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5-Fluorouracil mediated anti-cancer activity in colon cancer cells is through the induction of Adenomatous Polyposis Coli: Implication of the long-patch base excision repair pathway

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Abstract

Colorectal cancer (CRC) patients with APC mutations do not benefit from 5-FU therapy. It was reported that APC physically interacts with POL β and FEN1, thus blocking LP-BER via APC's DNA repair inhibitory (DRI) domain in vitro. The aim of this study was to elucidate how APC status affects BER and the response of CRC to 5-FU. HCT-116, HT-29, and LOVO cells varying in APC status were treated with 5-FU to evaluate expression, repair, and survival responses. HCT-116 expresses wild-type APC; HT-29 expresses an APC mutant that contains DRI domain; LOVO expresses an APC mutant lacking DRI domain. 5-FU increased the expression of APC and decreased the expression of FEN1 in HCT-116 and HT-29 cells, which were sensitized to 5-FU when compared to LOVO cells. Knockdown of APC in HCT-116 rendered cells resistant to 5-FU, and FEN1 levels remained unchanged. Re-expression of full-length APC in LOVO cells caused sensitivity to 5-FU, and decreased expression of FEN1. These knockdown and addback studies confirmed that the DRI domain is necessary for the APC-mediated reduction in LP-BER and 5-FU. Modelling studies showed that 5-FU can interact with the DRI domain of APC via hydrogen bonding and hydrophobic interactions. 5-FU resistance in CRC occurs with mutations in APC that disrupt or eliminate the DRI domain's interaction with LP-BER. Understanding the type of APC mutation should better predict 5-FU resistance in CRC than simply characterizing APC status as wild-type or mutant.

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Conflict of interest

Authors declare that there is no any conflict of interest of this article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.dnarep.2014.10.006>.

Keywords

5-Fluorouracil (5-FU); Adenomatous Polyposis Coli (APC); Long Patch Base Excision Repair (LP-BER); Colorectal Cancer (CRC)

1. Introduction

Colorectal cancer (CRC) ranks second in cancer related deaths and accounts for approximately 10–15% of all forms of cancer. Although there have been advances in screening and treatment options to reduce mortality, the prognosis of advanced forms of CRC remains very poor [1]. Mutations in the Adenomatous polyposis coli (APC) gene are associated with familial predisposition to CRC and are among the earliest events leading to the development of sporadic CRC [2]. APC is a 312 KDa protein of 2843 amino acids in its common isoform and is present in both the cytoplasm and nucleus [3]. Exon 15, containing nearly 75% of the protein coding sequence, is a common target of both germ line and somatic mutations [4]. Truncations of various lengths eliminating more than half of the C-terminus of APC are commonly observed. APC regulates Wnt signalling and β -catenin signalling, cell–cell adhesion, apoptosis, cell migration, chromosomal instability, cell cycle control and DNA repair [3,5–8]. The connection to DNA repair is intriguing, because we and others showed that DNA damaging agents increased APC expression in several cancer and normal cell lines [9–12,13].

The antimetabolite 5-fluorouracil (5-FU), and its prodrug capecitabine, remain a mainstay of standard therapy in colon cancer and are effective as a part of combination therapies that induce remissions [1]. However, resistance to fluoropyrimidines is quite common in tumours that recur, thereby rendering further usage ineffective. A better understanding of the mechanism of action of 5-FU is required to circumvent resistance. 5-FU exerts anti-proliferative effects through the inhibition of thymidylate synthase (TS), which decreases thymidylate levels and increases uracil incorporation into DNA [14]. In the reaction catalyzed by TS, the cofactor N^5,N^{10} -methylene tetrahydrofolate ($\text{CH}_2\text{H}_4\text{PteGlu}$) is the methyl and electron donor and also rate limiting in the reaction because its intracellular concentrations are lower than dUMP. The active 5-FU metabolite, FdUMP, forms a stable ternary complex with the active site cysteine of TS and $\text{CH}_2\text{H}_4\text{PteGlu}$, thereby suicide inhibiting dTMP synthesis [15]. Therefore, Leucovorin (folinic acid, LV) is administered clinically in combination with 5-FU to enhance its therapeutic effect, because LV is readily converted to $\text{CH}_2\text{H}_4\text{PteGlu}$ [16,17]. 5-FU can also be directly incorporated into RNA and DNA and alter transcription and replication, respectively [15]. Several lines of evidence support a role for APC in 5-FU mediated tumour killing in animal models and samples directly taken from CRC patients [18–20]. In the $Apc^{\text{Min/+}}$ mouse model, 5-FU monotherapy exerts cytostatic effects on intestinal tumour growth, but tumours resume growth upon cessation of treatment [18]. A recent report of a mutant Apc rat model noted no effect of 5-FU treatment on tumour number or size [20]. A small study of CRC patients treated with 5-FU chemotherapy showed that patients with somatic APC mutations did not benefit from the chemotherapy, also suggesting a link between APC status and the mechanism of action for 5-FU [19]. However, the mechanistic link remains poorly understood.

