Inhibition of survivin expression after using oxaliplatin and vinflunine to induce cytogenetic damage in vitro in lymphocytes from colon cancer patients and healthy individuals

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Abstract

Chemotherapy drugs usually inflict a lethal dose to tumour cells with the consequence that these cells are being killed by cell death. However, each round of chemotherapy also causes damage to normal somatic cells. The DNA cross-linking agent oxaliplatin which causes DNA double-strand breaks and vinflunine which disrupts the mitotic spindle are two of these chemotherapy drugs which were evaluated *in vitro* using peripheral lymphocytes from colorectal cancer patients and healthy individuals to determine any differential response. Endpoints examined included micronucleus (MN) induction using the cytokinesis-blocked micronucleus (CBMN) assay and pancentromeric fluorescence *in situ* hybridisation. Also, survivin expression was monitored since it regulates the mitotic spindle checkpoint and inhibits apoptosis. Oxaliplatin produced cytogenetic damage (MN in binucleated cells) via its clastogenic but also previously unknown aneugenic action, possibly through interfering with topoisomerase II, whilst vinflunine produced MN in mononucleated cells because of incomplete karyokinesis. Survivin expression was found to be significantly reduced in a concentration-dependent manner by not only oxaliplatin but surprisingly also vinflunine. This resulted in large numbers of multinucleated cells found with the CBMN assay. As survivin is upregulated in cancers, eliminating apoptosis inhibition might provide a more targeted chemotherapy approach; particularly, when considering vinflunine, which only affects cycling cells by inhibiting their mitotic spindle, and alongside possibly other pro-apoptotic compounds. Hence, these newly found properties vinflunine – the inhibition of survivin expression - might demonstrate a promising chemotherapeutic approach as vinflunine induces less DNA damage in normal somatic cells compared to other chemotherapeutic compounds.

Keywords: Oxaliplatin, vinflunine, survivin, micronuclei assay, western blot

1. Introduction

Human peripheral blood lymphocytes (PBL) have been used for over half a century to monitor various genetic effects due to being exceptionally sensitive for *in vivo* and *in vitro* induced cytogenetic damage. This resulted in specific WHO guidelines for monitoring genotoxic effects of carcinogens in humans, in particular describing the use of PBL as surrogate cells and genotoxicity endpoints as predictors of human cancer risk (1). As PBL travel the entire circulatory system, with PBL sub-populations having mean lifespans of 6 years (2), their individual and collective DNA integrity also reflects endogenously and exogenously induced damage from various stressors accumulated over time and distance. Hence, PBL originating from patients with cancer or with precancerous disease states have been shown to harbour an increased amount of cytogenetic damage while being increasingly more sensitive to DNA damaging agents than those from healthy individuals (3). The novel lymphocyte genome sensitivity assay for cancer diagnostics uses this differential PBL sensitivity to DNA a damaging stressor as a biomarker (4). Evaluating cytogenetic damage by detecting micronuclei (MN) *in vitro* in PBL cultures has been used for more than 20 years (5). In the cytokinesis-blocked MN (CBMN) assay, chromosomal acentric fragments from structural aberrations or even whole chromosomes can be recognized as extranuclear in the cytosol of cytokinesis-blocked binucleated cells (6) even linking these changes to cancer risks (7).

A genotoxic drug may not only cause cytogenetic damage but also impair proteins involved in apoptosis and cell cycle control. Survivin, the smallest member of the inhibitor of apoptosis protein (IAP) family, is a highly conserved protein being implicated in inhibiting apoptosis by interfering with various caspases, but it also plays a regulatory role for the mitotic spindle checkpoint, the promotion of angiogenesis and chemoresistance (8,9). Several mitotic kinases, including the three Aurora kinases, Aurora-A, -B and -C, regulate the progression of the cell through mitosis. Evidence has shown that the proto-oncogene survivin acts as a mitotic regulator being expressed during mitosis in a cell cycle dependent manner. It

functions as a subunit of the chromosomal passenger complex, which is essential for proper chromosome segregation and cytokinesis. In this complex, Aurora B acts as the enzymatic core, while survivin dictates chromosomal passenger complex localization (10). X-ray crystallography has revealed an unusual bow tie-shaped dimer with two α-helical extensions interacting with the microtubules through these α-helical extensions at the carboxyl termini (11). Survivin is uniquely placed at the border of both the cell-death machinery and mechanisms of cell cycle progression / microtubule stability linking mitotic spindle functions to apoptotic pathways (12). Expressed in the G2/M phase, survivin is up-regulated in almost all cancers but has low or no expression in most normal, differentiated adult tissues (13). In colorectal cancer (CRC), survivin over-expression is stimulated by TCF/β-catenin, linking enhanced cell proliferation with resistance to apoptosis (14) and probably also to chromosomal instability (15). This promotes tumourigenesis but also cancer progression, poor prognosis, shortened patient survival and resistance to chemo- and radiation therapies (16).

A number of molecules are able to modulate survivin expression and function in cancer cells through transcriptional mechanisms with an essential role for Sp1 sites and/or posttranscriptional mechanisms (17). The pro-apoptotic TP53 wild-type protein has been shown to effectively suppress survivin expression in normal cells by directly interacting with the TATA-less survivin promoter, while in cancer cells mutant TP53 contributes to over-expression of survivin due to its inability to bind the promotor, acting in concert with increased anti-apoptotic phosphatidylinositol 3-kinase/Akt signalling (17,18). The increase in survivin expression has been found to be maintained throughout the different CRC stages, i.e. during the mucosa-adenoma-carcinoma sequence (19). At the protein level, phosphorylation of survivin is important for its biological activity; hence function and over-expression strongly correlates with proliferative tumour activity indicating a possible role in cell cycle regulation and cancer progression (20). This makes IAP family members as a class of anti-apoptotic regulator proteins targets for novel therapeutic treatments (21,22).

Thus, survivin suppression can play a crucial role in such an approach, reversing suppression of apoptosis and increasing fidelity of chromosome segregation during mitosis.

Oxaliplatin, a second generation diaminocyclohexane platinum complex, is an antineoplastic agent which induces apoptosis in CRC cells. The biological activity of oxaliplatin is mainly based on its ability to form lethal DNA lesions, including interstrand DNA crosslinks and DNA-protein crosslinks (23), leading to the induction of apoptosis by finally inducing double-strand breaks as seen via persistent γ-H2AX foci (24). Oxaliplatin is also a potent inhibitor of survivin significantly reducing levels of protein in human CRC cell lines by p38 MAP kinase and the proteasome degradation pathway (25). However, the primary mechanism through which cell death occurs after exposure to oxaliplatin depends on the cell type and is not yet fully understood (26). Vinflunine on the other hand is a second generation Vinca alkaloid showing also anti-neoplastic activity in a wide spectrum of solid tumours. It acts by binding to tubulin and subsequently causes cells to arrest in mitosis (27). Vinflunine administration has been found to potentiate the anti-cancer activity of the platinum-based chemotherapeutic drug, cisplatin, in colon adenocarcinomas (28).

