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Interaction between celecoxib and docetaxel or cisplatin in human cell lines of ovarian cancer and colon cancer is independent of COX-2 expression levels

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ABSTRACT

Celecoxib, an inhibitor of cyclooxygenase-2 (COX-2), is being investigated for enhancement of chemotherapy efficacy in cancer clinical trials. We determined whether continuous exposure to celecoxib would increase the antiproliferative effects of a 1-h treatment with docetaxel or cisplatin in four human ovarian cancer cell lines. COX-2 protein could not be detected in these cell lines, because of which three COX-2 positive human colon cancer cell lines were included. Multiple drug effect analysis demonstrated additive to borderline antagonistic effects of celecoxib combined with docetaxel. Combination indices with values of 1.4–2.5 in all cancer cell lines indicated antagonism between celecoxib and cisplatin regardless whether celecoxib preceded cisplatin for 3 h, was added simultaneously or immediately after cisplatin. Apoptotic features measured in COX-2-negative H134 ovarian cancer cells and COX-2-positive WiDr colon cancer cells, such as the activation of caspase-3 and the number of cells in sub-G0 of the cell cycle, induced by docetaxel were increased in the presence of celecoxib, but were abrogated upon addition of celecoxib to cisplatin. Moreover, the G2/M accumulation in cisplatin-treated cells was less pronounced when celecoxib was present. Drugs did not affect p-Akt. Celecoxib upregulated p-ERK1/2 in H134 cells, but not in WiDr cells. Platinum–DNA adduct formation measured in WiDr cells, however, was reduced when celecoxib was combined with cisplatin. Taken together, our data demonstrate clear antagonistic effects when celecoxib is given concurrently with cisplatin, which is independent of COX-2 expression levels.

1. Introduction

The enzyme cyclooxygenase (COX) catalyzes prostaglandin synthesis from arachidonic acid. Two isoforms have been characterized of which COX-1 is a homeostasis protein constitutively expressed in a variety of tissues, while COX-2 expression is regulated by growth factors, cytokines and oncogenes. COX-2 overexpression can be found in both premalignant and malignant lesions. Induction of COX-2 has been shown to promote cell growth, inhibit apoptosis and enhance cell motility and adhesion [1,2]. Classical anticancer agents may upregulate COX-2 mRNA and protein levels in tumor cells, as has been described for cisplatin and taxanes [3–5].

Since COX-2 expression leads to a pro-survival effect, COX-2 inhibitors have been investigated for their potential to enhance chemotherapy efficacy. At first instance, non-steroidal anti-inflammatory drugs (NSAIDs) have been

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employed, but this class of agents also inhibits COX-1 function causing gastrointestinal injury. Thereafter, specific COX-2 inhibitors were developed to avoid side effects of NSAIDs related to COX-1 inhibition [6], such as celecoxib. Celecoxib specifically binds to COX-2, while little or no specific binding to COX-1 was observed [7]. Besides its anti-inflammatory activities, in relevant animal models of cancer celecoxib has been found to prevent colon tumor formation, to inhibit angiogenesis and to potentiate tumor response to radiation [8]. More specifically, celecoxib may enhance antitumor activity when combined with chemotherapy. As examples, celecoxib combined with docetaxel was more effective than docetaxel alone in the growth inhibition of A549 human lung tumors in nude mice [5]. The same combination potentiated apoptosis in human prostate cancer cells and had additive antitumor effects *in vivo* [9]. Combined with oxaliplatin it could enhance human colon cancer cell death *in vitro* [10]. Clinical trials have been conducted or are underway using celecoxib in combination with cytotoxic agents, such as gemcitabine and cisplatin in pancreatic cancer [11] and docetaxel in lung cancer [12]. Thus far, the benefit of celecoxib as an adjunct to chemotherapy regimens has not yet been established.

The most likely route of celecoxib to potentiate the efficacy of chemotherapy is considered via inhibition of COX-2. Recent experiments, however, provide evidence that celecoxib is also able to inhibit human cancer cell growth regardless of the presence of functional COX-2 [13–15]. It is, therefore, hypothesized that celecoxib improves chemotherapy outcome not only via COX-2 inhibition, but also by other, mostly unknown, mechanisms.

The functional activity of COX-2 and the effects of COX-2 inhibition have been mainly studied in colorectal cancer. COX-2 is overexpressed in approximately 90% of patients with colorectal adenocarcinomas [16]. A high level of COX-2 expression was correlated with more advanced stage and larger tumor size and might be related with reduced survival [17]. Various epidemiological studies have indicated that regular and prolonged intake of NSAIDs is associated with a 40–50% reduction in colorectal cancer incidence, most probably due to reduced prostaglandin synthesis as a consequence of decreased COX-2 activity [18]. Moreover, in experimental human colon cancer COX-2 inhibition potentiated the efficacy of cytotoxic agents, such as oxaliplatin, irinotecan and curcumin [10,19,20].

Although not frequently overexpressed, COX-2 can be detected in human ovarian tumors [21–23]. Expression of COX-2 was found to be associated with a significantly reduced median survival time [22] and levels were significantly higher in non-responding patients than patients responding to chemotherapy [23]. The well-known NSAID acetyl salicylic acid (aspirin) has been shown to inhibit OVCAR-3 human ovarian cancer cell growth *in vitro* [24]. Other studies in human ovarian cancer cell lines treated with the specific COX-2 inhibitor NS398, however, led to ambiguous effects; both induction of G0/G1 cell cycle arrest [25] and impairment of paclitaxel-induced apoptosis [26] have been reported. The exact role of COX-2 as a target for treatment in ovarian cancer remains to be elucidated.

In this study we investigated the possible synergism of celecoxib when combined with docetaxel or cisplatin in human ovarian cancer cell lines. Since these cell lines did not clearly express COX-2 protein levels, human colon cancer cell lines that contain COX-2 were included as control cell lines. We detected increased cytotoxic effects and apoptotic features when celecoxib was combined with docetaxel regardless of the expression levels of COX-2. Of interest, we consistently calculated antagonistic effects upon the combination of celecoxib with cisplatin, both in ovarian cancer as well as in colon cancer cells. We, therefore, investigated the mechanism of the antagonism between celecoxib and cisplatin. Evidence was found that celecoxib-treated cells were protected against the cisplatin-induced G2/M arrest as well as against platinum–DNA adduct formation in which p21 may play a role.

2. Materials and methods

2.1. Cell culture

Four human ovarian cancer cell lines: A2780, H134, OVCAR-3, IGROV-1 [27,28] and three human colon cancer cell lines: WiDr, HT29, SW1398 [29,30] were used for the experiments. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS, Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Bio-Whittaker, Verviers, Belgium) at 37 °C in 5% CO_2.

2.2. In vitro antiproliferative assay

The antiproliferative effects of docetaxel (kindly provided by Sanofi-Aventis, Antony, France), cisplatin (Bristol-Myers Squibb, Woerden, the Netherlands), celecoxib (kindly provided by Pfizer, Barceloneta, Puerto Rico) and combinations of docetaxel–celecoxib or cisplatin–celecoxib were measured in a 96-h antiproliferative assay. Cells were plated in quadruplicate in culture medium in 96-well plates at 3000 cells per well. After 24 h, cells were exposed to drug concentration ranges of docetaxel (1 h), cisplatin (1 h) or celecoxib (96 h). Docetaxel and cisplatin treatments were followed by a wash step and addition of fresh culture medium. Besides assessment of the antiproliferative effects of individual drugs, three different experimental designs were used: (1) a 3-h preincubation with celecoxib followed by a 1-h simultaneous exposure to docetaxel or cisplatin and a subsequent 96-h exposure to celecoxib, (2) a 1-h simultaneous exposure to docetaxel or cisplatin plus celecoxib and a subsequent 96-h exposure to celecoxib, (3) a 1-h exposure to docetaxel or cisplatin followed by a 96-h celecoxib exposure. Constant drug concentration ratios were applied in which docetaxel molar concentrations were 1000-fold lower than the celecoxib concentrations, while cisplatin and celecoxib concentrations were added in equal molar ratios.

