PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential

Ion Cristóbal¹, Rebeca Manso², Raúl Rincón², Cristina Caramés¹, Clara Senin³, Aurea Borrero¹, Javier Martínez-Useros¹, María Rodríguez¹, Sandra Zazo², Oscar Martinez-Aguilera¹, Juan Madoz-Gúrpide², Federico Rojo², Jesús García-Foncillas¹

¹Translational Oncology Division, Health Research Institute and Oncology Department, University Hospital “Fundacion Jimenez Diaz”, Autonomous University of Madrid, E-28040 Madrid, Spain.

²Pathology Department, IIS “Fundación Jiménez Diaz”, E-28040 Madrid, Spain.

³Medical Oncology Department, University Hospital of Vigo, E-36204 Vigo, Spain.

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Corresponding Author: Jesús Garcia-Foncillas, Translational Oncology Division, Health Research Institute and Oncology Department, University Hospital “Fundacion Jimenez Diaz”, Avda. Reyes Católicos-2 28040-Madrid, Spain. E-mail: jgfconcillas@gmail.com, Phone: +34-915504800.

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Abstract

Protein phosphatase 2A (PP2A) is a tumor suppressor that regulates many signaling pathways crucial for cell transformation. In fact, decreased activity of PP2A has been reported as a recurrent alteration in many types of cancer. Here, we show that PP2A is frequently inactivated in patients with colorectal cancer (CRC), indicating that PP2A represents a potential therapeutic target for this disease. We identified overexpression of the endogenous PP2A inhibitors SET and CIP2A, and downregulation of regulatory PP2A such as PPP2R2A and PPP2R5E as contributing mechanisms to PP2A inhibition in CRC. Moreover, we observed that its restoration using FTY720 impairs proliferation and clonogenic potential of CRC cells, induces caspase-dependent apoptosis, and affects AKT and ERK1/2 activation status. Interestingly, treatment with FTY720 showed an additive effect with 5-fluorouracil, SN-38 and oxaliplatin, drugs used in standard chemotherapy in CRC patients. These results suggest that PP2A activity is commonly decreased in CRC cells, and that the use of PP2A activators, such as FTY720, might represent a potential novel therapeutic strategy in CRC.
Introduction

Colorectal cancer (CRC) is a high prevalent neoplasia characterized by a progressive accumulation of genetic and epigenetic abnormalities that lead to cancer progression. Protein phosphatase 2A (PP2A) is a tumor supressor that regulates many signaling pathways (1-3), and its loss of function has been associated with cell transformation (4-5). PP2A is not a single entity but a heterotrimeric complex consisting of a scaffold PP2A-A subunit, a catalytic PP2A-C subunit, and a regulatory PP2A-B subunit that determines both the specific substrate and the localization of the holoenzyme. Two isoforms have been described for PP2A-A and PP2A-C, and at least 26 isoforms for PP2A-B (6-7). Therefore, PP2A can form a high number of different complexes that interact with many different substrates (5).

It has been reported that transformed cells use different mechanisms to inhibit PP2A, including alterations in any of the PP2A subunits, and also the overexpression of specific endogenous inhibitors (4, 8-9). Despite PP2A has been described as a novel therapeutic target in several tumor models (10-12), the importance and potential value of PP2A as a drugable tumor suppressor in CRC remains mostly underexplored. Interestingly, there are some evidences that would suggest a relevant role of PP2A deregulation in CRC development. Alterations affecting PPP2R1B have been reported in CRC, impairing the interaction between PP2A-Abeta and PP2A-C therefore inactivating PP2A (13-16). Furthermore, the tumor suppressor activity of PP2A seems to modulate the sensitivity of CRC cells to different treatments such as rapamycin (17), sphingadienes (18) or anti-angiogenesis therapies (19). Therefore, we hypothesized that PP2A would be deregulated and could represent a novel targeted therapeutic strategy in CRC.
In this study, we show that PP2A activity is reduced in colorectal cancer, and overexpression of the endogenous PP2A inhibitors SET or CIP2A were identified as possible mechanisms of PP2A inhibition in CRC. Interestingly, FTY720 treatment increased PP2A activity, affecting proliferation and clonogenic potential of CRC cells. In addition, FTY720-induced PP2A activation led to increased apoptosis and changes in the phosphorylation status of AKT and ERK1/2. Of importance, our data provides strong evidences that PP2A activation could be a promising therapeutic target in combination with drugs used in standard chemotherapy, such as 5-fluorouracil (5-FU), SN-38 or oxaliplatin (LOHP).
Materials and Methods

