Fluoxetine selectively induces p53-independent apoptosis in human colorectal cancer cells

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Abstract

Fluoxetine has been shown to induce anti-tumour activity. The aim of this study was to determine the effect of fluoxetine on HCT116+/+ and p53 gene-depleted HCT116-/- human colorectal cancer cells and the mechanisms, including potential p53-dependence, of its action. Fluoxetine-induced apoptosis was investigated by mitochondrial membrane potential assay, Annexin V assay, two-step cell cycle analysis using NC-3000™ system and pharmacological inhibition assays. Fluoxetine induced very selectively concentration-dependent apoptosis in human colorectal cancer cells by altering mitochondrial membrane potential and inducing translocation of phosphatidylserine to the outer membrane layer. Further evidence of the preponderance of apoptosis in fluoxetine-induced cell death is provided by the finding that the cell death was not blocked by inhibitors of parthanatos, a form of cell death that results from overactivation of the enzyme poly (ADP-ribose) polymerase (PARP) but is different from apoptosis. Data obtained indicate fluoxetine caused cell cycle event at Sub-G1 and G0/G1 phases in both cell lines. In terms of apoptosis, there is no significant difference between the responses of the two cell lines to fluoxetine.

In conclusion, fluoxetine's cytotoxicity induces mainly apoptosis and causes DNA fragmentation in human colorectal cancer cells, which seemed to be independent of the p53 protein, as no significant difference in death profiles in response to fluoxetine treatment was observed in both the p53-intact and the p53-deleted cell lines. Fluoxetine, therefore, has potential for being repurposed as a drug for the treatment of colon cancer and thus deserves further investigations in this context.

Key words

Fluoxetine, colon cancer, apoptosis, Annexin V, cell cycle, mitochondrial membrane potential, PARP

Abbreviations

DAPI (4′,6-diamidino-2-phenylindole); SSRI, selective serotonin reuptake inhibitor; JC-1 dye (5, 5, 6, 6-tetrachloro-1, 1, 3, 3-tetraethylbenzimidazol-carbocyanine iodide), MMP, mitochondrial membrane potential; TRAIL, tumour necrosis factor-related apoptosis-induced ligands; ROS, reactive oxygen species; Bcl-2 (B-cell lymphoma 2); c-Abl (Abelson murine leukemia viral oncogene homolog 1); HCT, human colon cancer cells, MCF-7, human breast cancer cells; A549, human lung cancer cells; A2780, human ovarian cancer cells sensitive to cisplatin; A2780-CP70, human ovarian cancer cells resistant to cisplatin.

1. Background

Colorectal cancer is one of the major worldwide causes of mortality. It is reported to be the third most common type of cancer throughout the world and the fourth most common cause of death, despite advances in therapy and increased screening rates (Shu Wang et al., 2017). Combinations of 5-fluorouracil (5-FU) and oxaliplatin (FOLFOX) or irinotecan (CPT-11; FOLFIRI) have improved response rates to chemotherapy in advanced colorectal cancer; however, resistance is still a major problem and an unmet clinical need. There is now some evidence that particular types of antidepressant drugs possess anti-tumour properties (Kannen et al., 2015; Coogan et al., 2009). Fluoxetine is currently prescribed as an anti-depressant and acts as a selective serotonin reuptake inhibitor (SSRI); it also reduces anxiety by regulating serotonin levels in the synaptic cleft. Some studies showed that selective serotonin reuptake inhibitors (SSRIs) possess potent apoptotic activity on different types of cells (Kannen et al., 2015). Treatment with fluoxetine was reported to reduce tumour cell proliferation, DNA synthesis or colony formation in human and mouse breast carcinoma cell lines (Volpe et al., 2003), although earlier studies in 1992 reported an increase in the number of mammary fibrosarcomas in mice which were treated with fluoxetine for 5 days, followed by an increase in the incidence of breast cancer after 15 weeks (Brandes et al., 1992). Later on, it was found that fluoxetine did not enhance pancreatic tumour proliferation but reduced lymphoma growth, modulating the T-cell-mediated immunity reaction via a 5-HT-dependent pathway (Jia et al., 2008; Frick et al., 2008). In some clinical studies, a 50% reduction of risk of colon cancer was reported in patients treated with fluoxetine (Coogan et al., 2009). Various animal studies also supported a reduction in colon cancer incidence and many different signalling pathways such as NF-κB, ROS formation and cell cycle arrest were reported as the likely mechanisms of action in a variety of different carcinoma cells (Koh et al., 2011; Tutton &

Barkla, 1986; Kannen et al., 2011, 2012; Lee et al., 2010, Krishnan et al., 2008; Stopper et al., 2014).