The number of viable cells was determined by addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma–Aldrich, Zwijndrecht, the Netherlands). The extinction of the formazan product was measured at 540 nm on a Multiscan plate reader (Thermo Biosciences, Breda, the Netherlands). Results were expressed in IC50

values, being the drug concentration responsible for 50% cell growth inhibition as compared to control cell growth.

2.3. Multiple drug effect analysis

All cell lines were exposed to combinations of docetaxel–celecoxib and cisplatin–celecoxib in the same way as described above for the antiproliferative assay. Concentrations of the drugs were added in constant molar ratios in quadruplicate. Using the MTT assay dose–effect curves were generated to obtain the IC50, IC70 and IC90 values of the combinations and to perform multiple drug effect analysis based on the method of Chou and Talalay [31]. The utilization of this method for the combination of cytotoxic agents has been described by our group [32]. Briefly, each fraction affected (FA) was calculated by comparing the absorbance values of drug-treated cell wells to the absorbance of control cell wells. A FA of 0.3 means for instance a reduction in growth of 30%. For each level of antiproliferative effect expressed as a particular IC value the combination index (CI) was calculated using the formula

$$\mathrm{CI} = \left[\frac{(D)_1}{(D_{1\text{-FA}})_1}\right] + \left[\frac{(D)_2}{(D_{1\text{-FA}})_2}\right] + \left[\frac{(D)_1(D)_2}{(D_{1\text{-FA}})_1(D_{1\text{-FA}})_2}\right]$$

In which $(D)_1$ and $(D)_2$ are the doses of the drugs in a fixed ratio, while $(D_{1\text{-FA}})_1$ and $(D_{1\text{-FA}})_2$ are the doses of individual drugs resulting in the effect 1-FA. Mean CI values of concentrations resulting in 50% growth inhibition (CI50), 70% growth inhibition (CI70) and 90% growth inhibition (CI90) were calculated to determine the presence of a drug interaction in the cell lines. $\mathrm{CI} < 0.9$ indicates synergism, $0.9 < \mathrm{CI} < 1.1$ indicates addition and $\mathrm{CI} > 1.1$ indicates antagonism.

2.4. Caspase-3 activity assay

The activity of caspase-3 was measured as described before [28]. In short, cells were plated in Petri dishes (one dish per treatment type) and grown to 75% confluence. H134 and WiDr cells were pretreated or not with celecoxib (3 h) followed by another hour with exposure to docetaxel or cisplatin. After washing, fresh culture medium was added with or without celecoxib. After 48 h, adherent and non-adherent cells were recovered and washed with phosphate-buffered saline (PBS, Bio-Whittaker). The pellet was resuspended in lysis buffer (10 mM Tris–HCl pH 7.6, 150 nM NaCl, 5 mM EDTA, 1% Triton X-100). After three freeze–thaw cycles samples were centrifuged at 14,000 rpm for 10 min. Protein concentrations were determined by the Bradford protein assay [33].

In a 96-well plate duplicate samples of 10 μg protein in a total volume of 20 μl were added to 80 μl of reaction buffer (100 mM HEPES pH 7.3, 10% sucrose, 0.1% Nonidet-P40, 10 mM DTT) complemented with 25 μM of DEVD-7-amino-4-methylcoumarin (DEVD-AMC; Sigma–Aldrich), which is a substrate of active caspase-3. After 60 min, the fluorescence generated by the cleaved substrate was measured at λ^{exc} 360 nm and λ^{em} 446 nm in a spectrafluor multiplate reader (Tecan, Gorinchem, the Netherlands). To calculate caspase-3 activity in the experimental samples an extrapolation curve was constructed using known concentrations of free AMC.

2.5. Cell cycle analysis

Cell cycle analysis and the measurement of the percentage of apoptotic cells were assessed by flow cytometry, using the Nicoletti protocol [34]. Cells were seeded in T25 flasks (one flask per treatment type) and grown to 75% confluence. Cells were pretreated or not with celecoxib for 3 h followed by another hour with exposure to docetaxel or cisplatin. After washing, fresh culture medium was added containing celecoxib or not. After 48 h adherent cells were trypsinized and collected together with the medium containing non-adherent cells. After washing with PBS/0.5% BSA and centrifugation (1500 rpm, 10 min) 5×10^5 cells were collected in FACS tubes. After centrifugation supernatant was removed and cells were resuspended in 400 μl Nicoletti buffer [50 μg/ml propidium iodide (PI; Sigma–Aldrich), 0.1% sodium citrate, 0.1% Triton X-100 and 1 mg/ml RNase A (Roche, Woerden, the Netherlands)]. Samples were incubated for 1 h at 4 °C followed by determination of the cell cycle distribution and the percentage of apoptotic cells with a FACScan flow cytometer (Calibur; Becton Dickinson, Alphen aan de Rijn, the Netherlands). Cellquest software (Becton Dickinson) was used for analysis.

2.6. Western blot

Cells were grown to 80% confluence in Petri dishes. For the analysis of baseline COX-2 protein expression all individual cell lines cells were collected at this point, while samples for analysis of p-Akt, p-ERK1/2 and associated COX-2 expression were pre-incubated for 3 h with celecoxib followed by another hour with exposure to docetaxel or cisplatin. After washing and another 24-h incubation period in the presence or the absence of celecoxib adherent and non-adherent cells were collected, washed with PBS and resuspended in lysis buffer (1% sodium desoxycholate, 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% SDS and 1% NP-40) supplemented with protease inhibitors [1 mM phenylmethylsulphonyl (Merck, Amsterdam, the Netherlands), 10 μM leupeptin (Sigma–Aldrich), 0.5 mM trypsin inhibitor (Sigma–Aldrich)] and phosphatase inhibitors [0.5 M sodiumorthovanedate (Sigma–Aldrich), 1 M sodiumfluoride (Merck)]. Protein concentrations were determined by the Bradford protein assay.

Protein samples of 50–100 μg were subjected to 10% polyacrylamide gel electrophoresis (130 V, 2 h). The separated proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Etten-Leur, the Netherlands) by electrotransfer (400 mA, 2 h). The blots were blocked with 10% milk (Protifar; Nutricia, Zoetermeer, the Netherlands) in Tris-buffered saline–Tween 20 (TBS–T: 10 mM Tris, pH 8.0, 150 mM NaCl, 0.0025% Tween 20) at room temperature for 1 h and incubated overnight at 4 °C with specific antibodies against COX-2 (mouse polyclonal C-20; SantaCruz, Heerhugowaard, the Netherlands), p-Akt (rabbit polyclonal; Cell Signaling Technologies, Leiden, the Netherlands), total Akt (rabbit polyclonal; Cell Signaling Technologies), p-ERK1/2 (rabbit polyclonal; Cell Signaling Technologies), total ERK1/2 (rabbit polyclonal; Cell Signaling Technologies), p53 (mouse monoclonal Pab 240; SantaCruz), p21/WAF1 (mouse monoclonal Ab-1; Oncogene, Boston, MA) or β-actin (mouse monoclonal; Sigma–Aldrich) diluted in 5% BSA/TBS–T. After the membrane was washed

three times with TBS–T, it was incubated with 5% milk/TBS–T, containing horseradish peroxidase (HRP)-linked anti-mouse (Dako, Amsterdam, the Netherlands) or anti-rabbit (Cell Signaling Technologies) IgG secondary antibody, for 1 h at room temperature. After three TBS–T washing steps of 15 min protein was visualized on photography film (Pharmacia, Uppsala, Sweden) by enhanced chemiluminescence.

