INHIBITION OF TELO2 SENSITIZES COLORECTAL CANCER CELLS TO CHEMOTHERAPIES

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Abstract

Colorectal cancer is the 2nd most killing cancer around the world. Because treatments generally involve DNA damage, high toxicity and undesirable effects are frequently observed, which lead to treatment suspension. Moreover, several resistances to chemotherapy have been reported. Therefore, the research for new therapeutic approaches has become a major challenge. In this project our aim was to test the therapeutic potential of the protein TELO2 in combination with chemotherapy in colorectal cancer. TELO2 impacts the DNA damage response by affecting the stability of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) proteins. Indeed, the three classically used chemotherapies: oxaliplatin, irinotecan and 5-Fluorouracil, are based on DNA damage production. We used a HCT116 derivative cell line with an auxin-inducible degron fused the endogenous TELO2 protein. We found additive effect with oxaliplatin, and synergistic effects with low doses of irinotecan and 5-fluorouracil. These results encourage to keep on researching for alternative approaches to enhance the response-rate to colorectal cancer treatments.

Introduction

Colorectal cancer (CRC) is the 2nd most killing cancer around the world and there were over 1.8 million new cases in 2018 (Compare GBD, 2019). CRC is the 2nd most frequent cancer in women and the 3rd in men. Because risk factors are usually related to western type of life such as highly fatty diet and sedentarism, a 10% of western deaths are produced by this malignant disease (Kuipers et al., 2015). Thanks to the application of screenings in the recent years early detection has improved, rising up to 62.7% the 5-year survival rate in the developed countries (Huang et al., 2017).

Few therapies are available for colorectal cancer patients, involving almost in every case surgery for resection of the affected area, radiotherapy and chemotherapy. With a successful resection of the tumor, cancer spreading can be controlled or even avoided. Chemotherapy is then used as a complementary treatment to reduce tumor or metastasis size, aggressivity or relapse (Abdolahi et al., 2018). Both chemotherapy and radiotherapy produce indiscriminate DNA damage. These damages lead to mitotic catastrophy and death of tumoral cells – which are characteristic by having an accelerated cell cycle. However, undesirable side-effects are very frequent, and toxicity in non-tumorigenic cells -specially in those with high proliferation characteristics such as epithelia, hair follicles and bone marrow- is often so severe that the treatment must be suspended. In early states of the cancer, adjuvant chemotherapy (an additional treatment used after the primary one) after surgery, can reduce the probability of recurrence. In late stages of the disease, when surgery is not an option, palliative chemotherapy is used to improve the patients' lifespan (ASCO, 2019). Resistance to chemotherapy has frequently been reported, involving mechanisms such as drug inactivation and efflux, inhibition of transport, increased expression of targeted enzymes and enhanced DNA damage response or catabolization of the drug (Leary et al., 2018). This situation leads the patients to reduced therapy options. Overcoming treatment-acquired resistance has become a major therapeutic challenge.

There are several drugs approved by the U.S. Food and Drug Administration (FDA). First option chemotherapies involve one drug alone, a combination of them, or a mixing with a sensitizer element, which makes the cells sensitive to the effect of the drug, for example, the nuclear factor-kB inhibitors (Nakanishi. and Toi., 2005). Combinations

usually guarantee a better response to the treatment. The three main DNA damage-inducers used in colorectal cancer are 5-fluorouracil - the most frequently used chemotherapy against CRC- oxaliplatin and irinotecan. There are also targeted therapies to attack specific genes and proteins contributing to cell growth and survival, and immunotherapy, usually used in combination with chemotherapy agents (Labianca et al., 2013).

5-Fluorouracil is a conventional cytotoxic antimetabolite that has been widely used as a chemotherapy for CRC since its first use in 1960. This chemical agent produces double-strand breaks in the DNA in a cell-cycle dependent manner. Its mechanism of action is based on blocking the synthesis of deoxynucleic triphosphate, a metabolite for nucleic acids (Longley et al., 2003). Oxaliplatin is a platinum-based compound which forms adducts by DNA cross-link and prevents replication and transcription. Its mechanism of action is cell cycle-dependent (Raymond et al., 2002). Irinotecan inhibits topoisomerase I, leading to DNA double strand breaks and cell death (Guichard et al., 1999).

The cellular reaction to chemotherapy's effect is known as DNA damage response (DDR). It is a highly entangled pathway, where the different types of DNA damage are sensed. The main transducers are Ataxia-Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3-related (ATR) kinases, which phosphorylate several targets, such as the effector kinases Check Point Kinases 1 and 2 (Chk 1 and Chk2). ATM is mainly activated by double strand breaks (Jiang et al., 2009), while ATR is activated by single strand breaks and replicative inhibition, such as stalled replication forks and anti-metabolite agents, which leads to the accumulation of intermediates (Cimprich and Cortez, 2008; Hurley and Bunz, 2007; Hurley et al., 2007). Yet there are cross-talks between the two signaling cascades, both leading to p53 phosphorylation. P53 is a well-known downstream key effector. Its phosphorylation enhances its stability and its function as a transcriptional factor. DDR maintains genome integrity, which is achieved through DNA repair during cell cycle arrest -to avoid aberrant mitosis- or apoptosis (Bouwman and Jonkers, 2012). Up-regulation of the DDR has been related to resistance to chemotherapy (Marin et al., 2012).

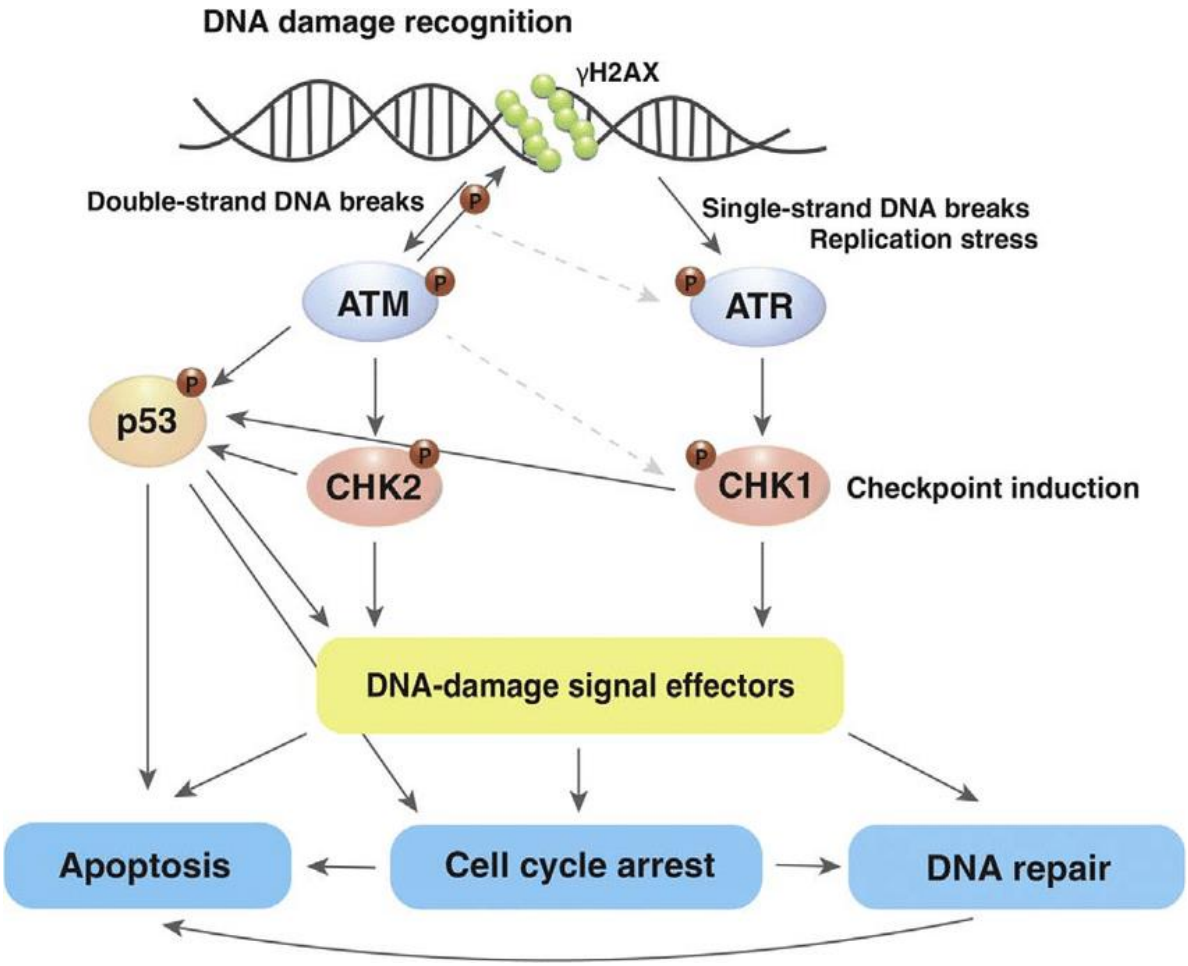


Figure 1: Simplified scheme of the DNA-damage response pathway. From Tšuiko et al. (2018). DNA damage and replicative stress produce DNA breaks. Double strand breaks activate ATM and single strand breaks activate ATR. When they are phosphorylated, both orchestrate the DNA Damage Response, by phosphorylating and activating their downstream targets CHK1 and CHK2, which in turn activate different DNA-damage effectors, finally leading to apoptosis, cell cycle arrest and DNA repair. They, together with ATM, phosphorylate and activate the tumor suppressor p53, which determines the final response of the cell, based on the level of DNA damage.