Although the above studies indicate differential effects of fluoxetine treatment in a number of cancer cell types, wider investigations of the mechanistic details of fluoxetine's anti-tumour activity in different cancer cell types are still required. Therefore, the present study was carried out, first to investigate if fluoxetine elicits anti-tumour activity in a variety of human tumour cell lines by selectively inducing apoptotic cell death and, second, to investigate if the mechanism of cell death induction by fluoxetine is influenced by the tumour suppressor p53 protein by assessing its effects on two human colon carcinoma cell lines, the HCT116+/+ cell line with intact p53 gene and the HCT116-/- cell line without the gene (p53 gene deleted).

2. Methods

2.1. Cell culture

The HCT116 +/+ human colorectal cancer cells with intact p53 gene and the HCT116 -/- human colorectal cancer cells with deleted p53 gene; ARPE19 (human retinal epithelial) and PNT2 (human prostate) non-carcinoma cell lines; A2780 (human ovarian carcinoma cells), A2780-CP70 (human ovarian carcinoma cells resistant to cisplatin), MCF-7 (human breast carcinoma cells), A549 (human lung carcinoma cells) were maintained according to the suppliers' guidelines. Cells were grown in T75 flasks containing DMEM (D2429) or RPMI supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and 200 µM sodium pyruvate. At 70% confluence, the medium within the flask was removed and the cell monolayer was washed with 10 ml of phosphate buffered saline (PBS) solution. This was followed with the addition of 2 ml of Trypsin-EDTA solution (0.05% Trypsin, 0.02% EDTA), after which the flask was

transferred into an incubator (5% CO_2, 37˚C) for three minutes. Once cells had detached, the appropriate cell media (10 ml) for the cell line was added to the flask to deactivate the trypsin enzyme and prevent damage to the detached cells through prolonged trypsin exposure. The resultant cell suspension was transferred into a 25 ml tube and centrifuged at a speed of 1200 RPM for 5 minutes. The supernatant was subsequently removed, and the remaining pellet was re-suspended in 10 ml of appropriate culture medium. Cells were then counted and the numbers were adjusted for the subsequent experiments.

2.2.Cell viability assay

Cells were seeded into 96-well plates at 2000 cells per well. After 24 hours of incubation, cells were treated with varying doses of fluoxetine hydrochloride (1 nM to 100 µM) or vehicle (sterile water) for 96 h, after which the MTT assay was performed as previously reported (Kumar et al., 2016; Blackburn et al., 2016) by addition of 10 µl of MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). Following 4 hour incubation with MTT, the content of each well was removed and the formazan crystals were dissolved in dimethylsulfoxide, DMSO (150 µL). Absorbance was then read at 540 nm on a Tecan Infinite 50 plate UV reader.

2.3.Mitochondrial membrane potential (MMP) assay

1 x 10^5 cells/ml were seeded into T25 flasks (total volume of 5 ml) and after 24 h elapsed cells were treated with vehicle control or fluoxetine at concentrations ranging from 10µM to 60µM and left in the incubator for a further 24 h. Cells were then subjected to the MMP assay according to the manufacturer's instructions. Briefly, the supernatant was removed and saved.

The cells in the flask were incubated with 500 µl of trypsin for 5 minutes and centrifuged for another 5 minutes at 1200 RPM. The supernatant was removed and the cell pellet was diluted in 2 ml of PBS. The NC-3000^{TM} vial cassette containing DAPI was used to determine the cell count. Samples with a cell count of 1x10^{6}cells/ml were subjected to 2.5 µg/ml Solution 7 (JC-1) and incubated for 20 minutes. The stained cells were then centrifuged at 400g for 5 minutes at room temperature. The supernatant was removed and the cell pellets were washed twice with 1ml of PBSThe samples were re-suspended with 250 µl of 'Solution 8'. An 8-chamber slide was used to load the samples with ~10µl, and the slides were put inside the NC-3000^{TM} system which was previously set to analyse cells and provide the values for apoptotic cells by choosing the correct programme according to the instructions for "Mitochondrial Potential Assay" on the NC-3000^{TM} system. After loading samples onto the slides and choosing the assay, the number of polarised/apoptotic cells was indicated.

2.4.Annexin V assay

2 x 10^{5} cells/ml of cells were seeded into T25 flasks containing 5 ml of complete media, which were incubated for 24 hours at 37^{o}C. After 24 h elapsed cells were treated with vehicle control or fluoxetine at concentrations ranging from 10 µM to 60 µM and left in the incubator for a further 24 h. Cells were then subjected to Annexin V assay according to the manufacturer's instructions. Briefly, 1 ml of each sample containing cell count of 4 x10^{5} cells/ml in media was transferred to an eppendorf tube. In a separate eppendorf tube, a mixture of 940 µl of Roche buffer plus 20 µl of Annexin V, 20 µl of Propidium Iodide, 500 µg/ml (Solution 16), and 20 µl of Hoechst 33342, 500µg/ml (Solution 15), was prepared. Cells were centrifuged at 400g for 5 minutes and the supernatant removed carefully without disturbing the pellet. Cells were re-suspended in 100 µl of the above mixture prepared earlier,

mixed well and incubated for a further 15 minutes. 30 µl of each sample was then loaded onto A2 slide and the data were analysed using NC-3000™.

