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Original Article

Platinum-(IV)-derivative satraplatin induced G$_2$/M cell cycle perturbation via p53-p21$^{waf1/cip1}$-independent pathway in human colorectal cancer cells

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Aim: Platinum-(IV)-derivative satraplatin represents a new generation of orally available anti-cancer drugs that are under development for the treatment of several cancers. Understanding the mechanisms of cell cycle modulation and apoptosis is necessary to define the mode of action of satraplatin. In this study, we investigate the ability of satraplatin to induce cell cycle perturbation, clonogenicity loss and apoptosis in colorectal cancer (CRC) cells.
Methods: CRC cells were treated with satraplatin, and the effects of satraplatin on apoptosis and the cell cycle were evaluated by flow cytometry. Western blot analysis was used to investigate the effects of satraplatin on cell cycle and apoptosis-related proteins. RT-qPCR was used to evaluate p53-related mRNA modulation.
Results: Satraplatin induced an accumulation of CRC cells predominantly in the G$_2$/M phase. Increased p53 protein expression was observed in the p53 wild-type HCT116 and LoVo cells together with p21$^{waf1/cip1}$ protein up-regulation. However, p21$^{waf1/cip1}$ protein accumulation was not observed in the p53 mutant HCT15, HT29, and WiDr cells, even when p53 protein expression was compromised, suggesting that the cell cycle perturbation is p53-p21$^{waf1/cip1}$ independent. Following a candidate approach, we found an elevated expression of 14-3-3σ protein levels in CRC cells, which was independent of the status of p53, further supporting the role of satraplatin in the perturbation of the G$_2$/M cell cycle phase. Moreover, satraplatin treatment induced apoptosis along with Bcl-2 protein down-regulation and abrogated the clonogenic formation of CRC cells *in vitro*.
Conclusion: Collectively, our data suggest that satraplatin induces apoptosis in CRC cells, which is preceded by cell cycle arrest at G$_2$/M due to the effect of 14-3-3σ and in a p53-p21$^{waf1/cip1}$–independent manner. Taken together, these findings highlight the potential use of satraplatin for CRC treatment.

Keywords: satraplatin; G$_2$/M phase; 14-3-3σ; p53; apoptosis; colorectal cancer

Introduction

The formation of DNA adducts by platinum analogs is recognized to be the most prominent effects in cells, blocking replication and transcription and inducing tumor cell death[1, 2]. The nature of the DNA adducts formed by these analogs affects a number of transduction pathways and triggers apoptosis and necrosis[3], thereby mediating cytotoxicity in these models. The most studied platinum analogs, cisplatin and oxaliplatin, have been used in the clinic for the treatment of various tumors[4], yet the acquisition of drug resistance remains a major obstacle in cancer management. Among the numerous platinum analogs, satraplatin has been shown to counteract *de novo* resistance and acquired resistance in some resistant human tumors[5, 6].

Satraplatin, also known as JM-216, is the first orally available active lipophilic platinum (Pt)-(IV) derivative that exhibits antiproliferative activity against various tumors[5, 6]. This drug is particularly attractive because of its convenience of administration, its milder toxicities (such as nephrotoxicity, neurotoxicity, and ototoxicity) and the lack of cross-resistance with cisplatin. Moreover, satraplatin demonstrates anti-cancer activity in tumors that are usually non-responsive to platinum drugs[7].

Upon diverse genotoxic stresses, DNA damage and other

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cellular lesions usually activate cell cycle checkpoints that inhibit cell cycle progression by decreasing the activity of key regulatory proteins[8], primarily including cyclin-dependent kinase (Cdk) complexes[9, 10]. After DNA damage signaling, the "p53 → p21$^{waf1/cip1}$ pathway" is activated[9, 10]. Activated p53 induces the expression of its downstream targets, including p21$^{waf1/cip1}$, which is a universal inhibitor of Cdks and is required to arrest cells at the G_1 and G_2 checkpoints[11–13]. The cells are then arrested at the G_1-S or G_2/M phase. We have previously reported cell cycle perturbation following satraplatin and oxaliplatin treatment in a panel of CRC cell lines that differ in their p53 gene status[14]. We observed that satraplatin induces cell cycle arrest at the G_2/M phase and that oxaliplatin induces arrest at the G_1 phase and results in apoptosis. On the basis of this observation, we proposed that satraplatin is a remarkably more potent activator of cell cycle and cell death proteins than oxaliplatin. We speculate that the perturbation of the G_2/M cell cycle phase may be a prominent effect of satraplatin treatment in CRC cells. Additionally, we demonstrate that p53-null cells, which are arrested in the same manner as wild-type cells, subsequently induce apoptosis with satraplatin treatment but not with oxaliplatin treatment.

The aim of this study was to elucidate the effect of satraplatin on modulators of the G_2/M cell cycle transition, apoptosis, and clonogenic formation. We show that satraplatin induces cell cycle arrest at G_2/M at least in part because of the effect of 14-3-3σ and in a p53-p21$^{waf1/cip1}$-independent manner. Moreover, satraplatin treatment affects DNA repair genes at the mRNA level. It also abrogated the clonogenic formation of CRC cells *in vitro*.

Materials and methods
Drug, chemicals, and reagents
Satraplatin was a kind gift from Agennix AG (formerly GPC Biotech AG), Fraunhoferstr, Germany. A satraplatin stock solution was prepared in 0.09% saline and stored at -20 °C. Antibodies were purchased from the following companies: anti-p53 (BD Pharmingen), anti-β-actin (Sigma), anti-Bax (Millipore), anti-Bcl-2 (Sigma), anti-p21$^{waf1/cip1}$ and anti-cyclin B1 (Santa Cruz) antibodies. Anti-rabbit and anti-mouse secondary antibodies were purchased from DakoCytomation, Denmark A/S. ECL detection reagents were purchased from Perkin-Elmer.

Cell culture
The HCT116 human CRC cell lines (wt, p53$^{-/-}$, and p21$^{-/-}$), HT29, HCT15, WiDr, and LoVo were tested for mycoplasma infection according to UKCCCR guidelines[15] and were cultured in monolayer in DMEM (HCT116, p53$^{-/-}$, p21$^{-/-}$, and HT29), DMEM:F12 (WiDr), RPMI-1640 (HCT15) and Ham's Nutrient mix F12 (LoVo) supplemented with 10%–20% FBS and 1% penicillin/streptomycin. No antibiotics were added prior to treatment. The cells were trypsinized and subcultured twice a week. All experiments were conducted at an early passage as previously described[14].

