Indole-3-carbinol enhances anti-proliferative, but not anti-invasive effects of oxaliplatin in colorectal cancer cell lines.

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This work was funded by the UK Medical Research Council, grant G0100872.

Abstract.

The primary aim of this study was to determine whether combination of the chemopreventive agent indole-3-carbinol with oxaliplatin would decrease proliferative index and invasive potential of human colorectal tumour cells.

Combination of the agents resulted in a 170-fold decrease in proliferative capacity in SW480 and SW620 cell lines, which was approximately 6-fold greater than for oxaliplatin alone. Decreased proliferation was attributed to enhanced S-phase cell cycle arrest for SW480, and increased apoptosis for SW620 cells.

The combined agents resulted in significantly increased E-cadherin levels in SW480 cells, and β -catenin levels in both cell lines (assessed by in-cell westerns). In SW480 cells confocal microscopy revealed an increase in membrane-associated β -catenin levels, with oxaliplatin treatments enhancing nuclear export and cytoplasmic localisation. In SW620 cells, all treatments increased membrane localisation of E-cadherin.

Whilst both oxaliplatin and I3C decreased invasive capacity of SW480 cells, this was not further enhanced by the combined treatment.

Key Words: Colorectal cancer, oxaliplatin, chemoprevention, indole-3-carbinol, E-cadherin, β -catenin.

1. Introduction.

Oxaliplatin is a third-generation, platinum-based compound, pivotal to combinatorial therapeutic regimes in the treatment of colorectal cancer (reviewed in [1, 2]).

Peripheral neuropathies present as the major dose-limiting factor within oxaliplatin-based regimes [3, 4], so it is of great importance to design treatment strategies that allow similar therapeutic indices, whilst decreasing the likelihood of toxic events. To this end, we have chosen to assess the effects of combining oxaliplatin with the diet-derived chemopreventive agent indole-3-carbinol (I3C). I3C is currently in clinical trial for treatment of recurrent respiratory papillomatosis [5, 6] and cervical intraepithelial neoplasia [7, 8] and has been shown to be well tolerated at doses up to 400mg/day [9]. Pharmacokinetic studies of I3C disposition have revealed systemic doses of up to 170 μ mol/L in tissue [10], which equates with *in vitro* efficacy particularly in the liver [11, 12] which along with lymph nodes [13, 14], is the major site for colorectal metastasis. I3C has been shown to exhibit both anti-proliferative and anti-invasive potential [15] via a variety of mechanisms, including up-regulation of E-cadherin and β -catenin at the cytoplasmic membrane [16, 17], concurrent with down-regulation of nuclear β -catenin levels.

In this study, we hypothesised that combination of oxaliplatin with I3C could enhance anti-proliferative effects of oxaliplatin at physiologically relevant concentrations, in SW480 (colorectal adenocarcinoma) and SW620 (lymph-node metastasis derived from same patient as SW480) cell lines, and alter markers of invasive capacity.

2. 1. Materials

Oxaliplatin (Sigma, Dorset, UK) was supplied as a lyophilised powder, and reconstituted immediately prior to use in 5% glucose. I3C was obtained from Sigma and reconstituted in DMSO. Antibodies against E-cadherin, α -catenin and β -catenin were from BD Transduction Laboratories (Oxford, UK), actin and cadherin-11 from Santa Cruz Biotechnology (CA, USA) and the Annexin V/FITC kit was from Bender Medsystems (Vienna, Austria). Collagen I was obtained from Cohesion (CA, USA), transwell filter inserts from Falcon (Oxford, UK) and Hema Gurr staining solution from BDH (Leicester, UK).

SW480 and SW620 cell lines (ATCC, Middlesex, UK) were cultured in DMEM. SW480 cells originally derived from a Dukes' type B primary colorectal adenocarcinoma, are tumorigenic in nude mice and are p53 and ras mutant. SW620 cells (Dukes' type C) were isolated from the same subject as the SW480 cells, being derived from lymph nodes following wide-spread metastasis from the colon. They are similarly p53 mutant and tumorigenic in nude mice [18]. Both cell lines required the presence of foetal calf serum (Invitrogen, Paisley, UK) in the medium, to a final concentration of 10%.