2.7. Platinum–DNA adducts

Platinum–DNA (Pt–DNA) adduct formation after treatment with cisplatin and/or celecoxib was analyzed as described previously [35]. WiDr cells were grown to 80% confluence and pretreated or not with celecoxib for 3 h followed by another hour with exposure to cisplatin. After washing and replacement with medium, with or without celecoxib, samples were collected immediately after cisplatin treatment (= 0 h) and at 2 h and 4 h. Samples containing 5×10^6 adherent and non-adherent cells were washed twice with ice-cold PBS. DNA was isolated using the QIAamp® DNA mini kit (Qiagen, Venlo, the Netherlands) according to the procedure supplied by the manufacturer. DNA content was estimated by measuring optical density at 260 nm using a NanoDrop® spectrophotometer (Nanodrop Technologies, Wilmington, DE). A solution of 0.165 M sodium chloride was added to the dissolved DNA to a total volume of 0.1 ml. A calibration curve was made using different concentrations of cisplatin (0–1.5 μM). Platinum content in Pt-containing standards and in DNA isolations of samples were measured using an atomic absorption spectrometer (SpectrAA-300; Varian, Middelburg, the Netherlands).

2.8. Statistics

The differences in the effects between docetaxel, cisplatin and these drugs combined with celecoxib on cell proliferation, caspase-3 activity and cell cycle distribution were statistically analyzed by one way ANOVA followed by the Bonferroni adjustment, using SPSS software (SPSS Inc. Chicago, IL). The level of significance was set at $p < 0.05$.

3. Results

3.1. Variable COX-2 expression levels in human tumor cell lines

For detection of COX-2 100 μg of protein of human ovarian cancer cell lines and human colon cancer cell lines was subjected to Western blot (Fig. 1). The human colon cancer cell lines WiDr, HT29 and SW1398 all expressed COX-2 protein under standard culture conditions, while no COX-2 protein could be visualized in the human ovarian cancer cell lines A2780, H134, OVCAR-3 and IGROV-1.

3.2. Additive to slightly antagonistic antiproliferative effects of celecoxib and docetaxel regardless of COX-2 expression levels

The antiproliferative effects of celecoxib, docetaxel and the combination of these two agents in human ovarian cancer and human colon cancer cell lines expressed as IC50 values are shown in the upper panel of Table 1. Sensitivity to celecoxib or docetaxel varied among the cell lines. No correlation between COX-2 expression levels and sensitivity to celecoxib was observed. As a group, tumor cells treated with docetaxel in combination with celecoxib required significantly less docetaxel to achieve IC50 as compared to IC50 concentrations of docetaxel alone ($p < 0.001$). When H134 was excluded from the calculations the difference between the groups was still significant ($p < 0.05$).

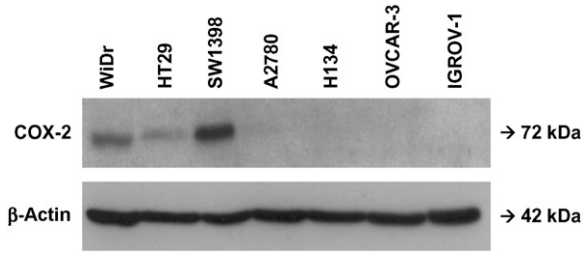

Fig. 1 – Basal protein levels of COX-2 in the panel of human ovarian cancer cell lines (A2780, H134, OVCAR-3, IGROV-1) and human colon cancer cell lines (WiDr, HT29, SW1398). Cells lysates were prepared from tissue culture and 100 μg of protein was analyzed by Western blot. The figure is representative for three separate observations. β-Actin is a loading control.

Three different celecoxib administration regimens in combination with docetaxel were used with constant drug ratios to determine the presence of a drug interaction in the cell lines. The combination of docetaxel and celecoxib (Table 2) proved to be additive in most cell lines analyzed, although borderline antagonism was calculated in A2780 and SW1398.

3.3. Antagonistic antiproliferative effects of celecoxib in combination with cisplatin regardless of COX-2 expression levels

The antiproliferative effects of celecoxib, cisplatin and their combination in human ovarian cancer and colon cancer cell lines expressed as IC50 values are shown in the lower panel of Table 1. As mentioned, celecoxib sensitivity did not correlate with COX-2 expression levels. Cisplatin sensitivity also varied among the cell lines. When combined with celecoxib, a significantly higher concentration of cisplatin was required to reach 50% growth inhibition in 5 out of 7 cell lines ($p < 0.05$). Multiple drug effect analysis confirmed the presence of antagonism between cisplatin and celecoxib independent of COX-2 expression levels. In all cell lines the CI values exceeded 1.4 for all three-treatment regimens and at each percentage of growth inhibition (Table 2).

Further investigation on the mechanism underlying the antagonism between celecoxib and cisplatin was carried out in the COX-2 negative ovarian cancer cell line H134 and the COX-2 positive colon cancer cell line WiDr. In the experiments docetaxel was included in the presence or absence of celecoxib for reasons of comparison. The relevant CI50, CI70 and CI90 values of the multiple drug effect analyses in these particular

Table 1 – Sensitivity of human ovarian cancer cell lines and human colon cancer cell lines, expressed as IC50 values[a], to a 1-h exposure of docetaxel or cisplatin (followed by a 96-h drug-free period) or to a 96-h exposure to celecoxib or the combination as determined by the MTT assay

Cell line	CXB alone μM ± S.D.	CXB in combination μM ± S.D.[b]	DOC alone nM ± S.D.	DOC in combination nM ± S.D.[b]
H134	36.9 ± 10.4	22.4 ± 9.4	64.3 ± 26.3	22.4 ± 9.4***
A2780	18.1 ± 7.4	12.1 ± 2.5	13.6 ± 4.4	12.1 ± 2.5
IGROV-1	27.5 ± 9.2	12.6 ± 5.7	17.5 ± 6.2	12.6 ± 5.7
OVCAR-3	12.1 ± 7.8	6.6 ± 2.3	8.5 ± 2.8	6.6 ± 2.3
WiDr	21.4 ± 8.2	6.9 ± 1.7	7.2 ± 1.6	6.9 ± 1.7
HT29	30.4 ± 4.3	8.3 ± 1.4	8.4 ± 2.1	8.3 ± 1.4
SW1398	33.8 ± 5.7	9.5 ± 4.1	10.7 ± 5.0	9.5 ± 4.1
Cell line	**CXB alone μM ± S.D.**	**CXB in combination μM ± S.D.[b]**	**CIS alone μM ± S.D.**	**CIS in combination μM ± S.D.[b]**
H134	36.9 ± 10.4	35.3 ± 9.7	15.5 ± 3.0	35.3 ± 9.7***
A2780	18.1 ± 7.4	23.6 ± 8.4	9.6 ± 4.7	23.6 ± 8.4**
IGROV-1	27.5 ± 9.2	28.2 ± 17.2	13.6 ± 10.3	28.2 ± 17.2*
OVCAR-3	12.1 ± 7.8	9.1 ± 6.2	7.3 ± 6.7	9.1 ± 6.2
WiDr	21.4 ± 8.2	54.1 ± 12.8	25.9 ± 8.6	54.1 ± 12.8**
HT29	30.4 ± 4.3	50.2 ± 1.1	25.7 ± 3.8	50.2 ± 1.1***
SW1398	33.8 ± 5.7	60.5 ± 15.6	44.5 ± 14.1	60.5 ± 15.6

CXB: celecoxib, DOC: docetaxel, CIS: cisplatin; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

[a] IC50, drug concentration resulting in 50% cell growth inhibition when compared to control cell growth; results are expressed as mean values ± S.D. of at least three separate experiments.

