it would seem obvious to also review prior studies to evaluate UNG as a predictive clinical predictive marker for pemetrexed response, we have been unable to find samples that allow this analysis. We are now collecting prospective samples to make this determination.

Other DNA glycosylases were not significantly associated with pemetrexed sensitivity and did not compensate for UNG loss suggesting UNG is the major glycosylase for uracil removal in pemetrexed-treated cells. The correlation between UNG expression and pemetrexed IC$_{50}$ remained marginally significant in multivariable regression models controlling for the expression of other BER genes and TYMS, with slight improvements in coefficients of determination. This finding introduces a level of genetic complexity to our discussion of pemetrexed response in lung cancer by showing that, while not significantly predictive of pemetrexed IC$_{50}$ alone, other BER and pathway specific genes contribute to pemetrexed response. To further clarify the combinatorial role of UNG and other genes, we propose future studies to determine pathway specific predictive gene
signatures for pemetrexed response in lung cancer tissue of patients receiving pemetrexed.

Prolonged cellular exposure to TYMS inhibitors results in growth arrest or resistance(227) and patients receiving pemetrexed ultimately progress. Continued clinical success of pemetrexed and other TYMS inhibitor chemotherapeutics therefore depends upon biomarker-based patient selection. Thymidylate synthetase (TYMS) levels have been studied for predictive value in pemetrexed sensitivity. Increased intra-tumor levels of TYMS – observed in highly proliferating tumors – limit dTTP pool depletion and contribute to TYMS inhibitor resistance.(192, 228) Additionally, in lung cancer models of acquired pemetrexed resistance, TYMS is consistently elevated.(209) Significant correlation between expression of TYMS and pemetrexed IC$_{50}$ in NSCLC cell lines(189, 192) and a modest survival advantage in patients(189) have also been reported. High TYMS expression has been reported in high-grade small cell carcinoma (SCLC).(190) However, TYMS has failed to predict SCLC response to pemetrexed combination therapy(196) and recent data suggests TYMS has less predictive value beyond second line therapy.(229) In our analysis, TYMS was significantly elevated in cell lines (PR1 and PR2) with acquired pemetrexed resistance. We did not, however, observe significant correlation between pemetrexed IC$_{50}$ and TYMS expression in our panel of cell lines.
Like TYMS, UNG expression is correlated with cellular proliferation. UNG-initiated BER has been observed at replication foci, illustrating coordination of DNA replication and repair of uracil-DNA. (75, 230) Despite observations of high UNG expression in rapidly proliferating cells, neither loss of UNG nor chronic pemetrexed exposure altered cellular doubling time. Thus the predictive value of UNG for pemetrexed response extends beyond the association of UNG with replication.

Fluctuations in UNG expression significantly impact pemetrexed sensitivity, consistent with the prior observation that glycosylase activity is a major rate-determining step of BER. (157) Recently, we reported that UNG\textsuperscript{−/−} DLD1 cells accumulated uracil and were hypersensitive to pemetrexed. (58) Here, through siRNA and shRNA knockdown of UNG we demonstrate the consistency of this phenotype in lung cancer cell lines, a clinically relevant model system. Decreased AP site formation in pemetrexed-treated UNG knockdown lung cancer cells suggests reduced uracil removal. Previous publications have suggested that substituting uracil for thymine reduces background DNA methylation, alters DNA structure and interferes with high-affinity protein-DNA interactions. (142) Heavily uracilated DNA may therefore impede access and/or activity of transcription factors and replication fork proteins. In our study, we’ve observed compromised replication fork stability in the absence of uracil excision by UNG. Sensitivity to pemetrexed in UNG deficient cells is rescued by supplemental thymidine, which
attenuates UNG induction response and AP site formation in parental cells.

Based on these data, we propose a novel hypothesis for thymine-less death in UNG deficient cells wherein lack of repair of misincorporated uracil leads to the collapse of DNA replication forks and triggers apoptosis.

That unrepaired uracil-DNA elicits a profound cytotoxic response in pemetrexed-treated human cancer cells was unexpected given the normal development of young UNG-/- mice(77, 132) and comparable sensitivity of UNG++ and UNG-/- MEFs to fluoropyrimidine TYMS inhibition.(121) Our data are consistent with earlier reports of a direct role for uracil misincorporation in pemetrexed cytotoxicity.(231) Indeed, RNA interference-mediated silencing of dUTPase, an enzyme responsible for maintaining low dUTP levels, significantly enhances pemetrexed cytotoxicity presumably due to increased dUTP incorporation.(231) At baseline, otherwise isogenic UNG-proficient and -deficient cells have comparable levels of DNA damage markers despite reduced capacity for uracil excision suggesting UNG loss is well tolerated in the absence of TS-inhibitor challenge. When exposed to pemetrexed, however, UNG-/- human cancer cells accumulate up to 40-fold more uracil compared to UNG++ controls(58). In contrast, only 1.5-fold and 8-fold increases in uracil were reported in 5-FU and FdUrd treated UNG-/- MEFs (121) and raltitrexed treated 293t cells expressing the bacteriophage UNG inhibitor Ugi,(147) respectively. These studies concluded that UNG activity did not impact TYMS sensitivity.(121, 147) We believe the
differential sensitivity to various TYMS inhibitors with UNG loss points to an as yet undetermined threshold of genomic uracil tolerance in mammalian cells. Such a threshold has been suggested by Luo et al (147) and is presumed to depend upon both the TYMS inhibitor used and the uracil excision capacity of the cells studied. We speculate previous observations of little correlation of UNG with TYMS sensitivity (121, 147) are due to uracil accumulation within tolerance levels in those model systems.

A clear advantage to the identification of UNG as a predictive marker for pemetrexed resistance is the ability to potentiate pemetrexed efficacy via BER inhibition. We show that UNG is induced by acute and chronic pemetrexed exposure in lung cancer cell lines and MX inhibition of BER restores pemetrexed sensitivity in chronically exposed cells. MX-bound AP sites are clastogenic, trapping TopoIIα in a cleavable complex resulting in DNA DSBs. (78) Cells with high TopoIIα expression are particularly sensitive to DNA damaging agents when combined with MX. Like UNG, TopoIIα levels are elevated in human lung cancer and are highest in small and squamous cell carcinoma. (216, 218) Therefore, the pemetrexed/MX combination is a rational strategy to overcome pemetrexed insensitivity in certain lung cancer subtypes and to restore sensitivity in cells that acquire resistance due to chronic pemetrexed exposure. Pemetrexed/MX combination therapy has been pursued in phase I clinical trials involving solid tumors (28) resulting in a partial response in 56% of patients enrolled. Among the
responders were 3 patients with squamous cell lung carcinoma and 1 patient with squamous oropharyngeal carcinoma that notably had high TYMS levels. (28) Based on these data, phase 2 and randomized controlled trials involving pemetrexed and methoxyamine are planned.

We have initiated studies of the transcriptional regulation of UNG in the context of relevant DNA damaging chemotherapy exposure (pemetrexed). Published reports have previously implicated E2F transcription factors in the cell cycle dependent regulation of the UNG gene. (75, 232, 233) In the present study, we observe an dissociation of E2F-1 and E2F-4 and an association of c-Myc and sp-1 transcription factors with the UNG nuclear promoter during pemetrexed exposure. Dissociation from the nuclear promoter precludes E2F-1 and E2F-4 as activating transcription factors of the UNG nuclear promoter during pemetrexed treatment. However, we cannot rule out that these transcription factors operate in a de-repression mode for UNG in this context.

The oncogene, c-Myc is de-regulated in most human cancers as is identified here as binding to the nuclear promoter of UNG during pemetrexed treatment. Constitutively active Myc-dependent mutagenic signaling cascades promote transcription of cell proliferation factors conferring a survival and growth advantage to tumor cells. (234) Amplification of c-Myc and n-Myc has been observed in small cell lung cancer and is higher in SCLC than NSCLC. (235, 236)
In lymphoma models, overexpression of c-Myc results in increased UNG levels, suggesting a link between the transcription factor and UNG gene expression. The possible relationship between c-Myc and UNG expression here is not surprising and implicates the transcription factor in UNG regulation during uracil misincorporation and accumulation. Targeting of c-MYC enhances pemetrexed mediated cell killing and decreases UNG baseline expression and induction. The convergence of several signaling pathways that are critical to tumor proliferation and survival at Myc makes targeting the oncogene an attractive strategy for anti-cancer therapeutic development. Myc inactivation and inhibition strategies have been illustrated to lead to tumor differentiation and sensitization to DNA damaging agent-induced apoptosis. Other studies have looked into exploiting Myc-driven transcription in tumors to produce selective toxicity. Though additional studies are needed, our data incentives future investigation of Myc as a target to selectively enhance tumor cell sensitivity to pemetrexed.

Tailoring chemotherapy based on histological subtype and biomarker expression is a favorable strategy for aggressive, treatment-refractory malignancies such as lung cancer. Our observations that UNG expression is elevated in experimental models of pemetrexed-resistant lung cancer and correlates with pemetrexed efficacy prompt us to propose investigation of UNG as a novel predictive marker for pemetrexed in human lung cancer. Moreover, because UNG loss and BER
inhibition with MX potently restore pemetrexed sensitivity in resistant cells, UNG-directed BER may be a novel therapeutic target, distinct from the folate metabolism pathway, for overcoming pemetrexed resistance in human lung cancer.
Chapter 4

Uracil DNA glycosylase (UNG) loss enhances DNA double strand break formation in human cancer cells exposed to pemetrexed

reproduced with permission from

Misincorporation of genomic uracil and formation of DNA double strand breaks (DSBs) are known consequences of exposure to TS inhibitors such as pemetrexed. Uracil DNA glycosylase (UNG) catalyzes the excision of uracil from DNA and initiates DNA base excision repair (BER). To better define the relationship between UNG activity and pemetrexed anti-cancer activity, we have investigated DNA damage, DSB formation, DSB repair capacity, and replication fork stability in UNG<sup>+/+</sup> and UNG<sup>-/-</sup> cells. We report that despite identical proliferation indices and DSB repair capacities in this isogenic cell pair, UNG<sup>-/-</sup> cells accumulated significantly more uracil and DSBs compared to UNG<sup>+/+</sup> cells when exposed to pemetrexed. ChIP sequencing for γ-H2AX confirmed fewer DSBs in UNG<sup>+/+</sup> cells. Furthermore, DSBs in UNG<sup>+/+</sup> and UNG<sup>-/-</sup> cells occur at distinct genomic loci, supporting differential mechanisms of DSB formation in UNG competent and UNG deficient cells. UNG<sup>-/-</sup> cells also showed increased evidence of replication fork instability (PCNA dispersal) when exposed to
pemetrexed. Thymidine co-treatment rescues S-phase arrest in both UNG<sup>+/+</sup> and UNG<sup>−/−</sup> cells treated with IC<sub>50</sub>-level pemetrexed. However, following pemetrexed exposure, UNG<sup>−/−</sup> but not UNG<sup>+/+</sup> are refractory to thymidine rescue suggesting that deficient uracil excision rather than dTTP depletion is the barrier to cell cycle progression in UNG<sup>−/−</sup> cells. Based on these findings we propose that pemetrexed-induced uracil misincorporation is genotoxic contributing to replication fork instability, DSB formation and ultimately cell death.
4.1 Introduction

Pemetrexed is a multi-target antifolate that inhibits several folate-dependent enzymes.(206) The major enzymatic target for pemetrexed is thymidylate synthase (TS), while dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT) are minor targets.(206) Inhibition of TS and other enzymes results in decreased nucleotide synthesis, which stalls DNA replication and leads to cell death. In addition to a global reduction in nucleotide synthesis, TS inhibition directly limits the methyl reduction of deoxyuridine monophosphate (dUMP) to form deoxythymidine monophosphate (dTMP). (113, 148, 237) The resulting imbalance in deoxyuridine and deoxythymidine nucleotide pools favors the utilization of dUTP in DNA replication and the accumulation of uracil in DNA.(148)

Uracil DNA glycosylase (UNG) is the major glycosylase for the removal of uracil from DNA and the initiation of base excision repair (BER). (77, 148, 157, 230, 238, 239) As a mechanism of TS-inhibitor induced cell death (or so called thymine-less death), it has been hypothesized that futile cycles of uracil excision by UNG, BER pathway repair activity, and dUTP re-insertion contribute to fragmentation of genomic DNA.(4, 237) Though expression of UNG is increased in the presence of TS inhibitors, little evidence is available to support the hypothesis that futile cycles of BER contribute to thymine-less death. If TS-
inhibitor induced genomic instability were reliant upon UNG excision of uracil, one would expect increased sensitivity to TS-inhibitors in cells with elevated UNG expression. However, pemetrexed-resistant subtypes of human cancer have statistically higher UNG expression than pemetrexed sensitive subtypes. (59) Additionally, overexpression of UNG does not sensitize cells to raltitrexed or plevitrexed, similar agents. (84)

Recently, we have reported that UNG is predictive of pemetrexed response in experimental human lung cancer models and that loss of UNG expression in human cancer sensitizes cells to pemetrexed. (59) These data imply that pemetrexed-induced genomic instability is mediated by accumulation rather than removal of uracil. The objective of the present study was to further investigate the pemetrexed hypersensitivity and DNA DSB formation observed in UNG⁻/⁻ cells in order to better understand the consequences of deficient uracil excision during pemetrexed exposure.

Herein, we show that despite equivalent rates of proliferation, DLD1 human colon cancer cells with stable knockout of UNG (UNG⁻/⁻) are hypersensitive to pemetrexed induced DNA DSB formation and cell death. With γ-H2AX chromatin immunoprecipitation sequencing (ChIP-Seq), we observed significantly greater number of γ-H2AX-associated genomic loci (DSB) in pemetrexed UNG⁻/⁻ cells compared to UNG⁺/+ cells. Additionally, ChIP-Seq data reveal distinct patterns of
DSB susceptible loci in UNG\textsuperscript{+/+} and UNG\textsuperscript{−/−} cells suggesting disparate mechanisms of DSB formation in UNG proficient and deficient cells. Using the complimentary approaches of CldU/IdU labeling and PCNA dispersal experiments, we show evidence of replication fork instability in pemetrexed-treated cells. UNG\textsuperscript{+/+} and UNG\textsuperscript{−/−} have similar basal levels of DNA repair proteins and DSB repair capacities, yet UNG\textsuperscript{−/−} cells show delayed recovery from pemetrexed induced DNA damage, including DSBs. Based on these findings we propose that the enhanced cytotoxicity observed in UNG\textsuperscript{−/−} cells is the result of intrinsic genotoxicity of misincorporated uracil, which persists in sufficient quantities in UNG\textsuperscript{−/−} cells to stall DNA replication, triggering cell death. We propose this as a new mechanism to explain thymine-less death in cells with limited UNG activity.

