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1 **Satraplatin (JM-216) mediates G2/M cell cycle arrest and potentiates apoptosis via**
2 **multiple death pathways in colorectal cancer cells thus overcoming platinum chemo-**
3 **resistance**

4

5 **Running title:** Satraplatin potentiates apoptosis in CRC cells

6

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9

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25 **Abstract**

26 **PURPOSES:** Satraplatin acts as a potent inhibitor of proliferation in castration-resistant
27 prostate cancer, yet the basic and molecular pharmacological mechanisms are still
28 unknown in all types of cancer including colorectal cancer (CRC). In an effort to explain
29 the mechanism of tumour sensitivity to satraplatin, the cytotoxic effects in a panel of
30 CRC cell lines was examined with regards to their p53 genotype in comparison to
31 oxaliplatin.

32 **METHODS:** CRC cell lines were chosen to ascertain the mechanism of satraplatin-
33 enhanced cytotoxicity. Cells were incubated with oxaliplatin and satraplatin for 24-72 h,
34 followed by the assessment of cell chemosensitivity with MTS. Western blot analysis
35 was used to detect the expressions of p53 related molecules Flow cytometry was used to
36 monitor cell cycle perturbation while qRT-PCR to detect mRNA and miRNAs activities.

37 **RESULTS:** Satraplatin treatment resulted an elevated increase in cell death *in vitro*
38 compared to oxaliplatin preceded by an acute arrest at G2/M phase, along with cyclin B1
39 and p21^{waf/cip1} up-regulation. It also exhibited 4 fold higher cellular platinum
40 accumulations compared to oxaliplatin. Satraplatin treatment induces p53 related genes
41 and its direct microRNA target of miR-34a independently. Thus, it potentiates apoptosis
42 via multiple death pathways including the caspase 8 cleavages and Fas protein
43 expression. The data suggests that the loss of p53 can increase oxaliplatin resistance but
44 not satraplatin resistance.

45 **CONCLUSION:** Further molecular biology studies are needed to identify the activity of
46 satraplatin in platinum resistant cancer models and to determine whether this orally

47 administered platinum analogue has synergistic effects in combination with other
48 chemotherapy agents.

49

50 **Key words:** Satraplatin. CRC. Apoptosis. Platinum. p53. Oxaliplatin. miR-34a

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52 **List of Abbreviations:** CRC: Colorectal cancer; DISC: death-inducing signalling
53 complex; miR: MicroRNA; Satraplatin: JM-216; IC: Inhibitory concentration; CRPC:
54 Castration-Resistant Prostate Cancer; UTR: Untranslated Region; platinum: p

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70 **Introduction**

71 The median progression-free survival of advanced colorectal cancer (CRC) patients has
72 increased since the introduction of oxaliplatin in combination with 5-Fluorouracil-
73 leucovorin in systemic chemotherapy treatment. However, as a single agent, oxaliplatin
74 has been less effective against transforming cancerous cells. Despite its proven activity in
75 combination treatments, the acquisition of drug resistance remains a major obstacle in
76 CRC management, ultimately leading to patients death [1].

77 Thus, many attempts have been made to scrutinize the mechanisms of chemo-resistance.
78 The mechanisms include increased DNA repair, impaired DNA adducts formation, over-
79 expression of copper transporters, enhanced drug detoxification and increased tolerance
80 for DNA damage [2-5].

81 The p53 gene takes part in cellular responses to DNA damage by regulating cell cycle
82 progression, cell death and DNA repair [6]. It is mutated in more than 50% of colorectal
83 tumours [7]. Furthermore, the functional prominence of this genome guardian was
84 correlated to cellular sensitivity and/or resistance to platinum agents. Although a clear
85 interpretation is lacking, it has been suggested that the decreased sensitivity to platinum
86 agents is a consequence of reduced or unfound expression of the p53 protein in these
87 tumour models [8].

88 One major process leading to chemotherapy resistance is the ability of cancerous cells to
89 evade apoptosis [9]. The development of drugs resistant cells resembles the stages of
90 tumour progression where molecular changes cause the evolution of cancerous cells that
91 proliferate due to their ability to escape programmed cell death [10,11].

92 Thus attempts have been made to synthesize a platinum agent with more efficient binding
93 sites in combination with other molecules to overcome the problems that arise with
94 current chemotherapy regimens, including oxaliplatin cross resistance. For instance,
95 satraplatin has been studied in a phase III clinical trial for Castration-Resistant Prostate
96 Cancer (CRPC) [12].

97 Satraplatin (also known as JM-216) or bis (acetato) ammine dichloro (cyclohexylamine)
98 platinum (IV) is the first platinum analogue available for oral administration. Satraplatin
99 exhibits a higher cytotoxic activity *in vitro* when compared to other platinum compounds
100 and also exhibits activity against some cisplatin-resistant human tumour models [13,14].

101 As earlier developed platinum agents typically demonstrate significant *de novo* resistance
102 and/or acquired resistance in tumour cells, satraplatin's uniqueness dwells on its putative
103 lack of cross-resistance [15-17]. Based on preliminary cell culture investigations on
104 various tumour types, satraplatin exhibited the ability to create DNA adducts that are
105 more resistant to recognition by DNA nucleotide excision repair enzymes and DNA
106 mismatch repair proteins [18-20]. Moreover, satraplatin-induced adducts are more stable
107 and do not bind to high mobility group 1 proteins, which recognize DNA damage.
108 Satraplatin also inhibits trans-lesion replication by certain DNA polymerases [21]. These
109 differences may provide a mechanism to overcome resistance to treatment observed in
110 other platinum compounds.

111
112 In an effort to investigate the underlying mechanism associated with the cytotoxicity of
113 satraplatin, we examined the efficacy of satraplatin compared to oxaliplatin using
114 colorectal cancer cell lines with regards to their p53 phenotype. Moreover to further
115 demonstrate the mechanisms of satraplatin and its effect to overcome the chemo

116 resistance of oxaliplatin in the absence of p53 gene, we further our analysis on three
117 representative CRC cells exclusively one with p53 wildtype (HCT116), one for mutant
118 p53 (HT29) and the final one for p53 knockout (HCT116). We further analyzed the cycle
119 perturbation and basic molecular mechanisms of apoptosis induced by satraplatin in these
120 cells.

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138 **Materials and Methods**

139 **Drugs, chemicals and reagents**

140 Oxaliplatin was purchased from Sigma (09512-5MG) and satraplatin was kindly gifted
141 by Agennix AG (formerly GPC Biotech AG), Fraunhoferstr, Germany. Stock solutions of
142 satraplatin and oxaliplatin were prepared in 0.09% saline solution stored at -20°C .
143 Antibodies were purchased from appropriate companies as indicated: anti-p53 and anti-
144 Bid (BD Pharmingen), anti- β -actin (Sigma), anti-Bax (Millipore), anti-BCL2 (Sigma),
145 anti-p21^{Cip1/WAF1}, anti-c-ABL, anti-Fas and anti-Cyclin-B1 (Santa Cruz), and anti-
146 Caspase 8 (Alexis Biochemical). Secondary antibody of anti rabbit and mouse were
147 purchased from DakoCytomation, Denmark A/S. ECL detection reagents from Perkin-
148 Elmer. miRNA analysis primer sets from Applied Biosystems.

149

150 **Cell culture**

151 HT29 were kindly provided by Dr. Isabella Faroni, University of Rome 'Tor Vergata'
152 and HCT116, HCT116 p53^{-/-} and HCT15 cells were obtained from Dr. Soddu, Regina
153 Elena Cancer Institute, Italy. WiDr and LoVo cells were given by Dr. Rossana Supino
154 from Istituto Nazionale Tumori, Milan, Italy and HCT116 p21^{-/-} were obtained from
155 Prof. Bert Vogelstein, from Ludwig Centre at John Hopkins, USA. All the cell lines were
156 tested for mycoplasma infection and were grown in monolayer cultures in DMEM
157 (HCT116, HCT116 p53^{-/-}, HT29, HCT116 p21^{-/-}), DMEM: F12 (WiDr), RPMI 1640
158 (HCT15) and Ham's Nutrients mix F12 (LoVo) supplemented with 10-20% FBS and 1%
159 penicillin streptomycin according to UKCCCR guidelines [22]. No antibiotics were

160 added to the medium prior to the treatment. The cells were trypsinized and passed once a
161 week and all the experiments were conducted at early passages.

