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Cisplatin and etoposide combined therapy changes heat shock proteins (HSPs) expression in tumor cells

Terapia złożona z cisplatyny i etopozydu zmienia ekspresję białek szoku termicznego (HSPs) w komórkach nowotworowych

SUMMARY

Chemical and physical stress induce synthesis of heat shock proteins (HSPs) in cells. These proteins are involved in protection of cells against pro-apoptotic stimuli, like hyperthermia, UV and several chemotherapeutics. Therefore, overexpression of HSPs in tumor cells may be closely associated with involving their malignancy and invasiveness. There is several evidences suggesting that combination of drugs may show synergistic anti-tumor effects. It is based on observations showing that combined therapy induce not only direct cytotoxic effects but delayed metabolic toxicity as well. It was also noted that three-dimensional (3D) cell culture models are more useful in modern *in vitro* oncological tests because they more closely reflect the situation *in vivo* than classic two-dimensional (2D) ones.

Therefore, we adopted 3D cell culture model on Spongostan® discs to analyze the combined effects of cisplatin (CDDP) and etoposide (VP-16) and/or heat shock (43°C) on HSP27, HSP72 and HSP73 expression in HeLa and Hep-2 human tumor cell lines and compare the results with those obtained from traditional 2D model.

The expression of HSPs was studied by immunoblotting. For drugs cytotoxicity analysis tetrazolium-based colorimetric assay (MTT) was used. Synergistic effect of drugs combination was observed in HeLa cells after 4h of incubation. In Hep-2 cells the effect was antagonistic. Drugs combination changed HSP27, HSP72 and HSP73 expression in tumor cells dependently on cell culture model and origin of cancer cells. Incubation of cells with drugs combination followed by heat shock (43°C) turned away the relations obtained in normal conditions (37°C) especially for inducible HSP27 and HSP72 proteins but remained unchanged for HSP73 that is expressed constitutively.

We suppose that the anti-tumor effect of drugs combination in some cancers may be a result of HSPs expression decrease which is then followed by increase of tumor cells sensitivity to therapy.

STRESZCZENIE

Chemiczne oraz fizyczne czynniki stresowe indukują w komórkach syntezę białek szoku termicznego (HSPs). Białka te zaangażowane są w ochronie komórek przed czynnikami działającymi proapoptotycznie, jak np. hipertermia, światło UV lub chemioterapeutyki. Nadekspresja białek HSP może być więc ściśle związana z nabywaniem złośliwości i zdolności inwazyjnych przez komórki nowotworowe. Istnieją doniesienia sugerujące, że synergistyczny efekt przeciwnowotworowy można uzyskać, stosując kombinację leków. Oparte jest to na obserwacjach wskazujących, że w trakcie takiej terapii indukowany jest nie tylko bezpośredni efekt cytotoksyczny, lecz również opóźniona jest toksyczność metaboliczna. Wskazano również, że trójwymiarowe (3D) modele hodowli komórkowych są bardziej użyteczne w nowoczesnych testach onkologicznych *in vitro* od klasycznych dwuwymiarowych (2D), ponieważ lepiej odzwierciedlają sytuację *in vivo*.

Zastosowaliśmy zatem 3D model hodowli komórkowej na dyskach Spongostanu® w celu przeanalizowania wpływu cisplatyny (CDDP) i etopozydu (VP-16) i/lub szoku termicznego (43°C) na wytwarzanie przez ludzkie komórki nowotworowe linii HeLa i Hep-2, białek HSP27, HSP72 i HSP73 oraz porównania z wynikami uzyskanymi w tradycyjnym modelu 2D.

Ekspresję HSP określono metodą immunoblotting. Analizę cytotoksyczności leków wykonano kolorymetryczną metodą z MTT. Po 4 godz. inkubacji obserwowano synergizm działania leków w stosunku do komórek HeLa. W przypadku linii Hep-2 efekt był antagonistyczny. Po inkubacji komórek z układem leków ekspresja HSP27, HSP72 i HSP73 ulegała zmianie w zależności od modelu hodowlanego oraz pochodzenia komórek nowotworowych. Inkubacja komórek z układem leków, a następnie podniesienie temperatury (43°C) odwróciło zależności uzyskane w normalnych warunkach hodowli (37°C), szczególnie w przypadku indukowalnych form białek HSP27 i HSP72. Zależności te pozostały niezmienne dla konstytutywnie wyrażonej formy HSP73.

