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Modulation of Apoptosis Protein Profiles – Role of P-gp in HeLa Cells Exposed to Doxorubicin

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Abstract. *As shown previously doxorubicin (1 μM) plus sulindac (50 μM) reduced the expression of ABCB1 (ATP-binding cassette, sub-family B (MDR/TAP), member 1) mRNA in HeLa cells and this effect was accompanied by increased apoptosis. The aim of this study was to define if the decrease of ABCB1 expression or blocking of P-glycoprotein (P-gp) can affect the expression of the apoptotic genes determined with use of quantitative real time polymerase chain reaction (qRT-PCR). Western blot was used for visualization of chosen pro- and antiapoptotic proteins. Doxorubicin was the main compound which affected the apoptotic genes. The effectiveness of the drugs in reducing of P-gp function has been shown as not being related to the regulation of apoptotic gene transcription. In this experimental scheme, regulation of apoptotic gene transcription depended on the kind of P-gp modulator.*

Non steroidal anti-inflammatory drugs (NSAIDs) are cyclooxygenase inhibitors; they are analgesic and anti-inflammatory (1). Epidemiological and experimental studies have shown that cyclooxygenase-2 (COX2) inhibitors such as NSAIDs are also effective chemopreventive agents helping to reduce the risks of many types of tumor, including colon, lung, prostate and gastric cancer (2).

The mechanisms underlying the antitumor activity of COX-2 inhibitors are thought to involve inhibition of COX-2 enzyme activity and induction of apoptosis, genetically controlled mechanisms of cell death regulating tissue homeostasis (2). There are many studies which show synergistic activity of the NSAIDs, such as sulindac, meloxicam, rofecoxib, indomethacin and anthracyclines in various types of cancer cells (3-6). Therefore, NSAIDs should be investigated as a treatment supplementary to chemotherapy.

We previously demonstrated that doxorubicin (1 μM) and sulindac (50 μM) given simultaneously reduced the mRNA expression of *ABCB1* in HeLa cells and this effect was accompanied by increased apoptosis as compared to the cells exposed only to doxorubicin (7).

Sulindac may affect apoptosis indirectly through the increase of intracellular level of doxorubicin due to quenching of *ABCB1* but there is also another possibility that such a drug combination as doxorubicin plus sulindac can change the expression of apoptosis proteins at the mRNA level, as was noted by the other authors in the case of treatment of the cells with sulindac alone (8-10). Overexpression of P-gp encoded by ABCB1 mostly reduces apoptosis. Among possible mechanisms of this reduction are: efflux of apoptogens from the cells, regulation of the cytosolic levels of apoptotic mediators, such as caspase-8 or FADD. It remains possible that P-gp may inhibit caspase activation, in particular caspase-3, by regulating chloride channel activity to stabilize intracellular K^+ and Na^+ concentrations, thereby inhibiting the early steps in apoptosis and subsequent caspase activation. It has been also hypothesized that P-gp may act as a primary anti-apoptotic molecule by reducing the pool of plasma membrane sphingomyelin which is hydrolysed to apoptogenic ceramide (11, 12). Liu *et al*. (13) reported P-gp/*ABCB1* overexpression as associated with increased survivin transcription. P-gp may also regulate the expression of miRNA16 and *BCL2* but the regulation is believed to be associated with the efflux of P-gp substrates which may likely be important for P-gp function in gene expression (14). Tsang *et al*. (14) mentioned that the mechanism by which P-gp regulates gene expression is not clear. There is mounting evidence that P-gp cannot protect against caspase-independent death stimuli (14).

The aim of this study was to define if the decrease in *ABCB1* expression by SUL or blocking of P-gp by verapamil can affect the expression of the apoptosis genes.

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Materials and Methods

Drugs. Doxorubicin was purchased from medac (Hamburg, Germany); sulindac and verapamil from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Cells. The human cervix adenocarcinoma cell line – HeLa was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal serum and penicillin (5mg/ml), streptomycin (5 mg/ml), amphotericin B (12.5 mg/ml) (Lonza, Walkersville, MD, USA). All cell cultures were mycoplasma-free.

Treatment protocol. For the studies the 24-h cell cultures were co-incubated for 24 h with doxorubicin at 1 μM with/without sulindac at 50 μM with/without verapamil at 40 μM (7). The dose of verapamil was chosen as the highest non-toxic dose for HeLa cells which was confirmed with enzymatic assay based on the conversion of hydrogenated tetrazolium salt into coloured compound (Gruber *et al*., unpublished data).

