Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells

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The platinum compound oxaliplatin has been shown to be an effective chemotherapeutic agent for the treatment of colorectal cancer. In this study, we investigate the molecular mechanisms of action of oxaliplatin to identify means of predicting response to this agent. Exposure of colon cancer cells to oxaliplatin resulted in G2/M arrest and apoptosis. Immunofluorescent staining demonstrated that the apoptotic cascade initiated by oxaliplatin is characterised by translocation of Bax to the mitochondria and cytochrome c release into the cytosol. Oxaliplatin treatment resulted in caspase 3 activation and oxaliplatin-induced apoptosis was abrogated by inhibition of caspase activity by z-VAD-fmk, but was independent of Fas/Fasl association. Targeted inactivation of Bax or p53 in HCT116 cells resulted in significantly increased resistance to oxaliplatin. However, the mutational status of p53 was unable to predict response to oxaliplatin in a panel of 30 different colorectal cancer cell lines. In contrast, the expression profile of these 30 cell lines, assessed using a 9216-sequence cDNA microarray, successfully predicted the apoptotic response to oxaliplatin. A leave-one-out cross-validation approach was used to demonstrate a significant correlation between experimentally observed and expression profile predicted apoptosis in response to clinically achievable doses of oxaliplatin (R = 0.53; P = 0.002). In addition, these microarray experiments identified several genes involved in control of apoptosis and DNA damage repair that were significantly correlated with response to oxaliplatin.

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Oxaliplatin is a third generation diaminocyclohexane (DACH) platinum compound that forms mainly intrastrand links between two adjacent guanine residues or a guanine and an adenine, disrupting DNA replication and transcription (Fink et al., 1997). Although the related platinum compounds, cisplatin and carboplatin, are generally ineffective in the treatment of colorectal cancer, oxaliplatin has been shown to be effective in the treatment of this disease and it is commonly used to treat patients unresponsive to 5-fluorouracil (5FU) based therapy. However, the details underlying the cytotoxic effects of oxaliplatin remain poorly understood.

Exposure of tumour cells to several chemotherapeutic agents, including oxaliplatin, has been shown to induce programmed cell death or apoptosis (Searle et al., 1975; Lowe and Lin, 2000; Gourrier et al., 2002; Johnstone et al., 2002). In mammalian cells, the signalling cascades leading to apoptosis can be divided into two broad groups. The intrinsic pathway is characterised by the central role of the mitochondria in the initiation of the caspase cascade executing the apoptotic program (Desagher and Martinou, 2000). Pro- and antiapoptotic Bcl-2 family members play a pivotal role in the intrinsic apoptotic cascade (Narita et al., 1998; Gross et al., 1999). Upon exposure to some apoptotic stimuli, proapoptotic Bcl-2 family members such as Bax, which normally reside in the cytosol, are relocated to the outer mitochondrial membrane where they lead to the release of apoptotic factors from the mitochondria and ultimately to caspase activation and an apoptotic cell death (Li et al., 1997; Zou et al., 1997). The tumour suppressor gene p53 has been shown to directly regulate the expression levels of Bax (Miyashita and Reed, 1995), and both p53 and Bax have been shown to be important determinants of the cellular response to chemotherapeutic agents (Bunz et al., 1999; Zhang et al., 2000). In the extrinsic pathway, however, caspase activation is initiated by death receptors on the cell surface (Ashkenazi and Dixit, 1998). Chemotherapeutic agents are known to induce apoptosis by either of these two mechanisms (Lowe and Lin, 2000; Johnstone et al., 2002).

While significant progress has been made in the identification of markers predicting response to 5FU and CPT-11, two additional chemotherapeutic agents commonly used for colorectal cancer treatment (Augenlicht et al., 1997; Bunz et al., 1999; Salonga et al., 2000; Arango and Augenlicht, 2001; Arango et al., 2001, 2003), there is great need for markers that allow discrimination of tumours that vary in their sensitivity to oxaliplatin. The tumour suppressor p53 has a pivotal role in determining the cellular sensitivity to a number of chemotherapeutic agents, including 5FU and CPT-11.
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MATERIALS AND METHODS

Cell culture
HCT116 colon carcinoma cells and isogenic derivatives with a targeted inactivation of p53 or Bax (Bunz et al, 1999; Zhang et al, 2000) were a gift of Dr Vogelstein (Johns Hopkins University School of Medicine). Cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1 × antibiotic/antimycotic (100 U ml⁻¹ streptomycin sulphate, 100 U ml⁻¹ penicillin G sodium and 0.25 μg ml⁻¹ amphotericin B), 100 μM nonessential amino acids and 10 mM HEPES buffer solution (all from Invitrogen Corporation, Carlsbad, CA, USA).

Cell cycle and apoptosis

For analysis of cell cycle, 2 × 10⁵ HCT116 cells were seeded on six-well plates in triplicate and allowed to attach for 24 h. For time-response studies cells were treated with 5 or 10 μM oxaliplatin (supplied by Sanofi-Synthelabo, New York, NY, USA) for 12, 24, 48 and 72 h. For concentration–response experiments, cells were treated for 72 h with 5, 10, 15, 20, 25 and 50μM oxaliplatin. Both attached and floating cells were harvested, washed twice with 2 ml of PBS and resuspended in 1 ml of Perm/Wash buffer (Pharmingen, San Diego, CA, USA) were added and cells were incubated at 4°C in the dark for 30 min, washed with Perm/Wash buffer and resuspended in 100 μl of Perm/Wash buffer. Samples were analysed using a Becton Dickinson FACScan, measuring logarithmic PE fluorescence in the FL-2 channel in a minimum of 10,000 cells.

Western blotting

HCT116 cells seeded in 75 cm² flasks were treated with 10 μM oxaliplatin for 0, 6, 12, 16 or 24 h and then rinsed with PBS twice, harvested and resuspended in 300 μl of RIPA lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate and 100 μM aprotinin). The cell suspension was vortexed and kept on ice for 30 min before cell debris was pelleted and the supernatant transferred to a new tube. SDS–polyacrylamide gel electrophoresis sample loading buffer (6 ×) was added to 20 μg aliquots and loaded on 15% tris-HCl precast gels (BioRad, Hercules, CA, USA). Fractionated proteins were transferred to a PVDF membrane (Amer sham, Piscataway, NJ, USA) and blocked with 10% nonfat milk for 1 h. Membranes were then probed at room temperature with the appropriate primary antibody in 5% nonfat milk for 1 h with the following antibodies: anti-p53 (Santa Cruz, DO-1, 1/7000), anti-p21(20/21)WAF1/SIP (Santa Cruz, H-164, 1/200) and anti-β-actin (Sigma, clone AC74, 1/1000). Membranes were washed three times with washing buffer (PBS with 0.1% Tween 20) and then probed with the appropriate peroxidase-conjugated secondary antibody for 1 h (all from Roche Diagnostics/Boehringer Mannheim Corporation, Indianapolis, IN, USA). The secondary antibody was washed three times with washing buffer and the signal was developed using ECL Plus Western Blotting Detection Method (Amer sham, Piscataway, NJ, USA). Detection was carried out using a Storm Phospholmage and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Protein levels were standardised using the signal from the β-actin probe.

Immunofluorescence analysis

Cells were cultured overnight on preferred glass coverslips (Fisher, Pittsburgh, PA, USA), and then treated with 10, 20 or 50 μM oxaliplatin. The cells were fixed for 15 min in 4% paraformaldehyde (Electron Microscopy Services, Ft Washington, PA, USA), permeabilised with 0.5% Triton X-100/PBS for 5 min and then incubated for 1 h in a 1% BSA/PBS blocking solution. To detect BAX, cells were incubated for 3 h with a rabbit polyclonal IgG that recognised the N-terminal region (Upstate Biotechnology, Lake Placid, NY, USA; 1: 100 dilution), followed by exposure to a goat Cy3-conjugated anti-rabbit secondary antibody (Amersham, Piscataway, NJ, USA). To detect mitochondria, a mouse monoclonal Hsp60 antibody was used (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1: 200), the binding of which was detected by a goat anti-mouse FITC-conjugated secondary antibody (Roche Diagnostics/Boehringer Mannheim Corporation, Indianapolis, IN, USA). Cytochrome c was detected utilising a mouse monoclonal antibody against the N-terminal region.
anti-cytochrome c IgG (Pharmingen, San Diego, CA, USA; 1:200) followed by exposure to a goat anti-mouse Cy5-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, USA). All secondary antibodies were used at a dilution of 1:200 for 1 h. All washes were performed with PBS. To visualise nuclei, cells were stained with DAPI (Sigma, St Louis, MO, USA; 4',6-diamidino-2-phenylindole). Fluorescent images were captured with a SPOT RT Diagnostic Instruments CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA) attached to a BX60 Olympus fluorescence microscope (Olympus, Melville, NY, USA). The proportion of apoptotic cells was quantified by scoring the number of cells simultaneously exhibiting Bax relocalisation and cytochrome c release in 200 cells.