BrdU cell proliferation assay
A BrdU (5-bromo-2'-deoxyuridine) incorporation assay (Calbiochem/Merck, Whitehouse Station, NJ, USA) was used to assess the antiproliferative effects of satraplatin according to the protocol of the manufacturer. Briefly, 10 000 cells/well were seeded in 96-well plates followed by satraplatin treatment of 0–50 μmol/L. After cells adhered to the plate BrdU was added (1:2000 dilution), and plates were incubated for different time intervals (24 h and 72 h). The plates were then washed, fixed with anti-BrdU antibody and a peroxidase goat anti-mouse IgG conjugate was added. Immunocomplex formation was measured using tetra-methylbenzidine solution, and the reaction was terminated with 2.5 mol/L sulfuric acid. The measured intensity was proportional to the amount of incorporated BrdU in the cells. Absorbance was measured at 450 nm using a Microplate Manager (Bio-Rad Laboratories, Inc) according to the protocol of the manufacturer. The absorbance results were expressed as relative to untreated control.

Cell cycle analysis and apoptosis
Cell cycle perturbations and the apoptotic fractions of untreated and treated cells following satraplatin treatment were defined by flow cytometry analysis of cells stained with propidium iodide as described previously[14].

Western blot analysis
Aliquots of whole cell extracts containing 20 μg of protein were used for Western blot analysis as previously described[14].

Clonogenicity assay
Approximately 300 cells were seeded into each well of a 6-well culture plate, which were treated and then incubated in 2 mL of medium for 10 d. Subsequently, the medium was removed, and the cells were fixed for 15 min in 0.5 mL of 0.5% glutaraldehyde. The fixing solution was removed, the wells were rinsed with PBS, the colonies were stained for 30 min in 0.5 mL of 1% (*w/v*) methylene blue solution in PBS, washed once again with water, and then photographed[16].

Quantitative PCR
p53 signaling pathway qRT-PCR array
For the p53 Signaling Pathway PCR Array analysis, RNA from HCT116 (wt, p53$^{-/-}$, and p21$^{-/-}$) treated and untreated cells were used to analyze the fold change in expression. A total of 1 μg of total RNA was reverse transcribed, and the cDNA was analyzed on a human p53 signaling pathway RT^2 Profiler PCR Array (SABioscience, Fredrick, MD, USA) using the Stratagene Real-Time PCR System (Mx3005). The human p53 signaling pathway RT^2 Profiler PCR Array profiles the expression of 84 genes that are known to be involved in p53 signaling pathway regulation. A complete list of the genes assayed by the array can be viewed at http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-027A.html. PCR amplification was performed with an initial 10-min step at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The SYBR green

fluorescent signal was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. The data were analyzed using the comparative cycle threshold method with normalization of the raw data to the normalizer on the array. The results are presented as n-fold changes *versus* the values in the untreated control cells.

mRNA isolation, cDNA synthesis and qRT-PCR for the expression of DNA damage-related genes

Total RNA was isolated with TRIzol reagent (Invitrogen, CA, USA). The total RNA integrity was determined by 1% agarose gel electrophoresis. cDNA synthesis was performed with the Superscript III cDNA synthesis kit (Invitrogen, CA, USA) using 1 μg of total RNA as the template according to the protocol of the manufacturer. The PCR amplification was performed in a final volume of 25 μL containing 12.5 μL of SYBR green (2×), 0.5 μL of primers that detect MLH1, MSH2, PMS2 and ERCC1 as previously reported[14] (10 μmol/L) and 1 μL of cDNA. The cycling conditions included preheating at 95 °C for 10 min and then 40 cycles of the following: 95 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s followed by a standard dissociation curve. Values that fell below 35 cycles were considered for the $2^{-\Delta\Delta Cq}$ determination.

Statistical analysis

The gene expression of ΔCq values from each sample were calculated by normalizing to ACTB and B2M, and the relative quantitation values were calculated according to the $2^{-\Delta\Delta Cq}$ method. Statistical analyses were performed using the SPSS statistical software package, version 12.0 (Chicago, IL, USA). Statistically significant differences in the expression level between the control and treated samples for each target were calculated using a paired Wilcoxon test. A comparison of the mean values of proliferation and the apoptosis levels in response to satraplatin and oxaliplatin treatment was performed using the Bonferroni *post hoc* multiple comparisons ANOVA test. [b]$P \leq 0.05$ value was considered significant, and [c]$P \leq 0.01$ value was considered highly significant.

Results

Anti-proliferative effects of satraplatin on CRC cells

The role of p53-p21[waf1/cip1] in the G_2/M cell cycle phase following satraplatin treatment was investigated in a panel of CRC cell lines that differ in their p53 genotype (HCT116[wt], LoVo[wt], HT29[mut], HCT15[mut], and WiDr[mut]) and isogenic HCT116 cells that were p53^{-/-} and p21^{-/-}. To assess the dose limiting effects of satraplatin on the rate of DNA synthesis, we performed a BrdU incorporation assay following satraplatin treatment for 24 and 72 h. As shown in Figure 1, we found that cells responded to satraplatin (when added below its IC_{50} value) in a dose- and time-dependent manner (Figure 1A). At a higher concentration (50 μmol/L), satraplatin decreased the proportion of S phase cells by about 90% in all seven cell lines tested (Figure 1B). Consistent with our previous findings on the effects of satraplatin on cell proliferation and apoptosis[14],