Uważamy, że w niektórych przypadkach przeciwnowotworowa aktywność kombinacji leków może wynikać z obniżonej ekspresji białek HSP, prowadzącej do wzrostu wrażliwości komórek nowotworowych na terapię.

K e y w o r d s: Heat shock proteins, cisplatin, etoposide, drugs combination, three-dimensional cell culture, Spongostan®

ABBREVIATIONS

2D – two-dimensional, 3D – three-dimensional, CDDP – cisplatin, DMSO – dimethyl sulfoxide, ECACC – European Collection of Cell Cultures, FBS – fetal bovine serum, GSH – glutathione, HSPs – heat shock proteins, MTT – 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, UV – ultraviolet radiation, VP-16 – etoposide.

INTRODUCTION

The aim of cancer therapy is the complete eradication of all tumor cells from the patient's body. However, cancer cells effectively develop biochemical mechanisms leading to cellular resistance to a particular anti-neoplastic agents (1, 4, 6). In order to overcome this problem the most common approach is the use of more than one drug to treat a cancer. This strategy is so-called combined chemotherapy. A particular drug may induce tumor cell apoptosis when used alone but can be more effective when used in combination with other anti-neoplastic agents (6). The combination of cisplatin (CDDP) and etoposide (VP-16) (PE program) is currently one of the most effective chemotherapy treatment of solid tumors and is suggested to be synergistic *in vitro*, in cell lines as well as clinically in small-cell lung carcinoma, non-small-cell lung carcinoma, testicular cancer, lymphoma and colorectal carcinoma (5, 11, 18, 22). However, cisplatin treatment increase gluthatione (GSH) level in cells that in turn cause increased heat shock proteins (HSPs), especially HSP27, expression and in consequence drug resistance of tumor cells (2, 8, 9).

HSPs are the group of proteins called molecular chaperones, protecting tumor cells against many stressors, among others chemotherapeutical drugs. Therefore, HSPs overexpression in cancer cells may increase their malignancy and invasiveness. However, the sensitivity of cells to cytostatics is not only drug and cell-type specific but also depends on the *in vivo* and *in vitro* environment. It is not surprising that three-dimensional (3D) models *in vitro* supersede classic two-dimensional (2D) cell cultures because in 3D cultures intercellular interactions are more physiological than in standard 2D monolayers. Moreover, they are close to *in vivo* conditions allowing cells to maintain their features as in natural tissues (8, 19).

Therefore, we adopted 3D cell culture model (cell culture on porous gelatin Spongostan® discs) to analyze the combined effects of cisplatin and etoposide and/or heat shock on HSP27, HSP72 and HSP73 expression and compare the results with those obtained in traditional 2D model.

MATERIAL AND METHODS

Cells and culture conditions. Human cervix carcinoma cell line (HeLa B, ECACC No 85060701), human larynx carcinoma (Hep-2, ECACC No 86030501) and human lung carcinoma (A549, ECACC No 86012804) cultured in RPMI 1640 medium supplemented with 5% FBS (fetal bovine serum) (Gibco™ Paisley, UK) (v/v) were used in this study. Cells were grown in Petri dishes (Nunc., Roskilde, Denmark) coated with Spongostan® derived gelatin (2D model) and incubated at 37°C in humidified atmosphere with 5% CO_2. In 3D model, porous gelatin Spongostan® discs (wet volume 0.11 cm^3) were used (Fig. 1 and Fig. 2). The discs were placed in siliconized tubes, seeded with cells and incubated at 37°C in water bath gyratory shaker (100 rpm).

Both in 2D and 3D models cells were seeded at a density of 5×10^5 cells/ml for heat shock proteins analysis and cytotoxicity.