2.2. Methods

2.2.1. Treatment of cells.

Cells were treated with oxaliplatin or I3C alone or in combination for times up to 144 hours. All treatments contained equivalent concentrations of DMSO, which did not exceed 0.1%.

2.2.2. Cell proliferation assay

Five thousand cells per well were seeded onto 12-well plates and treated with the appropriate concentration of agents for 144 hours. Cells were harvested and counted using a Beckman Coulter Z2 coulter particle count and size analyser (Beckman Coulter, Buckinghamshire, UK).

2.2.3. In-cell westerns

In-cell westerns are an emerging fluorescence-based technology which allows accurate quantitation of small changes to cellular protein expression.

Cells were seeded at 1×10^4 cells/well on a round-bottomed 96-well plate, left to adhere overnight and the wells treated for 24 or 48 hours with appropriate oxaliplatin/I3C treatments. Medium was removed, the cells fixed in 3.8% formaldehyde and permeabilised in 0.1% triton X-100 prior to blocking in Odyssey blocking buffer (LI-COR Bioscience, Cambridge, UK) overnight (4°C). Both primary and loading control antibodies (from differing species) were diluted in Odyssey blocking buffer and added to the wells simultaneously. Primary antibodies were incubated at room temperature for 2 hours prior to washing and addition of secondary antibodies (IRDye 680 and IRDye 800CW, LI-COR). Following further washes, plates were visualised and quantitated using the Odyssey infrared imaging system.

2.2.4. Preparation of nuclear extracts

Nuclear extracts were prepared as described by Plummer *et al* (1998).

2.2.5. Western Blotting

Cells were seeded at between 1 and 2.5×10^6 onto 9cm plates and treated with oxaliplatin or I3C for times up to 48 hours. Treated cells were lysed and samples (30 μ g protein) analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, followed by visualisation, using enhanced chemiluminescence (Amersham Life Science Ltd., Little Chalfont, UK). Blots were scanned using the Syngene Chemigenius II (Cambridge, UK) and quantified using the Genetools software.

2.2.6. Immunocytochemistry for E-cadherin and β -catenin

Cells were seeded at 4×10^4 cells/well on a Nunc 8-well chamber slide and left to adhere overnight prior to treating with appropriate concentrations of oxaliplatin +/- I3C for 24 hours. Medium was then removed from the chambers and the cells fixed in 3.8% formaldehyde. Following washing in PBS, cells were permeabilised in 0.1% triton X-100, washed and blocked overnight in 3% BSA. The cells were incubated in primary antibody for 3 hours, the slides washed, and then incubated in appropriate secondary antibody for a further hour prior to further washes and counterstaining with Hoechst. Slides were mounted using Slowfade Gold (Invitrogen) and visualised by confocal microscopy using a Leica TCS4D confocal imaging system.

2.2.7. Annexin V staining for apoptosis

This was based on the method described previously [19], and allowed determination of phosphatidylserine externalisation occurring during apoptosis.

2.2.8. Cell Cycle Analysis

The method was described previously [19]. In brief, 1×10^5 or 2.5×10^5 cells were plated onto 6-well plates, left to adhere overnight and then treated with appropriate concentrations of agents for 24, 48 or 72 hours. Adherent cells were trypsinised, washed x 2 in PBS and resuspended in 200 μ l PBS. Cells were fixed by the addition of 2ml ice cold 70% ethanol, whilst vortexing vigorously and incubated at 4°C for a minimum of 2 hours. Cells were pelleted (600xg for 10 minutes) and resuspended in 800 μ l PBS, whereupon RNase and PI were added to final concentrations of 0.1mg/ml and 5 μ g/ml respectively. The cells were incubated at 4°C overnight before analysis of DNA content, using the Becton Dickinson FACScan and Cell Quest software, with subsequent data analysis performed using Modfit LT software.

