Acta Poloniae Pharmaceutica – Drug Research, Vol. 67 No. 6 pp. 620–624, 2010 ISSN 0001-6837
Polish Pharmaceutical Society

POSSIBLE ROLE OF HSP60 IN SYNERGISTIC ACTION OF ANTHRACYCLINES AND SULINDAC IN HeLa CELLS

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Abstract: As was observed in the earlier studies, doxorubicin (DOX) induced apoptosis in HeLa cells and that effect was potentiated significantly by sulindac (SUL). The aim of the current work was to study the effects of DOX and SUL on *HSP60*, *HSF1* and HSP60 expression and the influence of DOX and SUL on HSP60 translocation. Expression of *HSP60* and *HSF1* was determined with QRT-PCR; the expression and localization of HSP60 were evaluated with Western blot. The 24-h cell cultures were co-incubated with DOX – 1 μM and/or SUL – 50 μM. The significant induction of *HSF1* and *HSP60* mRNA level was observed after exposure of the cells to DOX 1 μM. SUL 50 μM alone caused moderate increase in mRNA level. The significant decrease in expression of *HSF1* and *HSP60* was noted after DOX 1 μM and SUL 50 μM simultaneous treatment. HSP60 appeared in the higher levels in cytosol than in mitochondria No intracellular translocation was noted under treatment of the cells to DOX and/or SUL. In conclusion, the effects of *HSP60* and *HSF1* evoked in the cells depend on the inducer; proapoptotic action of DOX + SUL may correlate to the increased expression of *HSF1* and *HSP60*; *HSP60* mRNA level and the regulation of that protein expression depend on the apoptotic inducer; the role of HSP60 in apoptosis expressed in potential shift between mitochondria and cytosol is determined by the apoptotic inducer and the cell type.

Keywords: HSP60, *HSF1*, apoptosis, doxorubicin, sulindac

Most heat shock proteins (HSPs) have pro-survival fuctions. However, the role of HSP60, a mitochondrial matrix protein, is somewhat controversial with both pro-survival and pro-apoptotic functions reported. As was shown, in apoptosis induced by hydroxamic acid compound HSP60 accumulates in the cytosol with significant mitochondrial release. In contrast, in apoptosis induced by multiple other inducers, the cytosolic HSP60 accumulates without an apparent mitochondrial release (1).

HSP60 improves the vulnerability of pro-caspase-3 to proteolytic maturation and this represents an important regulatory event in apoptosis (1–3). As was noted by Samali et al. (3), the release of HSPs from mitochondria occurs simultaneously with the release of other proteins as cytochrome *c* prior to a loss of mitochondrial transmembrane potential.

The heat shock transcription factors (HSFs) were originally characterized as regulators of the expression of *HSP* genes. Among the family of HSFs, i.e., HSF1, 2 and 4, HSF1 is specifically responsible for the stress-mediated HSP induction (4).

NSAIDs are cyclooxygenase inhibitors; they are analgesic and anti-inflammatory (5). Epidemiologic and experimental studies have shown that COX-2 inhibitors such as NSAIDs are also effective chemopreventive agents, that help to reduce the risks of many types of tumors, including colon, lung, prostate and gastric cancers (6).

The mechanisms underlying the antitumor activity of COX-2 inhibitors is thought to involve inhibition of COX-2 enzyme activity and induction of apoptosis, genetically controlled mechanism of cell death regulating tissue homeostasis (6–9). There are many studies which show synergistic activity of the NSAIDs, such as SUL, meloxicam, rofecoxib, indomethacin and anthracyclines in various types of the cancer cells (10–13). Therefore, NSAIDs should be investigated as a treatment supplementary to chemotherapy.

As was observed in the earlier studies (14), doxorubicin (DOX) induced apoptosis in HeLa cells and that effect was potentiated significantly by sulindac (SUL) which was confirmed with TUNEL assay.

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The aim of the current work was to study the mechanisms of synergistic action in apoptosis induction observed previously in human cervix carcinoma cells – HeLa under DOX and SUL co-treatment (14). The following topics were considered:

- effects on DOX and SUL on *HSP60, HSF1,* and HSP60 expression;
- influence of DOX and SUL on HSP60 translocation.

