EFFECT OF FOLATE DEFICIENCY ON THE SENSITIVITY OF COLON CANCER CELLS TO 5-FLUOROURACIL BASED CHEMOTHERAPY

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Nutritional Sciences
University of Toronto

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2012

ABSTRACT

Folate is an essential cofactor in one-carbon transfer reactions including nucleotide biosynthesis, thereby playing an important role in DNA replication and repair. In cancer cells, folate depletion interrupts DNA synthesis, thereby causing cancer cell death. This has been the basis for chemotherapy using antifolates and 5-fluorouracil (5FU). We determined the effect of folate deficiency on the sensitivity of colon cancer cells to 5FU in a well established in vitro model of folate deficiency. Folate deficient cells had lower intracellular folate concentrations, had functional evidence of intracellular folate depletion, proliferated less, and had increased chemosensitivity to 5FU with and without Leucovorin. These data suggest that folate deficiency significantly enhances the sensitivity of colon cancer cells to 5FU based chemotherapy via changes in intracellular folate. Dietary or other strategies to deplete intracellular folate concentrations in colon cancer cells to enhance chemosensitivity to 5FU are worthy of further investigation.
ACKNOWLEDGEMENT

Alas, I have concluded yet another chapter (literally and metaphorically) in my life. I’d first and foremost like to extend my deepest thanks to Dr. Young-In Kim who gave me the opportunity to pursue a Masters under his supervision. You have given me endless support and guidance on matters both in and out of the lab. In particular, you have made the time exceptionally enjoyable with your oddly rational, blatantly honest and socially unacceptable joking. I will be passing on the torch of being your tech-guy to the next graduate student, I’m sure your computer and iPhone will behave in the meantime. Also, you can rest assured that you won’t ever see me again in “swim trunks” in your lab but I’m sure you’ll still find something to point out!

I’d like to extend sincere appreciation to Kyoung-Jin Sohn for all of the hands-on knowledge and powwow sessions we’ve had about our project. I know it wasn’t easy teaching someone with no wet bench experience but I have appreciated every effort you have made. I will miss being your go-to person for technological and parenting advice, but I’m sure Marco will do wonderful without me.

I would like to thank Dr. Michael Archer and Dr. David Hedley for their support and thoughtful insight throughout my project and for always keeping me on my toes during committee meetings.

Thank you to Pamela Plant for basically being my lab mom. Your guidance, calm and expertise will be sorely missed whenever I need to grab samples from the bottom of a pool of liquid nitrogen. Thank you to Christopher Spring for basically being a teenager in an
adult’s body and for guiding me through the process of flow cytometry. Your humor, knowledge of food and overall sarcasm will be greatly missed.

I’d also like to say thanks to individuals that I’ve had the pleasure of working with/near. Nancy: for always being there for me, good times and bad. Lisa: for laughing at basically everything I say, playing ping pong with me during incubations and using up all of my handcream. Lesley: for wonderful conversation about much girlier things than I’d care to admit to. Sung-Eun: for always knowing the exact answer for anything I’ve ever asked from you. Shaidah: for always posing questions. Michael K.: for RT-PCR help and lame jokes. Wing: for being unanimously voted the most Asian in the student room. Dave: for eating half a dozen donuts for dinner and wearing dress pants with a Mickey Mouse cotton tee. Jenn: for talking about the frustrations of medical school applications. Sagar: for rational powwows and logistics. Erica: for being in the lab at the same ridiculous hours that I’m in. To the rest of the Kim Lab: thank you for your support and friendly interaction.

Lastly, thank you to my family and friends who have supported me throughout this process. I am happy that this chapter is over but am excited and ready to start a new one.
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<tr>
<td>5FU</td>
<td>5-Fluorouracil</td>
</tr>
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<td>ACF</td>
<td>Aberrant Crypt Foci</td>
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<td>AICARFT</td>
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<td>APC</td>
<td>Adenomatous polyposis coli</td>
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<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
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<td>CIN</td>
<td>Chromosomal Instability</td>
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<td>CpG</td>
<td>Cytosine-Phosphate-Guanine</td>
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<td>CRC</td>
<td>Colorectal Cancer</td>
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<tr>
<td>DACH</td>
<td>1,2-Diaminocyclohexane</td>
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<tr>
<td>DCC</td>
<td>Deleted in Colorectal Cancer</td>
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<td>Dietary Folate Equivalents</td>
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<td>Deoxythymidine Triphosphate</td>
</tr>
<tr>
<td>DU</td>
<td>Deoxyuridine</td>
</tr>
<tr>
<td>dUMP</td>
<td>Deoxyuridine Monophosphate</td>
</tr>
<tr>
<td>dUST</td>
<td>Deoxyuridine Suppression Test</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>FA</td>
<td>Folic Acid</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FdUDP</td>
<td>Fluoro Deoxyuridine Diphosphate</td>
</tr>
<tr>
<td>FdUMP</td>
<td>Fluoro Deoxyuridine Monophosphate</td>
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<tr>
<td>FdUTP</td>
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<td>Folate Receptor</td>
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<td>Fluoro Uridine</td>
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<tr>
<td>FUTP</td>
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<td>GARFT</td>
<td>Glycinamide Ribonucleotide Formyltransferase</td>
</tr>
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<td>GCPII</td>
<td>Glutamate Carboxypeptidase II</td>
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<td>GGH</td>
<td>Gamma-glutamyl hydrolase</td>
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<td>HNPCC</td>
<td>Hereditary Nonpolyposis Colorectal Cancer</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
</tr>
<tr>
<td>LV</td>
<td>Leucovorin</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug Resistance Protein</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite Instability</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylenetetrahydrofolate Reductase</td>
</tr>
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<td>MTR</td>
<td>Methionine Synthase</td>
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<tr>
<td>MTRR</td>
<td>Methionine Synthase Reductase</td>
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<tr>
<td>NHANES</td>
<td>National Health and Nutritional Examination Survey</td>
</tr>
<tr>
<td>NTD</td>
<td>Neural Tube Defect</td>
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<tr>
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<td>Optical Density</td>
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<tr>
<td>OPRT</td>
<td>Orotate Phosphoribosyl Transferase</td>
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<td>para-aminobenzoic acid</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCFT</td>
<td>Proton Coupled Folate Transporter</td>
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<td>PRPP</td>
<td>Phosphoribosyl Pyrophosphate</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended Daily Allowance</td>
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<td>Roswell Park Memorial Institute</td>
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<td>SEPB</td>
<td>Surrogate End Point Biomarkers</td>
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<td>Sulforhodamine B</td>
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<td>Tetrahydrofolate</td>
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<td>Thymidine Kinase</td>
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<td>UK</td>
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<tr>
<td>UL</td>
<td>Upper Level</td>
</tr>
<tr>
<td>UP</td>
<td>Uridine Phosphorylase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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</table>
Chapter 1: Introduction

Folate is a naturally occurring water soluble B vitamin found in green leafy vegetables, legumes, organ meats, etc. Folic acid is the synthetic, monoglutamated and fully oxidized form of this vitamin which can be found in supplements as well as in enriched and fortified foods \[^1\]. Folate has two major biochemical functions. One is mediating the transfer of one-carbon units involved in biological methylation reactions and the other is nucleotide biosynthesis by acting as a methyl donor for thymidylate synthase (TS) to create deoxythymidine-5-monophosphate (dTMP) which is a precursor of DNA synthesis \[^2\]. Folate deficiency has been associated with an increased risk of a variety of diseases including certain cancers, anemia, cardiovascular disease, congenital defects, adverse pregnancy outcomes, and cognitive dysfunction \[^3-8\].

There is an overwhelming body of evidence demonstrating a significant protective effect of folic acid supplementation against the development of neural tube defects (NTDs) \[^9-13\]. As a result, Canada implemented mandatory folic acid fortification in 1998 which aimed to increase the mean folate intake by 100 μg/day \[^14\]. Because of mandatory folic acid fortification, serum folate levels in the Canadian population increased dramatically \[^15\]. Additionally, the use of supplements containing a minimum of 400 μg of folic acid has increased amongst North Americans. Currently, approximately 50% of the North American population consume at least one supplement per day, although the benefits are not well established \[^16\]. Recent studies have reported 15-32% of cancer survivors start multivitamin and supplement use after cancer diagnosis \[^17\]. As a result of both of fortification and
supplementation, folate deficiency has largely been abolished in the Canadian population and the percentage of the Canadian population with “high” levels has significantly increased \[^{18}\]. Despite the tremendous success of folic acid fortification in decreasing the rate of NTDs, concerns regarding the possible adverse effects of increased folate levels in the population have been raised.

Colorectal cancer (CRC) is an important disease among Canadians as it is the third most common cancer in both men and women. For overall mortality, it is ranked second in men and third in women. In 2012, it is expected that 23300 Canadians will be diagnosed with CRC with 9200 resulting deaths \[^{19}\]. Not only is it prevalent, it also adds a significant burden to the Canadian health care system \[^{20}\]. Similar to cancers, the etiology of CRC is multifactorial and contains a mix of genetic and environmental risk factors \[^{21}\]. Known risk factors for CRC include age, family history, prior colonic adenomatous polyps or adenocarcinoma, extensive and long duration of ulcerative and/or Crohn’s colitis as well as environmental factors such as diet, smoking, hormones, certain drugs, and physical inactivity \[^{22-26}\]. There are three types of standard treatment for colorectal cancer patients: surgery, chemotherapy, and radiation therapy \[^{27}\]. Adjuvant chemotherapy is usually administered along with surgery for advanced CRC (Stages 3 and 4). The specific treatment options that would be most beneficial are individualized and depend on the stage of the cancer, among others \[^{28}\]. 5-Fluorouracil (5FU) is the cornerstone of all colon cancer chemotherapeutic regimens, despite this, mechanisms of chemotherapy resistance can still occur.
5FU resistance can occur if there is any alteration in the natural metabolism of 5FU, for example, increased catalysis of 5FU would result in resistance. One potential mechanism of resistance is via cellular efflux transporters such as breast cancer resistance protein (BCRP) and the multidrug resistance protein (MRP) family that have the ability to transport antifolates as well as 5FU out of the cell, thereby causing resistance \[29\]. Cellular folate status can modulate the expression and activity of these efflux transporters thereby linking intracellular folate status with the potential for resistance \[30\]. It has been shown that folate deprivation can cause a decrease in MRP1 levels, thereby potentially lowering 5FU efflux and increasing 5FU sensitivity \[14\]. Therefore, our objectives were to determine the effects of folate deficiency on the sensitivity of colon cancer cells to 5-Fluorouracil, and to elucidate potential molecular mechanisms associated with any effects in an in vitro model of colon cancer and folate depletion.
Chapter 2: Literature Review

2.1 Colon Cancer

2.1.1 Incidence and Mortality

Colorectal cancer (CRC) is an important disease among Canadians as it is the third most common cancer in both men and women. In terms of mortality, it is ranked second in men and third in women. In 2012, it is expected that 23300 Canadians will be diagnosed with CRC with 9200 resulting deaths. Each day, 64 Canadians will be diagnosed with CRC and 25 will die from it\[19\]. During a man’s lifetime, he will have a 1 in 13 chance of developing CRC and a 1 in 28 chance that he will die from it. For women, they will have a 1 in 16 chance of developing CRC and a 1 in 32 chance of dying from it\[19\]. Since 1985, age-standardized incidence and mortality rates have steadily declined. However, due to the growth and aging of the Canadian population, the number of new cases has significantly continued to increase\[31\]. Despite this, the mortality rates have steadily declined 1.5%/year in males and 1.9%/year in females from a time span between 1997-2006. This decrease in mortality is likely due to a combination of improved prevention, early detection and treatment of CRC. Other potential factors include the inherent biological differences between men and women, differential gender responses to environmental factors as well as dietary and lifestyle pattern differences\[31\].

- 4 -
2.1.2 Risk Factors

Similar to other cancers, the etiology of CRC is multifactorial and contains a mix of genetic and environmental risk factors \(^{[21]}\). Known risk factors for CRC include age, family history, prior colonic adenomatous polyps or adenocarcinoma, extensive and long duration of ulcerative and/or Crohn’s colitis as well as environmental factors such as diet, smoking, hormones, certain drugs, and physical inactivity \(^{[22-26]}\). The following section describes some of these risk factors in more detail.

2.1.2.1 Molecular and Genetic Factors

Approximately 15% of all CRC cases are thought to arise due to a genetic predisposition \(^{[32]}\). CRC can also arise spontaneously (known as sporadic CRC) and is also caused by some genetic predisposition \(^{[33]}\). There two major familial conditions associated with CRC: Familial adenomatous polyposis (FAP) and Hereditary nonpolyposis colorectal cancer (HNPCC). Individuals with FAP and HNPCC are at an increased risk of developing CRC \(^{[34]}\).

2.1.2.2 Non Familial CRC (sporadic CRC)

More common than familial CRC, sporadic CRC can arise spontaneously through many genetic events spanning multiple years. The molecular genetics of familial CRC can be similar to that of sporadic CRC. Earlier studies showed that the order and accumulation of these molecular aberrations are important in CRC pathogenesis \(^{[35]}\). However, it has been suggested that the order is less important than the accumulation of these genetic
alterations. The transformation from normal colonic epithelium to adenoma to adenocarcinoma is correlated with acquired molecular changes in various oncogenes, tumor suppressor and mismatch repair genes.

In 1988, Vogelstein et al. proposed a stepwise molecular model of colorectal cancer pathogenesis referred to as the adenoma-adenocarcinoma model of carcinogenesis (Figure 2.1).

![Figure 2.1](image)

Figure 2.1 A scheme showing the relationship between normal crypts, aberrant crypts, adenomas, and adenocarcinomas or cancer. Permission obtained from publisher (Elsevier).

This model states that the progression of normal colonic epithelium to benign adenoma to malignant adenocarcinoma involves three major events:

1. in the early stages of polyp development, mutations occur in the adenomatous polyposis coli (APC) tumor suppressor gene found on chromosome 5q which ultimately leads to hyperproliferation of normal colonic epithelium.
2. in the middle stage of adenoma progression, mutations occur in the K-RAS oncogene.
3. in the late stage neoplastic transformation, mutations and deletions occur in the p53 tumor suppressor gene on chromosome 18q.
Since Volgelstein’s hypothesis, his model has been expanded to include at least two distinct pathways by which these molecular events can lead to CRC following the initial inactivation of APC (Figure 2.2). These pathways are the chromosomal instability (CIN) and microsatellite instability (MSI) pathways. These pathways are the chromosomal instability (CIN) and microsatellite instability (MSI) pathways lim. 

Figure 2.2 Genes involved in the pathogenesis of colon cancer. Reproduced with permission from Massachusetts Medical Society

1. Adenomatous polyposis coli (APC) tumor suppressor gene

The APC tumor suppressing gene is located on chromosome 5q21 and encodes a 2843 amino acid multifunctional protein that plays a role in cell adhesion, migration, proliferation, differentiation, apoptosis, signal transduction, microtubule assembly and chromosomal segregation. Losing the function of APC is one of the earliest events in CRC pathogenesis which ultimately leads to inappropriate cell proliferation. In sporadic CRC, somatic APC mutations are seen greater than 60% of the time. To further illustrate the early temporal nature of APC inactivation, APC but not K-RAS mutations have been observed in aberrant crypt foci (ACF). This suggests that APC mutations are an early event in CRC carcinogenesis. ACFs are the earliest identified neoplastic lesions in the colon,
are morphologically abnormal structures that are identified microscopically in the grossly normal colonic mucosas of human patients \cite{39}. However, it is important to note that not all ACFs have APC mutations, only those that become adenomas or adenocarcinomas \cite{43}. Because of its properties, the APC gene is considered a “gatekeeper” of CRC, where inactivation of this gene is necessary for colorectal carcinogenesis to proceed \cite{47}. Due to APC’s unique gatekeeper role, Min mice (\textit{Apc}^{+/−}) are frequently used to study the effects of \textit{Apc} gene inactivation to investigate the effects of nutrition and chemoprevention on CRC development and progression as these mice are prone to developing multiple intestinal adenomas \cite{50-52}.

The main tumor-suppressing function of \textit{APC} is mediated through its regulation of intracellular β-catenin levels \cite{53}. β-catenin is an essential intracellular component of cadherin adhesion complexes and is a critical factor in cell-cell adhesion and contact-associated growth \cite{53}. \textit{APC} has multiple functional domains which interact with β-catenin and other downstream effector proteins. Under normal conditions, glycogen synthase kinase (GSK)-3β forms a complex with and phosphorylates the \textit{APC}, β-catenin, and axin proteins \cite{54}. After β-catenin has been phosphorylated, it is destined for proteasomal degradation \cite{55}. However, if \textit{APC} is mutated, this process is prevented which results in increased levels of cytoplasmic β-catenin. This leads to subsequent overexpression of the Wnt signaling pathway and the transcription of tumor promoting genes (eg. \textit{C-MYC}, \textit{C-FOS}, \textit{CYCLIN D1}) \cite{56}. β-catenin can also interact with T-cell factor 4 (TCF-4) in the colonic epithelium and activate genes responsible for cell growth, and tumor formation such as \textit{C-MYC}, \textit{C-JUN}, and \textit{CYCLIN D1} \cite{56}. 
2. v-Ki-ras2 Kirsten rat sarcoma (K-RAS) viral oncogene homolog

Another common mutation found in 50% of colorectal tumors is the K-RAS oncogene in codons 12, 13, and 61 \[^{[57]}\]. K-RAS mutations appear to promote the early stages of adenoma-adenocarcinoma progression \[^{[58]}\]. RAS genes are critical components of the classical mitogen activated protein kinase (MAPK) signaling pathway and encode proteins that are involved in various cell processes such as signal transduction, cell proliferation, and differentiation in membrane bound cellular compartments \[^{[56]}\]. Oncogenic mutations of K-RAS lead to sustained K-RAS activation thereby increasing the interaction with the downstream MAPK signaling molecules, ultimately leading to increased cell proliferation \[^{[56, 59]}\].