2.2.9. Cell invasion assays

Cells were seeded at 2×10^6 /well on 9cm dishes and left to adhere overnight, prior to treatment with appropriate concentrations of agents for 24 hours. Transwell filter inserts (8 μ m pore size) were suspended in 12-well plates and coated with 0.1mg/ml collagen type I overnight. Treated samples were washed in serum free medium and 1×10^5 cells added to the top of the filters, with medium containing 10% foetal calf serum as the chemoattractant in the well beneath. Samples were incubated for 48 hours, and the average number of invading cells in 5 fields of view determined following Hema-Gurr staining. For each treatment, the same number of cells were seeded onto collagen in serum free medium, incubated for 48 hours and counted to adjust for treatment-induced discrepancies in cell number, using a Beckman Coulter Z2 coulter particle count and size analyser.

2.2.10. Statistics

All results were analysed using a oneway ANOVA, followed by Fisher's post hoc test.

3. Results

3.1. Cell proliferation

Efficacy of I3C and oxaliplatin both alone and in combination was initially assessed by cell proliferation over a 144 hour period. Oxaliplatin was used at a concentration close to the maximum achievable in patients [20], and I3C at concentrations documented in mice [11]. Proliferation was affected to a similar extent in both cell lines (figure 1). Oxaliplatin alone (5 μ M) significantly decreased proliferative capacity by up to 20-fold compared to the DMSO control (the IC₅₀ is approximately 2 μ M in SW480 [21, 22] and 0.4 μ M in SW620 cell lines respectively [23]). I3C alone decreased proliferation to a much lesser extent (exhibiting an IC₅₀ of approximately 130 and 110 μ M in SW480 and SW620 cells respectively). When in combination, proliferative capacity was decreased by 67- and 92-fold (oxaliplatin + 50 μ M I3C) and 173- and 187-fold (oxaliplatin + 100 μ M I3C) in the SW480 and SW620 cells respectively. At 72 hours, oxaliplatin alone caused a 5-fold decrease in SW480 and 14-fold decrease in SW620 cells, with a 10- and 40-fold decrease exhibited with the highest I3C combination in SW480 and SW620 cells respectively – data not shown. The mechanisms by which the combination treatments enhanced anti-proliferative capacity were further investigated by flow cytometry assessing apoptotic and cell cycle events.

3.2. Effects of combined treatment on apoptosis and cell cycle.

3.2.1. Apoptosis

SW480 cells underwent less apoptosis than their metastasis-derived counterparts (figure 2). Oxaliplatin alone resulted in a 7% increase in apoptotic cells following a 72 hour treatment, which was only increased to 9% upon combination with I3C in this cell line. In contrast, for the SW620 cells, oxaliplatin alone increased the number of cells undergoing apoptosis by 9%, which rose to 21% with the combined treatment (72 hours).

3.2.2. Cell cycle

Significant S-phase cell cycle arrest was observed in the SW480 cells with the combination only (figure 3), increasing from 15% up to 42% compared to the DMSO control following 48 and 72 hours combined treatment. In the SW620 cell line, oxaliplatin treatment resulted in a significant G₂/M arrest (14 % increase compared to DMSO control at 48 hours) which did not significantly alter following the combined treatment.

3.3. Cadherin and β -catenin expression

The effect of physiologically achievable concentrations of oxaliplatin on markers of adhesion and invasion has not been documented, with anti-invasive mechanisms for I3C only slightly better characterised. To this end, effects of both agents administered singly or in combination, on cadherin and catenin expression were investigated.

SW480 cells expressed low E-cadherin, high cadherin-11 and higher α - and β -catenin relative to SW620 cells, which expressed high E-cadherin but low cadherin-11 (figure 4a). Neither cell line expressed N- or P-cadherin (data not shown).