Base Excision Repair (BER) has also received attention as a cellular response to 5-FU treatment because enzymes in BER recognize and remove uracil and 5-FU from DNA [1]. In brief, DNA glycosylases cleave the glycosidic bond between the sugar and the base to form an apurinic/apyrimidinic (AP) site. AP endonuclease-1 (APE1) then cleaves the DNA backbone 5'- to the AP-site. The abasic-site is repaired either by single nucleotide gap (Short Patch, SP) – or Long Patch (LP) BER [21]. A key enzymatic step and important distinction between SP- and LP-BER regards how the fragmented sugar residue is removed to produce ligatable ends after nucleotide replacement. During SP-BER, DNA polymerase β (POL β) removes the 5'-deoxyribose phosphate intermediate by deoxyribose phosphate lyase (dRP lyase) activity [22,23]. When AP-sites are oxidized or reduced, the resulting deoxyribose moieties become resistant to β -elimination and cannot be removed by the dRP lyase activity of POL β . In this case, the modified AP-site is repaired via the LP-BER pathway in which POL β , δ or ϵ incorporates 2–15 nucleotides, displacing the strand containing the modified ribose. The DNA flap structure is cleaved by FEN1 [9,10,13,24]. Hence, FEN1 is crucial in LP-BER because it facilitates the removal of the modified ribose group [9,10,13,24].

Narayan et al. [9] showed that APC contains a DNA repair inhibitory (DRI) domain, a PIP-like box spanning amino acids 1245–1273. It was also shown that APC physically interacts with POL β as well as FEN1 via its DRI domain (Gln1256, Ile1259 and Tyr1262) and blocks strand displacement synthesis in LP-BER [13,24]. Therefore, APC has a direct role in regulating repair subpathway choice in BER. Only a few studies have examined BER components downstream of DNA glycosylase activity in response to fluoropyrimidine treatment, but these studies focused on components of SP-BER [25–28]. The contribution of LP-BER in the cellular response to 5-FU has remained unexplored. Interestingly, gene expression microarray analyses of 5-FU treated colon cancer cells have found alterations in the expression of genes of protein products implicated in LP-BER including FEN1 and PCNA [29–33]. These observations provided an impetus to examine the role of APC and LP-BER in the cellular response to 5-FU in different colon cancer cell lines possessing wild-type APC, or mutant APC variants containing or lacking the DRI domain. The study reveals the important role for the DRI domain of APC that inhibits LP-BER and causes sensitivity to 5-FU.

2. Materials and methods

2.1. Cell culture and treatment

The colon cancer cell lines HCT-116, LOVO and HT-29 obtained from ATCC (VA, USA, Cat# CCL-247, Cat# CCL-229 and Cat# HTB-38, respectively) were cultured in RPMI-1640 with 1% antibiotic (100 units penicillin and 1 mg streptomycin/mL in 0.9% normal saline) and supplemented with 10% FBS (HIMEDIA, India) in a humidified incubator in 5% CO₂ at 37°C. 5-FU, MTT, leucovorin (LV) and Tetrahydrofolate (THF) were purchased from Sigma Chemical Ltd. (St. Louis, MO, USA). A 1 mM stock of 5-FU was prepared in DMSO. A 1 mM stock of LV, THF and CaCl₂ was prepared in distilled water. 5-FU was diluted in RPMI-1640 and added to the cultures to achieve the desired final concentration during treatment. LV, CaCl₂ and THF were added at a final concentration of 1 μ M, 700 μ M and 80 nM, respectively in combination with 5-FU in each treatment (5-FU-LV-THF-CaCl₂) [17].

After cells reached 60–70% confluency, 5-FU-LV-THF-CaCl₂ treatment was for 48 hrs. The anti-APC antibody used for western blotting was from Calbiochem (Cat# OP44) and the anti-APC antibody used for immunoprecipitation was from Santa Cruz Biotechnology (Cat# SC-895). POLH and POL δ antibodies were procured from Abcam (MA, USA). DNA-LIGASE-I, APE and POL β antibodies were purchased from Novus Biologicals (CO, USA) and other antibodies were from Cell Signaling Technology (MA, USA).

2.2. Clonogenic assay

Clonogenic assays were carried out as described earlier [34–36]. Briefly, cells were treated with different concentrations of 5-FU-LV-THF-CaCl₂ for 48 h. After treatment, media was aspirated, cells were allowed to grow for 5–6 doublings in fresh media and the resulting colonies were stained with crystal violet. Colonies were counted using a gel documentation system (UVP, Germany). Data was calculated and presented as percent survival relative to mock treated control.

2.3. MTT assay

Anchorage dependent viability of cells was measured as described earlier [34–36]. Cells were exposed to different concentrations of 5-FU-LV-THF-CaCl₂ for 48 h. After treatment, MTT was added, the resulting purple formazan crystals were dissolved in a detergent solution and the colour intensity was measured spectrophotometrically using a microplate reader (Berthold, Germany) at 570 nm. The data was calculated and presented as percent viability compared to the untreated control.