The aim of this study was to investigate *in vitro* the cytogenetic effects of the cross-linking agent oxaliplatin and the spindle inhibitor vinflunine, both chemotherapeutic compounds, in PBL of healthy individuals and yet untreated colon cancer patients using the well-established and reliable cytokinesis-blocked micronucleus (CBMN) assay. In addition, survivin protein levels in PBL were assessed using Western blotting and were linked to the results from CBMN assay.

2. Methods

If not stated otherwise all chemicals were generally purchased from Sigma-Aldrich, UK.

2.1 Blood samples

Ethical approval was granted for healthy non-smoking individuals by the University of Bradford Research Ethics sub-committee (reference no.: 0405/8) and for colorectal cancer patients by the Bradford Royal Infirmary Hospital local Ethics Committee (Reference no.: 04Q1202/15). Peripheral blood was obtained after informed consent from 25 healthy non-smoking volunteers and 25 colorectal cancer patients (prior to undergoing treatment). This limited the number of patients due to the small sampling window.

2.2 CBMN assay

The CBMN assay used is based on Fenech's protocol (29). In brief, cultures were started with 500 µl of whole blood in RPMI 1640 with Glutamax-I supplemented with 15% foetal bovine serum, 1% penicillin-streptomycin solution) and 2.5% phytohaemagglutinin (chemicals from Invitrogen, UK) and incubated for 72 hours at 37 °C and 5% CO_2. Treatment with oxaliplatin (0.02, 0.2 and 2 µM) and vinflunine (0.06, 0.6 and 6 µM; LGM Pharma, FL, USA) was carried out 24 hours into the culture. Mitomycin C (0.4 µM) was used for the positive control. Cell viability was measured using the Trypan blue exclusion viability test (1:2 dilution with 0.4% Trypan blue; based on 100 evaluated cells) 1 hour after treatment. To exclude cytotoxic effects, any concentration producing cell viability below 75% was excluded (30). Cytokinesis was blocked using 6 µg/ml cytochalasin B after 44 hours. At the end of culture, cells treated with 110 mM KCl (15 min at 4 °C) and fixed with Carnoy's solution (1 part acetic acid and 3 parts methanol). For slide preparation, two drops (each 20 µl of the cell suspension) were dropped onto a clean glass slide and left to air-dry. Cells were stained using 5% filtered Giemsa (VWR, UK; in phosphate buffer, pH 6.8). Cover-slipped slides were used to evaluate the cells. The nuclear division index (NDI) based on 1,000 cells per concentration point was calculated according to the following formula: NDI = $\frac{M_1 + 2\times M_2 + 3\times M_3}{N}$

(M_1, M_2 and M_3 representing mononucleated, binucleated and multinucleated cells, respectively; N representing the total number of cells). The frequencies of induced MN were evaluated in 1,000 binucleated cells per concentration point. Frequencies of MN in mononucleated cells per concentration point were extrapolated to MN per 1,000 cells. Also, the frequencies of nucleoplasmic bridges (NPBs) and buds per 1,000 binucleated cells per concentration point were recorded. Statistical analysis was carried out using SPSS (version 13). NDI data were analysed by the Chi-Square Test, while MN formation was analysed using the Fisher's Exact Test.

2.3 Micronucleus fluorescence in situ *hybridisation (MN-FISH) assay*

The MN-FISH assay was carried out using unstained MN assay slides as previously described (31). In brief, target DNA was denatured for 3 min at 75 °C in 70% formamide (in 2x SSC, pH 7.0; Ambion) followed by dehydration in an ethanol series (70, 90 and 100%; 2 min each) and allowed to air-dry. The hybridization mix containing 2 µl biotinylated pan-centromeric DNA probe (32), 1 µl salmon sperm DNA (Invitrogen, UK) and 7 µl master mix (78.6% formamide, 14.3% dextran sulphate, 1.43x SSC, pH 7.0) was incubated for 10 min at 75 °C to allow denaturation of the DNA probe. This hybridisation mix (10 µl per slide) was then applied onto air-dried slides followed by overnight incubation at 37 °C and washing in 50% formamide (in 2x SSC, pH 7.0, 45 °C, 30 min) and in PN buffer (0.1 M Na_2HPO_4, 0.1 M Na H_2PO_4, 0.05% Igepal CA-640, pH 8.0, 45 °C, 10 min). Surfaces were blocked with PMN solution (5% non-fat dry milk powder in PN buffer, 0.1% NaN_3, pH 8.0, 10 min) prior to applying 5 µg/ml fluorescein-conjugated avidin per slide (30 min at room temperature). Slides were washed for 10 min in PN buffer, DNA stained with 0.05 µg/ml 4',6-diamidino-2-phenylindole (DAPI) and cover-slipped with antifade solution (0.2 M Trizma, pH 8.0, 90% glycerol, 2.33% 1,4-diazabicyclo[2.2.2]octane (DABCO)). A total of 100 binucleated cells containing MN were evaluated for the presence or the absence of pancentromeric signals (green fluorescence). For statistical analysis, the Fisher's Exact Test was used.

2.4 Western blot assay

Cultures were set-up as described for the CBMN assay but using separated lymphocytes. For lymphocyte separation, two parts of diluted heparinised blood (1:2 with saline, 0.9% NaCl) were carefully layered on top of one part of Lymphoprep (Axis-shield, Norway) without disturbing the Lymphoprep layer. After centrifugation (650 x*g*; 20 min), the white PBL layer directly above the Lymphoprep layer was then transferred into 10 ml of saline. After a further centrifugation (400x *g*; 15 min) and removal of the supernatant, the pellet was resuspended in culture medium.

