The Future of 5-Fluorouracil Treatment of Colorectal Cancer: Translating a Better Understanding into Improved Patient Care

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The Future of 5-Fluorouracil Treatment of Colorectal Cancer: Translating a Better Understanding into Improved Patient Care

By

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Paper Submitted in Partial Fulfillment

Of the Requirements for the Degree

Of Master of Science

Physician Assistant Studies

Augsburg University
August 3rd, 2017
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Abstract

5-fluorouracil (5-FU) is a pyrimidine analog that has been successfully employed in anticancer therapy for over forty years. Over the past several decades, researchers have characterized its cellular and clinical pharmacology and uncovered multiple mechanisms of action, which include the inhibition of thymidylate synthase (TS) and incorporation of its metabolites into DNA and RNA. The purpose of this quantitative systemic review was to summarize and evaluate what is known of these mechanisms to determine which are of most significance in the anticancer effects of 5-FU in metastatic colorectal cancer (mCRC) patients. A literature search of peer-reviewed articles dating back to the discovery of 5-FU did not find evidence to conclusively satisfy this inquiry, mainly due to a lack of relevant research in human models and uncertainty regarding the impact of DNA and RNA repair processes in tumor cells following drug administration. Overall, the literature did not support the current assumption that DNA-based mechanisms are definitively most responsible for the efficacy of 5-FU, and underscores the importance of better understanding how 5-FU promotes cell death and how tumors develop resistance to it. Future research could be directed toward further elucidating the mechanistic details of 5-FU treatment in humans, as well as discovering clinically useful biomarkers to better optimize and personalize metastatic colorectal cancer therapies.

Introduction

Worldwide, colorectal cancer is the 2nd and 3rd most common cancer in females and males, respectively. Metastatic disease at diagnosis is found in up to 25% of colorectal cancer patients, and up to an additional 60% of patients develop metastases following initial therapy. Once metastasis has occurred, treatment of colorectal cancer consists of chemotherapy to extend and improve quality of life, rather than to cure.
For decades now, the first-line chemotherapeutic for mCRC has been intravenous 5-FU, an anticancer agent believed to most significantly disrupt the DNA pathways of metastatic cancer cells. The most widely used dosing and duration regimens for 5-FU were developed in the late 1990’s. Other chemotherapeutics have since been added to these regimens, with the intention of amplifying its DNA-directed effects. However, more recent discoveries indicate 5-FU may rely heavily on RNA-directed mechanisms for its anticancer effects. If true, this would mean clinicians should utilize additional methods of combination therapy to better accentuate the drug’s RNA-based effects, rather than focus on reinforcing its impact on cancer cell DNA.

This review will summarize what is known about the mechanisms of 5-FU efficacy, while seeking to explore whether its effects are primarily due to DNA-based activity, or whether RNA-directed effects also play an important role. I hypothesize recent research shows 5-FU effects RNA pathways in mCRC in a fashion significant enough to warrant reconsideration of clinical combination strategies for colorectal cancer patients. Objectives of this review also include discussing research recommendations related to furthering our understanding of how 5-FU works, and briefly exploring the future of colorectal cancer therapy in terms of more specialized and personalized therapeutic options.

**Background: Literature Review**

**Discovery.** 5-FU is an example of a rationally designed fluoropyrimidine chemotherapeutic. The observation in 1954 that rat hepatomas incorporated radiolabeled uracil during nucleic acid biosynthesis more avidly than nonmalignant tissues implied that malignant cells would be more sensitive to cytotoxic agents that mimic uracil than are normal cells. Based on the hypothesis that uracil is preferentially used by cancer cells, and on speculation that a uracil analog might alter cancer cell metabolism, multiple uracil and uridine derivatives were
synthesized and then tested for antagonist activity against tumors. 5-FU was one such
discovered antimetabolite agent. While metabolites of other developed antimetabolite drugs
either inhibited essential biosynthetic processes, or incorporated into DNA or RNA, 5-FU
metabolites appeared to do both. Shortly after its discovery, the efficacy of 5-FU as a potential
anti-tumor drug was reported, and it has since been tested in numerous clinical trials and found
to exhibit DNA-based anti-tumor activity in patients.