162

163 **Cytotoxicity evaluation assay**

164 The concentration of oxaliplatin and satraplatin resulting in 50% inhibition of control
165 growth (IC₅₀) was calculated with an MTS assay (Promega). Three thousand cells per
166 well were seeded in 96-well plates and 24h later the cells were exposed to 200 µl of 0-
167 250 µM oxaliplatin and satraplatin. After 72 h of incubation, 20 µl of MTS solution was
168 added and the IC₅₀ concentrations were calculated using CalcuSyn Version 2.0. Since this
169 analysis is concerned with a comparison between oxaliplatin and satraplatin, the analysis
170 was done with drug concentrations controlled to be below IC₅₀ value (10 µM) for
171 practical considerations. At this concentration for both drugs, the percentage cell death
172 lies between 20%-40% for all cell lines tested.

173

174 **Intracellular platinum quantification**

175 Intracellular platinum (Pt) levels were measured following 2h incubation with 6.25 µM,
176 12.5 µM, 25 µM, 50 µM and 100 µM of satraplatin or oxaliplatin. Approximately 1x10⁶
177 cells were washed twice with 5 ml of cold phosphate-buffered saline (PBS) and the
178 monolayer was scraped into 2 ml PBS. Cellular Pt content was determined by flameless
179 atomic absorption spectroscopy (FAAS) using a Perkin-Elmer AAnalyst 800
180 (Beaconsfield, Bucks, UK). Cell aliquots were dissolved in 0.2% nitric acid and
181 automatically injected into the graphite furnace. The concentration of Pt in ng/mL was
182 measured by comparing Pt standards ranging from 5 to 500 ng/ml. Under these

183 conditions the limit of detection was between 5 and 10 ng Pt/ml. The cellular platinum
184 contents were then expressed as nmol Pt/ml normalized to 1×10^6 cells.

185

186 **Cell cycle analysis**

187 For cell cycle analysis 5×10^4 cells of HCT116, HCT116 p53 null and HT29 were seeded
188 in six well plates in triplicate and allowed to attach for 24h. For time response studies,
189 cells were treated with 10 μ M satraplatin and oxaliplatin for 48h and 72h. Both attached
190 and floating cells were harvested, washed twice with 1ml of PBS and re-suspended in
191 PBS containing 10 mg/ml of RNase and incubated for 1 hour. Later, 10-mg/ml
192 propidium iodide (Sigma p4170-100 mg) was added prior to the analysis. Cells were
193 analysed for DNA content using a FACScan flow cytometry (BD Biosciences, Mountain
194 View, CA). The proportion of cells in G0/G1, S phase and G2/M were quantified using
195 CellQuestTM software (BD Biosciences, San Jose, CA). The software directly calculated
196 the percentage of cells in each phase.

197

198 **Evaluation of apoptosis**

199 For apoptosis, assays cells were plated in 6-well plates and treated with 10 μ M of
200 satraplatin or oxaliplatin. Isolated nuclei were then analysed after 24, 48 and 72h of
201 treatment, using a FACScan flow cytometry (BD Biosciences, Mountain View, CA) as
202 previously described [23]. Briefly, detectors and amplifier gains for forward and
203 orthogonal scatter were adequately selected in order to simultaneously detect nuclei from
204 viable, apoptotic and necrotic cells. Events were gated on forward versus orthogonal
205 scatter in such a way that degraded DNA from cell debris or from doublets was excluded

206 and nuclei from viable, apoptotic and necrotic cells were assayed. Data acquisition and
207 analysis was performed using CellQuest™ software (BD Biosciences, San Jose, CA) on a
208 minimum of 5,000 events for each samples. The second method for evaluation of
209 apoptosis consisted in double staining of the cells with fluorescent annexin V and with PI
210 solution for the FACS data confirmation. To this purpose, the “Annexin V-FITC
211 Apoptosis Detection Kit” (BD-Bioscience Pharmingen, San Diego, CA, USA) was used
212 according to manufacturer instructions. Briefly, 5×10^5 cells were incubated for 15
213 minutes with annexin-V- fluorescein isothiocyanate (FITC) and then washed in annexin
214 buffer. Cells were then stained with PI solution and analysed immediately after staining
215 by flow cytometry using a FACScan flow cytometry and Cell Quest™ software. This
216 method is widely accepted to distinguish between early apoptosis and necrosis.

217

218 **Quantitative PCR**

219 **RNA isolation, cDNA synthesis and RT-PCR**

220 Total RNA was isolated with TRIzol reagent (Invitrogen, CA). The integrity of total
221 RNA was determined by 1% agarose gel electrophoresis. cDNA synthesis was carried out
222 with the Superscript III cDNA synthesis kit (Invitrogen, CA) using 1 µg of total RNA as
223 the template and specific reverse primers (Table 1) according to manufacturer
224 instructions. The PCR amplification was carried out in a final volume of 25µl containing
225 12.5 µl of SYBR green (2X), 0.5 µl both primers (10 µM), 1 µl of cDNA. Cycling
226 conditions included preheating at 95°C for 10 minutes and then 40 cycles of: 95°C for 30
227 seconds, 60°C for 30 seconds and 72°C for 45 minute, followed by standard dissociation

228 curve. Analysis below 35 cycles was considered for the $2^{-\Delta\Delta Cq}$ calculation according
229 to the MIQE guidelines [24].

230

231 **miRNA reverse transcription and qRT-PCR analysis**

232 Enriched microRNAs were extracted with mirVana™ Isolation Kit (Ambion Inc. TX).
233 Based on TARGET scan and PIC target, miRNAs that target our molecules were
234 selected. Based on the top predicted target, we identified 14 miRNAs involved with our
235 selected gene group focusing on cell cycle analysis, cell death and DNA repair system.
236 Applied Biosystems qRT-PCR primer sets for miRNA specific reverse transcription
237 including hsa-miR-150 (\rightarrow p53/ERCC1), hsa-miR-152 (\rightarrow MLH1/GADD45), hsa-miR-
238 128a (\rightarrow MLH1/BAX), hsa-miR-34a (\rightarrow BCL2), hsa-214 (\rightarrow BAX), hsa-miR-218
239 (\rightarrow BRCA1), hsa-miR-21 (\rightarrow MSH2), hsa-miR-106a (\rightarrow p21), hsa-miR-20, hsa-miR-148a
240 (\rightarrow GADD45), hsa-miR-370 (\rightarrow CLU), hsa-miR-326 (\rightarrow TP73), hsa-miR- 128b, hsa-miR-
241 17-3p and endogenous control RNU19 and RNU6B were utilized according to the
242 manufacturer's protocol. Briefly, the reaction master mix containing 5 \times RT Buffer, 5 \times
243 RT Primer, Array-Script™ Enzyme Mix and nuclease-free water was mixed with 50 ng
244 of input microRNA. The mixture was incubated for 30 min at 37°C, and then 10 min at
245 95°C. qPCR was carried out using the Stratagene Real-Time PCR System and Applied
246 Biosystems qRT-PCR miRNA Detection Kit (Ambion Inc. TX). The PCR master mix
247 containing Applied Biosystems 2 \times PCR Buffer (with FAM labeled Taqman probe), 1 μ L
248 FAM labeled specific primers and cDNA from process miRNA. RT products was
249 processed as follows: 95°C for 10 min, and then 95°C for 15 sec and 60°C for 30 sec for
250 up to 40 cycles (n = 2). Gene expression was calculated via a $2^{-\Delta\Delta Cq}$ method.