4.2 Materials and Methods

4.2.1 Cell lines and reagents. Pemetrexed was purchased from LC laboratories. Thymidine, raltitrexed and cisplatin were purchased from Sigma Aldrich. DLD1 cells were purchased from ATCC and expanded upon delivery into numerous vials of low passage cells for cryopreservation. Cell line characterization through ATCC is conducted through short tandem repeat (STR) typing. Re-authentication was not performed. DLD1 UNG\textsuperscript{−/−} are described by Guo et al (240) and were a kind gift from Sanford Markowitz (Department of Genetics,
Case Western Reserve University). Cells were maintained in complete DMEM (10% FBS, 2mM L-glutamine) at 37°C in a 5% CO₂ incubator.

4.2.2 Western blots. Protein extracts (30μg) were resolved by SDS-PAGE and transferred to PVDF membrane (Millipore). 5% milk in PBST blocked non-specific binding sites and membranes were incubated overnight at 4°C with primary antibody in 5% BSA/PBST, followed by secondary HRP-conjugated antibody in 2.5% milk in PBST at manufacturer specifications. Chromatin-bound proteins were extracted from formaldehyde cross-linked (1%) cells using the Pierce Chromatin Prep module (Thermo Pierce). Antibody sources: UNG-23936 (39 kDa band, nuclear UNG), Nbs1, DNA ligase IV, Ku70, BRCA1, MRE11, and PCNA (Abcam); Tubulin (Calbiochem); γ-H2AX, cleaved caspase 3, and Histone-H3 (Millipore); cleaved PARP, XRCC4, and secondary antibodies (Cell Signaling); and Rad51, p-chk1, chk1, p-cdc2, and cdc2 (Santa Cruz).

4.2.3 UNG activity assay. UNG activity was measured using 40-mer oligodeoxynucleotide duplex containing a single U:A mispair (Operon).

5′[HEX] GTAAAACGACGGCCAGTGU TTCTCGAGCTCGGTACCCGGGG (top)

3′-CATTTTGCTGCCGGTCACAGAAGCTCGAGCCATGGGCCCC[Cy5] (bottom)

Annealed oligonucleotide duplex was incubated with purified enzymes (1U) or whole cell extract (~2.5μg) at 37°C for 10 minutes. Products were resolved by denaturing polyacrylamide gels (20%), prior to visualization with a Typhoon 9200
fluorescence imager (Amersham Bioscience) and UNG activity was calculated (percentage of cut band relative to total oligonucleotide).

4.2.4 **Determination of cell growth rate/doubling time.** Cell counting with trypan blue exclusion determined cellular proliferation rates. Briefly, $1 \times 10^5$ cells were seeded in 60mm cell culture dishes and allowed to grow in typical cell culture medium for 24-96 hours. Cells were harvested by gentle trypsinization and both adherent and suspension cells were stained with trypan blue, counted using a hemacytometer and the number of viable cells (cells lacking trypan blue stain) was used to generate a growth curve. Each data point represents the mean of 3 independent experiments.

4.2.5 **Colony survival.** Colony survival was assessed by methylene blue staining of colonies formed after 10-day exposure to the drugs indicated. Only colonies containing $\geq 50$ cells were counted. Data points represent percent of colonies relative to untreated control averaged over 3 independent experiments.

4.2.6 **Annexin V staining.** Cells were stained with Annexin-V FITC and propidium iodide (PI) for evaluation of apoptosis by flow cytometry according to the manufacturer’s protocol (BD Pharmingen, San Diego, CA). Briefly, cells were harvested by gentle trypsinization and washed twice in cold PBS prior to staining with $5 \mu$l of Annexin V FITC reagent and $10 \mu$l of PI (5$\mu$g/ml) in 1x binding buffer
(10mM HEPES, 140mM NaOH, 2.5mM CaCl₂, ph7.4) for 30 min at room temperature in the dark. Apoptotic cells were determined using a coulter flow cytometer (EPICS-XL-MCL). Cell death calculations are based on sum of both early apoptotic (Annexin V positive, PI-negative) and late (Annexin V-positive, PI-positive) cells. Data are represented as the mean ± S.E.M.

4.2.7 Cell cycle and PCNA dispersal assays. PI staining of methanol fixed cells was used for cell cycle determinations. For PCNA dispersal assays, FITC-labeled PCNA antibody (PCNA-FITC, Abcam) was added for PCNA detection. Both uni-parameter (PI-only) and dual-parameter (PI + PCNA-FITC) analysis was performed on a coulter flow cytometer (EPICS-XL-MCL). Cell cycle histograms (PI) and PCNA dot plots (PCNA-FITC) were de-convoluted from ≥ 20,000 events using Flow Jo software.

4.2.8 Tumor growth assays in xenograft mice. 5x10⁶ tumor cells in early passage were injected into bilateral flanks of female NODSCID mice (~5 weeks old). When tumor volumes reached 100mm³, mice were divided into control and treatment cohorts (n = 6). Mice bearing tumors were treated with pemetrexed (150mg/kg) or 100ul sterile PBS (control) by daily intraperitoneal injection (IP) for 5 consecutive days. Tumor measurements were taken every 2 days and response was quantified by calculated tumor volume.
4.2.9 Comet assay under neutral conditions. Treated cells were processed for comet tail formation under neutral comet assay conditions according to the manufacture protocol for cell lysis and single cell gel electrophoresis (Trevigen). Tail lengths were recorded for at least 50 comets on 2 separate slides (~100 cells per treatment) using ImageJ software.

4.2.10 Oligonucleotide primer extension assays. HPLC purified oligonucleotide primers were purchased from Sigma Aldrich corresponding to the following primer sequences:

- **“5U”**: 3’- GTC GGC AGG TUU UUU CCC AAA-5’
- **“3U”**: 3’- GTC GGC AGG TUG UGU CCC AAA-5’
- **“1U”**: 3’- GTC GGC AGG TUG TGT CCC AAA-5’
- **“No U”**: 3’- GTC GGC AGG TTG TGT CCC AAA-5’

These primers were annealed in 1X annealing buffer (10mM TRIS, pH = 7.5, 50mM NaCl, 1mM EDTA) in a 1:1 ratio to the following truncated oligonucleotide sequence:

- **“Top”**: 5’- [A488]-CAG CCG TCC A -3’.

DNA polymerase (T4 DNA polymerase, a phage-derived replicative polymerase) was reacted with oligonucleotide duplex (500nM) in reaction buffer, mixed with 100μM dNTP mix (Sigma Aldrich) in the presence of 100mM Mg²⁺. Reactions were quenched at variable times (10-180 seconds) in 200mM EDTA. Products were resolved by 20% denaturing polyacrylamide gel electrophoresis and
analysis of band densities of primer and extension products was performed using a Typhoon scanner. Band densities corresponding to ≥ 12mer extension product were used to calculate “nM of extension product” according to the following equation:

\[
\text{nM reaction product} = 500 \times \frac{\text{Sum} \geq 12\text{mer band fluorescence}}{\text{Total fluorescence (all bands in reaction)}}
\]

Data at time 0 were subtracted from subsequent data points as baseline. Data represent the average of 3 independent experiments ± S.E.M.

**4.2.11 CldU/IdU pulse labeling and flow cytometry.** Cells were incubated for 45 minutes in media containing 50μM chlorodeoxyuridine (CldU) prior to drug treatments. Following treatment cells were washed to remove traces of drug using 1x sterile PBS and then labeled for 45 minutes with iododeoxyuridine (IdU, 50μM). For analysis, fixed cells were incubated in DNA denaturation solution (4N HCl, 0.1% Triton X-100 in PBS) for 15 minutes at room temperature followed by neutralization washes with 0.1M Na₂B₄O₇ buffer. Cells were then incubated for 30 minutes at 37°C in 1mg/ml RNAseA in PBS. Each sample was then labeled using rat anti-BrdU-FITC (detects CldU) and mouse anti-BrdU-APC (detects IdU) antibodies. Cells were counterstained with propidium iodide so that only cycling cells would be analyzed. CldU and IdU uptake and incorporation were determined using a BD LSR II flow cytometer. Dot plots of cycling cells (cells in G1/S/G2M) were de-convoluted from uni-variate analysis of PI staining. Bi-variate
dot plots of CldU/IdU content were obtained for cycling cells using Flow Jo software.

4.2.12 DNA double strand break repair assays. DNA double strand break assays were performed as described.(241) Briefly, cells were transiently transfected with either circular pDRGFP or ISCE1-linearized pDRGFR for homologous recombination (HR) assays or with circular pE_GFP-PEM1-Ad2 or ISCE1-linearized PE-GFP-PEM1-Ad2 for non-homologous end joining (NHEJ) assays. DNA repair plasmids were obtained from Elisa Tichy (University of Cincinnati) and plasmid stocks were expanded using bacterial transformation. Plasmid pDsRed express (Clontech) was co-transfected in each instance as a transfection efficiency marker. Transfections of 2.5x10^5 cells in 6-well culture dishes were carried out using Opti-MEM media and oligofectamine according to standard laboratory procedures. 5μg of pDsRed Express N1 and 40μg of construct DNA were utilized for transfections. 72-hours post transfection, cells were trypsinized and suspended in cold PBS containing 0.1% BSA for analysis by flow cytometry. Samples were analyzed using a BD LSRII (BD Bioscience). Dot plots for DsRed and GFP were acquired for ≥ 20,000 events using FlowJo software.

4.2.12 γ-H2AX ChIP Sequencing. Chromatin preparation, chromatin immunoprecipitation (ChIP), DNA purification, and library preparation for illumina
sequencing were performed as described.(242) ChIP was performed using commercially available antibody to γ-H2AX - p-S139 (Abcam 2893). Following ChIP-PCR, sequencing libraries were prepped from 30μl of ChIP or 500ng of input DNA. Sequencing was performed on an Illumina Genome Analyzer II at the Case Western University Genomic Core Facility. Referenced ChIP-seq datasets will be deposited in gene expression omnibus (GEO) at the time of publication.

4.3 Results

4.3.1 Loss of UNG expression hypersensitizes human cancer cells to pemetrexed. As we were interested in the impact of UNG expression on pemetrexed response in human cancer cells, we evaluated sensitivity to pemetrexed in UNG+/+ and UNG−/− colon cancer cells. These UNG−/− cells were engineered as described(240) and lack expression of both nuclear and mitochondrial UNG isoforms (Figure 4-1a). Loss of expression of UNG abolished in vitro cutting activity in the UNG/APE oligonucleotide cutting assay (Figure 4-1b) suggesting that in the absence of UNG, uracil is not efficiently removed from DNA.

UNG expression is coordinated with DNA replication.(75, 77, 230, 243, 244) This relationship prompted us to evaluate the impact of UNG loss on cellular proliferation rates for UNG+/+ and UNG−/− cells (Figure 4-1c). An identical number
of cells (1 x 10⁵) were seeded in 60mm culture dishes and incubated for 24-96 hours. Following incubation, cells were stained with trypan blue and the total number of viable cells per sample was determined using a hemacytometer. This simple experiment yielded identical proliferation rates for UNG⁺/⁺ and UNG⁻/⁻ cells, p=0.292 (Figure 4-1c). Clonogenic survival assays revealed UNG⁻/⁻ cells were 10-fold more sensitive to pemetrexed (IC₅₀ UNG⁺/⁺ = 205.8 nM and IC₅₀ UNG⁻/⁻ = 22.0 nM, p<0.0001, Figure 4-1d). UNG⁻/⁻ cells displayed cross sensitivity to the TS inhibitor, raltitrexed, p<0.0001 (Figure 4-1e) but not the cross-linking agent cisplatin, p=0.17 (Figure 4-1f). The impact of pemetrexed on cell killing was further analyzed by cleaved PARP measurements using western blot (Figure 4-1g) and Annexin V measurements using flow cytometry (Figure 4-1h) at 24-hours in cells treated with 0-100nM pemetrexed. UNG⁻/⁻ cells were significantly more sensitive to pemetrexed-induced apoptosis compared to UNG⁺/⁺ cells. Re-expression of UNG (UNGFLAG) rescued pemetrexed and raltitrexed sensitivity in UNG⁻/⁻ cells (Figure 4-1d-f) confirming that TS-inhibitor sensitivity in UNG⁻/⁻ cells is due to UNG loss.
Figure 4-1. Loss of UNG enhances pemetrexed sensitivity in DLD1 human colon cancer cells. (a) Western blot of UNG nuclear (top band, 39kDa) and mitochondrial (bottom band, ~36kDa) in UNG^{+/+} and UNG^{-/-} DLD1 human colon cancer cells. (b) UNG cutting activity assay using either purified enzyme (APE and UNG+APE lanes, 1U of each) or 2.5μg of whole cell extract from UNG^{+/+} and UNG^{-/-} cells. (c) Proliferation rates were determined by cell counting with trypan blue exclusion of non-viable cells over a period of 0-96 hours using a hemacytometer. Colony survival assays for UNG^{+/+} and UNG^{-/-} cells treated with pemetrexed (d), raltitrexed (e), and cisplatin (f) for 10-days. UNG^{-/-} cells are 10-fold more sensitive to pemetrexed, p<0.0001 and 4.3-fold more sensitive to raltitrexed, p<0.0001. Sensitivity to cisplatin was not significantly different for the two cell lines, p=0.17. UNG^{FLAG} cells are UNG^{-/-} cells that were transfected with a FLAG tagged UNG vector to induce re-expression of UNG. UNG^{FLAG} cells showed resistance to pemetrexed and raltitrexed similar to UNG^{+/+} cells (g) Western blot of apoptotic markers cleaved PARP and cleaved caspase 3 in UNG^{+/+} and UNG^{-/-} cells treated for 24hours with 0-100nM pemetrexed. (h) Percentage of Annexin V positive pemetrexed-treated UNG^{+/+} and UNG^{-/-} cells after 24 hour exposure to 0-100nM pemetrexed. (i) Tumor volumes of xenograft tumors grown in NODSCID mice that were treated for 5 consecutive days with pemetrexed (150mg/kg, intraperitoneal injection).
To evaluate the impact of UNG loss on tumor cell growth in vivo, UNG\(^{+/+}\) and UNG\(^{-/-}\) tumors were established as subcutaneous xenografts in NOD-SCID mice (Figure 4-1i). Treatment with 5 daily consecutive intraperitoneal (IP) injections of pemetrexed (150mg/kg) resulted in tumor quadrupling time of 7.83 ± 0.88 days in UNG\(^{+/+}\) tumors compared to 12.54 ± 0.42 days in UNG\(^{-/-}\) tumors, p<0.001. At day 20 pemetrexed treated UNG\(-/-\) tumors were 61% smaller than untreated UNG\(-/-\) tumors while UNG\(^{+/+}\) tumors were only 26% smaller with pemetrexed treatment compared to untreated controls (Figure 4-1i). Together these data indicate a significant increase in the anti-tumor effect of pemetrexed in UNG deficient tumor cells in vitro and in vivo.