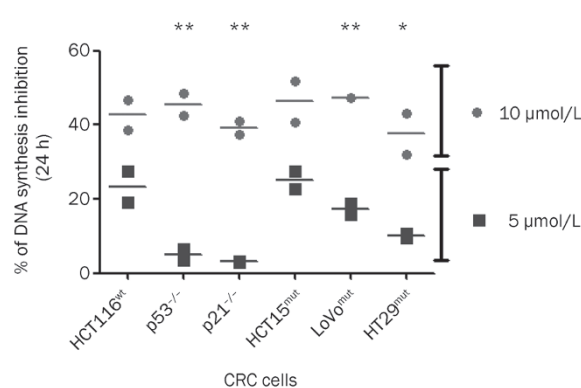| Cell line	p53 status	IC_{50} (μmol/L)
HCT116	Wild type	15.7±0.5
HT29	Mutant	27.2±3.1
HCT116p53**	Knockout	14.4±1.6
WiDr	Mutant	26.9±2.4
LoVo	Wild type	16.3±1.3
HCT15	Mutant	13.3±1.1
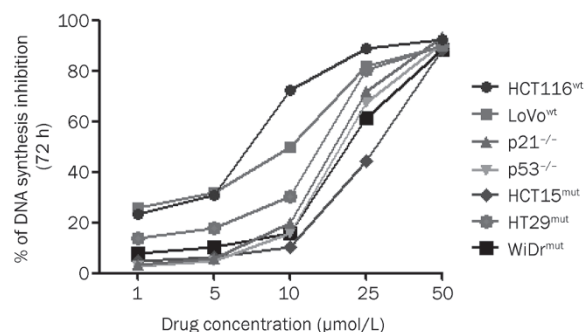
Figure 1. The IC_{50} value and the effect of satraplatin on CRC cells. (A) Table showing the IC_{50} value of satraplatin in a panel of CRC cell lines. (B) HCT116 (wt, p53^{-/-}, and p21^{-/-}), LoVo[wt], HT29[mut], HCT15[mut], and WiDr[mut] cell lines were treated with 0–50 μmol/L satraplatin, and the effect of satraplatin on DNA synthesis inhibition was determined by a BrdU incorporation assay at 24 and 72 h. At higher concentrations, all cells demonstrated about 90% inhibition, which indicates the optimal activity of satraplatin in CRC cells. (C) DNA synthesis was inhibited at least 50% by treatment with 10 μmol/L satraplatin, whereas a different pattern of inhibition was observed with 5 μmol/L satraplatin. The percentage of DNA synthesis inhibition was defined as the optimal density at a given drug concentration divided by the optimal density for untreated control cells multiplied by 100. Each point is the average of two independent trials; data are presented as the mean±standard deviation.

no evidence of cytotoxicity was observed when cells were exposed to lower drug doses (*ie*, ≤50 μmol/L). We also tested two drug concentrations, 5 and 10 μmol/L, that are lower than previously determined IC_{50} concentrations (Figure 1A)[14]. As expected, treatment with 10 μmol/L, but not 5 μmol/L,

satraplatin led to 36%–47% S phase inhibition as early as 24 h (Figure 1C). For example, 10 μmol/L at 72 h demonstrated that the wildtype p53 cells (HCT116 and LoVo) were more prone to S phase inhibition (61.06%±6.61%) than the mutant p53 HCT15, HT29, and WiDR cells (19.00%±4.02%, *P*<0.01). In addition, when comparing the 24 h *vs* the 72 h analysis, we noticed that all cells were more prone to S phase inhibition after as early as 24 h of treatment (43.08%±1.6%) but later returned to normal cell cycle progression, at least in mutant p53 cells but not in p53 wild-type cells. Thus, the BrdU incorporation assay confirmed the partial accumulation of S phase cells before cells exhibited a G_2/M phase perturbation.

Western blot analysis of proteins from HCT116[wt] and LoVo[wt] cells treated with 5 and 10 μmol/L satraplatin demonstrated a sustained increased level of p53 and p21[waf1/cip1] protein, particularly at 10 μmol/L (Figure 2A). In contrast, despite an elevated p53 protein level in HCT15[mut] and HT29[mut] cells, no expression of p21[waf1/cip1] was detected (Figure 2A, lower panel). To further confirm these data, we used the WiDr mutant cell line (Figure 2B) and showed no evident of p21[waf1/cip1] accumulation. Furthermore, when p53^{-/-} cells were treated with satraplatin, no evidence of p21[waf1/cip1] accumulation was observed (Figure 2A, upper panel), which indicates that p21[waf1/cip1] expression requires a p53-dependent mechanism. Moreover, an accumulation of p53 was observed in p21^{-/-} HCT116 cells treated at both concentrations (Figure 2), indicating that p53-dependent expression of p21[waf1/cip1] has no or only a modest effect on cell cycle perturbation.
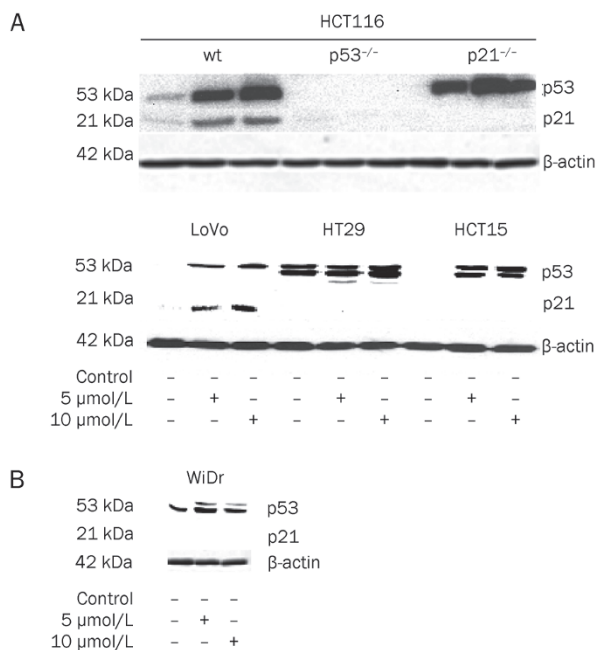

Figure 2. The effect of satraplatin on a clinically relevant concentration below its IC_{50} value on p53-p21[waf1/cip1] protein expression at 48 h. Whole cell extracts were prepared from HCT116 (wt, p53^{-/-}, and p21^{-/-}), HCT15[mut], HT29[mut], and WiDr[mut] cells treated with 5 and 10 μmol/L satraplatin for 48 h and subjected to Western blot analysis for the presence of p53 and p21[waf1/cip1] protein accumulation.

Satraplatin treatment induces G_2/M perturbation

Consistent with our previous study[14], we found that CRC cells treated with different satraplatin concentrations were past the initial entry into G_2 prior to DNA damage and were subsequently arrested (Figure 3A and Supplementary Table 1). The expression of cyclin B1, a marker of the G_2/M phase, was up-regulated following 5 and 10 μmol/L satraplatin treatment (Figure 3B). Cellular growth inhibition and cell cycle perturbation induced by satraplatin was evident at 10 μmol/L (Figure 3C, right panel) compared with 0.5 μmol/L (Figure 3C, left panel). Similar perturbations were observed in all six CRC cell lines when the cells were treated with low and high satraplatin concentrations, and this was independent of their p53 gene status (Figure 3C).

