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Celecoxib enhances doxorubicin-induced cytotoxicity in MDA-MB231 cells by NF- κ B-mediated increase of intracellular doxorubicin accumulation

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ARTICLE INFO

Article history:

Received 13 April 2006

Received in revised form 27 July 2006

Accepted 4 September 2006

Available online 9 November 2006

Keywords:

Celecoxib

NF- κ B

Cancer

Doxorubicin

MDR

ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) and cyclo-oxygenase (COX) inhibitors are anti-inflammatory agents that have also shown to be useful in anticancer therapy. In the present study, we show that the specific COX-2 inhibitor celecoxib enhances the inhibitory effect of doxorubicin (dox) on human MDA-MB231 breast tumour growth *in vivo* and *in vitro*. We also found that celecoxib increased the intracellular accumulation and retention of dox *in vitro*. Since the NSAID indomethacin and the specific COX-2 inhibitor NS398 did not affect the *in vitro* actions of dox, these effects are likely to be mediated via a COX-independent mechanism. It has been suggested that some COX-inhibitors can enhance the actions of cytostatics by overcoming multidrug resistance through the inhibition of ABC-transporter proteins. However, we found that the three main ATP-binding cassette (ABC)-transporter proteins, implicated in dox transport, were inactive in MDA-MB231 cells. Therefore, the finding that the P-glycoprotein (P-gp) blocker PSC833 also increased cellular accumulation of dox was unexpected. In order to unravel the molecular mechanisms involved in dox accumulation, we examined the involvement of NF- κ B, as this transcription factor has been implicated in celecoxib action as well as in chemoresistance. We found that celecoxib and PSC833, but not indomethacin or NS398, almost completely inhibited basal- and dox induced NF- κ B gene-reporter activity and p65 subunit nuclear translocation. Furthermore, the NF- κ B inhibitor PDTC mimicked the actions of celecoxib and PSC833 on cell growth and on intracellular accumulation of dox, suggesting that NF- κ B is functionally involved in the actions of these compounds. In conclusion, we show that structurally different compounds, among which are celecoxib and PSC833, increase the intracellular accumulation of dox and enhance dox induced cytotoxicity in MDA-MB231 breast cancer cells most likely via the modulation of NF- κ B activity.

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doi:10.1016/j.ejca.2006.09.010

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) and specific cyclo-oxygenase (COX)-2 inhibitors are widely used in the treatment of pain and rheumatoid arthritis and have shown promising results in the treatment of cancer in experimental and clinical studies.^{1,2} COX-2 is overexpressed in many malignancies and is involved in tumour development and growth. The effects of NSAIDs and specific COX-2 inhibitors on tumour cells include inhibition of cell proliferation, induction of apoptosis and reduction of cell motility and adhesion.^{2–10} Furthermore, both non-specific and specific COX-2 inhibitors have shown to significantly inhibit tumour angiogenesis.^{11–14} These anticancer properties make it worthwhile examining the possible benefit of combining NSAIDs and COX-2 inhibitors with conventional anticancer therapies, such as chemotherapy.

Several preclinical and clinical studies have explored and are currently exploring the therapeutic benefit of combining NSAIDs and specific COX-2 inhibitors with chemotherapeutics and have been shown to improve treatment outcome. For example, in experimental and clinical studies, the COX-2 inhibitor celecoxib has shown to enhance the anti-tumour efficacy of several cytostatics, such as that of irinotecan, doxorubicin (dox), bleomycin and 5-fluorouracil.^{15–18} The mechanism by which COX-2 inhibitors enhance the action of cytostatics is, however, not clear and it is suggested that this may involve mechanisms other than suppression of the COX-2 enzyme. For example, it has been proposed that COX-inhibitors modulate the resistance of tumours to chemotherapeutic drugs by affecting the activity of plasma membrane transporter proteins of the ABC-transporter family, which behave as energy-dependent efflux pumps for cytostatics. The three key mammalian transporters involved in the transport of anticancer agents, such as the anthracyclines, are P-glycoprotein (P-gp/ABCB1), multidrug-resistance protein-1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/MXR/ABCG2).^{19,20}

In recent years, much effort has been made to identify agents that are able to overcome MDR, in order to improve chemotherapeutic treatment. These agents, called chemosensitisers, belong to a variety of structural classes, such as calcium channel blockers, drug analogues, cyclic peptides and steroids.^{21,22} It has been suggested that COX-inhibitors may also act as chemosensitisers and can overcome MDR by inhibiting P-gp¹⁵ or MRPs.^{23–26} However, conclusive evidence for the actions of NSAIDs and specific COX-2 inhibitors on these transporters is lacking. Interestingly, apart from MDR, NF- κ B has recently been described as another COX independent molecular target for actions of NSAIDs and COX-2 inhibitors, such as aspirin, indomethacin and celecoxib.^{27–30} Moreover, NF- κ B has also shown to be involved in chemoresistance in different cancer types^{31–36}, suggesting a possible role of this transcription factor in the chemosensitising effect of COX-inhibitors.

In the present study, we addressed these issues both *in vivo* and *in vitro* by studying the effects of NSAIDs, specific COX-2 inhibitors such as celecoxib and specific pump inhibitors in combination with dox in the breast cancer cell line MDA-MB231.