**Growth/cytotoxicity assay**

The concentration of oxaliplatin resulting in 50% inhibition of control growth (GI50) in response to oxaliplatin was calculated using the sulphorhodamine B method according to the protocol used by the NCI *in vitro* Anticancer Drug Discovery Screen Program (Skehan *et al*., 1990). Ten thousand cells per well were seeded in 96-well plates and 24 h later exposed to 0, 0.0125, 0.025, 0.125, 0.25, 1.25, 2.5, 6.25, 12.5, 25, 50 and 125 μM oxaliplatin for 72 h. The GI50 concentrations were calculated as described (Skehan *et al*., 1990; Arango *et al*., 2003; Mariadason *et al*., 2003) using Prism 3.0 software (GraphPad, San Diego, CA, USA).

**Clonogenic assay**

One million cells were seeded in T25 flasks and 24 h later exposed to 0, 2.5, 3, 3.5 or 4 μM oxaliplatin for 9 h. Cells were then trypsinised and 500 cells reseeded in six-well plates in triplicate. Colonies were allowed to form for 2 weeks and then plates were washed and air-dried. Colonies were stained with 0.1% crystal violet, washed three times with distilled water, and air-dried. Plates were scanned using a Perfection 1250 scanner (Epson America Inc., Long Beach, CA, USA) and the number of colonies quantified using Total Lab 1.1 software (Nonlinear Dynamics, Durham, NC, USA).

**Assessment of p53 status**

The mutational status of the 30 colorectal cancer cell lines in the panel used has been previously reported (Mariadason *et al*., 2003). Sequencing of the hotspots for p53 mutations (exons 5–8) in T84 cells found no mutations (Mariadason *et al*., 2003). Moreover, Western blot analysis demonstrated that T84 cells have low basal levels of expression (defined as signal
diagonal
e are lower than the line of equality) for the left out cell line. Thus, the final analysis of observed apoptosis (ln(yi)) was plotted as a function of the predicted value (yi*), and a simple linear regression was constructed. The purpose of this regression analysis was to determine whether the predicted and observed values obeyed the equation yi = yi* (i.e., whether the points fall on the line of equality). If the prediction rule is true, then the observed and predicted values would be equal or nearly equal. The measure of linear fit was r, and the hypothesis of falling on the line of equality was tested by comparing the slope to unity and y intercept to zero.

**Microarray analysis**

The expression profile of the same panel of 30 colorectal cancer cell lines was assessed in using 9216-sequence cDNA microarrays from the Albert Einstein Cancer Center Facility as previously described (Mariadason *et al*., 2003). For each cell line, hybridisations were carried out in duplicate starting from RNA isolated from independent cultures. For each set of replicates, the mean expression value for each sequence was determined and entered into a final database for further analyses. The expression data for the 3725 sequences with a significant level of expression (defined as signal>background plus two standard deviations in the Cy5 and/or Cy3 channel) in at least one replicate for all 30 cell lines was used in subsequent analyses. All the databases are available on our website (www.au-genlitchlab.com).

‘Leave-One-Out’ cross-validation analysis

All leave-one-out analyses (Efron and Tibshirani, 1993) were performed using genes that showed a significant level of expression above background in each of the 30 cell lines (3725 of the 9216 genes on the arrays). First, from the 30 cell lines, cell line 1 was removed from consideration, leaving 29 cell lines for analysis. For these 29 cell lines, the Pearson correlation between the level of expression of each of the 3725 genes and the percentage of apoptosis induced by 10 μM oxaliplatin was computed, and the N genes highest absolute value correlations (i.e., corresponding to N genes) were selected. N was varied from the 10 to 200 best-correlated genes. As a control, N randomly selected genes were also analysed. To reduce the number of genes to a smaller set of variables, principal components analysis (PCA) was performed. PCA enables a large number of variables to be reduced to linear combinations of variables that can be used to predict an outcome. From the PCA, the principal components (PCs) having the 10 largest eigenvalues were selected. In general, these 10 PCs accounted for approximately 60% of the variance in the selected genes. Next a multiple regression model was developed using the 10 PCs to predict apoptosis, based on the 29 cell lines in the analysis. Once the regression equation was derived, the 10 PCs corresponding to the ‘left out’ cell line were computed and substituted into the derived regression equation to yield a prediction of apoptosis in the left out cell line. Thus, the final results for this first leave-one-out procedure were the predicted value of apoptosis for the left out cell line (yi*) and the observed value (yi).

After this first leave-one-out procedure was completed, the left out cell line was replaced in the data set, and cell line 2 was removed, once again leaving 29 cell lines in the data set with 1 cell line left out. The entire procedure was repeated for all 30 cell lines so that the final result was a set of predicted apoptosis values for each cell line that had been left out and the corresponding observed value. Each of these 30 leave-one-out procedures yielded 30 pairs of predicted and observed apoptosis values: yi*, y1, y2*, y2, …, y30*, y30.

To determine how well a given regression model predicted observed apoptosis in the left out cell line, the natural log of observed apoptosis (ln(yi)) was plotted as a function of the predicted value (ln(yi*)), and a simple linear regression was constructed. The purpose of this regression analysis was to determine whether the predicted and observed values obeyed the equation yi = yi* (i.e., whether the points fall on the line of equality). If the prediction rule is true, then the observed and predicted values would be equal or nearly equal. The measure of linear fit was r, and the hypothesis of falling on the line of equality was tested by comparing the slope to unity and y intercept to zero.
RESULTS

Oxaliplatin induces a G2/M cell cycle arrest and apoptosis

Exposure of an asynchronous culture of HCT116 colon carcinoma cells to clinically achievable concentrations (Ehrsson et al., 2002; Tassone et al., 2002) of the platinum compound oxaliplatin resulted in a significant time- and concentration-dependent accumulation of cells in the G2/M phases of the cell cycle and a reduction of cells in S phase (Figure 1A – D). These effects of oxaliplatin on cell cycle progression of proliferating HCT116 cells were accompanied by a significant proportion of cells showing signs of an apoptotic death, which was confirmed by the characteristic morphological changes observed in DAPI-stained nuclei of oxaliplatin-treated cells (Figure 2A and B). Quantification of the proportion of apoptotic cells by PI staining and flow cytometric analysis demonstrated that exposure of HCT116 cells to clinically achievable concentrations of oxaliplatin resulted in a significant time-dependent induction of apoptosis (Figures 1A and 2C) and that the proportion of apoptotic cells was concentration-dependent (Figures 1B and 2D).

The apoptotic cascade initiated by oxaliplatin is characterised by translocation of Bax to the mitochondria, cytochrome c release and caspase 3 activation

To investigate the molecular cascade of events following exposure to oxaliplatin, we utilised HCT116 cells, which have a wild-type p53 gene and express a functional Bax protein. Immunofluorescent staining of Bax in untreated HCT116 cells demonstrated a diffuse cytoplasmic localisation of Bax in most cells (Figure 3A). In agreement with a low incidence (<1%) of spontaneous apoptosis in untreated cultures (see Figure 2C), a small proportion of cells showed punctate Bax staining that was demonstrated to localise to the mitochondria by co-staining with Hsp60, a heat shock protein with mitochondrial localisation (Supplementary material Figure 1). However, exposure of HCT116 cells to 10 \( \mu \)M oxaliplatin resulted in a significant \((P<0.001)\) increase in the number of cells that exhibited a mitochondrial pattern of Bax staining (white arrowheads in Figure 3A). Translocation of Bax to the mitochondria has been implicated in the formation of the permeability transition pore and cytochrome c release from the mitochondria into the cytoplasm (Narita et al., 1998; Gross et al., 1999). Nonapoptotic untreated HCT116 cells exhibited a mitochondrial localisation of cytochrome c (Figure 3A). However, oxaliplatin treatment resulted in an increase in the number of cells showing a diffuse cytoplasmic cytochrome c immunostaining (yellow arrowheads in Figure 3A). The observed relocalisation of Bax and cytochrome c following treatment of HCT116 cells with oxaliplatin was concurrent (Figure 3A) and time- and concentration-dependent (Figure 3B and C). Extension of these analyses to other colorectal cancer cell lines showed that exposure of RKO, RW2982 and SW403 cells to 10, 20 or 50 \( \mu \)M oxaliplatin resulted in a significant \((P<0.01)\) increase in the number of cells showing Bax/cytochrome c re-localisation (Figure 3C).

