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Monoclonal antibody targeting MUC1 and increasing sensitivity to docetaxel as a novel strategy in treating human epithelial ovarian cancer

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ABSTRACT

The purpose of this study was to investigate the *in vitro* effect of anti-MUC1 monoclonal antibody (MAb) C595 alone and in combination with docetaxel, on the growth and survival of different epithelial ovarian cancer (EOC) cell lines. MUC1 expression was assessed on EOC cell lines (OVCAR-3, IGROV-1, A2780, CAOV-3, TOV-21G, TOV-112D, SKOV-3 and OV-90) using immunofluorescence labeling and flow cytometry. The effect of MAb C595 alone or in combination with docetaxel on the cell lines was studied by proliferation, colony and TUNEL assays. Our results indicate that all primary and metastatic EOC cell lines tested were positive to MAb C595 (MUC1); MAb C595 inhibited EOC cell proliferation in a MUC1- and dose-dependent manner; low-dose MAb C595 (1/2 of IC_{50}) combined with docetaxel greatly improved efficiency of cell killing in EOC cells and induced apoptosis; the additive effect of MAb C595 was further confirmed in colony forming assays; and cell death following single or combined treatments was associated with the release of cytochrome *c* and increased caspase-3 activity. These results suggest that MAb C595 used either alone, or combined with docetaxel, is an attractive strategy for targeting human EOC.

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy in the United States, resulting in an estimated 21,550 new cases and 14,600 deaths in 2008 [1]. Despite advances in surgery and chemotherapy over the past 20 years, overall survival has not changed significantly in patients with EOC. Although current approaches yielded responses in 60–80% of patients with advanced-stage disease, majority of EOC patients eventually relapse and become refractory to additional treatment. Conventional cancer chemotherapy often results in severe side effects related to non-specific modes of action. Studies evaluating various cytotoxic agents in recurrent EOC have found response rates of 10–28% with an accompanying progressive increase in the number of drug-resistant tumors [2]. Novel therapeutic strategies are urgently needed to improve the outcome for this deadly disease.

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MUC1 is a highly glycosylated type I transmembrane glycoprotein that is aberrantly over-expressed on the cell surface of multiple carcinomas including EOC [3]. Cancer-associated MUC1 has shorter and less dense O-glycan chains, exposing novel regions of the protein core [4]. This reduced glycosylation permits the immune system to access the peptide core of the tumor-associated underglycosylated MUC1 antigen (uMUC1) and reveal epitopes that are normally masked, allowing development of an antibody that discriminates between normal cells and adenocarcinoma cells. Enhanced levels of MUC1 expression by cancer cells may mask extra-cellular domains from immune surveillance, conferring a survival advantage on malignant cells and playing an important role in the ability of tumors to invade and metastasize [5]. Thus, tumor-associated MUC1 is a promising target for novel therapy.

Monoclonal antibody (MAb) C595 is an IgG_3, raised against the protein core of human MUC1 (urinary epithelial mucin1) [6]. Epitope mapping has shown that MAb C595 recognizes a tetrapeptide motif (RPAP) within the protein core of MUC1 that contains a large domain of multiples of a highly conserved 20-amino-acid-repeat sequence (PDTRPAPGSTAPPAHGVTSA) [6,7]. The reactivity of MAb C595 with synthetic peptides (a recombinant diabody fragment) containing this motif permits efficient antibody purification using peptide-epitope affinity chromatography, which, unlike other methodologies, enables exclusion recovery of functionally active antibody [8]. MAb C595 has been labeled with γ-emitting radioisotope (^{111}In) to test its capacity for cancer localization and identification in 19 patients with a clinical suspicion of ovarian malignancy, and achieved final accuracies of 79% and 64% compared with magnetic resonance imaging and ultrasound in relation to the final tumor histology [9]. We recently demonstrated that MAb C595 is strongly positive in over 90% of late stage of EOC sections, while no staining was found in normal ovaries [3]. After labeling with α-emitter (^{213}Bi), ^{213}Bi-C595 α-conjugate (AC) has been used to target single prostate [10], pancreatic [11] or ovarian cells [12] *in vitro* and regress pancreatic subcutaneous xenografts *in vivo* [13]. These results support the hypothesis that this MAb may be useful alone or in combination with other therapies to improve the treatment of the advanced EOC.

Docetaxel can stabilize tubulin polymerization and arrest cells primarily in the M and G2 phases of the cell cycle. Docetaxel has demonstrated significant activity in both pre-clinical and clinical studies for the treatment of numerous solid malignancies including EOC [14–16]. Docetaxel combined with a platinum compound (such as carboplatin) has become the systemic chemotherapy of choice for primary EOC, with high efficacy. However, dose-related toxicity and the eventual development of resistance are major issues requiring attention in a gynecologic oncology setting. Ultimately most of these patients will die of metastatic disease, and there is a need for more effective therapeutic strategies to control metastatic disease. Combination therapy specifically employing strategies such as a chemotherapeutic agent plus an antibody may reduce dose-limiting toxicity and improve treatment efficacy. A combination of docetaxel and MAb C595 may provide a novel alternative treatment option.

In this study, we hypothesized that MAb C595 can kill EOC cells and that treatment of EOC cells with combined therapy (MAb C595 and docetaxel) would reduce the dose of the cytotoxic component, increase the sensitivity EOC cells to chemotherapy and be more effective than either treatment alone. We found that MAb C595 binds all primary and metastatic EOC cell lines, and demonstrated that MAb C595 alone could kill EOC cells in a dose-dependent manner; this killing was also dependent upon MUC1 expression levels. Furthermore, low-dose MAb C595 (1/2 of IC_{50}) combined with docetaxel increased cellular sensitivity to the chemotherapy drug and reduced the dose required. Our results suggest that this combination therapy may be a useful novel treatment for advanced EOC.

2. Materials and methods

2.1. Drug

Docetaxel was purchased from Sigma–Aldrich, Pty Ltd., Castle Hills, NSW, Australia. The drug was first diluted in 100% ethanol and then in growth medium. The final ethanol concentration was 0.01% and the final concentrations of docetaxel were 0.001 nM–10 μM.

2.2. Antibodies

MAb C595 was kindly provided by Nottingham University (Nottingham, UK). Mouse anti-human IgG_3 isotype control was purchased from Zymed Laboratories INC (South San Francisco, CA, USA). Alexa Fluor-488 goat anti-mouse IgG was purchased from Molecular Probes (Eugene, Oregon, USA). Goat anti-mouse-fluorescein isothiocyanate (FITC) MAb was purchased from DakoCytomation (Glostrup, Denmark).

