

## RESEARCH PAPER

## Oxaliplatin responses in colorectal cancer cells are modulated by CHK2 kinase inhibitors

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**Background and purpose:** Checkpoint kinase 2 (CHK2) is activated by DNA damage and can contribute to p53 stabilization, modulating growth arrest and/or apoptosis. We investigated the contribution of CHK2 to oxaliplatin-mediated toxicity in a colorectal cancer model.

**Experimental approach:** We evaluated the ability of CHK2 small molecule inhibitors to potentiate oxaliplatin-induced toxicity. The role of CHK2 in oxaliplatin-induced apoptosis was investigated in HCT116 cells that were wild-type (WT) or KO for CHK2. Small molecule inhibitors of CHK2 were used in combination studies with oxaliplatin in this cell model.

**Key results:** In oxaliplatin-treated CHK2 KO cells, accelerated apoptosis was accompanied by attenuated p53 stabilization and p21<sup>WAF-1</sup> up-regulation correlating with increased Bax expression, cytochrome c release and elevated caspase activity. The higher levels of apoptosis in CHK2 KO cells were restored to control (WT) levels when CHK2 was re-introduced. This 'uncoupling' of p53 stabilization and Bax up-regulation in CHK2 KO cells suggested oxaliplatin-induced apoptosis was due to a p53-independent response. Combination studies revealed that CHK2 inhibitor II or debromohymenialdisine antagonized the responses to oxaliplatin. This inhibitory effect correlated with decreases in apoptosis, p53 stabilization and DNA inter-strand cross-link formation, and was dependent on the presence (but not activity) of CHK2.

**Conclusions and implications:** Combinations of CHK2 inhibitors with oxaliplatin should further sensitize cells to oxaliplatin treatment. However, these inhibitors produced an antagonistic effect on the response to oxaliplatin, which was reversed on the re-introduction of CHK2. These observations may have implications for the use of oxaliplatin in colorectal cancer therapy in combination with therapies targeting CHK2.

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**Keywords:** CHK2 inhibitor II; DBH; apoptosis; combination index; DNA cross-links; colorectal cancer

**Abbreviations:** ATM, ataxia telangiectasia mutated; ATR, ATM and rad3 related; Bcl-2, B-cell lymphoma 2; CHK1, checkpoint kinase 1; CHK2, checkpoint kinase 2; CI, combination index; CRC, colorectal cancer; DAPI, 4'-6'-diamino-2-phenylindole di-hydrochloride; DBH, debromohymenialdisine; DSB, double-strand break; IR, ionizing radiation; MRE11, meiotic recombination 11; MRN, MRE11 RAD50-NBS1 complex; NBS1, Nijmegen breakage syndrome; SMI, small molecule inhibitors; SRB, sulforhodamine-B; WT, wild-type

## Introduction

Colorectal cancer (CRC) is the second most common cancer in the Western hemisphere. While over 90% of patients are treated by surgical resection, nearly 50% relapse with recurrent disease and then undergo extensive chemotherapy

(Nelson *et al.*, 2001). Oxaliplatin is a third-generation platinum (Pt) compound that is as efficient as cisplatin at a lower metal dose than carboplatin (Mathe *et al.*, 1989). Oxaliplatin displays anti-tumour activity in a range of tumour types, including those intrinsically resistant to the first- and second-generation platinum compounds (Kelland, 2007). Oxaliplatin is a diaminocyclohexane carrier ligand-Pt complex that interacts with protein, RNA and DNA (Rabik and Dolan, 2007) leading to the formation of bulky mono-DNA adducts (Scheeff *et al.*, 1999) and intra- and inter-strand DNA cross-links. Oxaliplatin treatment also produces high levels of single- and double-strand breaks (DSBs) in DNA due to replication fork collapse and nuclease attack at the site of platinumated cross-links. Oxaliplatin is currently used in various combination schedules principally with 5-fluorouracil/

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leucovorin in advanced CRC. While response rates and progression-free survival has improved, efficacy is still limited and new therapies are urgently needed. Hence, a number of novel molecular targeted agents are currently being investigated in CRC, alone or in combination with standard chemotherapy (Saif *et al.*, 2006; Wils, 2007).

Conserved and coordinated molecular responses to DNA damage serve to delay cell cycle progression and allow damage repair and/or induction of apoptosis to prevent fixation of DNA mutations in the genome (Roos and Kaina, 2006). Two pathways that are activated following DNA damage involve ataxia telangiectasia mutated (ATM) and/or ATM and rad3 related (ATR) and result in the subsequent phosphorylation of their respective downstream targets checkpoint kinase (CHK)1 and CHK2. CHK1 is activated following impairment of replication progression while CHK2 responds to DNA DSBs (Bartek and Lukas, 2003). Such phosphorylation events are not mutually exclusive, and considerable crosstalk between the ATM-CHK2 and ATR-CHK1 pathways exists (Cuadrado *et al.*, 2006; Zaugg *et al.*, 2007).

Checkpoint kinase 2 may represent a potential target for anti-cancer therapy due to its functions in promoting p53-dependent cell cycle arrest, DNA repair and both p53-dependent and independent apoptosis (Pommier *et al.*, 2006). Combining CHK2 inhibitors with chemotherapeutic agents that damage DNA is therefore an attractive proposition (Antoni *et al.*, 2007). Several small molecule inhibitors (SMIs) of CHK1/2 are already in preclinical testing (CHIR124) or in phase I trials (XL844, PF-00477736, AZD7762) (Bucher and Britten, 2008).

In the present study, we investigated the effect of two SMIs of CHK2, debromohymenialdisine (DBH) and CHK2 inhibitor II, on the response of CRC cells to oxaliplatin. DBH was isolated from the marine sponge *Stylissa flabeliformis*, inhibits both CHK1 and CHK2, and prevents ionizing radiation (IR)-induced G<sub>2</sub> arrest and potentiates mitotic catastrophe-induced cell death (Curman *et al.*, 2001; Castedo *et al.*, 2004). CHK2 inhibitor II, a 2-arylbenzimidazole-based CHK2 inhibitor identified using a structure-based approach with a CHK2 homology model, demonstrates high selectivity against CHK2 (Arienti *et al.*, 2005). The effect of oxaliplatin on CRC cells alone and in combination with these two SMIs of CHK2 kinase was assessed. An isogenic pair of HCT116 colon carcinoma cell lines that do and do not express CHK2 were compared with assess this combination approach and to identify potential 'off target' effects of the CHK2 inhibitors.

## Methods

### Cell culture

HCT116 KO CHK2 and p53 KO cell lines and their respective wild-type (WT) controls (Jallepalli *et al.*, 2003) were kindly provided by Professor Bert Vogelstein (The Johns Hopkins University School of Medicine). Cells were maintained in McCoy's medium supplemented with 10% FBS, at 37°C and in 5% CO<sub>2</sub> in air. Cells were negative for mycoplasma infection.

Cells were transfected transiently using lipofectamine 2000 as described in the manufacturer's instructions. Gene expres-

sion was monitored by western blotting and optimum protein expression was observed 48 h post transfection.

### Drug treatments and irradiation

Stock solutions of oxaliplatin and cisplatin were prepared in sterile MilliQ water. Stock solutions of the CHK inhibitors were prepared in DMSO. Cells were exposed to 40 µM oxaliplatin either for a 1 h (pulse treatment) or for the duration of the experiment (continuous), unless otherwise noted. CHK inhibitors were added to cells 24 h prior to treatment with oxaliplatin and maintained throughout the remainder of the experiment. In some experiments irradiation (10 Gy) was applied using a 320 kV X-ray system (Gulmay Medical Ltd., Camberley, UK), at a dose rate of 1.37 Gy·min<sup>-1</sup>.

### Measurement of apoptosis

The percentage of apoptotic cells present within a culture was determined by harvesting both adherent and floating cells and staining with the fluorescent nuclear stain 4'-6'-diamino-2-phenylindole di-hydrochloride (DAPI); 200 cells were scored per sample and those exhibiting classic condensed and fragmented nuclear morphology defined as apoptotic. Caspase substrate-specific fluorogenic assays were used to determine the amount of activated caspases-2, 3 and 9 present before and following drug treatments. Triplicate samples of whole cell protein lysates (50 µg) were added to each well of a 96-well plate, incubated with 250 µM fluorogenic caspase substrate (VDVAD-AFC for caspase-2, Ac-LEHD-AFC for caspase-9 and Ac-DEVD-AMC for caspase-3) for 1–1.5 h at 37°C in the dark. Fluorescence was measured using a Fluostar optima plate reader (BMG LABTECH, Offenburg, Germany).