Flow cytometry. The cells were exposed to the drugs as described above, then scraped very gently and centrifuged at 1000 × *g* at 4˚C for 3 min. The resulting pellets were resuspended in Hank's buffer. The cell suspensions were removed to cytometry tubes and analyzed for cellular doxorubicin fluorescence by flow cytometry. Cytometric data were measured using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose CA, USA), analyzed by CellQuest software (BD Biosciences and WinMIDI 2.9 Joseph Trotter).

RNA isolation and qRT-PCR. RNA isolation was performed as two independent repeats for each sample and was carried out as described previously (7). qRT-PCR was performed with Prime Script RT Reagent Kit (TAKARA BIO INC, Japan) using a total RNA of 500 ng application per sample including incubation at 37˚C for 30 min and stopping with heat treatment (85˚C; 5 s).

Gene expression was assessed using a two-step qRT–PCR, including reverse transcription and quantitative PCR analyses and was performed with Mx3005P qPCR System (Stratagene, La Jolla, CA, USA). qPCR was carried out with SYBR Premix Ex Taq (TAKARA) with the application of 400 ng cDNA template into a sample-specific working solution prepared for 96-well StellArray plate according to Lonza Inc. (US) recommendations (15). Cycling parameters included preincubation at 50˚C for 2 min, initial polymerase activation at 95˚C for 30 s and amplification of 40 cycles (15 s at 95˚C and 1 min at 60˚C). For evaluation of specificity of the PCR products, a melting curve analysis of the amplification products was carried out by additional cycle at the end of amplification process (1 min at 95˚C, 30 s at 55˚C and 30 s at 95˚C). Before StellArrays, the amount of cDNA was normalized with that for β-actin (*ACTB*) gene. The primer sequences for *ACTB* gene were designed by Beacon Designer v.7.7 delivered by Beacon Designer 7 Software (PREMIER Biosoft International, Palo Alto, CA, USA) and included sequences: forward *ACTB*: 5'-TCGT GCGTGACATTAAGGAG-3'; and reverse *ACTB*: 5'-GAAGGAAG GCTGGAAGAGTG-3'. A variation between the CT values for ACTB, *i.e*. max. 1.0 was acceptable for the cDNA samples.

Gene expression. Evaluation of the results was carried out with Global Pattern Recognition™ Software (Bar Harbor Biotechnology, Inc. Trenton, ME, USA). For each gene tested a Global Pattern Recognition (GPR) fold change was calculated. The results with *p*-value ≤0.05 were accepted as being significant.

The panel of the genes analyzed in this study is presented in Table I.

Western blot. The cytoplasmic extracts preparation and western blot were proceeded as described by Gruber *et al*. (16). The assessed proteins were detected with use of primary monoclonal rabbit Ab for: BAX, 21 kDa (1:500, Epitomics, Burlingame, CA, USA); BCL-2, 26 kDa (1:250, Epitomics); Survivin, 16 kDa (1:250, Epitomics); primary monoclonal mouse Ab for β-actin, 42 kDa (1:1500, Abcam, Cambridge, UK) and with secondary goat anti-rabbit Ab (1:500, Epitomics) and goat anti-mouse Ab (1:500, Sigma, Saint Louis, MO, USA).

Results

Flow cytometry. To study the effectiveness of sulindac and verapamil as P-gp down-regulators, doxorubicin retention within cells was analyzed. When doxorubicin, as a fluorescent substrate, diffuses into the cells, P-gp actively pumps out the fluorochrome. Use of inhibitor along with the fluorescent marker increases the intensity of fluorescence which can be quantitatively analyzed. As shown in Figure 1, verapamil was the most effective for reducing P-gp function. The highest doxorubicin retention, reported as the highest value of fluorescence, was observed in the cells co-incubated with verapamil at 40 μM. Similar doxorubicin retention was shown after combination with doxorubicin (1 μM) plus verapamil (40 μM) plus sulindac (50 μM). The lowest doxorubicin retention was observed in the cells treated with sulindac 50 μM alone.

qRT-PCR. As shown in Table II combined treatment of HeLa cells with doxorubicin at 1 μM alone or in combination with sulindac at 50 μM and/or verapamil at 40 μM affected some genes related directly to apoptosis. All mRNA levels refer to the mRNA level determined in the intact cells.