Satraplatin increases 14-3-3σ expression in CRC cells

The results described above demonstrate that the presence of p21[waf1/cip1] and/or p53 following satraplatin treatment played a minor role in inducing cell cycle perturbation and apoptosis. To determine which genes significantly alter the cell cycle profile at the G_2/M phase, we used a candidate approach and found that 14-3-3σ mRNA and protein expression were elevated in CRC cells treated for 24 h (mRNA) and 48 h (protein) (Figure 4). We found a significant increase in 14-3-3σ mRNA expression in the wild-type p53 HCT116 cells but no significant increase in the p53^{-/-} and p21^{-/-} cells. At 48 h, elevated 14-3-3σ protein expression was detected after 5 and 10 μmol/L satraplatin treatment. We also observed that p53-null cells demonstrated an increase in 14-3-3σ that was independent of p53 action, particularly after treatment with 10 μmol/L satraplatin. Moreover, a similar protein expression pattern was observed in other CRC cell lines (Figure 4B, lower panel).

Satraplatin induces apoptosis in CRC cells

The cells that were G_2/M arrested also underwent apoptosis in a dose-dependent and a p53-independent manner (Figure 2 for p53 protein expression, Figure 5A for apoptosis by flow cytometry). A higher percentage of apoptotic cells was observed at 5 and 10 μmol/L (*P*<0.01) than at the lower concentrations of 50 and 500 nmol/L (Figure 5A). In particular, we found that the p53^{-/-} and p21^{-/-} cells showed a significant down-regulation of Bcl-2 protein expression (Figure 5B) after satraplatin treatment. In HT29[mut] and WiDr[mut] cells, no Bcl-2 protein accumulation was detected (Figure 5B, upper panel), whereas significant down-regulation of Bcl-2 protein expression was observed in HCT15[mut] and LoVo[wt] cells at 10 μmol/L (Figure 5B, lower panel).

In HCT116[wt] cells, 5 and 10 μmol/L satraplatin induced apoptosis by at least 16% (*P*≤0.05) and 40% (*P*≤0.01), respectively, *versus* only 5% in the untreated control. In Lovo[wt] cells, we observed 21% and 34% apoptotic fractions at 5 and 10 μmol/L satraplatin, respectively, *versus* 4.7% in the untreated control (*P*≤0.01). Moreover, in HT29[mut] cells, the same concentrations affected 15% and 27%, respectively, *versus* only 4% in the untreated control (*P*≤0.01). Finally, HCT15[mut] cells exhibited 26% and 36% apoptosis at 5 and 10 μmol/L satraplatin,

A   Cell cycle perturbation follwoing satraplatin treatment.

Cell lines	µmol/L	Cell cycle phase %		
		G_0/G_1	S	G_2
HCT116[wt]	0	78.98	4.97	14.27
	0.5	78.28	15.54	12.49
	10	34.06[b]	7.9	58.37[c]
HCT116 p21^{-/-}	0	52.89	14.52	32.97
	0.5	51.17	23.45	18.56
	10	10.9*	7.45	81.85[c]
HCT116 p53^{-/-}	0	77.01	6.11	17.03
	0.5	78.45	5.56	18.78
	10	22.75[c]	36.82[c]	41.24[c]
LoVo[wt]	0	60.7	15.62	22.03
	0.5	58.69	20.35	20.04
	10	27.45[b]	46.58[b]	18.54
HCT15[mut]	0	60.3	23.78	12.29
	0.5	58.08	23.62	14.59
	10	3.24[b]	42.15	54.94[b]
HT29[mut]	0	61.62	16.97	22.09
	0.5	58.86	15.89	24.78
	10	30.96[b]	23.01	46.36

[b]$P \leq 0.05$, [c]$P \leq 0.01$ treated *vs* untreated control.
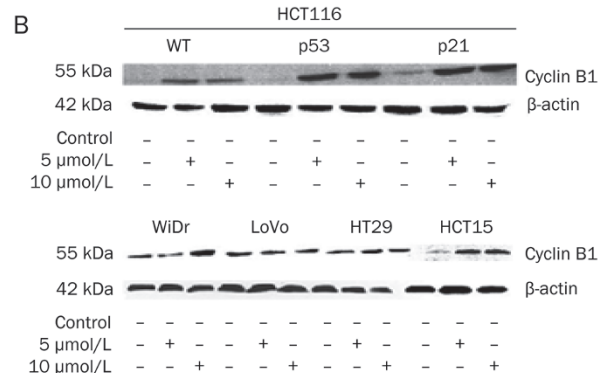

Figure 3. The effect of satraplatin-induced cell cycle perturbation at the G_2/M phase, apoptosis and cyclin-B1 expression in CRC cells at 48 h. (A) Table showing an accumulation of cells at each phase of the cell cycle following satraplatin treatment at lower and higher concentrations. (B) Cyclin B1 protein expression levels following treatment with 5 and 10 µmol/L satraplatin in CRC cells harvested at 48 h. The results shown are representative Western blots of at least two independent experiments. Equal loading was confirmed by β-actin detection. (C) The effects of various satraplatin concentrations on DNA synthesis and cell cycle progression in CRC cells. Typical cytograms are presented that represent the overlaps of the percentage of cells in each phase (G_0/G_1, S, and G_2/M) compared with untreated (control) cells (red background: untreated control cells; white background: treated cells) as determined by flow cytometry. This analysis indicates that the cells were substantially past the initial entry to G_2 after DNA damage and were subsequently arrested.