2. Materials and methods

2.1. Cell lines, chemicals and reagents

The human mammary carcinoma cell line MDA-MB231 was from the American Type Culture Collection (Rockville, MD) and was cultured in Dulbecco's modified Eagle's medium (Biochrom, Basel, Switzerland) and 10% FCS (p/s, Life Technologies, Breda, the Netherlands). Dox was from Pharmacia B.V., Woerden, The Netherlands. Indomethacin was from Bufa B.V., Uitgeest, The Netherlands; Sc-236, NS-398 and celecoxib were from Pharmacia, Skokie, USA. PSC833 was from Novartis, Basel, Switzerland. Probenecid was from Sigma, St. Louis, MO, USA. Ko143 (fumitremorgan C analogue) was a kind gift from A. van Loevezijn, Laboratory of Organic Chemistry, University of Amsterdam (Amsterdam, The Netherlands). The fluorescent pump substrates, Syto16 (for Pgp), calcein acetoxymethylester (for MRP1) and Bodipy-prazosin (for BCRP), were from Molecular Probes, Eugene, OR, USA, and actionomycin D (7-AAD) was from Pharmingen, San Diego, CA, USA. For transfections, Eugene 6 transfection reagent was from Roche, Basel, Switzerland, and the NF- κ B reporter construct NF- κ B-luc was from Stratagene, Amsterdam, The Netherlands, and Renilla luciferase (pRL-SV40) was from Promega, Madison, WI, USA. The NF- κ B inhibitor pyrrolidinedithiocarbamate (PDTCT) was from Sigma, Zwijndrecht, The Netherlands.

2.2. Animal study

Female BALB/c nu/nu mice were from Iffa Credo (L'Arbresle, France) and were housed in individual ventilated cages under sterile conditions according to the Swiss guidelines for the care and use of laboratory animals. Sterile food and water were provided *ad libitum*. At start of the experiment, the mice were ≈ 10 weeks old. MDA-MB231 cells (2×10^6 cells/100 μ l) were injected sc. in the left flank. After 2 weeks, animals containing a tumour with a volume (TV) of 100 ± 20 mm³ were selected and divided into four groups of eight mice each. TV was assessed by using a calliper measuring the two major diameters by the formula $TV = \pi/6 (d_1 \times d_2)^{1/2}$. Mice (8 per experimental group, $n = 8$) were treated with vehicle (DMSO), celecoxib (15 mg kg⁻¹), dox (0.5 mg kg⁻¹) or a combination of celecoxib and dox. All agents were injected i.p. in a total volume of 125 μ l. The mice were treated every other day for 30 days. TV and body weight were measured twice a week.

2.3. MTS assay

Cell proliferation was assessed by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) tetrazolium assay (Cell Titer96 Aqueous, Promega). For this, 2×10^3 cells per well were seeded in triplicate in 90 μ l culture medium in 96-well flat-bottom microculture plates and one day later the additives were added. After 4 d of culture, viable cells were determined by adding 20 μ l MTS to each well and measuring OD 490 (Thermomax, Molecular Devices) after 2 h incubation. The results

were expressed as the mean optical density (OD) of each 3-well set.

2.4. Dox accumulation assay

Cells (3×10^4) were seeded in 4-well Lab-Tek® glass Chamber Slides (Nalge Nunc International Corp. Naperville, IL) and incubated overnight after which additives were added for 24 h. Subsequently, the culture medium was removed and cells were washed three times with PBS. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed three times with PBS and mounted under glass coverslips with Vectashield (Brunschiwig, Amsterdam, The Netherlands). The cells were examined for doxorubicin fluorescence using fluorescence microscopy. Fluorescence was excited at 465–495 nm and detected at an emission maximum of 515–555 nm. Intracellular doxorubicin was quantified using computerised image analysis. Images of control and treated cultures were acquired using a colour CCD camera, digitised with a Matrix meteor frame grabber, filtered and analysed with Image Pro Plus 3.0. Cells (20) were measured and values were expressed as mean light intensity per cell \pm SD, corrected for background.

2.5. FACS analysis

P-gp, MRP and BCRP activities were measured using a green fluorescent probe assay with specific substrate/modulator combinations as previously described.^{20,37,38} In short, 3×10^5 cells/ml were incubated in DMEM at 37 °C for 45–60 min with either: (i) Syto16 (0.6 nM) in the presence or absence of PSC833 (2 μ M) for P-gp activity, (ii) calcein acetoxymethyl ester (1 nM) in the presence or absence of probenecid (3 mM) for MRP1 activity, or (iii) with Bodipy-prazosin (25 nM) in the presence of PSC-833 (2 μ M) with or without Ko143 (200 nM) for BCRP activity. Subsequently, they were incubated with 7-amino actinomycin D and flow cytometric analysis was performed on a FACScalibur (Becton Dickinson, San José, CA). P-gp, MRP and BCRP activities were expressed as ratio of substrate fluorescence with modulator present and substrate fluorescence without modulator present after subtraction of the fluorescence of the control (cells in accumulation medium alone). Since these functional assays are highly sensitive, the ratios below 1.5–2.0 in cell lines are thought to have little practical meaning in terms of drug resistance.^{37,38}

2.6. NF- κ B reporter assay

MDA-MB231 cells were seeded at a density of 1.25×10^4 cells/well in 24-well plates, and transiently transfected with 1 μ g of the reporter construct using Eugene 6 transfection reagent. The NF- κ B reporter construct NF- κ B-luc was used. To correct for transfection efficiency, 100 ng of Renilla luciferase was co-transfected. 24 h after transfection, test substances were added to the cells for another 24 h. Hereafter, luciferase assays were performed with the Dual-Luciferase Reporter assay system (Promega) according to the protocol. Cell lysate (5 μ l) was first assayed for firefly luciferase and then for Renilla luciferase activity, using the Wallac 1450 Microbeta Trilux

luminescence counter (Perkin-Elmer, Boston, MA, USA). Firefly luciferase activity was corrected for Renilla luciferase activity.