Both ATR and ATR are members of the phosphatidylinositol 3-kinase-related Protein Kinases family (PIKKs). PIKKs are a group of structurally related signal transducers involved in tumor suppression. They share a PI3K-related catalytic domain present in their C-terminal region, which phosphorylates serine/threonine residues (Lavin et al., 1995). There are six different PIKKs in mammals: the above-mentioned ATM and ATR, the DNA-dependent Protein Kinase catalytic subunit ataxia (DNA-PKcs), the Suppressor with Morphological effect on Genitalia 1 (SMG1), the Transformation/Transcription domain-associated protein (TRRAP), which is the only member without catalytic activity

and the mammalian Target of Rapamycin (mTOR). They all act in different pathways, including DNA damage response (ATM, ATR, DNA-PK) (Abraham, 2001; Kastan and Bartek, 2004; Shiloh, 2003), nonsense-mediated decay (an mRNA surveillance pathway) (SMG1) (Conti and Izaurralde, 2005), regulation of gene expression (TRRAP) (Herceg and Wang, 2005), and growth control according to nutrient and mitogenic signals (mTOR) (Hay and Sonenberg, 2004). No common substrate to all six proteins has been found.

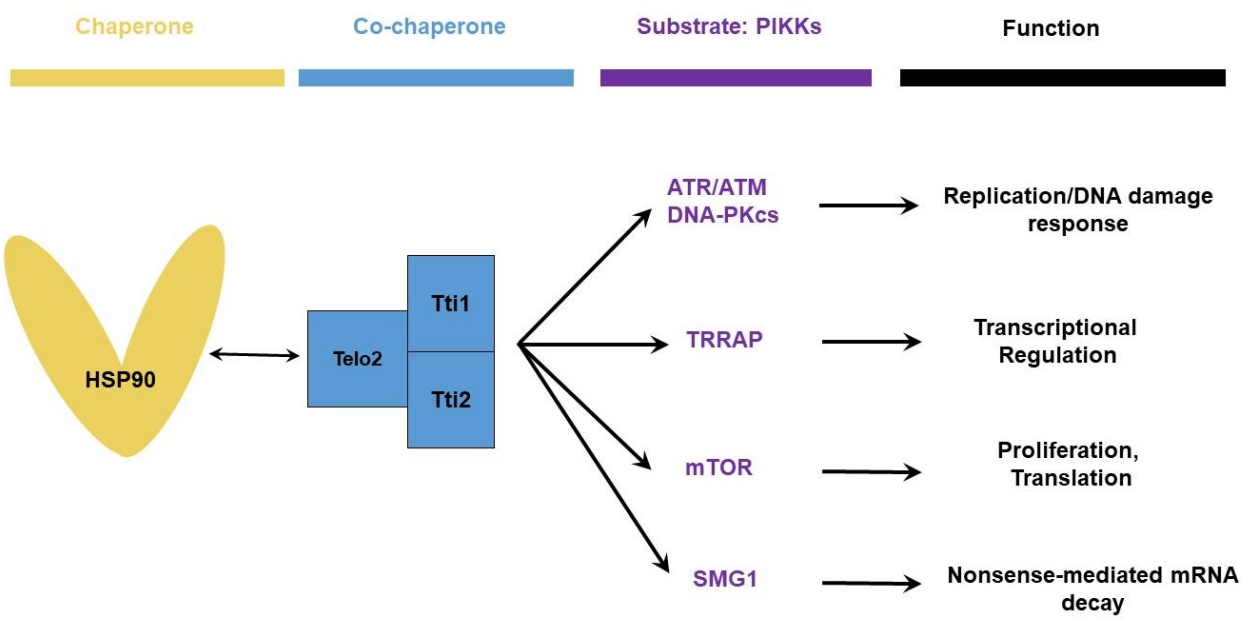


Figure 2. The triple TTT complex as a co-chaperone of HSP90. The components of the TTT complex work together with HSP90 to fold, maintain the stability of the six PIKKs and assembly them to complexes. The three subunits are needed for its function.

PIKKs share assembly and folding mechanisms, involving the Heat Shot Protein 90 (HSP90) and a trimeric complex known as TTT complex. This complex is composed of the co-chaperones Telomere Maintenance 2 (TELO2), TELO2- Interacting protein 1 and 2 (TTI1 and TTI2). Little is known about the architecture of TTT, but several groups have documented that each TTT component is necessary for the maturation and stabilization of the PIKKs. TTT is essential to fold, stabilize and incorporate the PIKKs into their functional complexes (Hurov et al., 2010; Takai et al., 2007). Thus, TTT appears as a hub to inhibit the activity of the PIKKs.

Inhibition of different substrates of the DNA-damage response cascade has proven to sensitize colorectal cancer cells to the effect of chemotherapy – and overcome resistance (Combes et al., 2019; Hosoya and Miyagawa, 2014; Ma et al., 2011). If the

DNA damage response fails to induce a cell cycle arrest, it will increase mitotic catastrophe and cell death. For this reason, several members of the cascade have been targeted to develop adjuvant therapeutics to improve the efficacy of the antitumor agents. Inhibition of ATR and its substrate Chk2 was shown to sensitize $p53^{-/-}$ murine lung adenocarcinoma cell lines to the effect of doxorubicin (Jiang et al., 2009). ATR inhibition sensitized $p53^{-/-}$ HCT116 colorectal cancer cell line to the effect of cisplatin (Sangster-Guity et al., 2011). Combination of different DDR inhibitors -including ATR, Chk1 and Wee1 inhibitors- were shown to synergize their cytotoxic effect in Mantle Cell Lymphoma and Diffuse Large B Cell Lymphoma, regardless their p53 status (Restelli et al., 2019).

Other agents have been shown to sensitize cell lines to the effect of antitumor agents. Ascorbic acid has been proved to chemosensitize colorectal cancer cells and inhibit tumor growth (Pires et al., 2018). Inhibition of autophagy enhanced synergistic effects of salidroside and anti-tumor agents against colorectal cancer (Li and Chen, 2017). Hypomethylating agents where shown to synergize the cytotoxic effect of irinotecan in CRC cells (Sharma et al., 2017).

The aim of this project is to evaluate the therapeutic potential of the co-chaperone TELO2 in combination with chemotherapeutic agents in CRC. We reasoned that inhibition of TELO2, by down-regulation all PIKK activities, including that of ATM and ATR, could sensitize colorectal tumor cells to chemotherapies. To test this hypothesis, we took advantage of a CRC cellular model developed in the lab., in which post-traductional depletion of TELO2 is achieved through an Auxin-inducible Degron (Holland et al., 2012; Nishimura et al., 2009). TELO2 degradation results in reductions of ATM and ATR levels. By measuring cellular protein production as an indicator of cell proliferation and growth, we addressed if inhibition of TTT could potentiate the effect of CRC chemotherapy 5FU, oxaliplatin and irinotecan. The inhibition of TELO2 showed some additive effect with oxaliplatin, irinotecan and 5-Fluorouracil.

Materials and Methods

Experimental model: colorectal cancer cell line with degradable TELO2

There are several ways to control protein expression. The more frequent are at the DNA level, modifying the sequence of the gene (Sauer and Henderson, 1988) or at the RNA level, suppressing mRNA traduction, especially with RNAi (Elbashir et al., 2001; Gossen and Bujard, 1992). However, depletion of a given protein using these techniques is rather slow (more than 24h), and depends on the protein stability, while incomplete depletion and off-targets are seen. For that reason, and because TELO2 is essential for cell survival, we used an inducible degron system, the AID (Auxin Inducible Degron) (Holland et al., 2012; Nishimura et al., 2009). TELO2 is the best characterized subunit of the TTT complex and is essential for the activity of the complex (Takai et al., 2010).