3. Tumor suppressor p53

The tumor suppressing p53 protein has a DNA binding capabilities and therefore can act as a transcriptional regulator thereby mediating cellular responses to stressful stimuli\[^{[60]}\]. Tp53 is known as the “guardian of the genome” because when the cell is exposed to DNA damage, heat shock, and metabolic changes, the p53 protein is activated which results in programmed cell death or cell cycle arrest to induce DNA repair mechanisms \[^{[61, 62]}\]. Mutations that inactivate Tp53 occur during the later stages of colorectal carcinogenesis and are found in about 50% of colonic tumors \[^{[57]}\]. These mutations are typically found in exons 5-8 and are commonly accompanied by a loss of the wild-type allele at chromosome 17p. The majority of Tp53 mutations occur in the sequence-specific DNA-binding region of Tp53 \[^{[63]}\]. These mutations disrupt the transcriptional regulation mediated by the p53 protein which leads to uncontrolled cell growth \[^{[56]}\].
2.1.2.1.2 Chromosomal Instability Pathway

About 85% of CRC cases are a result of chromosomal instability (CIN) alterations such as aneuploidy (prevalent changes in chromosomal number) as well as detectable molecular losses of sections of chromosomes 5q, 18q, 17p and a mutation in the K-RAS oncogene \[64\]. These changes may occur initially by loss of one allele at a chromosomal locus which is known as loss of heterozygosity (LOH), or homozygous deletion if both alleles are lost \[65\]. Many of these loci are associated with known candidate tumor suppressor genes such as β-catenin, APC, p15, p16, p53, DCC and SMAD4 \[41, 42, 66\].

2.1.2.1.3 Microsatellite Instability Pathway

The second major molecular pathway involved in colorectal tumorigenesis makes up the remaining 15% of CRC and consists of the MSI pathway \[67\]. DNA mismatch repair machinery is responsible for correcting mispairing DNA nucleotide bases and the minor insertions or deletions that frequently occurring during normal DNA replication. By doing so, mismatch repair machinery maintains the fidelity of genomic DNA \[68, 69\]. Cells with a single functioning wild-type allele have either near normal or normal repair activity. However, cells in which both alleles are nonfunctional will lack the ability to repair DNA replication mismatches leading to an increased mutation rate due to compromised DNA mismatch repair. Microsatellite DNA sequences are short (6 base pairs or shorter) repetitive DNA sequences of mono-, di-, and tri-nucleotides usually found in the non-coding intron regions of genes throughout the genome \[70\]. As a result of the repetitive nature of
these sequences, they have a higher tendency to be replicated incorrectly, resulting in MSI due to lack of MMR enzymes ensuring the fidelity of DNA. The resultant genomic instability is thought to be the cause of the quick accumulation of somatic mutations in tumor suppressor genes and oncogenes that have important roles in tumor initiation and progression \[^{71}\]. In addition, MSI tumors can be classified into three distinct classes of stability based on the percentage of unstable microsatellites: 1. MSI-high (>30-40%), 2. MSI-low (<30-40%), and 3. MSI-stable (0%) \[^{72}\]. The importance of MMR genes has led to several mouse models being developed in order to study them. These mice are typically homozygous recessive or knockout (KO) mice for a specific gene involved in MMR. For example, mouse models have been developed with PMS2, MLH, MSH2 and MSH6 KO mice \[^{73}\].

2.1.2.1.4 Familial Adenomatous Polyposis

FAP is dominantly inherited, autosomal and affects approximately 1 in 7000 people \[^{32}\]. This syndrome is characterized by the development of greater than 100 colorectal adenomas (polyps). According to polyp number, there are subgroup classifications: profuse (>2000), sparse (100-500), and attenuated (10-100) \[^{74}\]. These adenomas are considered premalignant and thus, have the potential to transform into malignant adenocarcinomas. Given that there are a large number of adenomas, there is a very high probability that some will undergo neoplastic transformation into invasive adenocarcinomas \[^{32}\]. This condition was first seen in the 18\(^{th}\) century and the genetic nature of it was suggested by the 20th century. By investigating the genetic components of FAP with linkage studies, in 1987, two
separate groups identified the adenomatous polyposis coli (APC) gene on chromosome 5q \[32, 45\]. As a result of this discovery, FAP was the first CRC-predisposing condition for which a causative gene was found \[75\]. Using mutational screening, upwards of 95% of patients with FAP have APC sequence changes \[76\]. Germline APC mutations are found in patients with FAP whereas somatic APC mutations are found in sporadic CRC\[77\].

2.1.2.1.5 Hereditary Nonpolyposis Colorectal Cancer

HNPCC is a dominantly inherited disease which typically occurs in early age \[78\]. It is associated with an increased risk of colorectal adenocarcinomas and several other cancers including cancer of the endometrium, small intestine, stomach, pancreas, thyroid, ovary, kidney, urinary tract, liver, biliary tree, and sebaceous skin tumors \[45, 75, 79\]. There are several components to the strict criteria for HNPCC and are as follows: 1. at least three relatives have histologically confirmed CRC and one of these is a direct first degree relative to the other two, 2. at least two consecutive generations are affected, 3. CRC must be diagnosed in one of the relatives under the age of 50. HNPCC is the most common cancer-predisposing condition among those with established causative genes \[80\]. To date, nine mismatch repair (MMR) genes have been identified: *MSH1*, *MSH2*, *MLH1*, *MLH2*, *MLH3*, *MLH4*, *MLH5*, *MLH6*, *PMS1*, *PMS2*, and *PMS3*. At least four of these genes have been associated with HNPCC (*MLH1*, *MLH2*, *MLH6*, and *PMS2*) with the former accounting for the majority of mutations in HNPCC \[45\]. Effective mismatch repair is sufficiently provided by one functioning wild-type allele. Most often, HNPCC patients have a germline mutation in one of the key MMR genes causing them to be heterozygous for the wild-type allele. After
a second somatic mutation, deletion or epigenetic silencing of the remaining wild-type allele, both alleles have become inactivated. When this event occurs, the rate of somatic mutation increases in the absence of MMR mechanisms and microsatellite instability (MSI) occurs.

2.1.2.2 Environmental Factors

Various environmental factors like drugs, alcohol, physical activity and diet influence the development of CRC (Table 2.1). Obesity, frequent consumption of saturated and animal fats, red meat, processed foods, simple sugars, and alcohol are all associated with increased risk of CRC. Conversely, consumption of fiber and calcium and are all associated with decreased risk of developing CRC\(^{[81-84]}\). Insulin resistance has been hypothesized as a possible link between obesity and CRC\(^{[85]}\). Although not uniform, these studies show that elevated serum glucose and insulin, and markers of insulin resistance, have been positively associated with CRC as well as colorectal adenomatous polyps with some studies also suggesting that metabolic syndrome also plays a role\(^{[86-90]}\).

Table 2.1 Food, Nutrition, Physical Activity, and Colorectal Cancer – Risk Factors. Adapted from and reproduced with permission from the World Cancer Research Fund International\(^{[91, 92]}\)

<table>
<thead>
<tr>
<th>Evidence</th>
<th>↓ Risk</th>
<th>↑ Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convincing</td>
<td>Physical activity, dietary fiber</td>
<td>Red meat, processed meat, alcoholic drinks (men), body fatness, abdominal fatness, adult attained height</td>
</tr>
<tr>
<td>Probable</td>
<td>Garlic, milk, and calcium</td>
<td>Alcoholic drinks (women)</td>
</tr>
<tr>
<td>Limited – Suggestive</td>
<td>Non-starchy vegetables, fruit, vitamin D, folate, selenium, fish</td>
<td>Iron, cheese, animal fats, sugars</td>
</tr>
</tbody>
</table>
Limited – No conclusion

Cereals (grains) and grain products, potatoes, poultry, shellfish and other seafood, other diary products, total fat, fatty acid composition, cholesterol, coffee, tea, caffeine, total carbohydrate, vitamin A, retinol, vitamin C, vitamin E, multivitamins, non-dairy sources of calcium, methionine, beta-carotene, lycopene, meal frequency, energy intake

2.1.3 Stages of CRC

The various stages (TNM and Dukes) and specific characteristics of each stage are summarized in Table 2.2

TABLE 2.2  Stages and specific characteristics of CRC, permission obtained from publisher (Informa Healthcare) [93]

<table>
<thead>
<tr>
<th>Tumor Stage</th>
<th>Specific Characteristics</th>
<th>5-year Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0 or Carcinoma in situ</td>
<td>Intraepithelial or invasion of the lamina propria with no extension through the muscularis mucosae into the submucosa (Tis) No regional lymph node metastasis (N0) No distant metastasis (M0)</td>
<td>90%</td>
</tr>
<tr>
<td>Stage 1 or Dukes’ A</td>
<td>Invasion of the submucosa and/or muscularis propria (T1-T2) No regional lymph node metastasis (N0) No distant metastasis (M0)</td>
<td>90%</td>
</tr>
<tr>
<td>Stage 2A or Dukes’ B1</td>
<td>Tumor invades through the muscularis propria into the subserosa, or into nonperitonealized pericolic or perirectal tissues (T3) No regional lymph node metastasis (N0) No distant metastasis (M0)</td>
<td>75-85%</td>
</tr>
<tr>
<td>Stage 2B or Dukes’ B2</td>
<td>Tumor directly invades other organs or structures by way of the serosa, and/or perforates visceral peritoneum (T4) No regional lymph node metastasis (N0) No distant metastasis (M0)</td>
<td>75-85%</td>
</tr>
<tr>
<td>Stage 3A or Dukes’ C1</td>
<td>Invasion of the submucosa and/or muscularis propria (T1-2) Metastasis in 1 to 3 regional lymph nodes (N1) No distant metastasis (M0)</td>
<td>40-60%</td>
</tr>
<tr>
<td>Stage 3B or Dukes’ C2</td>
<td>Tumor invades through muscularis propria into the subserosa, or into nonperitonealized pericolic or perirectal tissues (T3) and/or Tumor directly invades other organs or structures by way of the serosa, and/or perforates the visceral peritoneum (T4) Metastasis in 1 to 3 regional lymph nodes (N1) No distant metastasis (M0)</td>
<td>40-60%</td>
</tr>
<tr>
<td>Stage 3C or Dukes’ C3</td>
<td>Any level of tumor invasion (any T) Metastasis in 4 or more regional lymph nodes (N2) No distant metastasis (M0)</td>
<td>40-60%</td>
</tr>
<tr>
<td>Stage 4 or Dukes’ D</td>
<td>Any level of tumor invasion (any T) Any level of metastasis to regional lymph nodes (any N) Distant metastasis (M1)</td>
<td>5-7%</td>
</tr>
</tbody>
</table>

2.1.4 Treatment

There are three types of standard treatment for cancer patients: surgery, chemotherapy, and radiation therapy (Table 2.3). Surgery is the treatment of choice for all stages of CRC for both curative and palliative purposes. Chemotherapy can be administered systemically or site-specifically which will depend on the type and stage of CRC. Radiation therapy is only given in a site-specific manner. In the later stages of CRC, biological therapy can take the form of administration of monoclonal antibodies against epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF). These antibodies are meant to slow down cell proliferation and angiogenesis of the tumor. The specific treatment options that would be most beneficial are individualized and depend on the stage of the cancer, whether it is a recurrence, and the general health of the patient [28]. The treatment options for the various stages of CRC are summarized in Table 2.3 [28]. It is important to note that all colon cancer chemotherapy regimens are based on 5FU.
Table 2.3  Summary of treatment options for CRC by stage of disease. Adapted and reproduced with permission from the National Cancer Institute [28]

<table>
<thead>
<tr>
<th>Stage</th>
<th>Treatment</th>
</tr>
</thead>
</table>
| 0     | Local excision or simple polypectomy with clear margins  
Colon resection for larger lesions not amenable to local excision |
| 1     | Wide surgical resection |
| 2a & 2b | Wide surgical resection  
Following surgery, patients may be considered for clinical trials evaluating systemic or regional chemotherapy, radiation, or biological therapy  
Adjuvant therapy is typically not used for most patients |
| 3a & 3b | Wide surgical resection  
Chemotherapy regimens (FOLFOX4, Mayo Clinic, Levamisole, Capecitabine) |
| 4     | Surgical resection of locally recurrent cancer  
Surgical resection/anastomosis or bypass of obstructing or bleeding primary lesions  
Surgical resection of isolated metastases (liver, lung, ovaries)  
Chemotherapy regimens (eg, FOLFOX4, FOLFIRI, CAPOX, etc)  
Monoclonal antibodies against EGFR and VEGF  
Palliative radiation therapy  
Palliative chemotherapy |

2.2 Cancer Chemotherapy

2.2.1 5-Fluorouracil and Leucovorin

5-Fluorouracil (5FU) is a fluorinated pyrimidine and plays a vital role in CRC treatment. 5FU acts in via 3 major mechanisms (Figure 2.3).

1. Formation of a Ternary structure that inhibits TS activity

A major method of 5FU’s action is by inhibiting thymidylate synthase (TS), a key enzyme that is required for the de novo synthesis of thymidylate, a substrate that is required for DNA synthesis and replication [94-96]. After entering the cell, 5FU is converted to 5-fluoro-2’-deoxyuridine-5’-monophosphate (FdUMP) which subsequently forms a ternary complex with TS and its cofactor 5,10-methylenetetrahydrofolate. The irreversible formation of this
complex results in TS inactivity thereby stopping production of 2-deoxythymidine-5′-triphosphate (dTTP) which then inhibits DNA synthesis \(^{[95, 96]}\).

2. Incorporation into DNA, causing DNA damage

Secondly, FdUMP can be phosphorylated to become 5-fluoro-2′-deoxyuridine-5′-triphosphate (FdUTP) which can be used as a substrate by mammalian DNA polymerase which results in misincorporation of FdUTP into DNA. Under normal circumstances, misincorporated nucleotides can be removed by cellular DNA repair machinery. However, FdUTP is not an ideal substrate for uracil-DNA glycosylase \(^{[94]}\). Additionally, the depletion of dTTP pools as a result of TS inactivation, leads to further impairment of DNA repair systems. The results of FdUTP misincorporation into DNA are inhibition of DNA synthesis via single and double stranded DNA breaks.

3. Incorporation into RNA and disruption of RNA processing

Lastly, 5FU can be converted to 5-fluorouridine-5′-triphosphate (FUTP) which is extensively incorporated into RNA thereby disrupting normal RNA processing and function. It inhibits the processing of pre-rRNA to mature rRNA, disrupts the post-transcriptional modification of tRNAs, and inhibits the proper splicing of mRNA \(^{[95]}\). Therefore, the collective function of 5FU by multiple mechanisms, is to hinder DNA synthesis and repair mechanisms in order to induce apoptosis, cell cycle arrest and consequently tumor growth inhibition.

5FU is typically administered with leucovorin (LV; 5-formylTHF), a reduced folate and a precursor to 5,10-methyleneTHF which acts to stabilize the binding of 5FU to TS thereby increasing the stability of the ternary complex. Therefore, LV actually enhances the inhibition of DNA synthesis \(^{[97]}\). In patients with advanced CRC, 5FU and LV treatment
reduced tumor size by 50% or more in 20% of patients. The drug regimen also prolonged their median survival to 11 months from 6 months without treatment \(^9_{98}\).

**Figure 2.3** Metabolism of 5-Fluorouracil \(^9_{95}\). Reproduced with permission from the Nature Publishing group.

5FU can go through several metabolic pathways in order to act on its target. 5FU can be converted to FdUMP in order to inhibit TS thereby inhibiting thymidylate production and causing DNA damage. 5FU can be converted to FdUTP which directly incorporates into DNA thereby causing DNA damage. 5FU can also be converted to FUTP which directly incorporates into RNA thereby causing RNA damage.


2.2.2 Other Approved Chemotherapeutic Agents Used In Colon Cancer

While 5FU has remained the backbone of treatment for colon cancer for the last 40 years, in recent years, new drugs have been approved for colon cancer such as Irinotecan,
Oxaliplatin, Capecitabine, Bevacizumab, Cetuximab, and Panitumumab. Irinotecan is a prodrug that exerts its cytotoxic effect through its interaction with the topoisomerase 1 enzyme \(^{[99,100]}\). Topoisomerase 1 is vital for the uncoiling of DNA during replication and transcription. During this time, single-strand DNA breaks can occur and are normally transient and repaired. However, Irinotecan is able to stabilize these strand breaks thereby causing DNA fragmentation and subsequent cell death \(^{[99,100]}\). Oxaliplatin is a third-generation platinum-based chemotherapeutic agent with a 1,2-diaminocyclohexane (DACH) carrier ligand. The retention of the bulky DACH ring by activated oxaliplatin is thought to result in the formation of bulky platinum-DNA adducts, which are effective at blocking DNA replication thereby inducing cellular apoptosis \(^{[101]}\). Capecitabine is a prodrug that is selectively tumor-activated to its cytotoxic moiety, fluorouracil, by thymidine phosphorylase, an enzyme found in higher concentrations in many tumors compared to normal tissues or plasma. From this point, Capecitabine acts as 5FU and undergoes the same metabolism and same mechanism to cause tumor growth inhibition \(^{[102]}\).