2.7. Western blot analysis

In all, 10 μ g of cytoplasmatic or nuclear protein was prepared from cells treated under different conditions, separated on 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane by wet blotting. The membrane was blocked for 1 h at ambient temperature with 2% milk powder in PBST (PBS containing 0.1% (v/v) Tween-20), followed by primary antibody incubations (anti-p65 and anti-p50, Santa Cruz Biotechnology, Heerhugowaard, The Netherlands) overnight at 4 °C in 0.2% milk powder in PBST. The membrane was washed and primary antibodies were detected with rabbit anti-goat IgG conjugated to horseradish peroxidase and the bands were visualised with enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, England). Hereafter, blots were stripped using blot restore membrane rejuvenation kit (Chemicon, Hampshire, UK) according to the manufacturer's instructions. Hereafter, protein samples were checked for nuclear and cytoplasmatic specificity using anti-Lamin A (nuclear membrane structural component, Cell Signaling Technology, USA) and anti-protein disulphide isomerase (PDI, endoplasmatic reticulum protein, Stressgen Biotechnologies, Vic., Canada) antibodies.

2.8. Statistical analysis

The data are expressed as means \pm SD for comparing treated groups with control groups, a Student's t-test was used. A P-value of <0.05 was considered significant.

3. Results

3.1. MDA-MB231 tumour growth in vivo

Fig. 1 shows the effects of daily treatment with celecoxib (15 mg kg⁻¹) and dox (0.5 mg kg⁻¹) alone or in combination, on the subcutaneous growth of MDA-MB231 human mammary carcinoma xeno-transplants in nude mice. After 23 days of treatment, the average tumour volume of the mice treated with the combination of dox and celecoxib was, compared to controls, significantly suppressed ($P < 0.05$). Treatment with dox or celecoxib as single agents, with these doses, did not significantly affect tumour growth.

3.2. MDA-MB231 cell proliferation in vitro

Fig. 2 shows growth inhibition curves of MDA-MB231 cells by dox in the absence or presence of celecoxib (10 μ M) or indomethacin (10 μ M). Dox inhibited cell proliferation dose-dependently with an EC₅₀ of around 0.2 μ M. Co-treatment of the cells with celecoxib, which alone had no effect on cell growth, decreased the EC₅₀ to 0.06 μ M. In contrast, indomethacin did not affect the action of dox, and the same was true for the specific COX-2 inhibitor NS398 (10 μ M) (not shown). Moreover, the specific COX-2 inhibitor Sc236 (10 μ M), like

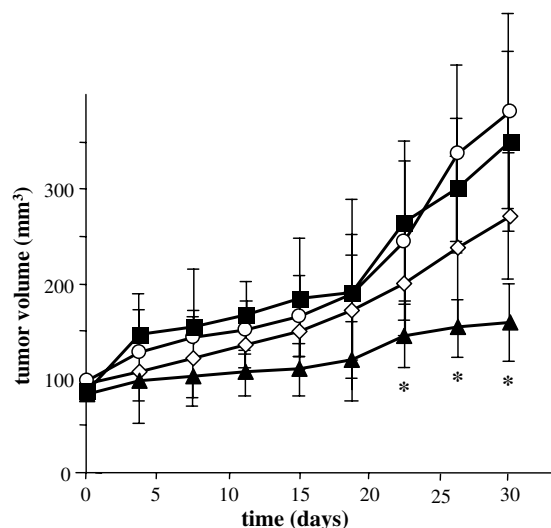


Fig. 1 – Effects of celecoxib and doxorubicin, alone and in combination on the growth of MDA-MB231 xenografts in nude mice. Vehicle (DMSO) (filled squares); celecoxib (15 mg kg⁻¹) (open circles); dox (0.5 mg kg⁻¹) (open diamonds) and celecoxib + dox (filled triangles). Values represent mean tumour volume. **P* < 0.05 (treatment versus control).

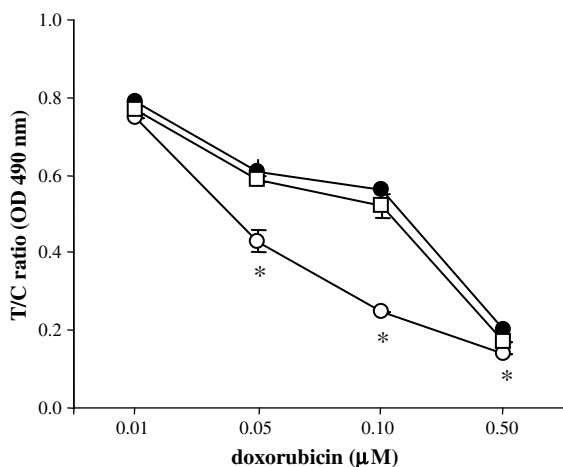


Fig. 2 – Effect of dox alone, or in combination with celecoxib or indomethacin, on in vitro MDA-MB231 proliferation (OD 490). Dox (0.01–0.5 μM) (filled circles); Dox + celecoxib (10 μM) (open circles) and Dox + indomethacin (10 μM) (open squares). Results are expressed as treatment versus control ratio (T/C-ratio); mean ± SD **P* < 0.05 (dox versus dox + celecoxib or versus dox + indomethacin).

celecoxib, enhanced the effect of dox, decreasing the EC₅₀ from 0.2 to 0.08 μM (not shown).