### Western blotting

Protein expression was determined by standard western blot protocols as previously described (Sambrook and Russell, 2001). Primary antibodies were CHK2, PARP and phospho-p53 Ser20, actin, GAPDH, cytochrome c, Bax-N20, aldolase-N15 and VDAC1-N18, p53 ab6 and p21. Detection was achieved using the HRP-conjugated secondary antibodies. Bands were visualized using an advanced chemiluminescence kit and quantified using a LAS-1000 plus imaging system (FujiFilm, Tokyo, Japan) and AIDA Bio software (Raytest Bio-imaging, Straubenhardt, Germany).

### Clonogenic survival

Exponentially growing cells were plated in six-well plates and seeded at a number that would create at least 200 colonies. Cells were then exposed to drug for either 1 h or continuously. After 10–15 days the colonies were washed and fixed with 70% methanol and subsequently stained with 1% methylene blue and scored. The surviving fraction was calculated and survival curves constructed.

### Growth inhibition

Cell lines were seeded at between 300 and 500 cells per well into 96-well plates and allowed to attach overnight at 37°C

before the addition of drugs. The degree of growth inhibition induced by these treatments was subsequently determined using the sulforhodamine-B (SRB) colorimetric assay (Rubinstein *et al.*, 1990) following 5 days of exponential growth. Growth inhibition curves were then constructed and  $IC_{50}$  values determined.

#### Combination index (CI)

Analysis of combination drug studies using conventional cytotoxics and CHK kinase inhibitors were performed using the CI method described by Chou and Talalay (1984). Cells were exposed to the prescribed SMI for 24 h prior to a 1 h pulse treatment with either oxaliplatin alone, inhibitor alone or fixed ratio combinations of both. Following drug treatment, fresh media plus inhibitor was added. Cells were then incubated for a further 48 h before fixing and staining with SRB. Determination of the CI values calculated at the  $IC_{50}$ ,  $IC_{75}$  and  $IC_{90}$  level of growth inhibition was carried out using the Calcsyn software package (v2.0, Biosoft, Cambridge, UK). A derived CI value of 1 ( $CI = 1$ ) indicates an additive effect while a  $CI < 1$  indicates synergy and  $CI > 1$  indicates antagonism respectively.

#### Subcellular fractionation

Cell cultures, including floating cells, were harvested and centrifuged for 5 min at  $100\times g$  and washed once with ice-cold phosphate-buffered saline. Samples were centrifuged at  $600\times g$  for 5 min at  $4^{\circ}C$  and the supernatant removed. The cell pellet was resuspended in isotonic buffer (10 mM HEPES pH 7.4, 0.22 M mannitol, 68 mM sucrose, 2.5 mM  $KH_2PO_4$ , 2 mM NaCl, 2 mM  $MgCl_2$  and 0.5 mM EGTA), containing a cocktail of protease inhibitors (0.1% v/v) and 0.1 mM PMSF. Cell suspension was homogenized on ice using a Dounce homogenizer. Mitochondria were resuspended in kinase buffer (50 mM Tris pH 7.5, 50 mM NaF, 10 mM b-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, 0.1 mM PMSE, 0.1%  $NaVO_4$  and 0.1% protease inhibitor cocktail. Samples were snap-frozen in liquid nitrogen and kept at  $-80^{\circ}C$ .

#### Comet-X assay

The comet-X assay was performed as described previously (Ward *et al.*, 2005). This assay is a semi-quantitative method of determining DNA inter-strand cross-links in single cells following embedding in agarose and alkaline unwinding and electrophoresis. The assay requires the introduction of a fixed number of DSBs (by IR exposure) into the DNA of cells that have been treated with a cross-linking agent. Cross-linking is expressed as a decrease in the fraction of DNA in the comet tail compared with irradiated only control cells (Ward *et al.*, 2005).

#### Statistical analysis

Differences between two sets of data were assessed by using Student's *t*-test. Statistical significance was assumed if  $P < 0.05$ .

#### Drugs and materials

Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA); oxaliplatin from Alexis (San Diego, CA, USA) and

cisplatin from Sigma (St. Louis, MO, USA). The CHK inhibitors, and VDVAD-AFC, Ac-LEHD-AFC and Ac-DEVD-AMC were obtained from Calbiochem (San Diego, CA, USA). The primary antibodies: CHK2 was from Neomarkers (Fremont, CA, USA); PARP and phospho-p53 Ser20 from Cell Signalling Technology (Boston, MA, USA); actin from Sigma; GAPDH from Abcam (Cambridge, MA, USA); cytochrome *c* from BD Biosciences (NJ, USA); Bax-N20, aldolase-N15 and VDAC1-N18 from Santa Cruz Biotech (Santa Cruz, CA, USA); p53 ab6 and p21 from Calbiochem (San Diego, CA, USA). The HRP-conjugated secondary antibodies were from Dako (Cambridge, UK) and the advanced chemiluminescence kit was from Perkin Elmer (Waltham, MA, USA). Sulforhodamine colorimetric assay and the protease inhibitors were obtained from Sigma (St. Louis, MO, USA).

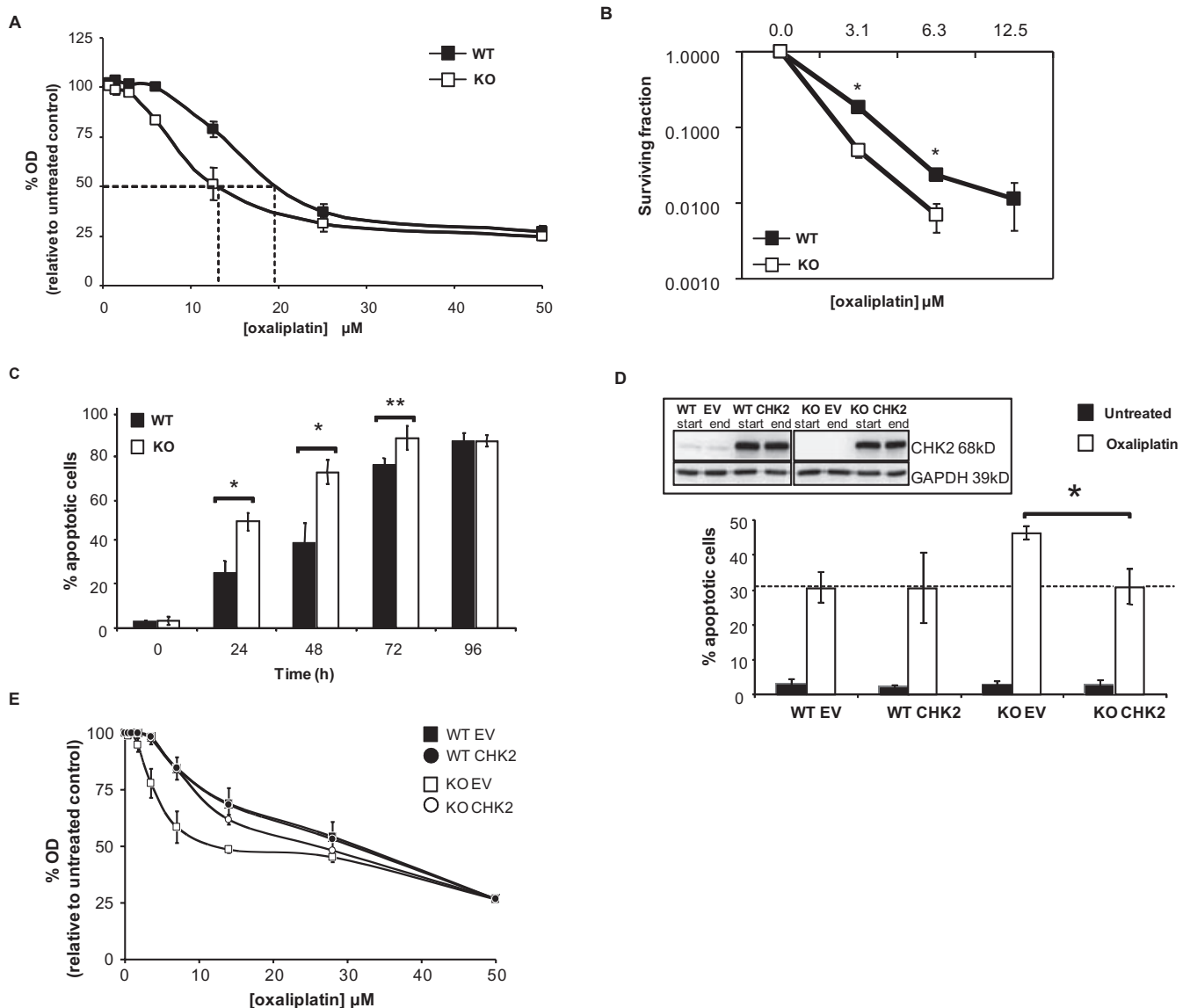
## Results

#### Sensitivity to oxaliplatin: growth inhibition and cell survival

A 1 h exposure to oxaliplatin led to a significantly greater growth inhibition of the CHK2 KO cell line compared with WT ( $P < 0.05$ ;  $IC_{50}$  14  $\mu M$  and 19  $\mu M$ , respectively; Figure 1A). Clonogenic assays following an 8 h oxaliplatin treatment also showed that the CHK2 KO cells were significantly more sensitive to oxaliplatin than the WT cells ( $P < 0.005$ ;  $IC_{50}$  6  $\mu M$  and 12  $\mu M$ , respectively; Figure 1B).