Expression of *HSP60* and *HSF1* expression was determined with QRT-PCR; the HSP60 expression and localization of HSP60 in the cells were evaluated with Western blot.

EXPERIMENTAL

Drugs

DOX was purchased from Fluka (Germany); SUL from Sigma, USA.

Cells

Human cervix carcinoma cell line – HeLa was purchased from American Tissue Culture Collection. Cells were grown in MEM supplemented with 10% fetal serum and antibiotic, antimycotic (10000 u. penicillin; 10 mg streptomycin, 25 μg amphotericin B per mL) (Sigma, USA). All cell cultures were mycoplasma-free.

Treatment protocol

For the studies on HSP60 induction and localization, the 24-h cell cultures were co-incubated with the chosen earlier (14) concentrations of the tested compounds, i.e., DOX – 1 μM and/or SUL – 50 μM for 24 h.

Protein extracts preparation

To obtain the cytoplasmic, nuclear and mitochondrial extracts the method of cell fractionation by differential centrifugation was used.

The cells were washed with cold PBS, scraped very gently, spinned and washed repeatedly at 12000 rpm for 3 min. The cell pellet was resuspended in buffer CEB (10 mM Tris-HCl pH 7.9; 60 mM KCl; 1 mM EDTA; 1 mM DTT, 0.4% Nonidet P-40) with a mixture of protease inhibitors (1 μg/mL leupeptin, 1 μg/mL pepstatin, 2 μg/mL aprotinin, 0.5 mg/mL benzamidine, 0.5 mM PMSF). Incubation took 15 min on ice with periodic vortexing. Unbroken cells and nuclei were removed by centrifuging the homogenates at 700 rpm for 10 min at 4°C (centrifugation No. 1; supernatant A; pellet A). The resulting supernatant A was centrifuged at 6600 rpm for 30 min at 4°C (centrifugation No. 2; supernatant B; pellet B). The received pellet A was resuspended in buffer NEB (20 mM Tris-HCl pH 7.9; 0.4 M NaCl; 1.5 mM $MgCl_2$; 1.5 mM EDTA; 1 mM DTT, 25% glycerin) with a mixture of protease inhibitors (1 μg/mL leupeptin, 1 μg/mL pepstatin, 2 μg/mL aprotinin, 0.5 mg/mL benzamidine, 0.5 mM PMSF). Incubation was as above. After centrifugation for 12000 rpm for 5 min at 4°C (centrifugation No. 3; supernatant C; pellet C) supernatant C – the nuclear extract, was obtained. After centrifugation No. 2, the purified mitochondria were separated from cytosol. The resulting supernatant B was the cytosol extract. Mitochondrial pellet B was resuspended in buffer TNC (10 mM Tris-HCl pH 7.9; 5 mM $CaCl_2$, 0.5% Nonidet P-40) with a mixture of protease inhibitors. The resulting suspension was the mitochondrial extract. The protein extracts were stored at –70°C.

Western blot

Samples containing 30 μg protein were combined with Laemmli buffer 1:1 and subjected to 12% SDS-polyacrylamide gel electrophoresis (110 V, 1 h, MGV-402, CBS Scientifical Company). Subsequently, proteins were transferred onto nitrocellulose membranes (Trans-Blot 0.45 μm) using Minitrans system according to the instruction manual. Blots were washed and the transfer was checked with Ponceau S (Sigma). Blots were blocked in PBS buffer containing 0.1 % Tween-20 (PBS-T) and 5% non-fat milk for 1.5 h. Next, the blots were incubated with primary monoclonal mouse Ab for: Hsp60 (1:60000, Abcam, 1 h, 4°C), for lactate dehydrogenase (LDH) (1:200, Abcam, overnight, 4°C), for β-actin (1:3000, Abcam, overnight, 4°C), for voltage-dependent anion channel (VDAC) (1:300, Abcam, overnight, 4°C). Subsequently, after washing with PBS-T (3×10 min), the blots were incubated with secondary anti-mouse Ab (1:500, Sigma, 1 h, 20°C). Bands were detected with Horseradish Peroxidase Assay Kit (Bio-Rad) according to the instruction manual. Densitometric analysis of band patterns was performed using Gel Doc 2000 (Bio-Rad).