2.3. Cell lines

Primary (OVCAR-3, IGROV-1, A2780, CAOV-3, TOV-21G and TOV-112D) and metastatic (SKOV-3 and OV-90) EOC cell lines (American Type Culture Collection (ATCC), Rockville, MD, USA) representative of different histological types were studied (Table 1). All tissue culture reagents were supplied by the Invitrogen Australia Pty Ltd., (Melbourne, VIC, Australia), unless otherwise stated. OVCAR-3, IGROV-1, A2780 and SKOV-3 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 50 units/mL penicillin and 50 units/mL streptomycin. OV-90 cells were maintained in a 1:1 mixture of MCDB 105 medium (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia) and 199 medium (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia), supplemented with 15% FBS, 50 units/mL penicillin and 50 units/mL streptomycin. CAOV-3 cells were maintained in DMEM with 10% FBS. TOV-21G and TOV-112D cells were maintained in DMEM with 15% FBS. All cell lines were maintained in a humidified incubator at 37 °C and 5%

Table 1
MUC1 expression in EOC cell lines assessed using immunofluorescence labeling (confocal microscopy) and flow cytometry.

Cell Line	Site of origin	MUC1 expression	
		[a]Confocal analysis	[b]Flow cytometry MFI ratio
OVCAR-3	Adenocarcinoma of ovary	3	26
IGROV-1	Adenocarcinoma of ovary	3	19
A2780	Adenocarcinoma of ovary	2–3	5
CAOV-3	Adenocarcinoma of ovary	3	69
TOV-21G	Clear cell	3	35
TOV-112D	Endometrioid	3	8
SKOV-3	Ascites, papillary serous adenocarcinoma of ovary	3	6
OV-90	Ascites, ovarian adenocarcinoma	3	24

[a] Confocal microscopy semi-quantitative grading scale: 0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining.
[b] Mean fluorescence intensity ratio = MFI from test/MFI from isotype control.

CO_2. Sub-confluent cells cultured for 48 h without a change of medium were harvested by gently rinsing flasks twice with Dulbecco's phosphate-buffer saline (DPBS) and then detached with 0.25% trypsin/0.05% EDTA in DPBS at 37 °C. Cells were collected and re-suspended in the appropriate buffer as described below.

2.4. Confocal microscopy for MUC1 expression

OVCAR-3, IGROV-1, A2780, CAOV-3, TOV-21G, TOV-112D, SKOV-3 and OV-90 cells were grown on glass coverslips (10^5 cells) for 24 h. After washing with Tris buffered saline (TBS) (pH 7.5), cells were fixed on coverslips in ice-cold methanol for 10 min at room temperature (RT). After rinsing in TBS, coverslips were incubated for 1 h at RT in mouse anti-C595 MAb (32 μg/mL), rinsed again in TBS, then incubated for 1 h at RT in Alexa goat anti-mouse 488, with a further rinsing in TBS. Cell nuclei were stained with propidium iodide (PI) (1:2000 dilution) (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia) for 1 min before coverslips were mounted on glass slides using glycerol (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia), and examined using confocal microscopy (FV 300/FV500 Olympus, Tokyo, Japan).

2.5. Flow cytometry

To detect whether the tumor-associated MUC1 is expressed on the cell surface of OVCAR-3, IGROV-1, A2780, CAOV-3, TOV-21G, TOV-112D, SKOV-3 and OV-90 cells, indirect immunofluorescence staining was performed. At each step, cells were incubated on ice. Briefly, 0.5–1.0×10^6 cells were washed twice with 4 mL cold DPBS containing 5% FBS (200 g for 8 min) before resuspension in 80 μL of cold DPBS plus 5% FBS. Cells were then incubated with either MAb C595 (32 μg/mL) or an irrelevant IgG_3 isotype control (32 μg/mL) for 45 min. After washing with DPBS, cells were re-suspended and incubated with a goat anti-mouse-FITC MAb for 30 min in the dark with shaking. The cells were washed again and re-suspended in 0.5 mL of DPBS supplemented with 5% FBS. PI was added (1 μg/mL) to the cells prior to analysis for identification of dead cells. Ten-thousand flow cytometry events were collected for each sample using a FACScan flow cytometer (Becton–Dickenson). Data was analysed using FlowJo (Tree Star, Ashland, OR, USA) flow cytometry analysis software.

2.6. In vitro cell growth assay

Cell growth was evaluated in EOC cell lines following treatment with single or combination therapies using MTT (3-(4,5-dimethylthlthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays. Briefly, 3000 cells were seeded in 96-well plates incubated in culture medium, RPMI-1640 with 5% FBS for 24 h. Cells were then treated with a range of concentrations of MAb C595 (5–125 μg/mL, test), MAb IgG_3 (5–125 μg/mL, non-specific control), docetaxel (10^{-5}–10^{-12} nM) or MAb C595 (1/2 IC_{50}, i.e. 25% cell killing) and docetaxel (10^{-5}–10^{-12} nM). The 1/2 IC_{50} doses were determined from previous MAb C595 cytotoxicity experiments. Cell viability was examined at 48 h post-exposure, when 20 μL of MTT (5 mg/mL, Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia) was added to a final concentration of 0.5 mg/mL and the cells were incubated for a further 4 h at 37 °C. Subsequently, 100 μL of DMSO (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia) was added and the plate was shaken for 20 min at RT to dissolve the formazan crystals. The absorbance (OD) was read at a wavelength of 562 nm on a BIO-TEC micro-plate reader (BIO-RAD, Hercules, CA, USA). Each experiment was repeated at least three times. Results represent the OD ratio of the treated and untreated cells.

2.7. Colony forming assays

OVCAR-3, IGROV-1, A2780 and OV-90 cells were used for colony forming assays as described previously with minor modifications [17]. Briefly, 500 cells/well were seeded in six-well plates for 48 h at 37 °C, 5% CO_2 and then treated with either MAb C595 or docetaxel alone at the respective IC_{50} concentrations, combined MAb C595 (1/2 IC_{50}) and docetaxel (IC_{50}), or vehicle control (100% ethanol). After 3 days treatment, the docetaxel or MAb-containing media was replaced with fresh media and all cultures were incubated for an additional 7 days until colonies were large enough to be clearly discerned. After washing with DPBS, colonies were stained with 3% methylene blue (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia) in 50% methanol for 30 min at RT. The colonies, defined

as groups of >50 cells, were scored manually with the aid of an Olympus INT-2 inverted microscope (Tokyo, Japan). Data from MAb C595, docetaxel or MAb C595 and docetaxel-treated cells were normalized against the vehicle treatment alone (scored as 100% colony forming ability). Cell survival curves were plotted, with mean and SE of at least three independent experiments.

2.8. Detection of apoptosis

OVCAR-3, IGROV-1, A2780 cells (0.5×10^6) were cultured in six-well plates for 24 h and then treated with MAb C595 and docetaxel alone, combined MAb C595 and docetaxel, or vehicle (100% ethanol and control MAb IgG_3) as described in the cell cycle analysis (see above) for 48 h. After treatments, apoptotic cells were observed by assessing nuclear morphology and TUNEL assay respectively.