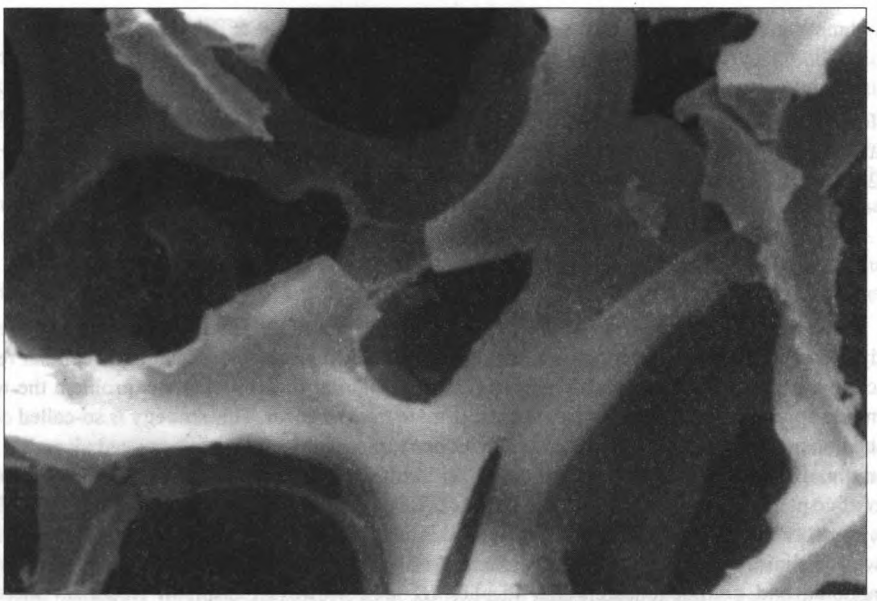

Fig. 1. Spongostan's® structure. Scanning electron microscopy micrograph. Magnification 1000×

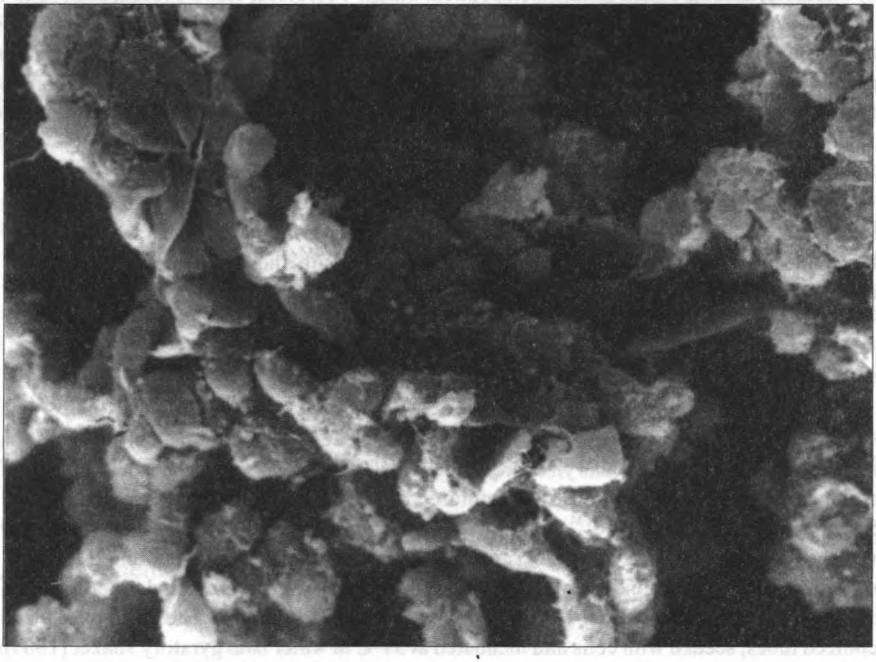

Fig. 2. HeLa cells cultivated on Spongostan®. Scanning electron microscopy micrograph. Magnification 1000×

Drugs treatment. Cisplatin (CDDP) and etoposide (VP-16) (Sigma, St. Louis, Mo, USA) at a final concentration of 5 μg/ml were used. For heat shock proteins analysis combination of 5 μg/ml + 5 μg/ml (CDDP+VP-16) was applied. Drugs alone were added at 10 μg/ml concentration. The chemioterapeutics doses have been stated on the basis of our previous experiments (7, 8) and *in vitro* toxicity tests performed by other authors (16, 20, 23, 24).

The drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma), but the final concentration of DMSO in the culture medium did not exceed 0.1%, which as indicated in previous experiments did not influence cells viability and HSPs expression. As control, cells were incubated with 0.1% DMSO.