**4.3.2 Increased DNA double strand break formation in UNG\(^{-/-}\) cells during pemetrexed exposure.** The formation of DNA double strand breaks is a long observed consequence of TS inhibitor exposure and so-called thymine-less death,(3, 4, 245) yet the mechanism of DSB formation has remained ill-defined. It has been hypothesized that futile cycles of uracil excision, BER and dUTP reinsertion contribute to DNA fragmentation and DSB formation in cells treated with TS-inhibitors.(4, 237) However this hypothesis lacks experimental support(84) and fails to explain the hypersensitivity to pemetrexed we observe in UNG\(^{-/-}\) cells (Figure 4-1). We therefore evaluated DNA DSB formation in our isogenic UNG\(^{+/+}\) and UNG\(^{-/-}\) cells to better understand the role of UNG in mediating DSB formation and thymine-less death during pemetrexed exposure.
Western blots for the DNA DSB surrogate marker, γ-H2AX, show significant induction in UNG−/− cells compared to UNG+/+ cells (Figure 4-2a). Importantly, even when the two cell lines are treated with equally cytotoxic concentrations of pemetrexed (IC₅₀, UNG−/− 25nM and UNG+/+ 200nM), induction of γ-H2AX protein expression appears greater in UNG−/− cells (Figure 4-2b, “P” lanes). As a complimentary experiment, we measured DNA double strand breaks using the neutral single cell gel electrophoreses (comet) assay. Pemetrexed treatment caused 1.85-fold longer comet tail lengths in UNG−/− cells compared to UNG+/+ cells, p<0.05 (Figure 4-2c-d). By providing a salvage pathway for the production of dTTP, the addition of supplemental thymidine should limit the impact of TS inhibition. Thymidine supplementation rescued pemetrexed-induced γ-H2AX induction (Figure 4-2b) and comet tail formation (Figure 4-2c) in both DLD1 UNG−/− and UNG+/+ cells, suggesting that in both cell lines the dUTP/dTTP nucleotide pool ratio drives DNA DSB formation. Of note, the increased formation of DNA double strand breaks in UNG−/− cells occurs despite equivalent expression of DNA DSB repair proteins and DSB repair capacities in UNG+/+ and UNG−/− cells (Figure 4-3).
Figure 4-2. Increased DNA DSB formation in UNG+ cells treated with pemetrexed. (a) Western blot for γ-H2AX was performed using whole cell extracts from UNG+/+ and UNG−/− cells treated with 25nM pemetrexed for 0-72 hours to compare sensitivity of the two cell lines to pemetrexed-induced DSB formation. (b) Western blot for γ-H2AX was performed using whole cell extracts from UNG+/+ and UNG−/− cells treated with IC50-level pemetrexed alone or in combination with 10μM supplemental thymidine for 24 hours. UNG+/+ cells were treated with 200nM pemetrexed and UNG−/− cells were treated with 25nM pemetrexed. (c-d) Single cell gel electrophoresis (comet) assay was performed under neutral conditions for UNG+/+ and UNG−/− cells treated with IC50-level pemetrexed alone or in combination with supplemental thymidine. At least 50 comet tail lengths were measured using NIH Image J software from 2 independent experiments for a total of ≥100 cells per drug treatment. Representative images are shown in (c) and the mean and SEM for this experiment is plotted in (d). Pemetrexed treatment caused 1.85-fold increase in comet tail length in UNG−/− compared to UNG+/+ cells, Ψ, and p <0.05 Thymidine rescued sensitivity to pemetrexed in UNG+/+(*, p<0.001) and UNG−/−(**, p<0.004) cells.
Figure 4-3. Comparison of DNA DSB repair capacity in UNG+/+ and UNG−/− cells. (a) Western blots comparing basal expression of proteins involved in DNA double strand break repair in UNG+/+ and UNG−/− cells. (b) Schematic for HR repair assay. Briefly, transient transfection of ISCE1 linearized pDR-GFP was used to measure homologous recombination efficiency. pDR-GFP contains 2 tandem but inactive GFP repeats, one of which contains an ISCE restriction enzyme cutting site. GFP fluorescence is activated by homology-mediated deletion between the two repeats during functional HR to generate a functional GFP. (c) Schematic for NHEJ repair assay. Briefly, pE-GFP-Pem1-Ad2 has non-functional GFP interrupted by Pem 1 intron into which there is inserted an adenovirus exon sequenced flanked by ISCE restriction enzyme cleavage sites. ISCE1 plasmid linearization generates non-complimentary ended DSB and removes the AD2 sequence. NHEJ allows juxtaposition of the 2 GFP fragments to reconstitute fluorescence. (d) Representative flow cytometry dot blots from untreated UNG+/+ and UNG−/− cells transfected with an empty vector, ISCE1 linearized pDR-GFP or ISCE1 linearized pE-GFP-Pem1-Ad2. pdSRed was co-transfected in each instance as a transfection efficiency control. Similar percentages of GFP+/dSRed+ cells were observed for UNG+/+ and UNG−/− cells for each transfection suggesting similar capacity for BER.
To further compare the mechanism of DSB formation and thus cell death in UNG$^{+/+}$ and UNG$^{-/-}$ cells, we performed γ-H2AX chromatin immunoprecipitation to map and compared the genomic distribution of DSBs in pemetrexed-treated UNG$^{+/+}$ and UNG$^{-/-}$ cells. Figure 4-4a shows representative signal tracks on chromosome 21 for pemetrexed treated UNG$^{+/+}$ and UNG$^{-/-}$ cells. ChIP seq experiments with cisplatin treated cells were performed as a positive control for double strand breaks. Pemetrexed treatment resulted in a 5.75 fold increase in distinct γ-H2AX bound genomic regions in UNG$^{-/-}$ cells compared to UNG$^{+/+}$ cells. Moreover, of the 5272 γ-H2AX enriched sequences identified in UNG$^{-/-}$ cells, only 0.0025% overlapped with γ-H2AX bound sites in pemetrexed-treated UNG$^{+/+}$ cells (Figure 4-4b). When a detailed analysis of genomic features of γ-H2AX-bound sequences was performed, DSBs were observed to map more frequently to transcription factor binding sites, CpG islands, DNAse clusters and putative origins of replication in pemetrexed-treated UNG$^{-/-}$ cells compared to UNG$^{+/+}$ cells (Table 4-1). In all, our data indicate that UNG$^{-/-}$ cells accumulate significantly greater number of DSBs in response to pemetrexed. Moreover, the differential chromosomal distribution of γ-H2AX enrichment in the genome suggests disparate mechanism of DSB formation in UNG proficient and deficient cells.
**Figure 4-4. γ-H2AX ChIP Sequencing in UNG+/+ and UNG−/− cells.** Cells were treated with IC₅₀ level pemetrexed (UNG+/+, 200nM and UNG−/−, 25nM) or with 20μM cisplatin for 24 hours and subsequently processed for ChIP sequencing as described in the materials and methods section. Cisplatin was utilized as a control DNA damaging agent that forms DNA double strand breaks. (a) Representative signal tracks showing γ-H2AX enrichment in chromosome 21 following drug exposure. Venn diagrams of the ChIP-Seq peaks generated comparing signal overlap for pemetrexed treated UNG+/+ and UNG−/− cells (b); pemetrexed treated and cisplatin treated UNG+/+ cells (c) and pemetrexed-treated and cisplatin-treated UNG−/− cells (d).
Table 4-1 Characteristics of γ-H2AX-enriched sequences (*, p<0.001)

<table>
<thead>
<tr>
<th>DNA feature analyzed for overlap</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encode clustered TFBS</td>
<td>35.1%</td>
<td>55.8%*</td>
</tr>
<tr>
<td>DNAse clusters</td>
<td>45.5%</td>
<td>62.8%*</td>
</tr>
<tr>
<td>CpG islands</td>
<td>4.5%</td>
<td>11.4%*</td>
</tr>
<tr>
<td>Lamin associated domains</td>
<td>33.9%</td>
<td>1.4%*</td>
</tr>
<tr>
<td>Caco-2 CTCF peaks</td>
<td>6.3%</td>
<td>11.0%*</td>
</tr>
<tr>
<td>HCT116 methyl sequencing peaks</td>
<td>5.1%</td>
<td>23.1%*</td>
</tr>
<tr>
<td>K562 and MCF7 identified origins of replication (246, 247)</td>
<td>12.7%</td>
<td>36.7%*</td>
</tr>
</tbody>
</table>
4.3.3 Delayed recovery from S-phase arrest in pemetrexed-treated UNG<sup>−/−</sup> cells. Pemetrexed-treated UNG<sup>−/−</sup> cells accumulate 40-fold more genomic uracil compared to UNG<sup>+/+</sup> cells.(58) Additionally there are data to suggest that heavily uracilated DNA alters DNA-protein interactions due to perturbations in basal genomic methylation, which may negatively impact replication and transcription.(142, 143) In ChIP seq experiments, we observed that DNA DSBs mapped more frequently to sites corresponding to putative human origins of replication (Table 4-1). Putative origins of replication in human cancer cells were identified as common sites pooled from published nascent DNA sequencing of K562 and MCF7 cells.(246, 247) We therefore surmised a mechanistic link between UNG loss, DNA double strand break formation and DNA replication instability.

Both UNG<sup>+/+</sup> and UNG<sup>−/−</sup> cells accumulate in S-phase in response to 24-hr exposure to IC<sub>50</sub>-level pemetrexed (Figure 4-5a). S-phase arrest was further suggested by phosphorylation of chk1(ser345) and cdc2( tyr15) (Figure 4-5b). Interestingly, however, UNG<sup>−/−</sup> cells show delayed recovery from S-phase arrest when pemetrexed is withdrawn (Figure 4-5a). Co-treatment with supplemental thymidine blocks S-phase accumulation in pemetrexed-treated UNG<sup>+/+</sup> and UNG<sup>−/−</sup> cells (Figure 4-5c). Culturing cells in medium containing supplemental thymidine after pemetrexed exposure accelerates recovery from S-phase arrest in UNG<sup>+/+</sup> cells but not UNG<sup>−/−</sup> cells (Figure 4-5d). Expression of replication
Figure 4-5 Delayed recovery from cell cycle arrest in pemetrexed-treated UNG<sup>−/−</sup> cells. (a) Cell cycle progression was monitored using propidium iodide staining and flow cytometry. UNG<sup>+/+</sup> and UNG<sup>−/−</sup> cells treated with IC<sub>50</sub> level pemetrexed (UNG<sup>+/+</ sup>, 200nM; UNG<sup>−/−</sup> 25nM) both show evidence of S-phase accumulation after 25 hours of exposure. When allowed to recover in drug free media, UNG<sup>+/+</sup> cells are capable of resuming cell cycle (return of G1 peak in +48 hrs sample) while UNG<sup>−/−</sup> cells are not. (b) Western blot from UNG<sup>+/+</sup> and UNG<sup>−/−</sup> cells treated for 0-72 hours with 25nM pemetrexed showing increased sensitivity in UNG<sup>−/−</sup> cells to pemetrexed induced phosphorylation of chk1 and cdc2, S-phase checkpoint kinases. (c) Cell cycle histograms for cells treated with supplemental thymidine. Cells were treated with 10µM thymidine for 24 hours (No Pem, +Thy), co-treated with IC<sub>50</sub> level pemetrexed and thymidine for 24 hours (24 hrs, + Thy) or treated with IC<sub>50</sub> level pemetrexed for 24 hours and allowed to recover for 48 hours in media containing 10µM thymidine (48 hrs post, +Thy).
checkpoint proteins and S-phase accumulation suggests replication instability contributes to DNA damage and cell death in pemetrexed treated cells. The inability of thymidine to facilitate restoration of cell cycle progression in UNG−/− cells after pemetrexed is withdrawn suggests that DNA damage caused by misincorporated dUTP is irreversible in the context of deficient uracil removal.

Oligonucleotide primer extension assays have been previously utilized to study the impact of uracil base substitutions on replication fork progression and stability. Eukaryotic and bacterial DNA polymerases can efficiently extend oligonucleotide primers containing a single U:A base pair. Because of these data, it has been generally accepted that uracil base substitutions are innocuous when they occur opposite adenine. Data using single uracil substitutions however, may not accurately reflect the genomic uracil burden of the pemetrexed-treated UNG−/− cell, which accumulates significant quantities of uracil-DNA. To more directly assess the impact of heavily uracilated DNA on polymerase extension efficiency and kinetics, we utilized oligonucleotide substrates containing up to 5 consecutive uracil mispairs (Figure 4-6a). For these experiments we used the phage-derived T7 polymerase, which has similar activity as human replicative polymerases and is commercially available. We observed attenuated polymerase primer extension kinetics using oligonucleotides containing 3 and 5 uracil substitutions (Figure 6b). Consistent
with published reports, polymerase kinetics were not significantly affected by a single uracil substitution.