3.3. Intracellular accumulation and retention of dox

Doxorubicin is an auto-fluorescent compound, which enables the visualisation of its intracellular presence by fluorescence microscopy. Fig. 3a shows the fluorescence of intracellular accumulated dox in MDA-MB231 cells after incubation for

24 h with different concentrations of dox (0.5, 1 and 5 μM) in the absence (A1, B1 and C1) or presence of celecoxib (50 μM) (A2, B2, and C2). As shown in the pictures and in the graphs, the intracellular fluorescence, which is mainly visible in the nuclei, is at all concentrations significantly (*P* < 0.001) higher in cells that were co-treated with celecoxib. Like celecoxib, the specific COX-2 inhibitor Sc236 (50 μM) also increased the cellular accumulation of dox (not shown). In addition, NS398 (50 μM) and indomethacin (50 μM) did not affect dox accumulation (not shown). Fig. 3b shows MDA-MB231 cells that were incubated for 24 h with dox (1 μM) followed by incubation for another 24 h in control medium (A) or in medium containing celecoxib (50 μM) (B), respectively. As shown in the pictures and the graphs, cells treated with celecoxib contained significantly (*P* < 0.001) more dox than those that were incubated in control medium during the second 24 h culture period.

3.4. Effects of ABC-transporter blockers on dox-suppressed cell growth and on intracellular dox accumulation

Dox is a substrate for proteins of the ABC-transporter protein family that enhance the efflux of cytostatics. Since our results would suggest that celecoxib and Sc236 modulate the cellular actions of dox via inhibition of these transporters, we examined their possible involvement on intracellular accumulation of dox and induction of cytotoxicity in MDA-MB231 cells using specific blockers.

Fig. 4 shows a dose-inhibition curve of dox in the absence or presence of the P-gp-blocker PSC833 (10 μM) on MDA-MB231 growth. PSC833 significantly enhanced the effect of dox on cell growth, decreasing the EC₅₀ from 0.2 μM to 0.08 μM. Moreover, PSC833 (50 μM) markedly increased the intracellular accumulation of dox (not shown). The MRP-blocker probenecid (0.1–5 mM) and the BCRP-blocker Ko143 (1–10 μM) did not affect the cell growth suppressive effect of dox, nor its intracellular accumulation (not shown).

3.5. FACSscan analysis for P-gp-, MRP- and BCRP-transporter activity

Table 1 shows the ratios for the activity of ABC-transporters P-gp, MRP1 and BCRP in MDA-MB231 cells under different treatment conditions. The measured ratio for each of the transporters, under all conditions, was hardly different from 1.0 and is considered to reflect a very low, if any, activity of these proteins since even very low resistant cell lines have a ratio of at least 2.0.^{20,37,38}

3.6. Effects on NF-κB activity

Fig. 5a shows the effects of dox (0.01–1 μM) and celecoxib (0.5–50 μM) on NF-κB-luc activity in MDA-MB231 cells. Dox dose-dependently stimulated NF-κB-luc activity and celecoxib dose-dependently inhibited it. Fig. 5b shows the effects of celecoxib, indomethacin and the P-gp blocker PSC833 (all at 50 μM) alone or in combination with dox (1 μM) on NF-κB-luc activity. Both celecoxib and PSC833 almost completely blocked basal – as well as dox induced NF-κB activity, while indomethacin had no effect. In addition, Sc236 (50 μM)