Total E-cadherin levels were significantly increased by 1.9-fold in the SW480 cell line following combined treatment of oxaliplatin with 250 μ M I3C at 48 hours (figure 4b). No significant changes in E-cadherin protein levels were detected in the SW620

cells. Interestingly, oxaliplatin alone significantly decreased expression of cadherin-11 by 20% in the SW480 cells (figure 4c), but this was not significantly decreased further when in combination.

Similar increases in β -catenin were observed in both cell lines following 24 hours treatment with oxaliplatin alone +/- I3C (figure 4d). At 48 hours, β -catenin levels were increased by up to 2.4-fold in the SW480 cells by oxaliplatin alone, and by up to 3-fold with the combination. In SW620 cells, increased treatment times did not result in significantly increased expression.

3.4. Localisation of E-cadherin and β -catenin

Following the observed changes in expression of E-cadherin and β -catenin it was important to investigate the localisation of these proteins, in order to better determine possible consequences with respect to adhesion and signalling capacity.

SW480 cells exhibited very low levels of E-cadherin, localised in a punctate pattern surrounding the nuclear membrane (figure 5a), which remained unchanged following any treatment. β -catenin localisation, however, was affected by all treatments. I3C alone induced increased expression at the cell membrane, whereas oxaliplatin enhanced nuclear to cytoplasmic export of β -catenin, which was augmented with the combination treatments.

E-cadherin localisation to the cell membrane was enhanced by all treatments in the SW620 cell line (figure 5b). β -catenin also appeared to exhibit increased localisation to the cell membrane in this cell line, particularly following oxaliplatin treatment.

To further assess localisation of β -catenin, nuclear extracts were made from both cell lines and analysed by western blotting (figure 5c). In agreement with confocal

microscopy data, nuclear β -catenin levels were decreased in both cell lines with all treatments by up to 36%, although there was no significant difference between treatments.

3.5. Assessment of invasive capacity.

In order to determine whether observed changes in cadherin and β -catenin localisation were pertinent to invasive potential in this system, invasive capacity of the cell lines was assessed using a modified Boyden chamber.

SW480 cells were able to invade through a collagen type I matrix following 48 hours incubation. Individually, oxaliplatin and I3C treatments decreased invasive capacity by ~70% (normalised to cell number), which was not enhanced by the combination. The SW620 cell line was not invasive through collagen at this time point.

4. Discussion

There is now an overwhelming body of evidence that I3C is able to exert anti-proliferative effects on many tissue types *in vitro*, including breast, colon and prostate [19, 24-28]. In recent years, a few studies have hinted at the potential for I3C to enhance chemotherapeutic regimes [12, 29, 30]. Yet to date, combinatorial options remain largely unexplored.

I3C was shown in this study, to greatly enhance the anti-proliferative effects of oxaliplatin in both the SW480 and SW620 cell lines, and this was found to be primarily by induction of S-phase cell cycle arrest in the SW480 and induction of apoptosis in the SW620 cells. Previously, we and others (reviewed in [31, 32] have found I3C to be effective at inhibiting survival signalling in colorectal and other cell

lines, but this is the first study to demonstrate increased efficacy when combined with platinum-based therapeutics. In agreement with the data presented in this study, oxaliplatin has previously been reported to induce apoptosis in SW480 [33] and G₂- and S-phase arrest [34] in SW620 cell lines. We have also previously shown enhanced anti-proliferative effects of curcumin combined with oxaliplatin, to be associated with increased caspase 8 activity and altered expression of G₂/M- and checkpoint- related cell cycle proteins, including p53, p21, survivin and cdc2 [35].