[b] Concentration of drug required in which the combination resulted in 50% cell growth inhibition; celecoxib exposure was 96 h, while docetaxel and cisplatin exposure was 1 h preceded by celecoxib for 3 h; constant molar drug ratios were applied in which celecoxib:docetaxel was 1000:1 and celecoxib:cisplatin was 1:1.

cell lines are illustrated in Fig. 2. In the next experiments as described below we made use of drug concentrations inducing 50% cell growth inhibition when combined, in which celecoxib preceded docetaxel or cisplatin with 3 h, and of the individual drugs at the same concentrations as in that combination (Table 1, drug concentrations required in the combination resulting in 50% cell growth inhibition).

3.4. *Celecoxib inhibits cisplatin-induced apoptosis*

Induction of apoptosis after drug exposure was analyzed in the caspase-3 activity assay and by calculation of the sub-G0 fraction of the cell population by FACS at 48 h after cells were treated with either docetaxel (1 h), cisplatin (1 h), celecoxib (continuous) alone or celecoxib in combination with docetaxel or cisplatin. The sub-G0 fraction was 3.3–4.0% in control H134 cells and 2.5–3.3% in control WiDr cells.

Docetaxel clearly induced apoptosis in both H134 and WiDr cells, while celecoxib potentiated pro-apoptotic features (Fig. 3A and B). In H134 cells the ratio of sub-G0 docetaxel-treated cells versus sub-G0 control cells significantly increased 1.75-fold ($p < 0.05$) in the presence of celecoxib. The same 1.7-fold increase was observed in caspase-3 activation in H134 cells. In WiDr cells the ratio of

Table 2 – Interaction between docetaxel or cisplatin in combination with celecoxib in human ovarian cancer cell lines and human colon cancer cell lines as calculated by multiple drug effect analysis

Cell line	Docetaxel CI[a]			Cisplatin CI[a]		
	Pre[b]	Sim[c]	Post[d]	Pre[b]	Sim[c]	Post[d]
H134	1.04 ± 0.07	0.89 ± 0.04	0.88 ± 0.05	2.39 ± 0.16	1.99 ± 0.14	1.77 ± 0.11
A2780	1.21 ± 0.13	1.29 ± 0.06	1.30 ± 0.06	2.18 ± 0.04	2.49 ± 0.18	2.03 ± 0.14
IGROV-1	1.11 ± 0.29	0.97 ± 0.13	1.23 ± 0.12	1.98 ± 0.14	2.20 ± 0.16	2.11 ± 0.16
OVCAR-3	0.93 ± 0.07	0.89 ± 0.11	0.92 ± 0.11	2.19 ± 0.50	2.07 ± 0.34	2.42 ± 0.53
WiDr	1.13 ± 0.05	1.19 ± 0.07	1.10 ± 0.04	1.88 ± 0.08	2.01 ± 0.11	2.36 ± 0.10
HT29	1.16 ± 0.04	1.19 ± 0.08	1.24 ± 0.05	1.64 ± 0.08	1.66 ± 0.09	1.77 ± 0.08
SW1398	1.18 ± 0.06	1.21 ± 0.03	1.22 ± 0.05	1.43 ± 0.12	1.48 ± 0.13	1.65 ± 0.09

[a] Values represent the combination indices (CI) of the fractions affected (FA) of 50%, 70% and 90% by the combination of docetaxel or cisplatin with celecoxib. Mean values ± S.E.M. are shown of at least three separate experiments. CI < 0.9 indicates synergism, 0.9 < CI < 1.1 indicates addition and CI > 1.1 indicates antagonism.

[b] Celecoxib continuously for 96 h and added 3 h before the 1-h exposure to docetaxel or cisplatin.

[c] Celecoxib continuously for 96 h and added simultaneously at the time of the 1-h exposure to docetaxel or cisplatin.

[d] Celecoxib continuously for 96 h, but added after the 1-h exposure to docetaxel or cisplatin.

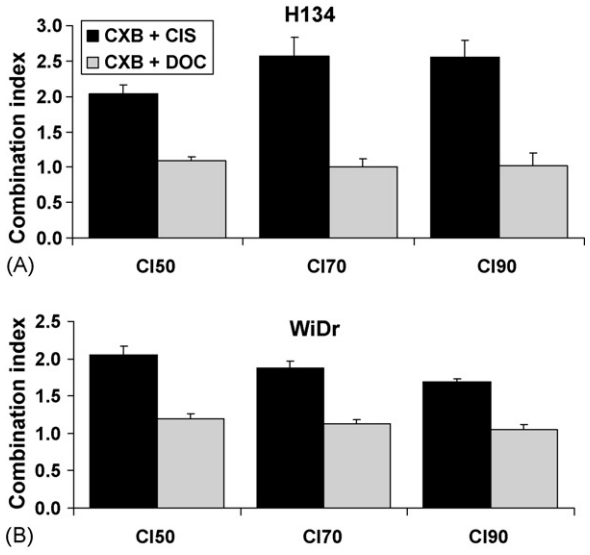

Fig. 2 – Mean values of the combination indices (CI) with affected fractions of 50% (CI50), 70% (CI70) and 90% (CI90) when docetaxel (DOC) or cisplatin (CIS) was combined with celecoxib (CXB) in constant ratios in H134 cells (A) and WiDr cells (B). Cells were treated with either docetaxel (1 h), cisplatin (1 h), celecoxib (continuous for 96 h) alone or celecoxib in combination with docetaxel or cisplatin (celecoxib preceded docetaxel or cisplatin exposure for a period of 3 h). Results of at least three separate experiments are shown. CI < 0.9 indicates synergism, 0.9 < CI < 1.1 indicates addition and CI > 1.1 indicates antagonism. Bars, S.D.

sub-G0 docetaxel-treated cells increased 1.4-fold in the presence of celecoxib, although the potentiating effect of celecoxib on the slight elevation of docetaxel-induced caspase-3 activity was less apparent.

Upon cisplatin treatment both activation of caspase-3 and the number of cells in sub-G0 increased in H134 and WiDr cells (Fig. 3C and D). When combined with celecoxib caspase-3 activation levels dropped in comparison with the levels in cells treated with cisplatin alone. In H134 cells the decrease was 1.5-fold. In WiDr cells this decrease was 2.5-fold and significant ($p = 0.001$). Moreover, in H134 cells the sub-G0 fraction also decreased 1.6-fold when celecoxib was added. In WiDr cells the cisplatin-induced sub-G0 fraction was higher in the presence of celecoxib, but the number of sub-G0 cells remained equal to that induced by celecoxib alone.

3.5. *Cisplatin-induced G2/M arrest is abrogated upon addition of celecoxib*

FACS analysis enabled us to also determine the cell cycle distribution 48 h after treatment. Celecoxib alone did not change the cell cycle distribution of H134 and WiDr cells. Moreover, neither docetaxel nor the combination of docetaxel with celecoxib was of influence on the cell cycle (Fig. 4A and B).

Of interest, cisplatin induced a G2/M accumulation in both H134 cells and WiDr cells (Fig. 4C and D). This G2/M arrest, however, was less pronounced when cisplatin was combined with celecoxib. In H134 the value of 66.5 ± 3.6% (mean ± S.D.)