Cell cultures

The human CRC cell lines RKO (ATCC CRL-2577), SW480 (ATCC CCL-228), WiDr (ATCC CCL-218), DLD-1 (ATCC CCL-221), HT-29 (ATCC HTB-38), LoVo (ATCC CCL-229) and SW620 (ATCC CCL-227) were purchased from American Type Culture Collection (ATCC) and were not cultured for more than two months. No authentication was done by the authors. Cell lines were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum (FBS) and were grown at 37°C in a 5% CO2 atmosphere. Media were supplemented with penicillin G (100 U/ml), and streptomycin (0.1 mg/ml). Cells were treated with the following reagents: 5-FU (1 µM) (Sigma), SN38 (50 nM) (Sigma), LOHP (1 µM) (Sigma), FTY720 (10 µM) (Calbiochem) and okadaic acid (OA) (2.5 nM) (Calbiochem).

Patient samples

The study comprised fresh frozen samples of 21 patients with CRC obtained from surgical specimens provided by the Biobank of Fundación Jiménez Díaz. Paired normal mucosa obtained from each patient was used as control. A pathologist confirmed that primary tumor tissues used in this work contained greater than 70% tumoral component. The ethical committee and institutional review board approved the project.

Direct nucleotide sequencing

Reverse transcription reactions were performed using SuperScript III (Invitrogen). The amplified PCR products were purified from agarose gel after electrophoresis using Ilustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequencing reactions were carried out by duplicates using forward and reverse primers to confirm
the results obtained and performed by the company Secugen, S.L. Results were analyzed using Chromas Version 2.4.1 and ClustalW2 bioinformatic tools.

Western blot analysis

Protein extracts were isolated using TRIzol Reagent (Invitrogen) following manufacturer’s indications, clarified (12,000xg, 15min, 4°C), denatured and subjected to SDS-PAGE and Western-blot. Antibodies used were mouse monoclonal anti-PP2A (clone 1D6, Upstate Inc.), rabbit polyclonal anti-PP2A (FL-309) (Santa Cruz Biotechnology Inc.), rabbit monoclonal anti-PP2AY307 (Epitomics), rabbit polyclonal anti-AKT, rabbit polyclonal anti-ERK1/2 (Cell Signaling Technology Inc.), goat polyclonal anti-SET, rabbit polyclonal anti-pAKT Thr308, rabbit polyclonal anti-pERK1/2 Thr202/Tyr204 (Santa Cruz Biotechnology), rabbit polyclonal anti-PPP2R2A (Abcam), goat polyclonal anti-PPP2R5E (Novus Biologicals), rabbit polyclonal anti-CIP2A and mouse monoclonal anti-β-actin (Sigma). Proteins were detected with the appropriate secondary antibodies conjugated to alkaline phosphatase (Sigma) by chemiluminescence using Tropix CSPD and Tropix Nitro Block II (Applied Biosystems).

Proliferation assay and cell viability

Cell proliferation was measured in triplicate wells by MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega), following the manufacturer’s indications.