Twenty-four hours after the start of the cultures, the medium was removed and cells were transferred into fresh medium; then treated with oxaliplatin (0.02, 0.2 and 2 µM) and vinflunine (0.06, 0.6 and 6 µM) prior to continuing the cultures for another 48 hours. Then cultures were centrifuged (500x *g*; 9 min) and pellets were washed twice in cold PBS) before being resuspended in RIPA buffer supplemented with protease inhibitor cocktail. After 30 min on ice and a brief sonication (5 s, 30 W), the suspension was centrifuged (12,000x *g*, 30 min) and the supernatant was collected. Total protein concentrations were then quantified by the method of Bradford (33). The polyacrylamide gel was prepared by overlaying the resolving gel (375 mM Trizma, pH 8.8, 0.1% SDS, 12% acrylamide/bis-acrylamide 19:1 (Bio-Rad, UK), 0.05% ammonium persulfate, and 20 µl TEMED) with the stacking gel (125 mM Trizma, pH 6.8, 0.1% SDS, 4% acrylamide/bis-acrylamide 19:1, 0.05% ammonium persulfate and 5 µl TEMED). Protein samples were mixed (1:2) with 2x Laemmli buffer (4% SDS, 10% mercaptoethanol, 20% glycerol, 125 mM Trizma, pH 6.8, 0.004% bromophenol blue), boiled for 5 min, shortly spun and then loaded onto the gel. For each experiment a negative control with extracted protein from untreated cultures was used, while Jurkat whole cell lysate (Abcam, UK) served as a positive control. A biotinylated protein ladder (9-200 kDa; Cell Signalling, UK) was used as a size reference. The polyacrylamide gel was run as a discontinuous system employing a XCell SureLock mini cell (Invitrogen, UK) and electrophoresis buffer (25 mM Trizma, 192 mM glycine, 0.1% SDS). The voltage was set to

50 V for the passage through the stacking gel and to 100 V for 2 hours through the resolving gel. After the electrophoresis, proteins were transferred to a nitrocellulose blotting membrane using the iBlot® Gel Transfer Device (25 V, 9 min; Invitrogen, UK). The membrane was incubated for 1 hour at room temperature with blocking solution (5% bovine serum albumin in TBS-T buffer) and then washed 3x for 10 min with only TBS-T buffer (150 mM NaCl, 20 mM Trizma, 0.1% Tween® 20, pH 7.4). Thereafter, the blotting membrane was incubated overnight at 4ºC on a shaker with the primary antibody (rabbit anti-Survivin monoclonal antibody ABfinity™ Recombinant (Invitrogen, UK); 1:2,000 in blocking solution). As an internal control the GAPDH rabbit monoclonal primary antibody (Invitrogen, UK) was used. Then, the secondary antibody (anti-rabbit IgG, horseradish peroxidase-linked antibody (Cell Signalling, UK); 1:3,000 in blocking solution) was added together with an HRP-conjugated anti-biotin antibody (Cell Signalling, UK; 1:1,000 in blocking solution) and incubation continued with gentle agitation for 1 hour before washing the membrane 4x in TBS-T buffer for 15 min each. For staining, the enhanced chemiluminescence (ECL) kit solution (Amersham ECL Prime Western Blotting Detection Reagent; GE Healthcare Life Sciences, UK) was used before the membrane was exposed for 2 min to an Amersham Hyperfilm X ray film (GE Healthcare Life Sciences, UK). The film was then immediately developed (manual process using developer and fixer). Relative protein expression was determined by densitometric analysis using Scion Image (Scion Corporation, Fredrick, MD, USA). GAPDH served as the protein loading control. Statistical analysis was carried out using SPSS (version 13). Data from three independent experiments were assessed for normality using normal probability plots. The significance between treatment concentrations was analysed by One–way ANOVA followed by the Dunnett's *Post Hoc* Test.

3. Results

3.1 Nuclear division index (NDI)

For determining the NDI, the frequencies of three different cell types were evaluated within a total number of 1,000 cells: mononucleated cells (MoNC), binucleated cells (BiNC) and multinucleated cells (MultiNC) containing three or four nuclei, respectively. It was shown that untreated PBL from healthy individuals and from CRC patients contained on average 26.1% and 25.5% MoNC, 72.8% and 70.8% BiNC as well as 1.1% and 3.7% MultiNC, respectively, resulting in NDI values of 1.73 and 1.82, respectively (Table 1).

Oxaliplatin (2 µM) treatment significantly increased NDI values from 1.73 to 2.39 ($p < 0.001$) in PBL from healthy individuals and from 1.82 to 2.42 ($p < 0.001$) in those from CRC patients driven by a highly significant concentration-dependent increase in MultiNC from both donor groups (45.8-fold, $p < 0.001$, and 15.2-fold, $p < 0.01$, respectively) (Table 1). Even the smallest concentration of 0.02 µM yielded a significant increase in MultiNC (18.5-fold, $p < 0.001$, and 7-fold, $p < 0.05$, respectively). At the same time the percentage of BiNC significantly decreased for the highest concentration by approximately one half (2.0-fold and 2.4-fold, respectively; $p < 0.01$). Even for the lowest concentration the numbers of BiNC were reduced (1.2-fold and 1.5-fold, respectively) but not above significance; although, this trend towards lower numbers of BiNC was significantly stronger ($p < 0.01$) in PBL from CRC patients when compared to those from healthy individuals. Only the lower concentration of oxaliplatin (0.2 µM) significantly ($p < 0.01$) decreased the number of BiNC more in PBL from CRC patients when compared to those from healthy individuals.

Vinflunine (0.6 µM) treatment significantly increased NDI values from 1.73 to 2.55 ($p < 0.001$) in PBL from healthy individuals and from 1.82 to 2.43 ($p < 0.01$) in those from CRC patients. Concomitantly, a highly significant concentration-dependent increase in MultiNC from both donor groups (52.0-fold, $p < 0.001$, and 15.4-fold, $p < 0.01$, respectively) was observed (Table 1). This increase in MultiNC was less prominent due to the 3.4-times higher baseline

frequency in PBL from CRC patients. The lower concentration of 0.06 µM also yielded a significant increase in MultiNC (15.1-fold, $p < 0.01$, and 6.1-fold, $p < 0.05$, respectively). Only at the higher concentration of 0.6 µM, the percentage of BiNC significantly decreased 1.7-fold and 2.5-fold, respectively ($p < 0.01$) showing a significantly greater reduction (1.5-fold, $p < 0.05$) in PBL from CRC patients. Only the 0.6 µM concentration of vinflunine significantly ($p < 0.05$) decreased the number of BiNC more in PBL from CRC patients when compared to those from healthy individuals.