Cytoplasmic cytochrome c has been shown to bind to other components of the apoptosome, resulting in caspase activation (Liu et al., 1996; Zou et al., 1997). A time-dependent Caspase 3 activation was demonstrated in oxaliplatin-treated cells by immunostaining with a fluorescently labelled antibody that binds specifically to active Caspase 3, and quantified using flow cytometry (Figure 4A). In agreement with this observation, treatment of HCT116 cells with oxaliplatin in the presence of different concentrations of z-VAD-fmk, a pan-caspase inhibitor, reduced oxaliplatin-induced apoptosis in a concentration-dependent manner (Figure 4B).

Figure 1 Effects of oxaliplatin on cell cycle. The cell cycle distribution of HCT116 cells was determined after, (A) exposure to 5 \( \mu \)M oxaliplatin for different times, and (B) treatment for 72 h with different concentrations of oxaliplatin. Representative experiments are shown. In panels (C) and (D) the number of cells in G0/G1, S phase and G2/M were quantified by PI staining and flow cytometric analysis. Mean of three experiments is shown.
Targeted inactivation of Bax decreased the apoptotic response to oxaliplatin

The cellular translocation of Bax to the mitochondria observed in HCT116 cells following exposure to oxaliplatin suggested a functional role for this Bcl2 family member in the apoptotic cascade initiated by oxaliplatin. To investigate the role of Bax in oxaliplatin-induced apoptosis, we utilised an isogenic HCT116 derivative line that differs only in the absence of a functional Bax gene. Propidium iodide (PI) staining and flow cytometric analysis demonstrated a significant ($P = 0.03$) time- and dose-dependent reduction in the number of apoptotic cells in HCT116 Bax$^{-/-}$ cells compared to isogenic Bax$^{+/+}$ cells following oxaliplatin exposure (Figure 5A and B). In agreement with this observation, HCT116 Bax$^{-/-}$ cells treated with 20 $\mu$M oxaliplatin for 24 h showed a significant ($P = 0.03$) reduction compared to isogenic Bax$^{+/+}$ cells in the number of cells with the cytotoxic staining pattern of cytochrome c characteristic of apoptotic cells (Figure 5C), further demonstrating an important functional role of Bax in oxaliplatin-induced apoptosis.

Oxaliplatin-induced apoptosis is not dependent upon Fas/FasL association in HCT116 cells

Bax relocalisation to the mitochondria, accumulation of cytochrome c in the cytoplasm and Caspase 3 activation are all events consistent with an intrinsic pathway of activation of apoptosis by oxaliplatin. To investigate the contribution of the extrinsic pathway in oxaliplatin-induced apoptosis, we used antibodies that specifically recognise either the Fas receptor (ZB4) or the Fas ligand (NOK-1) and disrupt Fas/FasL association and the subsequent induction of apoptosis by this pathway. Preincubation of HCT116 cells with either ZB4 or NOK-1 antibodies was effective in preventing apoptosis induced by recombinant human soluble Fas ligand (rhFasL; Figure 6A). However, pretreatment with ZB4 or NOK-1 antibodies did not affect apoptosis induced by oxaliplatin (Figure 6A), suggesting that this extrinsic pathway of induction of apoptosis was not activated by oxaliplatin in HCT116 cells.

Role of p53 in the response to oxaliplatin

More than 50% of colon tumours have a mutant p53 gene (Baker et al., 1990), and a functional p53 protein has been shown to be important for the cellular response to a variety of proapoptotic stimuli, including chemotherapeutic agents commonly used in the treatment of colorectal cancer, such as 5FU and CPT-11 (Bunz et al., 1999; Arango et al., 2001, 2003; Magrini et al., 2002). Here, we demonstrate that exposure of wild-type p53 HCT116 cells to oxaliplatin results in increased levels of p53 (Figure 6B), and consistent with this observation, the p53 target gene p21$^{waf1/cip1}$ was also upregulated (Figure 6B). The product of the p53 gene is a transcription factor that can either promote apoptosis, through different mechanisms such as Bax upregulation, or induce cell cycle arrest and DNA damage repair by means of the transcriptional upregulation of genes such as p21$^{waf1/cip1}$ and Gadd45. Therefore, we directly tested the role of p53 in the sensitivity of colon cancer cells to oxaliplatin using parental wild-type p53 HCT116 cells and isogenic HCT116 p53$^{-/-}$ cells. Relative to parental cells, p53-deficient HCT116 cells showed a time- and concentration-dependent protection from the apoptotic effects of oxaliplatin (Figure 7A and B). The reduced apoptotic effects of oxaliplatin in HCT116 p53$^{-/-}$ could be detected after 16 h of treatment as a 10-fold reduction in the number of cells exhibiting Bax translocation and cytochrome c release compared to parental HCT116 p53$^{+/+}$ (not shown). Moreover, the concentration of oxaliplatin necessary to cause a 50% growth inhibition (GI$_{50}$) after 72 h of exposure was four-fold higher in HCT116 p53$^{-/-}$ cells compared to parental HCT116 cells (2.04 ± 0.15 and 0.53 ± 0.04 $\mu$M respectively; $P < 0.0001$; Figure 7C). To investigate the long-term implications of the reduced apoptosis and growth inhibition in response to oxaliplatin in HCT116 p53$^{-/-}$ cells, we assessed the clonogenic potential of parental HCT116 p53$^{+/+}$ and isogenic HCT116 p53$^{-/-}$ cells 2 weeks after exposure to oxaliplatin for 9 h. This assay demonstrated that exposure of parental HCT116 p53$^{+/+}$ cells to concentrations of oxaliplatin ranging from 2.5 to 4 $\mu$M, resulted in up to 70% reduction in the number of cells with long-term clonogenic potential. In contrast, these concentrations of oxaliplatin had no effect on clonogenicity of HCT116 p53$^{-/-}$ cells (Figure 7D), further demonstrating that inactivation of p53 in colon cancer cells results in a significant protection from oxaliplatin cytotoxicity.

To further investigate the role of p53 in the response of colorectal cancer cells to oxaliplatin, we assessed the sensitivity to
this agent in a panel of 30 different colorectal cancer cell lines of known p53 mutational status (Mariadason et al., 2003). Of these 30 cell lines, 11 had a wild-type p53 gene, and the remaining 19 exhibited inactivating mutations in this tumour suppressor. Considerable variability was observed among different colorectal cancer cell lines in the number of apoptotic cells following 72 h treatment with 10 μM oxaliplatin (Figure 8A). Despite the clear role of p53 in the apoptotic response to oxaliplatin demonstrated using the HCT116 isogenic system that differs only in the presence or absence of a functional p53 protein, the mutational status of this
tumour suppressor gene could not predict the apoptotic response to 10 μM oxaliplatin (Figure 8A), suggesting that additional factors modulate sensitivity to this agent.

Response to oxaliplatin can be predicted using the expression profile of untreated colorectal cancer cells

Due to the great complexity of the molecular mechanisms determining response to oxaliplatin, analysis of the p53 mutational status is likely to be of limited predictive value in the clinical setting. As an alternative approach, we used cDNA microarray analysis to assess the levels of expression of 9216 sequences in the same panel of 30 colorectal cancer cell lines, and used the expression profile of untreated cells to make predictions concerning response to oxaliplatin.