A deoxyribonucleic derivative of 5-FU, 5-fluoro-2' deoxyuridine (FdUrd), was also
synthesized. In 1977, Corbett et al performed in vivo research on humans comparing FdUrd with
5-FU. Using various schedules of administration on patients with human colon tumors, these
experiments yielded more than three times as many tumor-free survivors following FdUrd
treatment. Since then, this derivative has consistently been shown to be more effective than 5-
FU in many cancer cell lines and rat models, and is FDA-approved for the treatment of hepatic
colon metastases. However, while FdUrd has exhibited better antitumor activity, 5-FU
provokes less systemic toxicity in humans and is less expensive, resulting in the restricted usage
of FdUrd. One of its common uses includes experimentally examining the effects of 5-FU as
an equivalent stand-in. However, there are many subtle chemical differences between the two
agents that raise questions as to the validity of such experimentation, which is crucial to keep in
mind when reviewing 5-FU research. This topic will be further addressed in the discussion
section.

Development of Treatment Schedules. As a small molecule, 5-FU generally exhibits
excellent absorption. However, its oral administration was abandoned decades ago due to
unpredictable catabolism and bioavailability. When 5-FU therapy was first developed in the
United States, it was usually administered intravenously via a bolus schedule. Later studies
showed that 5-FU was a time-dependent drug whereby greater cytotoxicity correlated with longer exposure. For example, the activity of 5-FU against HT-29 cancer cells in vitro was three times higher when the drug was present for longer times at lower doses as compared with higher doses for shorter times. As a result, therapeutic strategies in the late 1990’s were developed around infusion schedules to allow for longer-term systemic therapy. However, the resulting modest increase in efficacy did not immediately lead to changes in 5-FU-based treatment tactics due mainly to increased toxicity. As combination strategies incorporating agents that modulate the effects of 5-FU were developed, which allowed for improved patient safety alongside increased treatment intensity, non-bolus infusion schedules were utilized more. Currently, 5-FU is only used in combination with other therapeutics, which underlines the importance of finding the best complementary drugs to optimize results. FOLFIRI (5-FU, leucovorin, and irinotecan) and FOLFOX (5-FU, leucovorin, and oxaliplatin) therapies, each based on non-bolus infusion of 5-FU every other week for 6 months, have become the most commonly used standard regimens for late-stage colorectal cancer.

**Potential Mechanisms of Action.** Production of the active metabolites 5-fluorouridine-5′-triphosphate (FUTP), 5-fluorouridine-5′-monophosphate (FdUMP), and 5-fluoro-2′-deoxyuridine-5′-triphosphate (FdUTP) appears to be critical for 5-FU-based effects (Figure 1). FUTP is extensively incorporated into RNA, disrupting normal RNA processing and function. FdUMP and FdUTP, on the other hand, interfere with DNA processing and function. FdUMP also inhibits the function of thymidylate synthase (TS), an enzyme that is essential for DNA synthesis. In the scientific literature, TS inhibition is often referenced in terms of a significant DNA-based mechanism of 5-FU anticancer activity.
While both RNA- and DNA-directed effects have been associated with normal tissue toxicity and antitumor efficacy, the role of each in the outcome of 5-FU treatment remain to be established. To further complicate matters, the extent to which these mechanisms predominate in human tumor or normal cells varies across cell types and with different methods and doses of drug administration.\textsuperscript{1,6,14} The following is a summary of the cell line-, animal-, and clinical-based evidence for and against the significance of RNA- and DNA-based mechanisms following 5-FU treatment.