3.2 Micronuclei induction

The results for the MN formation are summarised in Table 1. The observed baseline damage in PBL from healthy individuals and from CRC patients was 3 MN and 10 MN per 1,000 BiNC and 2 MN per 1,000 MoNC, respectively, indicating a trend towards higher cytogenetic damage in these PBL. Only the oxaliplatin treatment of PBL from healthy individuals and from CRC patients with concentrations of 0.02, 0.2 and 2 µM resulted in the formation of MN in BiNC at frequencies of 1.5% and 3.6% (both $p < 0.01$), 2.7% and 4.1% (both $p < 0.001$) as well as 3.7% and 5.3% (both $p < 0.001$), respectively. Vinflunine did not induce MN in BiNC. However, in contrast to oxaliplatin, vinflunine treatment at concentrations of 0.06 and 0.6 µM significantly induced the formation of MN in MoNC in PBL from both groups (observed baseline frequencies of 0.2% and 0.15%, respectively). Vinflunine significantly increased the number of MN in MoNC to 8.2% and 10.1% ($p < 0.001$) as well as to 12.5% and 12.9% ($p < 0.001$), respectively, in PBL from healthy individuals and from CRC patients (Table 1). Furthermore, oxaliplatin treatment increased MN formation within MultiNC (data not shown), but such data were not taken into consideration (29).

Besides MN, also nucleo-plasmatic bridges (NPBs) and buds were recorded. Frequencies in were very low (e.g. 2 NPBs in 100,000 BiNC). No significant increases regarding these two endpoints were found. Although, Table 1 shows that buds were more frequent in lymphocytes from patients from colon cancer patients.

3.3 Investigation of the origin of MN formation by MN-FISH

Using the MN-FISH assay the mechanism of oxaliplatin-induced MN formation was investigated in order to differentiate between clastogenicity (acentric DNA fragments) and aneuploidy (missegregated chromosomes with a centromere) (29). For each of the oxaliplatin concentrations 0.02, 0.2 and 2 µM and for each of the two groups (healthy individuals and CRC patients) hundred aberrant BiNC that carry MN were evaluated (Figure 1). With increasing concentrations and for both groups ~60%, ~70% and ~80% of the MN were centromere-positive (C+MN). The results for PBL from healthy individuals or CRC patients, respectively, were all significantly different ($p < 0.001$) from the negative controls. The latter only showed levels below 10% for both groups and the majority of MN found in BiNC to be centromere-negative, thus, chromosomal fragments. Also, MN in MultiNC after treatment with oxaliplatin resulted from missegregated chromosomes as indicated by mainly centromere-positive MN (data not shown).

3.4 Inhibition of survivin expression by oxaliplatin and vinflunine in PBL from colon cancer patients

The survivin protein expression in PBL from colon cancer patients treated with 0.02, 0.2 and 2 µM oxaliplatin was evaluated. Untreated cells were used as a negative control to determine the cut-off value of survivin overexpression for comparison. For all tested oxaliplatin concentrations the expression of survivin was significantly reduced (Figure 2a). Survivin expression in PBL from colon cancer patients following treatment with different concentrations of oxaliplatin. The control consisted of untreated PBL and the significant differences were shown in relation to the control (Figure 2b). Lane 1 represents survivin expression in untreated PBL from colorectal cancer patients (negative control) while lane 2 represents survivin expression in Jurkat cells (positive control). Lanes 3-5 represent the survivin expression in treated PBL using 0.02, 0.2 and 2 µM oxaliplatin, respectively. Constitutively expressed GAPDH was used as loading control. The data presented result from three independent experiments using three male colon cancer patients ($n = 3$).

The reduction was concentration dependent. When normalised to the control levels, survivin expression in oxaliplatin-treated PBL was reduced by 10% (1.3-fold decrease, $p < 0.05$) at a concentration of 0.02 µM, by 39% at a concentration of 0.2 µM (1.6-fold decrease, $p < 0.01$) and by 72% (3.6-fold decrease, $p < 0.01$) at the highest concentration of 2 µM (Figure 2a). Vinflunine only produced at the higher concentrations of 0.6 and 6 µM a statistically significant reduction in survivin expression by 39% (1.6-fold decrease, $p < 0.01$) and by 85% (6.7-fold decrease, $p < 0.01$), respectively (Figures 3a and 3b). There was no statistically significant difference between the survivin expression levels of untreated and 0.06 µM vinflunine-treated PBL.

4. Discussion

It has been known that lymphocytes originating from cancer patients show a higher frequency of cytogenetic damage. They are also more sensitive to DNA damaging, genotoxic agents than PBL from healthy individuals allowing an explicit comparison between these different donor groups (3). Hence, this study uses PBL from healthy individuals and CRC patients to investigate two chemotherapeutic compounds, oxaliplatin and vinflunine. While the NDI, the percentages of MoNC and BiNC were found to be in the same range (Table 1), the percentage of MulitNC showed a trend to be higher in PBL from CRC patients. Also, the baseline MN frequency per 1,000 BiNC was slightly increased in PBL from healthy individuals compared to those of CRC patients. This increase, even though not significant, showed a trend towards higher cytogenetic damage in PBL from CRC patients as previously shown for MN induction or for genotoxic damage in the Comet assay (4).

Both investigated compounds follow two different modes of DNA damaging action: oxaliplatin acts via inducing significant mitochondrial oxidative stress (34) and DNA cross-links (35); thus, if not repaired correctly, the induction of DNA double-strand breaks, while vinflunine disturbs and inhibits the spindle apparatus (36) leading to missegregated chromosomes. As a consequence, oxaliplatin mainly creates structural aberrations whereas vinflunine mainly

induces numerical abnormalities. Our findings (Table 1) showed that with increasing concentrations of oxaliplatin (0.02, 0.2 and 2 µM) the induction of MN in PBL from healthy individuals and CRC patients significantly increased *in vitro* for all three concentrations (Table 1). At the highest concentration, this increase was 12.4-fold in PBL from healthy individuals and 5.3-fold in PBL from CRC patients (both, $p < 0.001$) being in the range of the positive control (0.4 µM mitomycin C). The higher fold-increase of MN in PBL from healthy individuals was due to the lower baseline damage in untreated PBL compared to PBL from CRC patients. The actual number of induced MN in PBL of the latter group was 1.4-times higher confirming earlier findings that PBL from cancer patients are showing higher baseline damage and greater sensitivity(3).