PP2A phosphatase activity assays

PP2A assays were performed with cell lysates (50μg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) as previously described (20).
Analysis of caspase activation

Quantification of caspase 3/7 activities in untreated or FTY720-treated CRC cells were carried out using the caspase Glo-3/7 assay kit (Promega Corp.). Briefly, $5 \times 10^3$ cells were plated in a white-walled 96-well plate, and the Z-DEVD reagent, the luminogenic caspase 3/7 substrate containing a tetrapeptide Asp-Glu-Val-Asp, was added with a 1:1 ratio of reagent to sample. After 90 minutes at room temperature, the substrate cleavage by activated caspase-3 and -7, and the intensity of a luminescent signal was measured by a FLUOstar OPTIMA luminometer (BMG Labtech). Differences in caspase-3/7 activity in FTY720-treated cells compared with untreated cells are expressed as fold-change in luminescence.

Colony-forming assay

Experiments were performed in 6-well plates were coated with 3ml of 0.6% soft agarose (Sigma) in medium. $1\times10^3$ cells were suspended in 0.3% agarose in medium and plated in triplicates over the pre-coated wells. Fresh medium was supplied once a week. After 15 days, colonies were stained with MTT (Methylthiazolyldiphenyl-tetrazolium bromide, M-5655, Sigma) for 4h at 37°C. Then, colonies were fixed by adding DMSO overnight at 37°C. Colony numbers were determined from triplicates and three independent experiments were carried out for each condition and cell line.

Ex vivo models

Tissue slices, which were not needed for diagnostic purposes, from primary colorectal tumors larger than 1.5cm of maximum diameter and normal adjacent colon mucosa were obtained from surgical specimens in patients newly diagnosed for adenocarcinoma. The samples were processed in sterile conditions immediately after surgical resection. Two slices ($2 \text{ cm}^3$) for tumor and normal mucosa were used for the
present study. One of the tumor and mucosa slides (designated as control samples) were put into culture medium, and another additional tumor and mucosa slices (designated as treated samples) were put on the same culture medium plus FTY720 at 10 µM. Incubation was performed in 24-well plates at 37°C in a constant atmosphere of 5% CO2 for 24 hours. At 24 hours, the specimens were fixed in 10% neutral-buffered formalin for 16 hours at room temperature and embedded in paraffin under vacuum conditions. These specimens were assayed for molecular markers as described in the IHC section.

**Immunohistochemistry**

Tissue sections (3µm) were placed on plus charged glass slides. After deparaffinization in xylene and graded alcohols, heat antigen retrieval was performed in pH9 EDTA-based buffer (Dako). Endogenous peroxidase was blocked by 0.03% hydrogen peroxide for 5 min. Slides were incubated with primary antibodies for 60 minutes at room temperature, followed of appropriate anti-Ig horseradish peroxidase-conjugated polymer (Flex+, Dako). Sections were visualized with 3,3′-diaminobenzidine as a chromogen. All stainings were performed in a Dako Autostainer. Sections incubated with non-immunized serum were used as negative controls.
Results

Reduced PP2A activity and SET deregulation are common events in CRC

To evaluate the importance of PP2A in CRC, we analyzed PP2A activation status in a series of 21 patients diagnosed of CRC. Patient characteristics are indicated in Supplementary Table 1. Interestingly, we observed a significant reduction of PP2A activity in 18 out of the 21 samples analyzed (Figure 1A, Supplementary Figure 1 and Supplementary Table 2). Since the 1D6 antibody against PP2A has been previously reported to recognize the demethylated fraction of PP2A (21-22), we performed these experiments with a second antibody against full length PP2A (FL-309), and similar results were observed in all cases except for P4 who was positive for PP2A inhibition only with the FL-309 antibody (Supplementary Figures 2 and 3). In order to investigate the possible causes of PP2A inhibition, we sequenced PPP2R1B in the 21 CRC patients. Information about the primers used for amplifying PPP2R1B cDNA segments is indicated in the Supplementary Table 3. Two silent heterozygous alterations were found: GAC (Asp) to GAT (Asp) at codon 391 in the patient 12 (P12), and TAT (Tyr) to TAC (Tyr) at codon 72 in patient 14 (P14) (Supplementary Figure 4). However, no missense mutations were identified in our cohort. In addition, we analyzed by western blot the expression of the PP2A endogenous inhibitor SET in these CRC patients. We found SET overexpressed in 13 out the 21 samples analyzed (Figure 1B and Supplementary Table 2). Taken together, these results would indicate that PP2A inactivation is a common event in CRC and that deregulation of the endogenous PP2A inhibitor SET would be a key contributing mechanism to PP2A inactivation in CRC.
CIP2A is frequently overexpressed and correlates with c-Myc levels in CRC