**RNA-Directed Mechanisms.** The 5-FU metabolite FUTP resembles uridine-5'-triphosphate (UTP) during RNA transcription and is recognized by RNA polymerases, leading to FUTP incorporation into all types of RNA. Research shows that this incorporation affects RNA metabolism in multiple ways. First, it leads to the inhibition of some RNA biogenesis. Rapidly growing cells, such as tumor cells, need vigorous RNA biogenesis pathways to continuously build up ribonucleoprotein complexes. FUTP incorporation stabilizes pseudouridylases in these complexes, inhibiting the conversion of uridine to pseudouridine. This limits the cell’s capacity to modify and produce RNA precursors, such as pre-mRNAs and rRNAs, which in turn limits RNA production.\textsuperscript{13} FUTP incorporation additionally disrupts post-transcriptional modification of tRNAs, and the assembly and activity of small nuclear RNA and protein complexes, thereby inhibiting the splicing of pre-mRNA, which also limits further RNA production.\textsuperscript{14} Second, RNA marked by FUTP incorporation is less likely to be recognized by surveillance machinery, and is thus not degraded at the appropriate times or levels. Third, 5-FU-based incorporation into RNA reduces the cellular levels of the nuclear exosome Rrp6, a complex that degrades RNA, preventing the efficient recycling of abnormal RNA transcripts. This results in the accumulation
of irregular RNA intermediates, which has been shown to associate with chromosomal instability for unknown reasons.\textsuperscript{13}

In summary, the activity of 5-FU at the RNA level is the result of at least three effects: the reduced synthesis of RNA and its precursors, the inhibition of the nuclear RNA surveillance pathway, and the increased levels of intermediate elements with mutagenic potential.\textsuperscript{13,14}

However, while FUTP incorporation into RNA accounts for over 90\% of overall 5-FU-based incorporation in most cells, the consequences of this accumulation have not been extensively studied and its true effects on RNA function remain poorly understood.\textsuperscript{10}

**Potential Clinical Significance of RNA-Directed Mechanisms.** As early as 1973, 5-FU was shown {	extit{in vitro}} to inhibit pre-RNA processing in rat hepatoma cells, which correlated with the stopping of protein synthesis and resulted in apoptosis processes.\textsuperscript{2} More recent murine and human cancer cell line studies have confirmed that 5-FU-based FUTP incorporation into RNA inhibits net RNA and protein synthesis in a concentration and time dependent manner, thought to be due mainly to inhibition of RNA biogenesis.\textsuperscript{13} Significant relationships between 5-FU incorporation into RNA, structural RNA modifications, and loss of clonogenic potential have also been shown in human colon and breast cancer cell lines.\textsuperscript{2} Such findings suggest that atypical RNA processing and maturation is an important aspect of 5-FU cytotoxicity.

However, other {	extit{in vitro}} evidence shows that 5-FU incorporation does not fully interfere with nuclear RNA maturation or functioning, including research illustrating that fluorinated nucleotides can be found in mature RNA, and can even remain for considerable periods of time in patient colon tumor RNA (at least 3 days)\textsuperscript{15} and in mice colon tumor RNA (at least 1 week),\textsuperscript{16} without detrimental effects. This suggests that, while incorporation into RNA may be quantitatively significant, it might not disturb normal RNA-based processes in these models.
Also, other colorectal cell line experimentation suggests that protein expression associated with 5-FU-based cytotoxicity is independent of 5-FU-based incorporation into RNA, furthering the notion that incorporation into RNA is not responsible for related cellular effects.\textsuperscript{16,17}

Clinically, it has been reported that there was no significant relationship between 5-FU metabolite incorporation into RNA and response to 5-FU treatment.\textsuperscript{10} Overall, the significance of 5-FU-mediated RNA alterations and the precise mechanism(s) by which they result in cytotoxic events remain uncertain both in cell lines and clinically. One reason for this is the general lack of understanding about the mechanisms of 5-FU-based RNA misincorporation; another is the lack of research designed to elucidate these mechanisms.\textsuperscript{10,17}