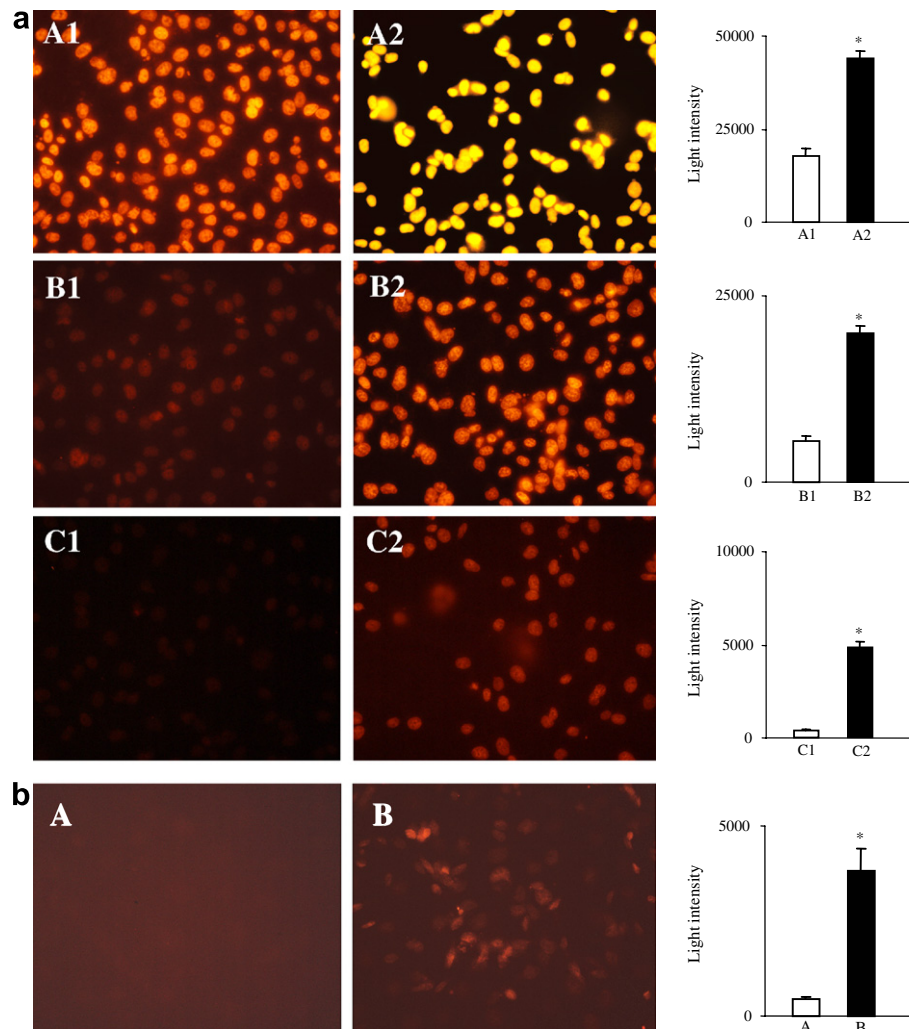


Fig. 3 – Effect of celecoxib on the intracellular accumulation of doxorubicin. (a) Shows fluorescent microscopic pictures of MDA-MB231 cells that were incubated for 24 h in a medium containing dox (5 μM) (A1); (1 μM) (B1) or (0.5 μM) (C1), or these concentrations of dox in combination with celecoxib (50 μM) (A2; B2; C2), respectively. Magnification: 200×. (b) Shows cells treated with dox (1 μM) for 24 h, followed by 24 h incubation in control medium (A) or medium containing celecoxib (50 μM) (B). Magnification: 200×. The bar graphs show, for all experimental conditions, the mean light intensity per cell, measured by computerised image analysis, expressed as mean ± SD * $P < 0.001$.

suppressed both basal and dox induced NF-κB activity and NS398 (50 μM) was totally ineffective (not shown).

3.7. NF-κB p65 and p50 subunit translocation

Fig. 6 shows Western blot detection of NF-κB subunit p65 in nuclear (N) and cytoplasmic (C) extracts from MDA-MB231 cells cultured for 24 h in the absence or the presence of different additives. As shown, the cytoplasmic extracts of cells that were cultured in the presence of dox (1 μM) (lanes 5–8) stained significantly stronger for p65 than those cultured in the absence of dox (lanes 1–4). Moreover, compared to control (lane 1), celecoxib (50 μM) (lane 2) and PSC833 (50 μM) (lane 4) significantly inhibited nuclear p65 staining, whereas indomethacin (50 μM) (lane 3) had no effect. Furthermore, dox strongly enhanced nuclear p65 staining (lane 5) which was almost totally suppressed by celecoxib (lane 6) and PSC833 (lane 8) and was unaffected by indomethacin (lane 7). In addition,

no effect of any of the additives on p50 nuclear translocation was observed, nor on IKKα, IKKβ and IκB-α (not shown).

3.8. Effects of PDTC on dox suppressed cell growth and on intracellular accumulation of dox

Fig. 7a shows the effects of dox in the absence or presence of PDTC (10 μM) on MDA-MB231 growth *in vitro*, and Fig. 7b shows its effect (10 μM) on intracellular dox accumulation. As shown, PDTC decreased the EC_{50} on cell growth from 0.2 μM to 0.09 μM and also significantly ($P < 0.001$) increased intracellular dox accumulation, see pictures and bar graph.

In addition, as expected, PDTC (10 μM) inhibited basal and dox (1 μM)-induced NF-κB-luc activity in MDA-MB231 cells from 82 ± 13 to 13 ± 4 and from 163 ± 18 to 39 ± 9 relative photon units, respectively (data not shown). Western blot detection of NF-κB subunit p65 in nuclear (N) and cytoplasmic (C) extracts from MDA-MB231 cells cultured for 24 h in the

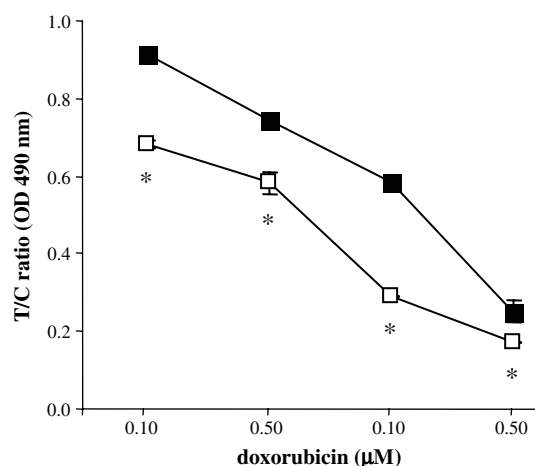


Fig. 4 – Effect of dox, alone or in combination with PSC833, on *in vitro* MDA-MB231 proliferation (OD 490). Dox (0.01–0.5 μM) (filled squares) or Dox + PSC833 (10 μM) (open squares). Results are expressed as treatment versus control ratio (T/C-ratio); mean ± SD *P < 0.05 (dox versus dox + PSC833).