1. Detection of nuclear morphology: Cells exposed to different treatments were stained with the DNA-binding agents acridine orange and ethidium bromide (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia) and examined with fluorescence microscopy (Zeiss, Gottingen, Germany). Apoptotic cells were characterized by morphology including nuclear condensation and fragmentation.
2. TUNEL assay: Treated cells (adherent and non-adherent) were collected, prepared as cytospins (3×10^4 cells/slide, Shandon Cyto-Centrifuge, Shandon, Pittsburgh, PA, USA) and air-dried overnight at RT. Cells were fixed in 2% paraformaldehyde at RT for 20 min. Apoptosis was assessed using the TUNEL method with the TdT-fragEL *in situ* apoptotic detection kit (Oncogene Research Products, Boston, MA) according to the manufacturer's instructions [18]. The specificity of TUNEL reactivity was confirmed with appropriate negative (TdT omitted from the labeling mix) and positive (treated HL-60 slides provided by the company) controls. Slides were examined using a Leica light microscope (Nussloch, Germany). The treatments were performed in triplicate with three experiments for test and control ($n = 3$).

2.9. Measurement of cytochrome c and caspase-3 activity

OVCAR-3, IGROV-1, A2780 cells treated with the same protocol as above were also assessed by commercial ELISA kits for cytochrome *c* and caspase-3 activity.

1. Cytochrome *c* assay: Briefly, trypsinized and non-adherent cells were pooled, washed three times with cold DPBS at 1000*g* for 5 min at 4 °C. The cells (1×10^6) were suspended in lysis buffer (250 mM sucrose 250, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 0.1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 20 mM HEPES/KOH, pH 7.5) for whole cell lysates. The supernatants and cytochrome *c* conjugate were added into the 96-well micro-plates coated with MAb specific for human cytochrome *c*. The procedure was performed according to the manufacturer's instructions (Cytochrome *c* Immunoassay Kit; R&D systems, Minneapolis, MN, USA). Sample absorbance was measured at 450 nm in a micro-plate reader (Bio-TEK, Winooski, Vermont, USA). A standard curve was constructed by plotting the absorbance values of diluted solutions of a cytochrome *c* standard. The results were compared with a standard curve and multiplied by the dilution factor. The amount was expressed as ng/mL.
2. Caspase-3 activity assay: Caspase-3 activity was determined using an assay kit, according to the manufacturer's directions (Caspase-3 Colorimetric Assay kits, R&D Systems, Minneapolis, MN, USA). Briefly, trypsinized and non-adherent cells from vehicle control and treated samples were pooled and washed twice with DPBS at 1500*g* for 5 min at 4 °C. Cell pellets (4×10^6) were re-suspended in lysis buffer and left on ice for 10 min. Lysed cells were centrifuged, supernatants collected and added to the reaction mixture containing dithiothreitol and caspase-3 substrate (*N*-acetyl-Asp-Glu-Val-Asp*p*-nitroanilide) and was incubated for 1 h at 37 °C. The absorbance of the chromophore *p*-nitroanilide was measured at 405 nm by a micro-plate reader (Bio-TEK, Winooski, Vermont, USA). Standard curves were obtained from the absorbance values of the *p*-nitroanilide standard reagent diluted in cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as the activity that produced 1 nmol of *p*-nitroanilide.

2.10. Statistical analysis

All numerical data were expressed as averages (mean), and the standard error (SE) was calculated. Data from treated and control groups were compared using the two-tail student's *t*-test. All *P* values were 2-sided. One way ANOVA, followed by the Dunnett's post hoc test was performed to determine the significance of differences between different treatment groups. $P < 0.05$ was considered significant. All statistical analyses were performed using the GraphPad Prism 4.00 (GraphPad, San Diego CA).

3. Results

3.1. Expression of MUC1 in EOC cell lines

All cell lines (OVCAR-3, IGROV-1, A2780, CAOV-3, TOV-21G, TOV-112D, SKOV-3 and OV-90) showed positive staining for MAb C595 (Fig. 1A). No immunolabeling was detected with isotype MAb IgG_3 (Fig. 1B) or when primary MAbs were omitted (not shown). The immunostaining results are summarized in Table 1, and show that high levels of target antigen (MUC1) are found in primary and metastatic EOC cell lines.

Distinct patterns of MAb C595 immunoreactivity for the EOC cell lines were found as shown in the representative flow cytometry (FCM) histograms (Fig. 1C). Positive shifts (increases in the intensity of fluorescence) were observed for all cell lines (Fig. 1C). These results indicate that the antigen recognized by MAb C595 is expressed on the

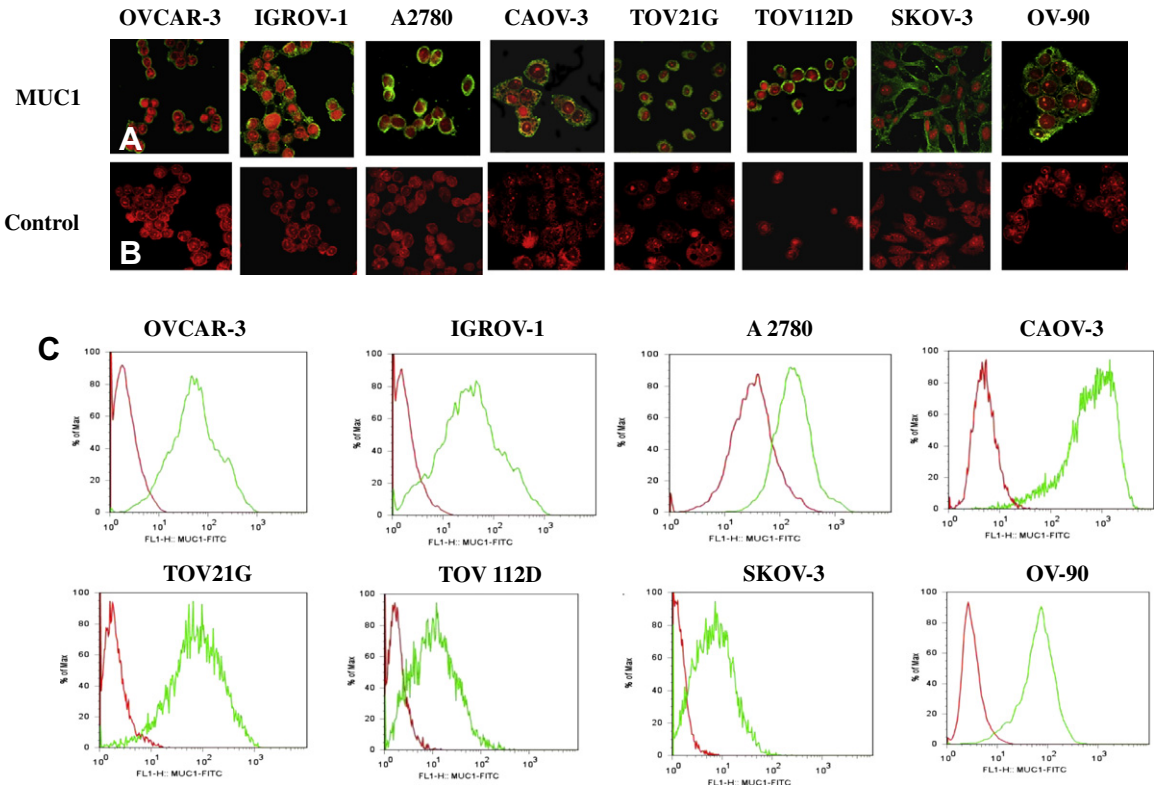

Fig. 1. Expression of MUC1 in EOC cell lines using confocal microscopy and flow cytometry (FCM). (A) Representative images showing variable cell surface immunolabelling with MAb C595 in primary and metastatic cell lines (green). (B) No immunostaining is seen for isotype IgG_3 controls for all cell lines. Nuclei are visualized using propidium iodide (red). Magnification: A and B 400×. (C) Expression of MUC1 in primary and metastatic cell lines assessed by flow cytometry (FCM) (right histogram). An obvious shift is seen for most cell lines. Data are presented as histograms, using mouse IgG_3 isotype control antibody for background fluorescence. FL1-H = log fluorescence intensity. The results are from three independent experiments ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surface of viable cells, and confirms our confocal microscopy observations. The ratio of mean fluorescence intensity (MFI) from test/isotype control for each cell line is summarized in Table 1.