2.4. Nuclear staining with DAPI

To measure apoptosis of the cells after treatment with 5-FU, DAPI nuclear staining was carried out as described earlier [34–36]. Cells were treated with 5-FU-LV-THF-CaCl₂ for 48 h followed by the addition of a DAPI staining solution. The stained nuclei were visualized with a fluorescence microscope (Nikon, Japan) at 40 $\times$ magnification. The number of apoptotic nuclei and total number of cells were counted manually and plotted.

2.5. Western blotting

Cells were plated on 100 mm tissue culture dishes and treated with 5-FU-LV-THF-CaCl₂ after reaching 70% confluency. Cells were washed with PBS, and nuclear extracts (NE) were obtained using a NE-PER kit (PIERCE Biotechnology, Cat# 78833). Proteins were separated by 10% SDS-PAGE and then transferred onto PVDF membranes, which were subsequently probed with specific antibodies according to the manufacturer's instructions [34–36]. For the proteasome inhibition study, the cells were pre-treated with MG-132 (5 μ M) for 3 h [37]. Densitometry analysis of each blot was performed using a gel documentation system (UVP, Germany) and the values were averaged.

2.6. Immunoprecipitation

The interactions between APC and FEN1, as well as APC and POL β , were measured in the nuclear extracts of colon cancer cells using untreated or treated with 5-FU-LV-THF-CaCl₂. BSA-blocked protein A-Sepharose 4B was incubated with anti-APC rabbit antibody or IgG

rabbit (for control) for 6 h at 4°C. Then, 150 µg of nuclear extract (NE) was incubated by rocking at 4°C with the beads overnight to capture the immunocomplex. To remove non-specifically bound proteins, the beads were washed once with lysis buffer, twice with 0.5 N LiCl₂, and lastly with PBS. Beads were then suspended in a SDS-sample loading buffer and the soluble proteins were separated by 10% SDS-PAGE, transferred onto a PVDF membrane, and probed with an anti-FEN1 and anti-POLβ antibody.

2.7. In vivo LP-BER assay

A plasmid based LP-BER assay was carried out as described previously [11,12]. This assay system utilizes a known type of DNA damage introduced into a p21P promoter whose activity is monitored to assess the efficiency of repair in the cell. This quick, sensitive and quantitative assay provides valuable information regarding repair capacity of cells. The modified plasmid shows poor promoter activity compared to the unmodified p21P plasmid when transfected into cells. Active repair processes in cells restore the promoter activity. As described previously, we randomly modified C residues in the p21P promoter to a reduced abasic site (R-p21P) plasmid by chemical and enzymatic modification [11,12]. In brief, a closed circular DNA containing the p21 (pGL2-p21) promoter downstream of the luciferase-reporter gene was deaminated by 3 M sodium bisulphite in the presence of 50 mM hydroquinone. Deamination modifies cytosine into uracil-residues (U-p21P) which is a substrate for SP-BER. The resulting U-p21P was further treated with uracil-DNA glycosylase (UDG), and the resulting abasic site was reduced with 0.1 M sodium borohydride (R-p21P). The reduced AP site is a substrate for LP-BER. After reaching 60–70% confluency, cells were transfected with 2.0 µg/ml of R-p21P cDNA and 0.5 µg/ml of β-gal using 7 µl/ml of Lipofectamine reagent. β-gal was used to determine the transfection efficiency of the cells (internal control). After acclimatization (5 h), the media was aspirated and replaced with fresh medium supplemented with 10% FBS and the cells were treated with concentrations of 5-FU-LV-THF-CaCl₂ described in each figure. After treatment, the cells were harvested and LP-BER activities were determined by measuring the luciferase gene-reporter activity of cellular lysate using a DLR luciferase assay instrument (Berthold, Germany).

2.8. Molecular modelling and docking studies

Molecular modelling studies were performed to explore a plausible binding mode of 5-FU with the DRI domain of APC. The detailed mechanism of protein modelling and ligand docking is mentioned in supplementary files under the heading (Molecular modelling and docking studies: Methodology).

2.9. Silencing of APC in the HCT-116 cell line

The wild-type APC gene was silenced in HCT-116 cells as described earlier [9,11,36]. Cells were grown on 60 mm tissue culture dishes until reaching 60–70% confluency and were transfected with 4 µg of the psiRNA-apc plasmid using lipofectamine. Cells were treated with 5-FU-LV-THF-CaCl₂ after which, they were harvested and the APC protein level was determined by Western blot.

2.10.. Over-expression of APC in LOVO colorectal cells

Cells were grown on 60 mm tissue culture dishes until reaching 60–70% confluency before transiently transfected with 3 µg pSAR-MT-APC plasmid using Lipofectamine transfection reagent [11]. The plasmid contains a zinc-inducible, full length cDNA for APC. The cells were treated with 50 µM ZnCl₂ for 12 h to induce APC expression, followed by treatment with 5-FU for another 48 h prior to harvest. Cellular lysates were examined by western blotting to measure APC expression.

2.11. Statistical analysis

The statistical analysis was carried out using GraphPad software and * $P < 0.05$ and ** $P < 0.005$ was considered as statistically significant.