24-hours exposure of HeLa cells to doxorubicin at 1 μM caused significant up-regulation of five out of 96 tested genes related to apoptosis, namely, *BCL6* (ca. 30-fold), *CD40* (ca. 60-fold); *FASLG* (above 230-fold), TNF (ca. 60-fold), *TNFAIP3* (called also *A20*) (160-fold) (Table II). Sulindac at 50 μM alone slightly induced overexpression of *TNF* gene only (more than 25-fold), similarly to verapamil at 40 μM (ca. 30-fold). Verapamil also induced A20 expression by over 50-fold. Sulindac plus verapamil led to overexpression of three genes: *FASLG* (20-fold), TNF (ca. 70-fold) and A20 (over 20-fold).

Combined treatment of HeLa cells with doxorubicin (1 μM) plus sulindac (50 μM) or with doxorubicin (1 μM) plus verapamil (40 μM) significantly induced the same genes as doxorubicin, although doxorubicin plus sulindac and doxorubicin plus verapamil additionally induced *GADD45G*

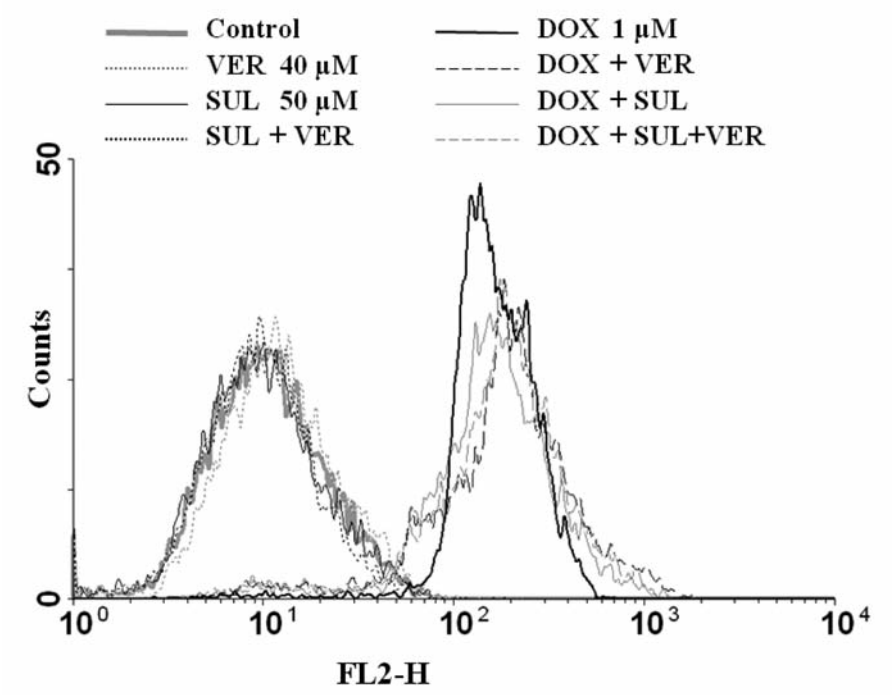

Figure 1. *Doxorubicin (DOX) retention in HeLa cells measured with flow cytometry. 24-H cell cultures were treated for 24 h with: culture medium (control); sulindac (SUL) 50 μM; verapamil (VER) 40 μM; SUL 50 μM + VER 40 μM; DOX 1 μM; DOX 1 μM + SUL 50 μM; DOX 1 μM + VER 40 μM; DOX 1 μM + SUL 50 μM + VER 40 μM.*

(over 130-fold and 60-fold, respectively) and *LTA* (ca. 30-fold and 25-fold respectively). It is worth noting that doxorubicin plus verapamil were weaker inducers of mRNA expression than doxorubicin plus sulindac. Under doxorubicin plus verapamil only *BCL6* and *LTA* expressed similar mRNA levels as those affected by doxorubicin plus sulindac. Both drug combinations strongly induced *TNF* and *A20* expression (over 200- and ca. 1800-fold for doxorubicin plus sulindac or ca. 150 and over 1300-fold for doxorubicin plus verapamil, respectively).

Simultaneous treatment of the cells with all three drugs significantly affected the same genes, except *LTA*, which was induced by doxorubicin plus sulindac and doxorubicin plus verapamil. The level of mRNA expression after exposure to the triple drug combination was the highest for *A20* gene (over 2000-fold) as compared to the control. In the case of the other genes, combination of three drugs was shown to have similar effect to doxorubicin plus verapamil. None of the drugs caused any significant decreases in any gene mRNA expression. All results presented in Table II were statistically significant ($p<0.05$).