2.5.Cell cycle analysis

1 x 10^6 cells/ml of cells were seeded into T25 flasks containing 5 ml of complete media and were incubated for 24 hours at 37ºC. After 24 h elapsed cells were treated with vehicle control or fluoxetine at concentrations ranging from 10 µM to 60 µM and left in the incubator for a further 48 h. Cells were then subjected to two-step cell cycle assay according to the manufacturer's instructions. Briefly, 1 ml of cells (1 x10^6 cells/ml) was transferred to an eppendorf tube. In a separate eppendorf tube, a mixture of 1960µl of lysis buffer (Solution 10) plus 40µl of 500µg/ml DAPI (Solution 12) was prepared. The eppendorf tubes containing cells were centrifuged at 400g for 5 minutes, supernatant was removed and cells were re-suspended in 250µl of the above mixture, mixed well and incubated at 37ºC for 5 minutes. 250µl of stabilization buffer (Solution 11) was then added to the cells and mixed well. 10µl of each sample was then loaded onto A8 slide and subjected to the two-step cell cycle assay using NC-3000™. The assay indicated the percentage of cells in each phase of a cycle.

2.6.Pharmacological inhibition of endogenous PARP to assess its involvement in fluoxetine-induced cell death

The HCT116 +/+ and HCT116 -/- cells grown in T75 flasks were each seeded, following PBS rinsing, trypsinisation, trypsin inactivation and cell counting, into opaque, flat-bottom 96-well (Falcon) plates or black, flat-bottom 96-well plates (Greiner Bio-One) at a density of 1 x 10^5 cells/ml (100 µl/well) and incubated for 24 h at 37ºC and 5% CO_2. They were then exposed to fluoxetine for 24 – 48 h in the absence and presence of a range of concentrations

of two highly-potent and selective PARP inhibitors, one of which is only used experimentally (DPQ) while the other is used clinically in cancer treatments (olaparib), with appropriate vehicle (DMSO) controls included. Two viability assays that depend on two different readouts – the MTT assay (absorbance-based) and the Alamar Blue (AB) assay (fluorescence-based) – were then used to assess the changes to viability induced following the treatments. The use of two assays relying on separate cellular mechanisms allows for the detection of any potential artefacts that might be inherent in either assay, for example, due to the confounding interactions of test compounds with the assay reagents or cellular targets. The MTT assay was carried out by adding 10 µl of MTT (5 mg/ml in PBS), warmed to 37^{o}C, to each well and incubating the plate for 3 h, following which the content of each well was aspirated and 100 µl of DMSO was added to solubilise the insoluble formazan. Absorbance was then read at 570 nm on a Clariostar plate reader (BMG LABTECH). The Alamar Blue (AB) assay was carried out as previously reported (Fatokun et al., 2013). Briefly, 10 µl of AB was added to each well and the plate was incubated for 3 h. It was then allowed to stand at room temperature for 5-10 min, after which fluorescence (of resorufin, the reduced and fluorescent form of AB) was read on a Clariostar plate reader (BMG LABTECH) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

2.7.Data presentation and statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) of n=4 separate experiments and each experiment was in triplicate (or as otherwise stated) and analysed using analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. A probability of $P<0.05$ was considered to be statistically significant as compared to control values; Tukey's comparison was used with 95% confidence. The cytotoxic selectivity ratios

(CSR, the IC_{50} of the non-carcinoma cells divided by the IC_{50} of the carcinoma cells) were calculated, where a value higher than 1 indicated cytotoxic preference for cancer cells and a value less than 1 indicated a cytotoxicity preference for normal cells.