The fact that some of the patients included in our series showed reduced PP2A activity without displaying SET deregulation prompted us to study the presence of alternative mechanisms to inhibit PP2A in CRC. We studied the levels of CIP2A, another endogenous PP2A inhibitor recently reported as deregulated in CRC (23). Interestingly, we found CIP2A overexpressed in 15 out 21 cases analyzed (Figure 2 and Supplementary Table 2). CIP2A acts inhibiting PP2A-mediated MYC dephosphorylation and proteolytic degradation (24). Furthermore, CIP2A has been proposed as a key c-Myc regulator in CRC (25) and, in concordance with this point, we observed a good correlation between CIP2A and c-Myc levels in our series (Figure 2). Therefore, these observations would suggest that CIP2A overexpression is another contributing mechanism to inhibit PP2A in CRC. To further investigate other possible mechanisms of PP2A inhibition in CRC we analyzed by western blot the expression of PPP2R2A and PPP2R5E. Interestingly, we found PPP2R2A downregulated in 9 out of the 21 CRC patients, and PPP2R5E downregulation in 3 out of 21 cases (Supplementary Table 2 and Supplementary Figure 5). These observations would suggest that downregulation of regulatory PP2A subunits such as PPP2R2A and PPP2R5E could be involved in the PP2A inhibition observed in CRC.

PP2A activation by FTY720 reduces cell viability in CRC cells

We next performed functional analyses in a series of 7 CRC cell lines to clarify the biological relevance of PP2A deregulation in CRC. We first analyzed the PP2A status and the expression levels of SET and CIP2A in our panel of CRC cell lines observing that all of them showed PP2A inhibition together with overexpression of both SET and CIP2A in comparison with normal controls (Supplementary Figure 6). In order to assess whether increased PP2A activity affects cell proliferation of CRC cells, RKO and LoVo
cell lines were treated with the PP2A activator FTY720 or vehicle (DMSO). Phosphatase assays to quantify PP2A activity levels confirmed that FTY720 treatment leads to PP2A activation (Figure 3A and Supplementary Figure 7). In addition, we pretreated RKO and LoVo cells with the PP2A inhibitor OA for 90 minutes, followed by incubation with FTY720 or vehicle for 24 hours. FTY720-induced PP2A activity in RKO and LoVo cells was inhibited by OA (Figure 3A). Western blot analysis showed that similar levels of PP2Ac protein were immunoprecipitated in the PP2A phosphatase assays (Figure 3B), suggesting that differences observed in PP2A activity are not due to changes in PP2Ac expression levels. We next analyzed the effect of PP2A activation on cell growth using MTS assay. We observed a decreased proliferation in FTY720-treated RKO and LoVo cells compared with vehicle-treated cells (Figure 3C). Similar results were obtained with the SW480, HT-29 and DLD-1 cell lines (Supplementary Figure 8). In addition, we observed that the impaired proliferation induced by FTY720 was partially rescued by the treatment with OA used at a concentration that inhibits PP2A but no other phosphatases (26) (Figure 3C and Supplementary Figure 8). Moreover, the images obtained after treatment with FTY720 using an optical microscope were in concordance with the MTS results (Supplementary Figure 9). Altogether, these results show that PP2A activation by FTY720 treatment induces toxicity in CRC cells.