Western blot. As was shown in Figure 2, exposure of cells to tested drugs, did not change the expression levels of BAX and BCL2. Most significant changes were noted for survivin levels: the highest expression of this protein was observed in the cells exposed to doxorubicin (1 μM) plus sulindac (50 μM) plus verapamil (40 μM), with significant increase also occurring after treatment with sulindac at 50 μM alone, after doxorubicin combined with sulindac at 50 μM and verapamil at 40 μM separately.

Discussion

On the basis of gene expression profiles tested in this study, it can be said that doxorubicin at 1 μM generates apoptosis through death receptors. This drug causes overexpression of genes encoding FASLG, CD40 and TNF. All three proteins belong to the TNF receptor superfamily members, which points to the extrinsic pathway of apoptosis being induced by doxorubicin. As was noted, doxorubicin at 1 μM after 24-h treatment strongly induced also the expression of *A20*, which is connected with proapoptotic function through NFκB inhibition or antiapoptotic through, among others, inhibition of p53 or BAX expression (17). Overexpression of *A20* upon treatment with doxorubicin at 1 μM, which was rather strong (ca. 160-fold) seems to be related to induction of apoptosis noted in HeLa cells (7) as well as that of *FASLG* gene, which reached a much higher level after doxorubicin treatment than A20 (above 230-fold). On the other hand,

Table I. *Panel of 96 genes analyzed with StellArray.*

Gene ID	Gene symbol	Function
207	*AKT1*	v-Akt Murine thymoma viral oncogene homolog 1
317	*APAF1*	Apoptotic peptidase activating factor 1
329	*BIRC2*	Baculoviral IAP repeat containing 2
330	*BIRC3*	Baculoviral IAP repeat containing 3
331	*XIAP*	X-linked inhibitor of apoptosis
332	*BIRC5*	Baculoviral IAP repeat containing 5
355	*FAS*	Fas (TNF receptor superfamily, member 6)
356	*FASLG*	Fas ligand (TNF superfamily, member 6)
572	*BAD*	BCL2-associated agonist of cell death
573	*BAG1*	BCL2-associated athanogene
578	*BAK1*	BCL2-antagonist/killer 1
581	*BAX*	BCL2-associated X protein
596	*BCL2*	B-Cell CLL/lymphoma 2
598	*BCL2L1*	BCL2-like 1
599	*BCL2L2*	BCL2-like 2
604	*BCL6*	B-Cell CLL/lymphoma 6
637	*BID*	BH3 interacting domain death agonist
638	*BIK*	BCL2-interacting killer (apoptosis-inducing)
662	*BNIP1*	BCL2/adenovirus E1B 19kDa interacting protein 1
663	*BNIP2*	BCL2/adenovirus E1B 19kDa interacting protein 2
664	*BNIP3*	BCL2/adenovirus E1B 19kDa interacting protein 3
665	*BNIP3L*	BCL2/adenovirus E1B 19kDa interacting protein 3-like
666	*BOK*	BCL2-related ovarian killer
835	*CASP2*	Caspase 2, apoptosis-related cysteine peptidase
836	*CASP3*	Caspase 3, apoptosis-related cysteine peptidase
840	*CASP7*	Caspase 7, apoptosis-related cysteine peptidase
841	*CASP8*	Caspase 8, apoptosis-related cysteine peptidase
842	*CASP9*	Caspase 9, apoptosis-related cysteine peptidase
958	*CD40*	CD40 molecule, TNF receptor superfamily member 5
1191	*CLU*	Clusterin
1603	*DAD1*	Defender against cell death 1
1611	*DAP*	Death-associated protein
1612	*DAPK1*	Death-associated protein kinase 1
1649	*DDIT3*	DNA-damage-inducible transcript 3
1676	*DFFA*	DNA fragmentation factor, 45kDa, alpha polypeptide
1677	*DFFB*	DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase)
54205	*CYCS*	Cytochrome c, somatic
2021	*ENDOG*	Endonuclease G
2261	*FGFR3*	Fibroblast growth factor receptor 3
2309	*FOXO3*	Forkhead box O3
2876	*GPX1*	Glutathione peroxidase 1
2932	*GSK3B*	Glycogen synthase kinase 3 beta
3091	*HIF1A*	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
3303	*HSPA1A*	Heat shock 70 kDa protein 1A
3479	*IGF1*	Insulin-like growth factor 1 (somatomedin C)
4049	*LTA*	Lymphotoxin alpha (TNF superfamily, member 1)
4055	*LTBR*	Lymphotoxin beta receptor (TNFR superfamily, member 3)
4170	*MCL1*	Myeloid cell leukemia sequence 1 (BCL2-related)
4671	*NAIP*	NLR family, apoptosis inhibitory protein
4790	*NFKB1*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
4982	*TNFRSF11B*	Tumor necrosis factor receptor superfamily, member 11b
5074	*PAWR*	PRKC, apoptosis, WT1, regulator
5366	*PMAIP1*	Phorbol-12-myristate-13-acetate-induced protein 1
5728	*PTEN*	Phosphatase and tensin homolog
5885	*RAD21*	RAD21 homolg *(S. pombe)*
5977	*DPF2*	D4,zinc and double PHD fingers family 2
6776	*STAT5A*	Signal transducer and activator of transcription 5A
6777	*STAT5B*	Signal transducer and activator of transcription 5B