3. Results

3.1. Involvement of APC in 5-FU mediated toxicity

To determine the involvement of APC in 5-FU mediated cytotoxicity, we chose three colorectal cancer cell lines; HCT-116 (expressing WT APC, 312 KDa), HT-29 (expressing two truncated versions of APC, 200 KDa and 120 KDa variants) and LOVO (expressing a 120 KDa truncated form of APC). A dose dependent decrease in colony forming ability was observed in all three cell lines after exposure to increasing concentrations of 5-FU (Fig. 1A). The LC₅₀ values for 5-FU in HCT-116, HT-29, and LOVO cells were 5, 8, and 26 µM, respectively. We also measured cell viability after 5-FU treatment using the MTT assay. Fig. 1B shows the viability curves of HCT-116, HT-29 and LOVO cells after 5-FU treatment. The dose causing a 50% reduction was 5 µM in the HCT-116 and HT-29 cells, but 25 µM in the LOVO cells. Apoptosis was also measured by DAPI nuclear staining after 5-FU exposure. Fig. 1C shows representative images and Fig. 1D shows the quantitation of apoptotic nuclei after 5-FU exposure. The number of apoptotic nuclei in 5-FU treated cells was significantly greater in HCT-116 cells (10-fold in comparison to untreated) and HT-29 cells (7-fold in comparison to untreated) whereas the number of apoptotic nuclei induced by 5-FU in LOVO cells was lower (3-fold in comparison to untreated) at a dose of drug, five times that used in the other two cell lines. Collectively, it can be concluded that HCT-116 and HT-29 cells are more sensitive to 5-FU in comparison to LOVO cells.

3.2. Expression of APC and selected DNA repair proteins in response to 5-FU

The more resistant LOVO cells only express the 120 KDa variant of APC. Because of previous results demonstrating the role of APC in LP-BER [9,10,12,13,24,38], we investigated the possibility that APC status was strongly influencing 5-FU induced death of colorectal cancer cells. Specifically, the expression of selected DNA repair proteins and APC was measured after exposure to 5-FU in HCT-116 and HT-29 cells (Fig. 2). The expression of the essential BER proteins POLβ, PCNA, APE and DNA-LIGASE-I did not change significantly compared to control in both cell lines (Fig. 2A and C). Interestingly, there was an almost 8-fold increase in the expression of full length APC in HCT-116 cells following treatment with 10 µM 5-FU, while an 11-fold down regulation of FEN1 was observed at the same concentration (Fig. 2A and B). WRN and POLH also decreased by 4- and 10-fold,

respectively, following treatment with 10 μ M 5-FU in HCT-116 cells. Fig. 2D shows that in HT-29 cells, FEN1, POLH, and POL δ were completely down regulated at concentrations of 5 and 10 μ M 5-FU, whereas the level of WRN remained almost the same (Fig. 2C). Interestingly, the expression of the 200 KDa form of APC in HT-29 increased up to 2.5-fold, while expression of the 120 KDa form was unaltered after 5-FU exposure (Fig. 2D). Taken together, the data suggests the longer, less truncated forms of APC regulate a key component of LP-BER.

3.3. Involvement of the DRI domain of APC in 5-FU mediated cytotoxicity

We have previously shown that FEN1 interacts with the DRI domain of APC and inhibits LP-BER activity in vitro [9]. The DRI domain of APC extends from amino acids 1245–1273, which is included in the 312 and 200 KDa APC forms expressed in HCT-116 and HT-29 cells, respectively, but not in the 120 KDa form expressed in LOVO (Fig. 3A). Fig. 3B shows the presence of the different APC forms in the three colorectal cancer cell lines. The expression of APC and FEN1 in nuclear extracts of LOVO cells treated with 5-FU was also measured. Interestingly, neither the 120 KDa form of APC nor FEN1 levels in nuclear extracts changed following 5-FU treatment of LOVO cells (Fig. 3C). Similarly, no change in the expressions level of WRN, POLH, POL δ , POL β , APE, DNA LIGASE-I and PCNA were observed (Fig. 3C). This data implies that the DRI domain of APC and inhibition of FEN1 is required for 5-FU mediated cytotoxicity in colon cancer cells.