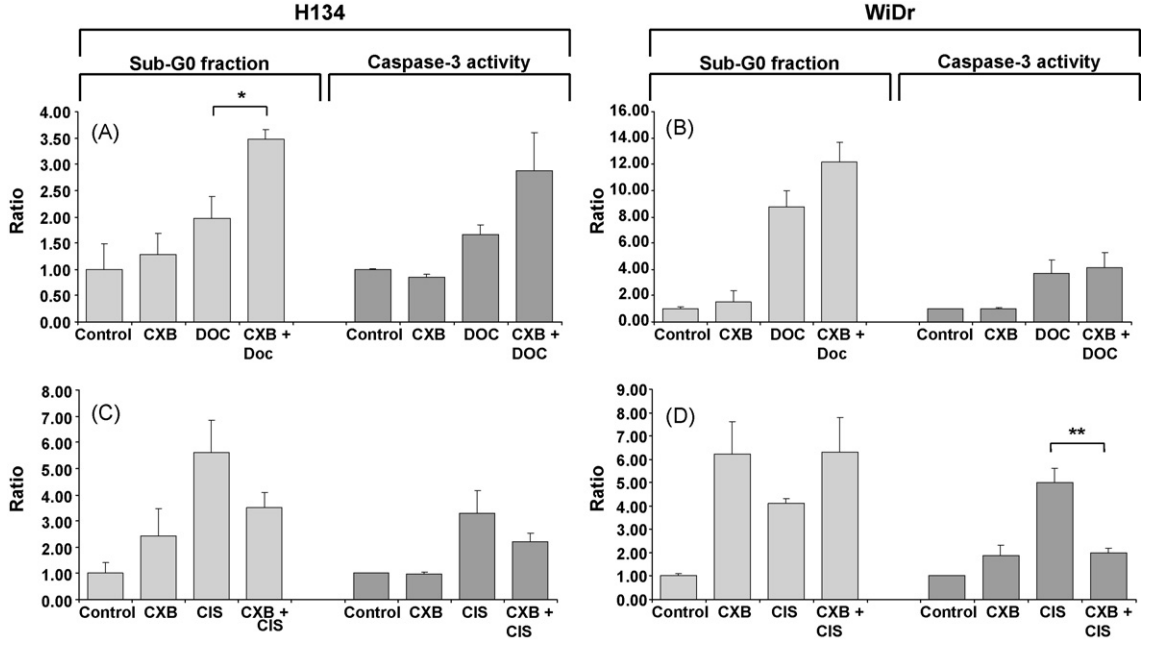

Fig. 3 – Apoptosis induction in H134 cells (A and C) and WiDr cells (B and D) after treatment with docetaxel (DOC; A and B) or cisplatin (CIS; C and D) alone or in combination with celecoxib (CXB). The left hand side of the individual figures shows the ratios of the sub-G0 fraction of treated cells as compared to that of control cells (set at 1.00) analyzed by FACS. The right hand side depicts the ratios of caspase-3 activity in cells after treatment as compared to that in control cells (set at 1.00). In both experiments cells were grown to 75% confluence and pretreated or not for 3 h with celecoxib prior to simultaneous treatment with docetaxel or cisplatin for 1 h. After washing, cells were exposed or not to celecoxib for an additional 48 h. Mean values are the results of three separate experiments. Bars, S.D.; $^{*}p < 0.05$; $^{**}p = 0.001$.

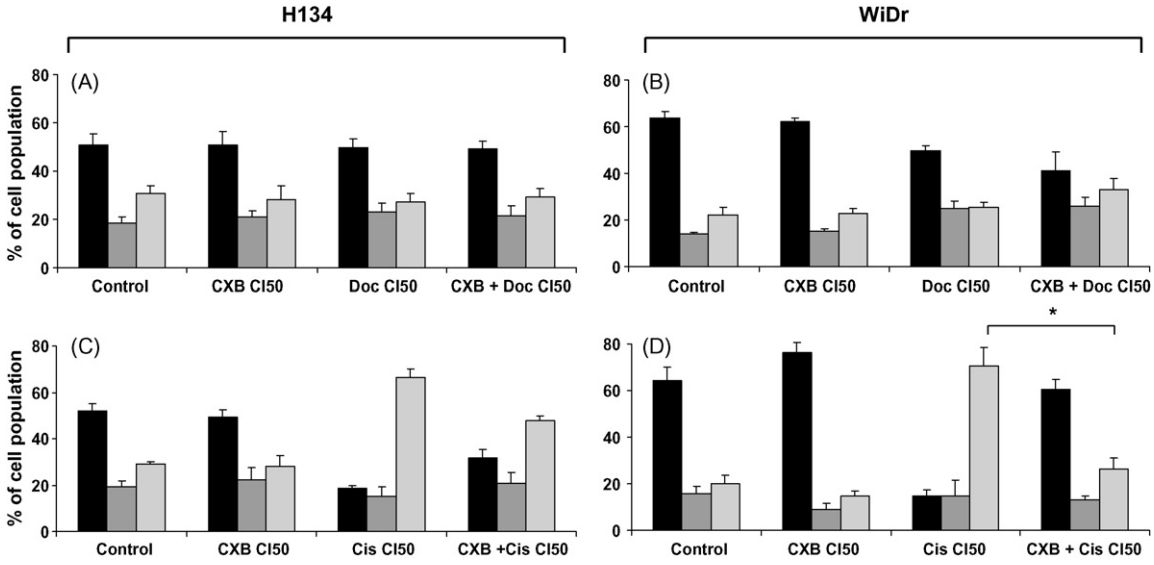

Fig. 4 – Cell cycle distribution in H134 (A and C) and WiDr (B and D) cells treated or not with docetaxel (Doc; A and B) or cisplatin (Cis; C and D) either alone or in combination with celecoxib (CXB). Cells were grown to 75% confluence and pretreated or not for 3 h with celecoxib prior to simultaneous treatment with docetaxel or cisplatin for 1 h. After washing, cells were exposed or not to celecoxib for an additional 48 h. Thereafter, samples were collected and analyzed by FACS. Mean percentages are shown of three separate experiments. Bars, S.D.; $^{*}p < 0.05$.

decreased to 47.7 ± 2.4% and the value in WiDr of 70.5 ± 7.8% significantly decreased to 26.2 ± 4.9% ($p < 0.05$).

3.6. *Effects of drugs on COX-2, p-Akt and p-ERK1/2 expression in H134 and WiDr cells do not explain antagonism between celecoxib and cisplatin*

The protein expression levels of COX-2, p-Akt and p-ERK1/2 in H134 and WiDr cells as visualized in Fig. 5 were investigated under similar conditions, but at earlier time-points as the above-mentioned experiments. Total protein levels of ERK1/2 and Akt remained unchanged after each treatment type (not shown).

The expression of COX-2 protein can be induced in certain tumor cells upon exposure to cytotoxic agents. In H134 cells, however, no COX-2 protein was detected before or after treatment with the various drugs (Fig. 5A and C). In WiDr cells treatment with cisplatin increased COX-2 protein expression levels, but expression of the protein was not affected by celecoxib or by docetaxel (Fig. 5B and D).

Inhibition of 3-phosphoinositide-dependent protein kinase-1 (PDK-1)/Akt signaling has been found to be a COX-2-independent consequence of celecoxib treatment in human prostate cancer cells [36]. In our experiments the levels of p-Akt were not affected by docetaxel, cisplatin, or celecoxib treatment, either alone or in combination.

Besides p-Akt we also investigated the effects of drugs on p-ERK1/2. In WiDr cells the levels of p-ERK1/2 were not affected (Fig. 5B and D). In the COX-2-negative cell line H134, however, the levels of p-ERK1/2 increased upon treatment with celecoxib. Increased levels of p-ERK1/2 were also evident in H134 cells after 4 h and 6 h upon treatment with docetaxel or cisplatin and levels diminished at 8 h.