Table I. *Continued*

Table I. *Continued*

Gene ID	Gene symbol	Function
7001	*PRDX2*	Peroxiredoxin 2
7040	*TGFB1*	Transforming growth factor, beta 1
7124	*TNF*	Tumor necrosis factor
7128	*TNFAIP3*	Tumor necrosis factor, alpha-induced protein 3
7132	*TNFRSF1A*	Tumor necrosis factor receptor superfamily, member 1A
7133	*TNFRSF1B*	Tumor necrosis factor receptor superfamily, member 1B
7157	*TP53*	Tumor protein p53
7186	*TRAF2*	TNF receptor-associated factor 2
7422	*VEGFA*	Vascular endothelial growth factor A
8539	*API5*	Apoptosis inhibitor 5
8717	*TRADD*	TNFRSF1A-associated *via* death domain
8737	*RIPK1*	Receptor (TNFRSF)-interacting serine-threonine kinase 1
8739	*HRK*	Harakiri, BCL2 interacting protein (contains only BH3 domain)
8772	*FADD*	Fas (TNFRSF6)-associated *via* death domain
8797	*TNFRSF10A*	Tumor necrosis factor receptor superfamily, member 10a
8915	*BCL10*	B-Cell CLL/lymphoma 10
9530	*BAG4*	BCL2-associated athanogene 4
9531	*BAG3*	BCL2-associated athanogene 3
9618	*TRAF4*	TNF receptor-associated factor 4
10017	*BCL2L10*	BCL2-like 10 (apoptosis facilitator)
10018	*BCL2L11*	BCL2-like 11 (apoptosis facilitator)
10912	*GADD45G*	Growth arrest and DNA-damage-inducible, gamma
23411	*SIRT1*	Sirtuin 1
9131	*AIFM1*	Apoptosis-inducing factor, mitochondrion-associated, 1
23604	*DAPK2*	Death-associated protein kinase 2
27113	*BBC3*	BCL2 binding component 3
27429	*HTRA2*	HtrA serine peptidase 2
28996	*HIPK2*	Homeodomain interacting protein kinase 2
29775	*CARD10*	Caspase recruitment domain family, member 10
51283	*BFAR*	Bifunctional apoptosis regulator
56616	*DIABLO*	Diablo, IAP-binding mitochondrial protein
57099	*AVEN*	Apoptosis, caspase activation inhibitor
64065	*PERP*	PERP, TP53 apoptosis effector
79370	*BCL2L14*	BCL2-like 14 (apoptosis facilitator)
94241	*TP53INP1*	Tumor protein p53 inducible nuclear protein 1
219699	*UNC5B*	Unc-5 homolog B (*C. elegans*)