251 **Western blotting**

252 All cells were seeded in 75 cm² flasks and treated with 10 μM oxaliplatin or satraplatin
253 for 24, 48 or 72 h, then rinsed with PBS twice, harvested and re-suspended in 150 ml of
254 Chaps 0.1% lysis buffer (1 M Tris-Cl pH 7.5, 5 M NaCl, 10% CHAPS, ddH₂O). The cells
255 suspension was vortexed and kept on ice for 30 min before cell debris was pelleted and
256 the supernatant transferred to a new tube. SDS–polyacrylamide gel electrophoresis
257 sample loading buffer was added to 20 μg aliquots, heated at 95 °C for 5 minute and then
258 loaded on 10% Tris-HCl gels (BioRad, Hercules, CA, USA). Proteins were transferred to
259 a nitrocellulase membrane (Amersham, Piscataway, NJ, USA) and blocked with 1% non-
260 fat milk and 1% BSA for at least 2 h. Membranes were then probed at 4°C with the
261 appropriate primary antibody in 0.1% T-TBS overnight with the following antibodies:
262 anti-p53 (1/500), anti-p21^{Cip1/WAF1} (1/200) and anti-β-actin (1/1000) anti-Bax (1/500),
263 anti-BCL2 (1/200), anti-c-ABL (1/200), anti-Fas (1/500), anti-Cyclin-B1 (1/200), anti-
264 Caspase 8 (1/500) and anti-Bid (1/500). Membranes were washed three times with
265 washing buffer (PBS with 0.1% Tween-20 for 10 minute interval). Then the filter was
266 probed with the appropriate peroxidase-conjugate secondary antibody for 1 h (from
267 DakoCytomation, Denmark A/S). The secondary antibody was washed three times with
268 washing buffer and the signal was developed using ECL Plus Western Blotting Detection
269 Method (Perkin-Elmer). Detection was carried out using a Molecular Imager ChemiDoc
270 XRS System, Bio-Rad laboratories (UK) Ltd. Protein levels were standardized using the
271 signal from the β-actin probe.

272

273 **Statistical analysis**

274 The gene expression ΔCq values of mRNA and miRNAs from each sample were
275 calculated by normalizing with internal control of ACTB and B₂M (for mRNA) and
276 RNU19 and RNU6B (for microRNAs) and relative quantitation values were calculated
277 according to the $2^{-\Delta\Delta Cq}$ method. The statistical studies were performed using a
278 statistical software system (SPSS, version 12.0 for windows, Chicago, IL, USA). The
279 statistically significant differences in expression level between control and treated
280 samples for each target were calculated using a paired Wilcoxon test. Comparison of
281 mean values of proliferation and apoptosis levels in response to satraplatin and
282 oxaliplatin treatment was carried out using the Bonferroni post hoc multiple comparisons
283 ANOVA test. * $p \leq 0.05$ value was considered significant while ** $p \leq 0.001$ value was
284 considered highly significant.

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296 **Results**

297 **Cytotoxic effect of satraplatin in the panel of colorectal cancer cells**

298 In order to evaluate the effective drug concentrations, a panel of six CRC cell lines with
299 different p53 genotypes were treated with various concentrations (ranging from 0-250
300 μM) of oxaliplatin or satraplatin for 72 h, before MTS assay. The IC_{50} value of each cell
301 line is shown in Table 2. The IC_{50} value ranged from 13 to 27 μM for satraplatin, and
302 from 19 to 96 μM for oxaliplatin. All p values between these two treatment groups
303 showed a significant difference in IC_{50} value except in HT29 cells where there was no
304 significant difference as shown in table 2. The general trend of MTS assays for HCT116,
305 HCT116 p53^{-/-} and HT29 cells are shown in Fig 1.1 (A-C). In all three cell lines,
306 satraplatin showed a higher percentage of cell inhibition based on MTS assay, compared
307 to oxaliplatin. For example, there was a statistically significant difference between both
308 drugs at doses from 10-50 μM in HCT116, p=0.001. Fig 1.2 (a-f) shows the cell
309 morphological changes at 10 μM of both drugs in two of the cell lines tested as described
310 in the figure legend.

311 To investigate the consequences of satraplatin treatment on cell cycle and apoptosis, we
312 used three different CRC cell lines with different p53 status; HCT 116 (wild type), HCT
313 116 (p53^{-/-}) and HT29 (mutant).

314

315 **Satraplatin treatment results in higher accumulation of platinum in CRC cells,**
316 **compared to oxaliplatin**

317 To investigate the cellular contents of platinum (Pt) in our model, we assessed different
318 concentrations of drug (0-100 μM) based on FAAS. Following the exposure of satraplatin

319 and oxaliplatin, the accumulation of satraplatin was higher in the cells tested compared to
320 oxaliplatin (Fig 2). For instance, when the cells were treated with 100 μ M of both drugs
321 accumulation of satraplatin was 387.4 ng/ml vs. 29.13 ng/ml for oxaliplatin in HCT116
322 cells, 316 ng/ml for satraplatin vs. 18.96 ng/ml for oxaliplatin in HCT16 p53^{-/-} cells.
323 Based in this graph, we found that the platinum incorporation into cells at 10 μ M, which
324 was the evaluation dosage for the subsequent experiments, was approximately 4 fold
325 higher for satraplatin compared to oxaliplatin.

326

327 **Treatment of CRC cells with satraplatin and oxaliplatin alters cell cycle profile**

328 To analyse the effect of satraplatin and oxaliplatin treatment on cell cycle, we treated
329 CRC cells with 10 μ M of each drug for 48 or 72 h. The cell cycle analysis of 3 CRC cell
330 lines following treatment with oxaliplatin and satraplatin are shown in table 3.

331 Satraplatin noticeably increased the relative number of cells in G2-M phase (57.08% vs.
332 14.42% for untreated cells at 72h, **p<0.001) in HCT116 cells while a minor alteration
333 was observed with oxaliplatin treatment. We also observed a similar alteration of cell
334 cycle in p53^{-/-} HCT116 cells following satraplatin treatment (50.85 % vs. 28.52 % for
335 untreated control at 72h, **p<0.001), while a similar pattern of cell perturbation was
336 observed with oxaliplatin treatment at 72 h where the cells accumulated in S phase. On
337 the other hand, in HT29 cells both drug treatments induced S phase cell cycle arrest,
338 Table 3.

339

340 **Satraplatin induces apoptosis in CRC cells**

341 To assess the effect of satraplatin treatment on apoptosis the three CRC cell lines were
342 treated with 10 μ M satraplatin and oxaliplatin for 24, 48 and 72 h and apoptosis was
343 assessed by flow cytometry. The results shown are the mean values \pm standard error (SE)
344 obtained from three independent experiments.

345 In HCT116 cells no significant increase in apoptosis was detected following treatment
346 with satraplatin for 24h, compared to untreated and oxaliplatin treated cells (data not
347 shown). However following treatment for 48h and 72h, a substantial increase in sub-G1
348 population was detected as compared to untreated cells (48h: 2.8 fold changed vs.
349 14.48%, untreated control; 72 h: 10.9 fold changed with satraplatin vs. 7.7 fold changed
350 with oxaliplatin to untreated control, $p < 0.001$ (Fig 3). A similar apoptotic profile was
351 also observed in HT29 and HCT116 $p53^{-/-}$ cells, although the difference between
352 satraplatin and oxaliplatin was also profound in HT29 cells following 48h and 72h
353 treatment. There were significant differences of apoptosis profile between these 3 cells
354 lines tested as shown in fig 3.

355

356 We confirmed these results by counting Annexin-V-PI (+) cells following satraplatin
357 treatment. This method also allowed us to detect early apoptotic (annexin-V+/PI-) and
358 late apoptotic/necrotic (annexin-V +/PI+) cells [23].

359 In HCT116 cells, 24 h of treatment with 10 μ M satraplatin, resulted in 0.20% annexin-
360 V+PI+ cells and 22.67 % annexin-V+/PI- cells vs. 0.14% annexin-V+PI+ cells and 7.55%
361 annexin-V+/PI- cells with oxaliplatin treatment, while in untreated controls 0.23%
362 annexin-V+PI+ and 9.32 % annexin-V+/PI- cells were observed. Similar results were

363 obtained in HT29 mutant cells and in p53^{-/-} cells as showed in Fig 4, which demonstrates
364 the efficacy of satraplatin on cell death induction as compared to oxaliplatin.
365 These data also indicate that satraplatin induces cell death in cells mainly, if not
366 exclusively, by apoptosis, with no or very little contribution by necrosis.

367

368 To study the biological effect of satraplatin on CRC cells cytotoxicity, cell cycle
369 distribution and apoptosis, first we analysed the p53 protein expression since our models
370 differ in p53 status. We observed a significant increase in p53 protein expression after 48
371 h of treatment of both drugs in HCT116 cells whereas no p53 protein accumulation was
372 observed in HT29 cell line as shown in Fig 5A. We observed a down regulation of p53
373 protein expression with both treatments in HT29 cell line. No p53 expression has been
374 shown in HCT116 p53^{-/-} cell line as expected. To clarify p53 expression and the action of
375 its downstream genes dependent and independently based on these drugs treatment, first
376 we analysed gene expression patterns at mRNA levels.