**FTY720 leads to an impaired clonogenic potential of CRC cells that is dependent on PP2A activation**

To further confirm the importance of FTY720 modulating CRC cell proliferation, we determined the effects on colony-forming ability of FTY720 treatment in CRC cells. Interestingly, FTY720-treated RKO and LoVo cells formed a markedly reduced number of colonies than controls (Figure 4). In concordance with the results obtained in the proliferation assays, we observed that the impaired clonogenic ability induced by
FTY720 was partially rescued by the treatment with OA, indicating that this effect is at least partially promoted by PP2A activation. Similar results were also obtained with the HT-29 and DLD-1 cell lines (Supplementary Figure 10). These results would suggest a potential therapeutic value for FTY720 treatment through PP2A activation in CRC cells.

*FTY720 induces apoptosis in CRC cells*

To further investigate the biologic effect of the FTY720-induced PP2A activation in CRC, RKO cells were treated with FTY720, and then we assessed apoptosis measuring activity levels of caspase 3 and 7. Vehicle-treated cells were used as controls. Consistent with its ability to increase PP2A activity and suppress cell growth, FTY720 showed a caspase-dependent proapoptotic effect, increasing caspase activity levels almost 7-fold in FTY720-treated RKO cells compared with vehicle-treated cells (Figure 5A). In addition, caspase activity in FTY720-treated cells was markedly reduced when cells were pretreated with OA. These results were confirmed in the LoVo cell line (Figure 5A).

*Molecular effects of PP2A activation in CRC cells after FTY720 treatment*

We next analyzed by western blot whether the FTY720 treatment had any effect in the phosphorylation status of previously described PP2A targets. Consistent with previous reports about the effects of PP2A activation in other tumor models (27), FTY720 treatment decreased phosphorylation (activity) of the PP2A targets AKT and ERK1/2 without affecting their expression levels (Figure 5B). Moreover, OA treatment rescued AKT and ERK1/2 phosphorylation in FTY720-treated RKO cells. Similar results were observed in LoVo (Figure 5B), HT-29 and SW480 cells (Supplementary Figure 11). Analysis in the HT-29 cell line showed decreased phosphorylation only for ERK1/2 (Supplementary Figure 11). Moreover, we observed that phosphorylation on tyrosine
307 of PP2Ac was not affected in cells treated with FTY720 compared with cells treated with vehicle (DMSO) (Supplementary Figure 11). Altogether, these results would indicate that the molecular mechanism of action of FTY720 involves the inhibition of AKT and ERK signaling.

We next analyzed PP2A, CIP2A and SET levels after treatment with FTY720 alone or in combination with OA in RKO and LoVo cell lines. Interestingly, western blot analysis showed that CIP2A decreased after FTY720 treatment and CIP2A levels were restored when cells where treated with FTY720 in combination with OA. No changes were observed in SET or PP2A expression. As expected, c-MYC levels decreased in correlation with CIP2A (Supplementary Figure 12). Altogether, these results would describe a novel mechanism of action of FTY720 through CIP2A and c-MYC deregulation.

FTY720 potentiates anti-tumor activity of 5-fluorouracil, SN-38 and oxaliplatin in CRC cells

In order to assess the effect of a combination between standard induction chemotherapy drugs used in CRC and FTY720, we treated CRC cells with either 5-FU, SN-38 or LOHP, alone or in combination with FTY720. Of importance, we observed that FTY720 enhanced the antitumor effects mediated by 5-FU (Figure 6A), SN-38 (Figure 6B) and LOHP (Figure 6C) treatments in the RKO and LoVo cell lines. These data were also confirmed in DLD-1 (Supplementary Figure 13) and HT-29 cells (Supplementary Figure 14). Therefore, these results showed that FTY720 treatment has an additive antitumor effect when combined with either 5-FU, SN-38 or LOHP in CRC cells.
FTY720 shows therapeutic effects in human CRC ex vivo models

To further investigate the potential therapeutic effects of FTY720 in CRC and assess cytotoxicity of this drug in normal colon cells we performed human CRC ex vivo models using five primary CRC tumors that were obtained from surgical specimens of patients newly diagnosed for adenocarcinoma. Immunohistochemistry analysis comparing samples of normal colonic mucosa and CRC from the same patient showed that FTY720 treatment induces an increased apoptosis (cleaved caspase-3) together with decreased proliferation (Ki-67) in tumor samples whereas no significant changes were observed in control samples (Supplementary Figure 15). These observations would support the observations made in vitro and indicate that cytotoxic effects of FTY720 are markedly reduced in normal colon cells.

