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Use of Small Molecule Fanconi Anemia Pathway Inhibitors as Sensitizing Agents to Laromustine.

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By Sam W. Marchant

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5/19/2021
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5/19/21
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Vitae

Sam Willard Marchant was born in Beverly, Massachusetts on April 30, 1999 to Wayne and Lorna Marchant. He grew up in Merrimac, Massachusetts and graduated from Pentucket Regional High School in 2017. As a Presidential Scholar, Sam began working in the Rice Lab during September of his first year at Colby and continued his research on the anti-cancer efficacy of laromustine for all academic terms and summers throughout his Colby career. He also spent a summer interning as a Biomedical Research Scientist for Reboot Rx. At Reboot Rx, Sam helped develop an AI Platform that synthesizes information from PubMed randomized controlled trials. Outside of research, Sam worked as a chemistry help center tutor his last three years at Colby. In addition to being a Presidential Scholar, Sam has been recognized as a Bixler Scholar; awarded the Phi Beta Kappa Scholastic Achievement Award, Colby College Physical Chemistry Award, Colby College Department of Chemistry Alumni Award, Colby College Department of Chemistry Stan Award, and AIC Biochemistry Award; and inducted to the Phi Beta Kappa Society. Sam majors in Chemistry with a concentration in Biochemistry and Physics and minors in mathematics. He will be graduating with a Bachelor of Arts in May 2021. Upon graduation from Colby, Sam will be attending the University of California San Diego to pursue a Ph.D. in Biochemistry and Biophysics.

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Lastly, I would like to thank all the amazing professors at Colby College. I want to give a special thanks to Professor Lindsey Madison, Professor Greg Drozd, and Professor Dasan Thamatoor. Lindsey and Greg, you both have truly guided my aspiration for my Ph.D research and Das you always bring life to Keyes when it is only you and me there on the weekends

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Abstract

Laromustine is an experimental chemotherapeutic sulfonyl hydrazine prodrug shown in clinical trials to be effective against acute myeloid leukemia. The mechanism of action of laromustine involves interstrand crosslinking, via chloroethylation, and enzyme inhibition, caused by carbamoylation. The work described herein aims to investigate whether inhibition of the replication-dependent interstrand crosslink repair Fanconi Anemia pathway further sensitizes cells to laromustine. By measuring metabolic activity immediately after drug exposure, we find laromustine to be equally as cytotoxic towards Fanconi Anemia deficient and wild type cells. However, through clonogenic assays we show Fanconi Anemia mutations sensitize cells to laromustine's anti-proliferative effect. Furthermore, we show curcumin, which has previously been reported as a Fanconi Anemia pathway inhibitor, is able to induce a similar sensitization to laromustine as observed in mutant cells. A similar effect is not observed for another reported inhibitor, pimozide. Further research expanding on these findings could inform combination therapies that could reduce therapeutic dose of laromustine.

Introduction

Laromustine [VNP40101M; Onrigin; Cloretazine; 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methylamino) carbonylhydrazine] is an experimental anticancer drug developed, along with a series of other sulfonylhydrazine DNA modifying agents, in 2001 by Alan Sartorelli in the Pharmacology Department at the Yale University School of Medicine.[1] These novel compounds were developed as alternatives to N-(2-chloroethyl)-N-nitrosoureas (CNUs). CNUs are a class of antineoplastic agents that exhibit strong anticancer activity against brain tumors, colon cancers, and lymphomas.[2,3] CNUs decompose into three reactive products, a chloroethylating, a carbamoylating, and a hydroxy ethylating species.[4,5] The hydroxy ethylating species has been found to a have a counterproductive effect in cells as it contributes to carcinogenesis[6] and to a resistance mechanism involving tumor-cell catalyzed denitrosation.[7] Sulfonylhydrazines were developed as drugs that retain the chloroethylating and carbamoylating activity, but lack the hydroxy ethylating activity of CNUs.

Laromustine was selected from the other novel alkylating agents for further development since it displayed significant broad-spectrum anticancer activity and therapeutic safety in preclinical trials.[1] Phase I trials found that laromustine exhibited significant activity against leukemia.[8] Subsequent Phase III clinical trials showed treatment with laromustine in combination with cytarabine increased the rate of complete remission from 19 % to 35 % in patients with first relapse acute myeloid leukemia (AML) in comparison to treatment with cytarabine alone.[9] However, further clinical research on laromustine was ceased because Phase III trials also found a decreased overall patient survival rate.[9] Nonetheless, the increased complete remission rate indicates further research on the biochemical mechanism of action of laromustine may provide the potential for new drug development and cancer treatments.

Laromustine is a prodrug that yields two reactive electrophiles *in situ* upon base-catalyzed activation (Figure 1). It breaks down into a chloroethylating, DNA interstrand crosslinking species (90CE) and a carbamoylating species (methyl isocyanate). Accessibility of laromustine analogues possessing only chloroethylating activity (90CE) and only carbamoylating activity (101MDCE) has allowed partitioning of laromustine's anticancer activity into contributions from each of its reactive subspecies.