3.7. *Celecoxib hampers the formation of cisplatin-induced Pt–DNA adducts and upregulates p21*

Formation of Pt–DNA adducts is the mechanism of cisplatin DNA damage. We selected WiDr cells to analyze the formation of adducts by atomic absorption spectrophotometry in samples obtained immediately at the end of the 1-h cisplatin treatment in the presence or absence of celecoxib as well as 2 h and 4 h thereafter (Fig. 6). Upon cisplatin treatment alone the concentration of Pt–DNA adducts was highest immediately after drug exposure. At this time-point Pt level was 0.13 pmol/μg DNA and this concentration was set at 100%. Pt–DNA adduct concentration was approximately twofold lower immediately after exposure to cisplatin when combined with celecoxib. The decrease in Pt–DNA adducts after withdrawal of cisplatin was more pronounced in the presence of celecoxib.

Celecoxib can induce expression of p21 [13], a key molecule in cell cycle arrest which is associated with facilitated repair of DNA damage [37]. In WiDr cells, we measured p21 levels 48 h after cells were treated with either docetaxel (1 h), cisplatin (1 h), celecoxib (continuous) alone or celecoxib in combination with docetaxel or cisplatin (Supplementary Fig. 1 online). The p53 protein was not clearly affected. Celecoxib induced the expression of p21, which was more pronounced upon combination with docetaxel. Of interest, cisplatin did not increase the p21 basal level or affect the p21 level already upregulated by celecoxib.

4. Discussion

In four human ovarian cancer cell lines we investigated the possible potentiating antitumor effect of celecoxib added to

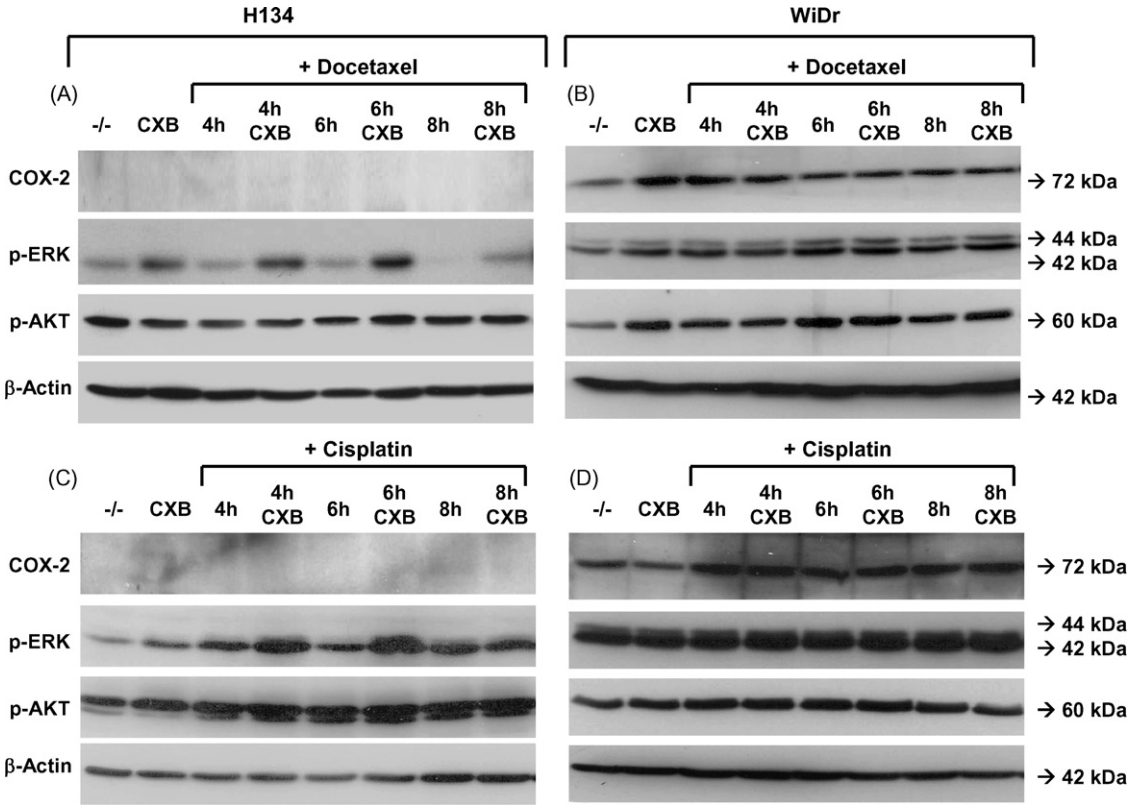

Fig. 5 – Protein levels of COX-2, phosphorylated ERK1/2 and phosphorylated Akt in H134 (A and C) and WiDr (B and D) cells after a 1-h treatment with docetaxel (A and B) or cisplatin (C and D) combined or not with celecoxib (CXB; celecoxib preceded docetaxel or cisplatin exposure for a period of 3 h). Samples were collected at baseline and at time-points 4 h, 6 h and 8 h after the end of docetaxel or cisplatin exposure in the presence or absence of celecoxib. Samples incubated with only celecoxib were obtained 3 h after celecoxib administration. Protein samples (50 μg) were analyzed by Western blot. Representative results of at least three separate experiments are shown.

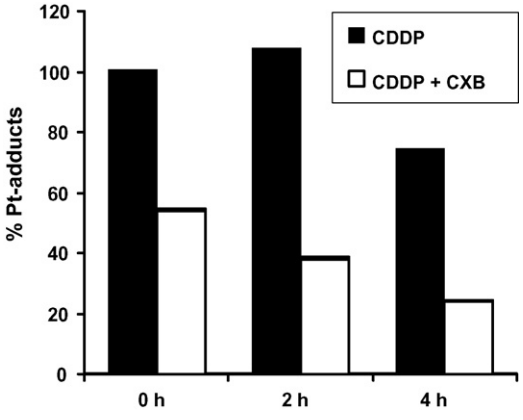

Fig. 6 – Pt-adducts in DNA of WiDr cells after a 1-h treatment with cisplatin (CDDP) alone or in combination with celecoxib (CXB; 3 h pre-incubation and present during the course of the experiment). Samples were collected immediately at the end of the 1-h cisplatin exposure (= 0 h) and at 2 h and 4 h thereafter. Concentrations of Pt-adducts in samples treated with cisplatin for 1 h are set at 100%. Representative results of one of two experiments are shown.

docetaxel or cisplatin, being drugs in use for the treatment of this disease. Since our cell lines did not express detectable levels of COX-2, we included three COX-2-positive human colon cancer cell lines in our experiments. Independent of COX-2 expression levels we calculated additive to slightly antagonistic effects for celecoxib added to docetaxel, but consistent antagonism when added to cisplatin. These effects were measured regardless whether celecoxib preceded these drugs, was added simultaneously or followed the drugs. The antagonism between celecoxib and cisplatin was accompanied with reduced caspase-3 activity, a diminished number of sub-G0 cells, abrogation of cisplatin-induced G2/M accumulation, the formation of less Pt–DNA adducts and celecoxib-induced upregulation of p21.

Drug exposure periods of 1 h for docetaxel and cisplatin were selected, because in patients these anticancer agents are given by short-term infusions. In our experiments, applied docetaxel concentrations were well below and concentrations of cisplatin were in range of peak plasma concentrations that are generally reached in patients. The peak concentration for docetaxel is approximately 4 μM upon infusion of 75–100 mg/m^2 for 1 h [38]. For cisplatin, a 1-h infusion of 75 mg/m^2 will lead to a maximum plasma concentration of approximately 14 μM of intact cisplatin [39]. When celecoxib is being combined with chemotherapy in the clinic, doses are given

as high as 400 mg on a twice-daily basis [11]. The peak concentration of celecoxib at steady state levels in children that received the drug at 250 mg/m^2 twice daily amounted to approximately 3 μM [40]. After single dosing C_{max} in adults resembles the C_{max} in children. In adults a half-life of celecoxib of approximately 9 h has been measured. Our experimental concentrations of celecoxib inducing 50% cell growth inhibition were four to 12-fold higher, because of which the translation of the data to the clinic should be handled with care.