**DNA-Directed Mechanisms.** 5-FU is believed to affect DNA processes through both the inhibition of TS and via direct misincorporation of metabolites.\textsuperscript{2} The inhibition of TS by FdUMP is a well-researched mechanism of DNA-based 5-FU action. TS is an enzyme responsible for the synthesis of deoxythymidine monophosphate (dTMP), which is necessary for downstream DNA replication and repair (Figure 2).\textsuperscript{2,17} Normally, in the presence of 5,10-methylene tetrahydrofolate (CH\textsubscript{2}-THF), TS and deoxyuridine monophosphate (dUMP) form a ternary complex that enables the transfer of a methyl group from CH\textsubscript{2}-THF to the C-5 position of dUMP, forming dTMP. FdUMP disrupts this conversion by forming a catalytically nonproductive, highly stable ternary complex with TS and CH\textsubscript{2}-THF, mainly due to the tight binding nature of its fluorine atom to TS.\textsuperscript{6} The ensuing reduction in levels of dTMP leads to downstream depletion of deoxythymidine triphosphate (dTTP), which induces fluctuations in the levels of other deoxynucleotides (dNTPs). Accordingly, TS inhibition therefore disrupts the normal ratios of dTTP, deoxyadenosine triphosphate, deoxycytidine triphosphate, and
deoxyguanosine triphosphate. These imbalances disrupt DNA synthesis and repair, and are thought to cause severe DNA damage.\textsuperscript{1,2,6,17}

As for misincorporation of metabolites, much more is known about the cellular responses to DNA-based incorporation of 5-FU activity than is known about the responses to its RNA-based mechanisms. Generally, the combined effects of dNTP imbalance and genomic misincorporation are thought to result in a variety of negative consequences affecting both DNA synthesis and the integrity of mature DNA, which lead to the activation of cell cycle checkpoints.\textsuperscript{14,18} More specifically, 5-FU-induced stalled DNA replication forks or damaged DNA is recognized by an assortment of molecules, cuing a signaling cascade that activates the kinases ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR). ATM and ATR then phosphorylate and activate the downstream serine and threonine checkpoint effector kinases, Chk1 and Chk2, which are responsible for initiating appropriate checkpoint responses. Significantly, Chk1 is essential for G2 arrest, which inhibits mitosis in response to DNA damage, stabilizes replication forks, and coordinates 5-FU-induced DNA repair.\textsuperscript{18} 5-FU incorporation is also thought to induce double-stranded DNA breaks, which primarily activate the ATM signaling pathway and lead to the blocking of cell cycle progression and mobilizing of DNA repair machinery.\textsuperscript{14}

In human tumor cells \textit{in vitro}, misincorporated uracil and 5-FU metabolites are mainly removed from DNA by base excision repair (BER) pathways following the activity of uracil-DNA glycosylases (UDGs).\textsuperscript{19} Generally, UDGs cleave an N-glycosidic bond to release the integrated metabolites. The resulting abasic site in DNA can then be cleaved creating a single-strand DNA break, which is then repaired by BER.\textsuperscript{1} Such processes have been identified in numerous human tumor cell lines following 5-FU administration, and very well may act to
protect tumor cells from DNA-directed destruction. This could indicate DNA-based pathways have little to do with the anti-cancer effects of 5-FU.\textsuperscript{19}

In addition to BER, mismatch repair (MMR) is a common cellular response to 5-FU-based DNA damage.\textsuperscript{20} MMR in human tumor cells initiates DNA re-synthesis along with polymerase and ligase activities, similar to those involved in BER.\textsuperscript{1} In studies, following 5-FU treatment, MMR improves DNA replication by correcting replication errors, such as base-to-base mismatches, and terminating strand exchange between mismatching sequences that occur following incorporation of dUTP and FdUTP.\textsuperscript{20} In these ways, MMR processes may also protect tumor cells from the DNA-directed effects of 5-FU treatment.