The cell line we used is TELO2-AID-YFP 620 (TAY620), a derivative from HCT116, established from a human colorectal carcinoma epitelium (Brattain et al., 1981). In this cell line, the post-traductional depletion of TELO2 is rapidly and reversibly induced upon auxin addition. TAY 620 was generated in the lab. by modification of the endogenous TELO2 gene locus using the CRISPR/Cas9 technique to fuse an Auxin-Inducible Degron (AID) and a Yellow Fluorescent Protein (YFP) in C-ter of TELO2 protein. Briefly, a recombining DNA template was created, constitute of a cassette encoding the AID and YFP, flanked by 800bp homology arms. Cas9 cut at the STOP of the TELO2 gene and the cassette was integrated by recombination. YFP+ clones were selected for an homozygous recombination of TELO2 (Figure 3A).

Upon auxin addition, AID recruits TIR1 (Transport Inhibitor Response 1). TIR1 is the vegetal F-box protein that promotes the interaction of the SCF complex with the AID-containing protein in presence of auxin. TIR1-encoding cassette was previously stably integrated into the HCT116 genome. TIR1 protein is integrated into the host SCF complex, which binds an E2 ubiquitin-conjugating enzyme and transfers the activated ubiquitin to the targeted TELO2 (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). Polyubiquitinated TELO2 is then degraded by the proteasome. (Figure 3B). Upon auxin removal, the process is reverted and the amount of TELO2 protein returns to normal levels (Detilleux, 2018). Auxin is a plant hormone that controls gene expression and development that is not present in mammals and has no effect on human cells (data not shown).

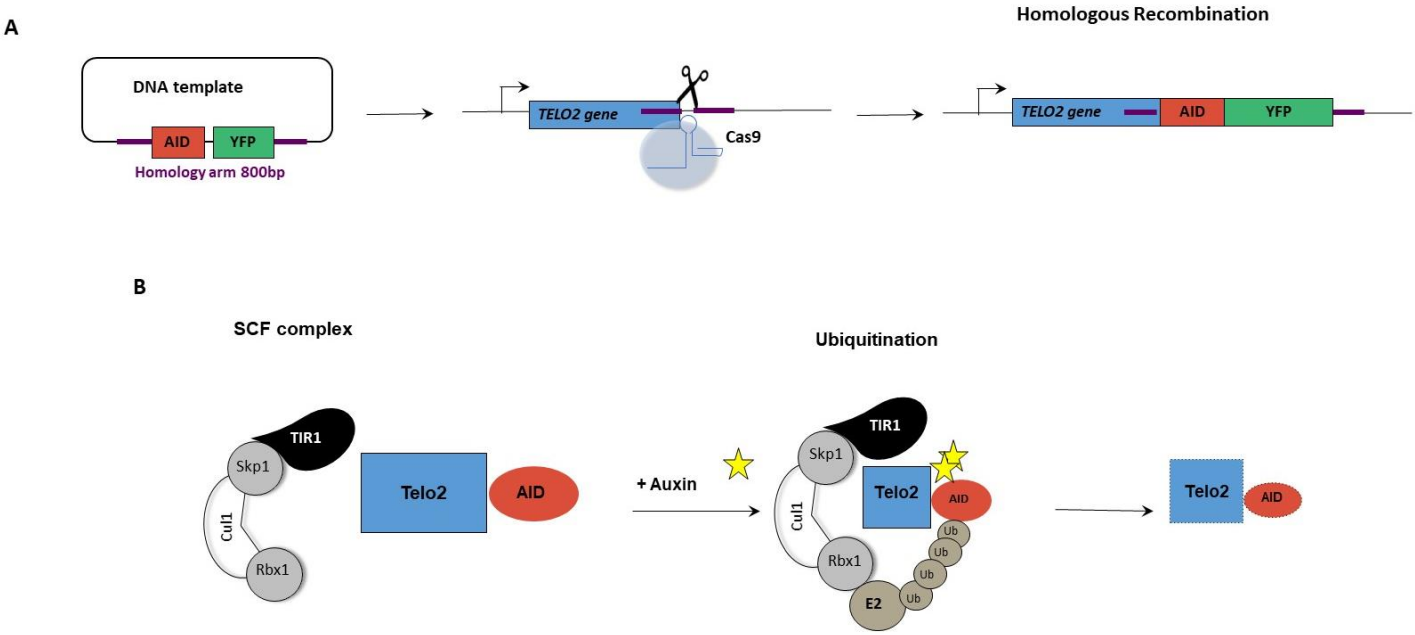


Figure 3. Creation of the cell line TAY620 and culture timepoints. A: Process of integration of the AID-YFP cassette with the CRISPR/Cas9 technique at the C-ter of the TELO2 protein **B**: Mechanism of TELO2 depletion upon auxin addition. The E3-uquituitin ligase SCF complex recruits a E2 ubiquitin-conjugating protein through the F-box protein Tir1 and polyubiquitination and degradation by the proteasome is done to the AID-targeted protein (TELO2). Adapted from Holland et al. 2012

Cell culture

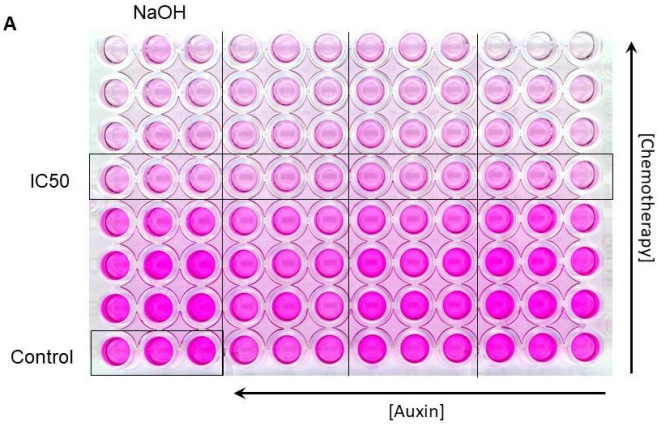
TAY620 cells were cultured in plastic plates with McCoy's Medium (Life Technology) with a final concentration of 10% Fetal Bovine Serum (Life Technology) and penicillin and streptomycin (Life Technologies). They were maintained in a humidified incubator at 37ºC with 5% CO2. Cells at 70-80% of confluency were passed by trypsinization. For proliferation and survival assays, 2000 cells/well were seeded in a final volume of 100ul in nineteen-six well plates.

Cell treatment

Several concentrations of four different treatments were used. The Auxin was indole-3-acetic acid (IAA; a natural auxin purchased from Sigma-Aldrich). To analyze the effect of the auxin alone, several 1:2 dilutions were tested from 1µM to 500µM. To study the effect of the combination with chemotherapy the concentrations tested were 5µM, 15µM, 25µM, 50µM, 500µM. The Oxaliplatin antitumor agent (gifted from C.Gongora, from Institut de Recherche en Cancerologie de Montpellier, Université Montpellier, CNRS)

Montpellier, France.) was tested at 0.075µM, 0.15µM, 0.3µM, 0.6µM, 1.2µM, 2.4µM, 4.8µM. The Irinotecan chemotherapy (gifted from C.Gongora) was used at 0.125nM, 0.25nM, 0.5nM, 1nM, 2nM, 4nM and 8nM. The 5-Fluorouracil (gifted from C.Gongora) treatment was used at 0.125µM, 0.25µM, 0.5µM, 1µM, 2 µM, 4µM and 8 µM. Cultures were done with every concentration of every treatment alone and in combination (auxin + chemotherapy). Negative controls were performed in every experiment with vehicle (NaOH 5mM).

A



B

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Plan 1	Cells seeding	Auxin and chemotherapy treatment	-	-	-	SRB assay - absorbance measure	-
Plan 2	Cells seeding and auxin treatment	-	Chemotherapy treatment	-	-	-	SRB assay- absorbance measure

Figure 4. Cell treatment A: Distribution of the chemotherapy and auxin treatments in the 96-wells plate. Growing concentrations of chemotherapy were added from the bottom to the top of the plate doubling the concentration at each line. The same volume of PBS was added to the bottom line (0µM of chemotherapy). Growing concentrations of auxin were added from the right to the left. The control was treated with PBS and NaOH. Technical replicates were done in every plate. **B:** Timelines of the two protocols that were used for the cell treatment. In both cases the cells were treated with chemotherapy for 4 days. In Plan 2, a pretreatment of 2 days was done with auxin, before adding the chemotherapy. Three biological replicates were performed for every chemotherapy treatment and for both plans.

Cell proliferation assays

For proliferation assays, 200.000 cells were first seeded in 12-well plates, and 500uM of Auxin added 24h after the seeding. Cells were detached using trypsin, and counted using a cell counter (Countess, Invitrogen) at 24h, 48h and 72h after auxin addition. Yet, this technique produces high inter-sample variability so we turned to the more effective sulforhodamine B assay, a colorimetric assay described by Orellana and Kasinski (Orellana and Kasinski, 2016).