respectively, whereas untreated cells showed just 10% ($P \leq 0.01$) apoptosis. We also found that p53^{-/-} cells responded in the same manner following 5 and 10 µmol/L satraplatin treatment, with 9% and 34% ($P \leq 0.01$) apoptosis, respectively, *versus* only 3% in the untreated control. However, in p21^{-/-} knockout cells, the percentage of apoptotic cells was 44% and 53% at 5 and 10 µmol/L satraplatin, respectively, *versus* only 5% in the untreated control ($P \leq 0.01$). We also observed that the cells responded in a dose- and time-dependent manner.
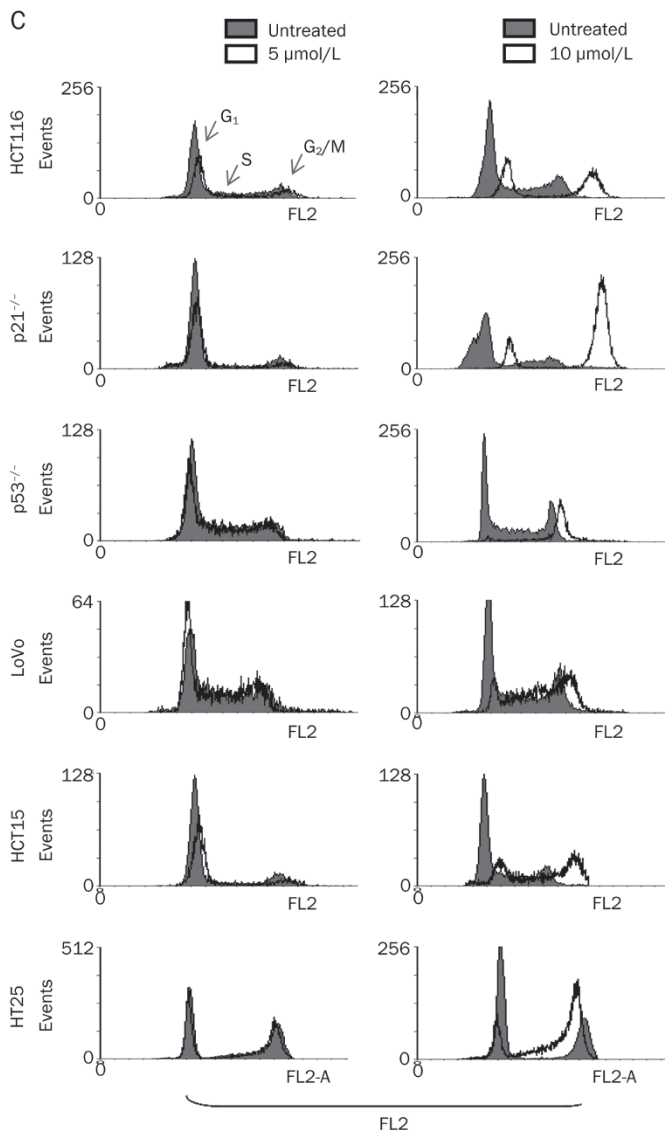
Satraplatin abrogated clonogenic formation in CRC cells
We also found that CRC cells, particularly HCT116 (wt, p53^{-/-}, and p21^{-/-}) (Figure 6, right panel), loss the ability to form colonies when treated with 5 or 10 µmol/L satraplatin as compared with 50 nmol/L treated cells (left panel). However, a partial clonogenicity loss was observed in all CRC cells when the cells were treated with 500 nmol/L satraplatin. This was also observed in the other CRC cell lines, including HCT15[mut], LoVo[mut], WiDr[mut], and HT29[mut] (data not shown).
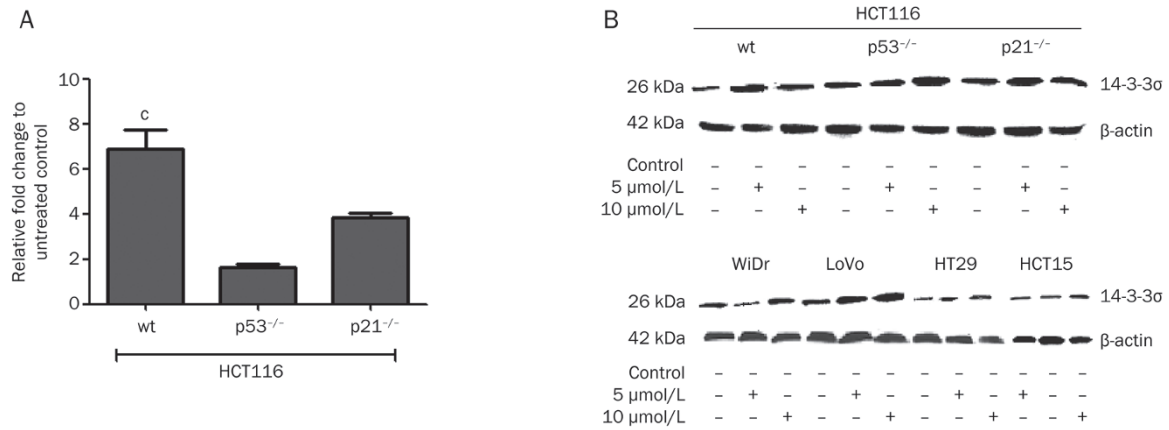
Figure 4. mRNA and 14-3-3σ protein levels following satraplatin treatment. (A) 14-3-3σ mRNA expression is markedly increased in HCT116 cells compared with isogenic p53^{-/-} and p21^{-/-} cells. Fold changes in this experiment are based on normalizing ACTB and B2M to untreated control cells. The results shown are representative of at least two independent experiments at 24 h. (B) 14-3-3σ protein levels indicated that the cells were arrested at the G_2/M phase. A marked increase in 14-3-3σ levels was also detected in p53^{-/-} cells, indicating an activation of cell cycle arrest in a p53-idependent manner at 48 h. The results shown are representative of at least two independent experiments. Equal loading was confirmed by β-actin detection. [c]$P<0.01$.
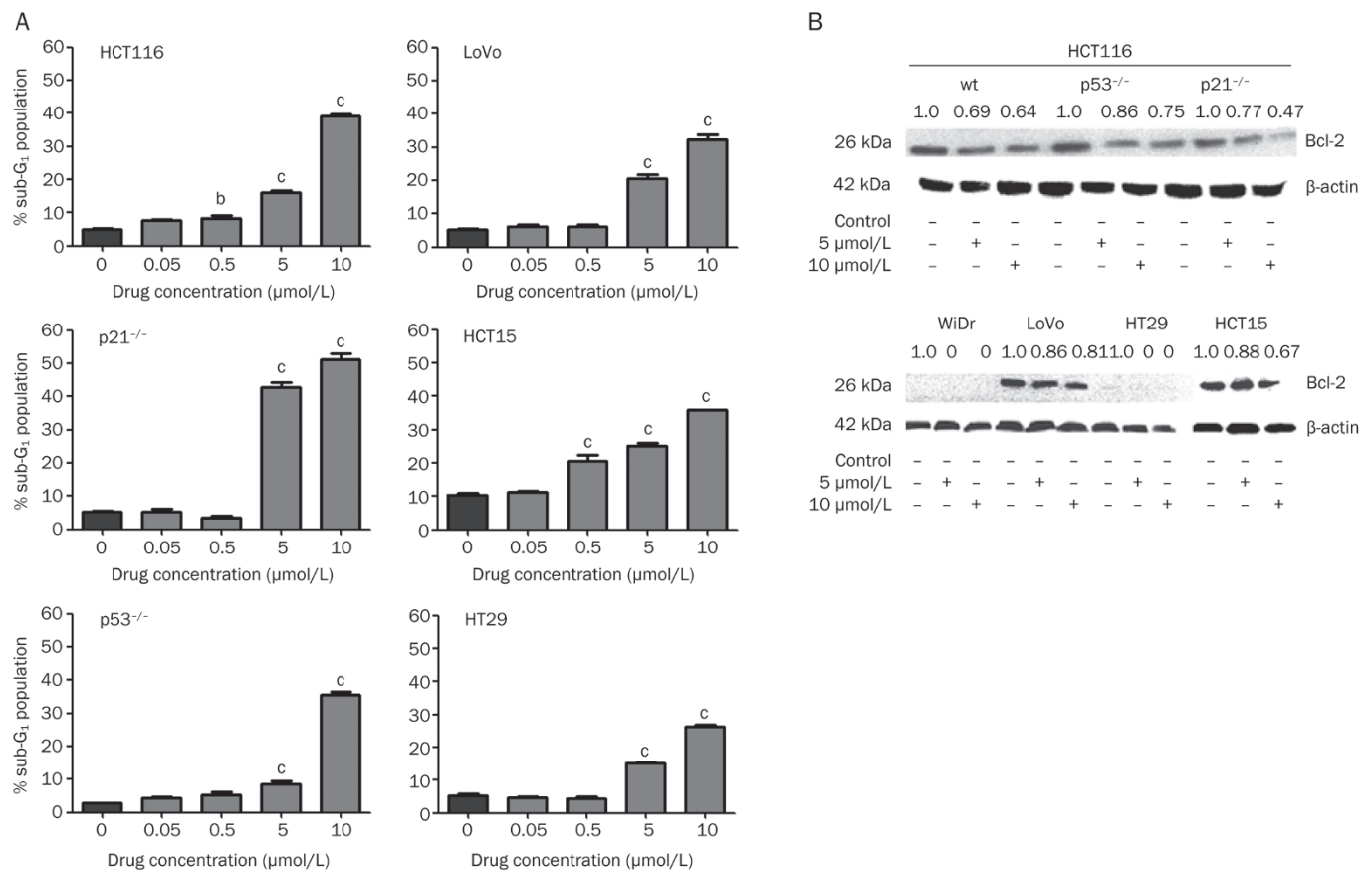