Discussion

PP2A is a human tumor suppressor that regulates the activity of several signaling proteins critical for malignant cell behavior. We report here that PP2A is frequently inactivated in CRC cell lines and patient samples. In addition, we demonstrate that FTY720-induced PP2A activation leads to cell growth inhibition, impaired clonogenic potential, caspase-dependent apoptosis, and changes in the activation status of downstream targets such as AKT and ERK1/2. Importantly, FTY720 treatment shows an additive effect with the chemotherapy reagents 5-FU, SN-38 and LOHP, suggesting that treatment with PP2A activators could be a novel therapeutic option in CRC in combination with standard chemotherapy.

To evaluate the importance of PP2A deregulation in CRC we quantified PP2A activity levels in 21 CRC patients (paired normal mucosa and tumor samples), observing a
reduced PP2A activity in 19 out of 21 CRC patient samples (Figure 1, Supplementary Figure 2 and Supplementary Table 2). PP2A assays were carried out with two different antibodies against PP2A (1D6 and FL-309) that showed similar results in all cases except in P4 (PP2A inhibition only with FL-309). Whereas the FL-309 antibody recognizes the full length PP2A, the 1D6 antibody has been reported to preferentially recognize the demethylated fraction of PP2A (21-22), and this could be an explanation for the discrepancy observed in this case P4 between the PP2A assays performed with these antibodies (Supplementary Table 2). In order to determine the molecular mechanisms responsible of this PP2A inhibition in CRC, we sequenced PPP2R1B but no missense mutations were found in our cohort. These results would confirm previous works reporting that cancer-associated mutations affecting the PPP2R1B gene are rare events in CRC (13-16). We then decided to assess the expression levels of the PP2A endogenous inhibitors SET and CIP2A, observing deregulation of SET in 13 out of 21 cases, and overexpression of CIP2A in 15 out of 21 CRC patient samples (Figures 1B and 2), which indicates that these alterations are recurrent events in CRC. Very interestingly, we observed no alterations in SET and/or CIP2A in our CRC cases without PP2A inhibition. Moreover, of 19 patients with reduced PP2A activity 6 cases showed CIP2A overexpression, 4 SET overexpression and 9 both SET and CIP2A deregulation (Supplementary Table 2). Data of PP2A activity, SET expression and CIP2A expression status have been indicated for each patient in the Supplementary Table 2. CIP2A is a protein that inhibits PP2A-mediated MYC dephosphorylation and proteolytic degradation then increasing c-Myc protein stability (24). In addition, it has been reported that CIP2A overexpression is associated with c-Myc expression in CRC (25), and we showed a correlation between CIP2A and c-Myc levels (Figure 2) that would confirm the previous observations made by Böckelman et al. Thoroughly, these
results would indicate that overexpression of SET and CIP2A are contributing mechanisms that could cooperate simultaneously to inhibit PP2A in CRC.