To analyze the effect of chemotherapy with TELO2 depletion, 96 well plates were used as in Figure 4A in triplicates (technical replicates). The sulforhodamine B assay relies on measuring the absorbance of a negatively charged pink amnioxantine that dyes proteins. The protein quantity is a measure of cell growth and proliferation altogether. It was always performed 4 days after chemotherapy addition. Two different experimental settings were tested, as explained in Figure 4B. On Plan 1, both the Auxin and the chemotherapy were added at the same time, 24h after cell seeding. On Plan 2, a pretreatment of Auxin was done for two days before adding the chemotherapy treatment. The percentage of proliferation were calculated as compared to the control, grown in McCoy without chemotherapy nor auxin, just NaOH.

Protein Extraction and analysis by Western blot

Cells were rinsed twice with 1ml of PBS, then directly lysed in the wells with RIPA lysis buffer composed of 150 mM NaCl, 50 mM Tris HCl pH7,5, NP40 1% and deoxycholate 0.5%, supplemented with anti-protease EDTA-free tablet (Roche). The lysates were transferred to 1.5ml Eppendorf tubes, sonicated and centrifuged at 12000 g at 4ºC to discard organelles and cell debris. Supernatant of these samples were stored at -20ºC.

We used the western blot technique as Burnette et al. described (Burnette, 1981) to evaluate the effect of the auxin on the protein levels. The Bradford method was used to measure the protein concentration and load equal amounts of protein across samples. The primary antibodies used were anti-TELO2 (Sigma; SAB110071 9), Tti1 (SCTB; sc-271638), ATM (2873 CST), ATR (139345 CST), TRRAP (a generous gift from L. Tora) and tubulin (B512 Sigma-Aldrich). Secondary antibodies coupled to HRP (Abcam) were used, and signal detected by chemoluminescence (GE).

Statistical analysis

All the statistical analysis was performed with the software IBM® SPSS® Statistics 24.0.0.0. The normality of the distribution of the quantitative variables was evaluated with Shapiro-Wilk and Kolmogorov-Smirnov tests. The variance homogeneity was tested with Levene test. Two-factor ANOVA was performed to compare quantitative variables between more than two groups. *Post hoc* analysis was performed using Tukey Test. A statistical significance of 0.05 was considered for all the analysis.

Results

NaOH has no detectable effect on TELO2 stability nor cell proliferation

Since NaOH was used as a vehicle to dilute the auxin as Holland et al. described (Holland et al., 2012), we first wanted to discard the possibility that NaOH would affect TELO2 levels per cell, cell viability or interfere with the chemotherapy. After 48h of treatment, the amount of TELO2 visualized in NaOH treated cells using the western blot analysis did not show significant differences as compared to PBS addition. The same results were obtained for Tti1 and Tubulin (Figure 5A). Besides, TAY620 cells were cultured for 4 days in McCoy medium, and NaOH 1mM or PBS were added, and combined with different concentrations of oxaliplatin (chemotherapy) (Figure 5B). Both groups of cells showed equivalent percentage of proliferation for every chemotherapy concentration from 0 to 2,4µM ($p > 0.05$), showing that NaOH treatment, at these concentrations, did not affect any of the characteristics considered in this study.

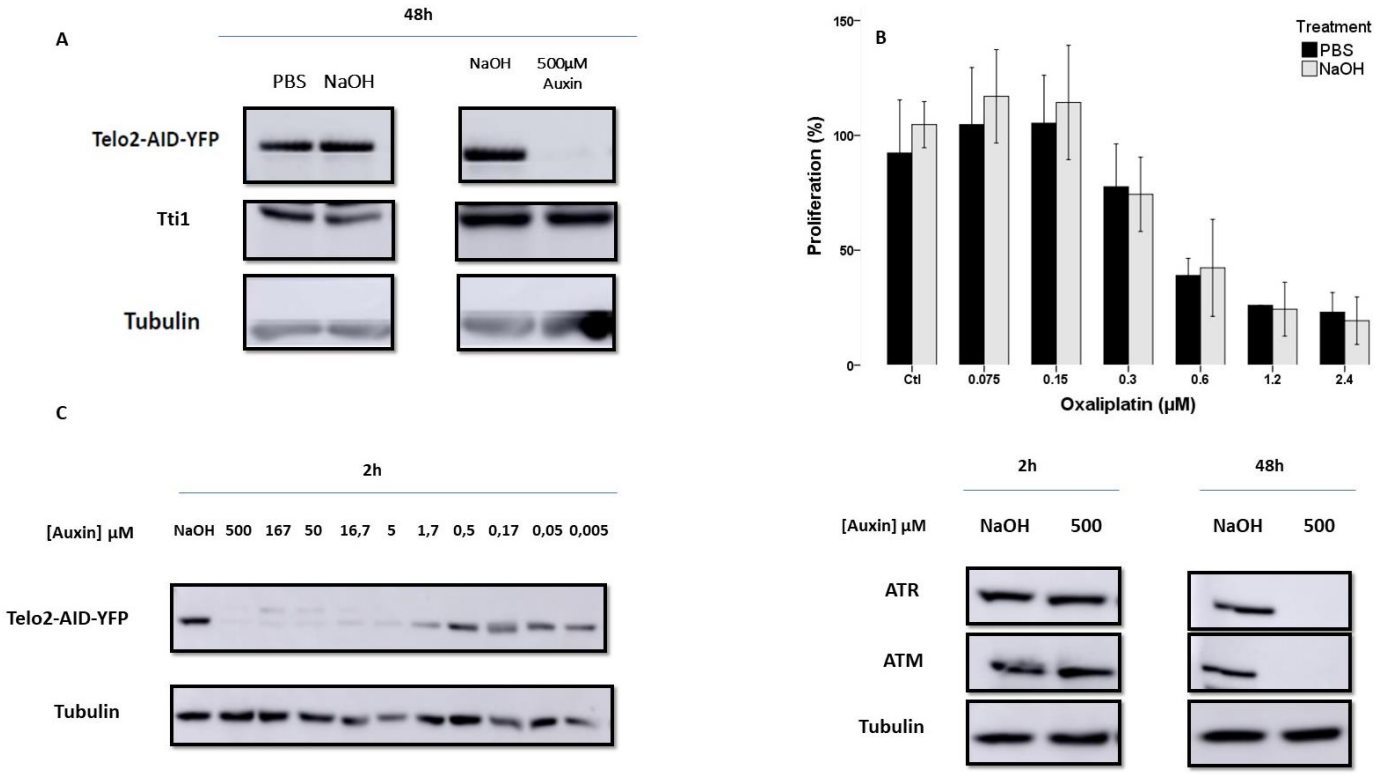


Figure 4. Protein levels and proliferation in cell line TAY620 after auxin and NaOH treatment; A: Protein quantities of the targeted protein (TELO2) and its cochaperone (Tti1) cultured with PBS (negative control), NaOH and 500µM of auxin treatment during

48h. **B:** Cell proliferation at different concentrations of oxaliplatin combined either wit PBS or NaOH. **C:** Protein quantity of TELO2 under different concentrations of auxin for 2h. **D:** Protein quantity of the PIKKs ATR and ATM under treatment of NaOH or 500µM of auxin at 2h and 48h. Tubulin was used as a loading control in every case.

Auxin depletes TELO2 in a specific and concentration-dependent manner

Western blot was performed to check levels of TELO2. Strong reduction of TELO2 was observed with a treatment of 500µM of Auxin during 48h. Contrarily, its interacting partner in the TTT trimeric complex Tti1, did not show any decrease when treated with auxin (Figure 5A). Different concentrations of auxin resulted in different levels of TELO2 depletion. Various concentrations were tested and TELO2 was analyzed after 2hours. From 500µM to 5µM, TELO2 was not detectable after 2 hours, from 1,7µM, lower amounts of TELO2 were observed (Figure 5C).

ATM and ATR levels are strongly decreased after 48h of treatment with Auxin

After 2h of treatment with 500µM of auxin, no decrease of ATM nor ATR levels were observed. When treated for 48h, strong decrease of ATM and ATM levels was found (Figure 5D). This is coherent with the fact that TELO2 is important for the accumulation of neo-synthesized PIKKs.

Auxin-mediated depletion of TELO2 starts to decrease cell proliferation by the 3rd day of treatment

The count of cells treated with 500µM of Auxin was equivalent to the NaOH-treated during the 2 first days ($p > 0.05$). The 3rd day the number of cells was statistically different to the parental ($p < 0.05$) (Figure 6A from *Detilleux et al. (2018)*), with a plateau at 50% growth inhibition. After 4 days of treatment with several concentrations of auxin, from 0µM to 500µM, we found that the percentage of proliferation of auxin-treated cells varied according to the concentration of auxin with a logarithmic relation (R^2=0.978). The IC50 found was 50µM (Figure 6B).