**Potential Clinical Significance of TS-Mediated DNA Mechanisms.** *In vitro*, a relationship between TS expression and DNA-mediated cytotoxicity after 5-FU treatment has been demonstrated in colorectal cancer cell lines according to a variety of measurement indicators, including TS enzyme activity, protein levels, and mRNA levels.\textsuperscript{2,6} For example, in colorectal tumors with low TS expression, ternary complexes of FdUMP, CH\textsubscript{2}THF, and TS were more efficiently formed than in tumors with high TS expression, which resulted in greater inhibition of DNA synthesis and increased cytotoxicity.\textsuperscript{14} Similarly, TS is overproduced in many 5-FU-resistant cell lines, implying that inhibition of this enzyme may be key to the antimetabolite’s efficacy.\textsuperscript{3} On the other hand, many other *in vitro* studies have shown no significant correlation between TS inhibition and 5-FU activity, and some have shown a negative correlation. Mice with advanced colon tumors, and treated with 5-FU, had a greater chance of survival than those treated with another TS inhibitor.\textsuperscript{2} In human colon cancer cell lines, suppression of proliferation continued in similarly inhibited cells even after the addition of exogenous thymidine, which should negate the effects of TS inhibition, as well as after
simultaneous treatment with thymidine and 5-FU.\textsuperscript{2,14} In both cell lines and tumors it was also found that treatment with 5-FU acutely induced TS expression, and similarly, that certain high concentrations of thymidine actually increased 5-FU antitumor activity.\textsuperscript{21} Such findings complicate the conclusion that TS inhibition correlates with DNA-mediated tumor cell death. In fact, since added thymidine does not always prevent the inhibitory effects of 5-FU on cell proliferation, and in some cases enhances cytotoxicity of the drug, there must be an alternative \textit{in vitro} mechanism of action for 5-FU.\textsuperscript{19,21}

The clinical role of TS in human tumors treated with 5-FU is also unclear. Patients with low TS gene expression in tumors have been shown to survive longer after 5-FU treatment than those with high tumor TS gene expression, which supports the notion that TS inhibition is critical to the agent’s effects in humans.\textsuperscript{15} Similarly, leucovorin (LV), which enhances TS inhibition by increasing the intracellular concentration of CH\textsubscript{2}THF, has been shown to improve \textit{in vivo} response to the drug.\textsuperscript{1} In fact, a series of landmark studies by Mayo Clinic illustrated that co-administration of 5-FU and LV improved quality of life, prolonged survival, and afforded higher response rates in patients as compared with 5-FU alone.\textsuperscript{10} Many other such studies have led to similar conclusions such that, at the present time, the combination of 5-FU and LV (with oxaliplatin or irinotecan) is considered standard chemotherapy for advanced colon cancer.\textsuperscript{3} Taken together, this indicates that TS concentration and inhibition are still currently accepted to correlate with 5-FU’s clinical activity.

Despite such evidence, the clinical significance of TS inhibition in patients has yet to be conclusively demonstrated. Although some studies suggest that TS concentration is a predictor of response to fluoropyrimidine treatment, this remains controversial. For example, in metastatic colon cancer, high levels of TS reflect advanced tumor progression as well as poor response to 5-
FU.\textsuperscript{15,22} The true correlation may thus be between poor response to the drug and advanced tumor progression, rather than between poor response and high levels of TS. Intrigued by such considerations, Showalter \textit{et al.} investigated the association between 5-FU and TS expression with a thorough literature survey and, in contrast to previous predictions, found no connection between TS and patient response.\textsuperscript{22}

**Potential Clinical Significance of Direct DNA Incorporation.** Several studies have explored incorporation of radiolabeled 5-FU and uracil metabolites into DNA using a variety of cell lines, cell culture systems, and human subjects.\textsuperscript{3,9,13,14,23} A majority of these studies provide evidence that substantial quantities of genomic 5-FU-based incorporation occurs following treatment, although it is not surprising that these amounts vary given the many murine and human, as well as \textit{in vitro} and \textit{in vivo}, models examined. Although some of this research reports that genomic misincorporation contributes to cytotoxicity, it is still unclear how significant uracil incorporation or 5-FU is to cell killing. This is mainly true because, as the rates of incorporation versus excision are not well determined in such studies, the interplay between misincorporation and DNA repair regarding cytotoxicity has yet to be established both in cell lines and clinically.\textsuperscript{14,17}

In fact, while 5-FU-based genomic incorporation is well-verified, the efficiency of subsequent DNA repair remains highly controversial both \textit{in vitro} and \textit{in vivo}.\textsuperscript{1} Despite a solid understanding of BER and much evidence that BER is activated following 5-FU administration, there is little known about how DNA lesions produced by uracil and 5-FU-based incorporation are specifically processed.\textsuperscript{19} In any case, the incorporation of dUTP and FdUTP into DNA has been correlated in human tumor cell lines and clinically with greater than expected levels of deleterious cellular effects, such as inhibition of further DNA synthesis, DNA fragmentations
due to single- and double-strand breaks, and mutations induced by 5-FU base pairing with guanine instead of adenine.\textsuperscript{24} It has also been reported that 5-FU exposure can restrain the DNA repair process in human tumor cell lines because of dTTP depletion, which leads to generation of fragmented DNA.\textsuperscript{20} Conversely, each of these points has been refuted in experiments involving murine and human cell lines.\textsuperscript{19,24}