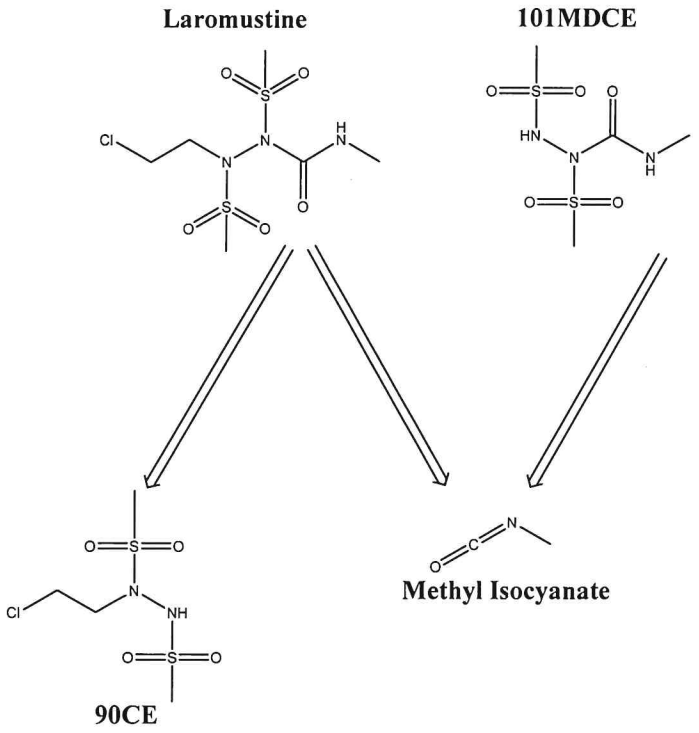

Figure 1. The base-catalyzed decomposition of laromustine into its two reactive subspecies: 90CE and methyl isocyanate.

The cytotoxicity of the 90CE analogue is shown to be significantly reduced by the expression of O^6-alkylguanine-DNA alkyltransferase (AGT).[10] AGT removes alkyl groups from the O^6 position of guanine in DNA by transferring the alkyl group to a cysteine residue in the active site of the protein. Therefore, the chloroethylating activity is believed to cause harmful DNA interstrand crosslinks (ICL) via chloroethylation on the O^6 position of guanine. The

chloroethylated guanine forms an ICL through two sequential reactions: first an intramolecular nucleophilic substitution results in the formation of N^1,O^6-ethanoguanine; next this molecule reacts with the N-3 position of the adjacent cytosine on the complementary DNA strand to yield a G-C etyl cross-link (Figure 2). [11–13]

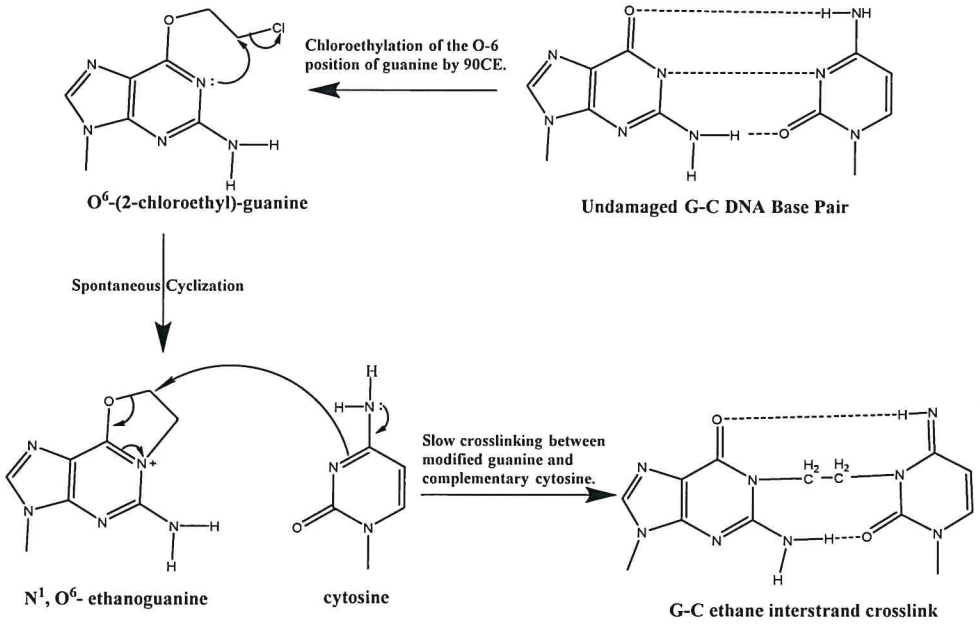

Figure 2. Mechanism of interstrand crosslink formation by a short-lived chloroethylating species formed by 90CE.[14]

The activity of the carbamoylating agent methyl isocyanate is less understood. However, in vitro, the analogue possessing only carbamoylating activity, 101MDCE, has shown cytotoxicity via modification of sulfhydryl groups on various macromolecules including proteins such as thioredoxin reductase and DNA repair proteins uch as DNA polymerase β and AGT.[13,15–17]

Previously, the ICL activity due to 90CE has been believed to be the major factor contributing to laromustine's antineoplastic activity.[13] However, recently we have shown the importance of laromustine's carbamoylating activity. Using DNA microarray analyses we demonstrated that methyl isocyanate is responsible for the majority of laromustine's gene dysregulation. Additionally, we used flow cytometry, PARP1 cleavage, and Caspase 3/7

experiments to find evidence that methyl isocyanate may be more critical to the activity of laromustine than previously appreciated.

Moreover, there appears to be synergism between the activity of methyl isocyanate and 90CE, as prodrugs yielding both 90CE and isocyanates have proven to yield more DNA ICLs, cause more apoptotic cell death, and thus to be more effective anticancer drugs, than 90CE alone.[18,19]It is proposed that this increased efficacy is a result of methyl isocyanate's inhibition of DNA repair protein, including AGT the protein responsible for preventing ICLs induced by 90CE by removing alkyl groups from alkylated DNA.[13]

ICLs are a highly cytotoxic form of DNA damage that induce chromosomal aberrations and interfere with the separation of DNA strands, thus making DNA inaccessible for replication, transcription, and repair.[10] DNA damage resulting from ICLs has been widely studied within the realm of chemotherapeutic agents. Previous research has shown that ICLs are involved in the mechanism of action of a variety of natural compounds, such as mitomycin C, and novel chemotherapeutic drugs, including laromustine, nitrogen mustards, nitrosoureas, and platinum-based compounds (*e.g* cisplatin).[20]