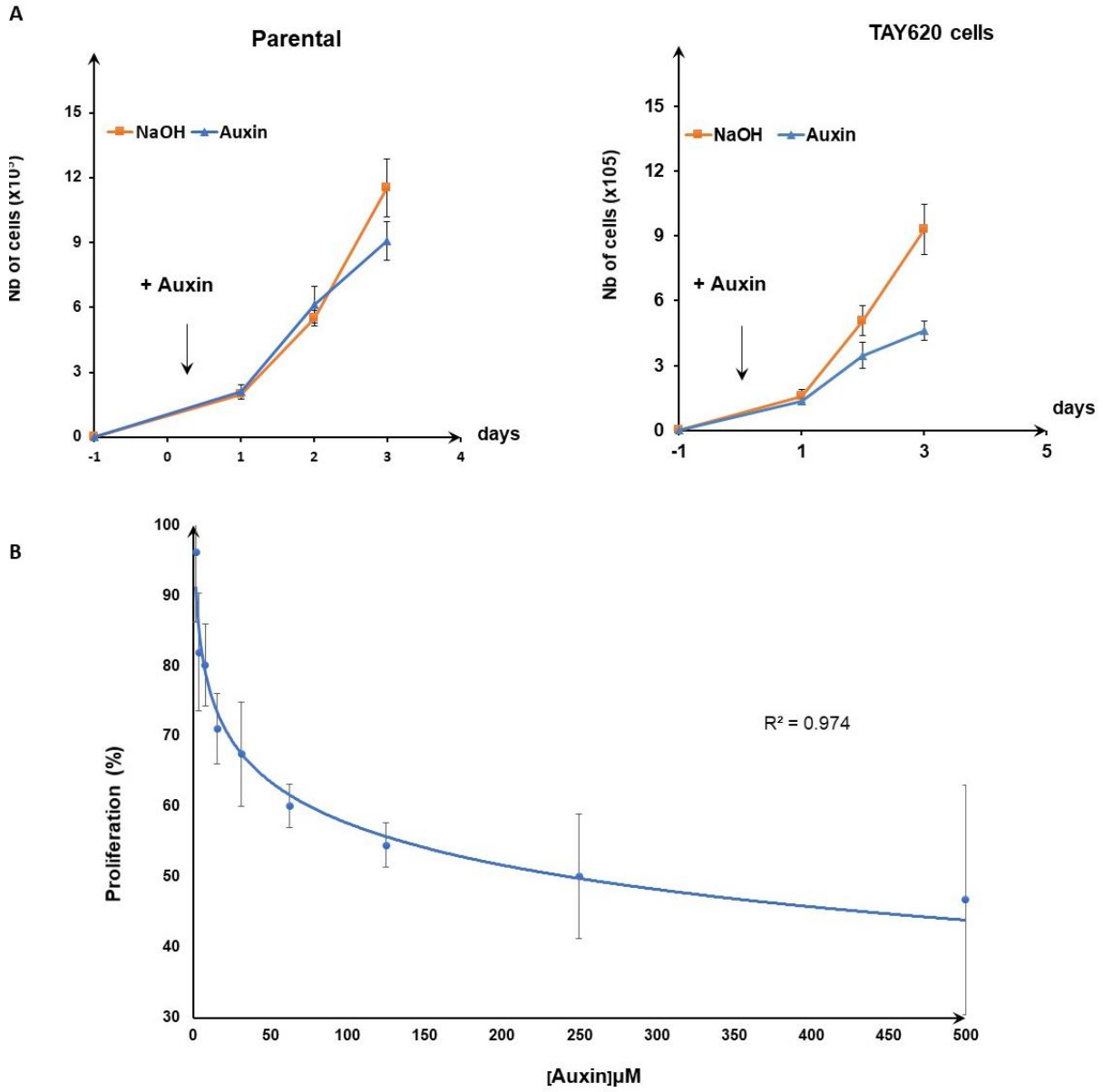


Figure 6. **TAY620 proliferation decreases when treated with auxin; A**: On the left the graph shows the number of parental (not genetically modified) cells counted both under treatment with NaOH and with Auxin. On the right there is the cell count of TAY620 cultures treated under the same conditions as the parental. **B**: The plot shows the percentage of TAY620 cells proliferation relative to the control (non-treated) after 4 days of treatment.

TELO2 depletion and oxaliplatin treatment

The first concentrations of auxin selected were 5µM (low dose) and 500µM (high dose). The reported Inhibitory Concentration 50% (IC50) of Oxaliplatin in HCT116 is 0.6µM (C.Gongora, personal communication). Our control culture showed the same IC50. The

cells did not show apoptotic phenotype. In every case, an addition of auxin resulted in a lower proliferation rate. The maximum effect observed is 80% inhibition of the proliferation. Three biological repetitions were performed, with 3 technical replicates each.

Untreated cells were set to 100% of proliferation. When low doses (0.075µM and 0.15µM) of oxaliplatin were added, no significant differences were found ($p > 0.05$). In fact, a slightly a higher proliferation, up to 114%, was observed. We hypothesized this effect could be due to the elimination of weaker cells that would benefit the fittest, remaining ones. From 0.3µM to 0.6µM of oxaliplatin, proliferation decreased and reach a plateau at 1.2µM to around 23%. For every concentration of oxaliplatin, from 0 µM to 0.6 µM, there was a significant difference in proliferation upon auxin treatments, and in between the two doses tested (5µM vs. 500 µM; except by 0.6µM where no differences were observed between both of auxin) (Supplementary table 1.1).

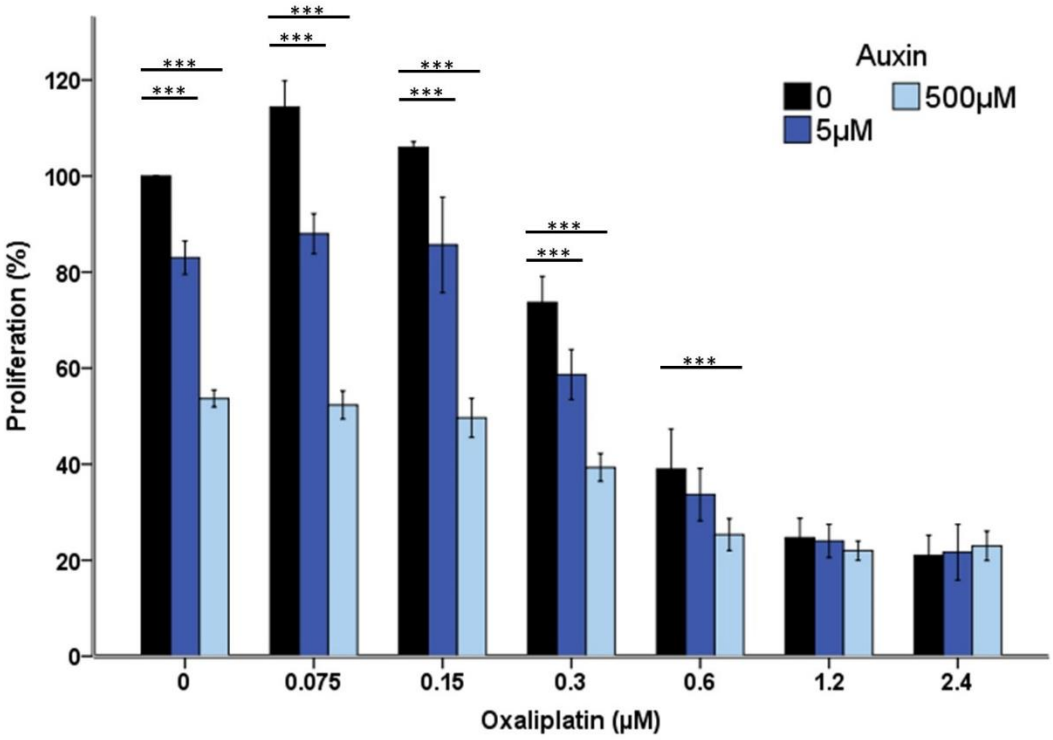


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Figure 7. Combined treatment with Oxaliplatin (4 days)

50.000 TAY620 cells were treated for four days with different doses of oxaliplatin, with 5 µM or 500µM of auxin (dark and light blue bars, respectively) or vehicle (0, black bars). After four days, protein content was measured by sulforhodamine B assay. Data are means from 3 different experiments ("biological repetition"), and for each experiment, measurements were done in triplicates. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$

For 0µM to 0,15µM of oxaliplatin, the chemotherapy did not change the effect of the auxin on cell proliferation. An addition of 5µM of auxin showed 84% of proliferation, while 500µM showed a 54%, but this effect was equivalent to the one observed with auxin alone. With 0.3µM of chemotherapy a 74% of proliferation was observed with no auxin. The addition of 5µM of auxin showed a 59% of proliferation – a 20% more of inhibition than the 0.3µM oxaliplatin alone –, and 500µM showed a 39% of proliferation – a 47% more of inhibition than the oxaliplatin alone. Treating with 0.6µM or more with oxaliplatin reached 41% of proliferation was found without auxin, and 28% was found with 500µM – a 32% more of inhibition than the oxaliplatin treatment alone (Supplementary tables 1.1 and 1.2). The effects observed were additive. From 1.2µM to more concentrated chemotherapy treatment, no differences were found between different concentrations of oxaliplatin and different additions of auxin.