2.8.Materials

Fluoxetine and the pan-caspase inhibitor Z-VAD-fmk were purchased from Tocris, UK. The PARP inhibitors DPQ (3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone) and olaparib were purchased from Sigma-Aldrich and Stratech Scientific Ltd., UK, respectively. Phosphate buffered saline (PBS), Solution 8 (1µg/ml 4′,6-diamidino-2-phenylindole (DAPI)), Solution 7 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide of 200µg/ml JC-1), 50 µg/mL Annexin V-CF488A conjugate, Annexin V binding buffer (10x concentrate), Solution 15 (500 µg/ml Hoechst 33342), Solution 16 (500 µg/ml Propidium Iodide), Solution 10 (Lysis buffer), Solution 11 (stabilization buffer), Solution 12 (500 µg/ml DAPI), NC-Slide A8™, NC-Slide A2™ glass slides and via-1 cassettes were bought from ChemoMetec, Denmark. NC-3000™ image cytometer was used to perform the assays. MTT and media for growing cell lines and all supplements were purchased from Sigma Aldrich, UK and Life Technologies, UK, and cell lines were purchased from ATCC.

3. Results

3.1.Effects of fluoxetine on cell viability

Pre-treatment with fluoxetine induced concentration-dependent cytotoxicity in all cells examined (Fig. 1). The cytotoxicity was ($p<0.001$) significantly greater in colon carcinoma cells, where IC_{50} values were 3.19 $\pm$ 0.23 µM and 4.73 $\pm$ 0.5 µM, for HCT116 -/- and HCT

116 +/+, respectively, as compared with 20.57 ± 2.4 µM and 24.14 ± 4.3 µM, for non-carcinoma cells ARPE19 and PNT2 cells, respectively (see Fig. 1 for effect of fluoxetine on HCT 116 +/+ morphology). Fluoxetine also significantly ($p<0.05$) induced cytotoxicity in A2780 and A2780-CP70 cells, with IC_{50} values of 10.39 ± 3.1 µM and 20.45 ± 2.8 µM, respectively, but less cytotoxicity in lung carcinoma cells, A549, with IC_{50} of 28.4 ± 2.0 µM (Fig. 2A). The results also indicated that, of all the cell lines tested, fluoxetine is more selective in inducing cytotoxicity in colon carcinoma cells as opposed to normal cells, although the level of selectivity is also higher than 1 in breast and ovarian carcinoma cells, both sensitive and resistant to cisplatin (Fig. 2B, C).

3.2.Effects of fluoxetine on mitochondrial membrane potential (MMP) in human colon cancer cells

Colorectal cancer cells that were exposed to different concentrations of fluoxetine were examined for changes in mitochondrial membrane potential (MMP) using the MMP assay protocol on the image cytometer NucleoCounter NC-3000™ system. This method allows identifying the level of live, apoptotic and dead cells. It has been done by measuring the mitochondrial transmembrane potential ($\Delta\psi m$), as its disruption is often linked to the early stages of apoptosis and the loss of it is associated with necrosis and apoptosis. The lipophilic cationic dye JC-1 (5, 5, 6, 6-tetrachloro-1, 1, 3, 3-tetraethylbenzimidazolcarbocyanine iodide) displays potential-dependent accumulation in the mitochondria. Healthy cells are recognized by JC-1 localization in the mitochondrial matrix due to negative charge formed by the intact mitochondrial membrane potential which induces red fluorescence. In cells that undergo apoptosis, mitochondrial potential collapses and JC-1 accumulates in the cytosol, establishing green fluorescence. Cells have also been stained with DAPI that recognizes necrotic and late

apoptotic cells which appear in blue fluorescence, resulting in decreased red/green fluorescence intensity ratio. Data obtained are as shown in the scatter plots in Figure 3, supported by histograms to additionally show the percentage of apoptotic cells in Figure 4.

Figure 3 shows that, as the concentration of fluoxetine increased (30.0 µM and 60.0 µM), the intensity of green colour was more evident, which was indicative of more cells in the apoptotic phase when compared to the cells which were treated with control or a lower concentration of fluoxetine (10.0 µM), which showed red fluorescence (live cells). This is also reflected in figure 4, where cells treated with 10.0 µM of fluoxetine showed comparable results to control cells in both cell lines, while as the concentration of fluoxetine increased in both cell lines the percentage of apoptotic cells increased. For example, at 60.0 µM of fluoxetine, the percentage of apoptotic cells significantly increased from 12.00 ± 1.53% to 47.33 ± 0.33% ($P<0.001$) in HCT116 +/+, and from 11.33± 1.67% to 31.33 ± 6.49% ($p<0.05$) in HCT116-/- (Fig 4). Interestingly there was a significant difference ($p<0.05$) between the number of apoptotic cells in the two cell lines when treated with 60.0 µM of fluoxetine. In summary, fluoxetine remarkably altered mitochondrial membrane potential in both cell lines. No significant difference was observed in the levels and nature of cell death between the two cell lines when treated with the lower concentrations of fluoxetine; however, at 60.0 µM fluoxetine induced lower percentage of apoptotic cells in HCT116 -/- compared to HCT116 +/+.