Bevacizumab consists of monoclonal antibodies that prevent or reduce angiogenesis thereby preventing or hindering cancer progression by binding to VEGF, a vital protein that stimulates angiogenesis. This prevents the interaction of VEGF to its receptors (Flt-1 and KDR) on the surface of endothelial cells which prevents blood vessel proliferation and subsequent tumor metastasis \(^{[103]}\). Cetuximab consists of monoclonal antibodies that bind to (EGFR) on both normal and tumor cells. EGFR is over-expressed in many colorectal cancers. Cetuximab competitively inhibits the binding of epidermal growth factor and TGF alpha, thereby reducing their effects on cell growth and metastatic spread \(^{[104]}\).
Panitumumab consists of monoclonal antibodies that bind specifically to EGFR on both normal and tumor cells, and competitively inhibits the binding of ligands for EGFR. Binding of panitumumab to the EGFR prevents ligand-induced receptor autophosphorylation and activation of receptor-associated kinases, resulting in inhibition of cell growth, induction of apoptosis, decreased pro-inflammatory cytokine and vascular growth factor production, and internalization of the EGFR\[^{105}\]. While all of these additional chemotherapeutic agents were found to be effective either alone or in combination with 5FU, they remain an addition to the backbone of 5FU chemotherapy for colon cancer.

2.2.3 5-Fluorouracil Resistance and Mechanisms

There are many possible forms and corresponding mechanisms for 5FU resistance. 5FU has to undergo a multitude of steps in order to act properly at its targets. Resistance can occur at virtually any step of 5FU metabolism if there are abnormalities with the pathway. In short, any mutation, aberration, or change in the pathway that prevents 5FU from entering, staying or reacting in the cell can cause 5FU resistance\[^{95}\]. One well documented mechanism of resistance lies in 5FU’s main enzymatic target: TS. In circumstances where TS is overexpressed, 5FU resistance can occur. For example, in a study by Longley et al., they induced the overexpression of TS using inducible promoters and they found a two fold increase in the IC\(_{50}\) of 5FU indicating that an increase in TS expression was associated with 5FU resistance\[^{106}\]. Additionally, it has been shown that mutations that alter the structure of TS negatively affect 5FU’s chemotoxicity\[^{107}\]. In a study by Tong et al., it was found that mutations in several highly conserved regions
(Lys47Glu, Asp49Gly, Gly52Ser) of TS were associated with antifolate resistance but were also cross resistant to 5FU, it is likely that the mutations prevented efficient binding of 5FU thereby lowering its toxicity \[^{108}\]. Additionally, site specific mutagenesis of these conserved regions confirmed that mutations in TS could confer resistance \[^{109}\]. Another potential mechanism of resistance is via cellular efflux transporters such as breast cancer resistance protein (BCRP) and the multidrug resistance protein (MRP) family that have the ability to transport antifolates as well as 5FU out of the cell \[^{29}\]. By increasing the expression or activity of these drug efflux transporters, 5FU metabolites are more effectively shuttled out of the cell and therefore cannot act on their targets, thereby causing resistance \[^{110}\].

2.3 Folate

2.3.1 Chemistry of Folate

Folate, also known as folacin or vitamin B\textsubscript{9}, is a general term used to describe a family of structurally and functionally similar compounds. Folic acid is monoglutamated and is the fully oxidized form of this vitamin which can be found in supplements as well as enriched and fortified foods. Folic acid consists of 3 distinct moieties: a 2-amino-4-hydroxypteridine (pterin) ring connected via a methylene bridge to para-aminobenzoic acid (PABA), which is peptide bound to glutamate (\textbf{Figure 2.4}) \[^{111}\]. Despite being able to synthesize all of the individual moieties, mammals lack the enzyme required to couple the pterin ring to PABA and because of this, they are unable to synthesize folate \textit{de novo} \[^{111}\]. Therefore, with
the exception being able of absorb some of the folate synthesized by intestinal microflora, mammals must obtain folate from their diet, in food or supplement form \([112,113]\).

![Figure 2.4](image)

\textbf{Figure 2.4} The chemical structure of tetrahydrofolate (THF) and its related derivatives, reproduced with permission from Elsevier \([2]\)

Folates found in food differ from folic acid in several different ways: the pteridine ring of folate is reduced when compared to folic acid, substituents like methyl donors at the N-5 and N-10 positions differ, and the number of glutamate residues can vary. While many forms of naturally occurring food folate exist, the primary ones include 5-methyltetrahydrofolate (5-methylTHF) and 10-formyltetrahydrofolate (10-formylTHF) \([114]\). Natural folates are very unstable and easily oxidized thereby losing their activity in foods over a period of days or weeks \([115]\). The steps of food processing from harvesting, storage, processing, and preparation can cause a loss of approximately 50-75% of food folate. Additional factors that alter bioavailability include the source of food as well as cooking method. On the other hand, folic acid is extremely stable due to its fully oxidized pterin ring, leading it to be very bioavailable, particularly on an empty stomach.
2.3.2 Folate Metabolism

Absorption of folate can occur along the entire small intestine but is most efficient in the jejunum\textsuperscript{[116]}. The majority of dietary folates are polyglutamated and therefore hydrolysis into their monoglutamated forms is required as folates with more than two glutamates are unable to cross the brush border membrane\textsuperscript{[117]}. This hydrolysis occurs in the lumen and is catalyzed by an apical brush border membrane enzyme called glutamate carboxypeptidase II (GCPII)\textsuperscript{[118]}. After entry into the enterocyte, folate is reduced to its biologically active tetrahydro form by dihydrofolate reductase (DHFR) and then methylated to 5-methyl THF by methylenetetrahydrofolate reductase (MTHFR) using 1,5-dihydro-flavin adenine dinucleotide (FADH\textsubscript{2}) as an electron donor which is then subsequently released into the portal circulation where it can reach target cells\textsuperscript{[117]}.

There are four different transport systems that mediate the cellular uptake of folate (Figure 2.5).

1. Reduced-folate carrier (RFC) is a saturable anion dependent membrane carrier that has a greater affinity for reduced folates (K\textsubscript{m}=1-5 \textmu M) versus folic acid (K\textsubscript{m}=100-200 \textmu M) and is ubiquitously in normal epithelial cells and cancer of epithelial origin\textsuperscript{[116, 119]}

2. Folate receptor (FR) has multiple isoforms (\(\alpha, \beta, \gamma\)) and is a specific folate binding protein that is often found on cell membranes. FR\(\alpha\) is the primary isoform involved in folate transport in epithelial membranes, it is moderately expressed in certain normal epithelial cells and its expression is markedly increased in several carcinomas such as cervical, breast and ovarian\textsuperscript{[119, 120]}. FR is found anchored to cell membranes
and facilitates uptake of folates by endocytosis. These receptors have a high affinity for many folates, particularly folic acid (Km<1 nm) and a less but still high affinity for 5-methylTHF (Km=3 nm) [119].

3. The proton-coupled folate transporter (PCFT) is a carrier-mediated folate transporter that mediates the absorption of folate in a low-pH environment characteristic of the small intestine. It is also widely expressed in other tissues and in solid tumors [121]. Additionally, the PCFT has similar affinities for both reduced (5-methyl THF, 5-formyl THF) and oxidized (folic acid) folates (Km=0.5-1.0 μM) [121].

4. Passive diffusion is another method of cellular folate entry, however, this has only been reported to occur in pharmacological doses of folate [116, 121].

In the circulation, folate exists exclusively in its monoglutamated forms with 5-methyl THF as the predominant form (Figure 2.5) [111]. On the other hand, intracellular folates can be elongated via the addition of up to nine glutamate residues via folypolyglutamate synthase (FPGS). Polyglutamation via FPGS acts as a cellular retention mechanism and a form of metabolic trapping to prevent efflux loss from the cell [117]. In addition to its metabolic trapping function, polyglutamation is also necessary for conversion of folates into their active coenzyme form. These polyglutamated folates serve as better substrates for folate dependent enzymes when compared to their monoglutamated counterparts [117].

Folate efflux can only occur after hydrolysis of the polyglutamates back into monoglutamate form which is catalyzed by Υ-glutamyl hydrolase (GGH). This allows folate to be transported out of the cell by RFC and PCFT [117].
2.3.3 Biochemical Roles of Folate

Folate has two major biochemical functions. One is mediating the transfer of one-carbon units involved in the methionine cycle, and biological methylation reactions \[^2\]. In the methionine cycle, 5-methyl THF transfers one methyl group to homocysteine to synthesize methionine \[^{122}\]. Methionine reacts with ATP to form S-adenosylmethionine (SAM) which is the primary methyl group donor for most biological methylation reactions.
including DNA\textsuperscript{5}. The remethylation of homocysteine to methionine is catalyzed by methionine synthase (MTR)\textsuperscript{2}. The second major function is in the nucleotide biosynthesis pathway. Folate enters the cell in the form of 5-methyl THF and is reduced by the enzyme methionine synthase reductase (MTRR) to THF\textsuperscript{2}. It is subsequently converted to 5,10-methyleneTHF by serine hydroxymethyltransferase (SHMT) which catalyzes the reversible interconversion of serine and THF to glycine and 5,10-methylene THF. 5,10-methylene THF is the methyl donor for the nonreversible methylation, catalyzed by thymidylate synthase (TS), of deoxyuridine-5-monophosphate (dUMP) to deoxythymidine-5-monophosphate (dTMP) which is a precursor of DNA synthesis\textsuperscript{2}. 5,10-methylene THF is then oxidated by dTMP to form dihydrofolate (DHF). DHF is metabolically inactive and is transformed to the metabolically active THF via DHF reductase (DHFR) activity\textsuperscript{123}. Both THF and 5,10-methylene THF can enter the purine biosynthesis pathway by the addition of a formyl group, further emphasizing folate’s role in nucleotide biosynthesis\textsuperscript{(Figure 2.6)}\textsuperscript{122}. The purine biosynthesis pathway consists of ten stepwise reactions that serve to convert phosphoribosyl pyrophosphate to inosine monophosphate, some enzymes of note are glycaminidate ribonucleotide formyltransferase (GARFT) and aminomimidazole carboxamid ribonucleotide formyltransferase (AICARFT) which serve as targets in certain antifolate drugs like methotrexate and permetrexed\textsuperscript{124}. 
Given the vital role folate plays in mediating these reactions, maintaining normal intracellular folate is important to ensure normal cell proliferation, maintain cellular integrity and essential cellular functions. A deficiency of folate may lead to several adverse outcomes including the misincorporation of uracil into DNA, DNA strand breaks, and impaired de novo purine synthesis and overall DNA instability, which can predispose cells to neoplastic transformations \(^{3,125}\). Folate is required as a cofactor in the successful conversion of dUMP to dTMP. Folate deficiency can cause an increase in the dUMP/dTMP ratio by lowering TS activity. As a result, DNA polymerase misincorporates uracil thereby prompting uracil-DNA glycosylase to excise the misincorporated uracil molecule. This enzyme causes transient single strand breaks which can result in a double stranded break if
these “nicks” are on opposing sides. Additionally, folate deficiency can lead to a lack of methyl groups which are required for biological methylation reactions \cite{123}. Without these reactions taking place, an accumulation of homocysteine, deficiency in intracellular SAM levels and buildup of SAH could lead to DNA hypomethylation with consequent functional ramifications \cite{123,125}. Global DNA hypomethylation of the coding and noncoding regions and demethylation of repetitive DNA sequences contribute to the development of cancer through the following mechanisms: chromosomal instability, increased mutations, mitotic recombination leading to loss of heterozygosity and promotion of rearrangements, aneuploidy, loss of imprinting; and up-regulation of protooncogenes \cite{2}.

2.3.4 Folate and Health

For quite some time, folate has been considered an ideal functional food component for disease prevention \cite{23,126}. There have been a number of observational epidemiological studies that have reported an association between folate deficiency and a risk of neural tube defects (NTDs), adverse pregnancy outcomes, neuropsychiatric disorders, anemia, atherosclerosis, and several types of cancer \cite{3,127-129}. As a result of these associations, many intervention studies were implemented to evaluate the benefits of folate for various diseases and health conditions. With the exception of NTD’s and anemia, folate supplementation did not clearly demonstrate any beneficial effects on cardiovascular disease and cognitive disorders. In many studies investigating cancer incidence as a secondary outcome, it was found that the risk of developing cancer was unchanged in the majority of studies, however, in some studies, a cancer promoting effect was observed \cite{130-}. 

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Additionally, there is evidence that suggests that folic acid supplementation could be associated with certain adverse outcomes including resistance to anti-folate based chemotherapy, decreased natural killer cell cytotoxicity and cancer promotion\textsuperscript{[133-135]}. In particular, a study by Mason et al., indicated the temporal relationship between folic acid supplementation in both USA and Canada. Interestingly, they found that there was an increase in 4-6 cases per 100000 individuals in 1998 when compared to the downwards trend prior to fortification\textsuperscript{[136]}. They suggested that this increase in colorectal cancer incidence could be wholly or partially as a result of the increased intake of folic acid as a result of fortification. As the body of evidence grows, it is clear that the relationship between folate and disease prevention is more complex than previously thought. While the data is not uniformly consistent, folic acid might have a potential tumor promoting effect.

2.3.4.1 Folate Intake and Requirements

Dietary reference intakes for folate are measured in terms of dietary folate equivalents (DFE). This adjusts for the nearly 50% lower bioavailability of food folate compared to folic acid\textsuperscript{[115]}. One DFE is equal to 1 μg of food folate, 0.6 μg of folic acid from fortified foods or supplements taken with food, and 0.5 μg of a supplement taken on an empty stomach. In North America, the recommended daily allowance (RDA) for folate is 400 μg/day of DFE for both men and women\textsuperscript{[115]}. The RDA increases to is 600 and 500 μg/day of DFE for pregnant and lactating women respectively, to meet the increased demands of the developing fetus and newborn child. While the exact safe upper limit (UL) of folate intake is unknown, general guidelines recommends no more than 1000 μg/day of
folic acid from supplements and fortified foods for men and women, including pregnant and lactating women. This level is deemed to be unable to mask vitamin B12 deficiency[115].

2.3.4.2 Folic Acid Fortification and Guidelines

Despite some controversy surrounding the health benefits of folate, the relationship between folate supplementation and decreased incidence of NTDs is considered one of the greatest public health successes. Using experimental and observational studies, the protective effect of folic acid supplementation during the periconceptional period against NTD development in the offspring has been clearly demonstrated[13,137-139]. In response to the overwhelming evidence, Canada and the United States mandated folic acid fortification of all white flour, cornmeal and enriched pasta starting in 1998[139]. White flour and cornmeal were fortified with 140-150 μg folic acid, while enriched pasta was fortified with 200 μg folic acid, per 100 g of food product. The goal of the fortification program was to increase daily consumption of folate by 100 μg/day or 30-70% the average folate intake among women of childbearing age without posing a risk to the general public[140]. Additionally, Health Canada and the Institute of Medicine recommend that all women who can become pregnant should consume 400 μg of folic acid from supplements or multivitamins in addition to food folate[115,139]. Since the implementation of fortification, NTDs have dramatically declined in both Canada and the United States (15-50%)[141-143]. A Canadian population based study reaffirmed the dramatic effect by demonstrating a decrease of 46% in the prevalence of NTDs from before fortification to full-fortification[140]. Guidelines are shown in Table 2.4.
Table 2.4  Recommended daily allowance for folate instituted by the Institute of Medicine \textsuperscript{[115, 144]}

<table>
<thead>
<tr>
<th>Population</th>
<th>Children</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-3</td>
<td>4-8</td>
</tr>
<tr>
<td>Age or demographic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDA (μg DFE/day)</td>
<td>150</td>
<td>200</td>
</tr>
</tbody>
</table>

2.3.4.3 Folate Status of North American Population

After mandatory folic acid fortification was implemented, blood levels of folate have increased compared to pre-fortification concentrations. Blood homocysteine levels, an accurate inverse indicator of folate status, have also significantly declined. In Ontario, a relative decline of between 59% and 77% in the rate of folate insufficiency (serum folate < 2 ng/mL or red blood cell folate < 124 nmol/L) was observed among adults \textsuperscript{[145]}. These increases in blood folate were mimicked in the United States. According to the National Health and Nutrition Examination Survey (NHANES), between 1988 and 2006, serum and RBC folate concentrations increased by 119-161% and 44-64% respectively (\textbf{Figure 2.7}) \textsuperscript{[15, 146]}. 

- 31 -
The success of folic acid fortification in improving folate status and reducing NTD rates is considered a public health triumph and provides a paradigm of collaboration between science and public health policy. Recent data from Canada suggests that folate deficiency in the Canadian population is virtually non-existent (Figure 2.8)\textsuperscript{[18]}.

**Figure 2.7** Median serum and RBC folate levels (ng/ml) in the U.S. population from 1988-2006\textsuperscript{[15, 146]}. Permission for reprint obtained from publisher (NCHS).

**Figure 2.8** Cumulative percentile distributions of red blood cell folate concentrations by age group among female participants in the Canadian Health Measures Survey, 2007–2009.
Folate concentrations for deficiency (305 nmol/L) and high folate concentrations (1360 nmol/L) are indicated by vertical lines \[18\]. Less than 1% of Canadians showed folate deficiency and 40% showed high folate concentrations. Copied under license from the Canadian Medical Association and Access Copyright.