In order to validate the accuracy of the predictions within the panel of 30 cell lines we used a ‘leave-one-out’ cross-validation approach. Here, one of the 30 samples is held out, and the N genes whose expression best correlates with the apoptotic response are selected using the remaining 29 cell lines. The apoptotic response in the 30th line is then estimated using the expression of those N genes and a predictor based on a multiple regression model (see Materials and Methods for details). This process is repeated 30 times holding out a different cell line in each iteration. In this way, a predicted value for the apoptotic response to oxaliplatin is obtained for each of the 30 cell lines, which can then be compared to the experimentally observed response to this agent. To optimise the predictive rule, we tested the effect of varying the number of N input genes from the 10-best through 200-best correlated with oxaliplatin-induced apoptosis (see Materials and Methods). This analysis demonstrated that selection of the 80 genes best correlated with oxaliplatin-induced apoptosis produced the most accurate prediction, with a highly significant correlation ($r = 0.53, P = 0.002$) between the observed and estimated response to oxaliplatin (Figure 8B). Importantly, the correlation between observed and estimated response to oxaliplatin by a group of 80 randomly selected genes was not significant ($P = 0.19$). This formally demonstrates that cDNA microarray based expression profiling can be used to predict response to oxaliplatin in colorectal cancer cells.

The ‘leave-one-out’ cross-validation approach adopted produced a slightly different list of 80 genes in each iteration, as a different cell line is held out in each round of analysis. A total of 254 genes were used in at least one of the 30 cycles, and 28 of them were present in all of the 30 gene lists used (Table 1). The expression profile of all 9216 genes in the cDNA microarrays used can be found on our website (www.augenlichtlab.com).
DISCUSSION

The platinum compound oxaliplatin is frequently used in the treatment of colorectal cancer patients that are resistant to 5FU, and can also be used in combination with 5FU or CPT-11, improving response rates and progression-free survival (Levi et al., 1993; Bertheault-Cvitkovic et al., 1996; Machover et al., 1996; de Gramont et al., 1997, 2000; Andre et al., 1999; Maindrault-Goebel et al., 1999; Giacchetti et al., 2000). Oxaliplatin disrupts DNA replication and transcription by forming intrastrand DNA adducts, but the downstream molecular events underlying the cytotoxic effects of this chemotherapeutic agent have not been well characterised.

Here, we show that exposure of HCT116 colon cancer cells to clinically relevant concentrations of oxaliplatin (Ehrsson et al., 2002; Tassone et al., 2002) greatly reduced the long-term clonogenic potential of these cells (solid line in Figure 7D). This was associated with an arrest of proliferating cells in the G2/M phases of the cell cycle (Figure 1). Consistent with this observation, there was a significant reduction in the growth rate of oxaliplatin-treated cells compared to control untreated cells (solid line in Figure 7C). Importantly, exposure of HCT116 cells to oxaliplatin resulted in a significant induction of apoptosis detectable as early as 24 h after treatment with the lowest concentration assessed (see Figure 2C). Although impairment of the growth of tumour cells is an important component contributing to the overall response to chemotherapy, cell death is the preferred method of elimination of malignant cells, since this is a terminal and irreversible mechanism. Clonal selection of tumour cells frequently results in the acquisition of mechanisms of evading apoptosis. Therefore, understanding of the molecular mechanisms involved in the induction of apoptosis after exposure to chemotherapeutic agents is important for two reasons: first, it can provide information regarding pathways that may be modulated to improve treatment efficacy, and second, it can lead to the identification of markers capable of predicting the probability of response to treatment.

In this study we demonstrate that exposure of four different colorectal cancer cells (HCT116, RKO, RW2982 and SW403) to oxaliplatin resulted in recruitment of Bax to the mitochondria, release of cytochrome c to the cytosol and Caspase 3 activation. Targeted inactivation of Bax in HCT116 cells resulted in a significant reduction in the number of cells displaying a cytosolic staining pattern of cytochrome c and terminal apoptosis following exposure to oxaliplatin. These results demonstrated an important functional role of Bax in the apoptotic cascade of events initiated by exposure to oxaliplatin and are in agreement with previous reports (Gourdier et al., 2002; Hayward et al., 2004). Moreover, it has been suggested that frameshift mutations in the G8 track of the Bax gene could contribute to the acquisition of resistance to oxaliplatin in HCT116 cells (Gourdier et al., 2002). Collectively, these observations suggest the potential of Bax as a genetic marker capable of predicting the probability of response of colorectal cancer patients to this important chemotherapeutic agent.

Bax relocalisation to the mitochondria, release of cytochrome c to the cytosol and Caspase 3 activation, are all events that are consistent with the induction of an intrinsic apoptotic pathway, characterised by the central role of the mitochondria in the initiation of the caspase cascade. Some chemotherapeutic agents, however, induce an apoptotic response through activation of the extrinsic pathway by promoting Fas receptor/Fas ligand association, which in turn leads to the formation of the death-inducing signalling complex (DISC) and the autocatalytic activation of pro-caspase 8. To investigate the contribution of this pathway to oxaliplatin-induced apoptosis, we utilised antibodies that bind to Fas or FasL, thus preventing the association of these two proteins. Although abrogation of Fas/FasL association completely prevented apoptosis induced by recombinant human Fas ligand, it had no effect on oxaliplatin-induced apoptosis, suggesting the predominance of the intrinsic pathway in the apoptotic program initiated by the platinum exposure in HCT116. However, Marchetti et al. (2004) have recently demonstrated the involvement of the extrinsic pathway in oxaliplatin-induced apoptosis in HCT15 colon cancer cells, suggesting that the role of this pathway may be tumour dependent.

Considerable progress has been made in the identification of genetic markers allowing prediction of colorectal cancer response to 5-FU and CPT-11, which together with oxaliplatin are commonly used for the treatment of these patients (Augenlicht et al., 1997; Bunz et al., 1999; Salonga et al., 2000; Arango and Augenlicht, 2001; Arango et al., 2001, 2003). However, despite recent efforts (Arnould et al., 2003; Rakitsa et al., 2003) it is currently not possible to accurately predict response to oxaliplatin.
p53 is mutated in over 50% of colorectal tumours, and the mutational status of this tumour suppressor has been shown to increase or decrease tumour sensitivity to a number of chemotherapeutic agents. Here we show that oxaliplatin-induced apoptosis was associated with upregulation of p53 protein levels, detectable within 6 h of treatment. This suggested a role of p53 in the apoptotic cascade initiated by oxaliplatin. Using an isogenic system we showed that targeted inactivation of p53 resulted in a four-fold increase in the GI50, reduced apoptosis and induced a significant increase in clonogenic potential after exposure to oxaliplatin, demonstrating that inactivation of p53 can lead to significantly increased resistance to oxaliplatin. However, there are some reports showing that p53 inactivation does not lead to increased resistance to oxaliplatin (Seo et al, 2002; Petit et al, 2003), suggesting that the role of p53 in the cellular response to oxaliplatin may be tumour dependent. To further investigate the role of p53 in sensitivity of colorectal cancer cells to oxaliplatin, we used a panel of 30 different cell lines of known p53 mutational status. We measured the induction of apoptosis following exposure of these 30 cell lines to clinically achievable doses of oxaliplatin, and found that cell lines with a wild type and mutant p53 gene did not significantly differ in their apoptotic response to oxaliplatin (see Figure 8A). Similar disparities have been reported for the role of p53 in 5FU response, depending upon whether isogenic cell lines or panels of colorectal cell lines were studied (Yang et al, 1996; Bunz et al, 1999; Violette et al, 2002). Therefore, despite the clear role of p53 in the response of colon cancer cells to oxaliplatin demonstrated using the HCT116 isogenic system, the multiple genetic and epigenetic differences that exist between tumours are likely to affect numerous pathways, thus modulating sensitivity to oxaliplatin. This could limit the clinical value of p53 and other single markers of response to treatment.

Simultaneous analysis of several independent markers predicting response to drug treatment has been shown to be advantageous over single markers (Salonga et al, 2000; Arango et al, 2001). Microarray analysis provides the means of assessing the levels of expression of thousands of genes simultaneously, and we and others have recently demonstrated that the expression profile of untreated tumour cells can be used to predict response of tumour cells to different chemotherapeutic agents in vitro and in vivo (Scherf et al, 2000; Kihara et al, 2001; Zembutsu et al, 2002; Mariadason et al, 2003). Here, we used a cDNA microarray-based

Figure 7 Role of p53 in sensitivity of colon cancer cells to oxaliplatin. (A) Fold induction of apoptosis in HCT116 p53+/+ and HCT116 p53−/− cultures after exposure to 25 μM oxaliplatin for different times, or (B) to different concentrations oxaliplatin for 72 h are shown. (C) Comparison of oxaliplatin-induced growth inhibition between HCT116 p53+/+ and p53−/− cells. (D) Clonogenic potential of HCT116 p53+/+ and p53−/− cells treated with 2.5–4 μM oxaliplatin for 9 h. Values shown are the mean of at least three different experiments ± s.e. of the mean.
approach to measure the expression levels of 9216 transcripts in a panel of 30 colorectal cancer cell lines for which the sensitivity to oxaliplatin was also assessed. The profile of expression of the 80 genes best correlated with sensitivity was used to predict the quantitative apoptotic response to oxaliplatin in these 30 cell lines. Using a 'leave-one-out' cross-validation approach we demonstrated a highly significant correlation between experimentally observed and microarray-predicted apoptotic response to oxaliplatin ($R = 0.53$, $P = 0.002$).