QRT-PCR

RNA isolation

RNA was extracted from HeLa cells using the Total RNA Mini Kit (A&A Biotechnology, Gdynia, Poland; www.aabiot.com) according to the manufacturer's protocol. Cells were harvested immediately after incubation. Before RNA isolation, cell cultures were initially washed with phosphate buffered saline solution (PBS) and then 1×10^6 cells per minicolumn were used. Finally, RNA samples were sus-

pended by RNase free H_2O and stored at -80°C. The RNA quality was preliminarily assessed as described previously (14). RNA templates before reverse transcription were additionally digested by DNase I (Fermentas UAB, Lithuania www.fermentas.com) according to Fermentas protocol for preparation of DNA-free RNA.

Quantitative real-time PCR

Gene expression was performed using a two-step QRT-PCR, including reverse transcription (RT) and quantitative PCR (QPCR) analyses and were performed with Mx3005P QPCR System (Stratagene, La Jolla, CA, USA). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used for RT according to the manufacturer's protocol. 500 ng of each RNA sample per 20 µL reaction volume were applied. QPCR was carried out with Fast SYBR Green master Mix (Applied Biosystems, USA) according to the manufacturer's recommendations. Cycling parameters comprised an initial polymerase activation: the 1st step (20 s at 95°C), the 2nd-step cycling for 40 cycles (3 s at 95°C and 30 s at 60°C). For specificity of the PCR products, a melting curve analysis of the amplification products was carried out by adding the 3th-step cycling at the end of amplification process (1 min at 95°C, 30 s at 55°C and 30 s at 95°C). SYBR green assays were performed using oligonucleotides primer pairs for *HSP60* gene (111 bp fragment) including sequences for Fw (forward) HSP60: 5'-TGCCAATGCTCACCGTAAG-3'; Rv (reverse) HSP60: 5'-ACTGCCACAACCTGAAGAC-3' and for *HSF1* gene (113 bp fragment) including sequences for Fw HSF1: 5'- GACCCATCATCTC-CGACATC-3' and Rv HSF1: 5'- CTCCTTGA-CACGCACCAG-3'. As a reference gene, *ACTB* gene, encoded β-actin was applied. Sequences for Fw ACTB1:5'-TCGTGCGTGACATTAAGGAG-3' and Rv ACTB1: 5'- GAAGGAAGGCTGGAA-GAGTG-3' primers for a 176 bp fragment were used. Pairs of primers for *HSP60*, *HSF*1 and *ACTB* genes amplification were designed using Beacon Designer program (Premier Biosoft Int.). Twenty-microliters reactions were optimized with Fast SYBR Green master Mix and cDNA inputs equivalent to the following amounts of total RNA 25 ng and 150 nM of HSP60, HSF1 and ACTB1 each forward and reverse primers. *ACTB* gene transcript level was used as a reference (normalizer). The relative quantity of *HSP60, HSF1* genes called as genes of interest (GOI) were calculated by individual comparison of its quantity achieved in HeLa treatment variants to the calibrator (untreated HeLa transcripts) and included calculation of the differences between the threshold cycle (Ct) of the normalizer assay and Ct of the GOI for each sample according to the formula $2^{-(DDCt)}$ (15). DDCt was defined as $(Ct_{GOI}-Ct_{norm})_{calibrator} - (Ct_{GOI}-Ct_{norm})_{unknown}$ (1). Before these experiments, assay optimization for all three genes was performed. Standard curves were generated using relative concentration *vs.* the threshold cycle. Primer efficiencies were evaluated by running standard curves with input amounts ranging between 100 ng and 0.1 ng including ten-fold dilution of RNA template by using the following equation: *E* (Efficiency) $=10^{(-1/slope)} -1$ (15, 16). The linear correlation coefficient (*Rsq*) of all these three genes ranged from 0.991 to 0.993. Based on the slopes of the standard curves, the amplification efficiencies of the standards ranged from 97.6% to 107.2%, (derived from the formula $E = 10^{-1/slope} - 1$) (15, 16).