#### Apoptosis

The levels of apoptosis following continuous exposure of WT and CHK2 KO HCT116 cells to oxaliplatin are shown in Figure 1C. In untreated controls, a basal level of apoptosis (3%) was seen over 96 h in both cell lines. Following 24 h of treatment with oxaliplatin, the levels of apoptosis increased in both the WT and CHK2 KO cell lines up to 96 h post treatment. The WT cells consistently showed a twofold lower level of apoptosis than the CHK2 KO cells after 24–72 h of treatment ( $P < 0.01$ ). However, after 96 h the WT and KO cell populations achieved identical levels of apoptosis (85%). Therefore, the lack of CHK2 resulted in an accelerated rate of apoptosis. To confirm that the accelerated apoptosis was a CHK2-dependent response to oxaliplatin, CHK2 was re-introduced to the KO cells by transient transfection. For a more valid comparison, CHK2 was also transfected into WT HCT116 cells thus inducing an overexpression of CHK2 in these two cell lines to an equivalent degree. Two days post transfection, cells were exposed to oxaliplatin for 24 h. The analysis of the subsequent induction of apoptosis is shown in Figure 1D. Expression levels of CHK2 (exogenous and endogenous) were monitored by western blotting at the start and end of treatment (Figure 1D, inset). Following oxaliplatin treatment, approximately 30% of both CHK2 WT cells transfected with the empty vector control and WT CHK2 showed levels of apoptosis similar to untransfected WT cells. CHK2 KO cells transfected with the empty vector alone exhibited a higher level of apoptosis (approximately 45%) than the corresponding transfected WT cells, consistent with the increased sensitivity of these cells. However, when CHK2 was overexpressed in the KO cells, a reversion to the WT

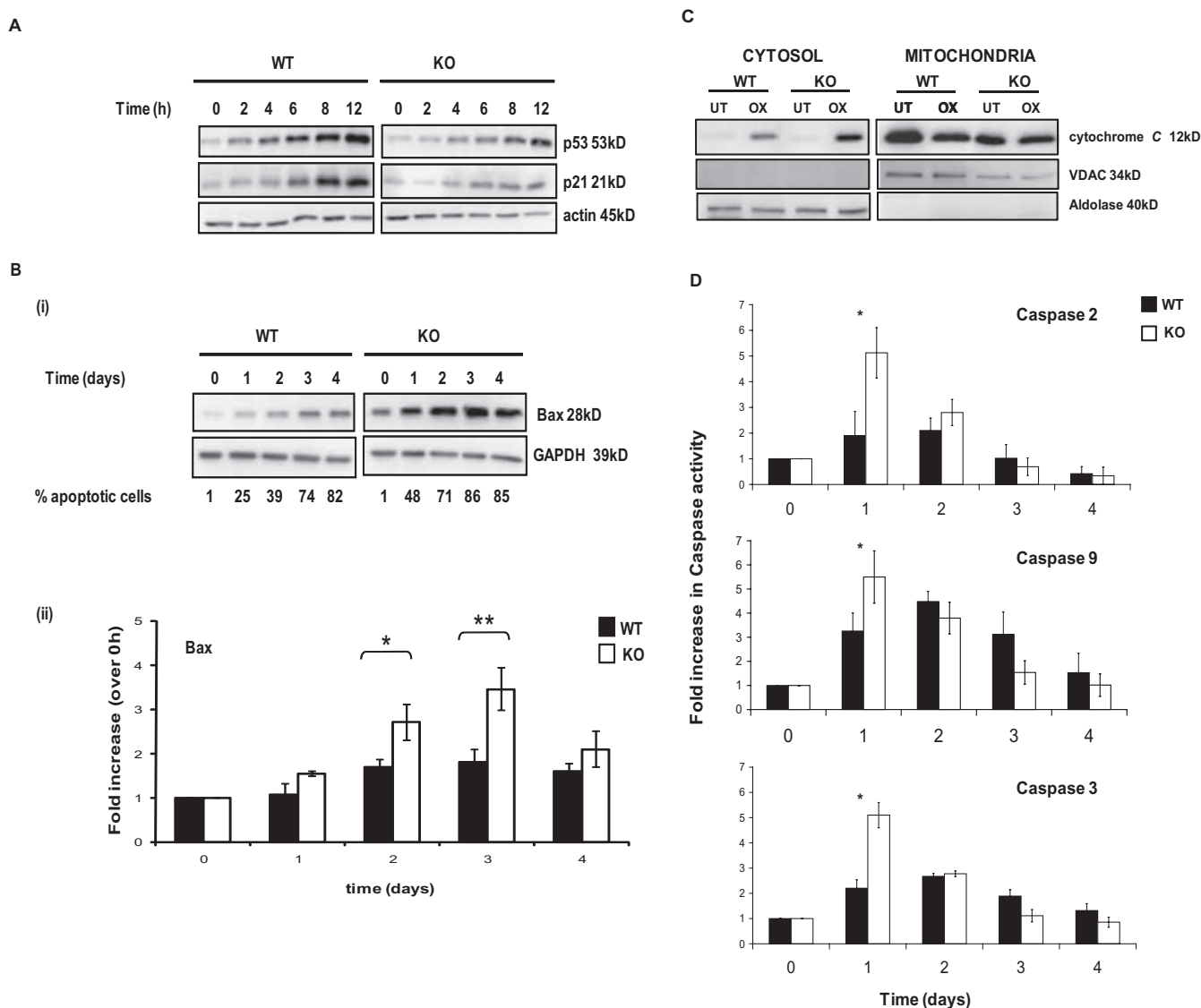


**Figure 1** Characterization of the effect of oxaliplatin on HCT116 checkpoint kinase 2 (CHK2) wild-type (WT) and KO cell lines. Responses of HCT116 CHK2 WT and CHK2 KO to treatment with oxaliplatin for 1 h. (A) Sulforhodamine-B (SRB) concentration–response curves, dashed lines indicate the  $IC_{50}$  doses. (B) Clonogenic survival curves. (C) Oxaliplatin-induced apoptosis kinetics for the CHK2 WT and CHK2 KO following 40  $\mu$ M continuous treatment with oxaliplatin. Data represent the percentage of apoptotic cells based on DAPI (4'-6'-diamino-2-phenylindole di-hydrochloride) stained nuclear morphology (condensation and fragmentation). (D) CHK2 WT or CHK2 KO cells were transfected with either empty vector (EV) or CHK2-expressing vector (CHK2) then exposed continuously to 40  $\mu$ M oxaliplatin or to vehicle control for 24 h. The percentages of apoptotic cells were determined as in (C). The data represented in (A–D) are the average of three independent experiments,  $\pm$ SE. \* $P$  < 0.05 and \*\* $P$  < 0.01, Student's  $t$ -test. Inset: western blot, representative of three independent experiments, showing expression of CHK2 in cell lines following transfection, just prior to oxaliplatin treatment (start) and 24 h following (end) of treatment. GAPDH was used as a loading control. (E) SRB concentration–response curves of HCT116 CHK2 WT and CHK2 KO transfected with either an EV or CHK2-expressing vector (CHK2) following treatment with oxaliplatin for 1 h (dashed lines indicate the  $IC_{50}$  doses).

sensitivity was observed. Levels of apoptosis reached 30%, compared with the empty vector-transfected KO cells ( $P$  < 0.05; Figure 1D). Therefore, the observed increased rate of apoptosis of CHK2 KO cells following oxaliplatin treatment was casually linked to the absence of CHK2. These observations were further supported by growth inhibition studies (Figure 1E). Oxaliplatin-mediated inhibition of growth in the KO empty vector-transfected cells was reversed in KO transfected CHK2 cells to similar levels to those observed in WT (empty vector- or CHK2-transfected) cells.

#### p53 stabilization, Bax levels and caspase activity

Consistent with the role of CHK2 as a p53 regulatory kinase, we observed a rapid increase in p53 expression in CHK2 WT cells 2 h following oxaliplatin treatment (Figure 2A). However, the onset of p53 stabilization in CHK2 KO cells compared with WT was delayed (8 h). Moreover, increased expression of the p53 transcriptional target and cyclin-dependent kinase inhibitor p21<sup>WAF-1</sup> was evident in WT cells by 6 h but only weakly expressed after 12 h in the CHK2 KO cells (Figure 2A).