To further confirm the importance of the DRI domain of APC in forming a functional complex with FEN1 and POL β in cells, we measured the interaction between APC and FEN1 or POL β by immunoprecipitation using nuclear extracts of the three colorectal cancer cell lines (Fig. 3D). As a positive control, lanes 4, 7 and 10 show nuclear extracts without immunoprecipitation from untreated cells of the respective lines. Immunoprecipitates of nuclear extracts were pulled down with APC, and then blotted for FEN1 or POL β . Lanes 2, 5, and 8 show immunoprecipitates of nuclear extracts from untreated HCT-116, HT-29 and LOVO cell lines, respectively. Lanes 3, 6, and 9 show the immunoprecipitates of nuclear extracts from the respective cell lines treated with 5-FU at the LC₅₀ concentration of each. The interaction seen between APC and FEN1 in HCT-116 (lane 2) and HT-29 (lane 5) was diminished after treatment with 5-FU for 48 h in HCT-116 (lane 3) and HT-29 cells (lane 6). In contrast, there was no interaction seen between APC and FEN1 in the absence or presence of 5-FU (lanes 8 and 9) in LOVO cells, which express the 120 KDa, APC form lacking the DRI domain. Lane 10 confirms the presence of FEN1 in nuclear extracts of LOVO cells. No FEN1 protein was detected in the control IgG pull-down (lane 1). Similarly, the interaction of POL β and APC remained unchanged in HCT-116 and HT-29 cells before and after 5-FU treatment, but no interaction was observed in LOVO cells. Lanes 4, 7, and 10 confirm the presence of POL β in the nuclear extract (Fig. 3D). We next sought to determine whether 5-FU directly interacts with the APC protein, specifically within its DRI domain, and what the atomic interactions are between the 5-FU ligand and residues of the DRI domain. In the absence of any 3D crystallographic or NMR structure for the DRI region (1245–1273) of APC, a homology model was constructed to obtain a 3D structure of the much larger region of APC encompassing amino acids 1141–1330. The initial model was subjected to refinement using full atomic MD simulations. During the course of simulation,

all important indices including density, temperature and total energy were monitored to ensure the stability of the system. The unperturbed profile of these parameters proved that the system has converged by the end of equilibrium and is stabilized at a simulation temperature of 300 K and pressure 1 of atm.

The C α backbone root-mean square deviation (RMSD) of the homology model of APC (amino acids 1141–1330) was plotted for the whole simulation run (2 ns equilibration + 15 ns production run) (Supplementary Fig. S1). There were large conformational changes during the initial phase of the simulation run, as reflected by continuous increase in the RMSD up to 8 ns of simulation time. But in the last 5 ns of simulation run, the RMSD converges around 8.5 Å. From this equilibrated trajectory (12–17 ns), 10 snapshots possessing minimum energy were extracted and analyzed for their PROCHECK and Errat plot statistics in order to choose the best model. The final selected model, presented in Fig. 3E, illustrates that the region of APC from amino acids 1141–1330 is mostly composed of loops (supporting the PSIPRED prediction). These loops confer flexibility to APC in its interactions with other proteins like POL β or FEN1. A small stretch of alpha helix (1258–1266) is present in the DRI domain, which could also play an important role during the molecular recognition of its interacting partners. Moreover, this DRI domain is exposed at the surface, which is a prerequisite to interact with other proteins, thus further validating the modelled structure. The secondary structure elements, Ramachandran and Errat plots of the MD refined homology model of APC were also generated (Supplementary Figs. S2–S4).

The 5-FU ligand was docked into the defined binding site of the MD refined and validated APC model using the Glide 5.5 programme incorporated in Schrödinger software. The best docked pose of 5-FU displayed a Glide score of 5.13 kcal/mol and E-Model score (combination of energy grid score, Glide score, and the internal strain of the ligand) of –27.78 kcal/mol, signifying good binding affinity with the DRI domain of the APC protein. The 2D interaction diagram of 5-FU (Supplementary Fig. S5) clearly shows that the binding site is mostly composed of polar amino acid residues of APC (Gln1237, Gln1260, Asn1300 and Thr1301), which can form electrostatic or hydrogen bonding interactions with the atoms of the ligand. In particular, the 3D binding model for the best docked pose of 5-FU (Fig. 3F) revealed that it can form four hydrogen bonds with the residues of APC. Both secondary amino groups at the N-1 and N-3 positions in 5-FU are capable of forming hydrogen bonds with the side chain carboxylate of Glu1257 (2.4 Å) and side chain carbonyl of Gln1237 (2.2 Å), respectively. Both hydrogen bond accepting carbonyls present at the C-2 and C-4 positions in 5-FU are capable of forming hydrogen bonds with the side chain amide of Gln1260 (2.3 Å) and side chain amide of Gln1237 (2.4 Å) respectively. In addition, 5-FU could also be involved in hydrophobic interactions with the alkyl side chain of Ile1259, providing extra stability to the protein–ligand complex. Previously reported site specific mutagenesis studies on the DRI domain of APC suggest that it interacts with POL β and FEN1 at Ile1259 and Tyr1262, since mutation of these residues to alanine abolished interactions as measured by yeast two hybrid analysis [9,24]. The molecular docking analysis here demonstrates that 5-FU forms multiple non-covalent contacts with Glu1257, Ile1259, and Gln1260 of the DRI domain, thus showing a molecular basis for interactions between APC and 5-FU.

3.4. 5-FU- induced APC suppresses LP-BER activity in CRCs

To further confirm that APC influences 5-FU cytotoxicity and gene expression in these CRC lines, APC was silenced in HCT-116 cells by transient transfection of a siRNA expression plasmid that targets APC (psiRNA-apc). APC expression decreased by approximately 70% in psiRNA-apc transfected cells (Fig. 4A). An MTT assay was performed to compare 5-FU sensitivity between HCT-116 and HCT-116-APC silenced cells. APC silenced cells were 5-fold more resistant to 5-FU (24 μ M) when compared to the parental HCT-116 cells (5 μ M), (Fig. 4B). The expression of the same DNA repair proteins were also measured in APC silenced cells after 5-FU treatment. Most notably, FEN1 levels remained unchanged in the APC silenced cells (Fig. 4C), in contrast to the decrease seen in parental HCT-116 cells (Fig. 2A and B). The other proteins including WRN, POL β , POL δ , APE, DNA LIGASE-I and PCNA remained unaltered by 5-FU treatment in the APC silenced cells (Fig. 4C and D).