Key to the controversy surrounding the effectiveness of DNA repair in 5-FU-based treatment, and thus the significance of DNA-based mechanisms, is the specific role of UDGs in human tumor cells. Each of the four UDG types has been variably implicated in cellular responses to 5-FU using different model systems.\textsuperscript{1} Importantly, it has been reported that the enzyme uracil N-glycosylase (UNG) is responsible for most of UDG activity involved following 5-FU treatment of human tumor cells.\textsuperscript{19,23} There is also evidence that UNG-initiated DNA repair protects normal and tumor cell lines through the excision of uracil and 5-FU metabolites, thus preventing any related effect following 5-FU therapy.\textsuperscript{24} Other \textit{in vitro} evidence suggests that UNG initiates repair following drug treatment, but in doing so creates a more toxic lesion. In this way, the repair enzyme contributes to cytotoxicity. For example, one study reported that 5-FU-mediated incorporation into DNA contributed to the formation of DNA strand breaks in both mouse bone marrow and human tumor cells, and that these breaks resulted from the removal of 5-FU metabolites by UNG.\textsuperscript{19} In direct opposition to this research, other murine and human cell line studies have reported that UNG has no role in the removal of 5-FU metabolites, suggesting that other UDGs may be involved.\textsuperscript{24} Overall, a direct correlation between 5-FU incorporation, DNA repair, and cytotoxicity has yet to be established \textit{in vitro} and \textit{in vivo}, and thus so does the significance of DNA-based mechanisms in 5-FU therapy.\textsuperscript{3,6}

\textbf{Methods}
This review was conducted as a quantitative systematic review of the scientific literature regarding the effects of 5-Fluoruracil in the treatment of colorectal cancer. It began as an exploration of treatment duration of IV 5-FU for late stage colorectal cancer. After finding only limited studies regarding treatment duration, the review was redirected to examine what appears to be a large question in the field.

The greatest difficulties included limiting the number of references among the available research, and correlating research that addressed the mechanisms of action of 5-FU to studies in various models aimed at specifically addressing colorectal cancer treatment. For included references, the primary outcome measure was impact on elucidating whether 5-FU exerted effects due to DNA or RNA-based mechanisms, which included articles that summarized the background of these mechanisms.

**Literature Search and Data Abstraction.** A literature search was performed using PubMed Central. Articles were searched for key words 5-Fluoruracil, 5-FU, colorectal cancer, DNA, RNA, and mechanism of action. The 5-Fluoruracil and 5-FU searches were crossed with the other search terms using the Boolean operator “AND.” The literature search was limited to English language articles.

Abstracts from searches were reviewed by a single reviewer to identify articles containing potential relevancy data. Full articles were obtained for all selected abstracts and were further reviewed for inclusion. The inclusion criteria applied at this step were: elucidation or discussion of the mechanism of action of 5-FU, use of 5-FU in cancer cell lines and animal models, use of 5-FU for late stage colorectal cancer in patients, and potential future uses of 5-FU. All full articles were also reviewed for references of interest. Abstracts and citations of selected references were obtained and reviewed according to the literature search procedure.
This step yielded most of the research included in this article. Finally, full articles were summarized, compared to one another, and organized according to relevance.

**Discussion**

Despite decades of research, the question remains as to which mechanisms are more important in 5-FU-based cytotoxicity, and furthermore, whether these mechanisms are the most important in both tumor and normal cells, both murine and human cells, and both cell lines and clinical models. There are several determinants of sensitivity to 5-FU that may help to explain why experimentation has failed to uncover the exact cellular responses. These include the model-specific availability of enzymes and co-factors involved in 5-FU metabolism and activation, the level of TS activity or expression, the extent of metabolite incorporation into RNA or DNA, and the ability of repair processes to neutralize toxic 5-FU metabolites.\(^{1,3,6,10,19}\) To complicate matters further, preclinical and clinical models suggest that toxicity, efficacy, and primary mechanism of action of 5-FU varies with the method and time of administration, whether 5-FU or a pro-drug is utilized, and which biomodulators are used in combination with 5-FU.\(^{2,6,9,25}\) The following sections summarize key evaluations of the current research and propose solutions as to how these considerations might be addressed in *in vitro, in vivo*, and clinical models.