**Figure 2** Kinetics of apoptosis of HCT116 wild-type (WT) and checkpoint kinase 2 (CHK2) KO cells induced by oxaliplatin. (A) Cells were continuously exposed to 40  $\mu$ M oxaliplatin for up to 12 h. Western blots (50  $\mu$ g protein per lane) show the kinetics of p53 stabilization and up-regulation of p21<sup>WAF-1</sup>. (B) (i) Western blots of HCT116 WT and CHK2 KO cells following continuous exposure to oxaliplatin for up to 4 days. (ii) Densitometry analysis of blots depicted in B (i). (C) Western blots showing cytochrome *c* levels in cytosolic and mitochondrial fractions from WT or CHK2 KO cells treated with 40  $\mu$ M oxaliplatin (OX) for 2 days. GAPDH was used as a loading control. Validation of purity and loading were confirmed using VDAC for the mitochondrial fraction and aldolase for the cytosolic fraction. (D) Fluorogenic measurement of active caspase-2, 3 and 9 levels in HCT116 WT and CHK2 KO cells during a 4 day continuous challenge with 40  $\mu$ M oxaliplatin. Blots are representative of three independent experiments. Actin was used as a loading control; error bars represent  $\pm$ SE. \* $P$  < 0.05 and \*\* $P$  < 0.025, Student's *t*-test.

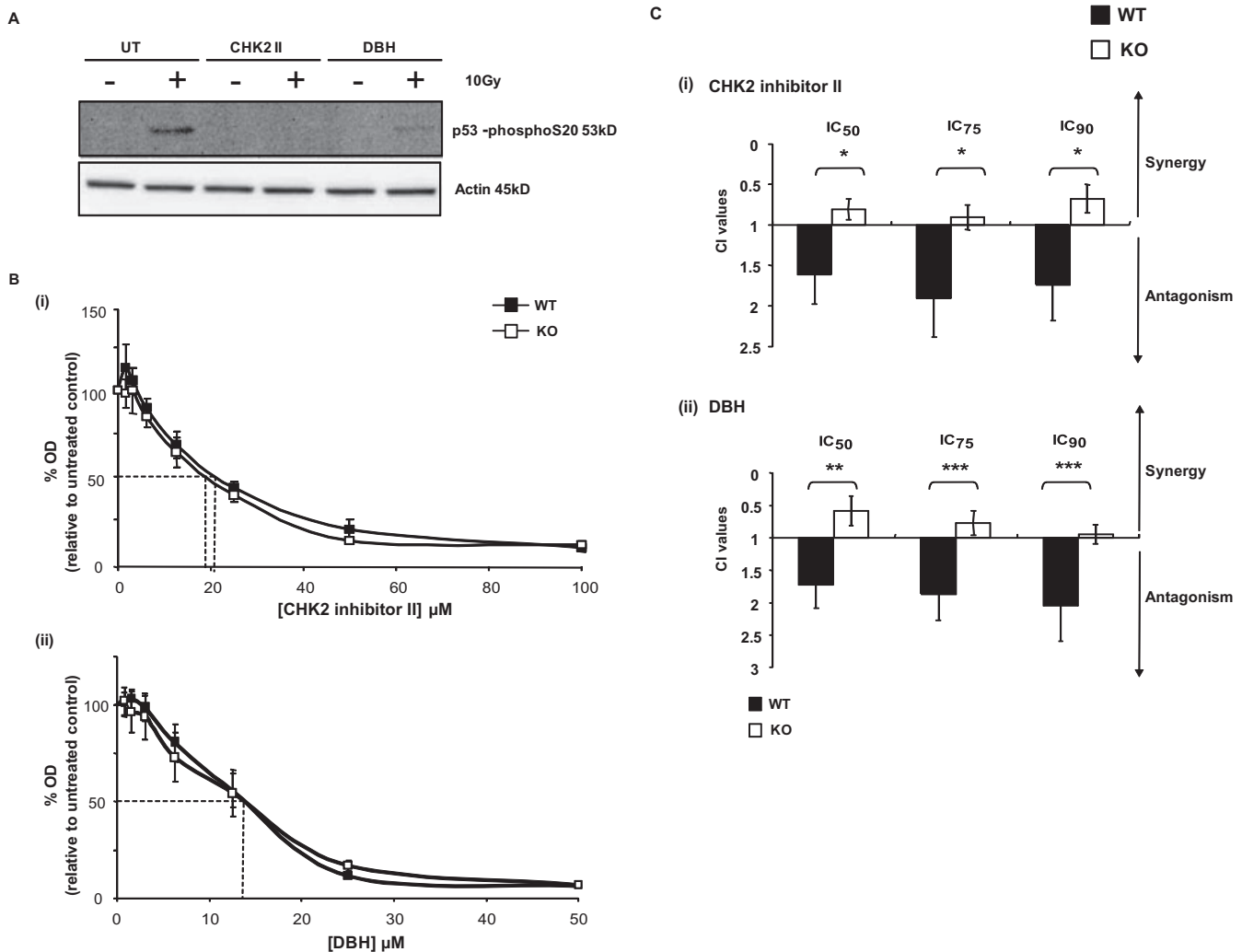
Analysis of the expression levels of several pro- and anti-apoptotic Bcl-2 (B-cell lymphoma 2) family members was conducted before and up to 4 days after oxaliplatin treatment to explore possible mechanisms whereby CHK2 KO cells were sensitized to oxaliplatin. The only striking observation was the up-regulation of the p53 target Bax; this occurred 1–4 days after oxaliplatin exposure in both WT and CHK2 KO cells. The magnitude of the increase in Bax was, however, consistently greater in the KO cells [Figure 2B (i)], correlating with the accelerated kinetics of apoptosis. The difference in the fold increase in Bax for WT and CHK2 KO

cells was significant at both day 2 and day 3 post treatment [ $P$  < 0.005; Figure 2B (ii)].

Checkpoint kinase 2 KO cells showed an increased release of cytochrome *c* from the mitochondria into the cytosol 48 h post drug treatment, when compared with WT cells (Figure 2C, left panel) consistent with an accelerated apoptotic response in these cells.

The levels of the activated initiator caspase-9 and the effector caspase-3 were measured to characterize further this drug-induced response. In addition activated caspase-2 levels were measured, as a number of studies have shown that caspase-2





**Figure 3** Effect of the checkpoint kinase inhibitors checkpoint kinase 2 (CHK2) inhibitor II and debromohymenialdisine (DBH) on the responses of the different cell lines to oxaliplatin. (A) Validation of CHK2 inhibition by CHK2 inhibitor II and DBH. CHK2 wild-type (WT) and CHK2 KO cells, either untreated (UT) or treated (CHK2II, DBH) were subsequently exposed to 10 Gy of ionizing radiation. Cells were harvested 30 min later and subjected to western blotting to determine the phosphorylation status of serine 20 on p53. Western blots are representative of two independent experiments and actin was used as a protein loading control. (B) Effect of a 72 h continuous exposure to either CHK2 inhibitor II (i) or DBH (ii) on CHK2 WT and KO cells. Cell population growth was determined using the sulforhodamine-B (SRB) assay as described and data plotted as a percentage of untreated controls. Data points are the average of three independent experiments error bars represent  $\pm$  SE. Dashed lines indicate the  $IC_{50}$  doses. (C) The Chou and Talalay combination index (CI) values following fixed drug concentration ratios of oxaliplatin and either (i) CHK2 inhibitor II (CHK2 II) or (ii) DBH. CI values are representative of the  $IC_{50}$ ,  $IC_{75}$  and  $IC_{90}$  effective doses. WT and CHK2 KO were treated for 1 h with oxaliplatin; inhibitors were added 24 h before and continuously following oxaliplatin treatment. Data shown are for the average of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.005$ , Student's *t*-test.

is activated as an early event following DNA damage (Zhivotovskiy and Orrenius, 2005).

The levels of all three active caspases were at a maximum 24 h following oxaliplatin treatment. However, the absolute levels were higher in CHK2 KO cells (Figure 2D). Taken together these results consistently indicate both an increase in the rate and levels of oxaliplatin-induced apoptosis in the absence of CHK2.

#### Effect of small molecule CHK inhibitors

Given the increased sensitivity of the CHK2 KO cells to oxaliplatin, combinations of CHK2 inhibitors with oxaliplatin might be expected to be synergistic. To test this hypothesis,

the effects of two SMIs of CHKs, a CHK2-specific inhibitor (CHK2 inhibitor II) and a CHK1/2 inhibitor (DBH), were investigated alone and in combination with oxaliplatin. In these experiments, the CHK2 KO cell line serves to inform on the off-target effects of these inhibitors.