Together with the overexpression of endogenous PP2A inhibitors and cancer associated mutations affecting structural PP2A subunits, downregulation of regulatory PP2A subunits has been described as a mechanism to inactivate PP2A in cancer cells (4, 9). Thus, we analyzed PPP2R2A and PPP2R5E, two regulatory PP2A subunits recently implicated in cancer (28-29) observing downregulation of these subunits in, respectively, 9 and 3 out of the 21 CRC cases (Supplementary Table 2 and Supplementary Figure 5). These alterations could contribute to explain some of the differences observed in the PP2A activity levels of the CRC patients. However, PP2A constitutes a large family of serine/threonine phosphatases and in this work we have only tested some of the potential mechanisms to inhibit PP2A. Although the catalytic and the scaffold subunits have two different variants, four unrelated families of PP2A regulatory subunits have been identified, including at least 26 different alternative transcripts and splice forms (5). Thus, PP2A has the ability to form a high number of different complexes with an also high number of potential alterations that could contribute to PP2A inhibition affecting any of these PP2A subunits. In fact, the cancerous cell shows a wide variety of molecular strategies to inhibit PP2A including hyperphosphorylation or downregulation of its catalytic subunit, mutations or downregulation affecting any of the scaffold or regulatory subunits, and the overexpression of endogenous PP2A inhibitors such as SET or CIP2A, but also others such as SETBP1 or SRC (20, 30). Therefore, further investigation would be necessary to clarify the particular importance of each mechanism and to identify other potential mechanisms involved in the PP2A inactivation observed in CRC.
In order to assess the potential therapeutic value of PP2A activation we used FTY720 to treat CRC cells. FTY720 is an immunosuppressor and a FDA approved drug for multiple sclerosis treatment. Interestingly, FTY720 has shown antitumor properties in several cancers. We considered this drug to treat CRC because together with its FDA approbation, Nagaoka et al. studied the effect of FTY720 phosphorylation in breast and colon cancer, observing an anticancer activity against two CRC cell lines (HCT-116 and SW620) (31). In addition, the pharmacological activation of PP2A using FTY720 has been proposed as a therapeutic alternative for future treatments in patients with some leukemias (27, 32). Interestingly, it has been recently reported that FTY720 targets SET and then mediates tumour suppression via PP2A activation in lung cancer (33). Our results show that PP2A inhibition plays an important role in CRC transformation, since the pharmacologic activation of PP2A in vitro and ex vivo using FTY720 reverse some of the malignant features of the CRC cells whereas cytotoxic effects seem to be markedly impaired in normal colon cells (Figures 3 to 5 and Supplementary Figure 15). Moreover, we observed that the molecular mechanism by which FTY720 is acting involves the inhibition of the AKT and ERK (Figure 5B), both PP2A targets, suggesting that this effect occurs via PP2A activation. In fact, we showed a PP2A activation after FTY720 treatment but, unexpectedly, we did not observed any change in the PP2A phosphorylation levels (Supplementary Figure 11), indicating that FTY720 activates PP2A by a mechanism alternative to the tyrosine-307 dephosphorylation. Interestingly, we observed that FTY720 led to decreased CIP2A levels (Supplementary Figure 12), which indicates that in addition to its recently reported blockade of SET (33), FTY720 could be acting decreasing CIP2A expression. Indeed, these two events are in concordance with the PP2A activation observed after the FTY720 treatment since SET and CIP2A are two endogenous PP2A inhibitors. Furthermore, our results also showed
that FTY720 exhibits additive antitumor effects with reagents used in standard chemotherapy such as 5-FU, SN-38 (active metabolite of irinotecan) and LOHP (Figure 6), suggesting that FTY720 is a good candidate for future trials in combination with standard chemotherapy reagents in patients with CRC.

In conclusion, we report that functional inactivation of PP2A is a common event in CRC. In addition, we show that functional loss of PP2A activity occurs through contributing mechanisms such as overexpression of the PP2A endogenous inhibitors SET and CIP2A, and downregulation of the regulatory PP2A subunits PPP2R2A and PPP2R5E. Moreover, the potent antitumor effects observed after restoration of PP2A activity with FTY720 indicates that PP2A inhibition is an alteration with high relevance in CRC pathogenesis. Finally, the fact that FTY720 treatment has shown an additive effect to chemotherapy drugs highlights PP2A as a molecular target with a potential value in therapies combined with PP2A activators in CRC.

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References


tumour suppression via activation of PP2A-RIPK1-dependent necroptosis.

Figure Legends

Figure 1. PP2A shows reduced activity in CRC patients. (A) Quantification of PP2A activity in CRC patients with normal mucosa and tumor samples; * P < 0.05; ** P < 0.01 (B) Western blot analysis of SET expression in CRC patient samples; + SET overexpression; N: normal mucosa; T: tumor sample.