However, chemotherapies yielding different crosslinking agents differ in the specific mechanism with which they induce crosslinks, their nucleotide base site specificity, the number of crosslinks they yield, and the cytotoxicity and bulk of the lesions they induce.[20,21] Laromustine, induces G-C crosslinks via 90CE induced chloroethylation at the O^6-position of guanine. These G-C crosslinks are relatively small lesions and thus it might be more difficult for DNA repair proteins to recognize them as opposed to more bulky ones.[14]

Herein, by investigating small molecule inhibitors of the Fanconi Anemia ICL repair pathway (Figure 3), we aim to reveal mechanisms by which cells mitigate the damage caused by

laromustine and possibly identify avenues for patient pre-screening or co-therapeutic strategies. Since many chemotherapies induce ICL, research here may be significant in aiding the development of combination therapies that involve any one of these crosslinking drugs.

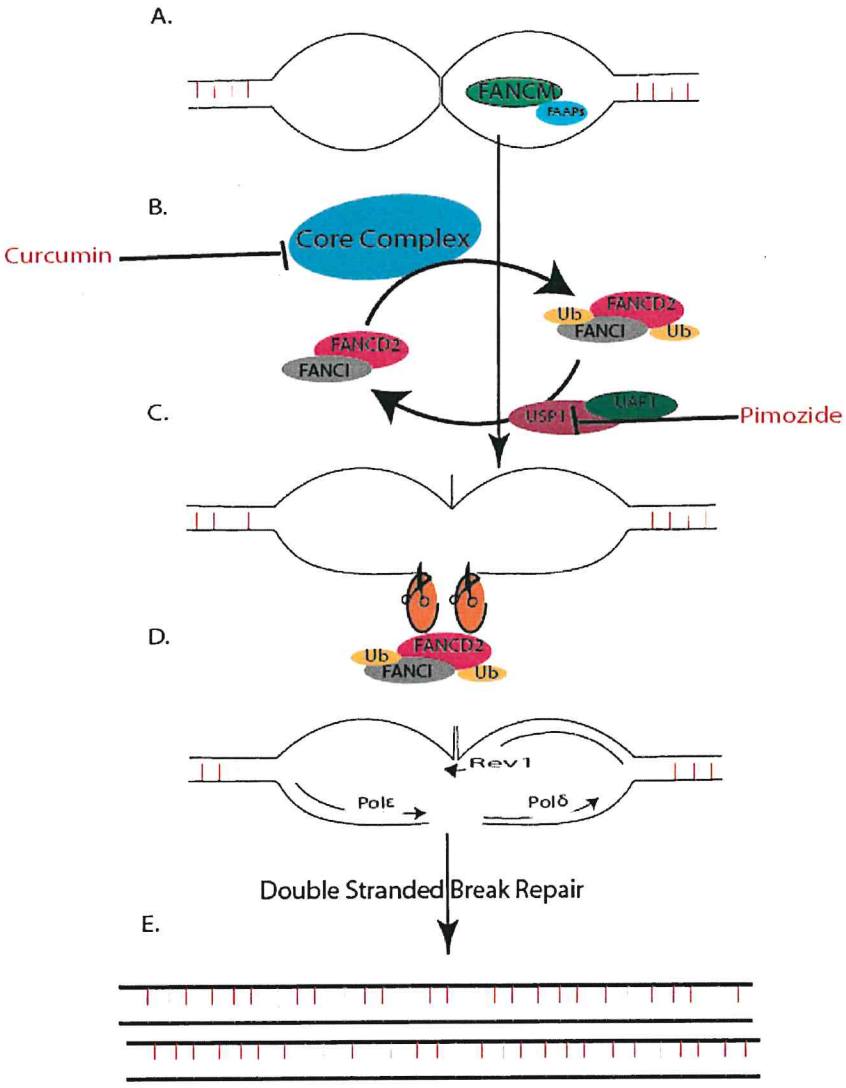

Figure 3. A brief overview of the ICL repair initiated by the FA pathway as described in the text. A) FANCM recognizes a stalled replication fork at the site of an ICL induced by some endogenous or exogenous agent. B) FANCM recruits the core complex which in turn leads to monoubiquitination of ID2 complex. Also shown here is the process of ID2 deubiquitination catalyzed by the USP1/UAF1 complex. Curcumin is proposed the inhibit the core complex and pimozide the USP1/UAF1 complex. C) Site specific endonucleases colocalize with ID2-ub to perform unhooking. D) Translesion synthesis polymerases such as Rev1 or DNA Polζ perform error prone replication around the unhooked crosslink, and traditional DNA replication polymerases DNA Polδ and DNA Polϵ on the strand that does not retain the crosslink. Not shown here are the other replication fork associated enzymes. E) Double stranded breaks caused by unhooking are repaired by homologous recombination to complete ICL repair and yield two daughter double helix DNA molecules.

The Fanconi Anemia (FA) Pathway is a replication-dependent ICL repair pathway used to detect and initiate ICL repair through promoting nucleotide excision repair, homologous recombination, and trans-lesion synthesis.[22] The major components of the FA pathway include twenty-two FA proteins known as complementation groups (designated FANCA through FANCW) and various FA-associated proteins (FAAP).[23]

Recognition of ICLs occurs at stalled replication forks by the anchoring complex, which contains FANCM and FAAPs. Upon detection of ICLs, the anchoring complex recruits the multisubunit FA core complex to the ICL. This core complex contains an enzymatic domain containing an E3 ubiquitin ligase, FANCL, which transfers an ATP-hydrolyzed ubiquitin attached to the E2 ubiquitin-conjugating enzyme FANCT (UBE2T) to a different complex involving FANCD2 and FANCI (ID2 complex), thereby monoubiquitinating ID2.