The phosphorylation status of Ser20 of p53 was examined in order to validate inhibition of CHK1 and/or CHK2 by the inhibitors in this cell model. This phosphorylation event is a well-established consequence of CHK1 and CHK2 activation (Shieh *et al.*, 1999; Chehab *et al.*, 2000; Hirao *et al.*, 2000). Consequently, following 24 h treatment at the  $IC_{50}$  concentration of each inhibitor alone, both CHK2 KO and WT cells were subjected to 10 Gy of IR and the phosphorylation status of p53 at Ser20 measured (Figure 3A). As predicted, p53 was

phosphorylated after irradiation in the absence of any inhibitor treatment. In the presence of CHK2 II, Ser20 phosphorylation was not detected, whereas further but not complete reduction of Ser20 phosphorylation was observed following DBH treatment.

The effects of continuous exposure to the CHK kinase inhibitors on cell population growth over a range of inhibitor concentrations was measured in both CHK2 WT and KO cells (Figure 3B). There was no significant difference in response between the two cell types observed for either inhibitor alone. The IC<sub>50</sub> values for the CHK2-specific inhibitor were 21  $\mu$ M and 19  $\mu$ M for WT and KO cells, respectively, while DBH gave an IC<sub>50</sub> value of 13  $\mu$ M for both cell lines. These experiments suggest that, in the absence of any DNA damage signal, the effects of these SMIs on cell population growth may be regarded as essentially CHK2-independent (off-target).

#### *Effect of small molecule CHK inhibitors in combination with oxaliplatin*

Any potential interaction of these SMIs in combination with oxaliplatin was evaluated using the fixed ratio CI index method. The CHK2 KO cells were included in the study to highlight CHK2-independent effects of such combinations. Inhibitors were added 24 h before a 1 h pulse of oxaliplatin and subsequently maintained throughout the experimental time course. Surprisingly, in CHK2-competent WT cells, across IC<sub>50</sub>, IC<sub>75</sub> and IC<sub>90</sub> fixed ratio concentrations, the SMIs were found to have an antagonistic effect on the response to oxaliplatin (Figure 3C). Conversely, an additive rather than antagonistic effect was observed in the CHK2 KO cells, suggesting a role for CHK2 in the antagonism observed in WT cells.

#### *Effects of the checkpoint inhibitors on the apoptosis induced by oxaliplatin*

In order to investigate further a potential cause for the antagonistic effects of the SMIs in WT cells, the extent of apoptosis in these cells was examined. Samples treated with the CHK2 II inhibitor or DBH alone exhibited less than 5% apoptosis in either cell line. In the WT cells, combinations of oxaliplatin and either CHK inhibitor resulted in a small but significant decrease ( $P < 0.05$ ) in the levels of apoptosis compared with oxaliplatin alone (Figure 4A). Conversely, the CHK2 KO cells presented no significant decrease in apoptosis with DBH and only a slight but significant decrease with CHK2 II.

Treatment using a more cytotoxic, continuous exposure protocol (48 h) still resulted in antagonism and therefore was unlikely to be due purely to a transient resistance mechanism induced by the inhibitors (data not shown). Apoptosis levels were determined for WT cells after continuous exposure to oxaliplatin in the presence or absence of the inhibitor DBH (Figure 4B). Continuous exposure to oxaliplatin alone led to a substantial induction of apoptosis (to 50%). By comparison, combination treatments lead to a highly significant reduction in apoptosis (3%,  $P < 0.005$ ). Substantial (DBH-treated) and almost complete (CHK2 II-treated) cleavage of the caspase

substrate PARP was observed when the cells were treated with the SMIs combined with oxaliplatin using this protocol (Figure 4B, lower panel).

#### *Effect of checkpoint inhibitors on oxaliplatin-induced DNA cross-links*

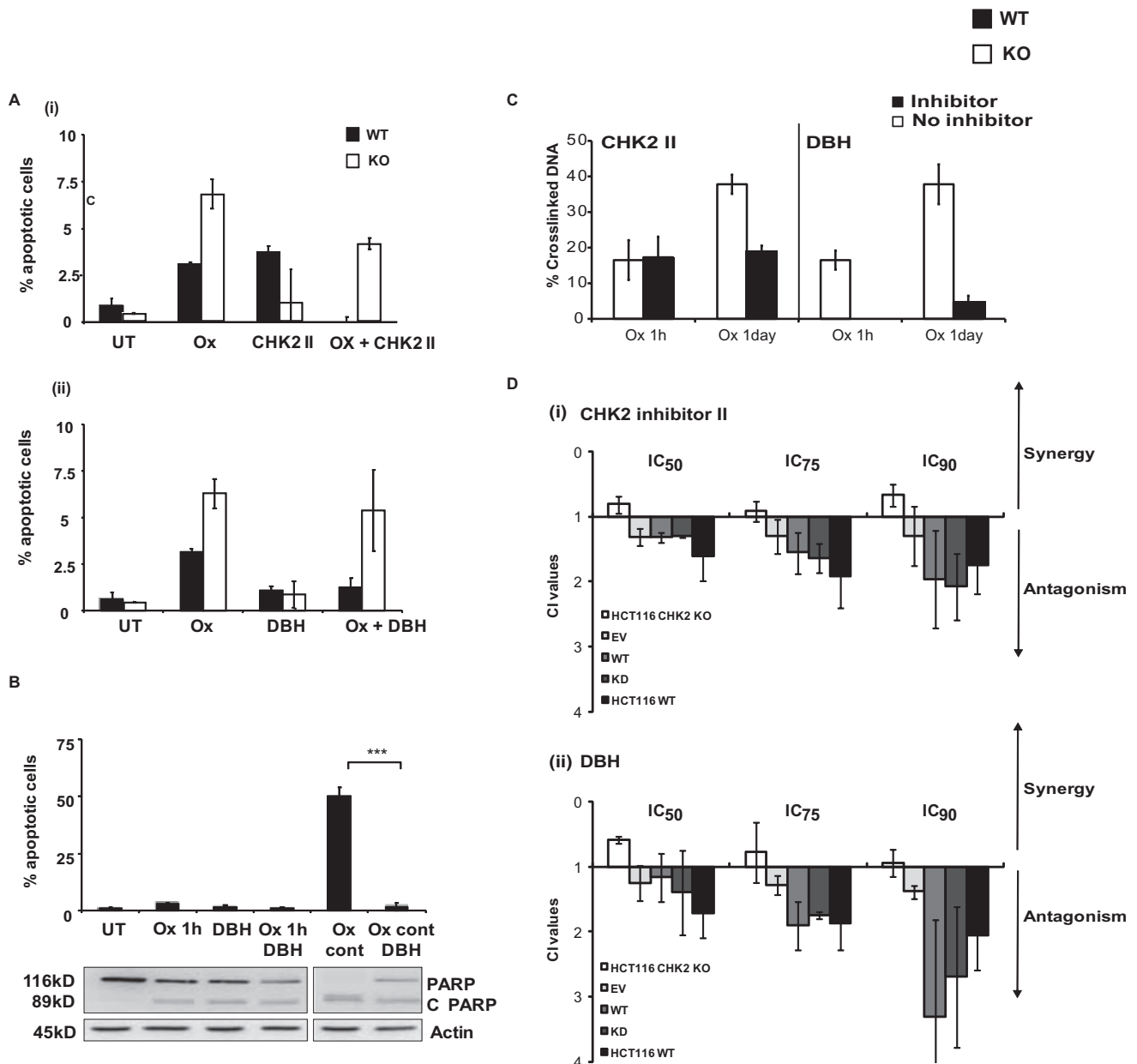
The next question we addressed in this study was whether or not CHK2 SMIs would influence the repair of oxaliplatin-induced DNA damage. Samples treated with oxaliplatin alone, CHK2 SMI alone or oxaliplatin and CHK2 SMI combinations were analysed using the comet-X assay. This single cell assay is capable of determining the extent of DNA cross-linking following drug treatment and can also inform on the rate and extent of repair of these lesions post treatment. HCT116 CHK2 WT cells were treated in scheduled combinations similar to those used in the CI experiments. Cells were harvested at 1 and 24 h following drug treatment (Figure 4C). Oxaliplatin treatment alone resulted in DNA inter-strand cross-linking with approximately 18% of the DNA cross-linked following a 1 h pulse and approximately 40% following a 24 h exposure. Differences were observed in the effects of the two inhibitors on the development of these DNA cross-links at the 1 h time point. Treatment with CHK2 II did not affect the amount of oxaliplatin-induced DNA cross-links, but DBH treatment abrogated oxaliplatin-mediated cross-link formation. The level of DNA cross-links 24 h following oxaliplatin treatment combined with either inhibitor showed a clear decrease when compared with oxaliplatin alone, more clearly so for DBH than for CHK2 II.