3.2. Growth inhibition of EOC cells by MAb C595 and docetaxel alone or in combination in vitro

To investigate whether MAb C595 alone can kill EOC cells or whether MAb C595 at a fixed concentration (1/2 IC_{50}) sensitizes EOC cells to docetaxel, eight EOC cell lines were examined by MTT proliferation assay. We first examined if MUC1-positive cell lines were responsive to different concentrations of MAb C595 after 48 h treatment. Our results indicated that MAb C595 alone markedly reduced cell viability of MUC1-positive cell lines in a dose-dependent manner which correlated with MUC1 expression (Fig. 2A). No cytotoxic effect was found for MAb IgG_3 (non-specific control) and vehicle (the same volume of DPBS) for all cell lines tested (data not shown). Cell survival following MAb C595 treatment was significantly less compared to MAb IgG_3 treatment for all MUC1-positive cell lines ($P < 0.01$, data not shown). CAOV-3 was the most MAb C595-sensitive cell line (IC_{50}: 8 μg/mL), while TOV-112D was the least sensitive cell line (IC_{50}: 63 μg/mL) (Fig. 2A). These results are consistent with the levels of MUC1 expression observed using immunofluorescence staining and FCM (Table 1). The IC_{50} (50% cell survival) for the different cell lines treated with MAb C595 are summarized in Table 2. These results indicate that MAb C595 can kill EOC cells *in vitro*.

Docetaxel treatment alone reduced cell viability in all cell lines tested, in a dose-dependent manner (Fig. 2B). Each cell line displayed a variable response to docetaxel after 48 h treatment, with IC_{50} ranging from 0.52 to 1.7 nM. We also investigated the effect of a fixed concentration of MAb C595 (4–31.5 μg/mL, 1/2IC_{50}, see Table 2) combined with a range of concentrations of docetaxel for each EOC cell line. After 48 h, the combined treatment markedly reduced cell survival of MUC1-positive cell lines compared to docetaxel alone (Fig. 2B and C). After using a published method to determine synergy or additivity [19], we established that MAb C595 potentiates the cytotoxic effect of docetaxel in an additive manner. Cell viability with docetaxel alone was significantly higher than with combined treatment of docetaxel and MAb C595 for MUC1-positive EOC cell lines ($P < 0.01$). For 100 nM docetaxel, cell viability was reduced to <12%, while for combined treatment with MAb C595 and docetaxel, cell viability was <4%. The effect of combined treatment was ~3 times higher

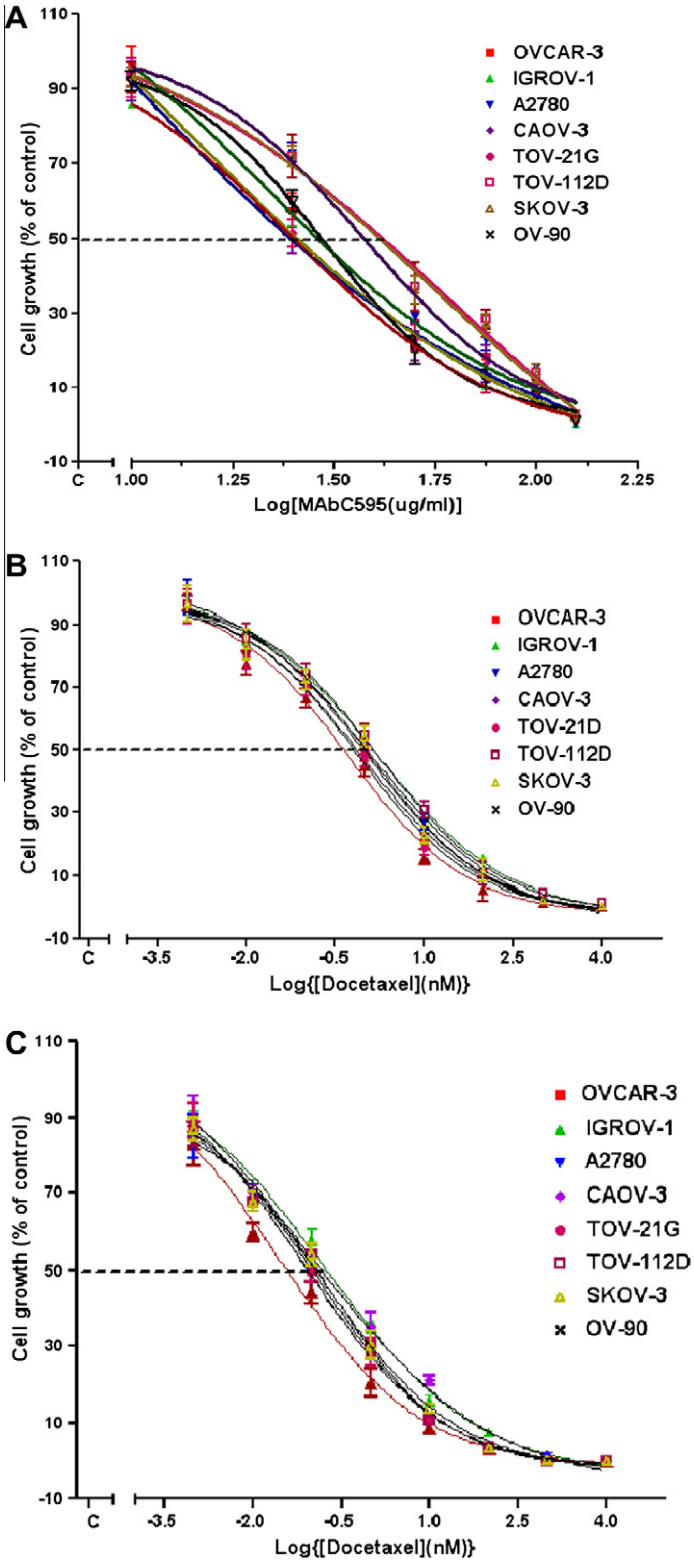

Fig. 2. Effect of MAb C595, docetaxel or combined MAb C595 and docetaxel on growth of EOC cell lines *in vitro*. Cell growth was assessed by MTT (3-(4,5-dimethylthlthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Representative MTT survival curves for different concentrations of: (A) MAb C595; (B) docetaxel and (C) combined docetaxel and MAb C595 (1/2 IC_{50}) 48 h post-treatment. Cell growth (measured as absorbance) is expressed as % untreated control (Mean% ± standard error (SE), $n = 3$). The results are from three independent experiments ($n = 3$).

than that of docetaxel alone, indicating that combined MAb C595 and docetaxel has an additive effect on MUC1-positive EOC cells.