377

378 **Satraplatin initiates p53 signalling pathway independently**

379 To further investigate the molecular cascade of events following the exposure to both
380 drugs, we selected appropriate genes involved in cell cycle regulation, DNA damage
381 response and transcription factor control based on the p53 signalling pathway. Using this
382 small array with a 15-gene simultaneous analysis after 48 h of treatment, we detected
383 significant changes in mRNA modulation. We detected a minor alteration in p53 mRNA
384 levels in HCT1116 (satraplatin: 1.6, p= 0.016 fold vs. oxaliplatin: 1.53 fold, p=0.007) but
385 not in HT 29 (satraplatin: -1.05 fold vs. Oxaliplatin: -1.07 fold). We also detected the

386 changes in expression levels of ERCC1, MSH2, BRCA1, GADD45, CLU and Bax genes
387 (Table 4.1). BCL2 expression was much more down regulated in HCT116 cells with 2
388 fold lower than oxaliplatin treatment whereas 12 fold lower in p53^{-/-} cells. Table 4.1
389 shows the fold-changed expression level in the three cells tested. Furthermore, we noticed
390 an up-regulation in Bax gene expression, further suggesting the apoptosis induction in
391 these cells by p53-dependent and independent manner.

392

393 **Effect on the levels of cell cycle related protein expression**

394 To elucidate the role of cell cycle regulating proteins, we investigated the expression of
395 cyclin B1 involved in G2/M cell cycle arrest. We found a marked increase in cyclin B1 in
396 HCT116 and p53 null cells exposed to 48 h satraplatin. A very low expression was
397 detected by oxaliplatin in HCT116 wild type and null p53 lines, whereas we could detect
398 the same expression for HT29 by both drugs (Fig 5B). We also detected a marked
399 increase in p21 expression at 48 h in HCT116 and the expression is much higher with
400 satraplatin treatment than oxaliplatin (Fig 5C). To confirm the G0/G1 arrest by
401 oxaliplatin, we looked at the expression of c-ABL gene. The expression is much higher
402 after oxaliplatin than after satraplatin treatment in HCT116 and null p53. This might
403 suggest that the G0/G1 block was activated upon the treatment with oxaliplatin, whereas
404 very low expression was detected after satraplatin treatment (Fig 5C).

405

406 **Effect on the levels of cell death pathway related protein expression**

407 Bax relocation to the mitochondrial, accumulation of cytochrome c in the cytoplasm,
408 caspase 8 activation and Bcl2 down-regulation are all events consisted with intrinsic

409 pathway of apoptosis activation. To elucidate the mitochondrial associated apoptosis
410 molecules, Bcl2 and Bax protein expression was noticed in our cell models. Bcl2
411 expression in both HCT116 and p53 null cells was much more reduced after 48 h with
412 satraplatin than oxaliplatin treatment. No Bcl2 protein expression was detected in HT29
413 with the same result as gene expression. On the other hand, the expression of Bcl2 on
414 oxaliplatin treatment was significantly reduced but a greater reduction was observed with
415 satraplatin treatment (Fig 6A, D). This further suggests that satraplatin induced apoptosis
416 might be by p53-dependent as well as an -independent manner. We also detected Bax
417 protein expression as well as an activation of truncated Bid in all the cells tested. Fas
418 protein expression was detected by both drug treatments mainly in HCT116 cells
419 suggesting that these cells might also undergo Fas-induced apoptosis (Fig 6A). To further
420 illustrate the activation of caspase family, we choose pro-caspase 8 in our analysis. Pro-
421 caspase 8 cleavage was observed with satraplatin treatment whereas only a very low
422 signal with oxaliplatin treatment (Fig 6 B).

423

424 **Differential expression of miR-34a exhibits apoptosis signals in CRC cells**

425 We conducted a differential miRNA expression analysis after satraplatin and oxaliplatin
426 based treatments. The expression of miR-34a which target molecule is Bcl2 is higher
427 after satraplatin than oxaliplatin treatment in HCT116 cells (2.65- vs. 1.89-fold). While a
428 down-regulation of miR-34a levels was observed in HT29 cells for both drug treatments,
429 in p53 null cells, we observed a 1.69-fold up-regulation with satraplatin, while a down-
430 regulation was observed with oxaliplatin treatment (Fig 6C). For both drugs, we also
431 detected a down-regulation of hsa-miR-214 that targets the UTR of Bax gene. HCT116

432 and HT29 cells also exhibited a down-regulation of hsa-miR-214 but very low expression
433 was observed in HCT116 p53 null cells. The differential expression of other miRNAs
434 analysed was pronounced in table 4.2

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455 **Discussion**

456 Colorectal cancer demonstrates elevated resistance to chemotherapy agents compared
457 with other human malignancies [25]. However, in a combination of 5-fluorouracil
458 leucovorin-oxaliplatin patients have gradually achieved improvement in clinical
459 outcomes along with increased median progression-free survival [26]. Despite this
460 improvement most platinum compounds eventually suffer from *de nova* resistance or
461 acquired resistance to the treatment over the time. In this study, we showed the effects of
462 oxaliplatin versus an orally administered platinum drug, satraplatin in CRC cell lines
463 which differ in p53 status on fundamental cellular pharmacological levels.

464 As documented before for prostate and other cancers [27, 28], satraplatin exhibited
465 comparable anti-proliferation effects on cell growth. Demonstrations of anti-proliferation
466 effect on our clonogenic model further support the action of satraplatin in a p53-
467 dependent and -independent manner.

468 There was a significant discrepancy in the uptake of platinum into the cells across the
469 three cell lines investigated. When we examined the contents of 10 μ M of both drugs, the
470 intracellular Pt levels were four fold higher with satraplatin. The cells displayed a greater
471 ability to tolerate higher levels of Pt with satraplatin compared to oxaliplatin. This may
472 be due to the greater lipophilicity of satraplatin enhancing its intracellular accumulation.

473 Flow cytometry analysis revealed that a slowdown in S phase transit was a prominent cell
474 cycle effect of satraplatin in these cells. This was accompanied by a marked accumulation
475 of cells in G2 phase in HCT116 and p53 null cells after 72 h. However, in HT29 cells, the
476 majority of the cells were still in S phase with no evidence of build up of cells in G2
477 phase at this point. Similar cell cycle perturbations were observed with cisplatin [29] and

478 other platinum agents [30,31]. Taken together, these data indicate that the accumulation
479 of cells in S phase is a general cell cycle effect of platinum drugs and depending on the
480 nature of the cell type as well as based on dose dependent which showed to be associated
481 with G2 cell cycle arrest [30-32]. In the p53 wild type and p53 null cells, accumulation of
482 cells in G2 coincided with the point at which significant apoptosis was occurring at 48
483 and 72 hours. It has been suggested that G2 arrest facilitates repair of DNA damage prior
484 to mitosis and depending on the success of repair or extent of DNA damage, cells
485 emerging from G2 either begin to cell cycle normally or engage in apoptosis [33,34]. Our
486 data suggests that the latter may be occurring in the HT29 cells, despite the repair of Pt
487 lesions induced by satraplatin.

488 Relatively more apoptotic cells were measured after satraplatin than oxaliplatin treatment
489 suggesting that satraplatin is a stronger inducer of apoptosis. To confirm our apoptosis
490 data measured by hypodiploid nuclei assessed by PI staining, we evaluated the
491 percentage of annexin and PI which the values were quite similar. When we correlated
492 our apoptosis data with the p53 gene status, we observed that even if HT29 cells that
493 have reduced p53 protein expression, satraplatin still induced apoptosis. This suggests
494 that the differences of p53 status in the cells play a role in the cell cycle arrest and
495 apoptosis inductions and adds further weight to the evidence that this process may be
496 p53-dependent as well as p53-independent.

497 The cell cycle determination and their validation at the protein level suggested that two
498 different mechanisms are responsible for oxaliplatin-induced G1 and satraplatin-induced
499 G2 transition control. Satraplatin induced an elevated protein expression of cyclin B1
500 after 48 h compared to oxaliplatin. It is now widely accepted that the G2-M transition is

501 partly governed by the CDK1-cyclin B complex [35,36]. Another fact is that p21^{waf1/cip1}
502 exerts a key role in controlling the G2 arrest by inhibiting the CDK1-cyclin B complex.
503 Protein analysis showed that p21^{waf1/cip1} expression was up regulated under both drug
504 treatments. Based on this data we assumed that the p21^{waf1/cip1} pathway may be involved
505 in early events of the satraplatin-induced G2 block and vice versa. Hata *et al.* (2005) has
506 also suggested that p21^{waf1/cip1} might play a role in exerting the activity of oxaliplatin in a
507 p53-dependent and -independent manner [37].