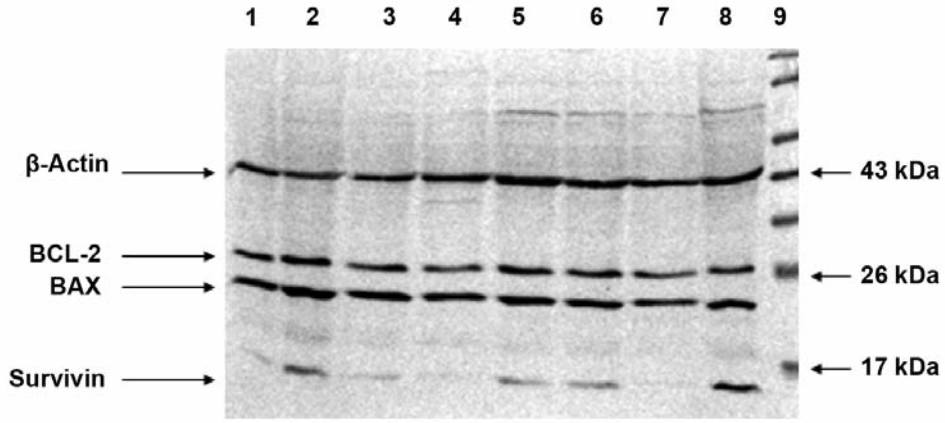

Figure 2. *Western blot. Cytoplasmic extract. 24-H cultured HeLa cells were exposed for 24 h as follows: Lane 1: control medium; lane 2: sulindac (SUL) 50 μM; lane 3: verapamil (VER) 40 μM; lane 4: doxorubicin (DOX) 1 μM; lane 5: DOX 1 μM + VER 40 μM; lane 6: DOX 1 μM + SUL 50 μM; lane 7: SUL 50 μM + VER 40 μM; lane 8: DOX 1 μM + SUL 50 μM + VER 40 μM; lane 9: Mw (kDa).*

Table II. *Apoptosis gene expression up–regulated by 24-h treatment of HeLa cells with sulindac (SUL) at 50 μM, verapamil (VER) at 40 μM alone or doxorubicin (DOX) 1 μM alone and in their different combinations.*

Drug	Gene	Function	GPR fold-change	*p*-Value
DOX 1 μM	*BBC3*	BCL2 binding component 3	3.10	0.028
	BCL2L14	BCL2-like 14 (apoptosis facilitator)	2.13	0.041
	BCL6	B-Cell CLL/lymphoma 6	30.53	0.002
	BIK	BCL2-interacting killer (apoptosis-inducing)	2.66	0.042
	BIRC3	Baculoviral IAP repeat containing 3	4.96	0.007
	CASP3	Caspase 3, apoptosis-related cysteine peptidase	3.13	0.016
	CASP7	Caspase 7, apoptosis-related cysteine peptidase	3.78	0.014
	CD40	CD40 molecule, TNF receptor superfamily member 5	58.8	0.000
	CLU	Clusterin	7.70	0.011
	DAPK1	Death-associated protein kinase 1	6.88	0.013
	DAPK2	Death-associated protein kinase 2	3.12	0.038
	FAS	Fas (TNF receptor superfamily, member 6)	3.18	0.015
	FASLG	Fas ligand (TNF superfamily, member 6)	232.67	0.000
	FGFR3	Fibroblast growth factor receptor 3	5.53	0.004
	GPX1	Glutathione peroxidase 1	2.66	0.037
	HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	4.21	0.012
	TNF	Tumor necrosis factor	59.98	0.007
	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	160.12	0.000
	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	2.98	0.029
	TP53INP1	Tumor protein p53 inducible nuclear protein 1	9.52	0.000
	UNC5B	Unc-5 homolog B (*C. elegans*)	5.44	0.020
SUL 50 μM	*TNF*	Tumor necrosis factor (TNF superfamily, member 2)	27.84	0.000
VER 40 μM	*BAG1*	BCL2-associated athanogene	2.39	0.041
	BCL2L1	BCL2-like 1	4.95	0.010
	BID	BH3 interacting domain death agonist	2.31	0.027
	DAP	Death-associated protein	2.36	0.018
	HRK	Hara-kiri, BCL2 interacting protein (contains only BH3 domain)	4.87	0.035
	TNF	Tumor necrosis factor (TNF superfamily, member 2)	29.19	0.001
	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	51.74	0.001
	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	16.97	0.00
SUL 50 μM + VER 40 μM	*FASLG*	Fas ligand (TNF superfamily, member 6)	20.04	0.001
	TNF	Tumor necrosis factor (TNF superfamily, member 2)	71.47	0.000
	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	22.00	0.001
	CD40	CD40 molecule, TNF receptor superfamily member 5	9.80	0.004
	DAP	Death-associated protein	2.03	0.038
	FGFR3	Fibroblast growth factor receptor 3	3.50	0.012
	HRK	Hara-kiri, BCL2 interacting protein (contains only BH3 domain)	3.35	0.047
DOX 1 μM + SUL 50 μM	*BCL2L10*	BCL2-like 10 (apoptosis facilitator)	6.85	0.02
	BCL2L14	BCL2-like 14 (apoptosis facilitator)	8.92	0.005
	BCL6	B-Cell CLL/lymphoma 6	36.35	0.000
	CD40	CD40 molecule, TNF receptor superfamily member 5	83.70	0.000
	CLU	Clusterin	6.98	0.015
	DAPK1	Death-associated protein kinase 1	5.85	0.021
	FAS	Fas (TNF receptor superfamily, member 6)	5.87	0.012
	FASLG	Fas ligand (TNF superfamily, member 6)	89.07	0.000
	FGFR3	Fibroblast growth factor receptor 3	8.86	0.002
	GADD45G	Growth arrest and DNA-damage-inducible, gamma	134.76	0.000
	HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	4.02	0.016
	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	28.75	0.002
	PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	4.65	0.010
	TNF	Tumor necrosis factor (TNF superfamily, member 2)	221.75	0.000
	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	1794.55	0.000
	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	3.79	0.028