Figure 5. Dose dependent of Bcl-2 protein expression levels during satraplatin sustained treatment in CRC cells. A) Following treatment with different satraplatin concentrations, the CRC cell apoptotic fractions were measured by FACScan analysis. The bar indicates that a greater accumulation of apoptotic cells was observed with a clinically relevant satraplatin concentration *vs* the untreated control cells. Data points indicate the mean percentages of apoptosis fractions from three independent experiments; bars, standard deviation. B) The cellular extracts were subjected to Western blot. Satraplatin treatment resulted in markedly decreased Bcl-2 protein expression. The results shown are representative of at least two independent experiments. Equal loading was confirmed by β-actin detection. [c]$P<0.01$.
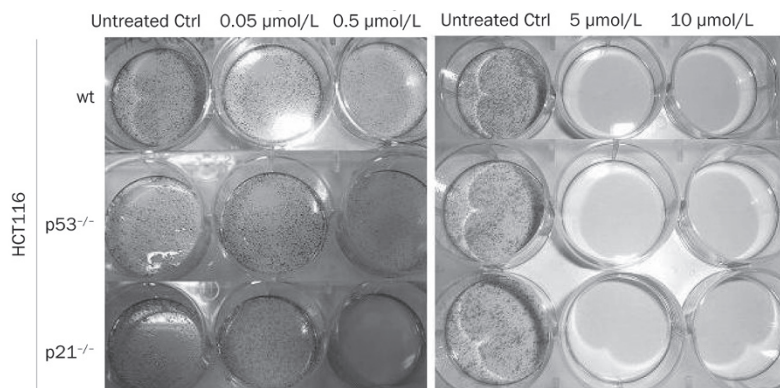
Figure 6. Clonogenicity loss following satraplatin induced DNA damage. Untreated- and satraplatin-treated cells were cultured in medium for two weeks and then fixed and stained with methylene blue for the clonogenicity assay. The results demonstrate that long-term satraplatin incubation leads to a loss of CRC cell clonogenicity potential.

G₂/M cell cycle arrest and apoptosis occurs in a p53-independent manner

To further elucidate the ability of satraplatin to perturb the G_2/M phase and subsequently apoptosis, we used a p53 signaling pathway qRT-PCR array containing 86 human p53 related genes. We compared the gene expression profiles of HCT116 (wt, p53$^{-/-}$, and p21$^{-/-}$) cells treated with 5 µmol/L satraplatin (Supplementary Table 2). We found that the cells responded independent of p53 activation and its related molecules. The up-regulation of Bax, BTG2, CDKN1A, FASLG, and MDM2 genes was observed in HCT116^{wt} cells but not in p53$^{-/-}$ and p21$^{-/-}$ cells. Moreover, we found that genes involved in DNA damage responses were also down-regulated together with the transcription factor NFKB1 (Supplementary Table 2).

Down-regulation of DNA damage repair genes following satraplatin induced apoptotic cells

To examine DNA damage repair gene activation, CRC cells were treated for 24 h with 5 µmol/L satraplatin and were tested for differential expression of mRNA encoding MLH1, MSH2, ERCC1, and PMS2. Interestingly, we found that all cells similarly responded to satraplatin treatment, with most of the DNA damage repair genes being down regulated, except for MSH1 and ERCC1 in HCT116^{wt} cells (Figure 7). We

also found that, based on our human p53 signaling pathway RT^2 Profiler PCR data, ATM and ATR expression was almost unchanged by 5 µmol/L satraplatin treatment in HCT116 (wt, p53$^{-/-}$, and p21$^{-/-}$) cells, whereas a 0.5-fold down-regulation was observed for E2F1 in HCT116^{wt} cells versus an up-regulation of 2.1-fold in the HCT116 p53$^{-/-}$ cells. Moreover, we found that XRCC5 expression was down-regulated in HCT116 (wt, p53$^{-/-}$, and p21$^{-/-}$) cells (Supplementary Table 2).