Table 2
IC_{50} for MAb C595, docetaxel and combined MAb C595 and docetaxel for EOC cell lines (MTT assay) ($n = 3$).

Cell lines	IC_{50} MAb C595 (μg/mL)	IC_{50} Docetaxel (nM)	[a]IC_{50} for docetaxel (nM) (combined with MAb C595 (μg/mL) (1/2 IC_{50}))
OVCAR-3	22	0.87	0.09
IGROV-1	25	1.55	0.18
A2780	37	0.52	0.03
CAOV-3	8	1.17	0.14
TOV-21G	15	1.12	0.09
TOV-112D	63	0.87	0.07
SKOV-3	54	1.70	0.20
OV-90	29	1.22	0.14

[a] IC_{50} for when a range of docetaxel concentrations is combined with a fixed concentration of MAb C595 (1/2 IC_{50}). The results (mean of IC_{50}) are from three independent experiments ($n = 3$).

3.3. Effect of single or combination therapy on colony formation by EOC cells

Colony formation assays were used to determine the long-term effects of MAb C595 alone or combined treatment on EOC cell growth. We first examined the colony forming capacity of different EOC cell lines and found that OVCAR-3, IGROV-1, A2780, CAOV-3, TOV-21G and OV-90 can form colonies after culture, while TOV-112D and SKOV-3 cells do not. We chose representative primary (OVCAR-3, IGROV-1 and A2780) and metastatic (OV-90) EOC cell lines for further experiments. For MAb C595 and docetaxel treatments alone, IC_{50} doses were used; for combined treatment, 1/2 IC_{50} MAb C595 and IC_{50} docetaxel was used, with the same volume of vehicle (100% ethanol and control MAb IgG_3) as the control. MAb C595 alone induced a 30–42% reduction in colony formation compared to controls, for OVCAR-3, IGROV-1, A2780 and OV-90 cells (MAb C595-positive), with 46–60% reduction for docetaxel (Fig. 3A). Combined MAb C595 and docetaxel markedly

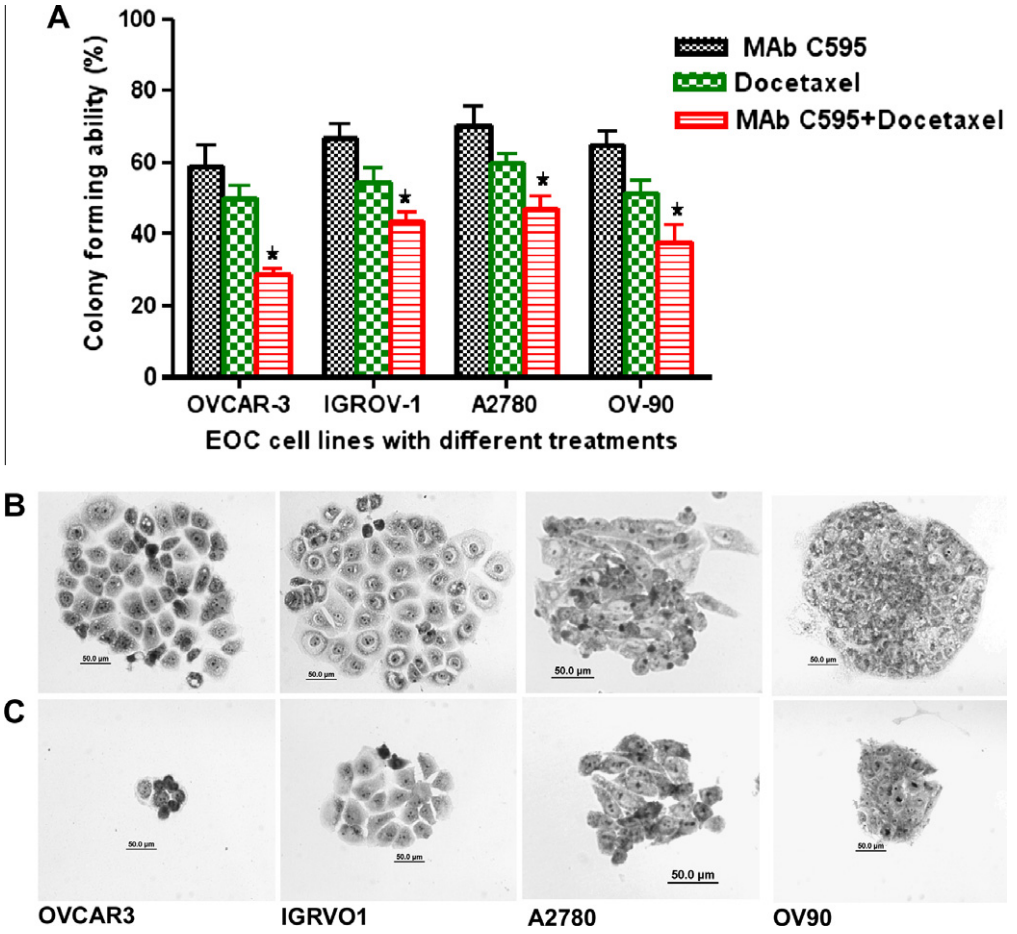

Fig. 3. Effect of MAb C595, docetaxel or combined MAb C595 and docetaxel on colony formation. OVCAR-3, IGROV-1, A2780 and OV-90 cells were seeded in six-well plates (300 cells/well) and treated after 48 h with vehicle (100% ethanol and the istotype MAb IgG_3 control), MAb C595 (IC_{50}), docetaxel (IC_{50}), or combined MAb C595 (1/2 IC_{50}) and docetaxel (IC_{50}) for 3 d. Following treatment, cells were cultured in growth medium for 7 d. After which, they were stained with 3% methylene blue in 50% methanol. Colonies containing ⩾50 cells were counted. (A) Results are presented as a percentage of control colony formation (100%). (Mean% ± standard error (SE), $n = 5$, $^*P < 0.05$ compared to MAb C595 or docetaxel alone). (B) Representative examples of colonies >50 cells are shown. (C) Colonies <50 cells are shown. The results are from five independent experiments ($n = 5$).

reduced colony formation of the four EOC cell lines by 72%, 57%, 54% and 64% respectively, compared to controls (Fig. 3A). There was a significant reduction in colony formation potential for the primary and metastatic cell lines with combined MAb C595 and docetaxel treatment, compared to either MAb C595 or docetaxel alone ($P < 0.05$). For the single or combination treatment, OVCAR-3 was the most sensitive cell line (Fig. 3A). In this study, only colonies with $\geqslant$50 cells after treatment were counted as positive (Fig. 3B) and small colonies <50 cells after treatment were not counted (Fig. 3C). These results further confirmed the MTT assay findings and provided evidence that MAb C595 can sensitize and enhance docetaxel-induced inhibition of colony formation by EOC cells.