508 In the present study, we also found that HCT116 p53 null cells did not show an induction
509 of p21^{waf1/cip1} expression as measured in p53 wild type cells, suggesting that the lack of
510 a functional p53 protein could lead to reduced p21 protein levels in response to
511 oxaliplatin and satraplatin exposure. To clarify the role of p21^{waf1/cip1} activation by mean
512 of both drug treatments on elevated cytotoxicity, HCT116 p21^{waf1/cip1} null cells were also
513 tested. Satraplatin again exhibited a higher anti-proliferation effect on oxaliplatin
514 compared to oxaliplatin. Indeed the IC₅₀ value of satraplatin was 6 μM vs. 37.5 μM for
515 oxaliplatin (data not shown). This further suggests that the cells after satraplatin treatment
516 endure apoptosis via a p21^{waf1/cip1}-independent pathway.

517 To better understand the apoptosis cascade, we evaluated cell death associated protein
518 expression. It well known that p53 is mutated in more than 50% in colorectal tumors, and
519 has been shown to have a role in modulating tumour sensitivity to a wide number of
520 chemotherapeutic agents [7]. Here we show that satraplatin/oxaliplatin-induced apoptosis
521 was associated with an up-regulation of p53 protein levels, detected at 48 h of treatment.
522 Using HCT116 p53 null cells we have shown that the targeted inactivation of p53
523 resulted in a 3-fold increase in the IC₅₀ in oxaliplatin- but not in satraplatin-treated cells.

524 Based on the p53 null model, we demonstrated that loss of p53 may acquire resistance
525 towards oxaliplatin but not towards satraplatin treatment. It was already published that
526 cell types having a mutant or inactive p53 and/or Bax gene(s) show increased resistance
527 to oxaliplatin treatment [7,8].

528 Furthermore, modulations of genes involved in the apoptosis response, such as Bax and
529 Fas, are part of each platinum drug mode of action signature, indicating a common cell
530 death mechanism [36]. We have demonstrated a down regulation of Bcl2 and an up
531 regulation of Bax and Bid after exposure of both drugs in a subset of cell lines. These
532 results demonstrate an important functional intrinsic pathway in the apoptotic cascade of
533 events initiated by both drugs and are in agreement with previous publications [4,38].

534 We also observed that satraplatin induced an apoptotic response through activation of the
535 extrinsic pathway by promoting Fas expression which in turn leads to the formation of
536 the death-inducing signalling complex (DISC) and the auto-catalytic activation of pro-
537 caspase 8. This phenomenon was further enforced by the involvement of the extrinsic
538 pathway by oxaliplatin-induced apoptosis in colon cancer cells [39].

539 In addition to the basic molecular and biochemical analysis, we also examined the
540 expression of certain miRNA on the response of cells to the platinum agent. MicroRNAs
541 are small non-coding RNA molecules, which are able to control protein expression. It has
542 been suggested that modification of miRNA gene expression could be another important
543 factor in the responses to drug treatment [40]. As documented before, hsa-miR-34a was
544 shown to induce apoptosis, cell cycle arrest, and senescence by p53 mediated activation
545 [41]. Thus, we have detected high expression of miR-34a with satraplatin treatment than
546 oxaliplatin in which miR-34a targeting Bcl2 protein expression. In fact we observed a

547 lower expression of Bcl2 with satraplatin than oxaliplatin. In addition, we also detected a
548 down-regulation of miR-214, which in turn targets Bax protein expression. This finding
549 further suggests the role of miRNAs in chemotherapy responses based on p53-dependent
550 and -independent routes.

551 In summary, this study highlights the molecular mechanisms underlying the cytotoxic
552 effects of satraplatin for the first time in colorectal cancer cells in an attempt to identify
553 different means of predicting response to this oral platinum agent. Our data suggests that
554 the effect of satraplatin on proliferating colorectal cancer cells induces a G2/M cell cycle
555 arrest and a molecular cascade of events consistent with an intrinsic-extrinsic mechanism
556 of apoptosis. Moreover, the cytotoxic effects of satraplatin were shown to be both p53-
557 dependent and -independent using an isogenic *in vitro* system. Further analysis is
558 necessary to determine if this orally available platinum agent has an effect in combination
559 with other chemotherapy agents compared with oxaliplatin-based combinations. This
560 could be a better solution for malignant cells that acquired resistance or *de nova*
561 resistance against the currently available chemotherapy regimens.

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570 **Conflict of interest statement**

571 The authors stated that there are no conflicts of interest regarding the publication of this
572 article.

573

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729 **Figure legends**

730 **Figure 1** Effect of satraplatin and oxaliplatin on cytotoxicity of representative colorectal
731 cancer cells. MTS assay curve demonstrating cytotoxic effect on both drug treatment.

732 **1.1 A-C:** Curves representing the average value for at least three independent
733 experiments in quadruplicate. Cells were treated for 72 h with increased concentration of
734 both drugs as described in material and method section. **1.2:** Cell morphology after 10-
735 μM exposure of satraplatin and oxaliplatin in HCT116 and p53 null. Representative
736 results are shown for HCT116 untreated ctrl (1.2a) HCT116 oxaliplatin (1.2b) and
737 HCT116 satraplatin (1.2c), p53 null untreated ctrl (1.2d), p53 null oxaliplatin (1.2e) and
738 p53 null satraplatin (1.2f) cells at 72 h after treatment. Arrows shows typical morphology
739 of cell undergoing apoptotic. Cells were treated as detailed in material and methods.

740

741 **Figure 2:** Quantification of cellular platinum accumulation by Atomic Absorbance
742 Spectrophotometer (AAS) following 2-hour incubation of satraplatin and oxaliplatin
743 (drug concentration range: 6.25-100 μM). Direct line indicates satraplatin and dot line
744 indicates oxaliplatin treatments.

745 Satraplatin in comparison to oxaliplatin resulted in more accumulation of Pt in each cell
746 line tested in reference to p53 gene status. This demonstrates that the uptake of platinum
747 is more efficient with satraplatin than oxaliplatin. Based on 10 μM treatment with both
748 drugs, the accumulation of satraplatin was 35-40 ng/ml whereas with oxaliplatin only
749 expected 3-10 ng/ml platinum for 10^6 cells. The accumulation of satraplatin in the cells
750 was 4 fold higher than after oxaliplatin treatment. Error bars= S.E of two independent
751 experiment. .

752 **Figure 3:** Apoptosis analysis using PI as described early by us (Matteucci et al., 1999)
753 on representative colorectal cell lines after the treatment with 10 μ M of satraplatin and
754 oxaliplatin for 48h and 72h.

755 The results are the means \pm S.E of the percentage of hypodiploid nuclei analyzed by flow
756 cytometry PI detection. In all three cell lines an increase of apoptosis cells were detected
757 based after satraplatin compared to oxaliplatin treatment.

758

759 **Figure 4** Evaluation of apoptosis analysis in representative colorectal cell lines after the
760 treatment with 10 μ M of satraplatin and oxaliplatin for 24h and 48 h by annexin V/PI
761 assay.

762 The results are the means \pm S.E of the percentage of hypodiploid nuclei analyzed by flow
763 cytometry after 24 hour of annexin in relation to 48 h of PI detection. Comparable
764 analysis was done to detect the apoptosis percentage based on both drugs treatment from
765 two independent experiments. Typical cytograms demonstrating satraplatin induces high
766 levels of apoptotic cells compared to oxaliplatin treatment at the same drug
767 concentration.

768

769 **Figure 5** Effect of oxaliplatin and satraplatin on p53 protein and cell cycle related protein
770 levels in a panel of wild type, knockout and mutant p53 gene carrying cells. Blot for actin
771 served as loading control.

772 **A.** Over expressed p53 protein by satraplatin and oxaliplatin treatment in HCT116 while
773 reduced expression levels were observed in HT29 cells with both treatments. **B.**
774 Expression of p53 downstream genes, p21^{waf/cip1}, and Cyclin B1, c-ABL were examined

775 following 48 h of treatment with both drugs at 10 μ M concentration. The expression of
 776 p21^{waf/cip1} is higher with satraplatin treatment while c-ABL expression was higher with
 777 oxaliplatin treatment. **C.** The expression of cyclin B1 is evidence at 48 which the
 778 expression is stronger with satraplatin treatment in HCT116 and p53 null cells while
 779 same levels of expression observed with oxaliplatin treatment in HT29 cells.