Importantly, celecoxib had an additional effect on the cytotoxic properties of docetaxel as demonstrated by the increase in the sub-G0 fraction and the activation of caspase-3 in H134 and WiDr cells. Docetaxel is known to stabilize microtubules, whereby the dynamic reorganization and depolymerization are inhibited. At higher concentrations inhibiting 90% of cell growth the drug will induce a G2/M arrest, which we have found earlier to be less evident at IC50 concentrations [28]. Our results are consistent with reports highlighting enhanced antitumor activity of docetaxel when combined with celecoxib in A549 lung tumors [5] and in human prostate cancer cells [9]. Subbaramaiah et al. [3,41] have reported that taxanes can stimulate transcription of COX-2 and stabilization of COX-2 mRNA. The group suggested that co-administration of a selective COX-2 inhibitor might therefore increase the efficacy of taxane-based chemotherapy. We did not find induction of COX-2 protein levels at the docetaxel concentrations applied, because of which other mechanisms should be explored as explanation for the increased apoptotic effects upon addition of celecoxib.

The antagonistic antiproliferative effects of cisplatin combined with celecoxib were accompanied by a reduction of the formation of Pt–DNA adducts in WiDr cells. Literature data on the interaction between COX-2 inhibitors and platinum compounds are not consistent. Inhibition of cisplatin-induced cytotoxicity has also been shown with another selective COX-2 inhibitor nimesulide in human head and neck cancer cell lines [42]. Another study in human colon cancer cell lines, however, has reported that specific inhibition of COX-2 by etodolac led to increased oxaliplatin-induced cell death [10]. Also, in human head and neck cancer cell lines increased chemosensitivity was observed when celecoxib was added to cisplatin [43]. It has to be mentioned that in the experimental design of the studies of Lin et al. [10] and Hashitani et al. [43] continuous exposure of platinum compounds was applied for periods of 24 h or 48 h, respectively, which may have abolished the antagonistic effects observed at the clinically more relevant 1-h exposure periods.

The best-known mechanisms of cisplatin resistance include reduced intracellular cisplatin accumulation, increased drug inactivation by thiol-containing molecules and increased DNA damage repair [reviewed in 44]. Reduced cisplatin accumulation or increased efflux by the presence of MRP2 seems unlikely since antagonism was present in all cell lines regardless the addition of celecoxib before or after cisplatin exposure. It is also unlikely that cisplatin has a direct interaction with celecoxib, since the compound does not contain a classic thiol group, but harbours a sulfur atom connected to two oxygen atoms and an amine group. Indeed, experiments (^{1}H NMR) performed by the group of Prof. Jan Reedijk, Leiden, the Netherlands, excluded a direct interaction between celecoxib and cisplatin (data not shown). The reduced Pt–DNA adduct formation upon the addition of celecoxib to cisplatin might indicate a direct effect of celecoxib on the formation or repair of the adducts.

We have shown earlier that cisplatin exposure can result in an accumulation of cells in the G2/M phase of the cell cycle [45], which was now counteracted by celecoxib. Consequently, the majority of cells resided in the G0/G1 phase, which might have been the result of increased DNA damage repair as was also suggested by the reduced amount of DNA–Pt adducts. Celecoxib can induce expression of p21 [13], a key molecule in cell cycle arrest and the repair of cisplatin-induced DNA damage [37]. An additional experiment in WiDr cells indeed showed p21 upregulation by celecoxib as well as by docetaxel, while cisplatin did not affect p21 levels as measured 48 h after drug exposure. It may be hypothesized that celecoxib-induced prevention of a G2/M arrest by cisplatin might give the cell more time to repair cisplatin-induced DNA damage via p21. Therefore, further analysis on the role of this protein should be carried out in isogenetic cells with and without functional p21.

Celecoxib induced p-ERK phosphorylation in H134, which may antagonize apoptosis pathways possibly reducing cisplatin efficacy [46]. It is, however, unlikely that this mechanism is responsible for cisplatin resistance, since ERK activation was absent in WiDr cells. A possible COX-2-independent mechanism of action of celecoxib described was inhibition of PDK-1/Akt signaling, thereby facilitating the apoptotic process [36,47]. In our study celecoxib had no effect on p-Akt, excluding a possible role for Akt in the interference with drug-induced toxicity.

Taken together, it is evident that the antitumor effects of celecoxib are not mediated only by inhibition of COX-2 activity. Celecoxib added to docetaxel resulted in increased cell death, but antagonistic effects were consistent for all combinations of celecoxib and cisplatin independent of COX-2 expression levels. Current clinical trials with celecoxib in combination with cisplatin require caution based on the antagonism observed in this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.09.005.

REFERENCES

[1] Cao Y, Prescott SM. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. J Cell Physiol 2002;190:279–86.
[2] Subbaramaiah K, Dannenberg AJ. Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol Sci 2003;24:96–102.
[3] Subbaramaiah K, Marmo TP, Dixon DA, Dannenberg AJ. Regulation of cyclooxgenase-2 mRNA stability by taxanes: evidence for involvement of p38, MAPKAPK-2, and HuR. J Biol Chem 2003;278:37637–4.