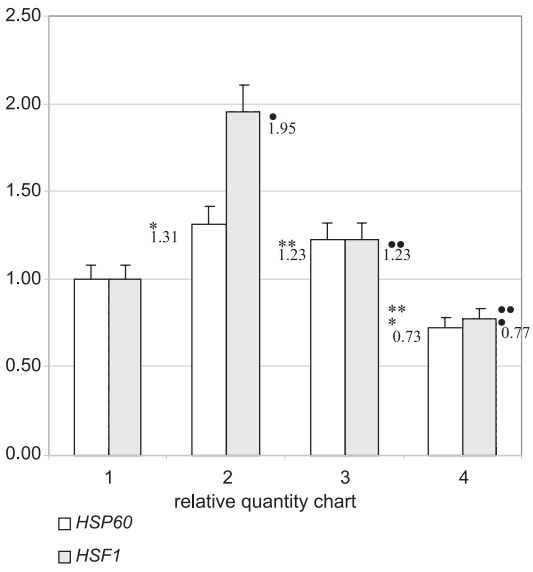

Figure 1. QRT-PCR. Effect of SUL on mRNA levels for *HSF1* and *HSP60* in HeLa cells treated as the following: Untreated (calibrator) – 1; DOX 1 µM for 24 h – 2; SUL 50 µM for 24 h – 3; DOX 1 µM + SUL 50 µM for 24 h – 4. Values are represented as the average fold change = $2^{-(DDC)}$ for HeLa treated samples compared with the calibrator, where the calibrator is assigned a value of "1". Results are represented as the mean ± SD of three independent experiments done in triplicates. *y-axis – mRNA expression level.* *p = 0.0010; ** p = 0.0056; •p = 0.0094 ••p < 0.0001

RESULTS

QRT-PCR

As was observed in the previous study (14), SUL and DOX significantly improved apoptosis induced by DOX in HeLa cells which was observed

with TUNEL assay. The mechanism of that effect was considered in the current work. To study the synergistic effect of the combined treatment with DOX and SUL, QRT-PCR assay was used to evaluate the level of expression of *HSF1* and *HSP60* genes in HeLa cells through measuring mRNA.

As is shown in Figure 1, in the case of *HSF1* and *HSP60* the significant induction (ca. 2-fold and above 1.3-fold, respectively) of mRNA level was observed after exposure of the cells to DOX 1 μM. SUL 50 μM alone caused rather moderate increase in mRNA level of *HSF1* and *HSP60* genes (only above 1.2-fold). The significant decrease in expression of these both genes, below the native level was noted after DOX 1 μM and SUL 50 μM simultaneous treatment.

Western blot

We first characterized the relative purities of mitochondrial and cytosolic fractions. The blots revealed that the cytosolic marker, lactate dehydrogenase (LDH), was detected only in the cytosolic preparations (Fig. 2). These results demonstrate that our subcellular fractions were relatively pure without significant cross-contamination. As shown in Figure 2, HSP60 appeared in the higher levels in cytosol than in mitochondria. No difference in the intensity of the bands corresponding to HSP60 protein was noted under treatment of the cells to DOX or SUL or DOX + SUL. So, there is not possible to assume any translocation of that protein between mitochondria and cytosol.

DISCUSSION AND CONCLUSIONS

The results observed in this study indicate the relation between *HSF1, HSP60* and apoptosis and their role in synergistic action of DOX and SUL noted by Gruber et al. (14). DOX 1 μM + SUL 50 μM, the complex which potentiated apoptosis in HeLa cells, induced also the decrease in *HSP60* expression. It points to the antiapoptotic role of HSP60, being proved also by Kirchhoff et al. (17), who noted the similar correlation in adult rat cardiac myocytes treated with DOX, and by Shan et al. (18), who reported that HSP60 overexpression significantly reduced DOX induction of proapoptotic proteins, like Bad, and inhibited caspase-3 in neonatal rat cardiomyocytes exposed to DOX. On the opposite, DOX alone, which also induced apoptosis in HeLa cells but in a moderate way, stimulated the expression of *HSP60* which, in turn, correlated with *HSF1* increased expression (however, the expression of these both genes did not change to the same extent). That was in agreement with proapoptotic role of HSP60 proved by the other authors (2, 3, 17). However, HSP60 translocation effect seems to be not characteristic for apoptotic inducers but probably is cell type dependent. Chandra et al. (1) noted that DOX stimulated cytosolic accumulation of HSP60 in LNCaP cells. In this study, DOX did not influence this protein shift.