To investigate the functional role of APC in LP-BER, repair was measured using a novel plasmid based LP-BER assay [9,11,13,24](see the diagram in Fig. 4E). HCT-116 and HCT-116-APC silenced cells were transfected with a substrate for LP-BER (R-p21P) and luciferase activity was measured after treatment with 5-FU (Fig. 4F). The amount of luciferase activity is directly proportional to LP-BER capacity within the cells. A significant decrease in relative luciferase activity (5-fold) was noted in HCT-116 cells treated with increasing concentration of 5-FU (Fig. 4F). However, the decrease in luciferase activity observed in HCT-116-APC silenced cells was significantly less (~40%) after the same 5-FU treatment (Fig. 4F). These results show that LP-BER activity is inversely associated with expression of APC and 5-FU inhibits LP-BER through APC. HCT-116 cells in which POL β was knocked down were also examined. The POL β knock-down HCT-116 cells were equally sensitive to 5-FU as the HCT-116 cells expressing POL β (Supplementary Figs. S6–S7), suggesting that SP-BER has no effect on 5-FU sensitivity in these cells expressing full length APC, which further supports the importance of LP-BER.

3.5. Over-expression of APC decreases LP-BER activity after 5-FU exposure in LOVO cells

Wild-type APC was overexpressed in LOVO cells by transient transfection with the pSAR-MT-APC plasmid [11]. Consistent with results seen in HT-29 cells, it was observed that 5-FU increased the level of wt APC (312 KDa) in transiently transfected LOVO cells while the level of 120 KDa APC remained constant (Fig. 5A). Viability was measured to compare the 5-FU sensitivity between LOVO cells and LOVO-APC over-expressed cells. APC over-expressing cells were found to be almost 2.5-fold more sensitive to 5-FU than the parental LOVO cells lacking the DRI domain of APC. The LC₅₀ value of the APC over-expressed LOVO cells was 11 μ M, whereas the LC₅₀ value was 25 μ M in parental LOVO cells (Fig. 5B). LP-BER activity was also measured in both parental LOVO cells and APC over-expressed LOVO cells after transfecting the R-p21P plasmid and then treating with 5-FU. There was no significant change in LP-BER activity after 5-FU treatment in parental LOVO cells, but there was significant decrease in the relative luciferase activity of APC over-expressing LOVO cells (Fig. 5C). This data is consistent with the notion that the DRI domain of APC suppresses LP-BER to affect 5-FU toxicity.

3.6. Proteasome mediated degradation of FEN1

We saw that FEN1 protein expression was reduced in HCT-116 and HT-29 cells after exposure to 5-FU (Fig. 2). To determine whether this reduction was due to proteasome mediated degradation of FEN1, we performed an experiment using MG-132 to inhibit the proteasome. Cells were pre-exposed with 5 μ M MG-132 for 3 h, followed by treatment with 5-FU for 48 h. It was observed that blocking the proteasome with MG-132 restored FEN1 levels even after 5-FU treatment in HCT-116, HT-29 and LOVO + pSAR-MT-APC cells (Fig. 6). Interestingly, no change in FEN1 protein expression was observed in the presence or absence of MG-132 in LOVO cells (Fig. 6)

4. Discussion

It is well established that 5-FU causes cancer cell death by misincorporation into DNA. Recognition of 5-FU in DNA is complex because MMR and DNA glycosylases of BER are capable of recognizing 5-FU in DNA [1,39,40]. Although several studies have shown that MMR deficient cells are resistant to 5-FU, the literature remains mixed regarding the clinical benefit, if any, of fluoropyrimidine therapy in patients with MMR defects (high microsatellite instability, MSI-H) [41–45]. Here, we compared cells differing in APC status to address the role of APC and LP-BER. Although, the role of APC in colorectal cancer is well known, the role of different truncated versions of APC and the response to chemotherapy is less understood. This has clinical relevance, given the widespread use of fluoropyrimidines such as 5-FU and its orally available prodrug, capecitabine, in the treatment of CRC [1]. APC is well known for its role in promoting apoptosis caused by DNA damage, but we identify a novel means of influencing cell death, in this case, inhibiting LP-BER repair. This role appears to be dependent on the DNA repair inhibitory domain (DRI) of APC, and independent of MMR.