Table 1 – Activity of Pgp, MRP and BCRP in MDA-MB231 cells.

	Pgp-activity		MRP-activity		BCRP-activity	
	-cxb	+cxb	-cxb	+cxb	-cxb	+cxb
Control	1.16	1.00	1.34	1.39	1.13	1.08
Doxorubicin (0.1 μM)	1.00	1.00	1.29	1.22	1.25	1.25

absence (lane 1) or the presence of PDTC (10 μM, lane 2), doxorubicin (1 μM, lane 3), and treatment with dox and PDTC (lane 4) shows that PDTC inhibits both basal and dox-enhanced nuclear p65 staining (Fig. 7c).

4. Discussion

In this study, we show that the specific COX-2 inhibitor celecoxib enhances the cytostatic effect of dox on MDA-MB231 tumour growth *in vivo* and *in vitro*. Celecoxib also significantly increased the intracellular accumulation and retention of dox *in vitro*. These effects of celecoxib were independent of COX-2 and of the activity of the ABC-transporters P-gp, MRP1 and BCRP and were most likely mediated by inhibition of NF-κB. Moreover, we show that the P-gp blocker PSC833, like celecoxib, increased the intracellular accumulation of dox and augmented the dox-induced cytotoxicity, independent of P-gp inhibition, mediated most likely via suppression of NF-κB activity.

Previous studies have shown that NSAIDs and specific COX-2 inhibitors are able to enhance the effects of certain cytostatic agents *in vitro* and *in vivo*. For example, celecoxib was shown to enhance the tumour growth inhibitory effect of dox in breast tumour bearing mice.¹⁵ In line with this, we found that the combined treatment of human breast carci-

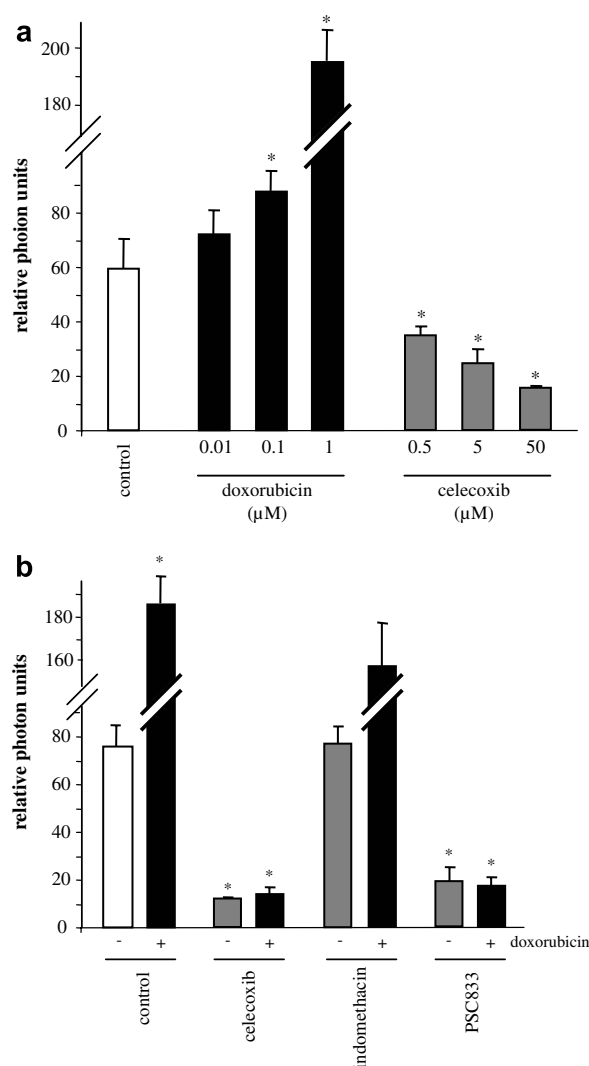


Fig. 5 – Effects of different additives on NF-κB-mediated luciferase expression (relative light units) in MDA-MB231 cells. (a) Dox (0.01–1 μM) (black bars) and celecoxib (0.5–50 μM) (grey bars). (b) Celecoxib, indomethacin and PSC833 (all at 50 μM) (grey bars) alone or in combination with dox (1 μM) (black bars). Results are expressed as mean ± SD *P < 0.05 (control versus treatment).

noma MDA-MB231 xenografts in nude mice with dox and celecoxib synergistically inhibited tumour growth. *In vitro*, these compounds also synergistically inhibited the proliferation of MDA-MB231 cells. The mechanism by which celecoxib as well as Sc236 enhanced the action of dox is most likely independent of suppression of COX, as we show that the specific COX-2 inhibitor NS398 and the NSAID indomethacin did not augment the cytotoxic actions of dox.