Monoubiquitination differs from the traditional polyubiquitination which is used to tag proteins for destruction by the proteosome. Instead, monoubiquitination is a stable protein modification like phosphorylation that acts in cell signaling, usually by altering a protein's activity, cellular location, or ability to form electrostatic interactions. Here, the monoubiquitination of ID2 is a key step in ICL repair which increases the ID2 complex's affinity for chromatin, causes colocalization of ID2 with downstream DNA repair enzymes at the ICL, and ultimately results in DNA lesion removal and repair.[22,23]

The first ICL repair step initiated by monoubiquitinated ID2 is recruitment of site-specific endonucleases (SSE) which perform ICL unhooking via nucleolytic incisions 3' and 5' to the lesion on one of the DNA strands. ICL unhooking uncouples the two complementary DNA strands by removing the ICL's covalent linkage, via the introduction of a single-stranded gap, to one of the DNA strands. Unhooking is followed by a bypass step where translesion synthesis polymerases,

recruited by the core complex as opposed to the ubiquitinated ID2 complex, perform error prone replication to fill in the single stranded nucleotide gap introduced by the nucleolytic incisions. Unhooking also causes double-stranded breaks that are repaired by homologous recombination proteins, which, like SSE, are recruited by monoubiquitinated ID2.[20,22–24] ID2 is subsequently deubiquitinated by the USP1-UAF1 complex. This step appears to be necessary for the completion of ICL damage reversal, as seemingly paradoxically, although USP1 ablation increases chromatin bound monoubiquitinated ID2, it also increases ICL sensitivity.[25]

There exist a variety of well-studied inhibitors of the FA pathway. The natural product curcumin and its analogs EF24, 4H-TTD, and MLN4924 inhibit this pathway by targeting the FA core complex through inhibiting an adaptor protein in the complex, FANCF. As a result of this inhibition ID2 monoubiquitination is blocked.[26,27] As one would expect, this activity of curcumin sensitizes cells to the crosslinking chemotherapy cisplatin[26] and EF24 sensitizes wild type, but not FA deficient, cells to ICL agent mitomycin C.[27]

Another potentially interesting class of FA pathway inhibitors are USP1 inhibitors, which include pimozide, C527, and GW7647.[22] Such inhibitors have been shown to inhibit K562 leukemic cell growth, decrease homologous recombination, increase ubiquitinated ID2, and sensitize cells to interstrand crosslinking chemotherapy mitomycin C and the topoisomerase I inhibitor camptothecin.[28] Among these USP1 inhibitors, of great intrigue is the antipsychotic pimozide which is traditionally used to treat tics caused by Tourette syndrome. Pimozide not only has been shown to inhibit deubiquitination of the ID2 complex via USP1, but it has also been proven to chemo sensitize via activity as a STAT-3 and STAT-5 signaling protein inhibitor and possess tumor inhibitory activity through inhibition of Ca^{2+} calmodulin, T-type Ca^{2+} channels, and σ-receptors.[29] Given its potential to exert tumoricidal activity and to sensitize cells

to chemotherapies through both the JAK/STAT signaling pathway and the FA pathway, pimozide is a promising candidate for combination treatment with previously established interstrand crosslinking chemotherapies.

Several factors led us to investigate whether the FANCF inhibitor curcumin and/or the USP1 inhibitor pimozide induce increased sensitization to laromustine. The first factor was the importance of the ID2 monoubiquitination/deubiquitination cycle to the completion of ICL repair. Additionally, a contributing element was the evidence of synergism between FA pathway inhibitors and other ICL chemotherapeutic drugs. The final determinant was the association of inherited FA pathway deficiencies with ICL sensitivity.

Here, we used PD20 cells as a model for FANCD2 deficiency in the FANCI-FANCD2 (ID2) complex, which as previously discussed, mediates ICL unhooking. These immortalized fibroblast cells are derived from a Fanconi Anemia patient deficient in FANCD2 due to biallelic expression of mutant FANCD2 with the paternal allele coding for FANCD2 with a single amino acid point mutation and the maternal allele coding for a truncated form of the protein due to a single nucleotide point mutation.[30] We employed clonogenic assays to assess whether FANCD2 deficiency in PD20 fibroblasts increases cytotoxicity of laromustine. Additionally, Western blots with FANCD2 antibodies were employed to confirm previously reported activity of each small molecule involved in inhibition of the ID2 monoubiquitination/deubiquitination cycle. Finally, by measuring cytotoxicity towards retrovirally recovered FANCD2 deficient cells (PD20 RV:D2), the ability of inhibitors to increase efficacy of laromustine was assessed.

Effective combination treatments involving laromustine and FA pathway inhibitors holds practical implications for the development of new combination therapies involving any crosslinking therapy and these inhibitors. The ability of such inhibitors to sensitize cells to ICLs

by increasing the yield of crosslinks for a given dose could aid in the more effective treatment of cancers and may allow oncologists to treat cancer patients with lower doses of these cytotoxins and thus potentially treat tumors with less severe side effects.

Material and Methods

Cell Culture

Human acute myeloid leukemia (HL60) cells were grown in RPMI-1640 medium with 0.1% gentamycin, and 10% fetal bovine serum. Human immortalized fibroblast FANCD2 deficient (PD20), FANCD2 deficient retrovirally empty (PD20 RV:E), and FANCD2 retrovirally corrected (PD20 RV:D2) were grown in Alpha-MEM medium with 10% FBS and 1% Penicillin-Streptomycin and maintained between 20–80% confluence. Cells were grown at 37°C with 5% CO_2 and 100% relative humidity. All incubations described are under these conditions unless specified otherwise.