TELO2 depletion and Irinotecan treatment

The concentrations used to treat the irinotecan were 5µM, 15µM, 25µM, 50µM and 500µM. 15µM and 500µM are not shown because the results were not biologically relevant. The reported IC50 for irinotecan in HCT116 is 1nM (C.Gongora, personal communication). No apoptotic signs were found after the treatment (Plan 1). In all conditions, an addition of auxin resulted in a lower proliferation. The maximum effect observed was an 80% of growth inhibition. Three biological replicates were performed, with different auxin conditions in each experiment.

The proliferation of the non-treated cells with irinotecan was set at 100%. No statistically significant differences were seen between 0nM and 0.125nM. With 0.25nM and 0.5nM, the proliferation decreased to 90%. From 1nM, to more concentrated chemotherapy, no differences were seen. The proliferation tended to a plateau at around 20% of cell proliferation. Statistical differences were found between the different concentrations of auxin when no chemotherapy was added, except between 5µM and 25µM (Supplementary table 2.1).

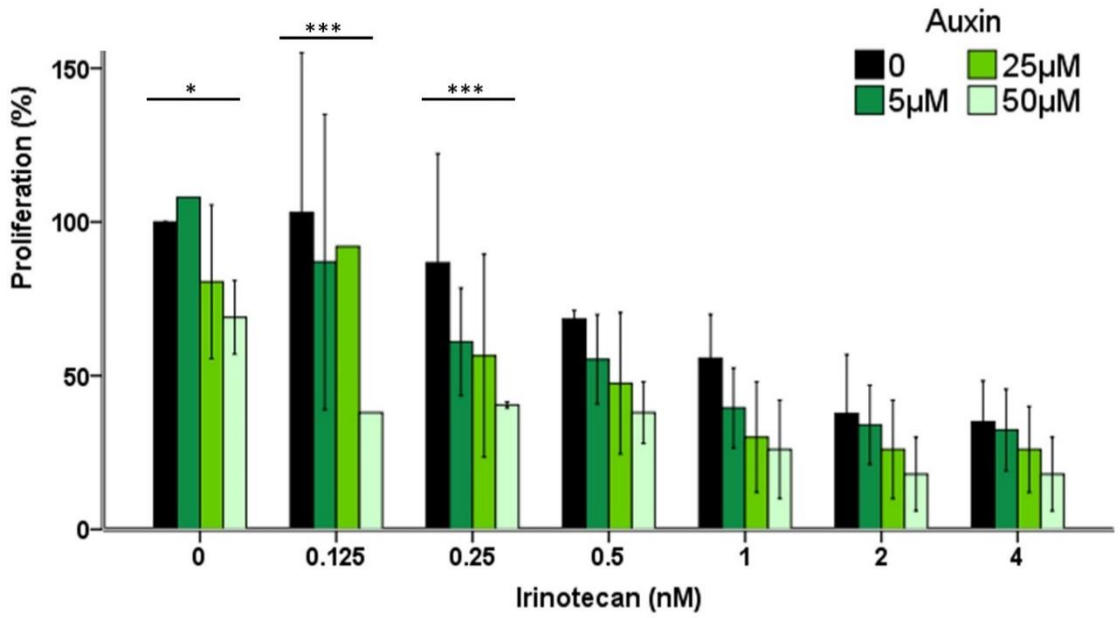

Figure 8. Combined treatment with Irinotecan (4 days)

50.000 TAY620 cells were treated for four days with different doses of irinotecan, with 5μM, 25μM and 50μM of auxin (from darker to lighter green bars) or vehicle (0, black bars). After four days, protein content was measured by sulforhodamine B assay. Data are means from 3 different experiments ("biological repetition"), and for each experiment, measurements were done in triplicates. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$

At every concentration of irinotecan, addition of 50μM of auxin showed a stronger inhibition in cell proliferation than just the presence of antitumor agent alone. An additive effect was found with 0.125nM and 0.25nM of irinotecan. More samples are needed to get better biological relevance and significance.

TELO2 depletion and 5-Fluorouracil treatment

Several concentrations of auxin were tested in combination with 5-FU: 5μM, 15μM, 25μM, 50μM and 500μM. The reported IC50 for 5-FU in HCT116 is 1μM (C.Gongora, personal communication). In our case, 2μM was found to be the IC50. The phenotype of the cells was not apoptotic after 4 days of treatment (Plan 1). Addition of auxin provoked a lowest proliferation in every case. The maximum inhibitory effect observed was an 80% of proliferation inhibition. The same case as with Irinotecan treatment was obtained with the biological relevance.

A 100% of proliferation was set with no chemotherapy. No significant difference was observed between 0µM, 0.125µM, 0.25µM and 0.5µM of 5-Fluorouracil. Nevertheless, a slight increase of the proliferation was found again, as in the case of the oxaliplatin. A 90% of proliferation was found with 1µM of 5-Fluorouracil. A 53% of proliferation was found with 2µM of 5-Fluorouracil, and a 27% with 4µM of the same agent (Supplementary table 3.1). The effects of those three concentrations are different between them also when compared to the non-treated. Statistically significant differences were found between the different concentrations of auxin alone, except between 5µM and 25µM.

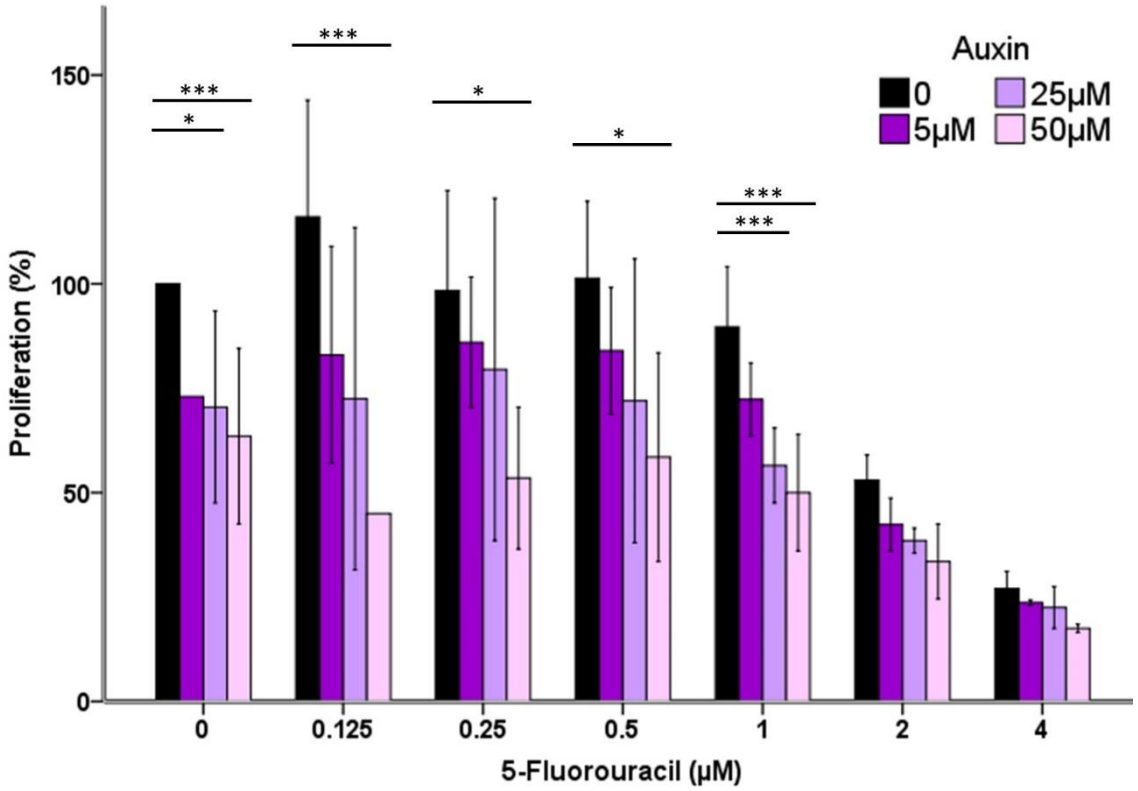

Figure 9. Combined treatment with 5-Fluorouracil (4 days)

50.000 TAY620 cells were treated for four days with different doses of 5-Fluorouracil, with 5µM, 25µM and 50µM of auxin (from darker to lighter purple bars) or vehicle (0, black bars). After four days, protein content was measured by sulforhodamine B assay. Data are means from 3 different experiments ("biological repetition"), and for each experiment, measurements were done in triplicates. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$

In the non-treated cells, addition of 50µM of auxin decreased the proliferation to 63%. The same effect was observed with 50µM in all the concentrations, from 125µM 0.5µM of 5-Fluorouracil. With 0.5µM of auxin the effect observed was small. In the case of 1µM, a proliferation of 57% was found with 25µM of auxin – a 44% of inhibition more than the chemotherapy alone -, and a 50% with 50µM – a 51% more of inhibition than the

chemotherapy alone (Supplementary tables 3.1 and 3.2). In both cases the effect observed was additive. No statistically significative differences in the proliferation were observed when adding auxin to either 2µM or 4µM of 5-Fluorouracil, but we think we should repeat it to get more robust data.