Only two studies have alluded to a role for I3C in inhibition of invasion, primarily through upregulation of E-cadherin/catenin complexes [36, 37]. E-cadherin plays an important role, not only in cell adhesion, but also as a tumour suppressor [38], reviewed in [39], making it an important therapeutic target. Regulation of E-cadherin at the epigenetic level often occurs via hypermethylation of its promoter and histone deacetylation, with transcriptional regulation influenced by the transcriptional repressors Snail and Slug [40]. We have recently found that oxaliplatin may be able to decrease expression of another transcriptional repressor of E-cadherin, Zeb 1 (unpublished), thought to be involved in epithelial to mesenchymal transition [41]. That these events may also be targeted by chemopreventive agents is evidenced by the ability of the green tea polyphenol, epigallocatechin gallate, to suppress DNA methylation via inhibition of DNA methyltransferases both *in vitro* [42] and *in vivo* [43], and for curcumin-mediated repression of histone acetyltransferase-dependent chromatin transcription [44]. This may provide a potential mechanism for reactivation of E-cadherin expression in tumour-derived cultures. Cadherin-11, a mesenchymal-associated cadherin, is expressed at high levels in the SW480 cell line. There is accumulating evidence that this cadherin may provide a suitable marker of increased

invasive potential [45, 46], the oxaliplatin-mediated decrease of which, would correlate well with the decreased invasive capacity observed in this cell line.

Whilst I3C alone does not induce significant overall increases in E-cadherin for either cell line, there is clearly an increase in membrane-bound E-cadherin in the SW620 cells when visualised by confocal microscopy. There are currently no reports as to how oxaliplatin may effect cadherin/catenin expression or localisation. Results presented here, demonstrating the ability of oxaliplatin to decrease cadherin-11 and nuclear localisation of β -catenin, and to increase levels of E-cadherin, provide additional support for first line treatments involving oxaliplatin-based therapy regimes. The fact that both I3C and oxaliplatin enhanced nuclear β -catenin export and increased levels of membrane-bound E-cadherin (SW620 cells) led us to investigate whether invasive potential of the cell lines was affected. Contrary to published data [18], the SW620 cell lines were found to be relatively non-migratory or invasive by 48 hours in agreement with Dhawan *et al.* who found invasion was only prevalent from 72 hours in this cell line [47]. SW480 cells exhibited moderate invasive capacity (in comparison to highly invasive J82 bladder tumour-derived cells – data not shown), which was inhibited by 70% with oxaliplatin alone. I3C displayed a similar ability to inhibit invasion, which was not significantly altered when combined with oxaliplatin. The observation that adenocarcinoma-derived cells were more invasive than the metastasis-derived cells is compatible with their lower E-cadherin and greater cadherin-11 expression. Batistatou *et al.* demonstrated 81% of lymph node metastases from colorectal cancer to be E-cadherin positive [48], with Imai *et al.* showing increased immunoreactivity for E-cadherin and β -catenin in metastatic ovarian lesions [49] suggesting E-cadherin levels decrease prior to invasion, with re-expression

occurring following metastasis. However, patients with higher levels of adhesion molecules in metastatic lymph nodes, compared to primary colorectal adenocarcinomas, demonstrated a poorer 5-year survival rate than those with levels similar to the primary carcinomas [50].

In conclusion, we have demonstrated that combining I3C with oxaliplatin greatly enhances the ability of the latter to decrease the proliferative rate of two colorectal cancer-derived cell lines. Both agents substantially decreased invasive capacity in their own right, although this was not enhanced when in combination, despite an increase in total E-cadherin levels, suggesting that other factors involved in the invasive process are being modulated by each agent. However, the marked decrease in proliferative capacity combined with a decreased nuclear signalling pool of β -catenin and increases in the tumour suppressor E-cadherin warrants further investigation regarding the potential of this combination. A number of studies have clearly demonstrated that I3C is biologically active in animals and humans [10] so it will be crucial to determine whether the effects reported here can also be replicated *in vivo* and most importantly, within a clinical context.

Acknowledgements.

This work was funded by the UK Medical Research Council, grant G0100872.

Conflict of interest statement.

There is no conflict of interest.

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Figure legends.