3.4. Morphological changes and evaluation of apoptosis after single or combination treatment

To investigate whether apoptosis is the pathway of cell death after treatment, we observed cell morphology changes and used TUNEL assay to evaluate the proportion of apoptotic cells for three EOC cell lines (OVCAR-3, IGROV-1 and A2780). After treatment with MAb C595 or docetaxel (IC_{50}) alone, or combined treatment with MAb C595 (1/2 of IC_{50}) and docetaxel (IC_{50}), obvious morphological changes were found in treated cell lines but not controls (Fig. 4). Cells exposed to combination therapy for 24 h displayed characteristic apoptotic morphology including rounding up and detachment from the flask surface, and condensed chromatin and cytoplasm (i.e., cells became

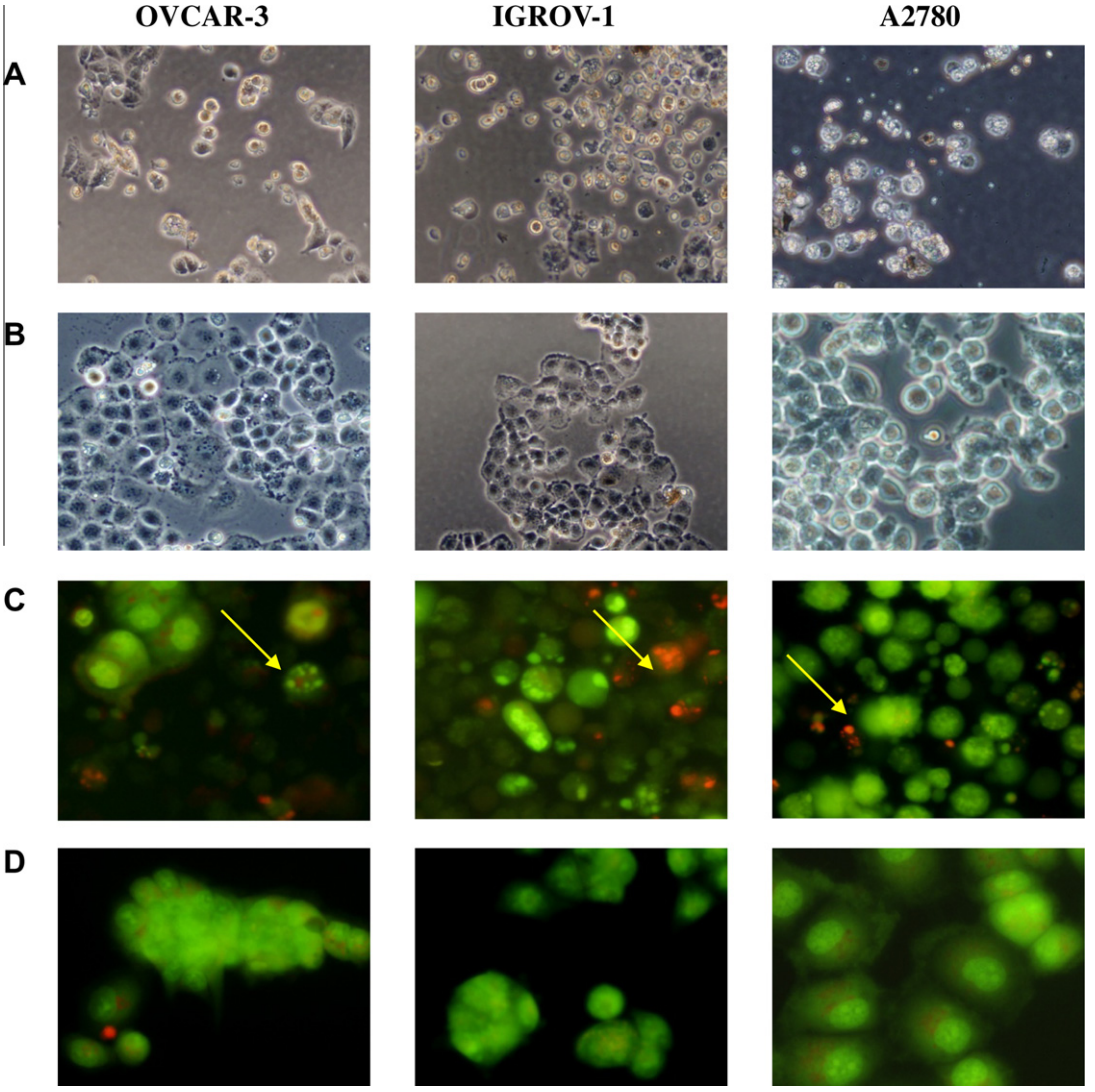

Fig. 4. Morphological changes and apoptosis in OVCAR-3, IGROV-1 and A2780 cells treated with combined MAb C595 (1/2 IC_{50}) and docetaxel (IC_{50}). At 24 h post-treatment (A) treated cells displayed rounding up and detachment from the flask surface, compared to (B) control untreated cells. (C) Condensed and fragmented nuclear chromatin (arrows) characteristic of apoptosis is clearly seen in treated cells using acridine orange/ethidium bromide staining and fluorescence microscopy. (D) Vehicle control cells appeared normal. Magnification 200×. The results are from three independent experiments ($n = 3$).