**Overcoming Discrepancies Among Results from Various Models.** The most obvious question concerning 5-FU therapy regards how the agent truly kills human tumor cells. Although countless studies have attempted to determine the specific mechanisms using cell lines, there are inherent disadvantages to this approach because lines grown in culture differ from real tumors in many ways. For example, there is much evidence that numerous genetic changes occur during both the induction and continuation of cellular growth on media.
disadvantage is that it is impossible to artificially mimic the environment of a tumor in vitro, as real tumors don’t grow in synthetic culture supplemented with animal sera. A third problem is that cell lines are composed solely of tumor cells, while real tumors contain many other types.\textsuperscript{6,10}

One way to overcome these concerns would be to explore and comparatively analyze mechanisms by conducting a comprehensive investigation in controlled model systems using cell lines derived from specific tumors that are treated with 5-FU, while keeping in mind the limitations of cell lines. An alternative method is to inoculate human tumors extracted from patients into an animal. Although it is preferable to inoculate cells into the same tissue type in which the tumor originally grew, it is possible to utilize another tissue or organ. For instance, it has proven worthwhile to seed human colon tumors into a mouse liver, as the liver is a common site of metastasis in humans.\textsuperscript{6} Such a model could then be utilized to test for specific mechanisms of 5-FU-based therapy, suggestions for which will be discussed below.\textsuperscript{2} Although these methods may be useful toward elucidating mechanisms, only clinical-based observations from patients will truly validate the efficacy and toxicity of 5-FU in humans.

**Elucidating the True Impact of 5-FU Incorporation into DNA.** One key consideration as to the importance of DNA-based pathways involves the common experimental use of FdUrd as a pro-drug for the elucidation of 5-FU-based mechanisms. As FdUrd has been highly correlated with DNA-directed mechanisms, subsequently, so has 5-FU. However, FdUrd and 5-FU vary in their pharmacological, therapeutic, and toxic attributes. As an example of these differences, in experiments involving 5-FU treatment of CHO-K1 cells, DNA incorporation has been reported to be only about 10\% of RNA incorporation.\textsuperscript{9} Conversely, following administration of FdUrd, DNA and RNA incorporation was approximately the same.\textsuperscript{9,10} Also, DNA strand breaks have been found to be more significant in FdUrd cytotoxicity
than 5-FU. Subsequently, because it has been common to equate 5-FU with its derivative for experimental purposes, it may be that some of the established knowledge that 5-FU has DNA-based mechanisms of cytotoxicity are based incorrectly on experiments utilizing FdUrd. The models outlined above could be utilized to more fully distinguish the effects of 5-FU from FdUrd, and in this way, gain a better understanding of DNA-based mechanisms regarding 5-FU. In any case, further analysis of both 5-FU and FdUrd may lead to both agents being more successfully modulated and incorporated into chemotherapeutic therapies.

**Elucidating the True Impact of 5-FU Incorporation into RNA.** A significant reason for our lack of comprehension concerning RNA-based 5-FU mechanisms is the paucity of research designed to elucidate these mechanisms. One suggestion is to conduct further studies in other genetic systems, which might identify novel genes, or reinforce the importance of known ones, involved in RNA-based effects. For example, genetic studies in yeast have identified critical elements in RNA-based mechanisms, such as nuclear RNA exosome subunit Rrp6p. Defects in Rrp6p were shown to cause high sensitivity to 5-FU. Research with yeast also indicated that although a DNA repair mutation triggered some sensitivity to treatment, Rrp6 mutations produced significantly greater sensitivity, potentially because of drug-induced RNA damage. Such yeast genes could correlate with homologous genes in humans, which might have at least similar importance in 5-FU activity. Future studies could then assess whether these homologous genes in humans play a role in the treatment of tumor cell lines, and ultimately, in real tumors.