In contrast, both evaluated concentrations of vinflunine (0.06 and 0.6 µM) did not show any increase in micronuclei above baseline frequencies. This clearly demonstrates the expected clastogenic action of oxaliplatin and that PBL from CRC patients are indeed more sensitive to cytogenetic damage caused by the action of oxaliplatin. Such micronuclei may either contain acentric chromosomal fragments or missegregated whole chromosomes that are defined by their centromeres (29); hence, using pan-centromeric fluorescence *in situ* hybridisation probes (31), the percentages of centromere-positive MN (C+MN) in BiNC were assessed in PBL of both donor groups (Figure 1) showing a highly significant ($p< 0.001$) concentration-dependent increase in C+MN to percentages of 56%, 78% and 82%, respectively, for the three oxaliplatin concentrations. There is also a tendency to even higher frequencies of C+MN when treating PBL from CRC patients with oxaliplatin (Figure 1). This result clearly demonstrates that oxaliplatin not only acts as a the well-known clastogenic compound (37), but unexpectedly also as an aneugen leading to missegregated whole chromosomes. So far, only cisplatin has been found to cause dose-dependent endoreduplication in Chinese hamster cells; hence, producing chromosome instability and polyploidy (38).

The frequencies of MN found in MoNC (Table 1) stayed for all three concentrations of oxaliplatin and the positive control at negative control levels. For vinflunine on the other hand, an extremely large, highly significant ($p < 0.001$) number of MN was observed in MoNC in PBL from healthy individuals (53.6 and 81.7-fold increase) and from CRC patients (50.5 and 64.5-fold) for both evaluated non-cytotoxic concentrations, evidently indicating that this compound acted in disrupting the spindle and inhibiting karyokinesis; thus, also impeding cytokinesis which resulted in a significantly increased number of MoNC and MN within them. This shows that the mechanism in which vinblastine causes aneuploidy is different from the novel aneugenic action found for oxaliplatin. Hence, oxaliplatin does not target the spindle apparatus like vinflunine but more likely interferes with DNA topoisomerase II and enzyme essential for segregating replicated chromosomes during mitosis (38,39). These different modes of action for both chemicals can also be observed by calculating the nuclear division index (NDI) using the numbers of MoNC, BiNC and MultiNC per concentration point (29); however, for oxaliplatin and vinflunine, this lead to unexpected results. All three oxaliplatin concentrations significantly increased the NDI in PBL from both donor groups (Table 1). The same was seen to an equal extent for vinflunine, except for the lower concentration in PBL from healthy individuals where the result did not reach significance. For both compounds, a NDI decrease would have been expected due to cell cycle inhibition caused by the induction of DNA damage (35) or spindle disruption (40). But it was indeed found that the NDI increases – driven by a significant 15-fold increase in the number of multinucleated cells (for percentages see Table 1) for the high concentrations of oxaliplatin and vinflunine. Usually, the occurrence of large numbers of MultiNC in the CBMN assay indicates an increased cell cycle rate. Hence, it was rather confusing at first why such an effect was observed as both compounds would normally induce cell cycle arrest due to the introduction of either DNA lesions (41) or the disruption of spindle microtubules (40). Interestingly, another platinum-based chemotherapeutic compound, satraplatin (42), did not show an increase in MultiNC using the same assay (data not shown).

Anti-tumour drugs can lead to a form of cell death in treated cells called mitotic catastrophe. Kondo proposed in 1995 a concept of a 'G1/G2 death circuit' where cells dying in the gap phases of the cell cycle might short circuit to M phase (43). Mitotic catastrophe is generally characterized by an aberrant mitosis and the formation of large cells containing multiple nuclei (44); hence, the increased frequency of multinucleated cells by oxaliplatin and vinflunine found in this study. At the molecular level, the unusual high number of induced multinucleated cells by oxaliplatin and vinflunine can be explained by the decreased expression of the anti-apoptotic protein survivin after *in vitro* treatment. Survivin belongs to the IAP family inhibiting apoptosis and promoting cell growth by microtubule stabilisation during mitosis (45). Survivin also interacts with other proteins such as aurora B in the cell functioning as a spindle checkpoint, regulating chromosome segregation and maintaining genomic stability (46). It has been demonstrated that a lack of survivin results in a disrupted cell division, mitotic catastrophe and subsequently in polyploidy (47). Survivin seems to be a key target of oxaliplatin (48) and expression of survivin was found to be significantly reduced for all three oxaliplatin concentrations (Figure 2a). When treating PBL with vinflunine a significant reduction of survivin expression was also observed but only for the concentrations 0.6 and 6 µM (Figure 3a). Although, the highest concentration of 6 µM vinflunine was cytotoxic in the MN assay when using established treatment times. It has been previously shown that survivin is upregulated in many cancers (49) where it is targeted as a molecular biomarker (50). The chemotherapeutic drug oxaliplatin was found to down-regulate survivin in colon cancer cells via p38 MAP kinase and proteasome degradation pathways (25) and aids to reduce resistance to chemotherapy (51). Inhibiting survivin significantly decreased tumour growth and induced apoptosis, hence it became an attractive target for CRC treatment (52) as over-expression of survivin together with down-regulated tumour suppressor miR-16-1 in CRC stem cells (CCSC) is thought to be one of the primary causes for therapy failure. Therefore, the search for new anti-proliferative agents which target survivin or miR-16-1 in CCSC is warranted. Prodigiosin, a compound isolated from the cell wall of *Serratia marcescens*, has been shown to strongly induce apoptosis in various cancer

cells by targeting survivin and miRNA-16-1, which makes it a potential candidate for a direct therapeutic approach against CCSC (53).

Our study confirmed oxaliplatin's and vinflunine's DNA damaging action on PBL from healthy individuals and CRC patients. For the first time, it has been shown that oxaliplatin also acts as an aneugen causing missegregation of chromosomes possibly by interfering with topoisomerase II as well as inhibiting survivin expression. Vinflunine on the other hand is a spindle inhibitor and thus a drug specifically acting only on cycling cells also inhibits survivin expression. Targeting the mitotic regulator and inhibitor of apoptosis survivin significantly inhibited its gene expression. As a consequence, a surge of multinucleated cells can be seen in the CBMN assay for both chemicals for both donor groups. PBL from cancer patients show higher frequencies due to their higher sensitivity when compared to those from healthy individuals; hence, both compounds are able to induce significant cytogenetic damage, oxaliplatin via inducing general DNA damage and vinflunine via only inhibiting specifically cycling cells. Oxaliplatin but also vinflunine significantly reduces the cells' anti-apoptotic potential by inhibiting survivin expression while causing cell cycle shortcuts. Future chemotherapeutic approaches using a smart mix of different concentrations of oxaliplatin and vinflunine might then be able to reduce harm to all somatic cells by only targeting cycling cancer cells.

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6. References

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Table legend

Table 1: The effects of oxaliplatin (OXP) and vinflunine (VFN) evaluated by the CBMN assay using PBL from healthy individuals and colorectal cancer patients. MoNC represent mononucleated cells, BiNC binucleated cells and MultiNC multinucleated cells. The highest vinflunine dose (6 μM) was cytotoxic. Data represent mean values. The results were normally distributed; therefore, the data were analysed by one-way ANOVA followed by Dunnett's post hoc test for significant differences compared to untreated PBL (* $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$). Significant differences of the impact of the compounds in PBL from healthy individuals and colorectal cancer patients were analysed by chi-square (χ^2) test ($^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$; n = 25).