**Figure 4-6** Uracil base substitutions attenuate polymerase extension efficiency *in vitro* and correspond to intracellular pemetrexed-induced replication instability. (a) Oligonucleotide primers containing 1, 3 and 5 uracil bases were used in primer extension assays to elongate a A488-labeled oligonucleotide primer. (b) Fluorescence intensity was used to determine the total amount of elongation product generated when the annealed oligonucleotide duplexes in (a) were incubated with T7 polymerase for variable time points. (c) Schematic for CldU and IdU pulse labeling of pemetrexed treated cells. Briefly, cells were incubated for 45 minutes with CldU (50μM) prior to treatment IC_{50} level pemetrexed (UNG^{+/+}, 200nM, UNG^{-/-} 25nM) for 24 hours. Cells were then allowed to recover in drug free media for 0-48 hours prior to incubation for 45 minutes with IdU (50μM). Cells were then stained with fluorescent antibodies that recognize CldU (rat-antiBrdU-FITC) and IdU (mouse-antiBrdU-APC). Representative data from flow cytometry sorting of CldU/IdU labeled cells is shown in (d) where the x-axis is CldU (FITC) and the y-axis is IdU (APC). (e) Cells were treated with IC_{50} level pemetrexed for 24 and 48 hours and subsequently stained with propidium iodide (PI, x-axis) to and FITC-labeled PCNA antibody (y-axis). (f) Cells were treated as in (e) and subsequently incubated in 1% formaldehyde for crosslinking and chromatin extraction for western blots of chromatin-bound PCNA and Histone H3 (loading control).
Intracellular replication fork progression is typically studied using CldU, IdU and BrdU in nucleotide incorporation assays. Using this approach in a flow cytometry-based pulse chase assay, we observed a decrease in post-pemetrexed treatment IdU incorporation in UNG<sup>−/−</sup> cells (Figure 4-6d) While this is consistent with decreased replication fork progression in pemetrexed-treated UNG-/- cells, these data alone could simply indicate that misincorporated uracil is an impediment to IdU incorporation due to competition for DNA polymerase and incorporation sites. Therefore, as a complimentary experiment, we assessed dispersal of the replication processivity factor, PCNA from chromatin using both flow cytometry (Figure 4-6e) and western blot (Figure 4-6f). Similar experiments have been used to analyze collapsing replication forks in etoposide and hydroxyurea-treated cells.(221, 222) In these assays, decreased PCNA staining of S-phase gated cells or chromatin is indicative of replication fork instability or fork collapse. Our data show increased PCNA dispersal in pemetrexed-treated UNG<sup>−/−</sup> cells compared to UNG<sup>+/+</sup> cells suggesting that pemetrexed treatment causes profound replication fork instability in UNG deficient cells (Figure 6e-f).

4.4 Discussion

We have previously shown that loss of UNG expression sensitizes human cancer cells to pemetrexed and that UNG expression predicts pemetrexed sensitivity in experimental models of human cancer.(58, 59) Here, we have analyzed the DNA
damage response to pemetrexed in UNG$^{+/+}$ and UNG$^{-/-}$ DLD1 human colon cancer cells to better understand the role of UNG in the mechanism of pemetrexed-induced DNA double strand break formation and cell death. We observed that loss of UNG hyper-sensitized DLD1 cells to pemetrexed in vitro and in vivo despite equivalent proliferation rates. This hypersensitivity is associated with increased replication fork instability and DNA double strand break formation, despite an equivalent capacity for DNA DSB repair in the two cells at baseline.

The formation of DNA DSBs in cells treated with TS inhibitors has been studied extensively for decades,(4, 115, 249-253) yet the precise role of uracil misincorporation and UNG excision of uracil in the mechanism of DNA DSB formation and cell death has not been sufficiently understood. The futile cycle hypothesis proposes that DSBs arise as a result of continuous cycles of uracil excision, followed by BER and uracil re-insertion that lead to chromosome fragmentation and double strand breaks. Experimental evidence from other labs,(84, 250, 254) and data presented herein, however suggests that futile cycles do not adequately explain thymine-less death. Overexpression of UNG, which should exacerbate futile cycles of UNG, does not enhance TS-inhibitor sensitivity.(84) Additionally, our data in human colon cancer and human lung cancer cells would suggest that uracil itself is intrinsically cytotoxic.(58, 59) UNG loss does not cause compensatory up-regulation of the other DNA glycosylases.
capable of uracil excision, so it is unlikely that futile cycles of BER are initiated to a significant degree in UNG−/− cells treated with pemetrexed. The relatively fewer DNA DSBs observed in UNG+/+ cells compared to UNG−/− cells treated with equally toxic concentrations of pemetrexed would suggest that, at least in the models examined, UNG activity limits rather than promotes pemetrexed-mediated DNA DSB formation and cell death.

Based on our data presented here and in recent publications, we bring forth a novel hypothesis for the mechanism of thymine-less death in UNG deficient cancer cells. In our model, uracil accumulates at critical levels near replication origins, stalling DNA replication fork progression and leading to fork collapse, DNA double strand break formation and cell death (Figure 1-7).

Several levels of experimental evidence support this model. First, we have observed substantial accumulation of genomic uracil, coordinated S-phase arrest, replication fork instability and DNA double strand break formation in UNG−/− cells. These data suggest that the accumulation of uracil is intrinsically cytotoxic. The intrinsic cytotoxicity of uracil may lie in the altered methylation status and secondary structure of DNA when heavily uracilated. Consistent with this view, primer extension assay results indicated attenuated polymerase kinetics when uracil is substituted for thymidine.
An alternative explanation for uracil-mediated DSB formation in UNG<sup>−/−</sup> cells is endonuclease cleavage at or near uracil-containing DNA or sites of stalled DNA replication. (144, 146) However, the activities of uracil-specific endonucleases have not been assessed in the context of pemetrexed treatment.

Secondly, we have noted increased sensitivity to pemetrexed-induced S-phase arrest, intra-S phase checkpoint activation and replication fork instability in UNG<sup>−/−</sup> cells compared to UNG<sup>+/+</sup> cells. S-phase arrest persists in UNG<sup>−/−</sup> cells, while cell cycle progression is restored when pemetrexed is withdrawn from UNG<sup>+/+</sup> cells. Co-treatment with supplemental thymidine limits pemetrexed-induced S-phase arrest in both UNG<sup>+/+</sup> and UNG<sup>−/−</sup> cells. However, supplementation of culture medium with thymidine has limited impact on cell cycle recovery from pemetrexed exposure in UNG<sup>−/−</sup> cells. Additionally, PCNA dissociates from chromatin in pemetrexed-treated UNG<sup>−/−</sup> cells, indicative of replication fork instability. Replication fork reversal and fork restart have been observed during dNTP depletion by hydroxyurea or thermal inactivation of ribonucleoside reductase, but not by thymine starvation (akin to TS inhibition). (255) Lack of replication fork reversal during thymine deprivation may explain the failure to recover from cell cycle arrest in UNG<sup>−/−</sup> cells and is consistent with our proposed model of uracil-mediated replication fork collapse in pemetrexed-treated UNG<sup>−/−</sup> cells.
Our data show that pemetrexed-exposed UNG\textsuperscript{+/+} cells still undergo S-phase arrest and cell death, albeit at significantly higher pemetrexed concentrations than UNG\textsuperscript{-/-} cells. Unlike UNG\textsuperscript{-/-} cells, UNG\textsuperscript{+/+} cells have low levels of genomic uracil during pemetrexed exposure\textsuperscript{(58)} suggesting that uracil is being efficiently and rapidly removed from DNA. While genomic uracil is not accumulating in UNG competent cells, dTTP levels are still aberrantly diminished during pemetrexed inhibition of TS. Low dTTP during TS inhibition is believed to alter the normal allosteric regulation of ribonucleotide reductase resulting in elevated dATP and decreased dGTP pools (Figure 1-6)\textsuperscript{(125)}. Hence, in UNG\textsuperscript{+/+} cells, S-phase arrest and cell death in response to pemetrexed may be a consequence of global nucleotide pool imbalance. Meanwhile, the absence of replication fork instability in UNG\textsuperscript{+/+} cells suggests that the uracil excision may prevent the development of catastrophically stalled DNA replication that collapse to form DNA double strand breaks.

Lastly, distinct patterns of genomic enrichment for γ-H2AX (DSBs) identified in this study suggest disparate mechanisms of DSB formation in UNG competent and deficient cells. Of interest, our analysis indicates that 36.7% of UNG\textsuperscript{-/-} and 12.67% of UNG\textsuperscript{+/+} γ-H2AX enriched sites overlap with putative human origins of replication\textsuperscript{(246, 247)} suggesting that UNG excision of uracil protects origins of replication from pemetrexed-induced DSB formation. It has been suggested that continued replication in thymine starved cells triggers destabilization of origins of
replication resulting in DSB at sites of origin firing. (250, 254) The fact that UNG deficient cells appear to be more sensitive to this mode of DSB formation is consistent with prior observations of coordination of UNG-initiated BER and identification of UNG complexed at replication foci. (75, 77, 230, 239)

We have utilized γ-H2AX enrichment in ChIP seq experiments to discern DSB locations in this study. Like UNG, H2AX is over-expressed in many cancers (Figure 4-7). Additionally, H2AX and UNG tend to be co-expressed pemetrexed-resistant human cancer types (Figure 4-8). (216, 218, 256) In actively dividing cells, deposition of endogenous un-phosphorylated H2AX occurs at certain genetic hotspots, such as transcription factor binding sites, and coincides with sites of endogenous DNA damage. (257) We did not observe an increase in basal γ-H2AX or comet tails in UNG−/− cells and so γ-H2AX enrichment around transcription factor binding sites in ChIP-Seq experiments is likely pemetrexed-induced. Still, some caution should be utilized with the interpretation of these data, since in some cases γ-H2AX may not always provide the precise location of DNA DSBs. Further assessment to confirm that the patterns of γ-H2AX enrichment observed in this study coincide with specific loci of DSBs are needed. Despite this caveat, the finding of significantly fewer γ-H2AX –enriched sequences in UNG+/− cells suggests that UNG protects from DSB formation in pemetrexed treated cells. Moreover, less than 1% overlap between UNG+/− and
UNG⁻/⁻ γ-H2AX enriched sites suggests different mechanisms direct DSB formation in the two cell lines.

Combined, the observation of fewer DSBs, reduced predilection of DSBs for origins of replication, lack of observed replication fork instability, and S-phase arrest that is adequately rescued with supplemental thymidine in UNG⁺/+ cells suggests pemetrexed-induced toxicity is more related to dTTP depletion than to dUTP misincorporation. In contrast, UNG⁻/⁻ cells, which accumulate genomic uracil,(58) have increased DSBs that localize to replication origins, show a significant degree of replication instability and have irreversible S-phase arrest, pemetrexed toxicity is more related to dU TP misincorporation (Table 4-2).

Together these findings highlight a critical role for genomic uracil accumulation and UNG expression and activity in mediating cellular response to pemetrexed. Aside from our data with pemetrexed, studies in UNG⁻/⁻ human cells and mouse embryonic fibroblasts have not illustrated significant sensitivity to TS-inhibition induced cell death. TS-inhibitor induced growth arrest has been observed without corresponding apoptosis. Additionally, one study has reported diminished DNA replication rates in UNG deficient cells even in the absence of drug treatment.

We speculate that these inconsistent findings may be explained by differences in the quantities of accumulating genomic uracil in the cell line/ TS-inhibitor models investigated. Indeed the sensitization index for UNG⁻/⁻ cells to raltitrexed was far
less than the sensitization index for UNG\(^{-}\) cell to pemetrexed suggesting that UNG expression and activity plays a greater role in pemetrexed vs. raltitrexed response. To address this, we propose future quantitative studies that compare genomic uracil and nucleotide pool levels in UNG\(^{-}\) and UNG\(^{+/+}\) cells treated with equally toxic concentrations of various TS-inhibitors.
Figure 4-7. **UNG and H2AX are over-expressed in many human cancers.** Published microarray data retrieved from Oncomine (Oncomine™, Compendia Biosciences). Analysis of gene expression for UNG and H2AX are shown. Red boxes indicate studies with significantly higher expression in cancer cells compared to normal cells for a given cancer type while blue boxes indicate significantly lower expression in cancer vs. normal. The numbers within the boxes indicates the number of independent studies with the observed trend. The threshold fold change is 2 (all data are log2 median centered) and p value significance was set at $1 \times 10^{-4}$. 

<table>
<thead>
<tr>
<th>Analysis Type by Cancer</th>
<th>UNG</th>
<th>H2AX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bladder Cancer</strong></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>Brain and CNS Cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Breast Cancer</strong></td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td><strong>Cervical Cancer</strong></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Colorectal Cancer</strong></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><strong>Esophageal Cancer</strong></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Gastric Cancer</strong></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Head and Neck Cancer</strong></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Kidney Cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Leukemia</strong></td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td><strong>Liver Cancer</strong></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Lung Cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lymphoma</strong></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Melanoma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Myeloma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other Cancer</strong></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><strong>Ovarian Cancer</strong></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Pancreatic Cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prostate Cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sarcoma</strong></td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Significant Unique Analyses** | 19  | 39   |
**Total Unique Analyses**       | 439 | 441  |
Figure 4-8 UNG and H2AX are co-expressed in human lung cancers. Published microarray data (216, 218, 256) retrieved from Oncomine database indicates that UNG and H2AX are co-expressed in human lung cancer subtypes. Specifically, both genes have increased expression in malignant vs. non-malignant lung tissue (compare 0 to 1-5) and both genes have significantly elevated expression in pemetrexed-resistant subtypes of lung cancer: small cell (group 3) and squamous cell (group 4) carcinomas.
### Table 4-2. Possible pathways to DSB formation and cell death in pemetrexed treated cells

<table>
<thead>
<tr>
<th>Possible route to cell death</th>
<th>Problem with hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stalled replication due to nucleotide pool aberrations caused by reduced dTTP</td>
<td>Pemetrexed-induced nucleotide aberrations have not been investigated.</td>
</tr>
<tr>
<td>Endonuclease cleavage of uracil and/or AP sites*</td>
<td>Products of APE incision are rapidly targeted by activities of Polβ and downstream BER.</td>
</tr>
<tr>
<td>Futile cycles of BER</td>
<td>UNG&lt;sup&gt;+&lt;/sup&gt;/ cells are more resistant than UNG&lt;sup&gt;−&lt;/sup&gt; cells suggesting excision of uracil protects from pemetrexed cytotoxicity.</td>
</tr>
<tr>
<td>Uracil-mediated replication fork stall and collapse</td>
<td>Attenuated replication kinetics have only been observed using non-human polymerases.</td>
</tr>
<tr>
<td></td>
<td>The currently is no available method to study impact of various degrees of uracil base substitution within living cells.</td>
</tr>
<tr>
<td>Uracil-mediated endonuclease cleavage*</td>
<td></td>
</tr>
</tbody>
</table>

*Endonucleases with uracil activity have not been studied in antifolate-mediated cell death.*
Chapter 5

Enhanced hematopoietic sensitivity in UNG−/− mice during antifolate response

We have previously shown that pemetrexed cell killing is enhanced through gene-targeted deletion of uracil DNA glycosylase (UNG). This observation spurred an interest in understanding the systemic effects of UNG loss in pemetrexed-treated animals. Uracil DNA glycosylase null (UNG−/−) mice are viable and young mice develop normally. The effect of DNA damaging chemotherapy on UNG−/− mice has not been tested. The predominant phenotype of UNG−/− mice is an increased propensity for B cell hyperplasia and B cell lymphoma development at 18-24 months of age. The redundancy of proteins with uracil DNA glycosylase excision activity may account for the lack of adverse effect of UNG loss on untreated young animals. In this work, we tested the thesis that young UNG−/− mice would show increased systemic toxicity when treated with pemetrexed compared to wild-type littermates. We report that UNG−/− derived murine cells are sensitive to pemetrexed-induced DNA damage including DNA double strand breaks in vitro. A mild and transient depletion of bone marrow cells in pemetrexed-treated animals corresponds to induction of increased genomic uracil and DSBs. Additionally, human lymphoblastoid cell lines (LCLs) derived
from patients with germline inactivating mutations in UNG were also more sensitive to pemetrexed-induced growth arrest and DSB formation compared to UNG wild type LCLs. Importantly, these data show the potential for increased pemetrexed sensitivity in non-malignant UNG deficient cells. Systemic down regulation or inactivation of UNG expression or activity may exacerbate dose-limiting hematologic toxicity in pemetrexed chemotherapy regimens.
5.1 Introduction

Spontaneous deamination of cytosine produces U:G mispairs at a rate of 100-500 per cell per day (73) and DNA polymerases can errantly incorporate uracil opposite adenine during DNA replication. Uracil in DNA is removed by the activity of uracil DNA glycosylase (UNG). Nuclear and mitochondrial isoforms of UNG are generated via alternative splicing of the first exon of the UNG gene on chromosome 12. (165) Nuclear UNG is the most abundant of the various enzymes with UNG excision activity and is the major glycosylase responsible for uracil that is misincorporated during DNA replication. (77)

We have recently shown that UNG activity mitigates sensitivity to pemetrexed by actively and efficiently removing genotoxic uracil lesions from DNA (Chapters 3 & 4). (58, 59) Based on these recent findings, we have initiated studies of UNG as a novel predictive biomarker (see chapter 8) and potential therapeutic target (see Chapter 6) for pemetrexed chemotherapy in human cancer. To better understand the systemic impact of UNG loss, we evaluated sensitivity to pemetrexed in mice with gene-targeted deletion in UNG.