2.3.4.4 Adverse Effects of High Folate Status

In general, folate has been regarded to be safe and has been shown to be vital for good health \[134\]. However, there are certain subgroups of the population that may have potential adverse effects due to the ingestion of folic acid containing supplements and fortified food products. For example, in the elderly population (>65 years of age), there is great concern that high levels of folic acid may mask vitamin B-12 deficiency which may lead to a delayed diagnosis and sub-acute degeneration of the spinal cord \[135\]. This masking is a result of the overlap in metabolic functions of folate and vitamin B12. Methionine synthase is vitamin B12 dependent and also functions in the folate cycle. This cycle is necessary to maintain the supply of the methyl donor S-adenosylmethionine, and if interrupted, it results in a reduction in a wide range of methylated products. One such important methylation product is that of myelin basic protein. Its reduction, as is seen in pernicious anemia and other causes of vitamin B12 deficiency, produces sub-acute combined degeneration of the spinal cord. Therefore, there is concern that as a result of increased folate status, one may not be able to see any clear signs of vitamin B12 deficiency such as anemia \[147\]. However, more recent studies have shown that consumption of fortified products and/or supplements contributes significantly to both folic acid and vitamin B-12 intake, making the prevalence of B-12 deficiency in all US adults, including the elderly, to be less than 0.1% \[148\]. High doses of folic acid (~ 200 μg) can also result in
unmetabolized folic acid in the serum\textsuperscript{[149,150]}. Due to the high levels of folic acid, the conversion from folic acid to reduced folate is overwhelmed and becomes the rate limiting step. This ultimately manifests itself as a build-up of unmetabolized folic acid in the serum which does not occur after consumption of naturally occurring food folate\textsuperscript{[149]}. Additionally, high levels of folic acid may interfere with the actions of antifolate drugs such as methotrexate, trimethoprim, pyrimethamin, sulfasalzine, colchincine, and trimetrexate\textsuperscript{[126]}. Other reported adverse effects include: epigenetic instability, decreased natural killer cell cytotoxicity, interference with zinc homeostasis and genetic selection of disease alleles (eg MTHFR C677T)\textsuperscript{[126,135]}. Furthermore, evidence from animal studies suggests that folic acid supplementation may promote the progression of (pre)neoplastic lesions to full blown cancer, which will be discussed in detail in section 2.4.2. In theory, high levels of serum unmetabolized folic acid could interfere with the metabolism, cellular transport, and regulatory functions of the natural folates by competing with the reduced forms for binding with enzymes, carrier proteins, and binding proteins\textsuperscript{[135]}. Despite the indisputable benefits of the folic acid fortification program in reducing the incidence of NTDs, there remains controversy surrounding the health benefits of folic acid fortification and supplementation. This is in part due to the inconsistency observed in the body of scientific evidence. It is likely that folic acid fortification and supplementation may benefit some with certain diseases and conditions, while being detrimental to others.
2.4 Folate and Colon Cancer

2.4.1 Epidemiological evidence

The results from epidemiological studies have not been consistent. Some of these studies support an inverse relationship between folate and the risk of CRC, where a deficiency increases and supplementation decreases the risk. Many epidemiological studies have explored this relationship between folate intake and the risk of CRC. The majority of these studies use dietary and total folate intake, including supplemental folic acid intake and the relationship between blood measures of folate and the risk of CRC or adenomas. Overall, these retrospective studies suggest a modest decrease in the OR of colorectal neoplasms in subjects with the highest intakes of folate compared to the lowest intakes. However, the data is not uniformly consistent, which may suggest that the relationship between folate and CRC is more complex than a simple inverse relationship.

Table 2.5 Summary of the case control studies of folate and colon cancer

<table>
<thead>
<tr>
<th>Study Ref.</th>
<th>Study Period</th>
<th>Location</th>
<th>Form of Folate (µg/day)</th>
<th>Gender</th>
<th>OR, HR (95% CI)</th>
<th>p-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freudenheim et al.</td>
<td>1975-1986</td>
<td>United States</td>
<td>Dietary &lt;210 vs &lt;340</td>
<td>Men &amp; Women</td>
<td>1.12 (0.66,1.19)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Ferraroni et al.</td>
<td>1985-1992</td>
<td>Italy</td>
<td>Dietary &lt;162 vs &gt;261.5</td>
<td>Men &amp; Women</td>
<td>0.52 (0.40,0.68)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Boutron Ruault et al.</td>
<td>1985-1990</td>
<td>France</td>
<td>Dietary &lt;309 vs &gt;359</td>
<td>Men &amp; Women</td>
<td>1.00 (0.50,2.00)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Slattery et al.</td>
<td>1991-1994</td>
<td>United States</td>
<td>Dietary &lt;120 vs &gt;210 per 1000</td>
<td>Men &amp; Women</td>
<td>1.2 (0.90,1.60)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
Levi et al. 2000\cite{155} & 1992-1997 & Switzerland & Dietary <173.8 vs >403 & Men & Women & 1.54 (0.77,3.10) & N.S.

LaVecchia et al. 2002\cite{156} & 1992-1996 & Italy & Dietary <197.6 vs >330.8 & Men & Women & 0.72 (0.61,0.85) & p<0.01

Le Marchand et al. 2002\cite{157,158} & 1994-1998 & United States & Dietary <252 vs >406 & Men & Women & 0.90 (0.62,1.30) & N.S.

Otani et al. 2005\cite{158} & 1998-2002 & Japan & Dietary <343 vs >485 & Men & Women & 1.30 (0.50,3.40) & N.S.

Kune et al. 2005\cite{159} & 1980-2981 & Austrailia & Dietary <246 vs >1367 & Men & Women & 1.24 (0.81,1.89) & N.S.

Murtaugh et al. 2007\cite{160} & 1997-2001 & United States & Plasma <441 vs >743 & Men & Women & 0.66 (0.47,0.92) & p<0.05

Kim et al. 2009\cite{161} & 2001-2004 & Korea & Dietary <179 vs >270 & Men & Women & 0.47 (0.32,0.69) & p<0.01

Glynn et al. 1996\cite{162} & 1988-1992 & Finland & Serum IQR 2.9-5.2 ng/ml & Men & 0.92 (0.42,2.00) & N.S.

Kato et al. 1999\cite{163} & 1985-1991 & United States & Serum <12.23 vs >31.04 nM & Women & 0.52 (0.27,0.97) & p<0.05

<table>
<thead>
<tr>
<th>Study Ref.</th>
<th>Study Period</th>
<th>Location</th>
<th>Form of Folate (μg/day)</th>
<th>Gender</th>
<th>RR, HR (95% CI)</th>
<th>p-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su et al. 2000\cite{129}</td>
<td>1975-1993</td>
<td>United States</td>
<td>Dietary &lt;103.3 vs &gt;249</td>
<td>Men &amp; Women</td>
<td>0.57 (0.34,0.97)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Harnack et al. 2002\cite{164}</td>
<td>1986-1998</td>
<td>United States</td>
<td>Dietary &lt;231 vs &gt;634</td>
<td>Women</td>
<td>1.12 (0.77,1.63)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Table 2.6 Summary of the cohort studies of folate and colon cancer
<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Country</th>
<th>Diet Measure</th>
<th>Gender</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flood et al. 2002</td>
<td>1995-1998</td>
<td>United States</td>
<td>Dietary &lt;142 vs &gt;272 per 1000 kcal/day</td>
<td>Women</td>
<td>0.86 (0.65,1.13)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Zhang et al. 2005</td>
<td>1992-2004</td>
<td>United States</td>
<td>Plasma &lt;259 vs &gt;614</td>
<td>Women</td>
<td>0.67 (0.44,1.03)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Ishihara et al. 2007</td>
<td>1990-1994</td>
<td>Japan</td>
<td>Dietary &lt;214 vs &gt;530</td>
<td>Men &amp; Women</td>
<td>1.20 (0.91,1.58)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>De Vogel et al. 2008</td>
<td>1986-2000</td>
<td>Netherlands</td>
<td>Dietary &lt;169 vs &gt;297</td>
<td>Men &amp; Women</td>
<td>0.93 (0.72,1.20)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Kabat et al. 2008</td>
<td>2000-2004</td>
<td>Canada</td>
<td>Dietary &lt;237 vs &gt;374</td>
<td>Women</td>
<td>0.89 (0.71,1.12)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Larsson et al. 2005</td>
<td>1987-2004</td>
<td>Sweden</td>
<td>Dietary &lt;150 vs &gt;212</td>
<td>Women</td>
<td>0.80 (0.47,1.36)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Schernhammer et al. 2008</td>
<td>1980-2002</td>
<td>United States</td>
<td>Dietary &lt;200 vs &gt;400</td>
<td>Men &amp; Women</td>
<td>0.75 (0.59,0.96)</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Giovannucci et al. 1995</td>
<td>1986-1992</td>
<td>United States</td>
<td>Dietary &lt;269 vs &gt;646</td>
<td>Men</td>
<td>0.86 (0.54,1.36)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Giovannucci et al. 1998</td>
<td>1980-1994</td>
<td>United States</td>
<td>Dietary &lt;200 vs &gt;400</td>
<td>Women</td>
<td>0.69 (0.52,0.93)</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Su and Arab 2001</td>
<td>1982-1992</td>
<td>United States</td>
<td>Dietary &lt;102.3 vs &gt;249</td>
<td>Men</td>
<td>0.40 (0.18,0.88)</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Terry et al. 2002</td>
<td>1980-1985</td>
<td>Canada</td>
<td>Dietary &lt;233 vs &gt;367</td>
<td>Women</td>
<td>0.6 (0.4,1.1)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Konings et al. 2002</td>
<td>1986-1992</td>
<td>Netherlands</td>
<td>Dietary &lt;168 vs &gt;266</td>
<td>Men &amp; Women</td>
<td>0.73 (0.46,1.17)</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Wei et al.</td>
<td>1976-1984</td>
<td>United States</td>
<td>Dietary &lt;200 vs &gt;200</td>
<td>Men &amp; Women</td>
<td>0.82 (0.66,1.03)</td>
<td>N.S.</td>
<td></td>
</tr>
</tbody>
</table>
In summary, evidence from epidemiological studies is inconsistent. Five out of sixteen studies showed a significant reduction in OR or HR. However, eleven out of sixteen studies showed no significant relationship between folate and OR or HR.

2.4.2 Evidence from Animal Models

Several animal models using chemical carcinogens and genetically predisposed rodent models have investigated the role between folate and CRC. The majority of these studies support a causal and inverse relationship between folate status and CRC risk. Animal studies have shown that folate possess a dual modulatory role in colorectal carcinogenesis which depends on both the timing and dose of the intervention.

**Table 2.7** Summary of studies of folic acid and CRC from chemical carcinogen animal models

<table>
<thead>
<tr>
<th>Study Ref.</th>
<th>Model</th>
<th>Dose (mg FA/kg diet)</th>
<th>Duration (weeks)</th>
<th>End Point</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cravo et al. 1992(^{175})</td>
<td>DMH (SD)</td>
<td>0, 8</td>
<td>20</td>
<td>Tumor incidence: Microadenomas</td>
<td>100% (0mg), 29% (8mg) p=0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Macroadenomas</td>
<td>86% (0mg), 43% (8mg) p=0.009</td>
</tr>
<tr>
<td>Kim et al. 1996(^{176})</td>
<td>DMH (SD)</td>
<td>0, 2, 8, 40</td>
<td>15</td>
<td>Tumor incidence: Microadenomas</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Macroadenomas</td>
<td>0mg – 70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2mg – 40%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8mg – 10%</td>
</tr>
<tr>
<td>Study</td>
<td>Model</td>
<td>Treatment</td>
<td>Dose</td>
<td>Tumor Incidence</td>
<td>Multiplicity</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------</td>
<td>-----------</td>
<td>------</td>
<td>-----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Wargovitch et al. 1996 [178]</td>
<td>AOM</td>
<td>Control, 2500, 5000</td>
<td>2</td>
<td>ACF</td>
<td>↑ vs control</td>
</tr>
<tr>
<td>Le Leu et al. 2000 [179]</td>
<td>AOM</td>
<td>0, 8</td>
<td>26</td>
<td>↑ with FA (SI &amp; colon, and SI alone)</td>
<td>NS</td>
</tr>
<tr>
<td>Le Leu et al. 2000 [180]</td>
<td>AOM</td>
<td>0, 8</td>
<td>12</td>
<td>ACF</td>
<td>↑ with FA</td>
</tr>
<tr>
<td>Lindzon et al. 2007 [181]</td>
<td>AOM</td>
<td>0, 2, 5, 8</td>
<td>34</td>
<td>NS</td>
<td>↑ with FA</td>
</tr>
</tbody>
</table>

In summary, evidence from animal studies suggest that folate can modulate colorectal carcinogenesis over a large range of dietary intakes. Modest deficiency can enhance, where FA supplementation of modest degree can suppress colorectal tumorigenesis. However, supraphysiological levels of FA may accelerate tumor progression. Thus, the effect of FA supplementation strongly depends on timing and dose of the intervention which conforms to its dual modulatory role.
2.4.3 Evidence from Human Intervention Trials

There are several small randomized, placebo controlled studies that have investigated the effect of folate supplementation on surrogate end point biomarkers (SEPB) of CRC in the colonic mucosa (Table 2.8). However, these trials do not provide conclusive evidence for the chemopreventative role of folate in CRC. Many of these trials have small sample sizes and the duration may not have been long enough to detect significant changes. Additionally, these studies use SEPB over actual CRC as an end point.

Table 2.8 Summary of randomized, double-blind, placebo-controlled clinical trials of folic acid chemoprevention of CRC. Modified from [2]

<table>
<thead>
<tr>
<th>Study Ref.</th>
<th>Participants (n)</th>
<th>Dose (mg FA)</th>
<th>Duration</th>
<th>End Point</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kim et al. 2001 [182]</td>
<td>Adenomas (n = 20)</td>
<td>5 mg/day</td>
<td>6 months and 1 year</td>
<td>Rectal mucosal genomic DNA methylation</td>
<td>57% increase in DNA methylation at 6 month (p = 0.001) but no difference at 1 year No significant difference in p53 strand breaks compared with placebo</td>
</tr>
<tr>
<td>Cravo et al. 1994 [183]</td>
<td>CRC or adenomas (n = 22)</td>
<td>10 mg/day</td>
<td>6 months</td>
<td>Rectal mucosal genomic DNA methylation</td>
<td>93% increase (p&lt;0.002)</td>
</tr>
<tr>
<td>Cravo et al. 1998 [184]</td>
<td>Adenomas (n = 20)</td>
<td>5 mg/day</td>
<td>3 months</td>
<td>Rectal mucosal genomic DNA methylation</td>
<td>37% increase in patients with 1, but not multiple, adenoma (p &lt; 0.05)</td>
</tr>
<tr>
<td>Khosraviniani</td>
<td>Adenomas</td>
<td>2 mg/day</td>
<td>3 months</td>
<td>Rectal</td>
<td>20% decrease in</td>
</tr>
<tr>
<td>Study</td>
<td>Type</td>
<td>Treatment</td>
<td>Duration</td>
<td>Outcome</td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------</td>
<td>-----------</td>
<td>----------</td>
<td>-------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Nagothu et al. 2003</td>
<td>Adenomas</td>
<td>1 mg/day</td>
<td>1 year</td>
<td>LOH of DCC, APC, p53, DCC protein expression, 100% protection of LOH of DCC (no effect on APC or p53)</td>
<td></td>
</tr>
<tr>
<td>Pufulete et al. 2005</td>
<td>Adenomas</td>
<td>400 µg/d</td>
<td>10 wk</td>
<td>Rectal mucosal genomic DNA methylation, 25% increase (p = 0.09)</td>
<td></td>
</tr>
<tr>
<td>Bruce et al. 2005</td>
<td>CRC or adenomas</td>
<td>3 mg/day</td>
<td>28 days</td>
<td>Biomarkers of insulin resistance, fecal calprotectin, C-reactive protein, 18% decrease in free fatty acid (p = 0.013); no effects on other markers</td>
<td></td>
</tr>
<tr>
<td>Biasco et al. 1997</td>
<td>Chronic UC</td>
<td>15 mg/day (folinic acid)</td>
<td>3 months</td>
<td>Rectal mucosal cell proliferation (LI), 44% decrease in LI in the upper 40% of the crypt</td>
<td></td>
</tr>
<tr>
<td>Paspatis and Karamanolis. 1994</td>
<td>Adenomas</td>
<td>1 mg/day</td>
<td>2 years</td>
<td>Adenoma recurrence, 40 and 46% reduction at 1 and 2 years, respectively (p = NS)</td>
<td></td>
</tr>
<tr>
<td>Cole et al. 2007</td>
<td>Adenomas</td>
<td>1 mg/day</td>
<td>6 years</td>
<td>Adenoma recurrence, Incidence: RR = 1.04 (95%)</td>
<td></td>
</tr>
</tbody>
</table>
In summary, the majority of the small intervention trials suggest that FA supplementation plays a preventative role in developing SEPB of CRC. However, more recent large intervention trials that use CRC occurrence or recurrence and colorectal adenomas as primary or secondary outcomes do not support this chemoprotective effect and actually show a potential tumor promoting effect.

2.4.4 Evidence from Meta-analyses

There are three pooled analysis from meta-analyses that investigated the effects of folic acid on colon cancer risk (Table 2.9). These meta-analyses consisted of observational case control and cohort studies, no randomized controlled trials were included. All three meta-analyses indicated a lower risk of developing CRC from pooling existing case control

<table>
<thead>
<tr>
<th>Study (Year)</th>
<th>Type</th>
<th>Dose (µg/d)</th>
<th>Duration (years)</th>
<th>Effect</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logan et al. 2008\textsuperscript{[192]}</td>
<td>Adenomas (n = 945)</td>
<td>500</td>
<td>3</td>
<td>Adenoma recurrence</td>
<td>No effect of folate supplementation. Any adenoma RR = 1.07 (95% CI 0.85–1.34). Advanced adenoma RR = 0.98 (95% CI 0.68–1.40).</td>
</tr>
</tbody>
</table>
and cohort studies. This data suggests that there is a slight protective effect of folic acid on colon cancer risk.