Chandra et al. (1) noted the dual role of HSP60, i.e., pro-death and pro-survival in dependence on cytosolic accumulation with or without concomitantly mitochondrial release, the mechanisms of which is not clear. The authors also reported that the observed accumulation of HSP60 is not related to the corresponding increase in its mRNA levels showing the transcriptional and/or post-transcriptional mechanisms. Our results indicate the similar effect. *HSP60* mRNA level was significantly increased upon exposure of DOX and SUL but no increased accumulation of this protein was noted. It is also worth noting that among these compounds only DOX generated apoptosis in HeLa cells. In turn, under treatment with DOX + SUL – the strong apoptotic inducer, *HSP60* mRNA level was significantly decreased and no difference in HSP60 expression was noted as compared to the control cells.

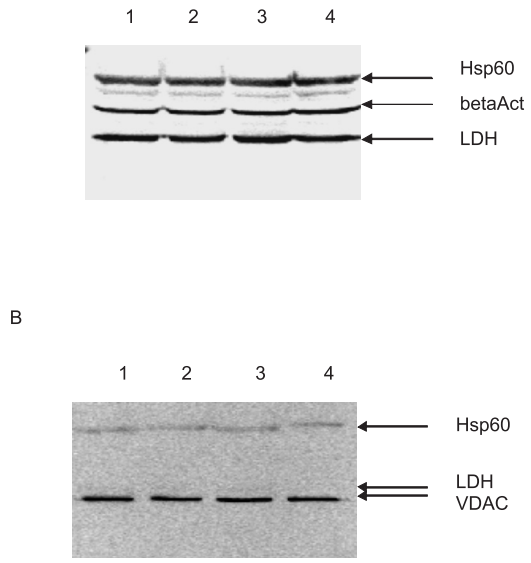

Figure 2. Cytosolic accumulation of HSP60 in the absence of obvious mitochondrial release. Cytosolic extracts – A. Mitochondrial extracts – B. Untreated HeLa cells – 1. HeLa cells treated with DOX, 1 μM for 24 h – 2. HeLa cells treated with SUL, 50 μM for 24 h – 3. HeLa cells treated with DOX, 1 μM + SUL, 50 μM for 24 h – 4. Equal amounts of cytosolic and mitochondrial extracts (30 μg/lane) were used in Western blotting. Loading control – β-actin, VDAC

The expression of *HSF1* potentiated by DOX + SUL alongside the intensified apoptotic processes may suggest some role of this transcription factor in apoptosis generated by DOX + SUL in HeLa cells.

Induction of *HSF1* and *HSP60* observed under treatment of HeLa cells with SUL does not seem to be related to apoptosis because this compound was not reported as apoptosis inducer (14) but confirms the effects noted by Batulan et al. (20) with other NSAIDs as sodium salicylate and niflumic acid.

The increase in *HSF1* expression induced by DOX besides apoptosis may be also connected with the increased expression of *MDR1* noted by Gruber et al. (14) in HeLa cells exposed to DOX. Such relation between these both genes was observed by Vilaboa et al. (4).

As was shown, cytosolic levels of HSP60 were higher than in mitochondria. HSP60 is the mitochondrial protein (17, 19). In agreement with the results obtained in this study Samali et al. (3) found HSP60 as detectable also in extramitochondrial sites of Jurkat T cells. Kirchhof et al. (17) reported HSP60 in cytosol fraction of rat cardiac myocytes but he underlined that the amounts of that protein are much smaller than in mitochondria (19–20% *vs.* 80%). Our results only in part confirm this notion. HSP60 was present constitutively in the cytosol of HeLa cells but the cytosolic levels of that protein were higher than in mitochondria. As was suggested by Chandra et al. (1), depending on the cell cycle status of cells, cytosolically synthesized HSP60 may not all be imported into mitochondria and therefore it can be detected in both fractions.

On the basis of the results noted in this study it can be concluded that: the effects of *HSP60* and *HSF1* evoked in the cells depend on the inducer; proapoptotic action of DOX + SUL may correlate to the increased expression of *HSF1* and *HSP60*; *HSP60* mRNA level and the regulation of that protein expression (transcriptional and/or post-transcriptional) depend on the apoptotic inducer; and the role of HSP60 in apoptosis expressed in potential shift between mitochondria and cytosol is determined by the apoptotic inducer and the cell type.

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