Discussion

Satraplatin, which was initially proven to have clinical activity against small-cell lung[17], ovarian[18], and prostate cancers[19], was developed for the treatment of castration-resistant prostate cancer and was found to significantly prolong disease progression-free survival[20]. Satraplatin disrupts DNA replication and transcription by forming intrastrand/interstrand DNA adducts; however, the downstream molecular events leading to G_2/M cell cycle perturbation and cell death have not been well characterized in CRC cells.

Here, we show that exposure of CRC cells to satraplatin reduced long-term clonogenic survival and induced cell cycle arrest and subsequently apoptosis. Our previous model suggested that the possible reasons for differences between oxaliplatin- and satraplatin-induced antitumor effectiveness might
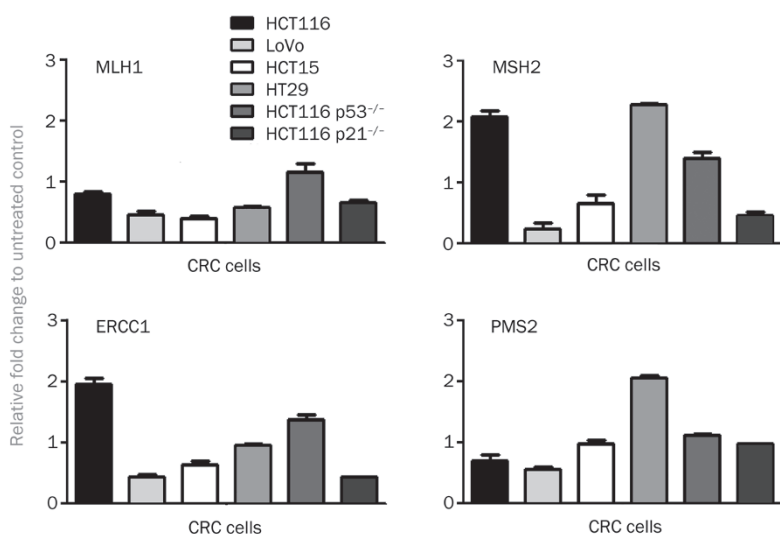

Figure 7. mRNA expression of the DNA repair enzymes ERCC1, MLH1, PMS2, and MSH2 in the CRC cell lines following 48 h satraplatin treatment. The CRC cells demonstrated unrepaired DNA damage owing to a down-regulation of the expression of DNA damage responsive genes in all cell lines tested. Only the expression of MSH1 and ERCC1 was up-regulated in the HCT116 wild-type cells. The fold change is based on the normalization of ACTB and B2M proteins in the treated vs the untreated control cells. The results shown are representative of at least two independent experiments.

be found at the level of cell cycle regulation[14]. Many studies have indicated that platinum agents predominantly reduce the DNA synthesis rate[4] and inhibit cell cycle progression at the G_1-S and/or G_2/M phases[21]. Using a BrdU incorporation assay, we found that the treatment of CRC cells with different satraplatin concentrations delayed the progression of cells in S phase and altered the G_2/M transition. Different drug concentrations perturbed the cells differently, particularly when cells were treated with lower drug concentrations. This finding could result from differences in cell characteristics that lead to different responses to treatment and the abilities of the drug to interact with its cellular target. More interestingly, the progression into mitosis was blocked. In addition, cells that were null for p53 and p21$^{waf1/cip1}$ also had a delayed cell cycle progression and G_2/M perturbation.

Since we observed a massive G_2/M phase arrest, we focused our analysis on p53–p21 involvement in the G_2/M transition as p53–p21 being a major regulator of the cell cycle. We found that satraplatin induced a significant accumulation of p53 protein in wild-type HCT116 and LoVo cells. In contrast, an accumulation of mutant p53 was also observed by Western blotting in HCT15 and WiDr cells. Bunz *et al* have reported the role of p53 at the G_2 checkpoint by inactivating the p53 gene with homologous recombination in a derivative of a human colorectal tumor model[12]. They demonstrated that few cells entered mitosis initially after exposure to ionizing radiation, which suggests that the G_2 checkpoint is intact. Therefore, they proposed that p53 is not required for the initial arrest but is essential for long-term arrest maintenance. However, it is possible that p53 can partially contribute to the initial arrest, and it is clear that other pathways can cause arrest when p53 is missing[12].