#### *Role of CHK2 in the responses to oxaliplatin and the effects of the SMIs*

In order to confirm the role of CHK2 in the observed antagonistic effects of the CHK inhibitors on oxaliplatin cytotoxicity, CHK2 was re-introduced into the CHK2 KO cells by transient transfection as described earlier. To elucidate the role of CHK2 activity in these events, CHK2 KO cells were also transiently transfected with a kinase dead mutant of CHK2 (D368A; which is catalytically inactive) (Xu *et al.*, 2002). The CI combination experiments were then repeated using these transfected lines. An antagonistic effect similar to or indeed higher than that observed in WT cells resulted upon re-introduction of WT CHK2 into the KO cells. Similarly, the expression of kinase dead CHK2 also resulted in substantial antagonism of the response to oxaliplatin (Figure 4D). These results suggest that the presence of the protein, but not necessarily its kinase activity, contributed to the observed antagonism.

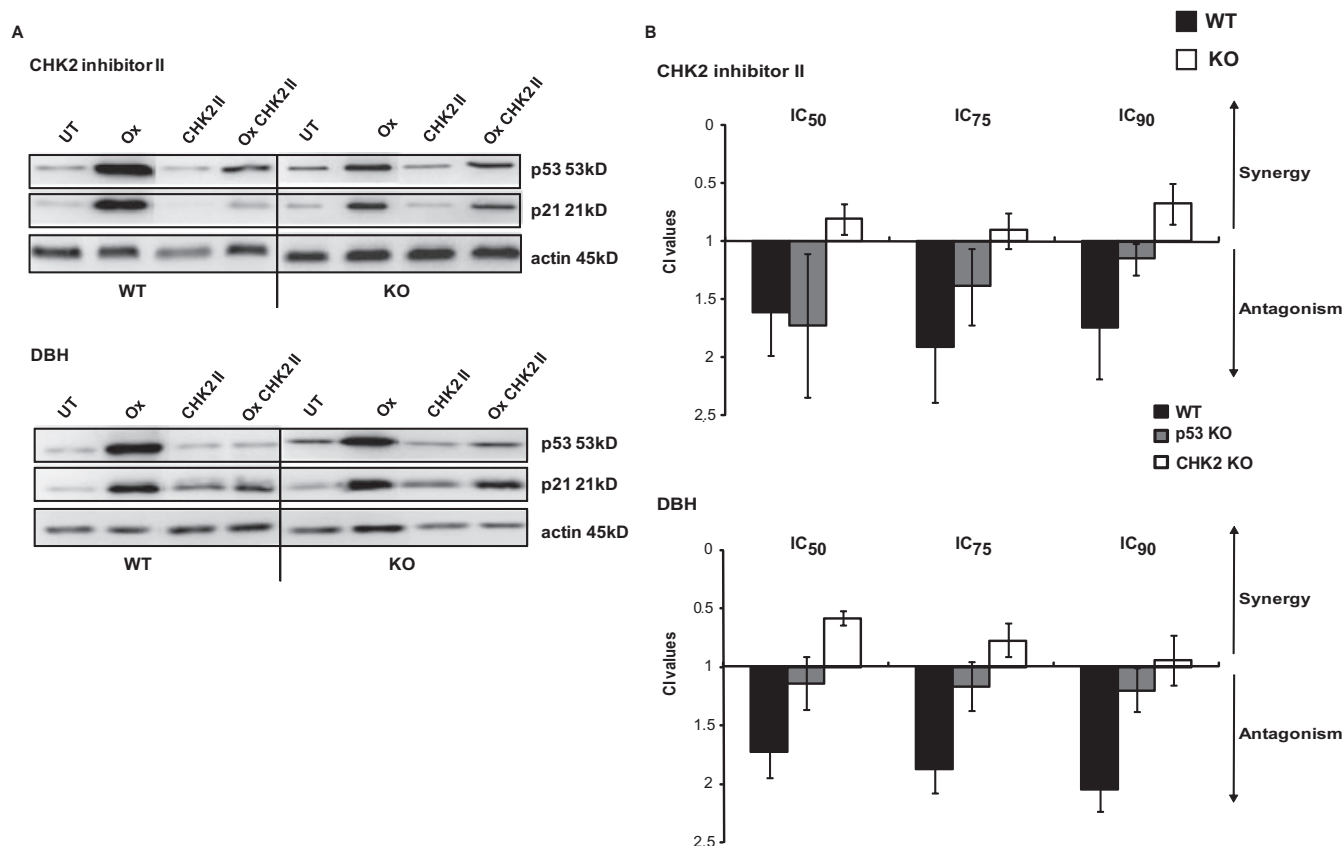
#### *Effects of CHK inhibitors on the stabilization of p53 induced by oxaliplatin*

We previously determined that p53 levels were increased in response to oxaliplatin treatment in these cell lines. To elucidate the pathways involved in the response to the checkpoint inhibitors used alone or in combination with oxaliplatin, both p53 stabilization and p21 up-regulation were analysed.



**Figure 4** (A) Apoptotic responses of HCT116 checkpoint kinase 2 (CHK2) wild-type (WT) and CHK2 KO cells following treatment with a combination of CHK inhibitors and oxaliplatin. HCT116 WT or CHK2 KO were either left untreated (UT) or treated with (i) oxaliplatin alone (20  $\mu$ M, Ox), CHK2 inhibitor II alone (19–21  $\mu$ M) or with the combination (Ox + CHK2 II), (ii) debromohymenialdisine (DBH) alone (13  $\mu$ M) or the combination of oxaliplatin and DBH (Ox + DBH). Oxaliplatin treatment was for 1 h and CHK2 II or DBH was added 24 h before oxaliplatin and continuously thereafter. (B) Effect of duration of exposure to oxaliplatin in combination with CHK2 inhibitor DBH in HCT116 WT cells on apoptotic response. HCT116 WT cells were either left untreated (UT), treated with DBH alone, oxaliplatin (20  $\mu$ M) alone [1 h or continuously (cont)] or in combination with DBH (13  $\mu$ M). DBH was added 24 h prior to oxaliplatin and maintained throughout the experiment. The percentage of apoptotic cells was determined by characteristic changes in nuclear morphology. Data are the average of three independent experiments; error bars represent  $\pm$ SE and \*\*\* $P$  < 0.005, Student's  $t$ -test. A western blot (representative of the 3 experiments) of PARP and cleaved PARP (C PARP, inset) levels are also shown. (C) Kinetics of DNA cross-linking after treatment of HCT116 WT and KO cells with oxaliplatin alone or in combination with CHK inhibitors and effects of the CHK2 status of the HCT116 cells. HCT116 WT cells were treated with oxaliplatin alone or in combination with CHK inhibitors and effects of the CHK2 status of the HCT116 cells. HCT116 WT cells were treated with oxaliplatin alone or in combination with CHK inhibitors and effects of the CHK2 status of the HCT116 cells. HCT116 WT cells were treated with oxaliplatin alone or in combination with CHK inhibitors and effects of the CHK2 status of the HCT116 cells. (D) Effect of re-introduction and overexpression of CHK2 on the response of WT and CHK2 KO cells to oxaliplatin and the combination of oxaliplatin and the checkpoint inhibitors. Both HCT116 WT and CHK2 KO cells were transfected with empty vector (EV), CHK2 wild-type (CHK2 WT) or CHK2 kinase dead (CHK2 KD) expressing vectors. Chou and Talalay combination index (CI) determinations using fixed drug concentrations ratios ( $IC_{50}$ ) were then performed following a 1 h oxaliplatin treatment in combination with either CHK2 inhibitor II or DBH. Inhibitors were added 24 h prior to oxaliplatin treatment and maintained throughout the experiment. Data plotted are the average percentage of apoptotic cells determined from three independent experiments; error bars represent  $\pm$ SE.





**Figure 5** The effect of the checkpoint inhibitors on oxaliplatin-mediated p53 stabilization and p21 up-regulation. (A) HCT116 wild-type (WT) and checkpoint kinase 2 (CHK2) KO cells were left either untreated (UT) or treated with either oxaliplatin (Ox), CHK2 II, debromohymenialdisine (DBH) or with a combination of the inhibitor and a 1 h pulse treatment of oxaliplatin. Inhibitors were added 24 h prior to, and continuously after, oxaliplatin treatment. The levels of p53 or p21 were assessed by western blotting. Actin was used as a protein-loading control. The western blot depicted is representative of three independent experiments. (B) Chou and Talalay combination index (CI) values following fixed drug concentration-ratios of oxaliplatin and either CHK2 II or DBH. CI values are representative of the IC<sub>50</sub>, IC<sub>75</sub> and IC<sub>90</sub> effective doses. WT, CHK2 KO and p53 KO cells were treated with oxaliplatin for 1 h. Inhibitors were added 24 h before and continuously following platinum treatment. Data shown are for the average of three independent experiments.