Table II. *Continued*

Table II. *Continued*

Drug	Gene	Function	GPR fold-change	*p*-Value
	TP53INP1	Tumor protein p53 inducible nuclear protein 1	5.91	0.009
	TRADD	TNFRSF1A-associated *via* death domain	5.36	0.013
	UNC5B	Unc-5 homolog B (*C. elegans*)	13.06	0.004
DOX 1 μM +	*BCL2L10*	BCL2-like 10 (apoptosis facilitator)	5.21	0.014
VER 40 μM	*BCL2L14*	BCL2-like 14 (apoptosis facilitator)	543.00	0.014
	BCL6	B-Cell CLL/lymphoma 6	30.38	0.01
	CD40	CD40 molecule, TNF receptor superfamily member 5	44.09	0.001
	CLU	Clusterin	5.23	0.015
	DAPK1	Death-associated protein kinase 1	5.73	0.019
	FAS	Fas (TNF receptor superfamily, member 6)	3.06	0.033
	FASLG	Fas ligand (TNF superfamily, member 6)	79.85	0.000
	FGFR3	Fibroblast growth factor receptor 3	5.00	0.017
	GADD45G	Growth arrest and DNA-damage-inducible, gamma	59.61	0.000
	HRK	Hara-kiri, BCL2 interacting protein (contains only BH3 domain)	6.31	0.010
	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	25.12	0.002
	PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	4.26	0.019
	TNF	Tumor necrosis factor (TNF superfamily, member 2)	151.16	0.000
	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	1349.50	0.000
	TRADD	TNFRSF1A-associated *via* death domain	4.21	0.010
	UNC5B	Unc-5 homolog B (*C. elegans*)	6.54	0.017
DOX 1 μM +	*BCL2L10*	BCL2-like 10 (apoptosis facilitator)	4.26	0.023
SUL 50 μM +	*BCL2L14*	BCL2-like 14 (apoptosis facilitator)	5.00	0.012
VER 40 μM	*BCL6*	B-Cell CLL/lymphoma 6	54.34	0.004
	CD40	CD40 molecule, TNF receptor superfamily member 5	39.66	0.010
	CLU	Clusterin	5.00	0.025
	DAPK1	Death-associated protein kinase 1	3.77	0.036
	FASLG	Fas ligand (TNF superfamily, member 6)	53.76	0.002
	FGFR3	Fibroblast growth factor receptor 3	7.02	0.005
	GADD45G	Growth arrest and DNA-damage-inducible, gamma	67.75	0.001
	HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	3.48	0. 029
	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	16.30	0.004
	TNF	Tumor necrosis factor (TNF superfamily, member 2)	147.24	0.000
	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	2227.07	0.000
	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	2.93	0.041
	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	17.66	0,042
	TP53INP1	Tumor protein p53 inducible nuclear protein 1	4.96	0.015
	TRADD	TNFRSF1A-associated *via* death domain	2.71	0.029
	UNC5B	Unc-5 homolog B (*C. elegans*)	10.21	0.011

stimulation of *A20* mRNA by non-apoptogenic agents verapamil or verapamil plus sulindac is rather related to cytoprotective effect of A20 induced upon *TNF* and *FASLG* stimulation. As was presented by Verstrepen *et al*. (17), the role of A20 in tumorigenesis might be cell type- and apoptogen- dependent. As was shown by the authors, this gene protected endothelial cells against apoptosis induced by TNF and FAS triggering, whereas in A549, HepG2 and HeLa cells, it was not able to block TNF-induced apoptosis.