Metabolic Activity Assay

96 well plates were seeded with 15,000 cells/well PD20, PD20 RV:E, or PD20 RV:D2. After incubating for 24 hr to allow cells to adhere, cells of each line were treated for 2 hr with either 100 μM laromustine, 50 μM laromustine, or equivalent volume DMSO. After equilibration of plates to room temperature, metabolic activity of cells was quantified, according to manufacture protocol, using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Luminescence was quantified using a SpectraMax M5 plate reader (Molecular Devices). Data were analyzed as average luminescence over 48 wells and errors were measured as standard deviations.

Clonogenic Assay

Six-well plates were seeded at 4,000 cells/well with PD20, PD20 RV:E, or PD20 RV:D2 cells. After incubating for 4 hr to allow cells to adhere, wells of each cell line were treated for 3 hr with either 100 μM laromustine or DMSO. Media in wells were refreshed with drug-free media and cells were incubated for 7 d. After incubation, cells were fixed and stained by incubating for 10 min with an aqueous solution of 6.0% glutaraldehyde (vol/vol) and 0.5% crystal violet

(wt/vol). Wells were then washed with water, dried at room temperature, then imaged using an Azure c600 imaging system. The clonogenicity was quantified using the ImageJ plugin, ColonyArea,[31] which determines the percentage of the well covered by stained cells. Colony area percentage for each laromustine treatment was then normalized for plating efficiency by dividing each percentage by the colony area percentage from the control well of the corresponding cell type.

Verification of Inhibitor Activity using Western Blotting

To verify previously reported inhibitors activity, 1×10^6 HL60 were incubated for 24 hr with 8 μM curcumin, 8 μM pimozide, or equivalent volume of DMSO. Concentrations of inhibitor were chosen based on reported ranges of concentration necessary for inhibition.[26,28] Samples were then harvested, washed with 1X phosphate buffered saline, and lysed using Pierce™ IP Lysis Buffer with EDTA containing protease inhibitors. Prior to storage at -70 C, lysates were concentrated via ultracentrifugation and concentrations were determined via a BCA assay.

Lysate samples were thawed and prepared for denaturing PAGE under reducing conditions by heating. PAGE was performed using 12 μg of lysate run on a NuPAGE™ 3 to 8%, Tris-Acetate, 1.0 mm, Mini Protein Gel with NuPAGE™ Tris-Acetate SDS Running Buffer. Sample preparation and electrophoresis were performed according to NuPAGE® Technical Guide.[32] Separated proteins were transferred to a nitrocellulose membrane via electrophoretic transfer. Nitrocellulose membrane was incubated at room temperature with FANCD2 (1436:1451) affinity purified rabbit polyclonal primary antibody at a concentration of 1 μg/mL. Membranes were then incubated at room temperature with Pierce® Fast Western Rabbit Optimized HRP Reagent and subsequently washed and incubated with Pierce® SuperSignal

West Dura Luminol/Enhancer Solution and SuperSignal West Stable Peroxide Solution at 1:1. An Azure c600 Imaging system was used to image resulting chemiluminescence.

Efficacy of Combination Treatment

To investigate the ability of curcumin and pimozide to sensitize cells to laromustine, 6 well plates were seeded at 4,000 cells/well PD20, PD20 RV:E, or PD20 RV:D2. After incubating for 4 hr to allow cells to adhere, cells were treated for 3 hr with 100 μM laromustine with 10 μM pimozide, 100 μM laromustine with 10 μM curcumin, or DMSO. After drug incubation, media was refreshed, and cells were incubated for 7 d. After incubation, staining/fixing of cells and quantification of colonies via ColonyArea plugin was performed as described above for the clonogenic assay.

Results

FANCD2 deficiency does not initially sensitize cells to laromustine treatment.

To investigate whether FANCD2 deficiency causes an increased cytotoxic effect of laromustine, a metabolic activity assay measuring ATP content of cell samples was performed. To quantify drug induced cell death, ATP content of cells immediately after 3 hr exposure to laromustine was measured and normalized relative to ATP content cells of the same line treated with DMSO. No difference was observed between the FANCD2 mutant PD20 and retrovirally recovered RV:D2 cells (Figure 4). Furthermore, independent of concentration, metabolic activity, relative to DMSO treated cells of the same lineage, was approximately 1.0. Therefore, laromustine exhibits little cytotoxicity towards either cell lines. We conclude that laromustine is largely ineffective at least in the short term at inducing cell death in immortalized fibroblast cells regardless of the presence of fully functional FANCD2.