Some previous studies using less specific or sensitive assays suggested that the enhancement of chemotherapeutic drug efficacy by COX-inhibitors may be due to inhibition of the ABC-transporters P-gp¹⁵ or MRP1.^{23–26} In our study, we show that celecoxib and Sc236 augmented the *in vitro* intracellular accumulation and retention of dox, also suggesting the possible involvement of one of the dox transporters in

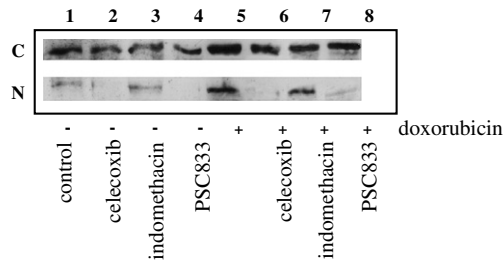


Fig. 6 – Western blot detection of NF- κ B subunit p65 in nuclear (N) and cytoplasmic (C) extracts from MDA-MB231 cells. MDA-MB231 cells were cultured for 24 h in the absence (lanes 1–4) or the presence of dox (1 μ M) (lanes 5–8) and in combination with celecoxib (lanes 2 and 6), indomethacin (lanes 3 and 7) or PSC833 (lanes 4 and 8) (all at 50 μ M).

this action. The activity of the key transporters of hydrophobic drugs, like the anthracycline dox, namely P-gp, MRP1 and BCRP can be inhibited by specific blockers, such as PSC833 for P-gp, probenecid for MRP1 and Ko143 for BCRP, respectively. Our results showed that, like celecoxib and Sc236, the P-gp blocker PSC833, but not the MRP-blocker probenecid, nor the

BCRP blocker Ko143, stimulated the accumulation of dox in MDA-MB231 cells and also synergistically augmented the inhibitory effect of dox on MDA-MB231 proliferation. Collectively, this would suggest the involvement of the P-gp transporter in the actions of celecoxib and SC236 on dox inhibited cell growth and intracellular accumulation of dox. However, by using a very sensitive functional assay for P-gp, using the fluorescent dye Syto-16^{20,38}, we showed that the MDA-MB231 cells revealed no P-gp activity under all treatment conditions, ruling out its involvement in the actions of celecoxib, Sc236 and PSC833. A finding consistent with this result was reported recently by Wang and colleagues³⁹, who showed that ABC-transporter proteins were absent in MDA-MB231 cells. Therefore, another classical MDR phenotype-independent mechanism must be involved in the actions of celecoxib, Sc236 and PSC833 on dox induced cytotoxicity in these cells.

Previously, NF- κ B has been shown to be involved in chemoresistance as well as to be a molecular target of celecoxib action. It has been shown that constitutive NF- κ B activation in tumours protects the cells against apoptotic stimuli, such as those induced by chemotherapeutic treatment. Using a transfection luciferase assay, we found that celecoxib could