UNG−/− mice were first described by Nilsen et al. (77) Despite the clear role for UNG in removing uracil from DNA, UNG−/− mice are viable, fertile and develop normally. (77) The predominant phenotype of UNG−/− mice is B cell hyperplasia
with corresponding gross enlargement of spleen and lymph nodes, and a 22-fold increased incidence of B cell lymphoma. (132) It is thought that redundancy of glycosylases with uracil excision capability (Table 1-2) may account for the lack of a more overt phenotype in young UNG<sup>-/-</sup> mice. (77) Indeed, combined gene-targeted deletion of SMUG1 and UNG in mice enhances mutagenesis and increases cancer predisposition. (258)

The development of B cell lymphoma in UNG<sup>-/-</sup> mice is presumably related to a role for UNG in antibody diversification. (131, 259) Adaptive immunity requires somatic immunoglobulin gene diversification within antigen stimulated B cells. Activating induced deaminase (AID) catalyzes deamination of cytosine to uracil in single stranded DNA in immunoglobulin variable (V) and switch (S) regions facilitating class switch recombination and somatic hyper mutation. Following deamination of cytosines, UNG is required for generating single strand breaks and therein facilitating mutations. (131, 260, 261) The second most abundant glycosylase, SMUG1, is down regulated in activated B cells making UNG indispensable as the glycosylase for antibody diversification activities. (262) Individuals with inactivating mutations in UNG show profound immunodeficiency owing to this impairment in adaptive immunity. (260)

To our knowledge, systemic toxicity of DNA damaging agents has never been evaluated in UNG<sup>-/-</sup> mice or the available UNG mutant human lymphoblastoid cell
lines. In this report, we describe increased in vitro and in vivo sensitivity of UNG⁻/⁻ murine cells to pemetrexed. Pemetrexed-treatment produces mild and transient marrow suppression in UNG⁻/⁻ mice, and not UNG⁺/+ mice. Similarly, UNG mutant human lymphoblastoid cells are more sensitive to pemetrexed compared to cells with normal UNG activity. Lymphoblastoid cell lines (LCLs) derived from patients with germline inactivating mutations in UNG accumulate uracil and show increased DNA damage compared to control cells,(263) suggesting the mechanism of cell death may be similar to what occurs in UNG⁻/⁻ malignant cells.(58, 59) Together these data illustrate the impact of UNG loss or inactivation on non-malignant cells and suggests the potential for UNG inhibition to exacerbate dose-limiting hematologic toxicity.

5.2 Materials and Methods

5.2.1 Cells and Reagents: Pemetrexed was obtained from LCC laboratories and working solutions were generated fresh from powder prior to experimental use. UNG mutant lymphoblastoid cell lines (LCLs)(263) were a generous gift from Ann Durandy (Hôpital Necker Enfants Malades, Service d'Immunologie et d'Hématologie Pédiatrique, Paris, F-75015, France). They were maintained in cell suspension in RPMI-1640 medium containing 10% fetal bovine serum, and 2mM L-glutamine at 37°C in 5% CO₂. Lymphoblastoid cells with wild type UNG activity were obtained from the Stem Cell Core facility of Case Western Reserve
University, Case Comprehensive Cancer Center. UNG^{+/+}, UNG^{+-}, and UNG^{-/-} MEF cells were derived from E14 embryos and maintained in sub-confluent cultures in complete DMEM containing 10% fetal bovine serum, and 2mM L-glutamine at 37°C in 5% CO₂. All MEF cell drug treatments and experiments were done at either passage 3-5.

5.2.2 MTT assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed using standard protocols. Briefly, cells were seeded in 24 well plates, allowed to adhere overnight, and treated with the indicated pemetrexed concentrations for 5 days. On day 5, media was replaced with serum-free control medium containing HEPES buffer and MTT, 1 mg/ml and incubated for 2 h at 37°C in 5%CO₂. Blue formazan metabolite was extracted from cells using DMSO and the optical density was read at 540nm with a spectrophotometer. For all experiments shown, data points are an average of 6 independent measurements.

5.2.3 Viability assay (Trypan blue exclusion). For LCLs and bone marrow cells, 5x10^5 cells were seeded in 25cm² flasks. DLD1 UNG^{+/+} and UNG^{-/-} cells were seeded in 24- well plates at a density of 1x10^4 per well. Duplicate samples of viable and dead cells were counted using a trypan blue dye and a hemacytometer for each individual treatment. Each experiment was performed in triplicate.
5.2.4 **Western blot analysis.** Whole cell extract (25 µg) was resolved by SDS-PAGE and immobilized on a PVDF membrane. Blocking in 5% milk/PBST was followed by overnight primary antibody incubation according to manufacturer instruction. Antibodies for cleaved PARP (cell signaling), cleaved caspase 3 (Abcam), p-chk1, chk1, p-cdc2, cdc2 (Santa Cruz), γ-H2AX (Millipore), and tubulin (Calbiochem) were used in this study. Incubation with appropriate secondary antibody (Cell Signaling) for 1 hour allowed for visualization of proteins with ECL reagent (Amersham Corp, Piscataway, NJ).

5.2.5 **Neutral Comet Assay.** Following drug treatment, cells were processed for comet tail formation using neutral comet assay conditions according to the manufacturers instructions (Trevigen). Comets were analyzed using ImageJ software. The lengths and intensities of the comet head and tail were recorded for at least 25 comets on two separate slides (total cell number ~50) per treatment and the average tail length was determined using Image J software.

5.2.6 **Glycosylase Activity Assay.** UNG activity (purified protein or whole cell extracts) was measured using a 40-mer oligodeoxynucleotide duplex containing a single uracil (Operon, Biotechnologies, Huntsville Al).

5’[HEX]  GTAAAACGACGGCCAGTGUATTCGAGCTCGGTACC CGGGG
3’  CATT TTGCTGCCGTCACAGAAGCTCGAGCCATGGGGCC [Cy5]
The fluorescent-labeled duplex oligonucleotides were incubated with 1 unit each of purified UNG (New England Biolabs, Iswich Ma) and APE (Trevigen, Gaithersburg, MD) or whole cell extract (0.5μg total protein) at 37°C for 15 min. The reaction was terminated with incubation at 95°C for 5 min. Reaction products were resolved by electrophoresis and visualized using a Typhoon 9200 fluorescent imager. UNG activity was calculated as a ratio of the cut to uncut band relative to UNG+APE purified enzyme control.

5.2.7 Abasic (AP) site detection. DNA extracts from lymphoblastoid cells were labeled with biotinylated aldehyde reactive probe (ARP) for chemiluminescent AP site detection as described in ref 59. Incubation of cells with UNG was accomplished by adding 1U of purified UNG enzyme (New England Biolabs) to 100μg of DNA prior to ARP labeling.

5.2.8 UNG knockout mice. A single breeding unit of UNG heterozygous mice were received from David Schatz (Yale University, New Haven, CT) with permission of Thomas Lindahl (London Research Institute, London, UK). Genotyping of live born mice confirmed recovery of UNG+/− and UNG−/− mice in the expected Mendelian ratios. Pemetrexed treatment. 6 week-old mice were subjected to 3 consecutive days of intraperitoneal (IP) bolus injections of 150-600mg/kg pemetrexed suspended in sterile PBS. Injection volumes of <200μl were used for each animal. Hemavet measurements of blood counts. Retro-
orbital collection of peripheral blood was performed on day 1, day 3, day 5 and day 10 of the drug treatment cycle. Whole blood was immediately analyzed using a Hemavet 950 machine (Drew Scientific, Dallas Texas). Colony forming unit assay. Bone marrow cells obtained from the hind limbs of treated mice were re-suspended in heparinized minimum essential eagles medium, counted using a hemacytometer and plated at 30,000 cells per ml in cytokine replete mouse methylcellulose (Stem Cell Technologies) and incubated at 37°C in 5%CO₂. Hematopoietic Colony Forming Units (CFU) were counted after 10 days. In vitro bone marrow cytotoxicity. For in vitro bone marrow analysis, cells were harvested from the hind limbs of untreated mice and 1x10⁶ cells were plated in 5 ml of RPMI-1640 media + 10% fetal bovine serum + 2mM L-glutamine and pemetrexed at the given concentrations.

5.2.9 Detection of Uracil Using HPLC/MS/MS Analysis. UNG⁻/⁻ and UNG⁻/+ bone marrow cells were harvested from pemetrexed treated mice as described above. Genomic DNA was extracted by phenol chloroform. 100μg of DNA was incubated with 10U of purified UNG enzyme (New England Biolabs) in 100ul of total reaction buffer at 37°C for 2 hours. The reaction products were dried at 35°C in a Turbovap under a stream of nitrogen and reconstituted in 150 μL 90% acetonitrile. Analyte was retained by an Atlantis HILIC Silica analytical column (2.1x100 mm, 3.5 μM) and eluted isocratically by a mixture of 90% acetonitrile and 10% 2.0 mM ammonium formate at a flow rate of 0.2 ml/min. The detection
was done by an API 3200 MS/MS mass spectrometer. Multiple-reaction-monitoring (MRM) data were acquired with the following parameters: m/z 111.0, 42.1 for uracil and m/z 113.3, 42.9 for uracil-1,3-15N2 (internal standard).

5.3 Results

5.3.1 Increased \textit{in vitro} sensitivity of UNG\textsuperscript{-/-} MEFs and primary bone marrow cells to pemetrexed. To characterize the role of UNG in pemetrexed response in non-malignant cells, we treated UNG\textsuperscript{-/-} primary mouse embryonic fibroblasts (MEFs) with pemetrexed for growth inhibition analysis by MTT assay (Figure 5-1a). UNG\textsuperscript{-/-} MEFs were 5.59-fold more sensitive to pemetrexed than UNG\textsuperscript{+/-} MEFs in 4 independent experiments (UNG\textsuperscript{+/-} IC50 = 115.9; UNG\textsuperscript{-/-} IC50 = 20.74 p <0.0001). Western blot analysis of whole cell extracts from MEF cells treated with 25nM pemetrexed evinced marked induction of the DNA double strand break (DSB) surrogate marker γ-H2AX and G1/S checkpoint activation (induction of p-chk1 and p-cdc2) in UNG\textsuperscript{-/-} cells that was absent in UNG\textsuperscript{+/-} cells (Figure 5-1b). Single cell gel electrophoresis (comet) assay confirmed significantly enhanced DNA DSB formation in UNG\textsuperscript{-/-} cells compared to controls (Figure 5-1c).