Our microarray experiments identified a number of genes and expressed sequence tags (ESTs) that are differentially expressed in cell lines that are sensitive and resistant to oxaliplatin. Among the 254 transcripts that were used in at least one of the 30 'leave-one-out' cross-validation loops, several of them have a known role in apoptosis (Table 1). The serine/threonine kinase Protein Kinase C alpha (PKCα) has been associated with cell survival and the suppression of apoptosis (Deacon et al., 1997; Orlandi et al., 2003). PKCα was represented twice in our cDNA microarray and both probes demonstrated that expression levels were lower in cell lines with a higher apoptotic response to oxaliplatin. This is in good agreement with previous reports demonstrating that PKCα levels modulate the cellular response to cisplatin and oxaliplatin (Johnson et al., 2002; Orlandi et al., 2003). The transcription factor NFκB has been shown to be frequently deregulated in colorectal tumours, and it is associated with increased proliferation and resistance to apoptosis induced by chemotherapeutic agents (Garg and Aggarwal, 2002; Orlowski and Baldwin, 2002). Here we found that the NFκB inhibitor epsilon (NFKBIE) is expressed at higher levels in the more sensitive cell lines. This is consistent with a recent report showing that downregulation of the transcriptional
| Accession | Name | Correlation | Count in LOOCV | Biological process (GO) |
|-----------|------|-------------|----------------|------------------------|
| AA455003  | General transcription factor III, polypeptide 1, 62 kDa | -0.77 | 30 | DNA repair |
| R89008    | Progester and adipoQ receptor family member III | -0.70 | 30 | Biological process unknown |
| AA417012  | Zinc-finger protein 336 | -0.67 | 30 | Regulation of transcription, DNA-dependent |
| AA412691  | Nuclear transcription factor Y, alpha | -0.67 | 30 | Regulation of transcription, DNA-dependent |
| AA030029  | Protein kinase C, alpha | -0.65 | 30 | Regulation of cell cycle |
| R05657    | Clone 23907 mRNA sequence | -0.64 | 30 | Biological process unknown |
| AA521025  | G protein pathway suppressor 1 | -0.62 | 30 | JNK cascade |
| AA055769  | Phosphoehomoarginyl carboxykinase 1 (soluble) | -0.62 | 30 | Gluconeogenesis |
| AA605765  | Microtubule-associated protein, RP/E6 family, member 2 | -0.62 | 30 | Cell proliferation |
| AA083385  | BTB (POZ) domain containing 1 | -0.62 | 30 | Biological process unknown |
| W32660    | RAP2A, member of RAS oncogene family | -0.61 | 30 | Signal transduction |
| R46662    | RAP2A, member of RAS oncogene family | -0.61 | 30 | Signal transduction |
| H14343    | Cell division cycle 25B | -0.60 | 30 | M phase of mitotic cell cycle |
| AA480071  | Transcription factor 7 (T-cell specific, HMG-box) | -0.60 | 30 | Wnt receptor signaling pathway |
| AA456014  | Hypothetical protein FLJ20580 | -0.60 | 30 | Biological process unknown |
| H09906    | Z,3'-cyclic nucleotide 3' phosphodiesterase | -0.60 | 30 | Synaptic transmission |
| N51260    | KIAA0240 | -0.60 | 30 | Biological process unknown |
| H56069    | Glutamate–cysteine ligase, catalytic subunit | -0.59 | 29 | Cysteine metabolism |
| AA489104  | Thyroid hormone receptor interactor 3 | -0.59 | 29 | Regulation of transcription, DNA-dependent |
| T77929    | Tripeptidyl peptide II | -0.59 | 29 | Proteolysis and peptidolysis |
| AA479196  | General transcription factor II, polypeptide 2, 30 kDa | -0.59 | 30 | Transcription initiation from Pol II promoter |
| AA430656  | Docking protein 4 | -0.58 | 29 | Biological process unknown |
| H30688    | Spectrin, beta, nonerythrocytic 2 | -0.58 | 29 | Vesicle-mediated transport |
| R0570     | Hypothetical protein KIAA1434 | -0.58 | 28 | Biological process unknown |
| R54358    | Ligase IV, DNA, ATP-dependent | -0.57 | 27 | DNA repair |
| H08548    | ATP citrate lyase | -0.57 | 27 | Coenzyme A biosynthesis |
| AA418811  | Fibrillin 1 (Marfan syndrome) | -0.57 | 26 | Skeletal development |
| AA410591  | Met proto-oncogene (hepatocyte growth factor receptor) | -0.57 | 28 | Cell proliferation |
| W86653    | FK506-binding protein 5 | -0.56 | 27 | Protein folding |
| H01150    | Hypothetical protein BC017169 | -0.56 | 27 | Transport |
| AA521490  | Linkman b1 | -0.56 | 27 | Biological process unknown |
| AA447691  | Phosphatidylmetaboloproteinase 1 | -0.56 | 27 | Activation of MAPERK kinase |
| R42187    | ESTs | -0.56 | 27 | Biological process unknown |
| H89996    | CCCTC-binding factor (zinc-finger protein) | -0.56 | 27 | Negative regulation of cell cycle |
| AA148536  | Nucleoporin 98 kDa | -0.56 | 25 | Intracellular protein transport |
| R01323    | Microtubil-associated protein 1 | -0.56 | 25 | Biological process unknown |
| AA452501  | Serine/threonine kinase 24 (STE20 homolog, yeast) | -0.55 | 25 | Signal transduction |
| T49159    | Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2 | -0.55 | 24 | Antiapoptosis |
| AA001918  | ESTs | -0.55 | 24 | Biological process unknown |
| N70759    | KIAA0996 protein | -0.55 | 24 | Biological process unknown |
| AA225797  | Zinc-finger RNA binding protein | -0.55 | 19 | Biological process unknown |
| AA521232  | ras-related C3 botulinum toxin substrate 2 | -0.55 | 20 | Small GTPase mediated signal transduction |
| AA463111  | Mesenchyme homeo box 1 | -0.55 | 21 | Development |
| AA074666  | ESTs | -0.54 | 15 | Biological process unknown |
| AA477888  | Rabaptin, RAB GTPase binding effector protein 1 | -0.54 | 14 | Endocytosis |
| AA701922  | Bystin-like | -0.54 | 15 | Cell adhesion |
| AA477771  | KIAA0076 | -0.54 | 19 | Biological process unknown |
| R76314    | ras homolog gene family, member G (rho G) | -0.54 | 15 | Positive regulation of cell proliferation |
| AA004484  | F-box only protein 9 | -0.54 | 17 | Protein modification |
| AA458840  | Cell death-regulatory protein GRIM19 | -0.54 | 9 | Apoptosis |
| AA036974  | Amine oxidase, copper containing 3 | -0.54 | 9 | Amine metabolism |
| R28280    | Solute carrier family 7, member 1, 1 | -0.54 | 0 | Amino acid metabolism |
| AA045192  | Retinoic acid receptor 1 (including osteosarcoma) | -0.53 | 8 | Negative regulation of cell cycle |
| H99215    | Discs, large homolog 3 (neuroendocrine-dlg, Drosophila) | -0.53 | 10 | Negative regulation of cell proliferation |
| T71757    | Heme oxygenase (decycling) 1 | -0.53 | 6 | Heme oxidation |
| AA453175  | Bridge integrator 1 | -0.53 | 6 | Biological process unknown |
| R12275    | Hypothetical protein FLJ21827 | -0.53 | 6 | M phase of mitotic cell cycle |
| AA476662  | M-phase phosphoprotein 6 | -0.53 | 6 | Biological process unknown |
| AA434322  | Proline arginine-rich end leucine-rich repeat protein | -0.53 | 6 | Skeletal development |
| AA459266  | Postmeiotic segregation increased 2-like 6 | -0.53 | 5 | Mismatch repair |
| H94592    | Guanine nucleotide binding protein (G protein), alpha activating activity polypeptide | -0.53 | 7 | G-protein coupled receptor protein signaling pathway |
| R99749    | B-cell CLL/lymphoma 6 (zinc-finger protein 51) | -0.53 | 7 | Cell growth and/or maintenance |
| AA621031  | KIAA0056 protein | -0.53 | 7 | Biological process unknown |
| AA232647  | Zinc-finger protein 161 | -0.52 | 5 | Regulation of transcription from Pol II promoter |
| AA485214  | Nucleobindin 2 | -0.52 | 5 | Biological process unknown |
| AA009773  | Polymerase (DNA-directed), delta interacting protein 3 | -0.52 | 3 | Biological process unknown |
| AA608730  | HBS1-like (S. cerevisiae) | -0.52 | 3 | Protein biosynthesis |
| H98688    | ESTs | -0.52 | 4 | Biological process unknown |
| Accession | Name                                                                 | Correlation* | Count in LOOCVb | Biological process (GO)c |
|-----------|----------------------------------------------------------------------|--------------|-----------------|------------------------|
| AA425908  | ADP-ribosylation factor interacting protein 2 (arfaptin 2)            | -0.52        | 3               | Small GTPase mediated signal transduction |
| AA424937  | Protein kinase C, alpha                                              | -0.52        | 4               | Regulation of cell cycle |
| AA017042  | HIV-1 Tat interactive protein, 60 kDa                                | -0.52        | 3               | Regulation of transcription, DNA-dependent |
| AA284338  | Chromobox homolog 5 (HP1 alpha homolog, Drosophila)                   | -0.51        | 2               | Cell proliferation      |
| AA40170   | Chemokine (C-C motif) ligand 7                                       | -0.51        | 3               | Antimicrobial humoral response |
| AA425089  | Clock homolog (mouse)                                                 | -0.51        | 4               | Circadian rhythm        |
| N58136    | Similar to KIAA0393 protein                                           | -0.51        | 2               | Biological process unknown |
| R24543    | Neuroepithelial cell transforming gene 1                              | -0.51        | 2               | Regulation of cell growth |
| R15708    | Insulin-like 4 (placenta)                                            | -0.51        | 2               | Cell proliferation      |
| W47073    | Solute carrier family 20 (phosphate transporter), member I            | -0.51        | 3               | Phosphate transport     |
| AA703187  | Solute carrier family 33 (acetyl-CoA transporter), member I           | -0.51        | 4               | Transport              |
| W79944    | UBX domain containing 2                                               | -0.51        | 2               | Biological process unknown |
| R32592    | Growth arrest-specific 2 like 1                                       | -0.51        | 3               | Cell cycle arrest       |
| AA406027  | CDS antigen (p56-62)                                                  | -0.51        | 2               | Cell proliferation      |
| W06875    | Hypothetical protein MGCI4186                                        | -0.51        | 2               | Biological process unknown |
| R14855    | F-box only protein 34                                                 | -0.50        | 2               | Biological process unknown |
| R01451    | Hypothetical protein KIAA1259                                         | -0.50        | 1               | Biological process unknown |
| AA449154  | Skeletal muscle and kidney enriched inositol phosphatase              | -0.50        | 2               | Actin cytoskeleton organization and biogenesis |
| H48420    | Prothymosin alpha                                                     | -0.50        | 1               | Biological process unknown |
| AA455043  | Holocarboxylase synthetase                                            | -0.50        | 2               | Protein modification    |
| AA232647  | Zinc-finger protein 161                                               | -0.50        | 2               | Regulation of transcription from Pol II promoter |
| R11490    | Translocated promoter region (to activated MET oncogene)              | -0.50        | 2               | Transport              |
| T58146    | ILA complex P5                                                        | -0.50        | 2               | Defense response        |
| H584366   | KIAA0334 gene, complete cds                                          | -0.50        | 2               | Biological process unknown |
| AA406269  | Nuclear factor IX (CCAT-binding transcription factor)                  | -0.50        | 2               | DNA replication         |
| R02346    | Small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen)       | -0.50        | 1               | RNA splicing            |
| H64850    | Clone 23612 mRNA sequence                                             | -0.50        | 1               | Biological process unknown |
| N74574    | Hypothetical protein MGCR084                                          | -0.49        | 3               | Biological process unknown |
| N26645    | Neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease) | -0.49        | 2               | RAS protein signal transduction |
| R26434    | Protein phosphatase 1, catalytic subunit, beta isoform                | -0.49        | 1               | Biological process unknown |