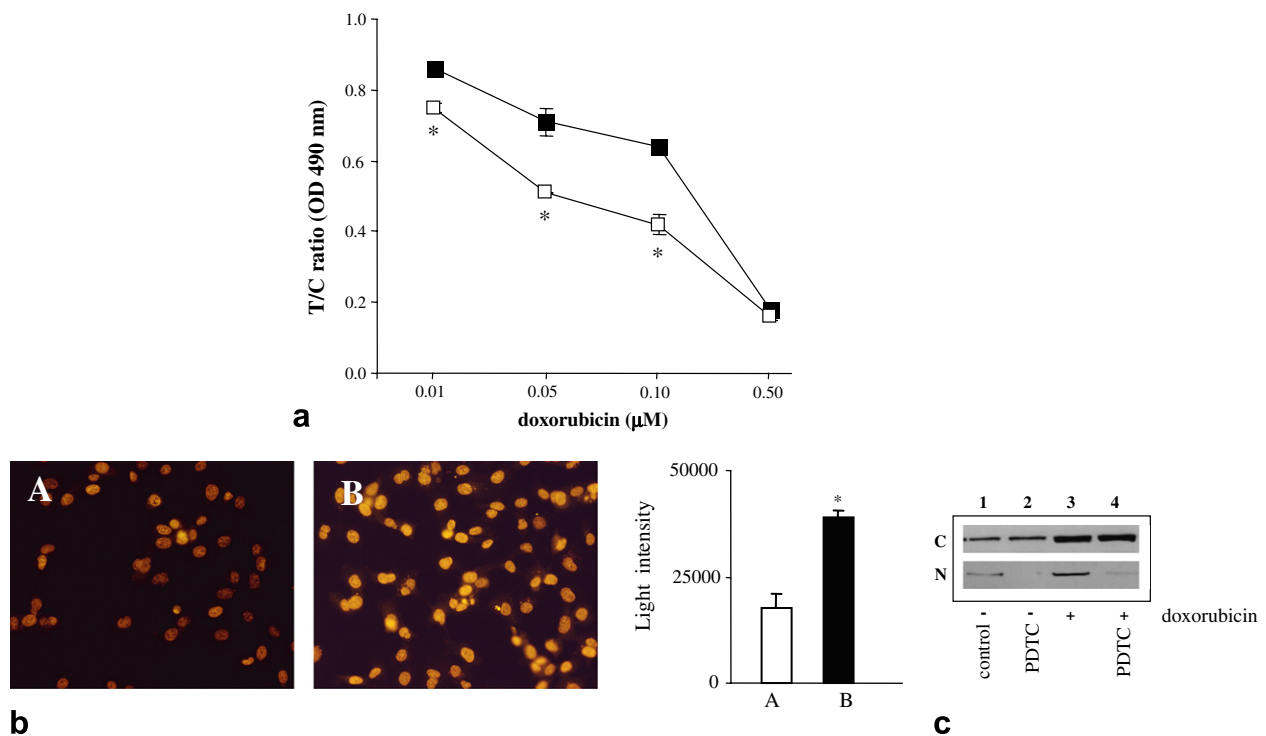


Fig. 7 – Effect of dox, alone or in combination with PDTC, on in vitro MDA-MB231 proliferation (OD 490) and effect of PDTC on intracellular dox accumulation. (a) Dox (0.010–0.5 μ M) (filled squares); dox + PDTC (10 μ M) (open squares). Results are expressed as treatment versus control ratio (T/C-ratio); mean \pm SD * $P < 0.05$ (dox versus dox + PDTC). (b) Fluorescent microscopic pictures of MDA-MB231 cells that were incubated for 24 h in a medium containing dox (1 μ M) (A) or dox in combination with PDTC (50 μ M) (B). Magnification 200 \times . The bar graph shows, for both experimental conditions, the mean light intensity per cell, measured by computerised image analysis, expressed as mean \pm SD * $P < 0.001$. (c) Western blot detection of NF- κ B subunit p65 in nuclear (N) and cytoplasmic (C) extracts from MDA-MB231 cells. MDA-MB231 cells were cultured for 24 h in the absence (lane 1) or the presence of PDTC (10 μ M, lane 2), or in the presence of dox (1 μ M, lane 3), and dox combined with PDTC (lane 4).

completely inhibit NF- κ B-luc activity, whereas dox increased it. This is in line with other studies, showing that celecoxib can inhibit and dox can specifically induce NF- κ B nuclear translocation and activation of its target genes. In addition we showed that, in contrast to indomethacin and NS398, celecoxib, Sc236 and PSC833 can inhibit basal and dox-induced NF- κ B activity. This is the first study that implicates NF- κ B as a molecular target for the actions of the P-gp blocker PSC833. PSC833 is in general thought to modulate cellular responses to chemotherapy exclusively via the suppression of P-gp drug pumping, although some reports already suggested the possibility that Pgp-independent effects might be involved in the chemomodulatory action of PSC833 on anthracycline cytotoxicity *in vitro* and in clinical studies.^{40–42}

Overall, the NF- κ B transfection studies were in complete concordance with findings in the MTS and dox intracellular accumulation assays. Moreover, Western blot analysis, showed that celecoxib, Sc236 and PSC833 all specifically inhibited constitutive and dox induced nuclear translocation of the p65 subunit of NF- κ B, and not the nuclear translocation of the p50 subunit, nor did these compounds affect IKK α , IKK β and I κ B- α . Inhibition of p65 translocation has been implicated in enhancing chemosensitivity of chemotherapeutic drugs^{43–45} however, the finding that only p65 is regulated is somewhat unexpected. Nonetheless, NF- κ B inhibitors, among which are PDTC, have shown to be able to specifically inhibit nuclear translocation of p65, independently of p50 and in concordance with our findings, Sc236 has been reported to suppress p65 nuclear translocation independent of IKK activity and I κ B- α gene transcription or degradation.^{46,47} The molecular mechanisms involved in this specific regulation of p65 are not well understood, but it is suggested that Sc236 may directly target proteins that facilitate the nuclear translocation of NF- κ B.

In order to further investigate the role of NF- κ B in the observed enhancement of the anti-tumour efficacy of dox, we combined the NF- κ B inhibitor PDTC with dox in the MTS and fluorescent dox accumulation assays. PDTC, which inhibited basal and dox-induced NF- κ B activity in our transfection assay, also inhibited basal and dox-enhanced p65 nuclear translocation using Western blot analysis and increased the anti-proliferative effect of dox in the MTS assay as well as the intracellular accumulation and retention of dox. The ability of NF- κ B activation to induce chemoresistance has previously been related to the escape of apoptosis, but the NF- κ B pathway is linked to many aspects of cell growth and apoptosis.^{34,45} Our results show that the inhibition of NF- κ B activation may be involved in modulating intracellular chemotherapeutic drug accumulation and/or transport. However, to irrefutably implicate NF- κ B as the sole and major target in the observed chemotherapeutic drug enhancement, further studies are warranted, as PDTC has been shown to not exclusively inhibit NF- κ B and also to be able to affect the ubiquitin-proteasome pathway.⁴⁸

Whether the enhanced intracellular dox accumulation is fully responsible for the observed, enhanced anti-tumour effect remains to be proven. The molecular mechanism linking the inhibition of NF- κ B activity with the enhanced dox accumulation and retention may be related to membrane-related actions of dox^{41,49} and is the subject of further investigations,

amongst which Suppression Subtractive Hybridisation-PCR⁵⁰ and micro array studies are planned to identify genes and biochemical pathways involved.

Taken together, our results show a new mechanism by which COX-inhibitors can overcome anticancer drug resistance and enhance chemotherapeutic drug efficacy. The molecular mechanism by which the inhibition of NF- κ B activity enhances the intracellular drug accumulation is the subject of further investigations and might have clinical implications.

Conflict of interest statement

None declared.

Acknowledgements

This study was supported by The Netherlands Organization of Scientific Research-Medical Sciences (Grant Number: PGN 902-17-090) (JvW, VWMvH and CL) and by the Dutch Cancer Foundation Koningin Wilhelmina Fonds (Grant Number: RUL2000-2196) (EvB, GvdP and CL).

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