3.3.Effect of fluoxetine on phosphatidylserine externalisation, Annexin V assay

In an attempt to further investigate the effects of fluoxetine, Annexin V assay was performed on both colon carcinoma cell lines. Annexin V assay detects phosphatidylserine externalization using Annexin V conjugate. Phosphatidylserine is a phospholipid located on

the cell membrane side facing the cytosol, and its translocation to the outer membrane layer is associated with early apoptosis. Annexin V is a protein that binds to phosphatidylserine in a calcium-dependent manner with high selectivity. However, Annexin V binds not only to early apoptotic cells but also to late apoptotic and necrotic cells. Therefore, cells have also been stained with Propidium Iodide (PI), which detects only late apoptotic and necrotic cells that have lost their membrane integrity. Furthermore, Hoechst 33342 has been added, which detects all cells. NucleoCounter® NC- 3000™ system detects violet light, which is total cell population stained with Hoechst 33342. Early apoptotic cells stained with Annexin V and Hoechst 33342 give violet and green fluorescence, while non-viable and late apoptotic cells stained with PI and Hoechst 33342 establish red and violet light. The fluorescence intensities of early apoptotic cells against non-viable cells are presented on a scatter plot (see figure 5). The histograms comparing the percentages of cells in both early and late apoptosis are shown in Figure 6. As the concentration of fluoxetine increased, the percentages of early and late apoptotic cells were increased in both cell lines in a concentration-dependant manner, reaching significance at 30.0 ($p<0.01$) and 60.0 µM ($p<0.001$) of fluoxetine, as compared to cells treated with vehicle control. For example, in cells treated with 60.0 µM fluoxetine, the percentage of cells in the early apoptotic phase increased from 2.0 ± 1.0% to 19.0 ± 2.0% in HCT116+/+, and from 5.5 ± 2.5% to 22.0 ± 1.0% in HCT116-/-. In addition, the percentage of late apoptotic cells remarkably increased from 7.5 ± 0.5% to 79.5 ± 1.5% in HCT116+/+, and from 4.0 ± 2.0% to 76.6 ± 1.5% in HCT116-/-. No significant difference was observed in the responses of the two cell lines when they were treated with the concentrations of fluoxetine.

In summary, fluoxetine altered membrane integrity and established the translocation of phosphatidylserine from inner to outer membrane layer in both cell lines.

3.4.Effect of fluoxetine on cell cycle arrest

In an attempt to investigate if fluoxetine induces cell cycle arrest, a cell cycle analysis was performed using the NucleoCounter® NC-3000™ system by rapid quantification of DNA content, which was measured using fluorescence reading of DAPI-stained cells. This assay determines cell sorting at different phases of the cell cycle. Scatter plots are shown in Figure 7. The cell population treated with 10.0 µM of fluoxetine was comparable to those in the control group in both cell lines at different phases of cell cycle. However, when cells were pre-treated with 30.0 and 60.0 µM of fluoxetine, cell population at sub-G1 was increased ($p<0.01$, $p<0.001$) in a concentration-dependent manner, which is indicative of DNA fragmentation (Fig 7). The percentage of cells increased from 2.3 ± 0.3 % to 15.0 ± 0.5% (30.0 µM) and 23.0 ± 2.6 % (60.0 µM) in HCT 116 +/+; and from 4.3 ± 1.2% to 18.6 ± 2.9 % (30.0 µM) and 28.3 ± 0.8% (60.0 µM) in HCT116 -/- cells (Fig 8). No significant difference was observed between the two cell lines when treated with the same range of concentrations of fluoxetine.

Cell percentage in G0/G1 phase was comparable in the control and 10.0 µM groups. However, a significant reduction ($p<0.01$) in G0/G1 was observed in the cells treated with 30.0 µM of fluoxetine, with values decreasing to 37.0 ± 0.7% and 41.0 ± 4.0% in HCT116 +/+ cells and HCT116-/- cells from control values of 58.0 ± 0.5 and 61.3 ± 1.8%, respectively. Increasing the concentration of fluoxetine to 60.0 µM further reduced ($p<0.01$) the cell populations in both cell lines, with 34.3 ± 4.2% for the HCT116+/+ cells and 32.0 ± 0.6% for the HCT-/- cells as compared to control values. At S phase, there was no noticeable difference between cells treated with 10.0 µM and 30.0 µM of fluoxetine and control, but a slight increase ($p>0.05$) was demonstrated when cells were treated with 60.0 µM of fluoxetine, where cell percentage increased from 14.6 ± 1.1% (30.0 µM) to 20.0 ± 1.5 % in HCT116+/+ and from 13.3 ±0.3% (30.0 µM) to 19.3 ± 0.6% in HCT116-/-. Cell percentages

in G2/M phase did not show a significant difference at different concentrations of fluoxetine as compared to control values in both cell lines (Fig 8).