Similar to MEF cells, primary bone marrow cells also showed increased sensitivity to pemetrexed. Viability of primary bone marrow cells treated with pemetrexed was assessed by trypan blue exclusion and counting of cells.
incubated with variable concentrations of pemetrexed for 48 hours (Figure 5-1d). Results indicated a 4.43-fold decrease in viability in UNG−/− bone marrow cells compared to UNG+/+ bone marrow cells, p = 0.017. Western blots of cleaved PARP and cleaved caspase 3 confirmed the initiation of apoptosis to a greater extent in UNG−/− bone marrow cells compared to UNG+/+ cells (Figure 5-1e). Like UNG−/− MEFs, UNG−/− bone marrow cells accumulated DNA double strand breaks corresponding to longer comet tail lengths when exposed to pemetrexed (Figure 5-1f).
Figure 5-1 Increased in vitro sensitivity of UNG⁺ MEFs and primary bone marrow cells to pemetrexed. (a) Growth was evaluated by MTT assay in mouse embryonic fibroblasts (MEF) cells from UNG⁺/⁺ and UNG⁻/⁻ cells treated for 5 days with 0-200nM pemetrexed. (b) Western blot for DNA double strand break surrogate marker, γ-H2AX ; and cell cycle checkpoint kinases p-chk1 and p-cdc2 in pemetrexed treated MEFs. (c) Neutral comet assay was performed on pemetrexed-treated MEFs. Representative images are shown (left panel). Comet tail length was calculated using NIH Image J software for ~50 cells per drug treatment and graphed in right panel. UNG⁻/⁻ MEFs show increased sensitivity to comet tail formation (DNA double strand breaks) during pemetrexed exposure compared to UNG⁺/⁺ MEFs. (d) Viability assay in primary bone marrow cells treated with 0-200nM pemetrexed for 48 hours. Viability was determined using trypan blue staining and cell counting with the aid of a hemacytometer. (e) Western blots were performed in primary bone marrow cells treated with 50nM pemetrexed in vitro for 0-48 hours. (f) Representative images from neutral comet assay of primary bone marrow cells treated with pemetrexed (left panel) and mean comet tail length from ~50 cells (right panel).
5.3.2 Increased *in vivo* sensitivity of UNG$^{-/-}$ mice to pemetrexed. To evaluate the systemic effects of UNG loss on pemetrexed sensitivity, we treated 6-week old UNG$^{+/+}$ and UNG$^{-/-}$ littermates with pemetrexed. Treatment was carried out according to the schematic in Figure 5-2a. Mice were subjected to 3 consecutive daily intraperitoneal (IP) injections of 150-600mg/kg pemetrexed. Because the anticipated toxicity of pemetrexed is hematologic,(264) peripheral blood counts were taken on days 1, 3, 5 and 10 of the pemetrexed treatment cycle (Figure 5-2a). Day 1 represents baseline peripheral blood counts, day 3 represents peripheral blood count during treatment, and days 5 and 10 represent acute and long-term recovery from pemetrexed exposure. In general, this treatment regimen was well tolerated by all of the animals in this study and only minor changes in body weight and peripheral blood count were observed (Table 5-1). The most profound changes in peripheral blood count were observed in UNG$^{-/-}$ animals treated with 600mg/kg pemetrexed. These animals had a 40% reduction in circulating total white blood cells on day 3 of treatment that recovered by day 5 (Figure 5-2b). This corresponded to a 60% reduction in circulating lymphocytes that was recovered at day 10 (Figure 5-2c). Histological examination revealed a paucity of lymphocytes in the spleens of UNG$^{-/-}$ but not UNG$^{+/+}$ mice (Figure 5-2d).
<table>
<thead>
<tr>
<th></th>
<th>Average pre-treatment body mass (grams)</th>
<th>Average post-treatment body mass (grams)</th>
<th>Spleen Mass (grams)</th>
<th>Average Blood Cell Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>WBC (K/μL)</td>
</tr>
<tr>
<td><strong>UNG</strong>&lt;sup&gt;1,4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=10)</td>
<td>23.21</td>
<td>N/A</td>
<td>0.080</td>
<td>9.70</td>
</tr>
<tr>
<td>Pemetrexed 150 mg/kg</td>
<td>22.10</td>
<td>21.87</td>
<td>0.085</td>
<td>9.29</td>
</tr>
<tr>
<td>Pemetrexed 300 mg/kg</td>
<td>23.18</td>
<td>23.05</td>
<td>0.1</td>
<td>9.15</td>
</tr>
<tr>
<td>Pemetrexed 600 mg/kg</td>
<td>23.27</td>
<td>24.20</td>
<td>0.091</td>
<td>7.23</td>
</tr>
<tr>
<td><strong>UNG</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=10)</td>
<td>23.10</td>
<td>N/A</td>
<td>0.087</td>
<td>9.18</td>
</tr>
<tr>
<td>Pemetrexed 150 mg/kg</td>
<td>22.19</td>
<td>21.33</td>
<td>0.080</td>
<td>6.29*</td>
</tr>
<tr>
<td>Pemetrexed 300 mg/kg</td>
<td>22.17</td>
<td>21.83</td>
<td>0.075*</td>
<td>5.2*</td>
</tr>
<tr>
<td>Pemetrexed 600 mg/kg</td>
<td>22.43</td>
<td>21.07</td>
<td>0.066*</td>
<td>5.61*</td>
</tr>
</tbody>
</table>

WBC - white blood cells; LY - lymphocytes; RBC - red blood cells; PLT - platelets
Mice received IP pemetrexed on Days 1, 2 and 3 and were sacrificed for bone marrow counts and CFU assay on day 4. Numbers given are average values from 10 mice. * indicates p<0.05 compared to UNG<sup>1,4</sup> of the same treatment group

Normal Ranges: WBC – 1.8-10.7 K/μL  LY – 0-9.3 K/μL  RBC-6.36-9.42K/μL  PLT-352-2972K/μL
Figure 5-2 Increased transient hematologic toxicity in pemetrexed treated UNG⁺ mice. (a) Mice were treated with pemetrexed for 3 consecutive days (days 1-3) as indicated in the schematic diagram. Blood was obtained from mice on days 1 and 3 immediately prior to pemetrexed dosing and on days 5 and 10. Blood counts were determined using a Hemavet 950 (Drew Scientific, Dallas, TX) and data for white blood cell count (b) and lymphocyte count (c) are shown. (d) Histopathology examination indicated a paucity lymphocytes in pemetrexed treated UNG⁻ mice.
We next investigated whether pemetrexed treatment was differentially impacting hematopoiesis through determinations of total bone marrow cell number and methylcellulose colony forming unit (CFU) assays from bone marrow cells harvested on day 4 of the treatment schedule outlined in Figure 5-2a.

Significantly fewer bone marrow cells were counted in UNG\(^{-/-}\) mice following pemetrexed treatment. When plated at equal density (3x10\(^5\) cells/ml) in cytokine supplemented methylcellulose, UNG\(^{-/-}\) cells grew significantly fewer CFU than UNG\(^{+/+}\) bone marrow cells. These data (summarized in Table 5-2) suggest that UNG loss particularly sensitizes mice to pemetrexed-mediated marrow suppression. When allowed to recover from pemetrexed treatment for 5 days, bone marrow cell numbers and CFU potential rebounded in the mice (Table 5-2).

Based on previous data regarding the mechanism of action of pemetrexed in UNG\(^{-/-}\) cells,(58, 59) we anticipated that lymphocyte depletion and marrow suppression observed were a direct result of genotoxic uracil accumulation in these cells during pemetrexed exposure. To confirm this, we measured the amount of uracil present in 100μg of DNA extracted from splenocytes or bone marrow cells in pemetrexed-treated animals (600mg/kg) using LC/MS. Significant uracil accumulation was observed in UNG\(^{-/-}\) but not UNG\(^{+/+}\) spleen and bone marrow cells in response to pemetrexed treatment (Figure 5-3a). Additionally, UNG\(^{-/-}\) cells show an increased induction of γ-H2AX with pemetrexed (Figure 5-3b), indicating formation of DNA double strand breaks coincident with uracil
accumulation *in vivo*. These findings are consistent with our hypothesis that uracil accumulation in UNG<sup>−/−</sup> cells triggers DNA double strand break formation and cell death. (59)

| Table 2. Pemetrexed-induced changes in bone marrow cell number and CFU potential |
|-------------------------------------------------|-----------------|-----------------|
|                                                 | Average Bone Marrow Cell Number *10<sup>6</sup> | Total CFU |
| UNG<sup>+/+</sup>                                |                 |                 |
| Control (n=3)                                   | 78.73           | 60.2           |
| Pemetrexed 150 mg/kg                            | 60.26           | 54.1           |
| Pemetrexed 300 mg/kg                            | 92.3            | 20.0           |
| Pemetrexed 600 mg/kg                            | 61.55           | 15.6           |
| Day 10 Recovery (600mg/kg)                      | 71.25           | 51.06          |
| UNG<sup>−/−</sup>                                |                 |                 |
| Control (n=3)                                   | 71.76           | 46.1*          |
| Pemetrexed 150 mg/kg                            | 45.1*           | 19.5*          |
| Pemetrexed 300 mg/kg                            | 45.45*          | 6.7*           |
| Pemetrexed 600 mg/kg                            | 33.9*           | 5.9*           |
| Day 10 Recovery (600mg/kg)                      | 65.12           | 38.59*         |

Mice received IP pemetrexed on Days 1, 2 and 3 and were sacrificed for bone marrow counts and CFU assay on day 4. Data are mean values (n=3) and * indicates p<0.05 compared to UNG<sup>+/+</sup> animals of the same treatment group.
5.3.3 Increased sensitivity to pemetrexed in UNG mutant human lymphoblastoid cells. The observation of mild, transient, yet statistically significant hematologic toxicity in UNG$^{-/-}$ mice prompted us to investigate the impact of diminished UNG activity on pemetrexed response in non-malignant hematologic cells of human origin. Biallelic germline somatic inactivating mutations in UNG have been characterized in 5 patients from France and
Japan.(260, 263) The predominant phenotype of these individuals is immune deficiency owing to a role for UNG in antibody diversification and B cell maturation. We were able to procure EBV-transformed lymphoblastoid cell lines from two of the three patients for which this mutation has been described courtesy of Anne Durandy, Paris, France.(263) These cell lines, P1 and P2, have a markedly diminished UNG cutting capacity compared to EBV-transformed lymphoblastoid cell lines from individuals with no reported hematologic defects, C1 and C2 and wild type UNG activity (Figure 5-4a). In 48-hour viability assay (Figure 5-4b), P1 and P2 were 10 to 30-fold more sensitive to pemetrexed than C1 and C2 (C1 IC50 = 141.2nM ; C2 IC50 = 87.9nM ; P1 IC50 = 8.26nM ; P2 IC50 = 4.34nM). Consistent with decreased cutting activity, UNG mutant LCLs (P1) had significantly more pemetrexed induced AP sites compared to wild type LCLs (C1). When DNA extract was incubated with purified UNG prior to analysis in the AP site detection assay, an enhancement of AP sites in P1 was noted, suggesting uracil retention in the DNA (Figure 5-4c). Sensitivity to pemetrexed was also marked by a significant enhancement of comet tail formation (DNA DSBs) in UNG mutant LCLs compared to LCLs with wild type UNG activity (Figure 5-4d).
Figure 5-4. Increased sensitivity to pemetrexed in human lymphoblastoid cells possessing inactivating mutations in UNG. (a) UNG activity assay in primary lymphoblastoid cell lines (LCLs). P1 and P2 are primary LCLs derived from patients with inactivating mutations in UNG. C1 and C2 are control lymphoblastoid cells from patients without hematologic or immunologic disorders. (b) decreased viability in human UNG mutant LCLs measured by trypan blue exclusion assay (c) AP site measurements in pemetrexed treated lymphoblastoid cells (d) neutral comet assay showing induction of DNA double strand breaks in human UNG mutant LCLs that is attenuated by 10μM supplemental thymidine.
5.4 Discussion

The data show that loss of UNG activity enhances pemetrexed-induced suppression of bone marrow cell production resulting in a mild decrease in circulating white blood cells and lymphocytes in mice. In *in vitro* studies, loss of UNG activity is associated with increased DNA double strand break formation, activation of a G1/S checkpoint, accumulation of uracil in DNA, and induction of apoptosis during pemetrexed exposure. Studies in human lymphoblastoid cells suggest that UNG activity may mitigate pemetrexed sensitivity in non–malignant cells of hematologic origin, particularly lymphocytes.

These findings are consistent with our earlier reports of pemetrexed hypersensitivity in UNG−/− human cancer cells (58, 59) and suggest that pemetrexed-induced cell death occurs via a similar mechanism of action in malignant and non-malignant UNG deficient cells. The observation of hematologic sensitivity in UNG−/− mice was not surprising given pemetrexed and similar anti-metabolites exhibit the bulk of their effects on rapidly dividing cells during early S-phase. (22) Additionally, the observation of UNG at replication foci, S-phase increases in UNG protein, and elevated levels of UNG in tissue with high turnover rates suggests coordination of DNA replication and repair. (75, 77, 202, 230)
Besides the transient hematologic sensitivity described, we observed no additional pemetrexed-related toxicity in UNG<sup>+/+</sup> or UNG<sup>-/-</sup> mice treated with pemetrexed. In preclinical studies desquamation of skin and mucosa, hypospermatogenesis, and enteropathy are additional reported toxicities of pemetrexed. In early mouse studies of pemetrexed toxicity and anti-cancer efficacy, it was noted that mice maintained on standard diet had circulating folic acid levels that were above physiologic levels in humans. Folate deprivation using a special diet enhanced hematologic toxicity in pemetrexed treated animals. Based on these findings in mice and later studies in human clinical trials, patients receiving pemetrexed receive folic acid supplementation as standard of care. For this reason, we declined to perform folate deprivation studies, as we believed they would overestimate combined toxicity of pemetrexed and UNG deficiency beyond clinical relevance.

UNG expression or activity may predict dose-limiting hematologic sensitivity in individuals receiving pemetrexed. To evaluate this possibility further, we propose to evaluate UNG expression and activity in the peripheral blood or bone marrow of patients receiving pemetrexed therapy for non-hematologic malignancy and to correlate resulting data with the occurrence of adverse events. Moreover, it is conceivable that pemetrexed may be particularly efficacious in lymphoid malignancies that develop in patients with either inactivating mutations in UNG or who have small nucleotide polymorphisms, which alter UNG activity or
expression. We propose future studies in UNG<sup>+/−</sup> mice, which will evaluate the efficacy of pemetrexed chemotherapy for the treatment and prevention of B-cell lymphoma. Additionally, syngeneic tumor grafts in UNG<sup>+/−</sup> mice may also be a relevant model system for evaluating whether UNG loss provides differential sensitization in malignant and non-malignant cells. These studies will inform whether the combination of UNG-directed therapy and pemetrexed provides a desirable therapeutic window.

Still, the mild and transient nature of hematologic sensitization observed in this study is encouraging for our efforts to develop a small molecule UNG inhibitor for use in combination with pemetrexed. Swift rebound of white blood cell and lymphocyte counts suggest that pemetrexed is not killing hematopoietic stem cells (HSCs). The most likely explanation is that HSCs are normally quiescent(267) and are only pushed into cell cycle by stress.(268) In mice treated with the TS-inhibitor 5-FU, cycling of HSCs began at 6-days post treatment in mice treated with the TS-inhibitor, 5-FU resulting in complete restoration of normal white blood cell counts in 12 days.(268) Because HSCs are not cycling during the 3 days of consecutive pemetrexed injections, they are likely protected from pemetrexed cell killing effects. Enhanced, and perhaps irreversible marrow ablation is expected to be observed if daily pemetrexed treatments are given for longer times or on a treatment schedule that would coincide with mobilization and cycling.
In sum, we have shown that despite the lack of an overt phenotype in untreated animals, UNG−/− mice have an increased sensitivity to pemetrexed-mediated bone marrow cell killing. These findings provide necessary foundation and understanding of the potential effects of systemic UNG loss or inactivation on pemetrexed sensitivity in vivo.
Chapter 6

Towards the development of a novel small molecule inhibitor of uracil DNA glycosylase (UNG)

UNG is the initiating enzyme of base excision repair (BER) of uracil in DNA that is misincorporated during DNA replication. Recently, we reported that pemetrexed sensitivity is strongly correlated with UNG expression in experimental lung cancer models. As a natural extension of our prior work, we initiated a screen for small molecule inhibitors of UNG for use in combination with pemetrexed in human cancer. In silico screening directed against the UNG active site was used to rank a 300,000 compound library; we employed a fluorescence-base high-throughput screening (HTS) assay to evaluate a batch of 100 of these compounds. Of the compounds tested, 11 had validated UNG inhibitor activity and the two compounds with the greatest anti-UNG activity are being pursued as lead molecules. Here, we report preliminary data describing the identification of compounds UNGi-1 and UNGi-2 through in vitro screening assays. Additionally, in initial intracellular growth inhibition assays using UNGi-2 are presented showing synergistic enhancement of UNG activity in the human lung cancer cell line, Calu-1. These data provide a promising foundation for our continued efforts to evaluate the use of UNG inhibitors as novel targeted therapy for use in combination with pemetrexed in human lung cancers.
6.1 Introduction

Uracil DNA glycosylase excises uracil from genomic DNA when it arises as a result of spontaneous cytosine deamination or due to polymerase misincorporation of dUTP during DNA replication. The excision activity of UNG has been associated with several biological processes relevant to human disease including: 1) mitigating the cytotoxicity of antifolate-induced uracil-DNA in human cancer;(58, 59) 2) generating tumorigenic chromosomal translocations;(269, 270) 3) participating in DNA strand break formation to facilitate class switch recombination in antibody diversification and B cell maturation;(259, 262, 271) and 4) participating in various role with the viral life cycles of cytomegalovirus(272), pox virus,(273, 274) herpes virus and type I human immunodeficiency virus (HIV1).(275) Small molecules that inhibit the activity of UNG are therefore of great scientific interest with a wide range of potential applications.