Additionally, to better understand the impact of 5-FU incorporation into RNA, it would be best to conduct a comprehensive analysis in a tumor model system using cell lines derived from tumors that are treated with 5-FU, paying special attention to RNA pathways. One
potential place to start is by further examining the OPRT-involved pathway through which FUTP is created during 5-FU anabolism. The favored use of this pathway in certain colon cancer cell lines and xenograft models was shown to correlate with higher sensitivity to 5-FU.\textsuperscript{2} Such preliminary results indicate that this may contribute to the specialized targeting of rapidly dividing cells in these systems to a greater degree than DNA-based mechanisms. Following up with greater research on members of this and other RNA-involved pathways may prove beneficial in better understanding the role of RNA misincorporation.\textsuperscript{2,19}

\textbf{Conclusion}

Despite therapeutic enhancements to 5-FU treatment over previous decades, new strategies are still needed. As with most chemotherapeutics, toxicity and resistance remain considerable limitations to its clinical use. To predict or overcome these issues, it is essential to better understand the mechanisms by which this agent promotes cell death and by which tumors demonstrate or become resistant to it. Accordingly, more research should be directed toward 5-FU, especially focused on illuminating both tumor killing and normal cell toxicity pathways that we know little about.\textsuperscript{3}

Previously, efforts to modify 5-FU cytotoxicity have centered on lessening its degradation and augmenting its activation, as well as increasing overall TS inhibition.\textsuperscript{22,25} Using high-throughput transcriptional profiling to identify downstream signaling pathways involved in cellular response to 5-FU will be a key step toward bettering our understanding of 5-FU-based mechanisms. In addition to identifying such interactions, high-throughput techniques may also identify novel therapeutic targets. Such potential targets would first have to be evaluated using \textit{in vitro} experimentation, perhaps by transgenic expression and antisense techniques, followed by studies on human tissues in animal models like those already suggested.\textsuperscript{6} Both uses of
transcriptional profiling will hopefully lead to the future development of combined chemotherapy regimens best designed to enhance the cytotoxic, and minimize the toxic, activity of 5-FU.

High-throughput technology could also be used directly as a therapeutic tool. While studies outlined in this review have identified some biomarkers that may predict tumor-cell sensitivity to 5-FU, identifying and analyzing additional factors will enhance our predictive capabilities even further. As ensuing clinical trials verify these biomarkers and lead to treatment that is more tailored to molecular phenotypes of tumor and patient, we should see increased tumor response rates, decreased rates of toxicities, and lower patient care costs.$^{1,2}$

However, it is important to keep in mind that determining the functional significance of biomarkers regarding clinical trials requires not only rigorous analytical tools, but also thorough attention to data management and ethical concerns, including the meticulous tracking of amounts and time schedules of drug administration, patient characteristics, tumor response, adverse effects, and long-term follow-up.$^2$ Also, because many biomarkers are likely to affect the outcomes of anticancer drug therapy, clinical trials should include large numbers of patients to produce meaningful results. If current trials include appropriate collection and storage of patient samples, documentation of therapy and results, and adequate study design, future analyses may be able to test which variants are predictive of anticancer outcome.$^6$ Perhaps in the coming years, clinical trials that target 5-FU therapy based on each patient's individual genetic constitution will be able to test whether such individualization truly benefits patients.

Indeed, the advent of high-throughput transcriptional profiling may have enormous therapeutic implications for cancer therapy. A better understanding of the molecular determinants of sensitivity to 5-FU may lead to more rationally designed treatment
combinations, the detection of new biomarkers and therapeutic targets, and enable the individualization of 5-FU-based patient treatment. It is conceivable that, soon, high-throughput techniques will be available for routine use in a clinical setting, and diagnosis and treatment of associated cancers will be tailor-made according to the expression profile of each patient. For now, our first step is to further our understanding of the mechanisms of 5-FU efficacy.
References


Appendices

Figure 1: Metabolism of 5-fluorouracil.²

Figure 2: Mechanism of thymidylate synthase inhibition by 5-fluorouracil.
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