Figure 2. CIP2A is overexpressed in CRC and correlates with c-Myc levels. Western blot analysis of CIP2A expression in CRC patient samples; + CIP2A overexpression; N: normal mucosa; T: tumor sample.

Figure 3. FTY720 treatment induces an impaired proliferation via PP2A activation. (A) Treatment with OA inhibits the FTY720-induced PP2A activity in RKO and LoVo cells. (B) Levels of immunoprecipitated PP2A (1D6 antibody) from the RKO and LoVo lysates used in the phosphatase assays. (C) The impaired cell viability induced by FTY720 is partially rescued by the treatment with OA.

Figure 4. Colony forming ability of untreated, FTY720-treated and FTY720/OA-treated RKO and LoVo cells. Colony-forming assay showing effects of FTY720 alone or in combination with OA in RKO and LoVo cells.

Figure 5. FTY720 induces caspase-dependent apoptosis together with changes in the phosphorylation status of PP2A targets. (A) Caspase 3/7 assays in RKO and LoVo cells untreated (DMSO), and FTY720-treated alone or in combination with OA. (B) Western blot showing AKT and ERK1/2 phosphorylation and expression after FTY720 treatment in RKO and LoVo cells.

Figure 6. FTY720 treatment shows an additive effect with 5-FU, SN-38 and LOHP treatments in CRC cells. MTS assays showing the effect of FTY720 and 5-FU (A),
SN-38 (B) or LOHP (C) alone or in combination in RKO and LoVo cells. Cells treated with vehicle (DMSO) were used as controls; * $P < 0.05$; ** $P < 0.01$. 
Figure 1

A

B

Figure 1
Figure 2
Figure 3

Panel A shows the PP2A activity in RKO and LoVo cells treated with different compounds: VEHICLE, FTY720, Okadaic acid, and FTY720 + OA. Statistical significance is indicated by P-values: P < 0.01, P = 0.027, P = 0.031, and P = 0.040.

Panel B includes immunoprecipitation (IP) and Western blot (WB) experiments for PP2A using the same treatments as in Panel A.

Panel C displays cell growth ratio over time (0h, 24h, 48h, 72h) for RKO and LoVo cells treated with Vehicle, OA, FTY720, and FTY720 + OA. Significant changes are marked with asterisks.*
Figure 4
**Figure 5**

A)

- **RKO**
  - Caspase 3/7 activity
  - VEHICLE: Low activity
  - FTY720: High activity
  - FTY720 + OA: Lower activity
  - Significance:
    - VEHICLE: P = 0.017
    - FTY720: P = 0.010
    - FTY720 + OA: P = 0.015

- **LoVo**
  - Caspase 3/7 activity
  - VEHICLE: Low activity
  - FTY720: Slightly higher activity
  - FTY720 + OA: Lower activity
  - Significance:
    - VEHICLE: P < 0.01
    - FTY720: P = 0.015
    - FTY720 + OA: P = 0.032

B)

- **RKO**
  - p-Erk1/2 (T202/Y204)
  - Erk1/2
  - p-Akt (T308)
  - Akt
  - β-Actin

- **LoVo**
  - p-Erk1/2 (T202/Y204)
  - Erk1/2
  - p-Akt (T308)
  - Akt
  - β-Actin
Figure 6

A RKO

B

C LoVo

Cell growth ratio (%)

0h 24h 48h 72h

Vehicle FTY720 5-FU 5-FU + FTY720

Cell growth ratio (%)

0h 24h 48h 72h

Vehicle FTY720 SN-38 SN-38 + FTY720

Cell growth ratio (%)

0h 24h 48h 72h

Vehicle FTY720 LOHPC LOHPC + FTY720

[Data and graphs showing cell growth inhibition over time for RKO and LoVo cell lines with various treatments.]
Molecular Cancer Therapeutics

PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential

Ion Cristobal, Rebeca Manso, Raul Rincon, et al.

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