Here, we report preliminary findings of studies involving the identification and evaluation of such compounds for application in human cancer. Other laboratories have investigated UNG inhibition using various strategies. The bacteriophage protein, Ugi structurally mimics the uracil-DNA and is a potent inhibitor of UNG activity in vitro.(158, 160, 276, 277) However, induced
expression of Ugi did not result in enhanced sensitivity to TS-inhibitor chemotherapy.\(^{(147)}\)

Using the bacteriophage protein as an example, uracil-DNA structural mimics have been pursued the design and synthesis of small molecule UNG inhibitors.\(^{(162, 164, 278-281)}\) In lieu of this approach, we used in silico screening to rank a library of 300,000 available drug-like small molecules for affinity to UNG active site. A batch of 100 compounds from this analysis were evaluated using a molecular beacon oligonucleotide substrate containing 9 U:A base pairs in an in vitro high throughput screening (HTS) assay. Preliminary data describing the in vitro validation, and intracellular toxicity are presented for the second most potent compound identified in the HTS screen, UNGi-2.

6.2 Material and Methods

6.2.1 Cells and reagents. Calu-1 and H1975 cells were obtained from ATCC and expanded and cryopreserved immediately upon delivery. Cells were cultured at 37°C and 5%CO\(_2\) in complete DMEM (10%FBS, + 2mM L-glutamine). Pemetrexed was purchased from LC laboratories. Compounds for UNG screen were provided by the Drug Discovery Center at the University of Cincinnati.
6.2.2 Screening for UNG inhibitor activity. A molecular beacon oligonucleotide containing 5’ FAM and 3’DABSYL moieties was purchased from Sigma Aldrich with the following sequence: 5’FAM-GCACUUAAGAAUUGCAAUUCUAAAGUC-DABSYL-3’.

This oligonucleotide forms a hairpin structure causing the DABSYL moiety to quench fluorescence of FAM. UNG enzyme (new England Biolabs) was incubated with inhibitor compound at variable ratios for 45 minutes in 96 well plate prior to the addition of oligonucleotide substrate in reaction buffer (20mM Tris, pH=7.8, 50mM KCl, 0.2mM MgCl₂, 1mM DTT). Fluorescence readings were taken every 150 seconds for a total reaction time of 1800 seconds using a Spectra Max M2 table top spectrophotometer and 490/520 excitation/emission wavelengths. Hairpin relaxation caused increased fluorescence relative to baseline (time 0). In the initial HTS screen, compounds with <60% inhibition were eliminated from analysis (82/100). Three false positives were eliminated in the second round of screening. Activities of the remaining 11 hits were validated using gel-based cutting assay. All screening reactions were conducted in triplicate.

6.2.2 Cell culture growth inhibition assay. Cells were seeded at 20,000 cells/well in 24 well plates for treatment with variable concentrations of pemetrexed alone and in combination with UNGi-2 (25μM). Cells were cultured in the presence of drugs for 5 days prior to staining with methylene blue. Cellular
density was determined by absorbance spectroscopy. IC$_{50}$ values were calculated using GraphPad Prism software.

6.3. Results

6.3.1 Optimization and validation of HTS assay methodology. An in silico screen was performed by Sichun Yang in the Center for proteomics and bioinformatics identifying 300,000 compounds with computed varying affinities for the UNG active site. We tested a batch of 100 high-ranking compounds using an in vitro high through-put screening (HTS) assay. We designed an oligonucleotide substrate for HTS screening of UNG inhibitory compounds based on a similar method in the literature.(280) This substrate, a hairpin oligonucleotide modified with a fluorescence moiety at the 5 prime end and a fluorescence quenching moiety at the 3 prime end will fluoresce when allowed to react with functional UNG enzyme (Figure 6-1).

Prior to screening compounds, we validated the conditions of this assay by testing the assay sensitivity to varying oligonucleotide (Figure 6-2a) and UNG enzyme concentrations (Figure 6-2b). Based on these experiments, 200nM oligonucleotide concentration and 0.25U of UNG enzyme were chosen as reaction conditions. Then as a proof of principle and to choose an appropriate
positive control compound for screening experiments, we performed the HTS assay after pre-incubating UNG for 30 minutes with 100μM of uracil-related compounds. Heat killed UNG, having no UNG activity, did not cause hairpin

![Diagram of molecular beacon substrate for HTS screening of compounds for UNG inhibitor activity](image)

**Figure 6-1. Principle of molecular beacon substrate for HTS screening of compounds for UNG inhibitor activity.** A hairpin oligonucleotide containing 9 U:A base pairs, and FAM and DABSYL modified 5 prime and 3 prime ends was used as the oligonucleotide substrate for UNG activity assays in a high throughput screen. Reaction of this oligonucleotide with UNG enzyme results in uracil removal and relaxation of the hairpin structure due to decreased base pairing. In the presence of UNG, fluorescence activity increases over time and can be measured using a spectrophotometer.
Figure 6-2. Optimization and validation of HTS assay. (a) Molecular beacon oligonucleotide (0-2000nM) incubation with purified UNG enzyme (1 U) for 0-1800 seconds in the absence of inhibitor compounds was used to test the HTS assay and determine an appropriate concentration of oligonucleotide. 200nM oligonucleotide was chosen for subsequent reactions. (b) HTS assay was carried out with 0-2U of UNG to determine the sensitivity of the assay to varying levels of the enzyme. Results show similar reaction kinetics for all concentrations of UNG tested, a 0.25 enzymatic unit of UNG was chosen for subsequent reactions. (c) HTS assay was conducted using 0.25 units of UNG, 200nM oligonucleotide substrate and 100μM of uracil-related compounds. Heat-killed UNG and non-inhibitor reactions were also performed as a positive (complete inhibition) and negative (no inhibition) controls, respectively. Uracil, the natural ligand of UNG was chosen as a positive control compound for subsequent reactions.
relaxation. Hairpin relaxation was also almost completely attenuated in the presence of uracil, the natural ligand of UNG. Interestingly other compounds investigated including chlorodeoxyuridine (CldU), iododeoxyuridine (IdU), and cyanodeoxyuridine (CNdU) had no inhibitory effect, while dUTP appeared to exacerbate hairpin relaxation in this assay.

Compounds were screened in 96-well plates spotted with 2μL of UNG inhibitor compounds. Results of primary screen yielded 18 compounds that inhibited 60% or greater of UNG activity. These 18 compounds were re-screened to eliminate false positives yielding 11 compounds with UNG inhibitory activity (Figure 6-3b). Of note, all compounds are while uracil analogs, they are all much less potent inhibitors of UNG than uracil (Figure 6-3b). The properties of the 11 “hit” compounds and 4 compounds with no UNG inhibitor activity are summarized in Table 6-1. Validation of HTS screening results was performed using UNG cutting assay. These data identified 4 compounds that inhibited UNG activity by 50% or greater. We chose to pursue compounds UNGi-1 and UNGi-2 for lead optimization and intracellular testing. UNGi-2 is commercially available allowing for immediate target validation and intracellular testing.

6.3.2. Determination of UNGi-2 IC_{50} in gel based cutting assays. UNGi-2 was commercially available so we chose to perform extended analysis of this compound first. UNG gel based cutting assay using 1 enzymatic unit each of
UNG and APE were performed in the presence of sequential dilutions (0-1mM) of uracil or UNGi-2. Relative UNG cutting activity was calculated as the percent of oligonucleotide cutting in the presence of inhibitor relative to the percentage of cutting in enzyme only control. Using a 5-minute reaction time, UNGi-2 inhibited UNG with an IC$_{50}$ of 241.6µM. As expected, uracil is a significantly more potent inhibitor of UNG (IC$_{50}$ = 0.13µM), p<0.0001 (Figure 6-4). Uracil has not been pursued as a candidate compound for intracellular assays because it would be immediately metabolized by pyrimidine salvage pathways upon uptake and sufficient intracellular machinery exist, such as the enzyme dUTPase, to limit the efficacy of unmodified uracil on cell killing. The efficient degradation of newly synthesized uracil nucleotide precursors is precisely the rationale for the combined uracil-tegafur (uracil + fluorouracil) regimen.(282)
Figure 6-3 Results of HTS screening of 100 compounds for UNG inhibitor activity. HTS assay was conducted using 0.25 U of UNG, 200nM oligonucleotide substrate and 1mM inhibitor in 96-well format. UNG was pre-incubated with inhibitor for 45 minutes at room temperature prior to the addition of oligonucleotide substrate in reaction buffer using a multichannel pipette. Results of the initial screen yielded 18 positives (compounds with >60% UNG inhibitor activity). The 18 compounds identified in (a) were rescreened to rule out false positives eliminating three compounds. (b) HTS assay kinetics for the 11 “hits”. (c) The 11 compounds that were identified in the HTS screen were subjected to gel-based oligonucleotide cutting assay. Percentage of cutting activity was determined by the ability of the compounds to impair the cutting of a 40-mer fluorescent labeled oligonucleotide containing a U:A base pair in a reaction containing 1 enzymatic unit each of purified UNG and APE.
<table>
<thead>
<tr>
<th>Compound name</th>
<th>Molecular Weight (g/mol)</th>
<th>Rank Score (virtual screen)</th>
<th>Non-polar VDS score (van der waals contact score) (kcal/mol)</th>
<th>Polar ES score (Electrostatic contact score) (kcal/mole)</th>
<th>ClogP</th>
<th>Molecular Refractivity (cm²/mol)</th>
<th>Polar Surface Area (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UNGi-1</td>
<td>278.26</td>
<td>77</td>
<td>-29.6</td>
<td>-9.5</td>
<td>0.444</td>
<td>67.95</td>
<td>108.97</td>
</tr>
<tr>
<td>UNGi-2</td>
<td>295.34</td>
<td>104</td>
<td>-31.5</td>
<td>-10.5</td>
<td>1.42452</td>
<td>74.54</td>
<td></td>
</tr>
<tr>
<td>UNGi-3</td>
<td>358.27</td>
<td>1805</td>
<td>-41.5</td>
<td>-5.9</td>
<td>-2.91</td>
<td>78.15</td>
<td>163.26</td>
</tr>
<tr>
<td>UNGi-4</td>
<td>385.29</td>
<td>6</td>
<td>-41.5</td>
<td>-14.1</td>
<td>2.748</td>
<td>83.72</td>
<td>179.64</td>
</tr>
<tr>
<td>UNGi-5</td>
<td>170.13</td>
<td>2</td>
<td>-18.3</td>
<td>-15.9</td>
<td>1.748</td>
<td>35.84</td>
<td>99.33</td>
</tr>
<tr>
<td>UNGi-6</td>
<td>131.09</td>
<td>54</td>
<td>-17</td>
<td>-12.7</td>
<td>-2.00275</td>
<td>27.01</td>
<td>95.66</td>
</tr>
<tr>
<td>UNGi-7</td>
<td>271.25</td>
<td>41</td>
<td>-27.4</td>
<td>13.6</td>
<td>0.889</td>
<td>62.76</td>
<td>131.32</td>
</tr>
<tr>
<td>UNGi-8</td>
<td>269.28</td>
<td>69</td>
<td>-29.1</td>
<td>-12.4</td>
<td>0.048</td>
<td>109.57</td>
<td></td>
</tr>
<tr>
<td>UNGi-9</td>
<td>292.26</td>
<td>105</td>
<td>-25.9</td>
<td>-19.5</td>
<td>-0.79076</td>
<td>131.14</td>
<td></td>
</tr>
<tr>
<td>UNGi-10</td>
<td>170.13</td>
<td>1</td>
<td>-18.3</td>
<td>-15.9</td>
<td>-2.1748</td>
<td>35.84</td>
<td>99.33</td>
</tr>
<tr>
<td>UNGi-11</td>
<td>260.21</td>
<td>49</td>
<td>-35.4</td>
<td>-12.5</td>
<td>3.1191</td>
<td>134.33</td>
<td></td>
</tr>
<tr>
<td><strong>Natural Ligand</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td>112.09</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>-1.056</td>
<td>26.03</td>
<td>58.2</td>
</tr>
<tr>
<td><strong>Non-Hits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UNGi-97</td>
<td>189.17</td>
<td>81</td>
<td>-18.8</td>
<td>-15.3</td>
<td>-2.855</td>
<td>41.11</td>
<td>135.51</td>
</tr>
<tr>
<td>UNGi-98</td>
<td>219.3</td>
<td>60</td>
<td>-26.1</td>
<td>-10.2</td>
<td>0.1574</td>
<td>82.05</td>
<td></td>
</tr>
<tr>
<td>UNGi-99</td>
<td>398.5</td>
<td>21</td>
<td>-37.6</td>
<td>-16.6</td>
<td>3.0702</td>
<td>116.2</td>
<td>71.03</td>
</tr>
<tr>
<td>UNGi-100</td>
<td>215.23</td>
<td>102</td>
<td>-28.2</td>
<td>-11.3</td>
<td>-1.4905</td>
<td>50.12</td>
<td>87.3</td>
</tr>
</tbody>
</table>
Figure 6-4. Comparison of UNG inhibitory effect of UNGi-2 and uracil in vitro using gel based cutting assay. Gel based cutting assay was carried out with UNG enzyme that was pre-incubated with uracil or with UNGi-2 for 30 minutes prior to addition of oligonucleotide, APE and reaction buffer. Data indicate the percentage of cutting activity for three independent experiments.
6.3.3 Intracellular activity of UNGi-2 in combination with pemetrexed. With the knowledge that UNGi-2 is active against UNG \textit{in vitro} in enzymatic assays, we next evaluated its activity within cells through growth inhibition studies. Cell growth was monitored by methylene blue staining of cells treated with variable levels of pemetrexed for 5 days. Combined treatment with UNGi-2 enhanced pemetrexed growth inhibition by 535.4- and 88.6-fold in Calu-1 and H1975 cells, respectively (p<0.0001, Figure 6-5).