[4] Kim SH, Hong KO, Chung WY, Hwang JK, Park KK. Abrogation of cisplatin-induced hepatotoxicity in mice by xanthorrhizol is related to its effect on the regulation of gene transcription. Toxicol Appl Pharmacol 2004;196: 346–55.
[5] Shaik MS, Chatterjee A, Jackson T, Singh M. Enhancement of antitumor activity of docetaxel by celecoxib in lung tumors. Int J Cancer 2006;118:396–404.
[6] Hawkey CJ. COX-2 chronology. Gut 2005;54:1509–14.
[7] Hood WF, Gierse JK, Isakson PC, Kiefer JR, Kurumbail RG, Seibert K, et al. Characterization of celecoxib and valdecoxib binding to cyclooxygenase. Mol Pharmacol 2003;63:870–7.
[8] Koki AT, Masferrer JL. Celecoxib: a specific COX-2 inhibitor with anticancer properties. Cancer Control 2002;9:28–35.
[9] Dandekar DS, Lopez M, Carey RI, Lokeshwar BL. Cyclooxygenase-2 inhibitor celecoxib augments chemotherapeutic drug-induced apoptosis by enhancing activation of caspase-3 and -9 in prostate cancer cells. Int J Cancer 2005;115:484–92.
[10] Lin J, Hsiao PW, Chiu TH, Chao JI. Combination of cyclooxygenase-2 inhibitors and oxaliplatin increases the growth inhibition and death in human colon cancer cells. Biochem Pharmacol 2005;70:658–67.
[11] El-Rayes BF, Zalupski MM, Shields AF, Ferris AM, Vaishampayan U, Heilbron LK, et al. A phase II study of celecoxib, gemcitabine, and cisplatin in advanced pancreatic cancer. Invest New Drugs 2005;23:583–90.
[12] Olsen SR. Taxanes and COX-2 inhibitors: from molecular pathways to clinical practice. Biomed Pharmacother 2005;59(Suppl 2):S306–10.
[13] Han C, Leng J, Demetris AJ, Wu T. Cyclooxygenase-2 promotes human cholangiocarcinoma growth: evidence for cyclooxygenase-2-independent mechanism in celecoxib-mediated induction of p21waf1/cip1 and p27kip1 and cell cycle arrest. Cancer Res 2004;64:1369–76.
[14] Maier TJ, Schilling K, Schmidt R, Geisslinger G, Grösch S. Cyclooxygenase-2 (COX-2)-dependent and -independent anticarcinogenic effects of celecoxib in human colon carcinoma cells. Biochem Pharmacol 2004;67: 1469–78.
[15] Patel MI, Subbaramaiah K, Du B, Chang M, Yang P, Newman RA, et al. Celecoxib inhibits prostate cancer growth: evidence of a cyclooxygenase-2-independent mechanism. Clin Cancer Res 2005;11:1999–2007.
[16] Wendum D, Masliah J, Trugnan G, Flejou JF. Cyclooxygenase-2 and its role in colorectal cancer development. Virchows Arch 2004;445:327–33.
[17] Sheehan KM, Sheahan K, O'Donoghue DP, MacSweeney F, Conroy RM, Fitzgerald DJ, et al. The relationship between cyclooxygenase-2 expression and colorectal cancer. J Am Med Assoc 1999;282:1254–7.
[18] Sinicrope FA, Gill S. Role of cyclooxygenase-2 in colorectal cancer. Cancer Metastasis Rev 2004;23:63–75.
[19] Yao M, Kargman S, Lam EC, Kelly CR, Zheng Y, Luk P, et al. Inhibition of cyclooxygenase-2 by rofecoxib attenuates the growth and metastatic potential of colorectal carcinoma in mice. Cancer Res 2003;63:586–92.
[20] Lev-Ari S, Strier L, Kazanov D, Madar-Shapiro L, Dvory-Sobol H, Pinchuk I, et al. Celecoxib and curcumin synergistically inhibit the growth of colorectal cancer cells. Clin Cancer Res 2005;11:6738–44.
[21] Klimp AH, Hollema H, Kempinga C, Van der Zee AG, De Vries EG, Daemen T. Expression of cyclooxygenase-2 and inducible nitric oxide synthase in human ovarian tumors and tumor-associated macrophages. Cancer Res 2001;61:7305–9.
[22] Denkert C, Kobel M, Pest S, Koch I, Berger S, Schwabe M, et al. Expression of cyclooxygenase 2 is an independent prognostic factor in human ovarian carcinoma. Am J Pathol 2002;160:893–903.
[23] Ferrandina G, Lauriola L, Zannoni GF, Fagotti A, Fanfani F, Legge F, et al. Increased cyclooxygenase-2 (COX-2) expression is associated with chemotherapy resistance and outcome in ovarian cancer patients. Ann Oncol 2002;13:1205–11.
[24] Drake JG, Becker JL. Aspirin-induced inhibition of ovarian tumor cell growth. Obstet Gynecol 2002;100:677–82.
[25] Denkert C, Fürstenberg A, Daniel PT, Koch I, Köbel M, Weichert W, et al. Induction of G0/G1 cell cycle arrest in ovarian carcinoma cells by the anti-inflammatory drug NS-398, but not by COX-2-specific RNA interference. Oncogene 2003;22:8653–61.
[26] Munkarah AR, Genhai Z, Morris R, Baker VV, Deppe G, Diamond MP. Inhibition of paclitaxel-induced apoptosis by the specific COX-2 inhibitor, NS398, in epithelial ovarian cancer cells. Gynecol Oncol 2003;88:429–33.
[27] Buick RN, Pullano R, Trent JM. Comparative properties of five human ovarian adenocarcinoma cell lines. Cancer Res 1985;45:3668–76.
[28] Kolfschoten GM, Hulscher TM, Duyndam MC, Pinedo HM, Boven E. Variation in the kinetics of caspase-3 activation, Bcl-2 phosphorylation and apoptotic morphology in unselected human ovarian cancer cell lines as a response to docetaxel. Biochem Pharmacol 2002;63:733–43.
[29] Marshall CJ, Franks LM, Carbonell AW. Markers of neoplastic transformation in epithelial cell lines derived from human carcinomas. J Natl Cancer Inst 1977;58: 1743–51.
[30] Noguchi P, Wallace R, Johnson J, Earley EM, O'Brien S, Ferrone S, et al. Characterization of the WIDR: a human colon carcinoma cell line. In Vitro 1979;15:401–8.
[31] Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22: 27–55.
[32] Temmink OH, Hoogeland MF, Fukushima M, Peters GJ. Low folate conditions may enhance the interaction of trifluorothymidine with antifolates in colon cancer cells. Cancer Chemother Pharmacol 2006;57:171–9.
[33] Kruger NJ. The Bradford method for protein quantitation. Methods Mol Biol 1994;32:9–15.
[34] Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139:271–9.
[35] Van Moorsel CJ, Pinedo HM, Veerman G, Bergman AM, Kuiper CM, Vermorken JB, et al. Mechanisms of synergism between cisplatin and gemcitabine in ovarian and non-small-cell lung cancer cell lines. Br J Cancer 1999;80:981–90.
[36] Kulp SK, Yang YT, Hung CC, Chen KF, Lai JP, Tseng PH, et al. 3-Phosphoinositide-dependent protein kinase-1/Akt signaling represents a major cyclooxygenase-2-dependent target for celecoxib in prostate cancer cells. Cancer Res 2004;64:1444–51.
[37] Ruan S, Okcu MF, Ren JP, Chiao P, Andreeff M, Levin V, et al. Overexpressed WAF1/Cip1 renders glioblastoma cells resistant to chemotherapy agents 1,3-bis(2-chloroethyl)-1-nitrosourea and cisplatin. Cancer Res 1998;58:1538–43.
[38] Bruno R, Hille D, Riva A, Vivier N, Ten Bokkel Huinink WW, Van Oosterom AT, et al. Population pharmacokinetics/pharmacodynamics of docetaxel in phase II studies in patients with cancer. J Clin Oncol 1998;16:187–96.
[39] Verschraagen M, Boven E, Ruijter R, Van der Born K, Berkhof J, Hausheer FH, et al. Pharmacokinetics and preliminary clinical data of the novel chemoprotectant BNP7787 and cisplatin and their metabolites. Clin Pharmacol Ther 2003;74:157–69.

[40] Stempak D, Gammon J, Klein J, Koren G, Baruchel S. Single-dose and steady-state pharmacokinetics of celecoxib in children. Clin Pharmacol Ther 2002;72:490–7.
[41] Subbaramaiah K, Hart JC, Norton L, Dannenberg AJ. Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2. Evidence for involvement of ERK1/2 AND p38 mitogen-activated protein kinase pathways. J Biol Chem 2000;275:14838–45.
[42] Czembirek C, Eder-Czembirek C, Erovic BM, Turhani D, Selzer E, Thurnher D, et al. Inhibition of cytotoxicity of cisplatin by cyclooxygenase-2 inhibitor nimesulide in head and neck cancer cell lines. Oncol Rep 2005;14:1523–6.
[43] Hashitani S, Urade M, Nishimura N, Maeda T, Takaoka K, Noguchi K, et al. Apoptosis induction and enhancement of cytotoxicity of anticancer drugs by celecoxib, a selective cyclooxygenase-2 inhibitor, in human head and neck carcinoma cell lines. Int J Oncol 2003;23:665–72.
[44] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22:7265–79.
[45] Kolfschoten GM, Hulscher TM, Schrier SM, Van Houten VM, Pinedo HM, Boven E. Time-dependent changes in factors involved in the apoptotic process in human ovarian cancer cells as a response to cisplatin. Gynecol Oncol 2002;84:404–12.
[46] Dent P, Grant S. Pharmacologic interruption of the mitogen-activated extracellular-regulated kinase/mitogen-activated protein kinase signal transduction pathway: potential role in promoting cytotoxic drug action. Clin Cancer Res 2001;7:775–83.
[47] Arico S, Pattingre S, Bauvy C, Gane P, Barbat A, Codogno P, et al. Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line. J Biol Chem 2002;277:27613–21.