| AA025850  | Myosin VA (heavy polypeptide 12, myoxin)                              | -0.49        | 1               | Transport              |
| H59208    | ESTs                                                                  | -0.49        | 1               | Biological process unknown |
| AA490721  | Splicing factor, arginine-serine-rich 9                               | -0.49        | 2               | mRNA splice site selection |
| R10604    | Spincerebellar ataxia 2 (olivopontocerebellar ataxia 2, autosomal dominant, ataxin 2) | -0.49        | 1               | mRNA splicing           |
| R95732    | DNA (cytosine-5')-methyltransferase 2                                 | -0.49        | 1               | DNA methylation         |
| AA149096  | Hemopoietic cell kinase                                               | -0.49        | 2               | Biological process unknown |
| AA489714  | Chromosome 9 open reading frame 60                                    | -0.49        | 1               | Biological process unknown |
| AA045387  | TAF12 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 20kDa | -0.49        | 2               | Regulation of transcription, DNA-dependent |
| AA485443  | Ring finger protein 41                                                | -0.48        | 1               | Biological process unknown |
| AA085676  | ADP-ribosylation factor guanine nucleotide factor 6                   | -0.48        | 1               | Biological process unknown |
| W58032    | Frizzled-related protein                                              | -0.48        | 1               | Wnt receptor signaling pathway |
| N71653    | Asparoacylase (aminoacylase 2, Canavan disease)                       | -0.48        | 2               | Aspartate catabolism    |
| AA004324  | Hypothetical protein BC07706                                           | -0.48        | 1               | Biological process unknown |
| AA441935  | Achaete-scute complex-like 1 (Drosophila)                            | -0.48        | 1               | Cell differentiation    |
| R22050    | Topoisomerase (DNA) II beta (180kD)                                   | -0.48        | 1               | DNA topological change  |
| W31074    | Fatty-acid-Coenzyme A ligase, long-chain 3                            | -0.48        | 3               | Fatty acid metabolism   |
| T72581    | Matrix metalloprotease 9                                              | -0.48        | 3               | Collagen catabolism     |
| AA453105  | Histone 1, H2ac                                                       | -0.48        | 1               | Nucleosome assembly     |
| AA988766  | WD repeat and SOCS box containing protein 2                           | -0.48        | 1               | Intracellular signaling cascade |
| AA495766  | Chromosome condensation 1-1                                           | -0.48        | 1               | Biological process unknown |
| AA489699  | COP9 constitutive photomorphogenic homolog subunit 8 (Arabidopsis)    | -0.48        | 1               | Biological process unknown |
| T65902    | Splicing factor, arginine-serine-rich 1 (splicing factor 2, alternate splicing factor) | -0.48        | 1               | mRNA splice site selection |
| AA446908  | Kinesin family member 3C                                             | -0.47        | 1               | Biological process unknown |
| AA047812  | Origin recognition complex, subunit 2-like (yeast)                   | -0.47        | 1               | DNA replication         |
| AA452566  | Peroxisomal membrane protein 3, 35 kDa (Zellweger syndrome)           | -0.47        | 1               | Peroxisome organization and biogenesis |
| R91948    | Pantothenate kinase 3                                                | -0.47        | 1               | Coenzyme A biosynthesis |
| T52894    | Myosin light chain 1 slow a                                           | -0.47        | 2               | Muscle development      |
| AA001749  | Microtubule-associated protein, RPE1 family, member 1                 | -0.47        | 1               | Cell proliferation      |
| AA130035  | Retinol 3                                                             | -0.47        | 1               | Biological process unknown |
| N79179    | Pyruvate dehydrogenase complex, lipoyl-containing component X, E3-binding protein | -0.47        | 1               | Biological process unknown |
| R1311     | T54 protein                                                           | -0.47        | 2               | Biological process unknown |
| AA447579  | Hypothetical protein FLJ11101                                         | -0.47        | 1               | Biological process unknown |
| AA454639  | F-box only protein 9                                                  | -0.47        | 1               | Protein modification    |
| Accession | Name                                                                 | Correlationa | Count in LOOCVb | Biological process (GO)c |
|-----------|----------------------------------------------------------------------|--------------|-----------------|--------------------------|
| AA476460  | Protein tyrosine phosphatase, receptor-type, Z polypeptide 1          | 0.33          | 1               | One-carbon compound metabolism |
| AA147642  | cDNA: FLJ21949 fis, clone HE04922                                   | 0.36          | 1               | Biological process unknown |
| AA479954  | Similar to RNA polymerase B transcription factor 3                    | 0.36          | 1               | Biological process unknown |
| N80382    | Phosphodiesterase 6D, cGMP-specific, rod, delta                      | 0.36          | 1               | Visual perception         |
| R88246    | Adrenergic, beta, receptor kinase 1                                  | 0.42          | 1               | Signal transduction       |
| AA150895  | Hypothetical protein MG26690                                        | 0.43          | 1               | Biological process unknown |
| AA680407  | Similar to ubiquitin binding protein                                  | 0.43          | 1               | Biological process unknown |
| AA0613998 | Chromosome 15 open reading frame 15                                 | 0.43          | 1               | Protein biosynthesis       |
| AA459572  | Protein phosphatase 1, regulatory subunit 7                         | 0.45          | 1               | Biological process unknown |
| AA401429  | Dynen light chain 2                                                  | 0.45          | 1               | Microtubule-based process |
| AA434130  | Thioredoxin reductase 2                                             | 0.44          | 1               | Response to oxygen radicals |
| AA453593  | BBI = malignant cell expression-enhanced gene/tumor progression-enhanced gene | 0.44          | 1               | Protein arginylation        |
| T77891    | ESTs                                                                 | 0.44          | 1               | Biological process unknown |
| N48137    | Glycophorin E                                                        | 0.44          | 1               | Biological process unknown |
| N30147    | CDNA FLJ34899 fis, clone NT2NE2018594                               | 0.43          | 1               | Biological process unknown |
| AA011185  | Hypothetical protein FLJ14431                                        | 0.43          | 1               | Metabolism                 |
| N8558     | Serine (or cysteine) proteinase inhibitor; clade A (alpha-1 antiproteinase, antithrypsin), member 4 | 0.43          | 1               | Acute-phase response       |
| AA005221  | Apoptosis, caspase activation inhibitor                               | 0.38          | 1               | Apoptosis                  |
| AA280677  | Zinc-finger protein 258                                              | 0.43          | 1               | Development                |
| AA026709  | Dedicator of cytokinesis 6                                           | 0.43          | 1               | Biological process unknown |
| AA487070  | Similar to CGI-55 protein (LOC152502), mRNA                          | 0.43          | 1               | Biological process unknown |
| AA284282  | Hypothetical protein FLJ30277                                        | 0.43          | 1               | Biological process unknown |
| T57859    | Natural killer cell group 7 sequence                                 | 0.42          | 1               | Biological process unknown |
| AA448501  | TBP-like 1                                                          | 0.42          | 1               | Regulation of transcription |
| N92085    | ESTs                                                                 | 0.41          | 1               | Biological process unknown |
| AA423867  | Multimerin                                                           | 0.41          | 1               | Blood coagulation          |
| AA443302  | ras homolog gene family, member E                                    | 0.38          | 1               | Small GTPase mediated signal transduction |
| H52361    | ESTs                                                                 | 0.37          | 1               | Biological process unknown |
| AA482198  | Mannose phosphate isomerase                                          | 0.36          | 1               | Carbohydrate metabolism    |
| H66030    | Centrosomal protein 1                                                | 0.26          | 1               | Biological process unknown |
| AA458472  | Major histocompatibility complex, class II, DQ beta 1                | 0.36          | 1               | Immune response            |
| AA447096  | Yippee protein                                                       | 0.35          | 1               | Biological process unknown |
| AA419164  | Retinoic acid receptor, beta                                         | 0.34          | 1               | Cell growth and/or maintenance |
| AA453849  | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isofrom 1 | 0.32          | 1               | ATP synthesis coupled proton transport |
| T96688    | PBX1 homedomain 1 homedobox 1                                       | 0.32          | 1               | Biological process unknown |
| R41732    | Mitogen-activated protein kinase 8 interacting protein 1             | 0.28          | 1               | Vesicle-mediated transport |
| AA419622  | Hypothetical protein FLJ10134                                        | 0.31          | 1               | Biological process unknown |
| AA128017  | Clone DNA:100312 VSSW1971 (UNQ1971), mRNA                           | 0.33          | 1               | Biological process unknown |
| R23810    | Full-length cDNA: Y109H09                                          | 0.33          | 1               | Biological process unknown |
| T90871    | EST                                                                  | 0.35          | 1               | Biological process unknown |
| AA005228  | ESTs                                                                 | 0.39          | 1               | Biological process unknown |
| AA004811  | ESTs                                                                 | 0.39          | 1               | Biological process unknown |
| R57947    | ESTs, Weakly similar to reverse transcriptase homolog                 | 0.39          | 1               | Biological process unknown |
| T95930    | ESTs                                                                 | 0.40          | 1               | Biological process unknown |
| AA058713  | ESTs                                                                 | 0.41          | 1               | Biological process unknown |
| T84865    | ESTs                                                                 | 0.41          | 1               | Biological process unknown |
| N46943    | GGA binding partner                                                  | 0.41          | 1               | Biological process unknown |