Pre-treatment with auxin

All the experiments performed following the timeline corresponding to the Plan 2 were discarded. The objective was to do a pretreatment of auxin for two days and, once TELO2 had already been successfully depleted, add the damaging stimuli. But an extreme toxicity with the high concentrations of both treatments for a prolonged incubation was observed. Apoptotic phenotypes were observed, and cells had no possibility of proliferation (data not shown). These experiments should be repeated with much milder concentrations of auxin.

Discussion

In this project, the recently developed TELO2-AID-YFP 620 cell line was evaluated to check if the depleting mechanism was effective and specific. A dramatic reduction of TELO2 was observed after 2 days of treatment with the highest concentration of Auxin (500µM). Less time or less concentration could be also used to produce a partial depletion, reducing to a low extent the amount of TELO2 protein. The depletion of TELO2 was independent from the expression levels of its complex partner Tti1, which means that the depletion of one of them is enough to disrupt the downstream pathway so that the cells enter the cell cycle with aberrant mitosis. Further research is needed in this direction, to decipher the molecular mechanisms of TTT mode of action.

We confirmed that the decreased levels of ATM and ATR come as a consequence of a specific depletion of TELO2, as expected according to previous studies that had related the amount of every subunit of the TTT complex to the levels of PIKK (Izumi et al., 2012). Strong depletion of ATM and ATR (at day 4) was observed after the specific depletion of TELO2 (at day 2). With this approach, the master regulators of the DNA damage response remain in a low level and we expect that the activation of the protective response of cell cycle arrest, DNA reparation and/or apoptosis come in a disorganized and non-effective manner.

We showed that a strong depletion of TELO2 decreases the cell survival to a different extent, according to the concentration of auxin. We hypothesize this effect is produced due to the lack of regulation of the different pathways in which the PIKKs are involved, that are apart from the DNA damage response (Figure 2). This dysregulation would weaken the cells to respond to different stimuli. To avoid that mortality, we propose to reduce the TELO2 quantity at levels, which are no relevant to cell survival. The objective is to use a concentration of auxin low enough not to directly harm the cells due to the reduction of PIKKs but to an extent that, when an external stimulus is present, the cells are not able to respond correctly. A treatment with an antitumor agent is an example of a stimuli that directly activates the DNA damage response, where ATM and ATR exert a crucial role.

We tested the main three current chemotherapy agents used in the clinical practice to overcome the colorectal cancer. The oxaliplatin showed additive interaction with the depletion of TELO2, specifically for the 0.3µM and 0.6µM, with both concentrations of

auxin tested (5µM and 500µM). When auxin was added to both chemotherapy agents, significantly statistical differences were observed in all cases ($p < 0.001$), except for the 5µM with 0.6 µM of oxaliplatin. which means the addition of auxin has a relevant and differential effect depending of its concentration. The irinotecan showed an additive effect when 50µM of auxin was added to the lowest doses tested (0.125nM and 0.25nM). Additive - yet not statistically significant – effects were observed when added to 0.5nM and 1nM. More replicates are needed to add confidence to these results. With the 5-Fluorouracil the same results than the Irinotecan were found, in this case the four concentrations (0.125µM, 0.25µM, 0.5µM and 1µM) were statistically significant when 5µM of auxin was added. Interesting results were obtained with 2µM and 4µM of 5-Fluorouracil – yet not significative. More replicates are needed to get better and more robust.

Our work gives preliminary results to study and analyze the therapeutic potential of the co-chaperone TELO2 to develop combinatorial therapies. Its depletion reduces the amounts of ATM and ATR, crucial regulators of the DNA damage response. Therefore, sensitization of the cells and a higher effect of the cytotoxic antitumor agents is observed. This outcome stresses the need to develop TELO2 inhibitors. Indeed, the best results were observed with low doses of 5-Fluorouracil (0.125µM-0.25µM) and Irinotecan (0.125nM and 0.25nM) and middle to low doses of auxin (50µM). TELO2 depletion in both treatments enhanced the effect at about 50% of the same concentration without auxin (Supplementary tables 2.2 and 3.2). With these results, our ultimate objective is to pursuit a translational approach. This strategy would allow us to slightly sensitize our cells to DNA damaging agents and, consequently be able to reduce the therapeutic doses of chemotherapy. This way, non-desirable effects and toxicity would be decreased.

It has been reported that inhibitors of the DNA damage response can enhance the activity of antitumor agents in $p53^{-/-}$ colorectal tumors, and some synergies have been found (Jiang et al., 2009; Ma et al., 2011; Sangster-Guity et al., 2011). However, the DNA damage inhibitors had limited succeed when the tumor was p53 wild type. Enhanced resistance to genotoxic agents was reported when ATM or its downstream target Chk2 were inhibited in $p53^{+/+}$ colorectal cells (Jiang et al., 2009). It has been described that potential effect of ATM inhibitors as chemosensitizing agents is just applicable in $p53^{-/-}$ tumors. Our results contradict this affirmation, because sensitizing effects have been found using an inhibitor of TELO2 in $p53^{+/+}$ colorectal cells. This could be because a large amount of studies had focused their work on inhibiting individually ATM, ATR or their downstream targets; but in this project, we targeted all of them at the

same time, by focusing the upstream regulator TELO2, which could have overcame this inconvenient. To confirm the biological relevance of this data, further research should be done in $p53^{+/+}$ colorectal cells, like the one we used (HCT116 p53 wt). To seek for stronger effects, $p53^{-/-}$ colorectal cells should be analyzed following this strategy and to increase the clinical relevance of the approach. Moreover, resistant cells with upregulation of the DNA damage response pathways should be tested to try to overcome the resistance, as Combes et al. did with oxaliplatin resistance upon ATR inhibition (Combes et al., 2019).

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Bibliography

Abdolahi, H.M., Asiabar, A.S., Azami-Aghdash, S., Pournaghi-Azar, F., and Rezapour, A. (2018). Cost-effectiveness of Colorectal Cancer Screening and Treatment Methods: Mapping of Systematic Reviews. Asia-Pacific journal of oncology nursing *5*, 57-67.

Abraham, R.T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes & development *15*, 2177-2196.

ASCO (2019). American Society of Clinical Oncology. https://wwwcancernet/cancer-types/colorectal-cancer/types-treatment

Bouwman, P., and Jonkers, J. (2012). The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance. Nature reviews Cancer *12*, 587-598.

Brattain, M.G., Fine, W.D., Khaled, F.M., Thompson, J., and Brattain, D.E. (1981). Heterogeneity of malignant cells from a human colonic carcinoma. Cancer research *41*, 1751-1756.

Burnette, W.N. (1981). "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Analytical biochemistry *112*, 195-203.

Cimprich, K.A., and Cortez, D. (2008). ATR: an essential regulator of genome integrity. Nature reviews Molecular cell biology *9*, 616-627.

Combes, E., Andrade, A.F., Tosi, D., Michaud, H.A., Coquel, F., Garambois, V., Desigaud, D., Jarlier, M., Coquelle, A., Pasero, P.*, et al.* (2019). Inhibition of Ataxia-Telangiectasia Mutated and RAD3-Related (ATR) Overcomes Oxaliplatin Resistance and Promotes Antitumor Immunity in Colorectal Cancer. Cancer research *79*, 2933-2946.

Compare GBD (2019). IHME Global Burden of Disease, The Institute for Health Metrics and Evaluation https://vizhubhealthdataorg/gbd-compare/.

Conti, E., and Izaurralde, E. (2005). Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. Current opinion in cell biology *17*, 316-325.

Detilleux, D. (2018). Functional characterization of the TRRAP pseudokinase and its chaperone TTTduring transcriptional regulation in colorectal cancer. Agricultural sciences.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. Nature *435*, 441-445.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494-498.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proceedings of the National Academy of Sciences of the United States of America *89*, 5547-5551.

Guichard, S., Terret, C., Hennebelle, I., Lochon, I., Chevreau, P., Fretigny, E., Selves, J., Chatelut, E., Bugat, R., and Canal, P. (1999). CPT-11 converting carboxylesterase and topoisomerase activities in tumour and normal colon and liver tissues. British journal of cancer *80*, 364-370.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes & development *18*, 1926-1945.