**Table 2.9** Summary of pooled analysis from meta-analyses investigating the effects of folic acid on colon cancer risk

<table>
<thead>
<tr>
<th>Study (Ref.)</th>
<th>Folate Measurement</th>
<th>Number of Studies</th>
<th>Summary RR/OR/HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kennedy et al. 2011</td>
<td>Dietary and Total intake</td>
<td>18 Case control</td>
<td>0.89 (0.74 - 1.02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 Cohort</td>
<td>0.92 (0.81-1.05)</td>
</tr>
<tr>
<td>Kim et al. 2010</td>
<td>Dietary and Total intake</td>
<td>13 Cohort</td>
<td>0.85 (0.77-0.95)</td>
</tr>
<tr>
<td>Sanjoaquin et al. 2004</td>
<td>Dietary and Total intake</td>
<td>9 Case control</td>
<td>0.76 (0.60–0.96)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 Cohort</td>
<td>0.75 (0.64-0.89)</td>
</tr>
</tbody>
</table>

### 2.4.5 Possible Mechanisms of Folate Mediated Carcinogenesis

#### 2.4.5.1 Dual Modulatory Role of Folate

Cancer develops at varying rates, but usually over long periods of time spanning decades. Folate may play an important role in several critical windows of cancer development. There has been clinical and animal studies suggest that folate possesses dual modulatory effects on cancer development and progression depending on the timing and dose of intervention (Figure 2.9) \[^{196,197}\]. It has been observed that in normal cells, modest supplemental levels of folate reduce, whereas folate deficiency increases, the risk of malignant transformation \[^{198-200}\]. In contrast, in preneoplastic and neoplastic cells, folate supplementation promotes, whereas folate deficiency suppresses, the progression of malignant transformation \[^{199,200}\]. While recent studies have enhanced our understanding of folate-mediated colorectal carcinogenesis, the specific underlying molecular
mechanism(s) remains to be elucidated. Potential mechanism(s) of folate may be explained by its two main biochemical roles, which are nucleotide biosynthesis and biological methylation.

![Diagram of Folate Deficiency and Supplementation](image)

**Figure 2.9** Dual modulatory role of folate in colon carcinogenesis. Folate deficiency in normal tissue predisposes them to neoplastic transformation, and modest supplemental levels suppress, whereas supraphysiologic doses of supplementation enhances, the development of tumors. In contrast, folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on the progression of established neoplasms. The ↑ symbol represents tumor promotion while the ↓ symbol represents tumor inhibition. Adapted from [2]. Copied under licence from the John Wiley and Sons Copyright Clearance Centre

2.4.5.2 DNA Synthesis and Repair

Folate is an essential co-factor in the *de novo* biosynthesis of purines and thymidylates which are vital for DNA synthesis, stability and integrity, and repair [23, 201, 202]. Evidence from *in vitro*, animal, and human studies suggests that folate deficiency is
associated with chromosomal and genomic instability, DNA strand breaks, uracil misincorporation, impaired DNA repair, and increased mutations, all of which may promote neoplastic transformation \cite{2}.

Folate deficiency in normal epithelium has also been linked to chromosomal anomalies such as gaps and breakage \cite{203}. DNA strand breaks in the molecular backbone of DNA would serve as a basis for disrupting chromosome integrity \cite{201}. Potential mechanisms for folate deficiency to induce such strand breaks may be the result of uracil misincorporation and impaired DNA repair. Folate deficiency leads to an imbalance in the pool of uridylate and thymidylate nucleotide substrates needed for DNA synthesis. As a consequence of increased uridylate concentrations, misincorporation of uracil instead of thymine bases into DNA occurs \cite{23}. While inappropriate nucleotide insertions are normally excised, there is evidence revealing folate deficiency impairs DNA excision repair, resulting in a greater number of mutations \cite{204}. However, folate supplementation has been shown to correct for some of these defects by enhancing DNA fidelity, maintaining DNA stability and integrity, and providing necessary DNA nucleotide precursors for DNA synthesis and replication \cite{205}. Thus, folate deficiency has generally been considered to promote, while folate supplementation may prevent or suppress neoplastic transformation in the normal epithelium by way of the DNA synthesis pathway. In cells with preneoplastic or neoplastic lesions on the other hand, folate deficiency has been shown to suppress the development and progression of cancer. A likely mechanism is folate depletion causes ineffective DNA synthesis in cells harboring neoplastic lesions and as a result, accelerated replication characteristic of cancer cells cannot occur \cite{125}. In fact, this has served as the foundation of
cancer chemotherapy using anti-folate agents such as methotrexate and 5-fluorouracil\textsuperscript{[206]}.

It is likely that the provision of adequate nucleotide precursors to rapidly dividing preneoplastic and neoplastic cells by folate supplementation may facilitate their growth and development into cancer\textsuperscript{[2]}. Therefore, folate deficiency is generally believed to suppress, while folate supplementation may promote the risk for cancer development and progression in cells already in the preneoplastic or neoplastic state.

2.4.5.3 DNA Methylation

DNA methylation is a heritable, tissue and species-specific, postsynthetic modification of mammalian DNA and is an important determinant of gene expression, genomic stability, and mutagenesis\textsuperscript{[207,208]}. Thus, the potential repercussions of aberrant DNA methylation patterns on cancer development and progression are greatly appreciated and have been a topic of immense interest in recent years. The process by which DNA methylation occurs is a dynamic one and is mediated by DNA methyltransferases (Dnmt1, 3a, and 3) that uses SAM to transfer methyl groups to DNA while demethylation is mediated by various mechanisms including 5-methyl demethylase\textsuperscript{[23]}. Folate’s role in DNA methylation is to ensure the adequate supply of SAM, the primary methyl group donor in biological methylation reactions (\textbf{Figure 2.6})\textsuperscript{[23]}.

Approximately 70\% to 90\% of cytosine residues in CpG dinucleotides are methylated in normal differentiated eukaryotic cells\textsuperscript{[209]}. Widespread loss of genomic DNA methylation in coding and non-coding regions as well as demethylation of repetitive DNA sequences has been consistently observed in tumorigenesis\textsuperscript{[210]}. Global hypomethylation is believed to
contribute to the development of cancer through the mechanisms listed in Table 2.10.

Several studies have shown that diets deficient in methyl donors, including folate, consistently cause genomic DNA hypomethylation and as a result, folate deficiency has been proposed to induce genomic DNA hypomethylation \[175, 211-214\]. In contrast, folate supplementation has been shown to significantly reverse or improve the extent of genomic DNA hypomethylation beyond its pre-existing state in animal and human studies \[184, 215\]. Thus, folate supplementation may have the capacity to prevent or reverse the consequences of genomic DNA hypomethylation and suppress neoplastic transformations \[2\].

**Table 2.10**  Consequences of folate deficiency and genomic DNA hypomethylation \[216\]

<table>
<thead>
<tr>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal instability, increased mutations</td>
</tr>
<tr>
<td>Reactivation of intragenomic parasitic sequences (transposons)</td>
</tr>
<tr>
<td>Mitotic recombination, loss of heterozygosity, promotion of rearrangements</td>
</tr>
<tr>
<td>Aneuploidy</td>
</tr>
<tr>
<td>Loss of imprinting</td>
</tr>
<tr>
<td>Up-regulation of protooncogenes</td>
</tr>
</tbody>
</table>

Neoplastic cells have also been observed to simultaneously contain gene-specific hypermethylated regions in parallel to genome-wide hypomethylation \[2\]. Methylation of promoter CpG islands serves as an important mechanism of gene silencing and inactivation \[216-220\]. Multiple genes that are inactivated by promoter CpG methylation in carcinogenesis have tumor-suppressor functions or play critical roles in the cell. Site-specific hypermethylation could result in the irregular control of the cell cycle, DNA damage repair, apoptosis, differentiation, angiogenesis, metastasis, growth-factor response, drug
resistance, and detoxification, resulting in cancer development\cite{220}. Over 60\% of tumor suppressor and mismatch repair genes have been shown to be methylated in their promoter regions in several cancers\cite{220}. In addition, not only are CpG dinucleotides the main target for DNA methylation, they are also mutational hot spots for germ line and acquired somatic mutations, which may contribute to carcinogenesis\cite{221,222}.

Folate supplementation may promote the progression of preneoplastic and neoplastic cells by enabling the \textit{de novo} methylation of promoter CpG islands of tumor suppressor or mismatch repair genes as well as creating mutation hot spots, leading to a sequelae of events as described above (\textbf{Figure 2.10})\cite{2}. Recent and emerging data have provided evidence in support of such a potential epigenetic mechanism to exist. Studies using viable yellow agouti mice pups and axin fused mice observed that maternal supplementation of methyl donors, including folic acid, were able to increase the methylation patterns at the promoter region of the agouti and axin fused genes, respectively, causing a permanent phenotypic change in the offspring\cite{223-225}. Thus, maternal exposures that could lead to epigenetic dysregulation of the offspring’s genome, such as folic acid, could potentially result in altered susceptibility to disease development in adulthood. However it is currently unclear if \textit{de novo} methylation of promoter CpG islands can occur with folic acid supplementation alone, if the observations are tissue and gene specific, or whether the effects can be attributed to in utero, perinatal or adult exposure\cite{2}. 

- 48 -
Figure 2.10  Distribution of CpG dinucleotides in the human genome and CpG methylation patterns in normal and tumor cells. In contrast to normal cells, cancer cells harbor widespread loss of methylation in the CpG-depleted regions where most CpG dinucleotides should be methylated and gene promoter area gains in methylation of CpG island sites. Open circles represent unmethylated while filled circles are methylated CpG sites. Boxes 1, 2, and 3 represent exons and line between exons are introns. X at the transcription start site represents transcriptional silencing. Copied under license from publisher John Wiley and Sons provided by Copyright Clearance Center [2]
Chapter 3: Rationale, Objectives and Hypothesis

3.1 Rationale

Colorectal cancer (CRC) is an important disease among Canadians as it is the third most common cancer in both men and women. In terms of mortality, it is ranked second in men and third in women. In 2012, it is expected that 23300 Canadians will be diagnosed with CRC with 9200 resulting deaths. Additionally, colon cancer is thought to cost $333 million per year with each patient costing $29110 on average \[226\]. As a result of its prevalence and cost, colon cancer adds a significant burden to the Canadian health care system. There are three types of standard treatment for cancer patients: surgery, chemotherapy, and radiation therapy. Surgery is the treatment of choice for all stages of CRC for both curative and palliative purposes. Chemotherapy can be administered systemically or site-specifically. There are many different adjuvant chemotherapy regimens for colon cancer (Table 2.3), however it is important to note that all colon cancer chemotherapy regimens are based on 5FU. Despite the number of adjuvant chemotherapeutic regimens, resistance mechanisms still exist in individuals. There are many possible forms and corresponding mechanisms for 5FU resistance. 5FU has to undergo many steps in order to act properly at its targets. Resistance can occur at virtually any step of 5FU metabolism if there are abnormalities with the pathway. In short, any mutation, aberration, or change in the pathway that prevents 5FU from entering, staying or reacting in the cell can cause 5FU resistance \[95\]. One potential mechanism of resistance is via cellular efflux transporters such as breast cancer resistance protein (BCRP) and the
multidrug resistance protein (MRP) family that have the ability to transport antifolates as well as 5FU out of the cell, thereby causing resistance\textsuperscript{[29]}. Cellular folate status can modulate the expression and activity of these efflux transporters thereby linking intracellular folate status with the potential for resistance. It has been shown that folate deprivation can cause a decrease in MRP1 levels, thereby potentially lowering 5FU efflux and increasing 5FU sensitivity\textsuperscript{[14]}.

3.2 Objectives

To determine the effects of folate deficiency on the sensitivity of colon cancer cells to 5-Fluorouracil, and to elucidate potential molecular mechanisms associated with this effect in an \textit{in vitro} model of colon cancer and folate deficiency.

3.3 Hypothesis

Folate deficiency will increase the sensitivity of colon cancer cells to 5-Fluorouracil by increasing expression of efflux transporters and by altering apoptotic pathways.
Chapter 4: Effect of Folate Deficiency on the Sensitivity of Colon Cancer Cells to 5-Fluorouracil Based Chemotherapy

4.1 Specific Objectives

■ To determine if folate deficiency can increase the sensitivity of colon cancer cells to 5-fluorouracil based chemotherapy in an in vitro model

■ To interrogate potential mechanisms by which folate deficiency affects the sensitivity of colon cancer cells to 5-fluorouracil based chemotherapy

4.2 Specific Hypothesis

Folate deficiency will increase the sensitivity of colon cancer cells to 5-Fluorouracil by increasing expression of efflux transporters and by altering apoptotic pathways

4.3 Materials and Methods

4.3.1 Cell Lines and Culture

HCT116, HT29 and Caco-2 (Table 4.1) (ATCC, Rockville, MD) cells were cultured in standard (control; replete) RPMI 1640 containing 2.3 μM folate (GIBCO Invitrogen Corporation, Carlsbad, CA) or in folate deficient RPMI 1640 (GIBCO Invitrogen Corporation, Carlsbad, CA). Growth medium was supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 0.1% fungizone. However, folate-deficient medium contained dialyzed FBS (GIBCO Invitrogen Corporation, Carlsbad, CA) (0 nm; deficient). Cells were maintained in standard conditions with incubation at 37°C, 95% humidity and 5% CO₂ and passaged every 3 days.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>HCT116</th>
<th>HT29</th>
<th>Caco-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Epithelial-like</td>
<td>Epithelial-like</td>
<td>Epithelial-like</td>
</tr>
<tr>
<td>Growth Properties</td>
<td>Adherent</td>
<td>Adherent</td>
<td>Adherent</td>
</tr>
<tr>
<td>Chromosome #</td>
<td>45 (stemline chromosome number is near diploid with the modal number at 45)</td>
<td>71 (stemline modal number) # is hypertriploid</td>
<td>96 (stemline modal number)</td>
</tr>
<tr>
<td>Gene Mutations</td>
<td>MLH1, MSH3 A8, MSH6 C8, K-RAS, p14ARF, p16, BAX G8, β-catenin, DCC, Axin2, CBF2, E2F4, GRK4, Helicase q1, MBD4 10A, RAD50 9A, RIZ 9A, TGFBR-II</td>
<td>p53, APC, p16</td>
<td>p53</td>
</tr>
</tbody>
</table>

4.3.2 Intracellular Folate Assay

Intracellular folate concentrations were determined by a standard microtiter plate assay using *Lactobacillus casei* (ATCC# 11578, Manassas, VA) as described previously[227].

Briefly, 20x10⁶ cells were trypsinized, washed twice with PBS and stored at -80°C in 1%
ascorbic acid with PBS until ready to proceed with the assay. Once thawed, cells were
sonicated 3x15 seconds while being kept on ice. Cell lysates were diluted with equal
volumes of 0.1M K$_2$HPO$_4$ and incubated with chicken pancreas conjugase for 2 hours at
37°C, which converts all polyglutamated folates to their corresponding diglutamate
derivatives$^{[227]}$. Aliquots of cell lysate in duplicate were incubated with *Lactobacillus casei*
in mylar-sealed 96-well microplates overnight for 16-18 hours. *L. casei* bacteria grow in
proportion to the amount of folate present and the turbidity of the media was measured
spectrophotometrically at a wavelength of 595 nm. A standard curve of known folate
concentrations was generated for each 96-well plate and used to calculate the intracellular
folate concentrations of the samples.

4.3.2.1 Folic acid standard preparation

Folic acid (10 mg) was dissolved in 10 mL of dH$_2$O with 5 μl of 10 N NaOH to a final
concentration of 1 mg folic acid/mL. The pH adjusted to pH 7 – 8 with HCl and the
concentration was verified with spectrophotometry (282 nm). The solution was diluted to 2
ng/mL with 0.1 M KPO$_4$ buffer (1.05 g KH$_2$PO$_4$, 0.4 g K$_2$HPO$_4$, 0.1 g sodium ascorbate, 100 mL
dH$_2$O, filter sterilized) and stored in aliquots at -80°C.

4.3.2.2 *Lactobacillus casei* stock preparation

200 μL of the *L.casei* stock (ATCC 7469) was incubated with *Lactobacillus* MRS broth
(200 mL) for 18 hours at 37°C. Under aseptic conditions, the cells were centrifuged and the
supernatant was decanted. The cell pellet was resuspended in 180 mL of *Lactobacillus* MRS
broth and 20 mL of 100% autoclaved cold glycerol was added. The solution was mixed well and stored in aliquots at -80°C.

4.3.2.3 Chicken pancreatic conjugase preparation

Chicken pancreas acetone powder (Difco 0459-12-2) was dissolved in 0.1 M KPO₄ buffer. The solution was incubated in a 37°C waterbath under a layer of toluene for 6 hours. The toluene was removed after this time and the solution was centrifuged in glass Corex tubes at 10000x g for 15 minutes. The supernatant was collected and an equal volume of tricalcium phosphate was added. Tricalcium phosphate was a rehydrated form of BioRad Gel HTP: 1 part HTP to 6 parts 0.1 M KPO₄ buffer per 10 g HTP. The mixture was stirred at 4°C for 30 minutes and centrifuged at 10,000x g for 30 minutes at 4°C. The supernatant was collected and cooled to 4°C. An equal volume of 95% ethanol was added, mixed, and incubated overnight at -20°C. The overnight solution was centrifuged at 10,000x g for 30 minutes and the supernatant was removed and resuspended in 50 mL of cold 1.0 M KPO₄ buffer. The solution was then mixed with 10 g of Dowex-1 (BioRad AG1 – X8) and stirred for 1 hour at 4°C. The product solution was filtered through Whatman #1 filter paper at 4°C, aliquoted, and stored at -80°C.