| AA677403  | Glycoprotein hormones, alpha polypeptide                             | 0.42          | 1               | Cell–cell signaling        |
| AA872379  | SMT3 suppressor of mif two 3 homolog 1 (yeast)                       | 0.42          | 1               | Biological process unknown |
| T96870    | ESTs                                                                 | 0.42          | 1               | Biological process unknown |
| AA610111  | Hypothetical protein FLJ21688                                        | 0.43          | 1               | Biological process unknown |
| AA400212  | A-kinase anchoring protein 28                                        | 0.44          | 1               | Biological process unknown |
| H23219    | ESTs                                                                 | 0.44          | 1               | Biological process unknown |
| H23124    | Olfactomedin related ER localized protein                            | 0.44          | 1               | Biological process unknown |
| AA447515  | MAX dimerization protein 4                                           | 0.44          | 1               | Negative regulation of cell proliferation |
| N62914    | EST                                                                  | 0.44          | 1               | Biological process unknown |
| T53928    | Insulin-like growth factor binding protein 7                         | 0.45          | 1               | Negative regulation of cell proliferation |
| AA421488  | CDNA FLJ26472 fis, clone KDN04506                                   | 0.46          | 1               | Biological process unknown |
| H93857    | Apolipoprotein B (including Apg(x) antigen)                          | 0.46          | 1               | Lipid transport            |
| N78306    | ESTs                                                                 | 0.46          | 1               | Biological process unknown |
| N32502    | ESTs                                                                 | 0.46          | 1               | Biological process unknown |
| AA488672  | Kruppel-like factor 7 (ubiquitous)                                   | 0.47          | 1               | Regulation of transcription from Pol II promoter |
| AA053815  | Hypothetical protein FLJ11767                                        | 0.47          | 1               | Biological process unknown |
| R02716    | Hypothetical protein FLJ14639                                        | 0.47          | 1               | Biological process unknown |
| AA701545  | Ribonuclease, RNase A family, k6                                      | 0.47          | 1               | RNA catabolism             |
| R93069    | ESTs                                                                 | 0.47          | 1               | Biological process unknown |
| Accession | Name                                                                 | Correlation | Count in LOOCV | Biological process (GO)                      |
|-----------|----------------------------------------------------------------------|-------------|----------------|---------------------------------------------|
| T54121    | Cyclin E1                                                           | 0.48        | 2              | Regulation of cell cycle                     |
| H57309    | Snail homolog 2 (Drosophila)                                       | 0.48        | 1              | Development                                  |
| T56948    | Pumilio homolog 1 (Drosophila)                                     | 0.48        | 1              | Biological process unknown                   |
| N93191    | Voltage-dependent calcium channel gamma subunit-like protein        | 0.48        | 1              | Biological process unknown                   |
| W33154    | Formin binding protein L                                            | 0.49        | 1              | Biological process unknown                   |
| T95113    | Viperin                                                             | 0.49        | 1              | Biological process unknown                   |
| AA679177  | Butyrate-induced transcript 1                                       | 0.49        | 2              | Activation of JNK                             |
| W89074    | ESTs                                                                | 0.49        | 1              | Biological process unknown                   |
| R97540    | Down syndrome critical region gene 3                                | 0.49        | 2              | Intracellular protein transport              |
| W60581    | Brain expressed, X-linked                                           | 0.49        | 2              | Biological process unknown                   |
| AA337352  | Paired box gene 1                                                   | 0.50        | 2              | Biological process unknown                   |
| T53389    | Fc fragment of IgG binding protein                                  | 0.50        | 1              | Biological process unknown                   |
| N53480    | Clone IMAGE:4794941, mRNA                                           | 0.50        | 3              | Biological process unknown                   |
| AA410604  | CDC16 cell division cycle 16 homolog (S. cerevisiae)                | 0.50        | 1              | Cell cycle                                   |
| N28287    | Ferredoxin                                                          | 0.50        | 1              | Steroid metabolism                           |
| R95893    | EST                                                                 | 0.50        | 1              | Biological process unknown                   |
| AA872397  | Lectin, galactoside-binding, soluble, 2 (galectin 2)                | 0.50        | 2              | Heterophilic cell adhesion                    |
| H72588    | ESTs                                                                | 0.51        | 2              | Biological process unknown                   |
| N92048    | ESTs                                                                | 0.51        | 3              | Biological process unknown                   |
| N54793    | ESTs                                                                | 0.51        | 3              | Biological process unknown                   |
| AA136699  | ESTs                                                                | 0.51        | 1              | Biological process unknown                   |
| AA131466  | Leukemia inhibitory factor receptor                                 | 0.51        | 4              | Cell surface receptor linked signal transduction |
| N33331    | Peroxisome proliferative activated receptor, delta                  | 0.52        | 3              | Lipid metabolism                             |
| AA668527  | Mucosal vascular addressin cell adhesion molecule 1                 | 0.52        | 5              | Cell adhesion                                |
| AA677006  | Lactotransferrin                                                    | 0.52        | 3              | Iron ion transport                            |
| AA459390  | Hypothetical protein FLJ22169                                       | 0.53        | 6              | Biological process unknown                   |
| W02624    | Kelch repeat and BTB (POZ) domain containing 2                     | 0.53        | 6              | Biological process unknown                   |
| AA984940  | Ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin) | 0.53 | 11 | RNA catabolism                                 |
| AA461180  | X-linked protein                                                   | 0.53        | 9              | Biological process unknown                   |
| R85763    | Full-length insert cDNA clone YP91F02                               | 0.54        | 11             | Biological process unknown                   |
| AA102068  | F-box and leucine-rich repeat protein 8                             | 0.54        | 13             | Biological process unknown                   |
| AA131429  | ESTs, Weakly similar to A-kinase anchor protein DAKAP550 [D. melanogaster] | 0.54 | 20 | Biological process unknown                   |
| AA009671  | ESTs                                                                | 0.54        | 21             | Biological process unknown                   |
| AA458965  | Natural killer cell transcript 4                                    | 0.55        | 19             | Cell adhesion                                |
| H51438    | ESTs                                                                | 0.55        | 24             | Biological process unknown                   |
| AA447593  | Dynemin, axonemal, light intermediate polypeptide 1                 | 0.55        | 26             | Cell motility                                 |
| T98717    | ESTs                                                                | 0.56        | 26             | Biological process unknown                   |
| AA485358  | Seven transmembrane domain protein                                 | 0.56        | 25             | Biological process unknown                   |
| AA022935  | Formin-like 2                                                       | 0.56        | 27             | Regulation of transcription, DNA-dependent   |
| H87770    | Full-length insert cDNA Y127F12                                     | 0.56        | 25             | Biological process unknown                   |
| AA024866  | Hypothetical protein FLJ32731                                       | 0.56        | 28             | Biological process unknown                   |
| AA053992  | Hypothetical protein MGC17791                                       | 0.57        | 28             | Biological process unknown                   |
| R92446    | Cytokine receptor-like molecule 9                                   | 0.57        | 28             | Biological process unknown                   |
| AA443971  | ESTs                                                                | 0.57        | 29             | Biological process unknown                   |
| N79813    | CDNA FLJ45930 fis, clone PLACE0700070                               | 0.59        | 28             | Biological process unknown                   |
| W80688    | Zinc finger, CW-type with coiled-coil domain 1                      | 0.59        | 29             | Biological process unknown                   |
| N72185    | ESTs                                                                | 0.60        | 30             | Biological process unknown                   |
| AA136666  | CDNA: FLJ22750 fis, clone KIAA0478                                  | 0.60        | 30             | Biological process unknown                   |
| AA133167  | KIAA1644 protein                                                   | 0.60        | 30             | Biological process unknown                   |
| T84703    | ESTs                                                                | 0.61        | 30             | Biological process unknown                   |
| AA029997  | Collagen, type IV, alpha 5 (Alport syndrome)                        | 0.62        | 30             | Biological process unknown                   |
| AA953975  | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon | 0.62 | 30 | I-kappaB kinase/NF-kappaB cascade |
| W88801    | Trans-prenyltransferase                                            | 0.63        | 30             | Isoprenoid biosynthesis                       |
| N70632    | Alcohol dehydrogenase, iron containing, 1                          | 0.65        | 30             | Metabolism                                   |
| AA701082  | Leucine-rich repeat transmembrane neuronal 2 protein                | 0.65        | 30             | Biological process unknown                   |
| W93482    | LOC374841 (LOC374841), mRNA                                         | 0.67        | 30             | Biological process unknown                   |
| R0886     | Stannicollin 2                                                     | 0.68        | 30             | Cell—cell signaling                          |