Neither of the inhibitors alone increased p53 or p21 protein levels in either cell line. In contrast, in the WT cells treated with oxaliplatin-CHK2 inhibitor II or DBH combinations showed lower levels of both p53 and p21 than those treated with oxaliplatin alone. In CHK2 KO cells, however, there was no clear reduction in the levels of these DNA damage responsive proteins when CHK2 II was combined with oxaliplatin; combinations of DBH and oxaliplatin did result in reduced stabilization of p53 but interestingly, still maintained a p21 response (Figure 5A).

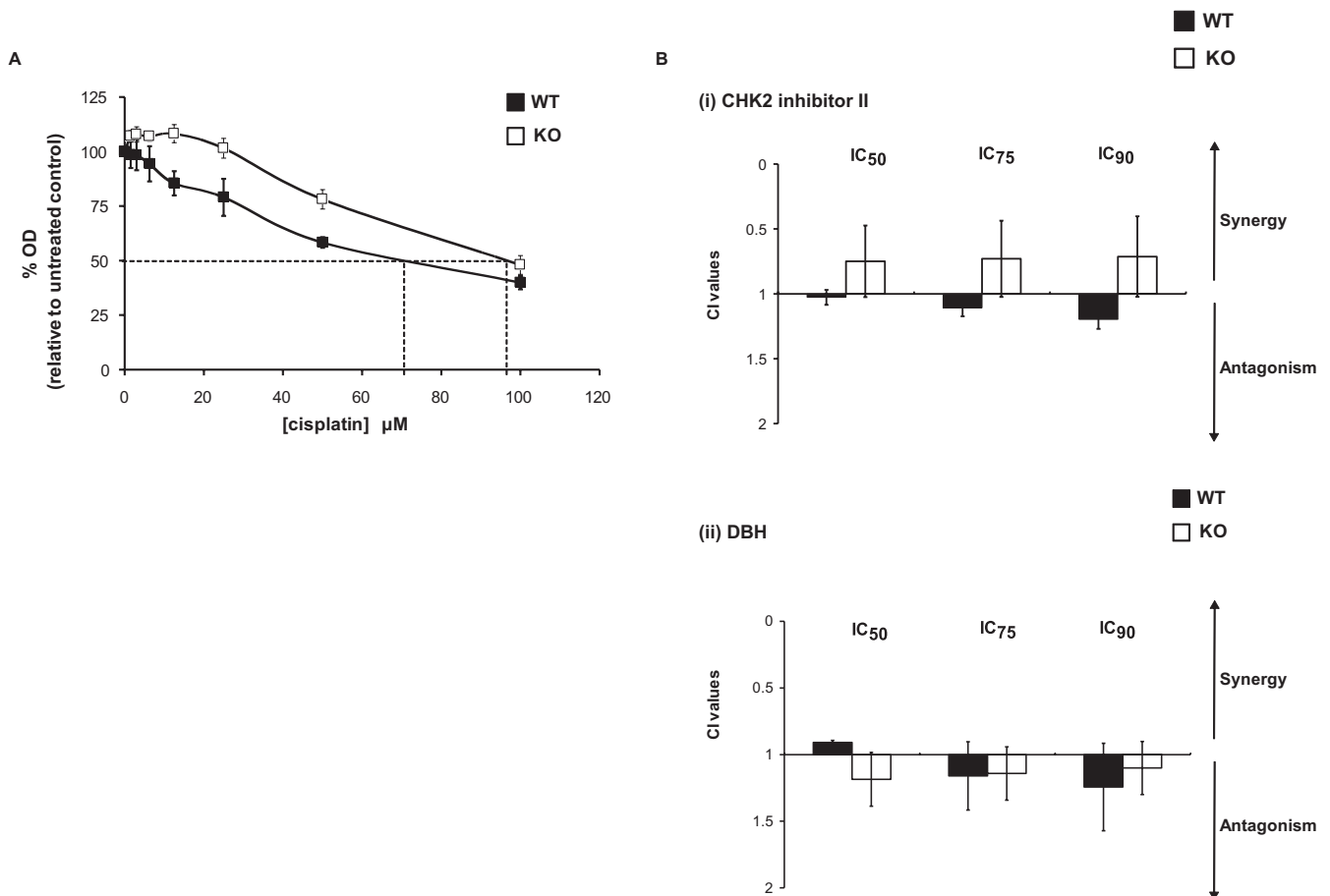
Because a robust p53 response was observed following oxaliplatin treatment and HCT116 p53 KO cells were significantly more resistant to oxaliplatin than WT cells (Boyer *et al.*, 2004; Hata *et al.*, 2005, and data not shown), we sought to clarify the role (if any) of p53 in the antagonist effects of the SMIs on the responses to oxaliplatin. CI assays were performed as described previously using p53 KO HCT116 cells (Figure 5B). In p53 KO cells the inhibitory effect of CHK2 inhibitor II on the antagonism induced by oxaliplatin was reduced whereas that induced by DBH was abolished.

#### Effect of absence of CHK2 and checkpoint inhibitors on cisplatin-induced toxicity

Checkpoint kinase 2 has recently been implicated in the promotion of cisplatin-mediated cell death in the HCT116 cell line model (Pabla *et al.*, 2008), so it was important to verify whether the CHK inhibitors can affect the response of these cells to cisplatin. The results depicted in Figure 6A show that CHK2 KO HCT116 cells are less sensitive to cisplatin-mediated growth inhibition compared with WT cells. CI assays were also performed as described previously and the results are shown in Figure 6B. Both CHK inhibitors had a slight antagonistic effect on cisplatin-mediated growth inhibition of HCT116 WT cells.

## Discussion and conclusions

Currently, there is widespread interest in combining cytotoxic agents with molecularly targeted inhibitors of cell cycle checkpoints and DNA repair pathways, in order to



**Figure 6** Effects of the checkpoint kinase 2 (CHK2) status of HCT116 cells on the response to cisplatin. (A) Response of HCT116 CHK2 WT and CHK2 KO following treatment with cisplatin for 1 h. Sulforhodamine-B (SRB) concentration–response curves, dashed lines indicate the  $\text{IC}_{50}$  doses. (B) Chou and Talalay combination index (CI) values following fixed drug concentration-ratios of cisplatin and either CHK2 II or debromohymenialdisine (DBH), as described in the legend of Figure 4.

promote cell cycle arrest, reduce DNA repair and trigger apoptosis. To test the potential of the checkpoint kinase CHK2 as one such target for combination studies, the effect of oxaliplatin in both CHK2-proficient (WT) and deficient (KO) colon carcinoma cell lines was studied. Oxaliplatin treatment produced a clear, differential, cytotoxic response between these cell lines, with CHK2 KO cell lines showing greater sensitivity. The responses observed in the HCT116 WT cells were broadly consistent with previous findings (Arango *et al.*, 2004; Boyer *et al.*, 2004); however, there are no previous publications on the effects of oxaliplatin on HCT116 CHK2 KO.

Cell cycle progression following oxaliplatin treatment in the two cell lines revealed no differences in oxaliplatin-induced G2/M arrest in the presence or absence of CHK2 (data not shown). The mechanism for the increased sensitivity in CHK2 KO cells was instead found to be an accelerated rate of apoptosis associated with an increased induction of Bax, despite decreased stabilization of p53. These observations suggest that a ‘un-coupling’ of Bax regulation from p53 stabilization occurs in this cell line. Evidence that CHK2 is not essential for the activation of a p53 response induced by other types of DNA damage, such as IR, has been reported previously (Ahn *et al.*, 2003; Jallepalli *et al.*, 2003). This exacer-

bated toxicity in the absence of CHK2 could potentially be mediated by caspase-2 as this caspase has been reported to be involved in DNA damage response (DDR), is constitutively present in the nucleus and activated by caspase-9 (Roos and Kaina, 2006). The data presented here show that activation of both caspase-2 and caspase-9 occurred alongside the increases in Bax activation and cytochrome *c* release and, hence, the involvement of caspase-2 warrants further investigation.

As with all stably genetically modified cell lines, these cells could have developed a compensatory protein expression as an adaptation response to the absence of the deleted gene. In our hands, cell division was normal and the levels of many DDR and apoptosis-related proteins were unaffected in these CHK2 KO HCT116 cells (data not shown). Furthermore, restoration of functional CHK2 into the KO cell line restored both the accelerated apoptosis and the response to oxaliplatin to that observed in the CHK2 WT cells (Figure 1D). Clearly the absence of CHK2 and not other compensatory mechanisms within these cells was responsible for the different responses to oxaliplatin. Taken together, activation of both caspase-2 and caspase-9 and increases in Bax point to an active and possibly dominant p53-independent mechanism in the CHK2 KO cells. Given this enhanced sensitivity of the CHK2 KO cell line to oxaliplatin, it was predicted that combining a CHK2

inhibitor with oxaliplatin would, in CHK2-proficient cells, result in an increase in the cytotoxic effects of oxaliplatin. However, attempts to confirm this resulted in reproducible antagonism of the toxic effects of oxaliplatin in WT cells with decreases in both apoptosis and DDR (Figure 3). Attempts explain these results in terms of cell cycle arrest failed to provide a satisfactory hypothesis as on treatment with the CHK inhibitors the oxaliplatin-induced G2/M arrest was abrogated to an equivalent extent in both WT and CHK2 KO cells (data not shown).