4.3.2.4 Folate Concentration Determination

3 μL of L. casei was inoculated in 3 mL of Lactobacillus MRS broth for 16 hours at 37°C. 500 μL of the overnight culture was inoculated with 2.5 mL of Lactobacillus MRS broth for 6.5 hours at 37°C to obtain an optical density (O.D.) reading greater than 1.8. On the day
of the assay, fresh 0.1 M KPO₄ buffer and folic acid media (9.4 g folic acid media, 0.05 g NaAscrobate, 100 mL ddH₂O, heated to dissolve, filter sterilized) were prepared. 150 μL of 0.1M KPO₄ buffer was added to all wells of the 96-well microtiter plate. An equal volume of folic acid standard was added to the representing wells and serial dilutions were performed to generate an 8 point standard curve. For the assay, 5-10 μL of cell extract solutions were added to the wells and brought up to 300 μL by adding 0.1 M KPO₄. Each sample was serially diluted three times in order to obtain four measurements. Standard and samples were run in duplicates. After 6.5 hours the prepared L. casei inoculums was diluted with folic acid media (24x) and diluted down further once again (40x). 150 μL of the 40x dilution was used to inoculate each well of the 96-well plate. The plate was covered with a mylar sealer, mixed and incubated at 37°C for 16 – 18 hours after which time the plate was read on a spectrophotometer at 650 nm. Softmax – 96 – software was used to calculate the absorption data and folic acid concentration determined for each sample.

4.3.3 Deoxyuridine Suppression Test

The deoxyuridine suppression test (dUST) was performed to verify that intracellular folate depletion was functionally significant. The dUST is used to assess the de novo synthesis of thymidylate (dTMP) on the basis of the competition between two pathways: the salvage pathway and the de novo pathway (Figure 4.1). The salvage pathway consists of phosphorylation of thymidine by thymidine kinase. The de novo pathway generates dTMP by methylating deoxyuridine (dU) monophosphate. Thymidylate synthase, the enzyme involved in this second reaction, requires folate as a substrate. Thus, the deoxyuridine
suppression test has been used as a functional assay for determining folate status at the cellular level, including colonic epithelial cells. In folate-replete cells, the incorporation of $[^3]H$– thymidine into DNA is suppressed by exogenous dU (Figure 4.1). In folate-deficient cells, the degree of suppression is less pronounced because of impaired de novo synthesis of dTMP and greater use of the salvage pathway resulting in higher $[^3]H$– thymidine incorporation into DNA.

The deoxyuridine suppression test was performed as described previously. Briefly, cells were harvested by trypsinization, washed with PBS and suspended in Hanks’ Balanced Salt Solution (HBSS, pH 7.4) (Sigma Chemical Co. St. Louis, MO) with 10mg/ml L-glutamine (Sigma Chemical Co. St. Louis, MO). Cellular suspensions were incubated with or without 2.4 mM 2’-deoxyuridine for 30 minutes at 37°C followed by incubation with 0.6 μM $[^3]H$– thymidine (NEN Life Science Products, Inc. Boston, MA) for 1 hour at 37°C. The reaction was stopped with the addition of 0.9% ice-cold saline. Following centrifugation and washing with saline, 10% trichloroacetic acid (Sigma Chemical Co. St. Louis, MO) was added to the cell pellet for DNA extraction. The extracted DNA was solubilized with Soluene 350 (Packard Bioscience, Groningen, The Netherlands), and transferred into a scintillation vial with 10 mL of CytoScint Scintillation Cocktail (ICN, Costa Mesa, CA) and the radioactive counts were determined. Additional reactions were performed with cellular suspensions undergoing preincubation (1 hour at 37°C) with 100 μM folinic acid. Each experiment was performed in triplicate and the deoxyuridine suppression test was repeated and confirmed.
The % dU suppression was expressed as \([\text{[^3]H}} \text{ – thymidine incorporation with dU}/[^3]H \text{ – thymidine incorporation without dU}] \times 100

![Folate Sufficiency](image1)

![Folate Deficiency](image2)

**Figure 4.1** Cellular *de novo* and salvage pathways under folate replete and folate deficient conditions. Under folate replete conditions, the *de novo* pathway dominates as a result of normal production of dTMP via TS. Under folate deficient conditions, the salvage pathway dominates as a result of impaired production of dTMP via TS. Abbreviations – THF: tetrahydrofolate, TS: thymidylate synthase and TK: thymidine kinase

4.3.4 Cellular Growth Rate and Proliferation

4.3.4.1 Growth Curve

3x10^6 cells were counted and seeded in 100 mm diameter plates containing either folate replete or deficient medium in triplicate. Cells were counted using trypan blue staining and hemocytometer counting every three days for twelve days.
4.3.4.2 $[^3]$H – Thymidine Uptake

Cell proliferation was determined using $[^3]$H – thymidine incorporation. All assays were performed in duplicate. Briefly, 500,000 cells were added to each well of a 24-well plate in 1 mL of appropriate medium. The cells were cultured for 24 hours at 37°C in 95% air and 5% CO$_2$ enriched environment. Cells were then washed with appropriate serum-free medium and incubated for another 24 hours. 16 hours before days 0, 6, and 12, 0.5 mL of $[^3]$H – thymidine (1 μCi/mL) was added to each well and left to incubate for 16 hours. Cells were washed twice with ice cold PBS and 1 mL of ice cold 5% trichloroacetic acid was added to each well. Cells were left at 4°C for 30 minutes and then washed once with PBS. Then at room temperature, 0.5 mL of 0.5N NaOH in 0.5% SDS was added to each well. Cells were solubilized, harvested and radioactivity counted using a scintillation counter with 500 μL of cell solution counted in 10 mL of scintillation fluid. $[^3]$H – thymidine uptake was expressed as the mean counts per minute of triplicate samples.

4.3.4.3 Doubling Time

Cells (8000 per well) were plated in 96-well plates and grown in RPMI-1640 medium with 10% fetal bovine serum for 72 hours. The cell population was determined using the sulforhodamine B (SRB) optical density (OD) measurement assay which uses an intracellular dye to stain cellular proteins in order to determine proliferation$^{[229]}$. Sulforhodamine B is an anionic aminoxanthene dye that forms an electrostatic complex with the basic amino acid residues of proteins under moderately acid conditions, which provides a sensitive linear response. The color development is rapid and stable and is readily measured at
absorbances between 560 and 580nm\textsuperscript{[230, 231]}. The growth rate constant $k$ was derived using an equation $N/N_0 = e^{kt}$, where $N_0$ is the optical density of cells at time zero and $N$ is the optical density of cells at 72 hours. The same equation was used to calculate the doubling time $t$ by setting $N/N_0 = 2$. All analyses were performed in triplicate, and three replicate experiments were performed.

4.3.5 \textit{In vitro} Chemosensitivity Assay

\textit{In vitro} chemosensitivity was determined using a modified SRB protein assay as described previously \textsuperscript{[230, 231]}. Briefly, 8000 cells per 100 μL of RPMI 1640 medium per well were seeded in triplicate in 96-well flat-bottom plates (Sigma Chemical Co. St. Louis, MO). After 24 hours, an additional 100 μL of RPMI 1640 medium containing 5FU (InvivoGen, San Diego, California) alone or in combination with LV (Sigma Chemical Co. St. Louis, MO) was added, and cells were cultured for an additional 72 hours. The concentration of 5FU was varied with concentrations ranging from 1.5x10\textsuperscript{-6} to 25x10\textsuperscript{-6} M, whereas the concentration of LV was held constant at 5x10\textsuperscript{-6} M. After 72 hours, cells were fixed with 70% trichloroacetic acid (TCA) and stained with SRB protein dye. The dye was solubilized, and the OD of the solution was measured at 595 nm. The results were expressed as a percentage of cell survival based on the basis of the difference between the OD at the start and end of the drug exposure, according to the formula:

\[
\text{Survival} = \frac{[(\text{OD}_{\text{Drug}}/\text{OD}_{\text{Start Drug Exposure}}) - 1]}{[(\text{OD}_{\text{No Drug}}/\text{OD}_{\text{Start Drug Exposure}}) - 1]} - 60 -
\]
4.3.6 Thymidylate Synthase Catalytic Enzyme Activity Assay

The catalytic activity of thymidylate synthase was determined by the $^3$H release that occurred during the conversion of [5-$^3$H]-dUMP to dTMP, as described $^{[232]}$. Briefly, 10 μM [5-$^3$H]-dUMP and 350 μM methyleneTHF (final concentration) were added to 150 μg of total cellular protein in a total volume of 50 μL of Tris–HCl buffer (pH 7.4) for 1 hour at 37 °C. The reaction was ended by the addition of 50 μL of 35% trichloroacetic acid. After the addition of 250 μL of 10% activated charcoal in 0.2 M HCl, which bound the unreacted [5-$^3$H]-dUMP, the mixture was centrifuged for 30 minutes, the supernatant was collected, and the amount of radioactivity in the supernatant was measured.

4.3.7 Flow Cytometry

For all flow cytometry experiments, HCT116, HT29 and Caco-2 cells were grown in 12-well plates until confluent, defined as day 0. Cells were subjected to either folate replete or deficient medium for 12 days. Medium was changed on days 3, 6 and 9. Cells were harvested at 12 for all flow cytometry experimental analysis.

4.3.7.1 CFSE Staining of Cells for Cell Proliferation

Carboxyfluorescein diacetate, succinimidyl ester, often called CFSE is a fluorescent cell staining dye. CFSE passively diffuses into cells and is colorless and nonfluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well retained and can be fixed with
aldehyde fixatives. Excess unconjugated reagent and by-products passively diffuse to the extracellular medium, where they can be washed away \[^{233}\].

24 hours prior to day 0 and 12, 1 mL of 5 μM CFDA SE (Vybrant CFDA SE Cell Tracer Kit; Invitrogen) in PBS was added to each well and cells were incubated at 37°C for 10 minutes. Cells were then washed twice with 1 mL of PBS and fresh medium (either replete or deficient) was added to each well to get rid of excess CFDA SE. After 24 hours, cells were trypsinized and subsequently read on the MACSquant flow cytometer.

4.3.8 mRNA Expression with RTPCR

4.3.8.1 RNA Extraction (Qiagen #73304, all reagents and buffers used are proprietary)

HCT116, HT29, and Caco-2 cells were grown in 100 mm round flat bottom dishes until confluent. Cells were washed three times with 10 mL of PBS. 1mL of QIAzol Lysis reagent was added to each confluent dish and cells were scraped off using a cell scraper and put into an eppendorf tube. Cells were incubated at room temperature for 5 minutes to allow the complete dissociation of nucleoprotein complex. 200 μL of chloroform was added and the tube was shaken vigorously for 15 seconds. The homogenate was then incubated for 3 minutes at room temperature and then centrifuged at 12000 x g for 15 minutes at 4°C. The upper aqueous phase of the cells was transferred to a new tube and 600 μL of 70% ethanol prepared with RNase free water was added. The sample was mixed thoroughly and 700 μL of the sample was transferred to an RNeasy mini spin column which was then placed into a 2 mL collection tube. The sample was centrifuged for 15 seconds at 10000 RPM at room temperature and the flow-through was discarded. 350 μL of RW1
buffer was added to the RNeasy spin column and centrifuged for 15 seconds at 10000 RPM to wash the membrane and the flow-through was discarded. 10 μL of DNase 1 stock solution was added to 70 μL of RDD buffer and was mixed gently by inversion and pulsed in the centrifuge briefly. 80 μL of the DNase incubation mix was added directly to the RNeasy spin column and was placed on the benchtop for 15 minutes. 350 μL of RW1 buffer was added to the RNeasy spin column and centrifuged for 15 seconds at 10000 RPM, and the flow-through was discarded. 500 μL of RPE buffer was added to the RNeasy spin column and centrifuged for 15 seconds at 10000 RPM to wash the membrane, the flow-through was then discarded. The RNeasy spin column was placed in a new 1.5 mL collection tube and 30 μL of RNase-free water was added directly to the spin column membrane, the RNA was eluted via centrifugation for 1 min 10000 RPM.

4.3.8.2 Production of cDNA

Template RNA at a concentration of 150 ng/μL was reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen). In short, 7x gDNA wipeout buffer, reverse transcriptase, Quantiscript RT buffer, RT Primer Mix, RNase-free water, and template RNA were centrifuged for 10 seconds at 1400 rpm and then placed immediately on ice to thaw. 2 μL of gDNA wipeout buffer, 10 μL of template RNA, and 2 μL of RNase free water were brought to a 14 μL volume. The tubes were placed in an Eppendorf Mastercycler DNA Engine Thermal Cycler PCR and incubated for 2 minutes at 42°C. The Denature Template RNA Reaction Mix was then placed immediately on ice. The reverse-transcription mastermix was prepared on ice. This mastermix consisted of: 4 μL of 5x
Quantiscript RT Buffer, 1 μL of RT Primer Mix, and 1 μL of Reverse-transcription master mix, bringing the total reaction volume to 20 μL. The mixes were incubated at 42°C for 30 minutes, and then 95°C for 5 minutes to inactivate the Reverse Transcriptase. The reverse-transcription reactions were then stored at -20°C until use for quantitative real-time polymerase chain reaction.

4.3.8.3 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time PCR was conducted using the ViiA-7 Real Time PCR System (Applied Biosystems). A literature search was conducted to find primer sequences for the genes of interest. These primers were then verified for sequence specificity using the Basic Local Alignment Search Tool, or BLAST. Selected primers were synthesized at from Integrated DNA Technologies (Coralville, Iowa, United States). The primers normalized to 100 μM in IDTE pH 8.0 or in LabReady format, were diluted in IDTE pH 8.0 to 50 nM. A list of primer sequences used is shown in Table 4.2.

Table 4.2  List of RT-PCR primer sequences used

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Study Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5’-TGACGGGGTCACCCACACTGTGCCCATCTA-3’</td>
<td>5’-CTAGAAGCATTTGCGGTGGACGATGGAGG-3’</td>
<td>[234]</td>
</tr>
<tr>
<td>DHFR</td>
<td>5’-ACCTGGTTCTCCATTCTGAG-3’</td>
<td>5’-CCTTGTGGAGGTTCTTGAGT-3’</td>
<td>[235]</td>
</tr>
<tr>
<td>TS</td>
<td>5’-CCAAACGTGTGTTCTGGAAGG-3’</td>
<td>5’-GCCCTCCACTGGAAGCCATAA-3’</td>
<td>[235]</td>
</tr>
<tr>
<td>MRP 1</td>
<td>5’-ATGTCACTGGAATACCAGC-3’</td>
<td>5’-GAAGACTGAATCCCTTCCCT-3’</td>
<td>[236]</td>
</tr>
<tr>
<td>MRP 5</td>
<td>5’-CGAAGGGTTGTGATCCTT-3’</td>
<td>5’-GTTCACCATGAAAGGCTTGT-3’</td>
<td>[237]</td>
</tr>
<tr>
<td>MRP 8</td>
<td>5’-GTTCACCATGAAAGGCTGT-3’</td>
<td>5’-GTTCACCATGAAAGGCTGT-3’</td>
<td>[234]</td>
</tr>
</tbody>
</table>
The reactions were run in triplicate on MicroAmp Optical 384-well plates (Applied Biosystems) and their amplifications were tracked by SYBR Green fluorescent dye (Applied Biosystems). The genes of interest were optimized to either a 10 nM mastermix concentration per well, or a 50 nM mastermix concentration per well. The 10 nM mastermix composed of: 4.9 μL SYBR Green, 3.9 μL double-distilled water, 0.3 μL sense primer, and 0.3 μL antisense primer. The 50 nM mastermix contained volumes of 4.9 μL, 4.4 μL, 0.06 μL, and 0.06 μL respectively. A cDNA volume of 0.6 μL was added to each mastermix bringing the reaction volume for both concentrations to 10 μL.

Completed plates were spun at 1200 rpm for 2 minutes at 4°C Celsius and then placed in the ViiA-7 Real Time PCR system. The reaction conditions for stage one were as follows: 2 minutes at 50°C, followed by 10 minutes at 95°C to activate the polymerase. This stage was followed by 40 cycles beginning with 15 seconds at 95°C to denature the target strand followed by 1 minute at 60°C to allow for the polymerase to anneal to and extend the target strand. Finally a melt curve followed consisting of 15 seconds at 95°C then 1 minute at 60°C.

Results were analyzed using the comparative threshold (Ct) method and normalized relative to the quantification levels of the housekeeping gene β-actin. Since the experiments were performed in triplicate, the average of the three Ct values for each gene was taken yielding the Ct mean value. The Ct mean value of β-actin was subtracted from the Ct mean value of each gene of interest and the difference yielded ΔCt.
In order to determine relative quantification, the ΔCt values of the HCT116, HT29, and Caco-2 grown in folate replete medium, or ΔCt₁, served as internal controls for the ΔCt values of HCT116, HT29, and Caco-2 cells grown in folate deficient medium, or ΔCt₂ respectively. The ΔCt₂ values were divided by the respective ΔCt₁ values to yield a fold change in mRNA expression relative to the appropriate internal control.