Figure 1. Cell counts for SW480 and SW620 following 144 hours treatment with oxaliplatin +/- I3C. Graph shows fold decrease. $n = 3 \pm \text{SEM}$. * = significant difference from DMSO control (^), a = significant difference from oxaliplatin alone, b = significant difference from equivalent concentration of I3C alone as analysed by ANOVA followed by Fisher's post hoc test.

Figure 2. Annexin V/PI staining for SW480 and SW620 following 48 and 72 hours treatment with oxaliplatin +/- I3C showing % apoptotic population. Black bars = 48 hours, grey bars = 72 hours. $n=3 \pm \text{SEM}$. * = significant difference from DMSO control (position indicated by ^), a = significant difference from oxaliplatin alone, b = significant difference from equivalent concentration of I3C alone as analysed by ANOVA followed by Fisher's post hoc test.

Figure 3. Cell cycle distribution for SW480 and SW620 following 48 and 72 hours treatment with oxaliplatin +/- I3C. Black bars = G1, white bars = S, grey bars = G2/M. Graph shows % cells in each population. $n=3 \pm \text{SD}$. * = significant difference from DMSO control (^), a = significant difference from oxaliplatin alone, b = significant difference from equivalent concentration of I3C alone as analysed by ANOVA followed by Fisher's post hoc test.

Figure 4. E-cadherin, cadherin-11 and β -catenin levels in SW480 and SW620 cells. (a) representative western blots showing basal levels of cadherin and catenin expression in SW480 and SW620 cells ($n=3$), (b) shows fold change in E-cadherin

expression for SW480 and SW620 following 24 or 48 hours treatment with oxaliplatin +/- I3C. (c) shows representative western blot for cadherin-11 and actin loading control in SW480 following 24 hour treatment with oxaliplatin +/- I3C (n=3). (d) shows fold change in β -catenin expression for SW480 and SW620 following 24 or 48 hours treatment with oxaliplatin +/- I3C, n = 6 +/- SD. * = significant difference from DMSO control (^), a = significant difference from oxaliplatin alone, b = significant difference from equivalent concentration of I3C alone as analysed by ANOVA followed by Fisher's post hoc test.

Figure 5. E-cadherin and β -catenin localisation in SW480 and SW620 cells.

(a) shows E-cadherin and β -catenin localisation in SW480 and (b) shows E-cadherin and β -catenin localisation in SW620 cells by confocal microscopy following 24 hours treatment with 5 μ M oxaliplatin +/- 250 μ M I3C. n=2. (c) Nuclear β -catenin levels in SW480 and SW620 following 24 hours treatment with 5 μ M oxaliplatin +/- 250 μ M I3C as determined by western blot. n=3. Chart shows semi-quantitative analysis of densitometry for western blots. Black bars = SW480, grey bars = SW620. n = 3, +/- SD, * = significant difference from DMSO control as analysed by ANOVA followed by Fisher's post hoc test.

Figure 6. Invasion of SW480 cells through collagen I matrix after 48 hrs incubation following 24 hours pre- treatment with 5 μ M oxaliplatin +/- 250 μ M I3C. Chart represents mean of 5 fields of view per treatment (x40 magnification) normalised to cell number, and presented as percent of DMSO control. n = 3, +/- SD. * = significant

difference from DMSO control as analysed by ANOVA followed by Fisher's post hoc test.

Figure 1

Figure 2

SW480

SW620

5μM oxali
μM I3C

- - + - - - + + +
- - - 50 125 250 50 125 250

^

Figure 3

SW480

SW620

Figure 4a

Figure 4b

Figure 4c

Figure 4d

Figure 5b

E-cadherin

β -catenin

SW620

DMSO

250 μ M I3C

5 μ M oxali

5 μ M oxali/
250 μ M I3C

Figure 5b

E-cadherin

β -catenin

SW620

DMSO

250 μ M I3C

5 μ M oxali

5 μ M oxali/
250 μ M I3C

Figure 5c

Figure 6