780

781 **Figure 6:** Effect of both drugs on cell death related protein and microRNA expression of
 782 miR-34a. Equal protein amounts from unexposed cells have used as loading control and
 783 blot for actin served as normalization gene.

784 **A.** Basal protein levels of p53 downstream genes on cell death related molecules of Bax,
 785 Bcl2, Bid and Fas on a representative colorectal cancer cells at 48 h treatment with 10
 786 μ M of oxaliplatin and satraplatin. **B.** Pro and active Caspase 8 after the treatment of cells
 787 with both drugs. **C.** Fold change miRNA expression of hsa-miR-34a, which is inhibiting
 788 the transcription of Bcl2 and **D. Semi-quantitative analysis of fold change to untreated**
 789 **control of Bcl2 protein expression following satraplatin and oxaliplatin treatments at 48**
 790 **h.**

Table 1 Primers list for mRNA RT-PCR

Genes	Primer Set	Length (bp)
MLH1	Forward: 5' TTTACAACATAGCCACGAGGA 3' Reverse: 5' CTATCAGTTCTCGACTAACAGCA 3'	221
P21waf1/cip1	Forward: TCACACCATGACAAGACTCTC Reverse: AAATGCCAGTCACTTAGTACAG	281
PMS2	Forward: 5' CTCATAGCACCTCAGACTCTC 3' Reverse: 5' GGAAATCAGTTTAGCCCTTTCAG 3'	140
BCL2	Forward: 5' GGATTGTGGCCTTCTTTGAG 3' Reverse: 5' CCAGGAGAAATCAAACAGAGG 3'	205
BRCA1	Forward: 5' CTGCTCAGTAATGAACACTGG 3' Reverse: 5' CAGCATCCATTGAGAATCCC 3'	218

B2M	Forward: 5' CAGCGTACTCCAAAGATTTCAG 3' Reverse: 5' GTCAACTTCAATGTCGGATGG 3'	110
MSH2	Forward: 5' TTCCATACAGAGGAACTAGGAC 3' Reverse: 5' AACTGCAACCTGATTCTCCA 3'	300
BAX	Forward: 5' TTTGCTTCAGGGTTTCATCC 3' Reverse: 5' TGTTACTGTCCAGTTCGTCC 3'	145
TP73	Forward: 5' AAACCCAGACTTATCTCAGACAG 3' Reverse: 5' GTCTTGCTGCCATATACACC 3'	130
ERCC1	Forward: 5' AATTTGGCGACGTAATTCCC 3' Reverse: 5' GGGATCTTTCACATCCACCT 3'	169
14-3-3-sigma	Forward: 5' CTCAGTAGCCTATAAGAACGTG 3' Reverse: 5' AGTAGTCACCCTTCATCTTCAG 3'	250
CLU	Forward: 5'TGATCCCATCACTGTGACGG Reverse: 5' GCTTTTGGCGTATTCCTGC	120
β -actin	Forward: 5' GGACTTCGAGCAAGAGATGG 3' Reverse: 5' AGCACTGTGTTGGCGTACAG 3'	242
TP53	Forward: 5' GTCTGGGCTTCTGCATTCT 3' Reverse: 5' AATCAACCCACAGCTGCAC 3'	120
GADD45	Forward: 5' GAGAACGACATCAACATCCTG 3' Reverse: 5' GAATGTGGATTCGTCACCAG 3'	152

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Table 2 IC₅₀ value of Satraplatin and oxaliplatin in a panel of colorectal cancer cell lines

Cell line	Tp53 Status	IC ₅₀ μ M		IC ₅₀ p values
		Satraplatin	Oxaliplatin	*
HCT116	Wildtype	15.7 \pm 0.5	21.5 \pm 1.3	0.0020
HT-29	Mutant	27.2 \pm 3.1	33.1 \pm 2.7	0.1199
HCT116 p53 ^{-/-}	Knockout	14.4 \pm 1.6	95.7 \pm 2.7	0.0001
WiDr	Mutant	26.9 \pm 2.4	36.2 \pm 2.3	0.0084
LoVo	Wildtype	16.3 \pm 1.3	19.4 \pm 0.9	0.0274
HCT15	Mutant	13.3 \pm 1.1	20.4 \pm 2.3	0.0085

*p value represents difference in IC₅₀ between satraplatin and oxaliplatin treatment

792

793

Table 3 Effect of satraplatin and oxaliplatin on cell cycle perturbation in representative colorectal cancer cells at 10 μ M

	HCT116			HCT116 Tp53 ^{-/-}			HT29		
	Untreated	Oxaliplatin	Satraplatin	Untreated	Oxaliplatin	Satraplatin	Untreated	Oxaliplatin	Satraplatin
48 hours									
G0/G1	69.95	56.04	23,13**	67.47	65.32	26,56**	62.2	37.52*	17.64**

S	17.56	25.94	26.28	11.33	21.29	52,32**	34.94	53.58	51,89*
G2/M	13.49	18.02	50.59**	21.37	13.29	21.08	2.86	8.9	30,47**

72 hours

G0/G1	69.55	57.48	21.05**	58.86	40.54	13,58**	42.93	11.13*	10.01**
S	17.03	19.15	21.88	12.9	52,56**	35,95*	53.68	84.64**	68.87*
G2/M	14.42	24.37	57,08**	28.52	7.03	50,85**	2.39	4.23	31.12

794

795 Result are representative of at least three independent experiment. This data demonstrated that satraplatin
796 treatment arrested cell in G2/M phase in wildtype p53, and full S phase arrest in mutant p53 cells.

797 Treatment vs. untreated CTRL

798 *p<0.05

799 **p<0.001

800

801

Table 4 mRNA and miRNA of satraplatin vs. oxaliplatin at 48 hour post treatment

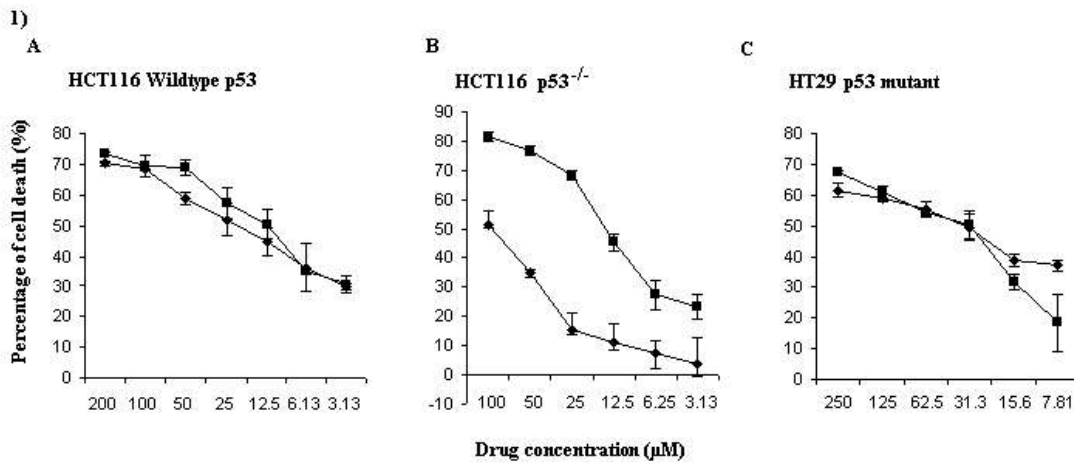
4.1							Fold Change (mRNA) to untreated CTRL					
No Gene symbol	HCT116		HCT116 p53^{-/-}				HT29					
	Oxaliplatin	Satraplatin	Oxaliplatin	Satraplatin	Oxaliplatin	Satraplatin	Oxaliplatin	Satraplatin				
	1 ERCC1	2.41	1.78	1.18	1.11	1.25	1.14					
2 MLH1	1.45	1.22	1.61	1.38	1.05	-1.29						
3 MSH2	-3.13	-1.7	1.8	1.23	2.13	1.37						
4 p53	1.53	1.6	ND	ND	-1.07	-1.05						
5 BRCA1	-2.8	2.36	-1.44	-1.55	1.19	1.47						
6 PMS2	2.2	1.44	-1.26	-1.05	1.38	1.31						
7 Tp73	1.3	1.49	1.39	1.66	1.37	-1.12						
8 GADD45	2.57	1.88	1.6	1.2	1.09	1.45						
9 p21	2.39	1.83	1.31	1.99	1.04	1.09						
10 BCL2	-2.68	-4.33	-1.29	-13.38	ND	ND						
11 BAX	2.59	2.18	1.06	1.13	1.2	1.22						
12 14-3-3-SIGMA	3.96	4.77	-2.39	1.6	-1.51	1.42						
13 CLU	1.17	2.67	-1.77	2.22	1.94	3.42						