We previously showed that expression of the *APC* gene is induced in several cancer cell lines upon exposure to DNA-alkylating agents and a cigarette smoke carcinogen [9,11,12], which ultimately leads to cancer cell death. APC has a PIP-like box (DRI domain) spanning a region from amino acids 1245–1273, which is responsible for interactions between PCNA and other DNA repair/replication proteins (reviewed in. [46,47]). The DRI domain consists of a sequence QXX(h)XX(a)(a), in which “X” may be any amino acid, and “h” and “a” represent hydrophobic and aromatic ring side chains respectively ([9], reviewed in [46,47]). The Meta-MEME programme was used to identify the conserved DRI domain, which identified the motif between amino acid residues 1245 and 1273 in APC [9,48]. We previously showed that LP-BER activity is inversely related to APC expression in different breast cancer cell lines [11,12]. Here, we show that 5-FU induced APC, and we link APC-mediated regulation of FEN1, a key component of LP-BER, in the response of CRC to 5-FU therapy. Immunoprecipitation studies confirmed that 5-FU treatment disrupted the physical association between the full length or 200 KDa forms of APC and FEN1, which promotes proteasome-mediated degradation of FEN1. A schematic diagram of 5-FU mediated cytotoxicity has been provided in supplementary Fig. S8.

To our knowledge, this is the first report detailing the involvement of LP-BER in response to 5-FU. A number of studies have examined the role of several DNA glycosylases in response

to 5-FU, with mixed conclusions regarding the relative contributions to 5-FU toxicity [49–58]. One important experimental point to consider regards the fact that our experiments included LV, the activated folate used clinically with 5-FU to enhance its antitumor activity, because N^5,N^{10} -methylene tetrahydrofolate is rate limiting in the reaction. The results presented here bring a new perspective in considering the role of BER in response to TS inhibitors. Specifically, in cells with full length or the 200 KDa truncated form of APC, DNA glycosylase activity would be predicted to have no influence because of the downstream inhibition of FEN1. The importance of FEN1 in colorectal carcinogenesis is known from studies showing that *APC*^{Min/+} mice heterozygous for FEN1 suffer from accelerated tumorigenesis [59]. Mutations in FEN1 have been identified in human tumours, and mice expressing a mutant form of FEN1 suffer from chronic inflammation and cancer [60]. In a clinical setting, the results presented here would predict that in tumours with mutant APC, those retaining expression of APC variants with DRI domain are sensitive to fluoropyrimidines, whereas those tumours lacking APC or with severely truncated forms of APC will be resistant to fluoropyrimidine treatment.

The observation that POLH expression decreased with increasing concentration of 5-FU in the presence of APC forms containing the DRI domain, but was unchanged in cells lacking the DRI domain of APC is a novel, intriguing observation suggesting a potential functional interaction between POLH and APC. It further suggests that POLH may possibly participate in an as yet reported role in LP-BER. These suggestions require further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

5-FU	5-fluorouracil
CH₂H₄PteGlu	N^5,N^{10} -methylene tetrahydrofolate
TS	thymidylate synthase
APC	Adenomatous Polyposis Coli
CRC	colorectal cancer
BER	base excision repair
LP-BER	long patch base excision repair

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Fig. 1.

5-FU mediated cytotoxicity in colon cancer cells (a) clonogenic cell survival assay. The cells were treated with increasing concentrations of 5-FU-LV-THF-CaCl₂ for 48 h, followed by incubation in fresh media for 5–6 doublings as described in Materials and Methods section. (●), (▲) and (■) represent HCT-116, HT-29 and LOVO cells, respectively. The graph was prepared in log-log format. Error bars represent SEMs, and statistical significance was determined by paired *t*-test *P < 0.05. Data was compared between untreated control with respect to treatment. (b) MTT cell viability assay. Cells were plated in 96 well plates for 24 h prior to treatment, and then treated as described in Materials and Methods section. (●), (▲) and (■) represent HCT-116, HT-29 and LOVO cells, respectively. The graph was prepared in log-log format. Error bars represent SEMs, and statistical significance was

determined by paired t -test $*P < 0.05$. Data was compared between untreated control with respect to treatment. (c) Representative images of apoptotic nuclei as visualized by DAPI staining of colon cancer cells after exposure to 5-FU-LV-THF-CaCl₂. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at 40× magnification. (d) A bar graph showing quantitation of the apoptotic nuclei; Error bars represent SEMs, and statistical significance was determined by paired t -test $*P < 0.05$. Data was compared between untreated control with respect to treatment.

Fig. 2.

(a) Expression pattern of selected BER proteins in HCT-116 cells after exposure to 5-FU-LV-THF-CaCl₂. Nuclear extracts were prepared and analyzed by Western blotting as described in Materials and Methods section. Images shown represent one of the three different experiments. α-Tubulin served as the loading control. (b) The table shows the fold change in protein expression with respect to untreated control for each protein by densitometric analysis. Error bars represent SEMs, and statistical significance was determined by paired *t*-test *P < 0.05. Data was compared between untreated control with

respect to treatment. (c) Expression pattern of select BER proteins in nuclear extracts of HT-29 cells after exposure to 5-FU-LV-THF-CaCl₂. α -Tubulin served as the loading control. (d) The table shows fold change in protein expression with respect to control by densitometric analysis. Error bars represent SEMs, and statistical significance was determined by paired *t*-test *P < 0.05. Data was compared between untreated control with respect to each treatment.