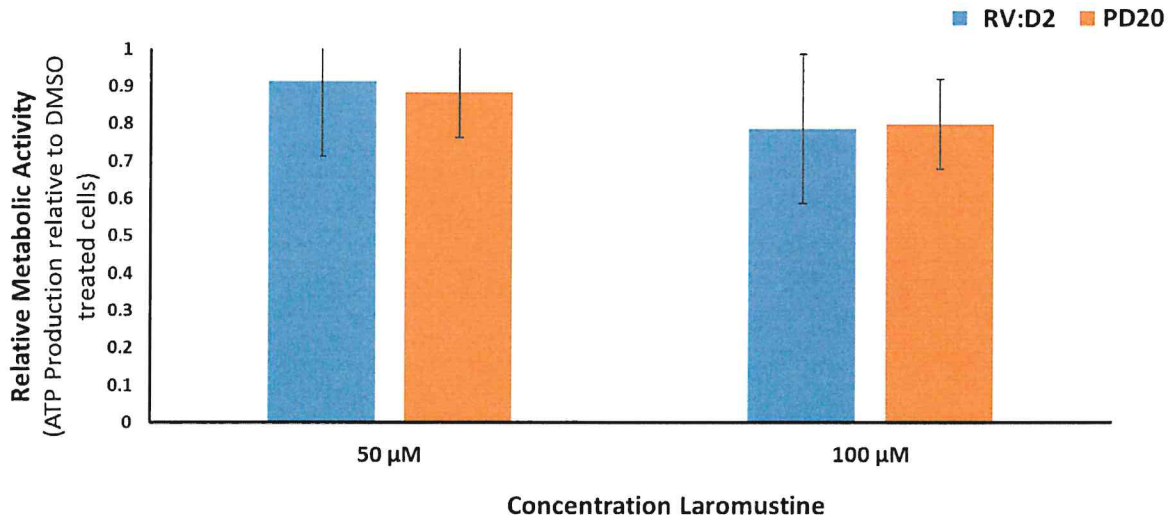

Figure 4. ATP production, relative to DMSO control of the same cell line, of FANCD2 mutant PD20 and retrovirally recovered PD20 RV:D2 cells immediately following 3 hr treatment with 50 or 100 μM laromustine.

FANCD2 mutation causes decreased clonogenicity following laromustine treatment.

Since the Fanconi Anemia deficiencies are expected to increase laromustine cytotoxicity due to formation of lethal ICLs, it is possible that increased efficacy might be more apparent in clonogenicity of cells. To investigate the possibility of increased anti-proliferative effect, after 3 hr exposure to 100 μM laromustine, cells seeded at low density were allowed to grow for 7 d and the area of wells covered by colonies normalized to the area of wells covered by untreated cells was quantified using the ImageJ plugin ColonyArea.[31]

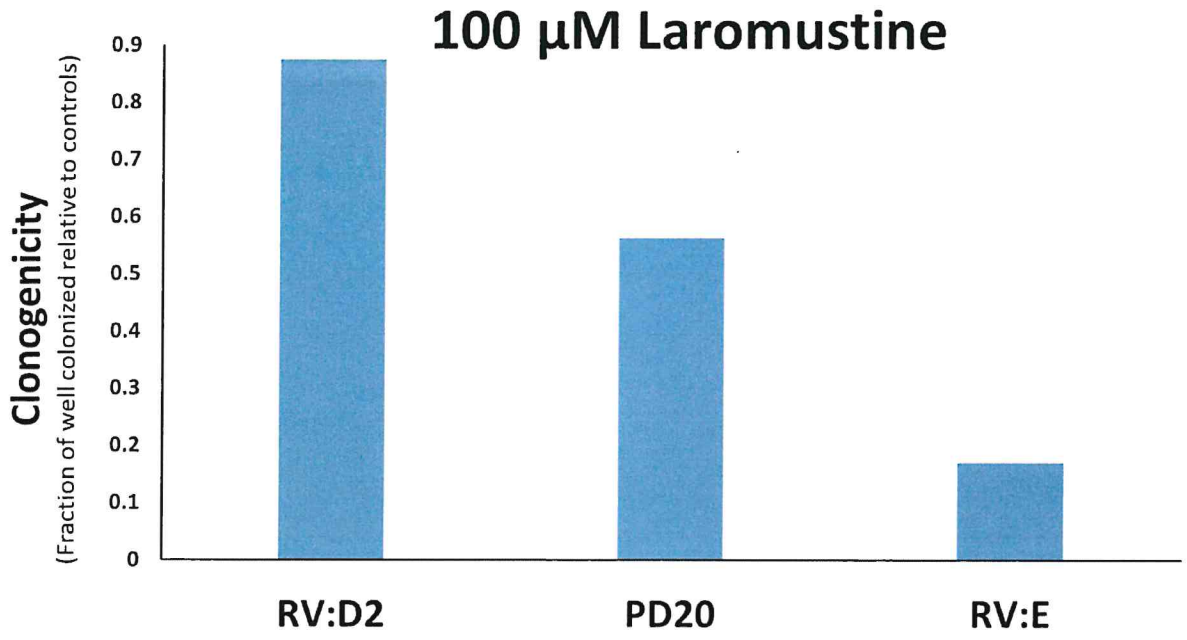

Figure 5. Clonogenicity of cells from each line after 3 hr exposure to 100 μM laromustine. Clonogenicity is quantified using crystal violet staining. Values are reported as fraction of the well colonized after 7 d incubation by cells in the treatment group normalized by the fraction of well area colonized in wells seeded with untreated cells of the same cell line. Cell lines are as follows: PD20 are the FANCD2 mutant immortalized fibroblasts, RV:D2 cells are the retrovirally recovered PD20 cells, and RV:E are the PD20 cells with a retrovirally empty plasmid.

As seen in Figure 5, FANCD2 mutant PD20 cells exhibit a decreased ability to form colonies as compared to the retrovirally recovered RV:D2 cells. The FANCD2 mutant RV:E cells with an empty retrovirally introduced plasmid have a further reduced clonogenicity.

However, this indicates that wild type FANCD2 cells may be more clonogenic than is observed for the retrovirally corrected PD20 RV:D2 cells. Therefore, the discrepancy in anti-proliferative effect between cells with endogenously functioning FANCD2 genes and those with mutant FANCD2 genes might be greater than is observed here.

ID2 Ubiquitination State in Inhibitor Treated Cells.

Next, to investigate the effect of curcumin and pimozide on FANCD2 ubiquitination, Western blotting with anti-FANCD2 antibodies of HL60 leukemia cells treated with and without inhibitor was performed. We expected to observe two bands in each lane: one corresponding to deubiquitinated FANCD2 and the other to ubiquitinated FANCD2-ub. Based on the previously reported FANCD2 ubiquitination inhibitory activity of curcumin,[26,27] we hypothesized that the proportion of FANCD2-ub: FANCD2 in curcumin treated cells would be decreased relative to DMSO-treated control. Alternatively, since pimozide has been shown to inhibit FANCD2-ub deubiquitination,[28,29] we theorized the ratio of FANCD2-ub:FANCD2 in pimozide treated cells would be increased. However, no second higher molecular weight band was found in either of the treatments nor the control (Figure 6). We conclude that the Western blot protocol used herein requires optimization to prevent degradation of post-translational modifications during lysis or sample prep.