3.5.Effect of PARP inhibitors on fluoxetine-induced toxicity and cell death

In response to toxic insults, cells die through activation of a variety of death paradigms, of which apoptosis and necrosis are examples. It is therefore important in mechanistic studies to establish whether the profile of cell death induced by a toxic agent is consistent with the dominance of a particular paradigm or with substantial co-activation of a number of different forms of cell death. We, therefore, investigated the potential involvement of PARP, which is associated with the DNA damage response and mediates a unique form of cell death (parthanatos), in fluoxetine-induced toxicity. Two highly-potent and selective pharmacological inhibitors of PARP, one of which (DPQ) is an experimental agent, while the other (olaparib) is a licensed drug, were each tested against fluoxetine. Data obtained using two different viability assays, the MTT assay and the Alamar Blue (AB) assay, were similar. The results further confirmed that fluoxetine induced concentration-dependent reduction in the viability of both the HCT+/+ and HCT-/- cells and there was no difference in the sensitivities of both cells to fluoxetine (Figure 9, A and B). Neither DPQ (7.5 – 60 µM) nor olaparib (10 nM - 10µM) was able to modulate fluoxetine-induced cell death (Figure 9, C-F).

4. Discussion

Colorectal cancer is one of the major worldwide causes of mortality. Currently, there is no single treatment available that could cure the cancer or combine high potency and/or high specificity with little or minor side effects. This study investigated the effect of the anti-depressant drug fluoxetine on human carcinoma cell lines, with a view to identifying the

mechanisms and potential specificity of its cytotoxicity and thus its anti-tumour potential, especially against colorectal cancer. In the first part of the study, the cytotoxicity induced by fluoxetine was assessed using a variety of human carcinoma cell lines. Results indicated that fluoxetine induced concentration-dependent cytotoxicity, which was highly selective for human colon, breast and ovarian carcinoma cells versus non-cancerous cell lines. The IC_{50} values for fluoxetine cytotoxicity in both colon carcinoma cell lines HCT+/+ (in which the p53 gene is intact) and HCT-/- (in which p53 is deleted) are comparable, indicating that the p53 gene is unlikely to play a role in fluoxetine-induced cytotoxicity in these cell lines. An attempt was then made to use these two human colon carcinoma cells to further understand the mechanisms underlying fluoxetine-induced cytotoxicity. Several past investigations have provided compelling evidence that p53 protein status can have a profound effect on the susceptibility to apoptosis induced by a variety of apoptotic stimuli (Lowe et al. 1993, Fisher, 1994). Such studies have shown that deletion or mutation of p53 diminished the apoptotic response to chemotherapeutic drugs and /or increased resistance to anticancer therapies (Lowe et al., 1993; Harris, 1996). However, whilst p53 is required for DNA damage-induced apoptosis in some cell types, its presence and integrity may even be counteractive for drug sensitivity in other cells. For example, increased sensitivities to chemotherapeutic drugs were shown in inactive p53 primary mouse fibroblasts, human breast cancer cells (MCF-7), human papillomavirus-type-16 E6 (HPV16 E6) and testicular germ cell tumour cells (Hawkins et al., 1996; Fan et al., 1995; Burger et al., 1997, 1998). Induction of p53-independent apoptosis was shown to be via ceramide through the sphingomyelin-signalling pathway and Fas-dependent mechanisms (Jarvis et al., 1996; Burger et al., 1999). Later studies also reported cell death via p53-independent pathways via p73 activation, which is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage (Irwin et al., 2003; Ozaki and Nakagawara, 2005; Roos and Kaina, 2006). In addition, induction of

apoptosis via a p53-independent pathway has been reported for other compounds such as rapamycin in non-small cell lung cancer (Miyake et al., 2012). Such studies demonstrated a down-regulation of the expression levels of Bcl-2, which leads to an increase in the level of cytochrome c from mitochondria, which in turn would activate caspase cascades. Another possible target for fluoxetine could be TRAIL (tumour necrosis factor-related apoptosis-induced ligands) receptors/pathway, as TRAIL-induced apoptosis has been reported in colon cancer cell lines, which was not dependent on p53 (Galligan et al., 2005). Overall, the present study supports the unlikely involvement of p53 in mediating fluoxetine cytotoxicity, indicating the activation of an apoptotic pathway independent of p53 in HCT116 human colon carcinoma cells.