4.2							Fold Change (miRNA) to untreated CTRL					
No Gene symbol	HCT116		HCT116 p53^{-/-}				HT29					
	Oxaliplatin	Satraplatin	Oxaliplatin	Satraplatin	Oxaliplatin	Satraplatin	Oxaliplatin	Satraplatin				
	1 hsa-miR-150 -->Tp53/ERCC1	1.61	2.06	-2.04	-1.31	-2.56	-1.04					
2 hsa-miR-152 -->MLH1/GADD45	1.34	2.53	-1.53	2.05	-1.21	1.64						
3 hsa-miR-128a -->MLH1/BAX	1.41	-1.04	1.07	-1.06	-1.41	-3.01						
4 hsa-miR-34a -->BCL2	1.89	2.66	-1.24	1.69	-1.33	-1.84						
5 hsa-miR-214 -->BAX	-1.06	-1.48	-2.21	-2.78	-1.17	-1.18						

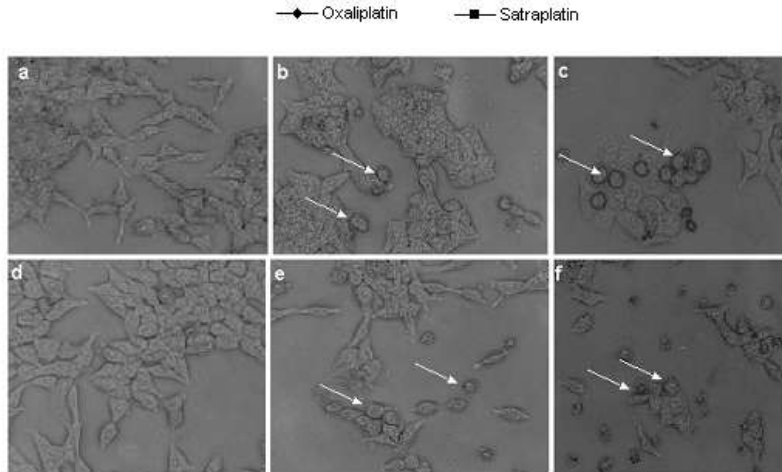
6 hsa-miR-218 -->BRCA1	-1.58	-1.51	1.02	-1.14	-1.15	30.69
7 hsa-miR-21-->MSH2	1.19	1.25	-1.42	-1.34	-1.64	-2.36
8 hsa-miR-106 --> p21	2.25	1.09	1.14	1.06	-1.18	-1.84
9 hsa-miR-20	1.39	1.62	-1.23	1.38	-1.89	-1.34
10 hsa-miR-148a --> GADD45	-1.94	-1.25	-1.41	1.19	-2.14	1.46
11 hsa-miR-370 --> CLU/cyclin E2	10.55	7.51	1.85	-2.25	-6.82	-6.02
12 hsa-miR-326 --> Tp73	4.14	8.16	-2.21	-1.37	-1.22	1.03
13 hsa-miR-128b	-1.95	-2.02	2.46	2.26	1.24	-1.56
14 hsa-miR-17-3p	-1.03	1.5	1.47	2.46	-1.63	-1.02

802

Figure 1

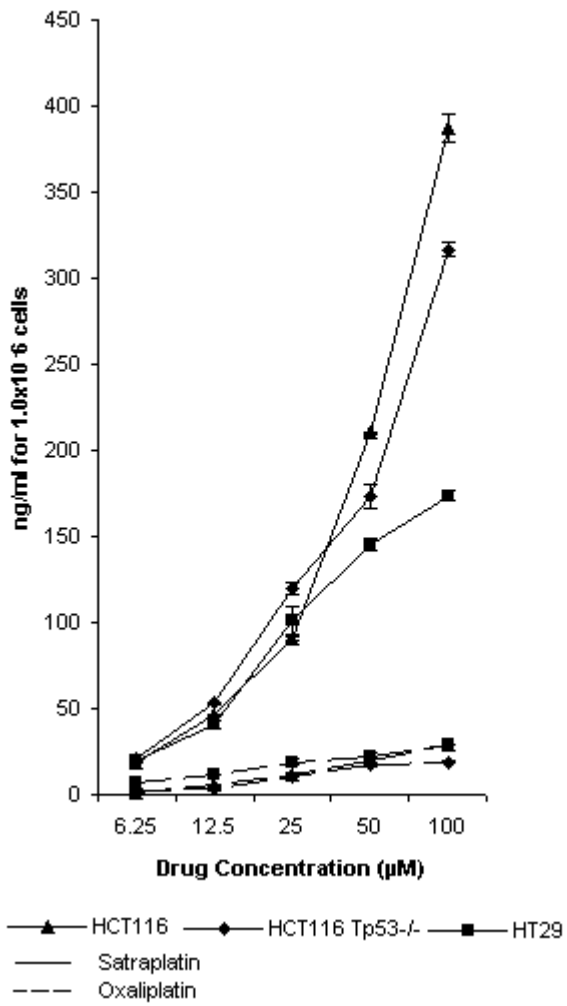


2)



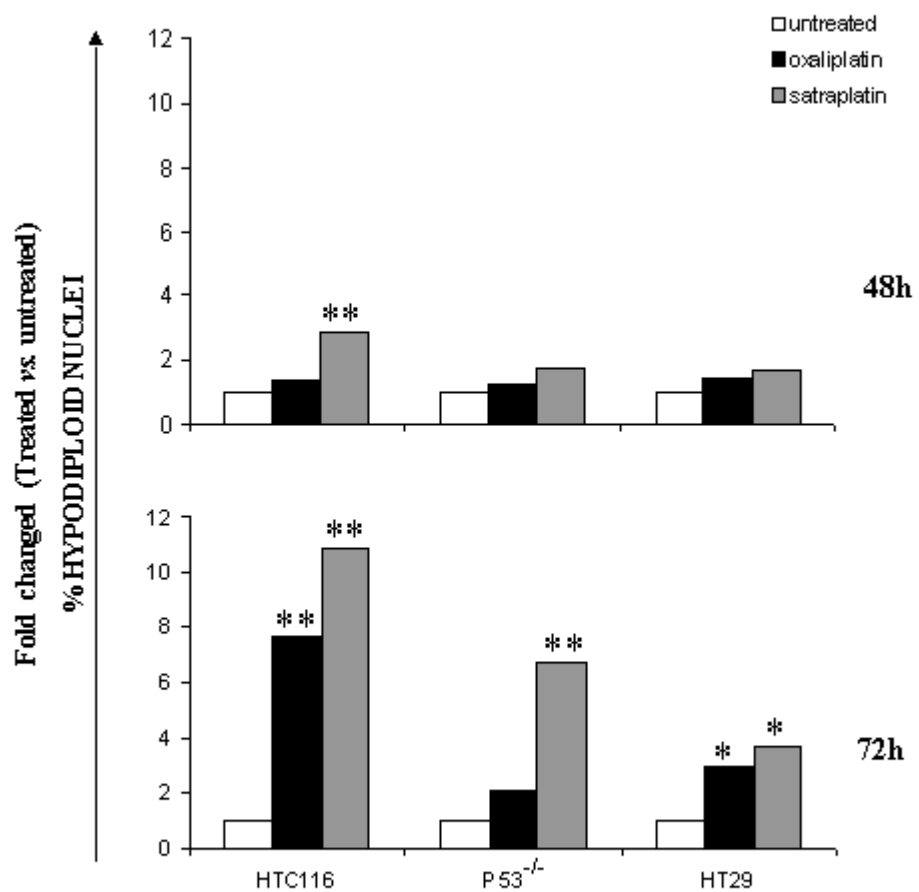
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Figure 2