*Correlation coefficient between expression levels in 30 colorectal cancer cell lines (Ln) and the corresponding proportion of apoptotic cells after 72 h exposure to 10 μM oxaliplatin (Ln). The correlation of all 9216 genes can be found in our website: www.augenlichtlab.com. *Number of times used in the leave-one-out cross-validation process. *Biological process from Gene Ontology.
activity of NFκB significantly sensitises colorectal cancer cells to cytotoxic effects of oxaliplatin (Rakitina et al., 2003). In addition, the recently identified apoptosis inhibitor AVEN (Chau et al., 2000) was found to be expressed at higher levels in cell lines that showed reduced apoptosis in response to oxaliplatin.

The cytotoxic activities of oxaliplatin are believed to be linked to DNA damage, and the levels of expression of the DNA repair endonuclease ERCC1 (excision repair cross-complementing 1) have been shown to be inversely correlated with response to oxaliplatin (Shirota et al., 2001; Arnould et al., 2003). Although ERCC1 was not represented in the 9K chips used in this study, our microarray analyses identified at least four genes involved in DNA repair mechanisms that were significantly correlated with the ability of oxaliplatin to induce apoptosis in colorectal cancer cells (see Table 1). The histone acetyltransferase HATATIP (HIV-1 Tat interactive protein, 60 kDa), postmeiotic segregation increased 2- (see Table 1). The histone acetyltransferase HATATIP (HIV-1 Tat interactive protein, 60 kDa), postmeiotic segregation increased 2-

In summary, this study investigates the molecular mechanisms underlying the cytotoxic effects of oxaliplatin in colorectal cancer cells in an attempt to identify different means of predicting response to this chemotherapeutic agent. We demonstrate that exposure of proliferating colorectal cancer cells to oxaliplatin induces a G2/M arrest and a molecular cascade of events consistent with an intrinsic mechanism of apoptosis. Moreover, the cytotoxic effects of oxaliplatin were shown to be Bax and p53 dependent using and isogenic in vitro system. Importantly, we demonstrate that the expression profile of untreated tumour cells can be used to predict response to oxaliplatin, and that this approach outperforms the accuracy of p53 mutational status as a predictive marker. The efficacy of this microarray-based approach to predict response to oxaliplatin remains to be confirmed in vivo. Collection of tumour samples from suitable patient populations is currently ongoing at our institution to test the value of these approaches, although completion of the study is dependent upon prolonged follow-up periods to assess response to therapy.

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