\textbf{Figure 6-5 Intracellular toxicity of UNGi-2 in combination with pemetrexed} (a) Calu-1 and (b) H1975 cells were treated with variable amounts of pemetrexed alone or in the presence of 25\mu M UNGi-2. Cells were stained with methylene blue as in (c), the degree of staining was measured by absorbance spectroscopy and translated into a percentage of viable cells.
6.4 Summary

These initial data validate the utility of a molecular beacon oligonucleotide substrate for high throughput screening of UNG inhibitor compounds from a library of small molecules. Initial data indicate that UNGi-2 inhibits UNG activity \textit{in vitro} and significantly enhances pemetrexed sensitivity in Calu-1 and H1975 human lung cancer cells.

UNGi-2 was toxic in the absence of pemetrexed. Given that we observe no increased DNA damage in shRNA targeted H1975 cells or UNG$^{-/-}$ DLD1 cells. Compounds that produce minimal cell killing when administered alone but enhance cell killing in combination with pemetrexed are preferred. Studies to determine the manner and mechanism of cell death in cells treated with the UNGi-2 inhibitor are planned. These studies will include an evaluation of DNA damage marker induction, AP site formation and genomic uracil measurements. More importantly however, we have initiated additional screens of structurally similar compounds in the original 300,000 compound library for use in additional HTS screening and target validation assays. Additionally we are seeking the involvement of synthetic and medicinal chemists to optimize the 2 hit compounds from this screen for increased potency. It is anticipated that combined these efforts will produce a smaller library of compounds with validated in vitro UNG activity that can be tested for intracellular, in vivo studies towards clinical development (Figure 6-6).
Figure 6-6. Continued steps towards identification of UNG inhibitor for clinical development from a library of lead compounds. Combined analysis of compounds structurally similar to the “hits” identified through HTS screening and chemical modification of most promising lead molecules will be used to generate a library of lead compounds with validated *in vitro* UNG inhibitor activity. From there we will proceed towards identification of a compound with potential clinical activity through Intracellular target validation and mechanistic studies, in vivo toxicity analysis and proof of concept studies in human xenograft tumors.
Chapter 7

Summary and conclusions

7.1 Summary of major findings

The present work has focused on elucidating a role for the BER pathway and uracil excision by the enzyme, uracil DNA glycosylase, in the cellular response to pemetrexed induced DNA damage. The possibility of enhancing anti-cancer activity of pemetrexed through inhibition of UNG/BER was explored in detail. Key research findings include:

1. The novel observations that UNG is induced in cells as a result of both acute and chronic pemetrexed exposure and that UNG expression is correlated with cellular sensitivity (IC50) to pemetrexed.

2. Elucidation of an alternative mechanism of pemetrexed toxicity in cancer cells that is typified by uracil accumulation, replication fork stalling, intra S phase checkpoint activation, and subsequent replication fork collapse that is rescued by thymidine supplementation.

3. The novel observation that UNG+/− mice are susceptible to transient, yet significant hematopoietic sensitivity when exposed to pemetrexed.
4. The proposal of UNG/BER inhibition via down regulation of UNG expression or methoxyamine inhibition of BER to override acquired and native pemetrexed resistance in human lung cancer.

5. The initiation of studies, that stand to identify novel inhibitors of the UNG enzyme for use in combination with pemetrexed to enhance anti-cancer activity.

7.2 Limitations of this research

No scientific endeavor is ever perfect and this research has several limitations. First, a quantitatively determined threshold of uracil tolerance still eludes us. With \textit{in vitro} primer extension assays we have observed significant attenuation of polymerase activity with 3 and 5 but not 1 uracil base substitutions. This would suggest that cells have an ability to cope with infrequent uracil bases, but are sensitive to heavily uracilated DNA. This study however, was performed \textit{in vitro} and needs to be confirmed with a comparable intracellular assay. Such studies are key to rectifying reports of limited impact of UNG loss in certain cell/TS-inhibitor models.
Secondly, we have not directly addressed the likelihood of complex gene expression patterns that govern pemetrexed sensitivity in lung and other cancer cells. We alluded to this possibility in Chapter 3 by showing in multivariable regression models that UNG gene expression when paired with the expression of other pathway specific genes can, in some cases, be more predictive of pemetrexed response than UNG alone. Moving forward, it is important that UNG expression be considered in the context of other pathway-specific resistance markers, such as other genes in the BER pathway, other DNA repair genes, dUTPase, ribonucleotide reductase, and pemetrexed enzymatic targets. Aside from expanding our understanding of genes that predict pemetrexed response, these studies would also improve our understanding of genetic interactions within cells. For example, in methotrexate-treated cells, combined deficiency in mismatch repair (MMR) and BER is synthetically lethal. Because deaminated cytosines that form U:G mispairs are targeted by MMR machinery, one can imagine that dual inhibition of MMR may enhance spontaneous uracil accumulation (U:G) while BER inhibition targets the misincorporated uracil (U:A). Shifting our focus towards understanding the expression of UNG in the context of other potentially relevant genes will certainly aid us in identifying additional synthetic lethal relationships that may be exploited in the treatment of human cancer.
7.3 Conclusions

Significantly, we have shown that the dominant hypothesis of futile cycles of BER excision and repair of uracil is inaccurate, at least in the models studied. As such, this work bolsters UNG activity as a major driver of pemetrexed resistance. We illustrate the genotoxic nature of uracil accumulation and the potential of this lesion (uracil-DNA) to contribute to double strand break formation in UNG deficient systems. By comparing UNG competent and UNG deficient cells, we have observed that unresolved genomic uracil (in UNG–/– cells) is more cytotoxic – generating sufficiently more DNA double strand breaks – than TS-inhibitor-induced nucleotide aberrations (occurring in UNG+/- cells). In all, we have provided a substantial foundation for clinical studies that: a) seek to target UNG/BER to potentiate pemetrexed cytotoxicity and b) seek to determine a clinical value for UNG and BER pathway gene expression in predicting clinical response to pemetrexed and other antifolates/TS inhibitors.
Chapter 8

Future directions

In order to extend the understanding of the pathways and mechanisms identified in this work and to address the limitations of this research, our continued investigative efforts will be focused towards 4 major areas:

1. **Determine a threshold of uracil burden in human cells.**
   In all of the cell lines tested, we have observed increased sensitivity to pemetrexed when UNG expression was knocked down or knocked out. This coupled with increased uracil retention (measured either by mass spectrometry or indirectly by AP sites) suggest that cells are sensitive to uracil accumulation. However, plotting pemetrexed IC$_{50}$ against UNG RQ value suggests that there is a threshold level for UNG expression and therefore uracil tolerance. A precise determination of the threshold of uracil burden within cell lines will augment our understanding of uracil as a genotoxic lesion in DNA. Studies involving direct dosing of dUTP, dUTP analogs or plasmids containing a precise number of dUTP residues into UNG$^{-/-}$ cells can be utilized to determine if cellular sensitivity to uracil-DNA occurs only at a particular threshold level.
Thresholds of uracil tolerance may vary widely across cell lines and tumor cell types. Therefore, studies that determine threshold uracil burdens for cell sensitivity and that correlate the degree of UNG inhibition or knockdown with UNG expression will be performed in multiple cell lines.

We have also considered the possibility that different TS-inhibitors are distinct in the degree of dUTP/dTTP nucleotide pool imbalance and thus, the level of genomic uracil incorporation that is observed. To address this, we plan to directly measure nucleotide pool levels in situ using Raman spectroscopy in cells treated with clinically and experimentally relevant antifolates and TS inhibitors, in collaboration with Dr. Paul Carey (Case Western Reserve University, Department of Biochemistry). We expect that these data will clarify the differential effects of these agents on nucleotide pools and uracil burden in human cancer cells.

Finally, based on ChIP sequencing data obtained in this study, an identification of double strand break “hot-spots” may point to chromosomal regions that are particularly sensitive to increased uracil levels. Alternatively, regions of the DNA that are protected from uracil accumulation and therefore double strand break formation can also be defined. Experiments to identify key genetic sequences that directly influence sensitivity to pemetrexed when uracil is misincorporated could
also improve our understanding of this mechanism of antifolate induced cell death.

2. Clarify the effectiveness of BER inhibitors for targeting pemetrexed-resistant tumor populations

a. We believe that BER inhibition is an effective strategy for re-sensitizing tumor cells that possess native or acquired pemetrexed resistance. Initial clinical trial data from pemetrexed/MX combinations suggests that responses to chemotherapy may be achieved even in squamous cell carcinoma, a classically pemetrexed-resistant histological subtype of lung cancer. (28) Based these findings in conjunction with our experimental data, we propose the evaluation of UNG/BER gene expression in individuals who progress following pemetrexed chemotherapy or maintenance therapy (acquired resistance) and individuals who have treatment refractory small cell or squamous cell carcinoma (native resistance). High UNG/BER levels in the tumors of these patient populations may indicate that individual is a candidate for pemetrexed/BER inhibition combination.

b. Tumor heterogeneity and experimental evidence of tumor initiating cells (TICs) represents a significant challenge to treating human cancer. TICs are chemotherapy resistant populations of cancer cells that are present at
low frequency in the bulk tumor but are capable of tumor repopulation (relapse) and metastasis. (283-286) Cancer chemotherapy triggers the expansion of tumor initiating cell populations via an unknown mechanism. (283) Additionally, chemotherapeutic resistance in these cells may be related to enhanced capacity for DNA repair. (283, 287) Since we have established that increased BER capacity is a feature of native and acquired resistance to pemetrexed, we have begun to study DNA repair status and the impact of BER inhibitors in CD133+ human lung cancer cells, a putative TIC population. In preliminary experiments, we have observed expansion of CD133+ cells with pemetrexed treatment. These CD133+ cells have increased expression of genes belonging to BER, HR and NHEJ pathways suggesting that resistance to chemotherapy may be related to an increased capacity for DNA repair. In addition to increasing overall sensitivity to pemetrexed, DNA repair inhibition with methoxyamine decreased the frequency of A549 CD133+. To better determine the impact of pemetrexed and DNA repair inhibition on tumor-initiating cells steps will be taken to a) validate that CD133+ cells are tumor initiating through standard intracellular and in vivo assays; b) determine dependence of TIC population expansion during chemotherapy on DNA repair up-regulation through measurements of TIC cell frequency in DNA repair deficient cells (i.e. clonogenic isolates of UNG+ deficient cells); c) determine impact of UNG/BER inhibition on TIC stem cell phenotype (pre-and post pemetrexed
treatment). When complete, these studies will allow an understanding of the role of DNA repair and particularly UNG/BER in the tumor initiating cell response to pemetrexed based chemotherapy. Based on data accumulated, it may be reasonable to consider evaluating the impact of methoxyamine, PARP inhibition or UNG inhibition on tumor-initiating cells in human patients receiving pemetrexed and methoxyamine in planned Phase II and randomized clinical trials.

3. **Optimization of lead UNG inhibitor compounds towards anti-cancer drug development.**

We’ve reported marked sensitization to pemetrexed when UNG levels and activity are reduced suggesting that an inhibitor of UNG may be effective at potentiating pemetrexed anti-tumor effect. In Chapter 7 we describe the results of *in silico* screening, *in vitro* high throughput screening and intracellular lead validation towards the development of a novel class of inhibitors of UNG. In collaboration with the Drug Discovery Center at the University of Cincinnati, we are conducting additional screening of compounds with similar chemical structures in addition to structure optimization to establish a library of viable lead compounds. From the library we will use intracellular, in vivo and xenograft studies to select compound(s) for clinical development.
4. **Clinical investigation of relationship between UNG expression and tumor response to pemetrexed, other antifolates/TS-inhibitors and additional anti-metabolites that provoke UNG excision activity (i.e. fludarabine).**

The relationship between UNG expression and cellular sensitivity to pemetrexed in cell lines, and differential expression of UNG skewed towards higher expression in pemetrexed-resistant lung cancer histological subtypes suggests UNG is potentially exploitable as a biomarker for predicting pemetrexed response in lung cancer. A small cohort of human lung biopsy specimens from patients with pemetrexed-treated adenocarcinoma is presently being evaluated for UNG expression by immunohistochemistry (collaboration with Dr. Nooshin Hashemi-Sandraei, Department of Solid Tumor Oncology, Taussig Cancer Center, Cleveland Clinic). Prospective investigation of UNG and other pathway specific biomarkers in pemetrexed-treated lung cancer are planned in collaboration with Dr. Afshin Dowlati (Division of Hematology and Oncology, University Hospitals, Case Medical Center). Expression levels will then be correlated with clinical data for each sample to determine if UNG levels are at all correlated with tumor regression, survival, and adverse events in patients treated with pemetrexed.
Studies of UNG expression as predictive biomarker for hematologic malignancies treated with fludarabine alone and in combination with methoxyamine are underway in collaboration with clinical investigators at University Hospitals Case Medical Center (Dr. Paolo F. Caimi, Principal Investigator) and Translation Research and Pharmacology Core Facility (Dr. John Pink, Case Western Reserve University). We propose the inclusion of UNG expression interrogation as a clinical correlate for all clinical trials involving pemetrexed, 5-fluorouracil, raltitrexed, methotrexate, fludarabine and other agents that provoke UNG excision activity.
References


20. van Triest B, Pinedo HM, van Hensbergen Y, Smid K, Tellemann F, Schoenmakers PS, et al. Thymidylate synthase level as the main predictive


64. Min A, Im SA, Yoon YK, Song SH, Nam HJ, Hur HS, et al. RAD51C-deficient cancer cells are highly sensitive to the PARP inhibitor olaparib. Mol Cancer Ther 2013; 12: 865-77.


116. Sedwick WD, Kutler M, Brown OE. Antifolate-induced misincorporation of deoxyuridine monophosphate into DNA: inhibition of high molecular weight DNA
125. Kunz BA, Kohalmi SE, Kunkel TA, Mathews CK, McIntosh EM, Reidy JA. International Commission for Protection Against Environmental Mutagens and


197


151. Fischer F, Baerenfaller K, Jiricny J. 5-Fluorouracil is efficiently removed from DNA by the base excision and mismatch repair systems. Gastroenterology 2007; 133: 1858-68.


262. Di Noia JM, Rada C, Neuberger MS. SMUG1 is able to excise uracil from immunoglobulin genes: insight into mutation versus repair. EMBO J 2006; 25: 585-95.