Figure legends

Figure 1: Fluorescence *in situ* hybridization (FISH) of oxaliplatin induced MN in binucleated cells. The figure shows the percentage of micronuclei originated from a whole chromosome as indicated by positive pancentromeric probe signal (C+MN) after treatment with oxaliplatin. Data were analysed with a Fisher's Exact Test for statically differences (*** $p < 0.001$; $n = 25$).

Figure 2a: Survivin expression in PBL from colon cancer patients following treatment with different concentrations of oxaliplatin. The control consisted of untreated PBL. Significant differences in relation to the control (* $p < 0.05$, ** $p < 0.01$). The data presented result from three independent experiments using three male colon cancer patients ($n = 3$).

Figure 2b: Western blot of survivin expression in PBL from colon cancer patients following treatment with different concentrations of oxaliplatin. Lane 1: survivin expression in untreated PBL from colorectal cancer patients (negative control); lane 2: survivin expression in Jurkat cells (positive control); lanes 3-5: survivin expression in treated PBL using 0.02, 0.2 and 2 µM oxaliplatin, respectively. Constitutively expressed GAPDH was used as loading control.

Figure 3a: Survivin expression in PBL from colon cancer patients after treatment with different concentrations of vinflunine. The control consisted of untreated PBL. Significant differences in relation to the control (** $p < 0.01$). The data presented result of three independent experiments using three male colon cancer patients ($n = 3$).

Figure 3b: Western blot of survivin expression in PBL from colon cancer patients after treatment with different concentrations of vinflunine. Lane 1: survivin expression in untreated PBL from colorectal cancer patients (negative control); lanes 2-4: survivin expression in treated PBL using 0.06, 0.6 and 6 µM vinflunine. Constitutively expressed GAPDH was used as loading control.

Table 1

	Lymphocyte treatment	BiNC (%)	MultiNC (%)	NDI	MN	NPBs	Buds	MN in 1,000 MoNC
					in 1,000 BiNC			
Healthy Individuals	**Control**	72.8	1.09	1.73	3	0.02	0.0	1.53
	0.4 µM MMC	61.13	1.51	1.61	51	3.15	0.24	2.04
	0.02 µM OXP	60.03	20.2***	2.13*	14.62**	0.05	0.0	1.06
	0.2 µM OXP	38.4**	49.3***	2.37**	26.88***	0.04	0.0	2.04
	2 µM OXP	36.9**	49.9***	2.39***	37.14***	0.08	0.0	3.11
	0.06 µM VFN	60.03	16.5**	1.92	4	0.73	0.3	82***
	0.6 µM VFN	41.8**	56.6***	2.55***	3	1.02	0.5	125***
	6 µM VFN	-	-	-	-	-	-	-
CRC patients	**Control**	70.8	3.7	1.82	10	0.02	0.34	2
	0.4 µM MMC	61.13	2.8	1.66	59.33	0.05	1.07	2.52
	0.02 µM OXP	47.4^{++}	26.03*	2.01**	36**	0.12	0.52	3.10
	0.2 µM OXP	31.2**	53.1**	2.39***	41***	0.30	0.83	3.79
	2 µM OXP	29.7**	56.4**	2.42***	53***	0.22	0.95	4
	0.06 µM VFN	57.1	22.5*	2.14*	13	0.07	0.0	101***
	0.6 µM VFN	28.9** $^{+}$	57.1**	2.43**	10	0.09	0.33	129***
	6 µM VFN	-	-	-	-	-	-	-

Figure 1

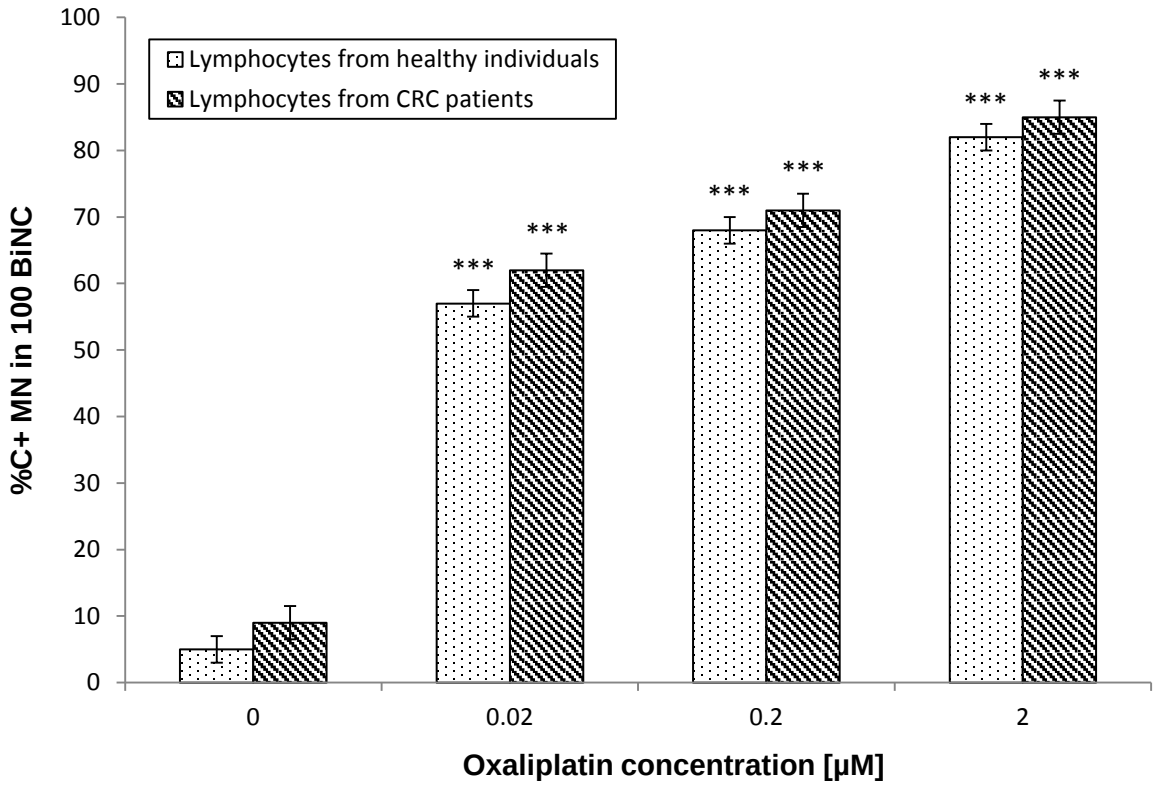

100
90
80
70
60
50
40
30
20
10
0
%C+ MN in 100 BiNC
Lymphocytes from healthy individuals
Lymphocytes from CRC patients
***
***
***
***
***
***
0
0.02
0.2
2
Oxaliplatin concentration [µM]