We detected a marked increase in cyclin B1, a marker for G_2/M cell arrest, in all CRC cell lines tested, and this was independent of the p53 gene status. Our data suggest that p53-independent pathways could inhibit cdc2 activity in response to DNA damage. In relation to this hypothesis and by following a candidate approach, we found that satraplatin increased the expression of 14-3-3σ, a conserved phosphoserine binding protein in CRC cells. We noticed a similar alteration even in p53-null and mutant cells, which further delineates the action of this protein in a p53-independent manner. We believed that 14-3-3σ may become a key regulatory modulator in the G_2/M checkpoint that may form a cytoplasmic complex with cdc2 and sequester cdc2/cyclin B1 in the cytoplasm. Hermeking *et al* have shown that 14-3-3σ overexpression in HCT116 CRC cells causes most cells to arrest in the G_2 phase[22]. Thus, we assumed that satraplatin-induced 14-3-3σ contributes to the G_2/M accumulation. We also observed that, following satraplatin-induced cell cycle perturbation, the cells eventually undergo apoptosis in a dose- and time-dependent manner. We have reported that satraplatin treatment induces the accumulation of apoptotic cells even in the p53-null model, thus overcoming chemoresistance by a p53-independent mechanism[14]. Generally, the protection of cells against apoptosis requires the down-regulation of proapoptotic activities or the up-regulation of anti-apoptotic mechanisms[23]. The Bcl-2 family is an important regulator of cellular apoptosis[24]. Thus, we investigated Bcl-2 involvement in the mitochondrial (intrinsic) pathway to determine whether the Bcl-2 family was functioning as upstream initiators of apoptosis. Notably, the satraplatin effects on Bcl-2 expression in CRC cells (HCT15^{mut}, LoVo^{wt}, and WiDr^{mut} and p21$^{-/-}$) were consistent with our previous findings in HCT116 (wt and p53$^{-/-}$) and HT29^{mut} cells[14]. However, these effects did not only affect Bcl-2-dependent apoptosis because the mutant HT29 and Lovo cells significantly underwent apoptosis, which further suggests that CRC cells could also undergo Bcl-2-independent apoptosis following satraplatin treatment. In addition to the Bcl-2-apoptosis analysis, we also demonstrated with the long-term clonogenicity survival analysis that the cells lose the ability to form colonies. Remarkably, we found that all cells, particularly the p53$^{-/-}$ and p21$^{-/-}$ cells, lost their clonogenicity potential in a dose-dependent manner. Finally, satraplatin mediated apoptotic activity is believed to be linked to DNA damage, and the levels of DNA damage repair genes have been shown to be correlated with the response to the platinum agent[4]. We hypothesized that the DNA damage inflicted by satraplatin may be repaired by the nucleotide excision repair (NER) and/or the mismatch repair (MMR) pathways with kinetics similar to that of the DNA damage repair that is induced by cisplatin and oxaliplatin[25, 26]. Accordingly, we showed that the mRNA expression levels of ERCC1, MLH1, MSH2, and PMS2 were significantly down-regulated in CRC cells. In addition to these results, Vaisman *et al* and Fokkema *et al* have suggested that satraplatin-induced adducts, compared with adducts formed by other platinum drugs, do not bind to high mobility group 1 protein, which recognizes the DNA damage caused by cisplatin and inhibits translation replication by certain DNA polymerases. These differences may provide a mechanism by which some platinum resistance may be overcome by satraplatin treatment in this model[27, 28].

Thus far, *in vitro* and *in vivo* data have indicated satraplatin efficacy in the treatment of various cancer models[7, 14]. However, the ability of satraplatin to induce G_2/M cell cycle arrest and subsequently apoptosis in CRC cells suggests that it may have a wide range of clinical applications. In Figure 8, we illustrated the molecular mechanism of satraplatin action on cell cycle perturbation. In summary, we hypothesize that satraplatin-induced cell cycle arrest is likely executed via 14-3-3σ in a p53-p21$^{waf1/cip1}$-independent manner (Figure 8). Furthermore, cell death may also be related to the reduced expression of DNA repair enzymes.

In conclusion, the ability of satraplatin to induce different modes of cell cycle regulation and cell death in different types of CRC cells at relatively low concentrations indicates that it may be highly efficacious in killing tumor cells and warrants further *in vivo* clinical investigation.
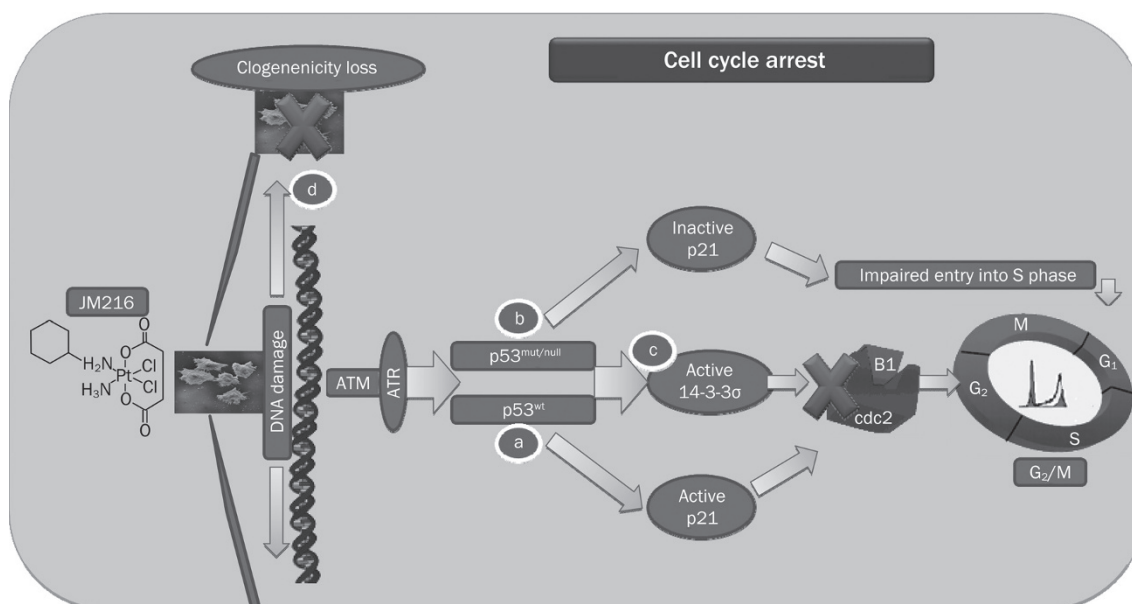
Acknowledgements

Figure 8. A flow chart depicting how 14-3-3σ induces cell cycle arrest at G_2/M, which may not rely on $p21^{waf1/cip1}$ and p53-dependent pathway in CRC cell models following satraplatin treatment. (A) Upon DNA damage caused by satraplatin treatment, we assumed that the ATM and ATR genes are activated, which induces the downstream signaling of cell cycle and cell death modulators. This results in p53 accumulation, which induces the expression of its primary cell cycle modulator, $p21^{waf1/cip1}$, an inhibitor of Cdks, and its activation sequesters cyclin B1/cdc2 complexes, which can lead to an accumulation of cells at the G_2/M transition. Alternatively, p53 could also be activated through 14-3-3σ (a conserved phosphoserine binding protein) to induce an accumulation of cells at G_2/M by sequestering the cyclin B1/cdc2 complexes. (B) In cells that lack p53 or contain mutant p53, we observed that there was also a G_2/M perturbation that was independent of $p21^{waf1/cip1}$ activation because we first identified impaired S phase entry and then identified arrest at G_2/M. (C) These cells might also activate 14-3-3σ independently of p53 action because $p53^{mut}$ and $p53^{null}$ cells demonstrated an elevated expression of 14-3-3σ and subsequently arrested at G_2/M. (D) Loss of clonogenicity patterns were observed following satraplatin induced DNA damage, which led to cell mortality.

Author contribution

Supplementary information

Supplementary tables are available at Acta Pharmacologica Sinica website of NPG.

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