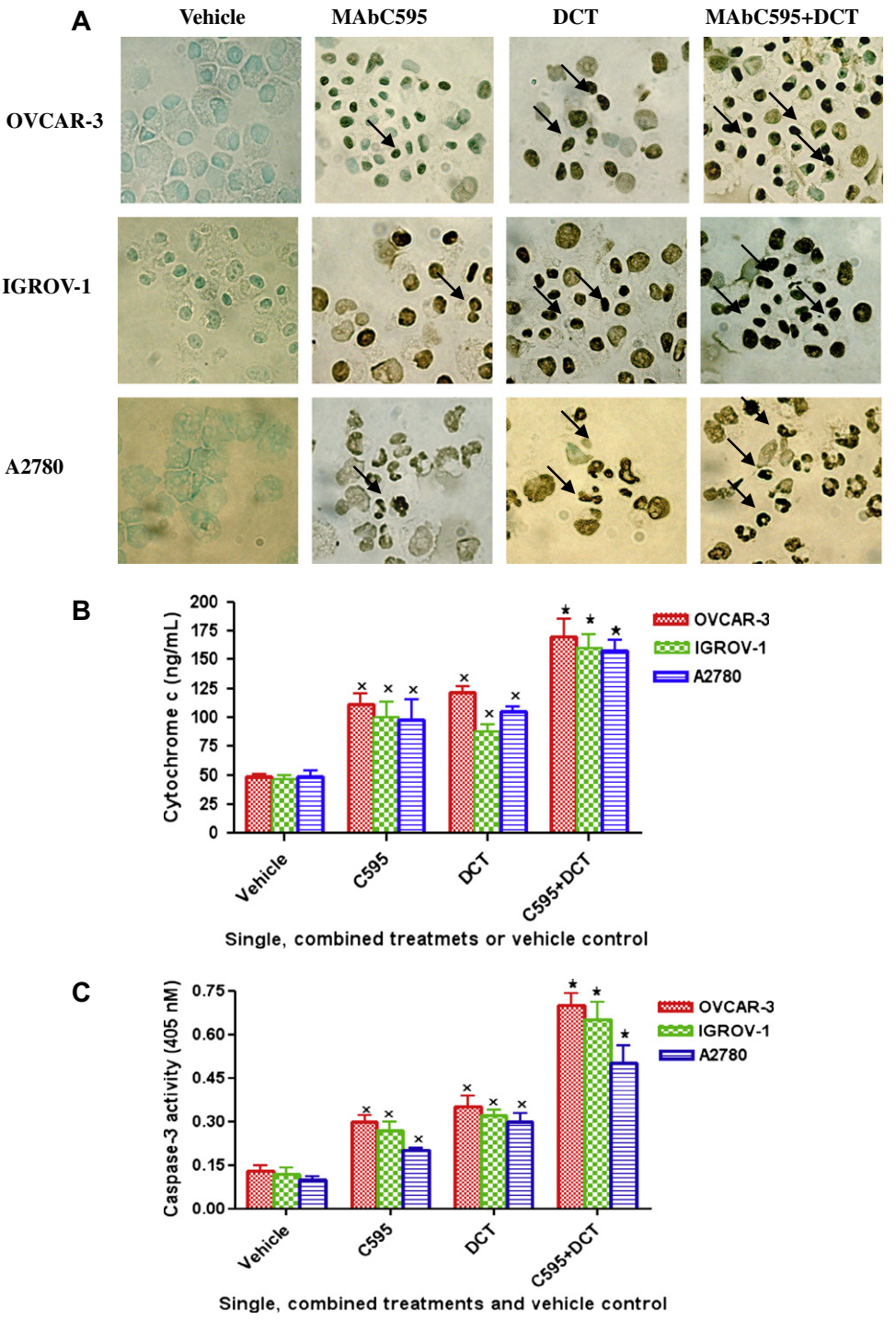

Fig. 5. TUNEL assay, cytochrome *c* and caspase-3 activity after treatment with vehicle control, MAb C595, docetaxel or combined MAb C595 and docetaxel. (A) OVCAR-3, IGROV-1 and A2780 cells were treated with MAb C595 (1/2 IC_{50}), docetaxel (IC_{50}), combined MAb C595 (1/2 IC_{50}) and docetaxel (IC_{50}), or vehicle control. After 48 h, treated cells were fixed and processed for TUNEL assay and visualized by light microscopy. Arrows indicate typical apoptotic cells (dark brown colour) with condensed or fragmented nuclei; control cells show normal morphology for each cell line (Original magnification × 20 for all images). (B) Induction of cytochrome *c* release in OVCAR-3, IGROV-1 and A2780 cells treated with vehicle control, MAb C595, docetaxel or combined MAb C595 and docetaxel. (C) Caspase-3 activity in OVCAR-3, IGROV-1 and A2780 cells treated with vehicle control, MAb C595, docetaxel or combined MAb C595 and docetaxel. Activity was measured using ELISA 48 h post-treatment. (Mean ± SD, $n = 3$. $^{\times}P < 0.05$ compared to vehicle control; and $^{*}P < 0.05$ compared to MAb C595 or docetaxel alone. DCT: docetaxel). The results are from three independent experiments ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rounded, shrunken, and detached) (Fig. 4A), while untreated control cells and those treated with the non-specific control MAb IgG_3 did not exhibit apoptotic morphology (Fig. 4B). The characteristic morphological changes in treated cells were confirmed by acridine orange/ethidium bromide staining, which showed typical features of apoptosis, including nuclear condensation and fragmentation (Fig. 4C). These changes did not occur in untreated control cells (Fig. 4D). Induction of apoptosis following 48 h treatment was further evaluated by TUNEL assay. Representative results are shown in Fig. 5A. Untreated cells showed typical viable cell morphology, MAb C595-treated cells showed some evidence of apoptosis; docetaxel-treated cells showed moderate levels of apoptosis; however many apoptotic cells were seen following combination therapy (Fig. 5A). These observations are consistent with either single or combination treatment inducing apoptosis in EOC cells; low-dose MAb C595 greatly increased the numbers of apoptotic cells.

3.5. Effect of MAb C595 and docetaxel on cytochrome c and caspase-3 activity

Release of cytochrome *c* has been shown to lead to caspase-3 activation and apoptosis. We therefore investigated cytochrome *c* and caspase-3 activity following different treatments. The changes in cytochrome *c* and caspase-3 activity were similar following treatments. At 48 h, compared to the vehicle (100% ethanol and control IgG_3) treated cells, there was a 5–6-fold increase in caspase-3 activity in cells treated with combination therapy and a 2–2.5-fold or 2.5–3-fold increase in single MAb C595 or docetaxel-treated cells, respectively (Fig. 5C). These results suggest that the three EOC cell lines are sensitive to single or combined treatment and that combination treatment induced increased caspase-3 activity, consistent with results for cytochrome *c* release.

OVCAR-3, IGROV-1 and A2780 cell lines showed a 3–4-fold increase in cytochrome *c* activity compared to the vehicle-treated cells following combined treatment for 48 h (Fig. 5B). However, treatment with MAb C595 or docetaxel alone produced only a 2–3-fold or 1.9–2.5-fold increase in cytochrome *c* activity respectively, when compared to the vehicle-treated cells (Fig. 5B). Cytochrome *c* activity was significantly increased with combined treatment compared to either treatment alone ($P < 0.05$). These results suggest that single or combined treatment can induce increased cytochrome *c* activity.

4. Discussion

In the present study, we investigated whether MAb C595, alone or combined with docetaxel, would be more efficient than docetaxel alone in killing EOC cells that express MUC1 antigens. Initially, we demonstrated MUC1 expression on all human primary and metastatic EOC cell lines using MAb C595. MUC1 is an important marker of malignancy and is a target for several immunotherapies currently under investigation [20]. The level of MUC1 expression has been shown to correlate with the degree of breast tumor differentiation, estrogen receptor status, and clinical outcome of breast cancers [21]. Gao et al. [22] recently demonstrated that MUC1 knockdown induced cellular apoptosis concomitant with reduced Bcl-XL and sensitized cells to cisplatin treatment; they also found inhibited tumor growth and metastasis in an orthotopic mouse model of lung cancer by activating apoptosis and inhibiting cell proliferation *in vivo*. Raina et al. [23] have reported that a MUC1 inhibitor (GO-201) that binds to the MUC1 cytoplasmic domain could target the MUC1 oncoprotein and effectively induce human breast cancer cell death *in vitro* and in tumor animal models. Taken together, these data suggest that MAb C595 has the potential to target MUC1-positive cancer cells and increase sensitivity to current chemotherapeutic agents.