Figure 2a

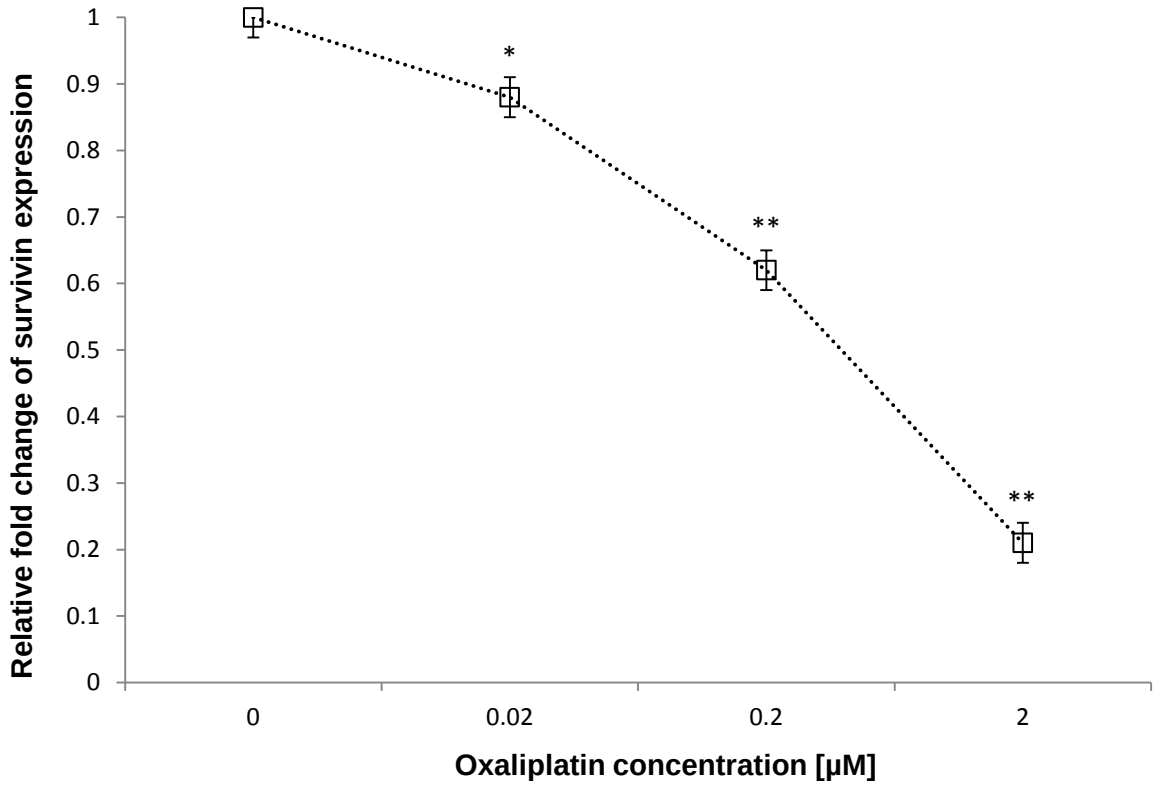

Relative fold change of survivin expression
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
**
0
0.02
0.2
2
Oxaliplatin concentration [μM]

Figure 2b

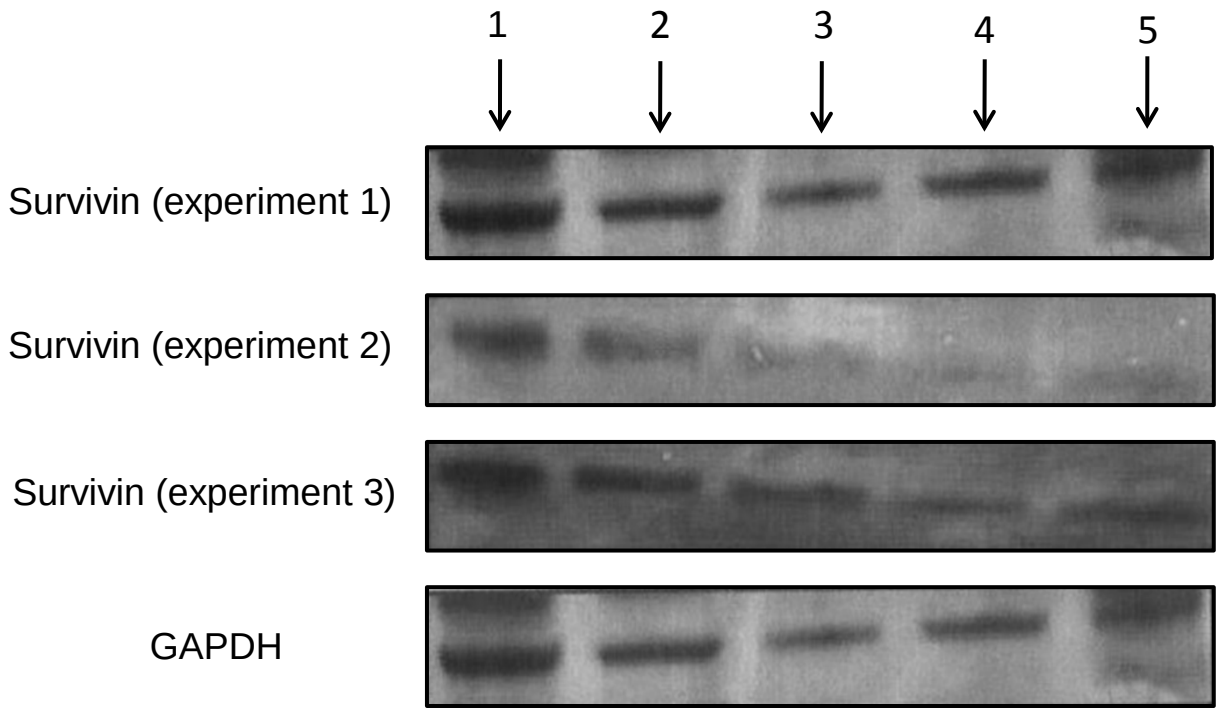

1
2
3
4
5
Survivin (experiment 1)
Survivin (experiment 2)
Survivin (experiment 3)
GAPDH