The results obtained with doxorubicin in qRT-PCR and in western blot are in agreement with Suzuki *et al*. (18), who noted that doxorubicin did not significantly change the intracellular concentrations of BCL2, BAX and BAD in HL-60 cells, although the treatment time in that case was much shorter (6 h) than in the present study (24 h). Lüpertz *et al*. (19) reported the increase of BAX in colon cancer cells under treatment with doxorubicin at 1 μM but given as a bolus (3 h). The authors suggested that doxorubicin is able to induce cell death by apoptosis only at particular doses and under specified treatment conditions, and imply a completely different cellular response following bolus or continuous exposure to DOX.

As was shown earlier (7), combined treatment of HeLa cells with doxorubicin plus sulindac enhanced apoptotic

processes and this event is reflected in the gene expression profiles. Doxorubicin plus sulindac caused strong induction of proapoptotic *TNF* and *GADD45G* genes. *A20* gene expression also reached a much higher level (ca. 1800-fold) as compared to the mRNA level in untreated cells. mRNA fold change of *A20* was positively correlated with apoptosis observed in HeLa cells treated with doxorubicin and doxorubicin plus sulindac (7). It suggests a proapoptotic function of *A20*, which may be the specific response of HeLa cells to doxorubicin or doxorubicin plus sulindac exposure. Increases in *A20* mRNA expression alongside P-gp modulator addition indicate the dependence of the level of mRNA expression of the genes affected on intracellular doxorubicin concentration.

The slight influence of sulindac on the transcription of the genes related to apoptosis is consistent with the effects reported by Gruber *et al*. (7), *i.e*. sulindac alone at the concentration of 50 μM was not apoptogenic in HeLa cells and overexpression of *TNF* which has been evoked as the effect of sulindac has no significance. Most literature data indicate the apoptogenic functions of sulindac. Liu *et al*. (20) noted such a process in HepG2 cells, Park *et al*. (21) in HT-29 cells and Scheper *et al*. (22) in oral squamous carcinoma (SCC) cells. In turn, Wu *et al*. (23) noted apoptosis induced by sulindac depended on the differentiation of the cells. Han *et al*. (24) showed that induction of apoptosis by sulindac is cell dependent. The authors treated breast cancer cells with sulindac at 1600 μM for 48 h and no apoptotic processes were reported. Worth noting is the fact that the gene expression profile depends on the dose and time of exposure of the cells to the drug. In our study, sulindac was used at 50 μM for 24 h and under such conditions, this drug was not apoptogenic. On the other hand, sulindac used by the other authors at the concentration range of: 100-4000 μM for 48 or 72 h did affect antiapoptotic and proapoptotic genes and these effects were reflected in apoptosis induction in neoplastic cells by sulindac alone (8, 9, 22, 23). The effect of sulindac frequently pointed out by the other authors, *i.e*. down-regulation of survivin gene (9, 10, 22) was not noted in our study. On the contrary, western blot showed strong activation of this protein under non-apoptogenic dose of sulindac which suggests some posttranslational changes at the protein level but this is unclear. As was shown in a previous study (7), sulindac significantly attenuated the expression of *ABCB1* and the same P-gp membrane pump. The are some reasons for which we suggest that intensified apoptosis noted under treatment with doxorubicin plus sulindac and the influence of these drug combination on apoptosis genes expression may be related rather to doxorubicin retention inside the cells than with the direct correlation between P-gp modulation and apoptotic genes expression. These reasons are: the lack of significant influence of sulindac on the mRNA levels of apoptosis genes; the fact that verapamil as the most effective down-regulator of P-gp slightly affected the apoptosis gene profile; sulindac or verapamil alone, or the combination of these drugs did not affect the same genes; doxorubicin plus verapamil were weaker inducers of mRNA expression than doxorubicin plus sulindac. The results obtained in this study are inconsistent with these of Tsang *et al*. (14), who noted that *ABCB1* expression correlated with *BCL2* in hepatocellular carcinoma cells. Notarbartolo *et al*. (25) and Liu *et al*.(26) observed the decrease of survivin (*BIRC5*) and *IAP* mRNA under verapamil treatment. None of these effects were noted in this study.

In this study, the main compound which affected the apoptosis genes was doxorubicin. Sulindac and verapamil only modified the level of the mRNA expression in the same genes. The effectiveness of the drugs in reducing P-gp function does not appear to be related to the regulation of transcription of apoptosis genes.

It can be concluded that in this experimental scheme, the efficiency of P-gp modulators such as sulindac and verapamil in regulation of transcription of apoptosis genes depends on the intracellular apoptogen retention which is directly related to the the kind of the P-gp modulator.

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