This antagonism could not simply be a 'off target effect' independent of CHK2 as it was not observed in the CHK2 KO cells and was subsequently restored upon reintroduction of CHK2 to these cells (Figure 4). Furthermore, expression of a kinase dead CHK2 in the KO background also reinstated this antagonism, suggesting that the observed effect in the CHK2 WT cells may not be dependant upon CHK2 kinase activity. When the drug combination experiments were conducted in a CHK2 functional but p53 KO background, we found the combined drug effects additive and not antagonistic (Figure 5), suggesting a potential requirement of p53 signalling for the observed antagonism. In the CHK2 KO cell line p53 stabilization was decreased but not abrogated and oxaliplatin-mediated apoptosis still occurred. However, in the presence of the CHK inhibitors p53 stabilization was more extensively compromised and apoptosis was reduced to a substantial degree. It is thus plausible that the levels of p53 may need to exceed a certain threshold for p53-dependent apoptosis to occur. This is particularly relevant when the amount of protein, and not its activation status, is of importance, as is the case for the transcription-independent apoptogenic role of p53 at the mitochondrial level (Chipuk and Green, 2006). In the studies presented here the concentrations of the CHK inhibitors used in both the p53 phosphorylation assays and the CI index assays were based on the IC<sub>50</sub> for cell growth and not those required solely for kinase inhibition. Often high extracellular concentrations are needed to elicit a sustained biological response, as is clearly the case in a number of *in vivo* studies and early phase I clinical trials (Curman *et al.*, 2001; Arienti *et al.*, 2005; Ashwell *et al.*, 2008; Bucher and Britten, 2008; Goldstein *et al.*, 2008). The results presented here are consistent with these and other reports.

Clearly in the clinical situation, the doses applied are indeed higher, approaching the MTD based on toxicology. The durability of these agents and their PK characteristics then determine what intracellular concentrations can be actually achieved within tissues. At these higher doses the potential for off-target effects of these inhibitors increases and this may have substantial effects on the pharmacology of the drug combinations used.

To investigate further the underlying pathway(s) responsible for this antagonistic effect, a mechanistic approach was adopted to measure DNA damage and repair following drug combinations. The comet-X assay has been used by us and others to measure DNA cross-links in individual peripheral blood mononuclear cells following treatment with bi-functional alkylating agents (Ward *et al.*, 2005). In this study, DNA cross-linking of up to 30% was measured in CHK2-proficient cells treated with oxaliplatin alone, similar

to levels described previously (Almeida *et al.*, 2006). Both the time and extent of this cross-linking were significantly affected by both SMI CHK inhibitors. That the reduction in the initial amount of DNA cross-linking occurred immediately following treatment with oxaliplatin plus DBH (Figure 4C) suggests that the transport of oxaliplatin (uptake) was impaired; however, further investigations to confirm this were not undertaken. Intriguingly, similar early effects on oxaliplatin toxicity have previously been reported when combined with other kinase inhibitors such as the Bay 43-9006, Iressa and Zactima (Xu *et al.*, 2003; Heim *et al.*, 2005; Troiani *et al.*, 2006). In all of these studies, the scheduling of the kinase inhibitor with oxaliplatin was critical, antagonism only being demonstrated, as in our study, when the inhibitor was present before and during oxaliplatin treatment. In the case of Bay 43-9006, a reduction in the intracellular levels of oxaliplatin and DNA-platinum adducts was demonstrated (Heim *et al.*, 2005). These authors did not comment on the finding that pretreatment with oxaliplatin before the inhibitor, a potentially synergistic combination, still resulted in an antagonistic effect despite no reduction in platinum uptake or adduct formation. Presumably, a mechanism unrelated to platinum transport was invoked in this instance. Whereas a reduced cellular uptake of platinum could account largely for the antagonism observed with DBH combinations, no such early effects were observed for the CHK2 II inhibitor combined with oxaliplatin. Indeed, this combination appeared to result in lower amounts of DNA cross-links when measured 24 h post treatment, possibly indicating enhanced DNA repair (Figure 4C). A recent study has also implicated CHK2 signalling in cisplatin-induced apoptosis (Pabla *et al.*, 2008). These authors observed that CHK2 signalling to apoptosis after platinum-induced DNA damage was compromised in the HCT116 CHK2 KO cells, with an associated decrease in caspase activity. In our hands, the CHK2 KO cells also showed this decreased sensitivity to cisplatin (Figure 6A). We explored this effect further by examining the response to cisplatin in the presence of the CHK2 inhibitors (Figure 6B). In the CHK2 WT cells, the response to cisplatin, unlike that to oxaliplatin, was only modestly affected by the presence of either CHK2 inhibitor. Oxaliplatin uptake is mediated through organic cation transporters while cisplatin is not, further supporting the possibility that the inhibitory effects of the CHK2 inhibitors on the response to oxaliplatin may be associated with effects on drug transport.

Exposure of cells to oxaliplatin produces bulky DNA adducts and DNA cross-links. DSBs frequently result from repair of these lesions. (Raymond *et al.*, 2002; Sancar *et al.*, 2004). DSB lesions activate the ATM/ATR pathways and sequester the meiotic recombination 11 (MRE11 RAD50-NBS1 complex, MRN), which transiently binds to the chromatin adjacent to DSB lesions. Activated CHK2 transiently co-localizes at foci of DNA damage and is subsequently released following its phosphorylation on Thr68 by ATM. CHK2 is then free to activate downstream substrates (Yang *et al.*, 2002; Stevens *et al.*, 2003; Zhang *et al.*, 2004). Thus, CHK2 acts as a mobile sensor and activator of DDR and checkpoint control (Lukas *et al.*, 2003; 2004).

As in other CRC cell lines and primary tumours, the HCT116 cell line used in this study possess MRE11 inactivating mutations with an associated reduced expression of the other MRN complex members (Giannini *et al.*, 2002). These MRE11 mutations were found to be associated to a mismatch repair and S-phase checkpoint defects. However mismatch repair deficiency does not affect the cellular responses to oxaliplatin-induced DNA damage (Raymond *et al.*, 2002; Kelland, 2007). MRE11 mutations in HCT116 cells have been closely associated with defective ATM and CHK2 activity in replication stress and replication-dependent DSBs (Takemura *et al.*, 2006; Wen *et al.*, 2008). These HCT116 cells express substantial levels of CHK2 but this CHK2 is only weakly activated, thus substantially impacting on the ability of these cells to respond to drugs targeted at DNA. They are, however, capable of both p53-dependant and p53-independent apoptosis (Ahn *et al.*, 2003; Jallepalli *et al.*, 2003; Carrassa *et al.*, 2004). It is unclear how these MRE11-CHK2 defective cells deal with DNA damage and we hypothesize that in MRE11-deficient cells CHK2 may still be recruited to sites of DNA damage but it is poorly activated. As a consequence CHK2 is unable to phosphorylate its targets (promyelocytic leukaemia and breast cancer susceptibility-associated protein 1), thus becoming fixed at sites of DNA damage and less able to spread damage recognition, a process currently understood to be essential for DDRs. The resultant accumulation of un-repaired DNA lesions would eventually activate apoptosis via p53. In the absence of CHK2, the DDR following oxaliplatin treatment is further impaired and apoptosis is induced principally, more rapidly possibly, in a p53-independent manner. The balance between these two pathways could be influenced by the presence of the CHK2 protein and be independent of its kinase activity. The observed antagonism of the response to oxaliplatin in CHK2 competent cells could therefore, in part, be explained in terms of an off-target effect of the CHK2 inhibitors on p53, as evidenced by the reduced antagonism observed in p53 KO cells. Re-introduction of the CHK2 protein into the CHK2 null cells switches the bias from p53-independent to p53-dependant apoptosis. Further studies in other relevant cell lines that represent varied CHK2 phenotypes (WT, loss-of-expression or loss-of-function mutants) as well as normal cells are required to clarify this phenomenon.

Clearly these observations show that, in this HCT116 cell line at least, the presence and/or activity of CHK2 can differentially modulate the outcome of treatment with oxaliplatin. In addition, these observations highlight some possible confounding effects of combining novel SMI of CHK kinases with existing chemotherapeutic strategies.

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## Conflicts of interests

None.

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