4.3.9 Statistical analysis

Comparison of means between folate replete cells and folate deficient cells were determined using Student’s t-test. For in vitro chemosensitivity, all experiments were performed in sextuplet and repeated in five different experiments. Chemosensitivity results presented are representative data from three experiments. Results obtained from all assays were consistent between experiments and trends were reproducible. Unless otherwise noted, results were considered statistically significant if two tailed p-values were <0.05. Analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL)

4.4 Results

4.4.1 Effect of Folate Deficiency on Folate Measurements

4.4.1.1 Intracellular Folate Assay

Intracellular folate concentrations of cells cultured in folate deficient medium were significantly lower (by 74-92%) than those of the corresponding cells cultured in folate replete medium (p<0.05; Figure 4.2). Despite the nearly complete absence of folic acid in
the medium, the folate deficient cells demonstrated measurable levels of intracellular folate (Figure 4.2). Intracellular folate concentrations of the folate replete and folate deficient cells were markedly different among the 3 cell lines (Figure 4.2).

![Figure 4.2](image)

**Figure 4.2** Intracellular folate concentrations of HCT116, HT29 and Caco-2 cells cultured in folate replete (2.3 μM) and folate deficient (0 μM) RPMI 1640 medium at days 0, 6 and 12. Intracellular folate concentrations were determined by a standard microtiter plate assay using *Lactobacillus casei* after cellular folate extraction and subsequent treatment with chicken pancreas conjugase. Values are expressed as the mean and standard deviation of duplicate measurements.

* - Asterisks indicate significant difference between groups within the same time point
abc, xyz – Letters indicate significant differences among the same cell line at different time points
p< 0.05, statistically significant compared to corresponding cells
4.4.1.2 Deoxyuridine Suppression Test

To determine whether the observed degree of intracellular folate depletion in the folate deficient cells was functionally significant, the deoxyuridine suppression test was performed. The folate deficient cells were significantly less suppressed by exogenous deoxyuridine, resulting in significantly higher $[^3]$H – thymidine incorporation into DNA, compared with the corresponding folate replete cells ($p<0.01$; Figure 4.3) suggesting significant functional intracellular folate depletion in folate deficient cells. This effect was abolished by preincubation with folinic acid (Figure 4.3).

![Figure 4.3 Deoxyuridine Suppression Test](image-url)

The deoxyuridine suppression test was used to verify that intracellular folate depletion was functionally significant in HCT116, HT29 and Caco-2 cells cultured in folate replete and folate deficient medium. In folate replete cells, the incorporation of $[^3]$H – thymidine into DNA is suppressed by exogenous deoxyuridine, whereas in folate deficient cells, the degree of
suppression is less pronounced because of impaired de novo synthesis of thymidylate and greater use of the salvage pathway (i.e., higher $[^3]$H – thymidine incorporation). Values are expressed as the mean and standard deviation of duplicate measurements as a % of control (identical cell conditions without dU added).

* - Asterisks indicate significant difference between folate replete and folate deficient cells $p<0.05$, statistically significant compared to corresponding control cells

4.4.2 Cellular Growth Rate and Proliferation

4.4.2.1 Twelve Day Growth Curves

Cells grown in folate deficient medium for 12 days grew despite the nearly complete absence of folic acid in the medium (Figure 4.4). Compared with corresponding cells cultured in folate replete medium, the folate deficient cells demonstrated significant progressive retarded growth (Figure 4.4). The growth rates of the folate replete and deficient cells were markedly different among the 3 cell lines (Figure 4.4).
Cells grown in folate deficient medium for 12 days continued to proliferate despite the nearly complete absence of folic acid in the medium (Figure 4.5). Compared with corresponding cells cultured in folate replete medium, the folate deficient cells demonstrated significant progressive retarded growth (p<0.05; Figure 4.5).
Figure 4.5  

$[^3]$H] – Thymidine uptake assay for HCT116, HT29 and Caco-2 cells cultured in folate replete and folate deficient RPMI 1640 medium at days 3, 6, and 12. Medium was changed every 3 days and proliferation is expressed as % of control (folate replete condition). All cell lines grown in folate deficient medium exhibited progressively retarded proliferation over time (p<0.05)

* - Asterisks indicate significant difference between groups within the same time point
xyz – Letters indicate significant differences among the same group at different time points
p< 0.05, statistically significant compared to corresponding folate replete cells

4.4.2.3 Doubling Time

Across all cell lines, cells grown in folate deficient medium had significantly longer doubling time by (on average 5 days) when compared to corresponding cells cultured in folate replete medium (p<0.05). The doubling times for the folate replete and deficient cells were similar among the 3 cell lines (Figure 4.6).
p< 0.05, statistically significant compared to corresponding folate replete cells

4.4.3 *In vitro* Chemosensitivity

*In vitro* chemosensitivity was determined using a modified SRB protein assay. For all three cell lines, cells grown in folate deficient medium showed significantly higher sensitivity to 5FU and 5FU+LV as shown by the decreased % of surviving cells compared to cells grown in folate replete medium (p<0.05). The effect was more severe for high concentrations of 5FU (25 μM vs 1.5 μM) and for regimens also including LV (*Figure 4.7 and 4.8*). The effect of folate deficiency on the chemosensitivity to 5FU was more pronounced in HCT116 compared to HT29 and Caco-2.
Figure 4.7  *In vitro* chemosensitivity (5FU alone) for HCT116, HT29 and Caco-2 cells cultured in folate replete (2.3 μM) and folate deficient (0 μM) RPMI 1640 medium. Across all 3 cell lines, cells cultured in folate deficient medium showed increased sensitivity to the drug as shown by decreased % cell survival compared to cells cultured in folate replete medium. The results were expressed as a percentage of cell survival based on the basis of the difference between the OD at the start and end of the drug exposure, according to the formula:

\[
\text{Survival} = \frac{[(\text{OD}_{\text{Drug}}/\text{OD}_{\text{Start Drug Exposure}}) - 1]}{[(\text{OD}_{\text{No Drug}}/\text{OD}_{\text{Start Drug Exposure}}) - 1]}
\]

* - Asterisks indicate significant difference between groups within the same cell line \( p<0.05 \), statistically significant compared to corresponding folate replete cells.
survival based on the basis of the difference between the OD at the start and end of the drug exposure, according to the formula:

\[
\text{Survival} = \frac{[\text{OD}_{\text{Drug}}/\text{OD}_{\text{Start Drug Exposure}}] - 1}{[\text{OD}_{\text{No Drug}}/\text{OD}_{\text{Start Drug Exposure}}] - 1}
\]

* - Asterisks indicate significant difference between groups within the same cell line \( p<0.05 \), statistically significant compared to corresponding folate replete cells

4.4.4 Thymidylate Synthase Catalytic Enzyme Activity Assay

The catalytic activity of thymidylate synthase was determined by the \( ^{3}H \) release that occurred during the conversion of \( [5-^{3}H]-\text{dUMP} \) to \( \text{dTMP} \). Cells grown in folate deficient medium for 12 days continued to exhibit some TS enzyme activity despite the
nearly complete absence of folic acid in the medium (Figure 4.9). There were no significant differences in TS activity across all cell lines and all time points (p=N.S.; Figure 4.9). It is important to note that the p values for HCT116, HT29 and Caco-2 respectively for day 12 compared to day 0 were 0.06, 0.17, 0.37.

![Figure 4.9 Thymidylate Synthase Catalytic Enzyme Activity Assay for HCT116, HT29 and Caco-2 cells cultured in folate deficient RPMI 1640 medium at days 6, and 12 compared to day 0 (replete). Medium was changed every 3 days and enzyme activity is expressed as pmol/hr/mg protein. There were no significant differences in TS activity across all time points in all cell lines.](image-url)
4.4.5 Flow Cytometry

4.4.5.1 CFSE Staining of Cells for Cell Proliferation

Figure 4.10  Analysis of day 12 HCT116 cells grown in folate replete medium. A visible left shift is seen in the bottom right panel when compared to Figure 4.11 suggesting more later generational cells
Figure 4.11  Analysis of day 12 HCT116 cells grown in folate deficient medium. A visible right shift is seen in the bottom right panel when compared to 4.10 suggesting less later generational cells.
Figure 4.12  Analysis of day 12 HT29 cells grown in folate replete medium. A visible left shift is visible seen in the bottom right panel compared to Figure 4.13 suggesting more later generational cells.
Figure 4.13  Analysis of day 12 HT29 cells grown in folate deficient medium. A visible right shift is seen in the bottom right panel when compared to Figure 4.12 suggesting less later generational cells.
Figure 4.14  Analysis of day 12 Caco-2 cells grown in folate replete medium. A visible left shift is seen in the bottom right panel when compared to Figure 4.15 suggesting more later generational cells.
Figure 4.15  Analysis of day 12 Caco-2 cells grown in folate deficient medium. A visible right shift is seen in the bottom right panel when compared to Figure 4.14 suggesting less later generational cells.

4.4.6 mRNA expression via RT-PCR

The relative mRNA expression (fold change) of select genes of interests of cells grown in folate deficient medium relative to cells grown in folate replete medium is shown in Table 4.3. These genes were chosen on the basis of importance. TS and DHFR are vital enzymes in folate metabolism, additionally, TS is also one of the main targets of 5FU. MRPs
1, 5 and 8 have all shown 5FU efflux capability and therefore are investigated in order to
detect any potential changes as a result of folate depletion. Bcl-2 is an important player in
many cellular processes with emphasis on apoptosis.

**Table 4.3** Summary of mRNA expression of select genes of interest

<table>
<thead>
<tr>
<th>Gene</th>
<th>HCT116</th>
<th>HT29</th>
<th>Caco-2</th>
<th>p value (HCT116, HT29, Caco-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>1.03</td>
<td>0.94</td>
<td>0.98</td>
<td>0.97, 0.92, 0.98</td>
</tr>
<tr>
<td>DHFR</td>
<td>0.92</td>
<td>0.94</td>
<td>0.97</td>
<td>0.94, 0.93, 0.96</td>
</tr>
<tr>
<td>MRP1</td>
<td>0.66</td>
<td>0.79</td>
<td>0.75</td>
<td>0.069, 0.082, 0.091</td>
</tr>
<tr>
<td>MRP5</td>
<td>0.96</td>
<td>0.98</td>
<td>0.65</td>
<td>0.92, 0.98, 0.11</td>
</tr>
<tr>
<td>MRP8</td>
<td>0.91</td>
<td>0.82</td>
<td>0.86</td>
<td>0.88, 0.75, 0.77</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>1.09</td>
<td>0.94</td>
<td>1.15</td>
<td>0.94, 0.91, 0.96</td>
</tr>
</tbody>
</table>

4.5 Discussion

In this study, an *in vitro* model of folate deficiency using three human
adenocarcinoma cell lines, HCT116, HT29 and Caco-2, was employed to examine the effects
of folate deficiency on the sensitivity of colon cancer cells to 5FU and to briefly interrogate
potential mechanisms by which this effect may occur. These three cells lines were chosen
as they display different phenotypic and molecular characteristics (*Table 4.1*). We chose to
perform a time-course experiment in which changes in intracellular folate and proliferation
were assessed at varied time points between days 0-12 following exposure to folate
deficient RPMI 1640 medium. Our rationale for performing a time-course experiment was
to achieve a better idea of timing for the significant fluctuations in intracellular folate in
both the cells grown in folate replete medium as well as the cells grown in folate deficient
medium. Additionally, the time course experiment allowed us to paint a clearer picture of
the sometimes large changes in proliferation between specific days. Overall, the time-
course experiments were employed in order to achieve greater temporal resolution to elucidate the activity of these cells as a result of exposure to folate deficient medium.

Folic acid was the chemical of choice for a number of reasons including its stability, ease of use, and the fact that it is used to create RPMI1640, the medium of choice for all experiments. It is important to note that in the folate replete medium, the concentration of folic acid is 2.3 μM. This concentration was used as it was the standard concentration of folic acid in normal RPMI1640 medium considered to be sufficient for regular cell growth. While it is considered the control concentration in all experiments, it is not considered to be of nutritionally relevant range in humans. Conversely, in the folate deficient medium, the concentration does not reach 0 μM. This is because the dialysis process for the dialyzed fetal bovine serum added to the folate deficient medium does not filter out 100% of the folic acid. Therefore, the final concentration of the folate deficient medium used for all experiments becomes less than 0.6 nM. It is also important to note that all cells that were eventually subjected to folate deficient conditions, were grown in standard folate replete RPMI 1640 medium in order to reach desired confluency or total amount of cells in order to start the experiment. For instance, the thymidylate synthase catalytic enzyme activity experiments required over 70 full 100 mm diameter confluent plates of cells. When day 0 was defined, cells that would eventually be exposed to folate deficient RPMI 1640 medium were gently washed multiple times with PBS in order to most optimally rid free folate in the dishes in order to mimic true deficiency. Without this precaution, cells newly exposed to folate deficient medium would have had leftover folate from the necessary growing phase prior to the experiment which may have altered future experimental results. This was
shown to be the case in a pilot study (data not shown) that investigated the differences in proliferation as measured by a twelve day growth curve between cells grown in folate replete medium, unwashed cells grown in folate deficient medium and washed cells grown in folate deficient medium. As expected, the cells grown in folate replete medium showed the highest growth. Interestingly, the unwashed cells grew significantly better than the washed cells, suggesting that the washing protocol was indeed effective at minimizing unwanted exogenous folate in the surrounding medium. In order to maximize our chances of observing any effect of folate deficiency on mRNA expression, and flow cytometry, we chose to concentrate our attention on the differences between folate replete and folate deficient cells after 12 days of exposure.

4.5.1 Effect of Folate Deficiency on Folate Measurements

4.5.1.1 Intracellular Folate Levels

Despite being grown in folate deficient RPMI 1640 for 12 days, we were still able to observe detectable levels of intracellular folate in these cells. The intracellular folate levels across all three cell lines exhibited similar patterns of significance with the exception of Caco-2. In HCT116 and HT29, cells grown in folate replete conditions had significantly higher levels of intracellular folate at day 12 when compared to either days 0 or 6. However, this was not found to be the case in Caco-2 as there was no difference in intracellular folate levels across all time points for cells grown in folate replete medium. As expected, in HCT116, HT29 and Caco-2, cells grown in folate deficient medium had lower levels at day 12 compared to day 0 (87%, 89%, and 89% respectively). These decreases in
intracellular folate were more severe compared to similar previous work that showed a 57% and 58% drop for HCT116 and Caco-2 respectively\[^{238}\]. This is most likely a result of more initial washing of the cells with PBS which resulted in less unwanted leftover folic acid from the cell growing phase prior to the start of the experiment. Interestingly, in HCT116 and HT29 but not in Caco-2, cells grown in folate replete medium had an increase in intracellular folate of 35% and 44% respectively after 12 days. This may have been due to a cellular mechanism that recognized the presence of excess folic acid and increased the expression of membrane bound folate carriers in order to take advantage of excess resources for proliferation. It is well known that in certain cancerous tissues (ovarian, cervical and breast), folate receptors such as FRα are upregulated. However, in this case, it appears that HCT116 and HT29 can increase their intracellular folate levels in response to a supraphysiological level of folic acid. Overall, the absolute levels of folate were comparable between HCT116 and HT29 however, Caco-2 had higher levels throughout the time course experiment. This is most likely a result of cell morphology and physiology as Caco-2 is noticeably larger than HCT116 and HT29 which suggests a greater amount of initial storage of folate to begin with. Furthermore, as later experiments confirmed, the growth rate of Caco-2 is also noticeably slower than HCT116 and HT29.

4.5.1.2 Deoxyuridine Suppression Test

The deoxyuridine suppression test was used to verify that intracellular folate depletion was functionally significant in HCT116, HT29 and Caco-2 cells cultured in folate replete and folate deficient RPMI 1640 medium. In this case, functional significance is
defined as a significant change in folate metabolism in the cell, not only a significant change in intracellular folate. Across all three cell lines, in the folate replete cells, the de novo pathway is the predominant pathway as abundant deoxyuridine suppresses the incorporation of $[^3]H$ – Thymidine into DNA. Whereas in folate deficient cells, the degree of suppression is much less pronounced because of impaired de novo synthesis of thymidylate (as a result of low folate levels) and greater use of the salvage pathway (i.e., higher $[^3]H$ – thymidine incorporation). As expected, preincubation of folate deficient cells with folinic acid severely decreased the dependence on the salvage pathway and therefore resulted in significantly lower $[^3]H$ – thymidine uptake in these cells. When analyzed at a significance level of $p<0.05$, both preincubated and non preincubated folate deficient cells showed a statistically significant increase in $[^3]H$ – thymidine uptake, albeit, the folate deficient cells without folinic acid preincubation still exhibited much higher salvage pathway activity. However, when these same results were analyzed with a significance of $p<0.01$, the folate deficient cells that underwent folinic acid preincubation showed no difference compared to folate replete cells. This analysis was also run in order to emphasize the rescuing ability of preincubation with folinic acid in the cells grown in folate deficient medium. By these analyses, we can conclude that folate deficient cells without folinic acid preincubation were less suppressed than folate deficient cells that underwent folinic acid preincubation. These results make biological sense as any folate deficient cells that undergo folinic acid preincubation get their folate coenzymes restored thereby shifting thymidylate synthesis from the salvage pathway to the de novo pathway. However, it is important to note that 1 hour incubation with folinic acid was not enough time for folate deficient cells to regain
complete *de novo* pathway usage as shown by the still significant increase in $[^3]H$ – thymidine in folate deficient cells compared to replete cells in the folinic acid preincubation condition.