The efficacy of anti-MUC1 MAb C595 has been reported in oral squamous cell carcinomas (OSCCs), where MAb C595 induced complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) to OSCC cells. This effect was strongly correlated with MUC1 expression [24]. Furthermore, a humanized anti-MUC1 MAb (huHMFG-1) induced strong ADCC to breast cancer cells and is currently being used in a clinical trial for breast cancer [25]. Our data demonstrated that MAb C595 can inhibit growth of MUC1-positive EOC cells *in vitro* in a dose-dependent manner. The efficacy of cell killing (IC_{50}) was also related to cell surface expression of MUC1 (see Table 2), with no effect in controls (isotope control IgG_3) (data not shown), consistent with MAb C595 specifically targeting MUC1-positive EOC cells.

Docetaxel is now considered the preferred chemotherapeutic agent for EOC [15,26]. The most widely described mechanism by which docetaxel achieves this effect is through its activity as a mitotic spindle poison, disrupting microtubule dynamics and inducing G2/M cell cycle arrest, a downstream effect thought to be related to the phosphorylation of Bcl-2 [27]. Thus, we hypothesized that MAb C595 may increase the sensitivity of EOC cells to docetaxel, as well as being directly cytotoxic to the cells. After addition of low-dose MAb C595 to different concentrations of docetaxel, the IC_{50} was greatly reduced compared to docetaxel alone (Table 2 and Fig. 2). In a parallel study, we compared a docetaxel-sensitive EOC cell line (1A9, parental line) with a docetaxel-resistant EOC cell line (PTX10). After treatment with combined therapy (low-dose MAb C595 and different concentrations of docetaxel), docetaxel-resistant PTX10 cells showed increased sensitivity to docetaxel (unpublished data). Results obtained reveal that the IC_{50} for docetaxel used in combination therapy is much lower than for docetaxel alone. The toxicity of MAbs was also less compared to that of chemotherapeutic agents. This has implications clinically, where combined treatment may reduce the dose of docetaxel, with a better clinical outcome. The enhanced response was observed for both primary and metastatic EOC cell lines, as well as the docetaxel-resistant cell line, indicating that combined treatment (MAb C595 + docetaxel) could be a valid option for patients with localized, metastatic or drug-resistant EOC disease. These results further suggest that MAb C595 can not only directly kill EOC cells but can also sensitize the response of EOC cells to docetaxel.

Colony formation assays provide a more appropriate measure of the long-term effects of potential therapeutic agents, assessing the capacity of cells to retain proliferative potential after treatment, a characteristic that clinically facilitates tumor recurrences in patients. In the present study, we showed that treatment with MAb C595, docetaxel or combined MAb C595 and docetaxel inhibited the colony forming potential of primary and metastatic EOC cell lines; combined treatment was consistently more effective than either agent alone (Fig. 3). Taken together with the MTT results, these observations suggest that MAb C595 may sensitize cells to docetaxel and promote docetaxel-induced suppression of colony formation, although the mechanisms involved are not known.

To gain further insight into the mechanisms of action of MAb C595 especially when combined with docetaxel, we examined induction of cell death and cytochrome *c* and caspase-3 activity. We found a 2–6-fold increase in cytosolic cytochrome *c* and caspase-3 activity for all treatments. Efflux of cytochrome *c* from mitochondria to cytosol to form complexes with Apaf-1 and caspase-9 with subsequent activation of the caspase-3, are critical events in apoptosis [28]. Kolfschoten et al. [29] demonstrated that docetaxel is able to activate caspase-3, and induce Bcl-2 phosphorylation and apoptosis in ovarian cancer cells with a prolonged G2/M arrest. Caspase-3 and mitochondrial release of cytochrome *c* are highly regulated by the Bcl-2 family of proteins, including the death agonists Bax, Bak, Bad and Bcl-Xs, and antagonists Bcl-2, Bclw, Bcl-xL and Mcl-1 [30–32]. In a separate experiment using immunocytochemistry, we found a decrease of Bcl-2-positive EOC cells (24–46%), but marked increase of Bax-positive EOC cells (32–68%) after MAb C595 or docetaxel alone, or combined MAb C595 and docetaxel for 48 h (unpublished data). These results further suggest that it is possible that single or combined treatments may suppress Bcl-2 and translocate Bax to mitochondria, with subsequent mitochondrial release of cytochrome *c* into the cytosol and activation of death effector-caspase-3.

The mechanisms of action for MAb C595 on MUC1-positive EOC cells in the current study remain unclear. It remains to be determined whether cell surface bound MAb C595 can be internalized in cancer cells, and subsequently trafficked intracellularly, as seen for example for MAb J591 [33]. In the present study, we found that MUC1 expression levels were reduced after treatment with MAb C595 alone or MAb C595 combined with docetaxel but not in docetaxel treatment alone in EOC cell lines using immunostaining (data not shown). In our recent *in vivo* animal model study, we further confirmed that the MUC1 expression was reduced after treatment with MAb C595 or MAb C595 combined with docetaxel in OVCAR-3 xenograft tumors (unpublished data). These results suggest the MAb C595 can affect MUC1 expression after treatment. One possible reason explanation may be that MAb C595 binding of MUC1 may either block or stimulate a particular cell membrane molecule (e.g. growth factor receptor) through its cytoplasmic tail, inhibiting tumor growth as seen for example for a MUC1 inhibitory peptide (MIP) [34]. Bitler et al. [34] demonstrated that intracellular MUC1 peptides can act in a dominant-negative fashion, blocking both MUC1/β-catenin and MUC1/EGFR interactions, inducing ligand-dependent reduction of EGFR levels in breast cancer *in vitro* and in an animal model. Another possibility is that in the presence of MAb C595, targeting MUC1-C oligomerization may block nuclear localization of MUC1-C and induce growth arrest and death of EOC cells like a MUC1 inhibitor (GO-201) [23]. This would imply that targeting MUC1 signalling pathways by MAb C595 may reduce cancer proliferation, migration and invasion of metastatic EOC cells. Given that low-dose MAb C595 can increase the effects of docetaxel, it is possible that MAb C595 has an additive effect with docetaxel, in addition to a direct effect on EOC cells. We are currently investigating MAb C595 mechanisms of cell killing in ovarian cancer cell lines.

In summary, we have demonstrated inhibition of EOC cell proliferation by MAb C595 in a dose- and MUC1-dependent manner. Low-dose MAb C595 combined with docetaxel greatly enhanced killing of MUC1-positive EOC cells. This combination therapy may provide a treatment strategy to improve anti-cancer efficacy while reducing the toxicity often seen in patients treated with docetaxel alone. In particular, our results may provide a basis for reducing the dose of docetaxel required, without adversely impacting on treatment efficacy. Additionally, since the mechanisms described are not EOC-specific, this combination may also prove beneficial for other MUC1-positive malignancies commonly treated with docetaxel, including advanced prostate and breast cancers. Taken together, our data indicate that combined MAb C595 and chemotherapy offer opportunities for novel therapeutic strategies in EOC and other cancers.

5. Conflicts of interest

None declared.

Acknowledgments

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