804

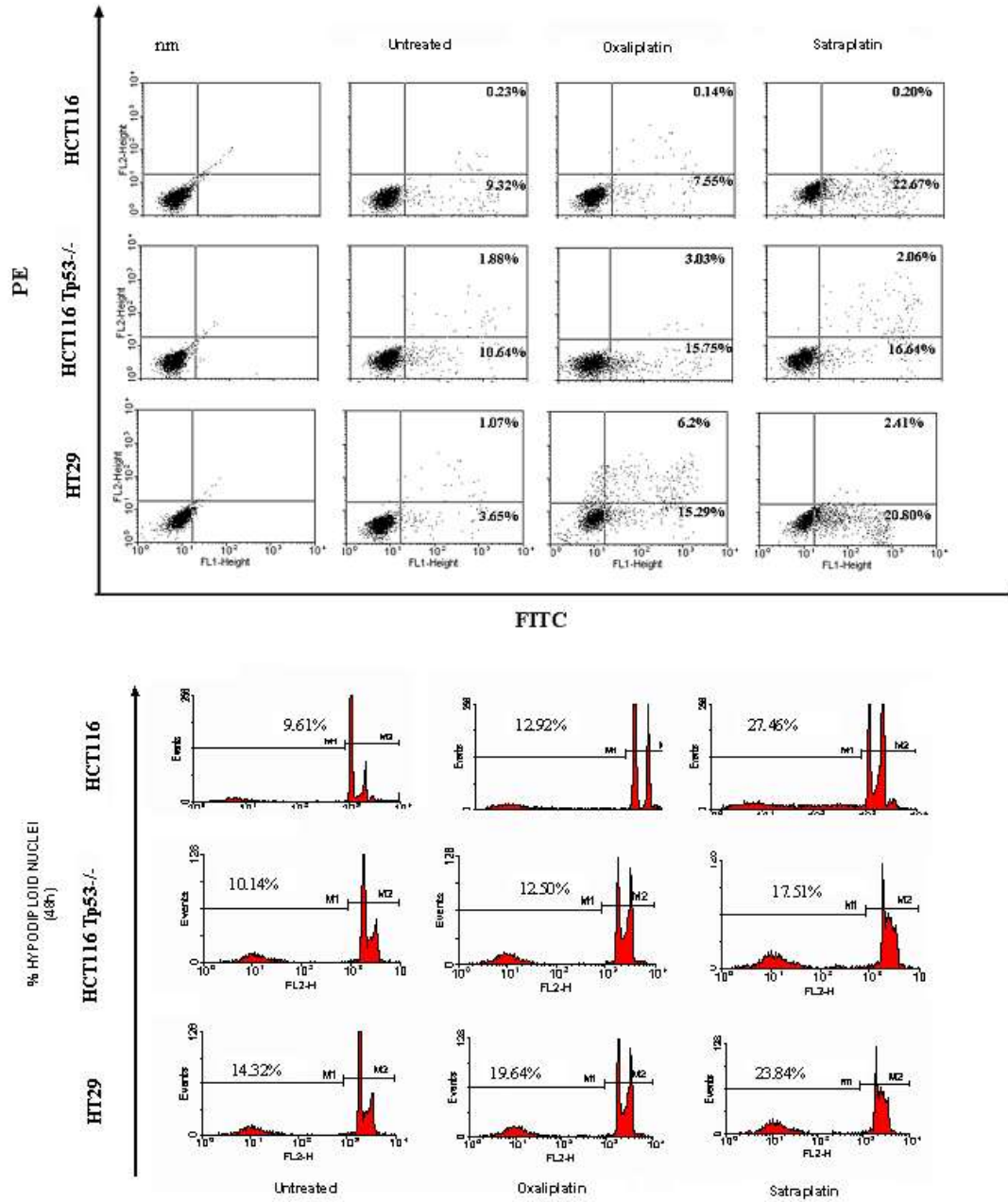
Figure 3



* $p \leq 0.05$; ** $p < 0.001$ treated vs untreated control

805

Figure 4



806

Figure 5

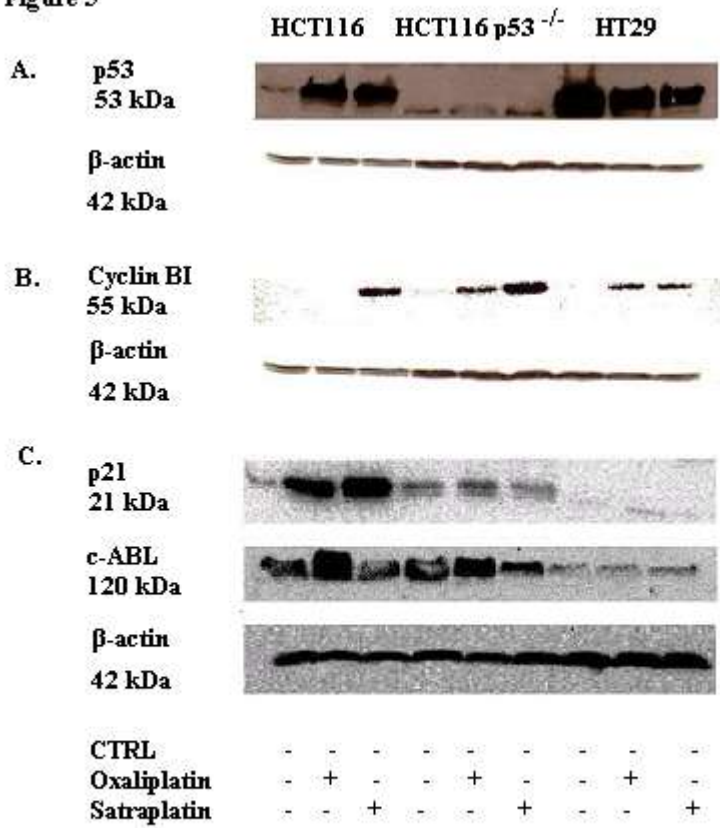
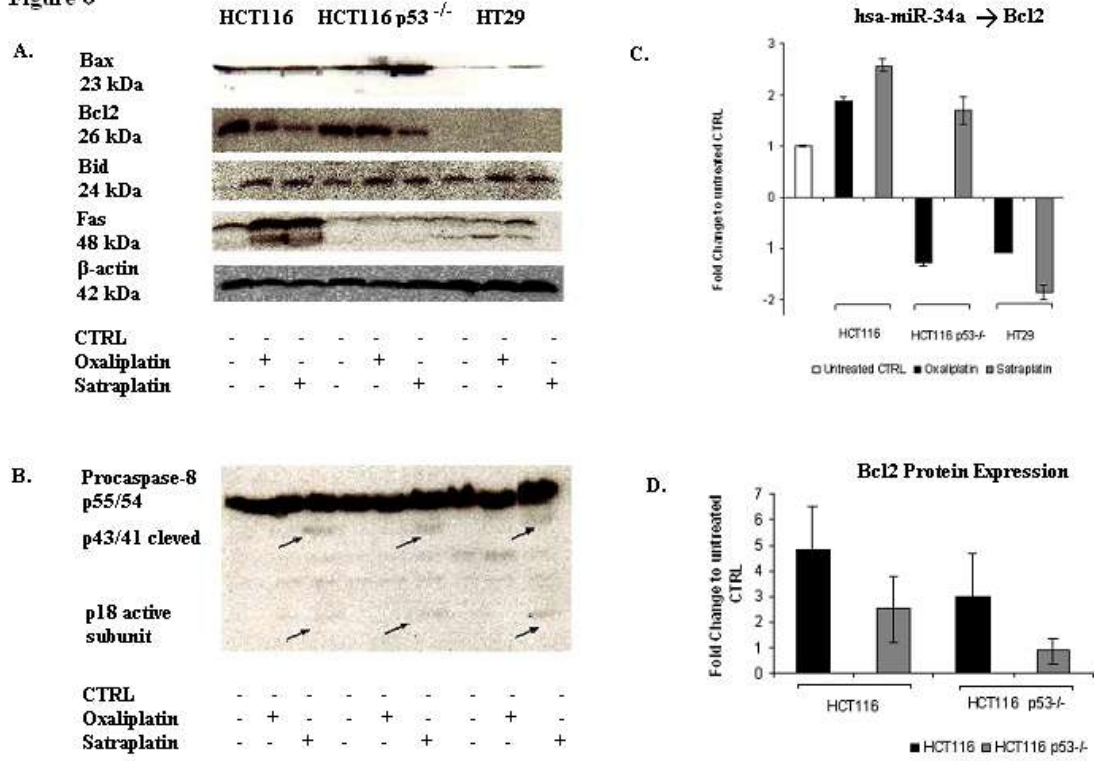


Figure 6



808