4.5.2 Cellular Growth Rate and Proliferation

4.5.2.1 Growth Curves

Since folate is vital for nucleotide biosynthesis, it was expected that cells grown in folate deficient medium would grow significantly slower compared to cells grown in folate replete medium. Growth curves indicated that this was true among all three cell lines. The shape of the growth curves differed slightly among the cell lines. HCT116 and HT29 growth curves appeared to exhibit a very similar pattern, albeit with different numbers of cells. In these two cell lines, there was a significant departure in proliferation between days 9 and 12 where folate replete cells outnumbered folate deficient cells. At day 9, the fold difference was 4 and 5 and by day 12, the fold difference became 11 and 16 for HCT116 and HT29 respectively. Additionally, at day 3, there was no significant difference in growth between folate replete and folate deficient cells for HCT116 and HT29. Even in the absence of virtually all folic acid in the medium for three days, cells in the folate deficient medium still continued to grow unperturbed when compared to cells in the folate replete medium. During these three days, it is likely that these cells without a source of exogenous folate used all of their intracellular stores in order to continue proliferating at a normal rate. Conversely, Caco-2 cells grew differently compared to HCT116 and HT29, not only in absolute numbers of cells, but also the pattern. Caco-2 had a far more gradual growth of
cells both in folate replete and deficient conditions. The fold change at days 3, 6, 9 and 12 were 1.2, 1.4, 1.5, and 2.0 respectively indicating a gradual change. The ability of Caco-2 to continually grow in folate deficient conditions, albeit at a slightly retarded rate, likely relies on two key factors: initial storage and rate of use. First, as a result of Caco-2 being morphologically larger, intracellular folate data confirmed that on day 0, Caco-2 had 2.3 and 1.6 times more intracellular folate than HCT116 and HT29 respectively. Therefore, the initial stores of folate were higher in Caco-2 compared to other cells, this presumably would help under conditions of folate deficiency. Second, despite having the same initial seeding density among all three cell lines, Caco-2 grew more slowly over the span of 12 days when compared to HCT116 and HT29. Therefore, in combination with higher initial storage, the rate of proliferation (therefore DNA biosynthesis) is also lower. These two factors likely explain the difference in proliferation patterns in Caco-2 compared to HCT116 and HT29.

4.5.2.2 $[^{3}\text{H}]$ – Thymidine Uptake Assay

Thymidine uptake is a proxy indicator for proliferation via the salvage pathway of thymidylate production. As expected, across all three cell lines and every time point (days 3, 6 and 12), the cells grown in folate deficient medium showed significantly less $[^{3}\text{H}]$ – thymidine uptake versus cells grown in folate replete medium. In this particular proliferation assay, all three cell lines exhibited a different pattern. In HCT116, folate deficient cells were 82%, 16% and 16% of control on days 3, 6 and 12 respectively. $[^{3}\text{H}]$ – thymidine incorporation was still remarkably high after 3 days but dropped significantly by day 6 and maintained until day 12. In HT29, folate deficient cells were 31%, 15% and 15%
of control on days 3, 6 and 12 respectively. The pattern of $[^3]$H – thymidine incorporation is identical to that of HCT116 with the exception of a even more severe drop at day 3. In Caco-2, folate deficient cells were 73%, 61% and 43% of control on days 3, 6 and 12 respectively. This gradual pattern was unique to Caco-2 in that, there were no steep drops of $[^3]$H – thymidine incorporation. Similar to the growth curve data, Caco-2 cells grown in folate deficient medium appear to have a more gradual change in proliferation and are therefore more resistant to changes in folate depletion as a result. Again, this is likely a result of Caco-2 having higher initial stores of folate as well as a lower rate of use as a result of a lower rate of proliferation.

4.5.2.3 Doubling time

Doubling time is an inverse indicator of proliferation. Not surprisingly, across all three cell lines, cells that were grown in folate deficient medium showed significantly slower doubling times, with cells grown in folate deficient medium taking 6.2, 8.9 and 4 hours longer to cycle compared to cells grown in folate replete medium for HCT116, HT29 and Caco-2 respectively. Interestingly, the doubling time of Caco-2 cells was altered the least by the condition of folate depletion. This again lends credence to the idea that Caco-2 cells are the most resilient to folate deficient conditions as a result of higher intracellular folate levels in conjunction with a lower rate of folate use as a result of slower proliferation.
4.5.3 In Vitro Chemosensitivity

Chemosensitivity was interrogated by a modified SRB protein assay. Concentrations for 5FU were selected based on initial range finding experiments (data not shown) as well as to mimic human tissue levels\textsuperscript{[239]}. Across all three cell lines and both drug regimens, cells that were grown in folate deficient medium showed significantly increased sensitivity to 5FU either alone or with LV. This effect was dependent on the dose of 5FU as well as the presence of LV. However, the largest differences in chemosensitivity were found to be cells that were exposed to 25 \( \mu \text{M} \) of 5FU and 5 \( \mu \text{M} \) of LV, the difference in \% cell survival between folate replete and deficient conditions were 22.9\%, 10.2\% and 7.3\% for HCT116, HT29 and Caco-2 respectively. From these results, it is clear that the decreased intracellular folate pool has positive effect on the sensitivity of these cells to 5FU based regimens.

Having earlier established that Caco-2 exhibits the most resistance to folate deficient conditions, it is not surprising that the beneficial effect of folate status on the efficacy of 5FU is least convincing in this cell line. The majority of the literature regarding the relationship between intracellular folate status and chemosensitivity deals with antifolates such as methotrexate (MTX). One of the key differences between antifolates and 5FU is that antifolate activity is dependent on its ability to become polyglutamated by FPGS. The addition of polyglutamates retains antifolates within the cell and makes them better substrates for target enzymes such as DHFR. However, because 5FU is not polyglutamated, its activity does not depend on its ability to compete with folates for FPGS with the exception of its cofactor LV which binds to the ternary structure of TS better if polyglutamated. Therefore, the predominant explanation for the relationship between
folate status and 5FU chemosensitivity may lie in the relationship between intracellular folate and expression of 5FU efflux transporters.

4.5.4 Thymidylate Synthase Enzyme Activity Assay

Despite showing no statistical significance in TS activity across all three cell lines, there was a noteworthy trend towards lower TS activity in day 12 compared to day 0 in both HCT116 and HT29. It is unclear whether the results of this experiment truly represent the biochemistry of TS and its relationship to folate depletion. It is possible, if not probable, that this lack of significance is a result of being underpowered. For this current study, two confluent 100 mm dishes for each unique sample were used and counted with the scintillation counter. In order to truly determine the relationship between TS activity and folate depletion, future studies require a larger number of replicates to minimize type 2 error. Despite the small sample size, the difference in TS activity between days 0 and 12 is only marginally insignificant in HCT116 (p=0.06). However, Caco-2 did not display large differences in TS activity between days 0 and 12, again reinforcing the idea that Caco-2 appears to be the most resilient to conditions of folate depletion.

4.5.5 Flow Cytometry

4.5.5.1 CFSE Staining of Cells for Cell Proliferation

CFSE passively diffuses into cells, is colorless and nonfluorescent until its acetate groups are cleaved by intracellular esterases to yield highly fluorescent amine-reactive CFSE. The ester group of CFSE reacts with intracellular amines which form fluorescent CFSE.
conjugates that are well retained in the cell. Excess unconjugated CFSE passively diffuses out of the cell into the extracellular medium and can be washed away. The dye-protein adducts are inherited by daughter cells and are not transferred laterally to adjacent cells in a population. Therefore, in the histograms comparing FITC fluorescence vs # of events a shift either left or right can be seen when comparing cells within the same cell line grown in folate replete and deficient medium. A shift to the left (lower fluorescence intensity) indicates that cells with lower intensity (later generational cells) were detected, this indicates that the cells were more proliferative within the same cell line. Conversely, a shift to the right (higher fluorescence intensity) indicates that cells with higher intensity (early generational cells) were detected, this indicates that the cells were less proliferative within the same cell line. Across all three cell lines, cells grown in folate replete medium showed a clearly visible left shift when compared to cells grown in folate deficient medium. This is unsurprising as we have already seen through growth curves, doubling time and \[^{3}\text{H}\] - thymidine incorporation that folate plays a vital role in the cell’s ability to biosynthesize nucleotides used for cell proliferation and division. This experiment further confirms the vital role of folate in cell proliferation.

4.5.6 mRNA expression via RT-PCR

It has been shown that increased levels of intracellular folate induce anti-folate efflux pumps such as breast cancer resistance protein (BCRP) and multidrug resistance proteins (MRP). Specifically, MRP 1, 5 and 8 have shown the ability to pump out 5FU from the cell, which would result in resistance. Therefore, it is hypothesized that as a result of
increased levels of intracellular folate, MRP expression and activity increase which lead to
great efflux of 5FU and its metabolites, ultimately leading to 5FU drug resistance.

Despite showing no statistically significant difference between cells grown in folate
deficient medium compared to cells grown in folate replete medium for all the candidate
genes of interest, there was a noteworthy trend towards downregulation of MRP1 in cells
grown in folate deficient medium across all cell lines. This is in accordance with previous
data where breast cancer cells grown in low folate conditions exhibited a 5 fold decrease in
MRP1 expression \([14]\). However, because of the borderline insignificance and the
consistency in direction and fold change across all three cell lines, it is likely that the
statistical insignificance is a result of being underpowered. In this experiment, samples
were run in triplicate, however it may be necessary to increase the number of replicates in
future experiments to potentially confirm the effect of folate deficiency on MRP1
expression in these cells.

4.6 Conclusion

Cells grown in folate deficient medium showed lower intracellular folate levels,
evidence of functional folate depletion, less proliferation via growth curves, \([^{3}\text{H}] –
thymidine uptake, doubling time and CFSE fluorescence, higher sensitivity to 5FU with and
without LV when compared to cells grown in folate replete medium. There was no
significance in TS activity across all three cell lines but HCT116 and HT29 showed borderline
insignificance most likely as a result of low sample size. There were also no significant
differences in mRNA expression via RT-PCR in any of the selected genes of interest, however, there is a promising near-significant trend for the downregulation of MRP1 that is consistent among all cell lines which is consistent with both previous literature and my hypothesis of folate levels regulating the expression/activity of 5FU efflux transporters.

We found evidence of Caco-2 showing the most resistance to folate deficient conditions such as higher intracellular folate stores, more proliferation and less 5FU sensitivity. These findings are in accordance with research that shows Caco-2 having higher absorption rates \( (V_{\text{max}}) \) for folic acid in conditions of low folate (1-2 nm) \([240]\). Interesting, this same study indicated that steady-state mRNA levels of RFC, PCFT and FRα actually decreased under folate deficient conditions \([240]\). However, they did not interrogate the spatial expression of these transcripts. This seemingly paradoxical finding may be explained in part by RFC and PCFT’s ability to also act as folic acid efflux transporters. Therefore, lowering expression on the basolateral end of the cell may act to reduce folic acid efflux thereby adapting to low folate conditions.

Overall, these data suggest that folate deficiency significantly enhances the sensitivity of colon cancer cells to 5FU based chemotherapy possibly due to MRP1 downregulation. Dietary or other strategies to deplete intracellular folate concentrations in colon cancer cells to enhance chemosensitivity to 5FU are worthy of further investigation. Given the dramatically increased dietary intake and blood levels of folate in North America resulting from folic acid fortification and supplementation, whether or not high folate status would affect chemotherapy for colon cancer also warrants future studies.
Chapter 5: General Discussion and Future Directions

5.1 General Discussion and Summary

Folate is an essential B vitamin that is required for thymidylate and purine synthesis as well as transfer of one carbon units in biological methylation reactions \[^{241}\]. Folate cannot be made by humans and therefore must be consumed in the diet. It plays an important role in cancer development, progression and treatment. Alterations of folate status have been associated with various diseases including certain cancers, NTDs, anemia, cardiovascular disease, adverse pregnancy outcomes, and cognitive dysfunction \[^{3, 23, 242}\].

Folate deficiency has been shown to possess the ability to delay or diminish the progression of established neoplastic foci \[^{243}\]. This has been the basis for antifolate based chemotherapy (MTX, 5FU) that interrupts folate metabolism in order to delay or suppress cancer growth. The ability of folate to suppress tumor cell growth and proliferation lies in one of its main biological functions: nucleotide biosynthesis. The transfer of one carbon groups mediated by folate is necessary for the \textit{de novo} synthesis of thymidylate and purines. Therefore, folate plays a vital role in DNA synthesis and repair as well as maintenance of DNA integrity and stability\[^{244}\]. Additionally, folate deficiency can induce DNA strand breaks, increase uracil misincorporation, impair DNA repair and lead to apoptosis. Its effect on the chemosensitivity of colon cancer cells to 5FU based chemotherapy was investigated in this thesis.
Transformed human adenocarcinoma cell lines (HCT116, HT29 and Caco-2) were cultured in folate deficient medium were used as a model of folate deficiency. All cells cultured in folate deficient medium showed significantly lower intracellular folate concentrations and less suppression by exogenous deoxyuridine using \[^{3}\text{H}]\) – thymidine incorporation in the deoxyuridine suppression test. These results indicate that the model of folate deficiency was indeed effective at altering both intracellular levels of folate as well as biochemical function. Cells grown in folate deficient medium exhibited less proliferation as shown by cellular growth curves, doubling time assays and \[^{3}\text{H}]\) – thymidine uptake assays. Most interesting was the effect of folate deficiency on chemosensitivity of these colon cancer cells. In all cell lines, it was found that cells grown in folate deficient medium exhibited significantly higher sensitivity to 5FU compared to cells grown in folate replete medium. This was true for both 5FU alone and 5FU + LV drug regimens. Unfortunately, we were unable to detect statistical significance in the thymidylate synthase catalytic enzyme activity assay which would have further added to the resulting functional data. However, future studies may be able to address this statistical power issue. Additionally, flow cytometry was employed to investigate proliferation in these cells after 12 days of culture. We found a clearly visible left shift in cells grown in folate replete medium when compared to deficient medium, reinforcing the idea that folate deficient cells proliferate less than folate replete cells.

The findings from this study contribute to the current understanding of the effect of folate deficiency on the sensitivity of colon cancer cells to 5FU based chemotherapy. Based on findings from this proof of principal study, folate deficiency may be an interesting
avenue towards increase the efficacy of 5FU based chemotherapy in colon cancer. Therefore, dietary or other strategies to deplete intracellular folate concentrations in colon cancer cells to enhance chemosensitivity to 5FU are worthy of further investigation.

5.2 Future Directions

This study used an in vitro model which investigated the effects of folate deficiency on the sensitivity of colon cancer cells to 5FU based chemotherapy. A natural progression would be an in vivo study with folate replete and folate deficient diets being fed to mice prone to developing adenomas. The effectiveness of a standard regimen of 5FU and LV would then be measured. Additionally, a xenograft model using immunocompromised nude mice could be fed different levels of folate deficient (mild, moderate and severe) and folate replete diets and the effectiveness of a standard regimen of 5FU and LV could be measured.

As part of this project, we investigated two concentrations of folic acid in the medium. As proof of principle we chose two extreme concentrations of folic acid in order to maximize our chances of seeing an effect on chemosensitivity. While these concentrations are easily seen in medium used for cell culture work, they are far outside of the scope of human physiological range of 15-50 nM serum folate\[^{15, 146}\]. Therefore, this beneficial effect of folate deficiency on chemosensitivity to 5FU may be altered if cells were grown at a more human physiologically relevant concentration. Future studies would

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investigate intermediate concentrations such as 25 nM and 50 nM in order to see if these effects on chemosensitivity remain constant.

Additionally, similar experiments could be conducted using 5-methyl THF (the bioactive form of folate) instead of the synthetic form of folic acid. It is unclear whether or not the addition of 5-methyl THF as opposed to folic acid would have a significant effect on both regular cell processes as well as chemosensitivity to 5FU. While 5FU is the cornerstone of all CRC chemotherapy, there are other approved chemotherapies like Irinotecan, and Oxaliplatin. It is unclear what effect folate deficiency would have on the chemosensitivity to these drugs. The effect of folate can be unpredictable as shown in a human study by Vogelzang et al., he found that patients with malignant pleural mesothelioma who took folic acid had significantly increased cisplatin antitumor activity and decreased toxicity despite being biochemically unrelated\textsuperscript{[245]}. As most CRC chemotherapy regimens now consist of a variety of drugs in combination with 5FU, testing the effects of multiple drugs using this model of folate deficiency and chemosensitivity testing would give valuable information.

Since it is the gold standard of research, a double-blind, placebo-controlled study of folate deficiency and 5FU chemosensitivity could give strong evidence of this potentially beneficial effect of increased chemosensitivity to 5FU. However, it would be unethical to subject patients to folate deficiency given the multitude of associated maladies and risks. Therefore, an observational study would be the study design of choice. However, folate deficiency has been virtually abolished in Canada and therefore there would be no subjects to compare to\textsuperscript{[18]}. Future human observational studies would most likely be conducted in
countries without folic acid fortification and high rates of supplement use in order to capture a subsection of the population that may be folate deficient.

This study suggests that folate deficiency significantly enhances the sensitivity of colon cancer cells to 5FU based chemotherapy. Dietary or other strategies to deplete intracellular folate concentrations in colon cancer cells to enhance chemosensitivity to 5FU are worthy of further investigation. Given the dramatically increased dietary intake and blood levels of folate in North America resulting from folic acid fortification and supplementation, whether or not high folate status would affect chemotherapy for colon cancer also warrants future studies.
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