Figure 3a

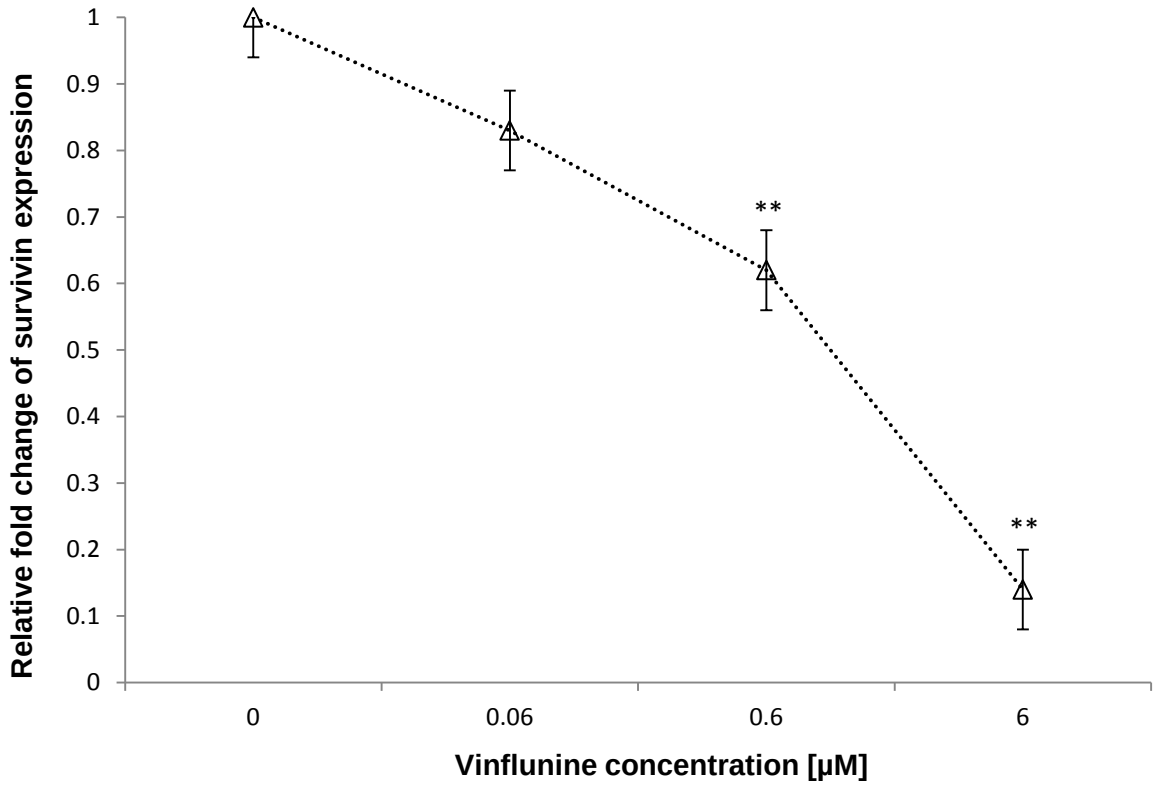
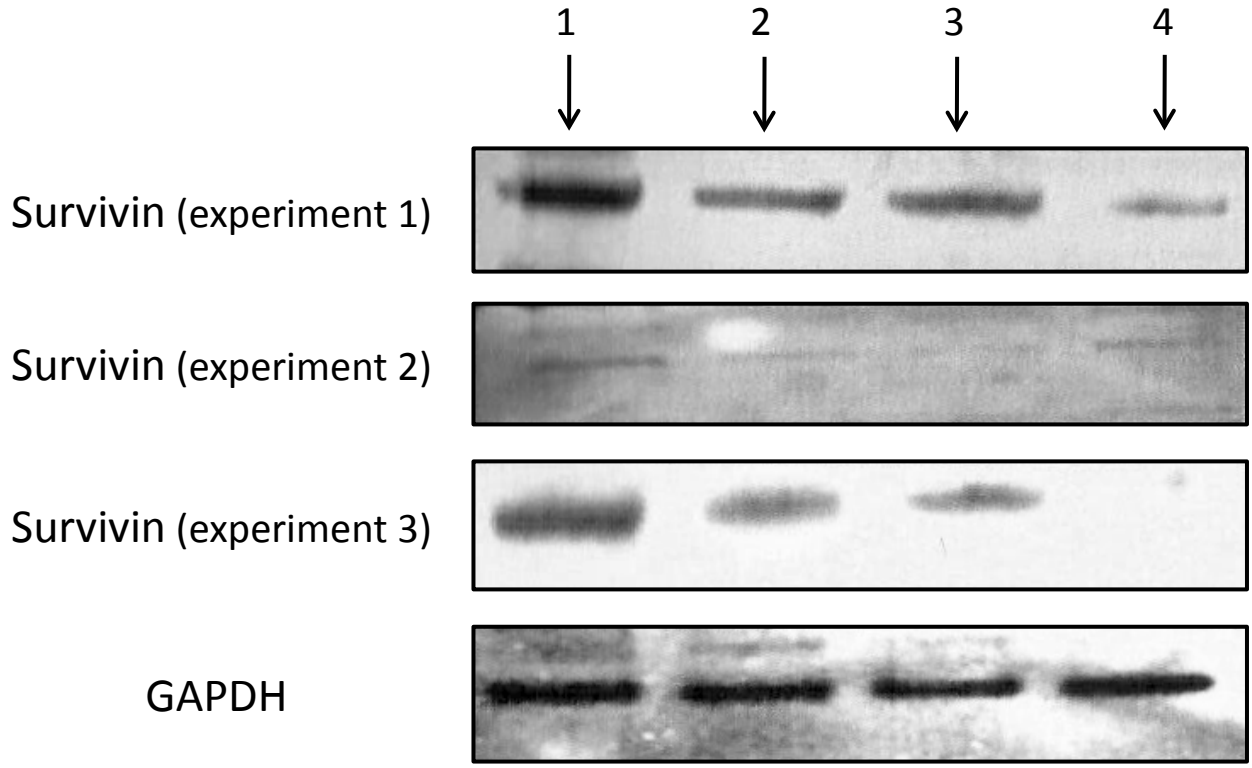

Relative fold change of survivin expression
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
**
0
0.06
0.6
6
Vinflunine concentration [µM]

Figure 3b


1
2
3
4
Survivin (experiment 1)
Survivin (experiment 2)
Survivin (experiment 3)
GAPDH