Fig. 3.

(a) Cartoon showing the different lengths of APC present in HCT-116, HT-29 and LOVO cells. The DRI domain in APC extends from amino acids 1245–1273 and is present in HCT-116 and HT-29, but absent in LOVO cells. (b) Expression of APC in the different colon cancer cell lines. HCT-116 expresses full length APC (312 kDa), HT-29 expresses two truncated forms of APC (200 kDa and 120 kDa), while LOVO only expresses the 120 kDa form of APC. (c) Expression pattern of BER proteins in nuclear extracts from LOVO cells after exposure to 5-FU-LV-THF-CaCl₂. α-Tubulin served as the loading control. The number

above each panel is the fold change relative to control. (d) Detection of an interaction between APC and FEN1 or POL β . Nuclear extracts (150 μ g) from HCT-116, HT-29 and LOVO cells were immunoprecipitated with anti-APC antibody and immunoblotted with anti-FEN1 antibody and anti-POL β antibody. Lanes 4, 7, and 10 contain 40 μ g of nuclear extracts from each respective untreated cell line as a control for the nuclear presence of FEN1 and POL β . Lanes 2, 5 and 8 are from untreated control cells for the respective cell lines. Lanes 3, 6, and 9 are from the respective cell lines treated with 5-FU-LV-THF-CaCl₂ for 48 hrs at their respective LC₅₀ concentrations. Lane 1 is negative control with IgG only. (e) Cartoon representation of the final MD refined homology model of APC (amino acids 1141–1330). Starting (Lys1250) and ending (Ile1269) residues of the DRI domain (1250-KVSSINQEAIQTYCVEDTPI-1269) are shown in sticks. (f) Binding pose of 5-FU with the final MD-refined homology model of the DRI-containing APC fragment (1141–1330). Yellow dashes represent hydrogen bonding. Protein is shown in *cyan* colour and ligand in magenta colour. Atom colouring is as follows: Carbons of protein- *cyan*, Carbons of ligand – *magenta*, Nitrogen – *blue*, Oxygen – *red*, Hydrogen – *white*.

Fig. 4.

(a) APC protein expression in HCT-116 and HCT-116 + psiRNA apc treated cells. α -Tubulin was used as the loading control. (b) Anchorage-dependent cell viability of HCT-116 and HCT-116-APC silenced cells after 5-FU-LV-THF-CaCl₂ exposure. The graph was prepared in log-log format. The dashed line represents parental HCT-116 cells and the solid line represents HCT-116-APC silenced cells. Error bars represent SEMs, and statistical significance was determined by paired *t*-test. Data was compared between untreated control with respect to treatment *P < 0.05. (c) Expression pattern of selected BER proteins in the

nuclear extracts of HCT-116-APC silenced cells after exposure to 5-FU-LV-THF- CaCl_2 . α -Tubulin served as the loading control. (d) The table shows fold change in protein expression with respect to control by densitometric analysis of the data shown in (C). Error bars represent SEMs, and statistical significance was determined by paired t -test $*P < 0.05$. Data was compared between untreated control with respect to treatment. (e) Schematic representation of plasmid based LP-BER assay. The covalently closed circular DNA (PGL2-Luc-p21 promoter) was chemically and enzymatically modified to produce the substrate for SP and LP-BER, respectively. Luciferase activity is measured according to protocol described in methods and material. Relative luciferase activity is directly co-related to the repair capacity of cells. (f) Bar graph showing the relative luciferase activity from the R-p21P plasmid in parental HCT-116 and HCT-116-APC silenced cells after 5-FU-LV-THF- CaCl_2 treatment. The black bars represent data from parental HCT-116 cells and grey bars represent the HCT-116-APC silenced cells Error bars represent SEMs, and statistical significance was determined by paired t -test $*P < 0.05$. Data was compared between untreated control with respect to treatment.

Fig. 5.

(a) APC protein expression in LOVO and LOVO + pSAR-MT-APC cells after 5-FU-LV-THF- CaCl_2 treatment. α -Tubulin was used as the loading control. (b) Anchorage-dependent cell viability of LOVO and APC over-expressed LOVO cells after 5-FU-LV-THF- CaCl_2 exposure. The graph was prepared in log-log format. The dashed line represents parental LOVO cells and the solid line represents APC over-expressed LOVO cells. Error bars represent SEMs, and statistical significance was determined by paired t -test $*P < 0.05$. Data was compared between untreated control with respect to treatment. (c) Bar graph showing the relative luciferase activity in LOVO and APC over-expressed LOVO cells transfected with the R-p21P plasmid after 5-FU-LV-THF- CaCl_2 treatment. Error bars represent SEMs, and statistical significance was determined by paired t -test. Data was compared between untreated control with respect to treatment ($**P < 0.005$).

Fig. 6.

Expression of FEN1 protein. Cells pre-exposed with the proteasome inhibitor MG-132 prior to 5-FU-LV-THF-CaCl₂ treatment. The number above each panel is the relative fold change measured by densitometric analysis.