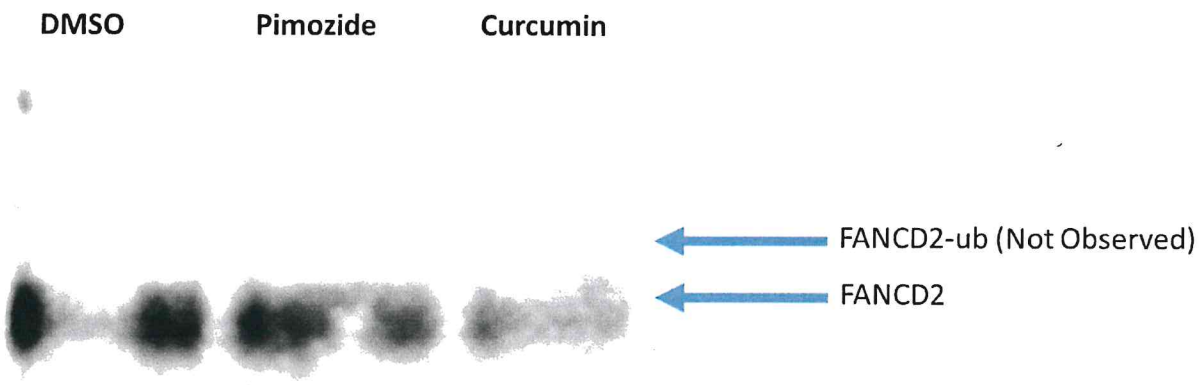

Figure 6. Deubiquitinated FANCD2 and ubiquitinated FANCD2-ub protein levels in HL60 cells treated with previously reported ubiquitination/deubiquitinating cycle inhibitors pimozide or curcumin.

Curcumin, but not pimozide, recovers decreased clonogenicity found in FANCD2 mutant cells following laromustine treatment.

Despite our inability to verify inhibition by curcumin and pimozide, to not disregard the previously reported activity,[26–29] we again utilized clonogenic assays to investigate whether the reported inhibitors could sensitize wild type FANCD2 cells to laromustine. We aimed to see if combination treatment of retrovirally recovered cells would recover the increased anti-proliferative effect of laromustine in FANCD2 mutant cells.

As seen in Figure 7, combination treatment with pimozide did not affect laromustine's anti-proliferative efficacy. In particular, pimozide did not reduce discrepancy in clonogenicity between laromustine treated FANCD2 mutant PD20 and recovered RV:D2 cell lines. This could indicate the previously reported deubiquitinating inhibitory activity of pimozide was not replicated in our studies or that having FANCD2 constituently in the monoubiquitinated state does not interfere with ICL repair. It is possible that when FANCD2 is unable to be deubiquitinated, cells upregulate expression to synthesize new proteins in response to ICL stress.

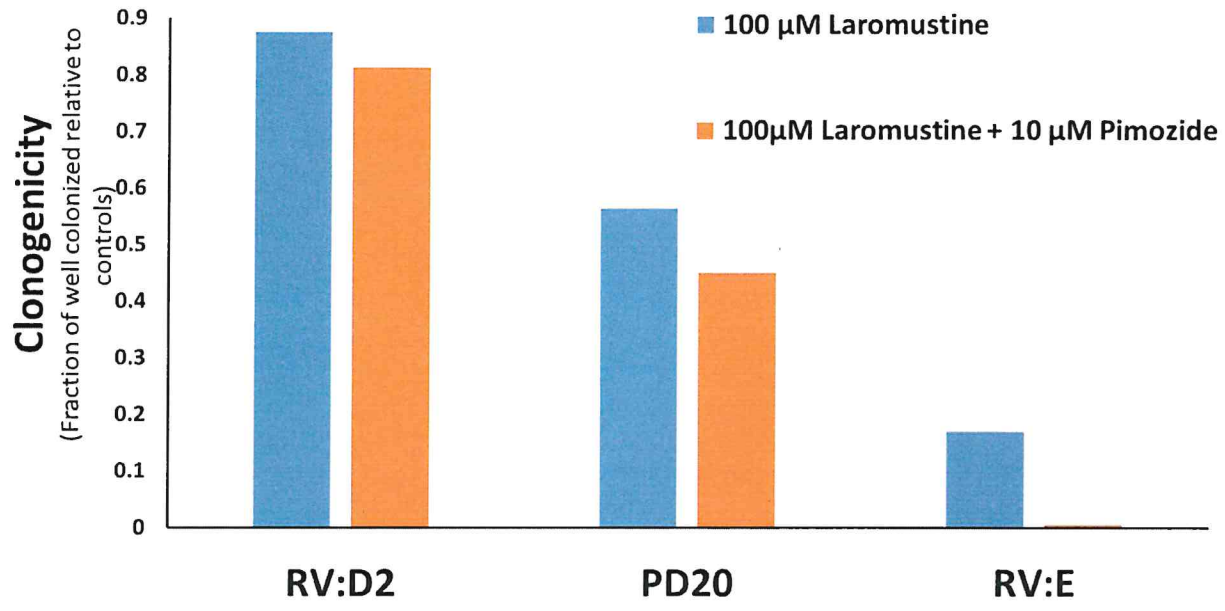

Figure 7. Clonogenicity of FANCD2 deficient PD20, retrovirally recovered RV:D2, and retrovirally empty RV:E cells following 3 hr treatment with 100 μM laromustine in combination with 10 μM pimozide. Clonogenicity is quantified by crystal violet staining after 7 d incubation and values are reported as normalized to untreated controls of the same line. Data from laromustine alone (Figure 5) is also shown for comparison.