This study also investigated whether fluoxetine-induced cytotoxicity is related to altering mitochondrial membrane potential. The finding indicated a major change in mitochondrial membrane potential in colon carcinoma cells, in response to fluoxetine. This finding is in line with previous studies which showed induction of apoptosis through mitochondrial membrane dysfunction in hepatocellular carcinoma cells (Mun et al., 2013). A very similar effect has also been found in human neuroblastoma cells, in which apoptosis was induced by mitochondrial membrane potential alteration which involved ROS accumulation (Choi et al., 2017). ROS accumulation has also been reported in human ovarian carcinoma cells treated with fluoxetine (Lee et al., 2010). Cancer cells were found to increase the activity of lactate transporters alkalizing their ipH (intracellular pH) (Cairns et al., 2011; Daniel et al., 2005). This process leads to hyperpolarization of mitochondrial membrane potential, which allows the tumour to use oxidative phosphorylation and glycolysis (Jones and Schulze, 2011). Previous studies have shown that fluoxetine inhibits tumour energy generation machinery, reducing proliferation in cancer cells (Kannen et al., 2015). This was found together with reduced angiogenesis and impaired tumour development (Kannen et al., 2015). The

mitochondrial membrane depolarization was promoted by reducing the activity of lactate- and glucose-related transporters (Edinger and Thompson, 2002). Furthermore, studies have demonstrated that lactate efflux from glycolysis is regulated by cell-surface glycoprotein (Cardone et al., 2005; Slomiany et al., 2009). In hypoxia, lactate transporters alkalise ipH of cancer cells, thus generating energy and promoting cell growth (Cardone et al., 2005; Beasley et al., 2001; Silva et al., 2009). Researchers have found that fluoxetine slightly decreased ipH in HT29 cells (Kannen et al., 2015). Therefore, future studies should be focused on hypoxic colon cancer cells to confirm that fluoxetine decreases ipH and reduces the activity of lactate transporters in them. In addition, fluoxetine could be tested in combination with bevacizumab, a monoclonal antibody that exerts anti-tumour activity by targeting vascular endothelial growth factor (VEGF), thus impairing angiogenesis in the tumour. Furthermore, *in vivo* study has shown that the anti-apoptotic protein Bcl-2 was significantly decreased in tumours in fluoxetine-administered mice (Frick et al., 2011). Therefore, future experiments could investigate the influence of the Bcl-2 protein and gene on the other mechanistic pathways underlying the anti-tumour activity of fluoxetine.

In the present study, further confirmation of induction of apoptosis by fluoxetine came from Annexin V study where externalization of phosphatidylserine due to progressive loss of membrane integrity was observed. This is in line with previous studies which reported externalization by fluoxetine treatment of phosphatidylserine in Jurkat cells (Charles et al., 2017), as well as human oral cancer cells (Lin et al., 2014) and the HD29 human colon cancer cell line (Kannen et al., 2012). The later study also indicated an effect of fluoxetine on cell cycle in the $G0/G_1$ phase (Kannen et al., 2012). Our study also indicated a sub- G1 event, which was indicative of DNA fragmentation/apoptotic event (Wadman, 2013). Our study showed a concentration-dependent effect associated with fluoxetine treatment in both cell lines. The number of cells with fragmented DNA in sub G1 phase increased in comparison

with untreated controls. This observed increase in DNA fragmentation was accompanied by a reduction in the percentage of cells treated with higher concentrations of fluoxetine in the G0/G1 phase, further suggesting a cell death by apoptosis without indication of a cell cycle arrest in G0/G1 phase over 48 h contact time with fluoxetine.

Additional studies using experimental and clinically-used pharmacological inhibitors of PARP, an enzyme that mediates a distinct, non-apoptotic cell death modality, provided evidence that supports the fact that fluoxetine toxicity induces predominantly apoptosis, as the PARP inhibitors had no effect on the toxicity. It should be emphasised that, in all assays performed in the present study, deletion of the p53 gene did not lead to any differential effect of fluoxetine on mitochondrial membrane potential, Annexin V staining and cell cycle events in the human HCT116 colon carcinoma cell line.

Colorectal cancer is often caused by the mutation of SMAD4 tumour suppressor gene (Ormanns et al., 2017); therefore, future work should also consider using human colorectal cancer cells with intact and deleted SMAD4 gene, as well as those in which other tumour suppressor genes such as APC, DCC, MLH1 and MLH2 are intact or have been deleted.