Herceg, Z., and Wang, Z.Q. (2005). Rendez-vous at mitosis: TRRAPed in the chromatin. Cell cycle *4*, 383-387.

Holland, A.J., Fachinetti, D., Han, J.S., and Cleveland, D.W. (2012). Inducible, reversible system for the rapid and complete degradation of proteins in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America *109*, E3350-3357.

Hosoya, N., and Miyagawa, K. (2014). Targeting DNA damage response in cancer therapy. Cancer science *105*, 370-388.

Huang, W.W., Hsieh, K.P., Huang, R.Y., and Yang, Y.H. (2017). Role of cyclooxygenase-2 inhibitors in the survival outcome of colorectal cancer patients: A population-based cohort study. The Kaohsiung journal of medical sciences *33*, 308-314.

Hurley, P.J., and Bunz, F. (2007). ATM and ATR: components of an integrated circuit. Cell cycle *6*, 414-417.

Hurley, P.J., Wilsker, D., and Bunz, F. (2007). Human cancer cells require ATR for cell cycle progression following exposure to ionizing radiation. Oncogene *26*, 2535-2542.

Hurov, K.E., Cotta-Ramusino, C., and Elledge, S.J. (2010). A genetic screen identifies the Triple T complex required for DNA damage signaling and ATM and ATR stability. Genes & development *24*, 1939-1950.

Izumi, N., Yamashita, A., Hirano, H., and Ohno, S. (2012). Heat shock protein 90 regulates phosphatidylinositol 3-kinase-related protein kinase family proteins together with the RUVBL1/2 and Tel2-containing co-factor complex. Cancer science *103*, 50-57.

Jiang, H., Reinhardt, H.C., Bartkova, J., Tommiska, J., Blomqvist, C., Nevanlinna, H., Bartek, J., Yaffe, M.B., and Hemann, M.T. (2009). The combined status of ATM and p53 link tumor development with therapeutic response. Genes & development *23*, 1895-1909.

Kastan, M.B., and Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.

Kepinski, S., and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature *435*, 446-451.

Kuipers, E.J., Grady, W.M., Lieberman, D., Seufferlein, T., Sung, J.J., Boelens, P.G., van de Velde, C.J., and Watanabe, T. (2015). Colorectal cancer. Nature reviews Disease primers *1*, 15065.

Labianca, R., Nordlinger, B., Beretta, G.D., Mosconi, S., Mandala, M., Cervantes, A., Arnold, D., and Group, E.G.W. (2013). Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology : official journal of the European Society for Medical Oncology *24 Suppl 6*, vi64-72.

Lavin, M.F., Khanna, K.K., Beamish, H., Spring, K., Watters, D., and Shiloh, Y. (1995). Relationship of the ataxia-telangiectasia protein ATM to phosphoinositide 3-kinase. Trends in biochemical sciences *20*, 382-383.

Leary, M., Heerboth, S., Lapinska, K., and Sarkar, S. (2018). Sensitization of Drug Resistant Cancer Cells: A Matter of Combination Therapy. Cancers *10*.

Li, H., and Chen, C. (2017). Inhibition of autophagy enhances synergistic effects of Salidroside and anti-tumor agents against colorectal cancer. BMC complementary and alternative medicine *17*, 538.

Longley, D.B., Harkin, D.P., and Johnston, P.G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. Nature reviews Cancer *3*, 330-338.

Ma, C.X., Janetka, J.W., and Piwnica-Worms, H. (2011). Death by releasing the breaks: CHK1 inhibitors as cancer therapeutics. Trends in molecular medicine *17*, 88-96.

Marin, J.J., Briz, O., Monte, M.J., Blazquez, A.G., and Macias, R.I. (2012). Genetic variants in genes involved in mechanisms of chemoresistance to anticancer drugs. Current cancer drug targets *12*, 402-438.

Nakanishi., C., and Toi., M. (2005). Nuclear factor-κB inhibitors as sensitizers to anticancer drugs. Nature Reviews Cancer *5*, 297-309.

Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T., and Kanemaki, M. (2009). An auxin-based degron system for the rapid depletion of proteins in nonplant cells. Nature methods *6*, 917-922.

Orellana, E.A., and Kasinski, A.L. (2016). Sulforhodamine B (SRB) Assay in Cell Culture to Investigate Cell Proliferation. Bio-protocol *6*.

Pires, A.S., Marques, C.R., Encarnacao, J.C., Abrantes, A.M., Marques, I.A., Laranjo, M., Oliveira, R., Casalta-Lopes, J.E., Goncalves, A.C., Sarmento-Ribeiro, A.B*., et al.* (2018). Ascorbic Acid Chemosensitizes Colorectal Cancer Cells and Synergistically Inhibits Tumor Growth. Frontiers in physiology *9*, 911.

Raymond, E., Faivre, S., Chaney, S., Woynarowski, J., and Cvitkovic, E. (2002). Cellular and molecular pharmacology of oxaliplatin. Molecular cancer therapeutics *1*, 227-235.

Restelli, V., Chila, R., Vagni, M., Lupi, M., Tarantelli, C., Spriano, F., Gaudio, E., Bertoni, F., Damia, G., and Carrassa, L. (2019). DNA damage response inhibitor combinations exert synergistic antitumor activity in aggressive B cell lymphomas. Molecular cancer therapeutics.

Sangster-Guity, N., Conrad, B.H., Papadopoulos, N., and Bunz, F. (2011). ATR mediates cisplatin resistance in a p53 genotype-specific manner. Oncogene *30*, 2526-2533.

Sauer, B., and Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proceedings of the National Academy of Sciences of the United States of America *85*, 5166-5170.

Sharma, A., Vatapalli, R., Abdelfatah, E., Wyatt McMahon, K., Kerner, Z., A, A.G., Singh, J., Zahnow, C., S, B.B., Yerram, S*., et al.* (2017). Hypomethylating agents synergize with irinotecan to improve response to chemotherapy in colorectal cancer cells. PloS one *12*, e0176139.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nature reviews Cancer *3*, 155-168.

Takai, H., Wang, R.C., Takai, K.K., Yang, H., and de Lange, T. (2007). Tel2 regulates the stability of PI3K-related protein kinases. Cell *131*, 1248-1259.

Takai, H., Xie, Y., de Lange, T., and Pavletich, N.P. (2010). Tel2 structure and function in the Hsp90-dependent maturation of mTOR and ATR complexes. Genes & development *24*, 2019-2030.

Tan, X., Calderon-Villalobos, L.I., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M., and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature *446*, 640-645.

Supplementary data

		Auxin (µM)		
		0	5	500
Oxaliplatin (µM)	0	100	84	54
	0.075	114	88	51
	0.15	105	86	50
	0.3	74	59	39
	0.6	41	34	28
	1.2	25	24	22
	2.4	21	22	22

Table 1.1 Oxaliplatin proliferation assay (%). For all the tables, the statistically significative results (compared to the chemotherapy alone) are highlighted in a dark color.

		Auxin (µM)		
		0	5	500
Oxaliplatin (µM)	0	-	16	46
	0.075	-	23	55
	0.15	-	18	52
	0.3	-	20	47
	0.6	-	17	32
	1.2	-	4	12
	2.4	-	-5	-5

Table 1.2 Inhibition (% from the one produced by the oxaliplatin alone) when auxin is added to each concentration of oxaliplatin. Combined effect.

		Auxin (µM)			
		0	5	25	50
Irinotecan (nM)	0	100	108	80	69
	0.125	103	87	73	39
	0.25	90	61	57	51
	0.5	69	56	48	38
	1	54	39	30	26
	2	38	34	25	18
	4	35	32	25	19

Table 2.1. Irinotecan proliferation assay (%).

		Auxin (µM)			
		0	5	25	50
Irinotecan (nM)	0	-	0	20	31
	0.125	-	16	29	62
	0.25	-	32	37	43
	0.5	-	19	30	45
	1	-	28	44	52
	2	-	11	34	53
	4	-	9	29	46

Table 2.2 Inhibition (% from the one produced by the irinotecan alone) when auxin is added to each concentration of irinotecan. Combined effect.

		Auxin (µM)			
		0	5	25	50
5Fluorouracil (µM)	0	100	74	71	63
	0.125	116	83	71	45
	0.25	99	86	80	54
	0.5	102	84	70	58
	1	90	74	57	50
	2	53	42	38	33
	4	27	24	23	17

Table 3.1 5-Fluorouracil proliferation assay (%).

		Auxin (µM)			
		0	5	25	50
5Fluorouracil (µM)	0	-	26	29	37
	0.125	-	28	39	61
	0.25	-	13	19	45
	0.5	-	18	31	43
	1	-	18	37	44
	2	-	21	28	38
	4	-	11	15	37

Table 3.2 Inhibition (% from the one produced by the 5-Fluorouracil alone) when auxin is added to each concentration of 5-Fluorouracil. Combined effect.