On the other hand, curcumin sensitized cells to laromustine (Figure 8). Curcumin combination treatment caused a drastic reduction in the clonogenicity of PD20 RV:D2 and PD20 cells. Since the reduction was observed in both FANCD2 mutant cells and retrovirally recovered cells, it is possible that sensitization is working through a mechanism other than inhibition of FANCD2 ubiquitination. However, another rationale is that the minimal activity of the mutant FANCD2 protein in the PD20 cells is further reduced by curcumin. It is important to note however, that these findings are preliminary and need to be replicated to verify and understand the error in the measurements.

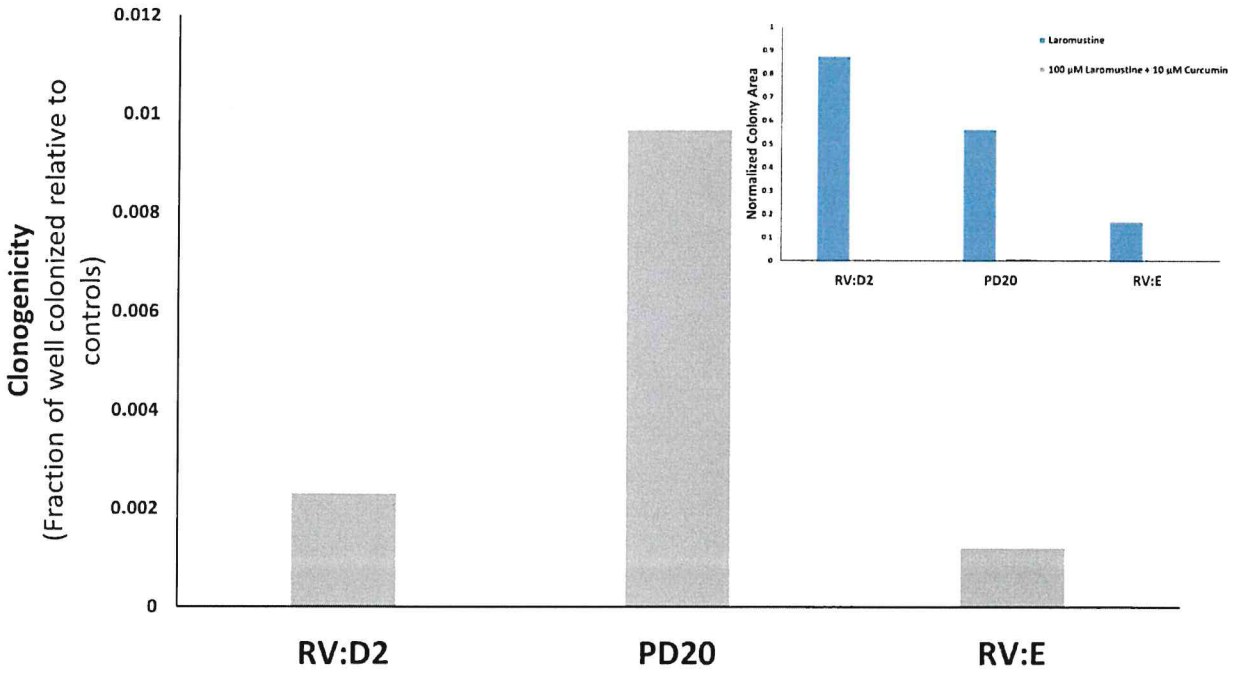

Figure 8. A) Normalized fraction of wells covered by colonies for FANCD2 deficient PD20, retrovirally recovered RV:D2, and retrovirally empty RV:E cells following 3 hr treatment with laromustine in combination with curcumin and 7 d incubation. In set shows combination treatment results plotted on the same axis as laromustine alone.

Discussion

As a result of these studies, we have shown that decreased activity of FANCD2 sensitizes cells to laromustine's anti-proliferative effect. We also find that sensitization does not reveal itself in an immediate cytotoxic effect. We propose therefore that FA deficiencies will only reveal themselves once ICLs are able to interfere with DNA replication. Furthermore, we have found that in cells able to express fully functioning FANCD2 protein, curcumin, but not pimozide, is able to recover the increased anti-proliferative effect of laromustine. We hypothesis that this increased efficacy is a result of curcumin's ability to inhibit FANCD2 ubiquitination.[27] Unfortunately, our Western blots were unable to confirm this inhibitory activity. Optimization of our lysate handling to prevent degradation of the monoubiquitin post translational modification during lysis or sample prep is required to verify inhibitory activity.

These results are promising as they indicate combination treatments that could reduce the therapeutic dose of laromustine and other bifunctional alkylating agents. The ability to treat malignant tumors with lower chemotherapy dosages can mitigate the severity of the adverse side effects intrinsic to such therapies. Therefore, future research focused on combination treatment with curcumin is.

This project lends itself to clear next steps. First our clonogenic results should be replicated and the ability of combination treatment to act synergistically to reduce cancer proliferation should be tested in cancer cell lines. Next, the ability of curcumin to reduce the LD50 of laromustine should be investigated by generating a dose-response curve in leukemia cells. Finally, measurements of ICLs would reveal if the increased anti-proliferative effect in FANCD2 mutant and wild type FACND2 cells treated with curcumin is a result of increased ICL

yield. Potential routes to determine ICLs include thermal melt experiments, Alkaline Comet Assay, or cytogenetics.[33–35]

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