5. Conclusion

The present study shows that fluoxetine induces anti-proliferative effects in a variety of human carcinoma cell lines, with higher selectivity for tumour cells versus normal cells in colon, breast and ovarian carcinoma cells than in lung carcinoma cells. Further experiments focusing on colon carcinoma cells indicated the establishment of apoptotic events as a result of a change in mitochondrial membrane potential and translocation of phosphatidylserine. Further evidence for the induction of apoptosis came from cell cycle analysis where an increase in the cell population at sub-G1 was indicative of DNA fragmentation/apoptosis-

related event. Apoptosis was also confirmed as the predominant pathway that mediates fluoxetine-induced cell death. The present study did not find evidence for the involvement of the p53 gene in the cytotoxicity induced by fluoxetine in the human colon carcinoma cells HCT 116 +/+ and HCT116 -/- . The only difference between the two cell lines was observed when fluoxetine was added at the highest concentration of 60 µM in the measurement of the mitochondrial membrane potential. Therefore, these findings suggest that fluoxetine is likely to induces p53-independent apoptosis through mitochondrial pathway that leads to DNA fragmentation depending on the concentration used. In other words, as revealed in the present study, p53 is unlikely to be of primary importance for the sensitivity of the tested human colon carcinoma cells to the cytotoxic effects of fluoxetine. The study provides new insights into some key molecular mechanisms underpinning the cytotoxicity of fluoxetine and demonstrates a potential for its repurposing for cancer treatment. The knowledge furnished by the work is valuable to developing more efficacious and targeted anti-cancer treatments.

6. Conflict of Interest

The authors declare no conflicts of interest.

7. References

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Figure 1. Representative photomicrographs showing the effects of fluoxetine on HCT 116 +/+ and HCT 116 -/- (human colon carcinoma parental and p53 deleted, respectively) cells under the light microscope (resolution 20x); a) control (0µM), b) 10µM, c) 30µM and d) 60µM of fluoxetine after 24 hours exposure time.

Figure 2. Histobars indicating (A) the IC50s and (B, C) the selectivity for fluoxetine-induced cytotoxicity compared to normal /non-cancerous cells, ARPE19 and PNT2 in a variety of human carcinoma cells: colon, HCT116 +/+ and HCT116 -/-; breast, MCF-7; lungs, A549; ovarian (cisplatin sensitive), A2780; ovarian (cisplatin-resistant), A2780-CP70; and normal /non-cancerous cells, ARPE19 and PNT2 cells. Each bar represents the mean ± s.e.mean of n=4.

Figure 3. Representative scatter plots indicating the red/green intensity and DAPI fluorescence of HCT116 +/+ and HCT116-/- cells treated for 24 h with 0.0 µM, 10.0 µM, 30.0 µM and 60.0 µM of fluoxetine.

Figure 4: Histograms showing the percentage of apoptotic colorectal cancer cells (HCT116+/+ and HCT116-/-) after 24 h exposure to fluoxetine (10.0 µM, 30.0 µM, and 60.0 µM) or control, captured using the MMP assay. Each column represents mean ± s.e.mean, n=3. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the control and # $p<0.05$ compared to HCT116 +/+.

Figure 5. Representative scatter plots indicating the green/red light intensity of stained HCT116+/+ and HCT116-/- cells treated for 24 h with 0.0 µM, 10.0 µM, 30.0 µM and 60.0 µM of fluoxetine.

Figure 6. Histograms showing the percentage of early apoptotic and late apoptotic cells after performing Annexin V assay on colorectal cancer cells (HCT116+/+ and HCT116-/-). Data set is based on 24 hours fluoxetine exposure at 0.0 µM (Control), 10.0 µM, 30.0 µM and 60.0 µM of fluoxetine. Each column represents mean ± s.e.mean, n=3. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the control.

Figure 7. Representative scatter plots indicating the percentage of HCT116+/+ and HCT116-/- cells in Sub-G1 (M1), G0/G1 (M2), S (M3) and G2 (M4) phases after 48 hours of exposure to different concentrations (0.0 µM, 10.0 µM, 30.0 µM, 60.0 µM) of fluoxetine.

Figure 8. Histograms showing the percentage of cells in Sub-G1, G0/G1, S and G2 phases after performing two-step cell cycle assay on colorectal cancer cells (HCT116+/+ and HCT116-/-). Data set is based on 48 hours exposure to fluoxetine at 0.0 µM (Control), 10.0 µM, 30.0 µM, and 60.0 µM. Each column represents mean ± s. e. mean of n=3.* $p<0.05$, **$p<0.01$, *** $p<0.001$ compared to the control.

Figure 9. Concentration-dependent effects of fluoxetine (FXT) on the viability of the HCT +/+ and HCT -/- cells, as quantified by (A) the MTT assay (absorbance-based), and (B) the Alamar Blue (AB) assay (fluorescence-based); and the lack of effect on fluoxetine-induced cell death of the highly-potent and selective inhibitors of PARP-mediated cell death, DPQ (an experimental agent) and olaparib (OPB, used in the clinic), as quantified for DPQ by (C) the MTT assay and (D) the AB assay; and for olaparib by (E) the MTT assay and (F) the AB assay. Values represent the mean ± s.e.mean of n=3. ***$p<0.0001$ compared to the control.