Heat shock treatment and HSPs detection. After 24h of culture, the tumor cells were incubated with drugs for 4h at 37°C before heat shock (30 min., 43°C) and than for 2.5h at 37°C (totally for 7h). Control cells and those treated with drugs without heat shock were incubated only at 37°C for 7h. After incubation, the cells were detached from Petri dishes by short trypsinization. In 3D model the cells were released from the carrier by digestion of Spongostan® discs in 0.25% trypsin solution. The amount of HSPs in the cells after drug and/or heat shock treatment were performed by western blotting. Semiquantitative densitometric analysis of immunoblotts were determined as described in detail previously (8).

Two independent experiments were performed.

The MTT assay. The cells were seeded in a 96-well microculture plates for 24h and than treated with drugs for 4h. The absorbance was measured in a microplate reader by the use of drug cytotoxicity MTT assay as described previously (21).

Analysis of interactions. The effect of two drugs combination was analyzed using the interaction index according to Kerry et al. (12). The index, denoted by A, is defined by the isobolar relation

$$A = x/X + y/Y$$

Where X and Y are the doses of drug X (alone) and Y (alone), respectively and (a, b) is the combination dose that gave the specified effect levels. It was established, that if the interaction index value is equal to 1 it indicate pure additive interaction. If the index is lower than 0.7, it indicates synergistic interactions, whereas greater than 1.3 it reflects antagonistic dependences. The values ranging between 0.7 and 1.3 indicate additive relations between the analyzed drugs (14, 25).

Statistical analysis. Significance levels were calculated using one-way ANOVA test with the post-hoc Bonferroni's test. Significance was analyzed between culture models of the same cell line and between the same culture model of different cell lines. Differences were considered significant at $p < 0.001$.

RESULTS

Anti-tumor activity of cisplatin (CDDP) and etoposide (VP-16) were analyzed after their separate and combined administration to culture medium. Synergistic effect of 5 μg/ml + 5 μg/ml combination (CDDP+VP-16) was observed after 4 h of drugs incubation with HeLa cells but was antagonistic when analysis was performed with Hep-2 cells as measured by MTT assay (Table 1).

Cisplatin and etoposide, administered separately, had slight effect on HSP27, HSP72 and HSP73 expression in comparison to control (100%) in HeLa and Hep-2 cells. When administered together only in HeLa cells cultivated in 2D

Table 1. The ratio of anti-tumor activity of cisplatin (C) (5 μg/ml) and etoposide (E) (5 μg/ml) measured by MTT assay

Quotient: sum of C and E activity administered separately/activity of C+E administered simultaneously	5C+5E/5+5
HeLa	0.56 ± 0.08
Hep-2	1.79 ± 0.03

5+5 – drugs administered simultaneously; 5C+5E – drugs administered separately; Quotient lower than 0.7 indicate synergism of drugs combination activity; Quotient greater than 1.3 indicate antagonism of drugs combination activity.

model increased HSP27 (50 ± 4% over control level) was observed when compared to results obtained in monodrug tests. Drugs combination decreased HSP27, HSP72 and HSP73 expression in HeLa cells and increased in Hep-2 cells cultivated in three-dimensional (3D) conditions when compared to two-dimensional (2D) models (Fig. 3A-C). Comparison of the appropriate culture models of two analyzed cell lines showed that Hep-2 cells expressed lower HSPs quantities in 2D conditions but higher in 3D model than HeLa cells. After cells pretreatment with drugs combination and subsequent exposition to 43°C for 30 min. the level of proteins increased. The exception was only Hep-2 cell culture in 3D conditions, where after heat shock HSP72 and HSP73 expression decreased and HSP 27 remained unchanged as compared to counterpart unshocked model. Moreover, the relations between 2D and 3D models observed in normal conditions for inducible HSP27 and HSP72 were inversed in heat shocked models. However, in the case of HSP73 the interdependences between culture models remained unchanged and looked like in unshocked cultures (Fig. 4A-C).

DISCUSSION

A common strategy for achieving improved response and cure rates in cancer patients is the use of drug combinations. Improved therapeutic effect could be obtained only when two or more drugs with different mechanisms of action and characterized by different toxicity values are combined (16). There are distinguished four most important types of interactions between two drugs in the mixture: pure additivity, supra-additivity (synergy), indifference and infra-additivity (antagonism) (15). The isobologram analysis of drug-drug interactions may be helpful to prepare a clinical rationale for the optimal administration schedule of drugs combination. Among others one of the most effective treatment of selected solid tumors is cisplatin (CDDP) and etoposide (VP-16) combination. We observed that selected concentration system of CDDP and VP-16

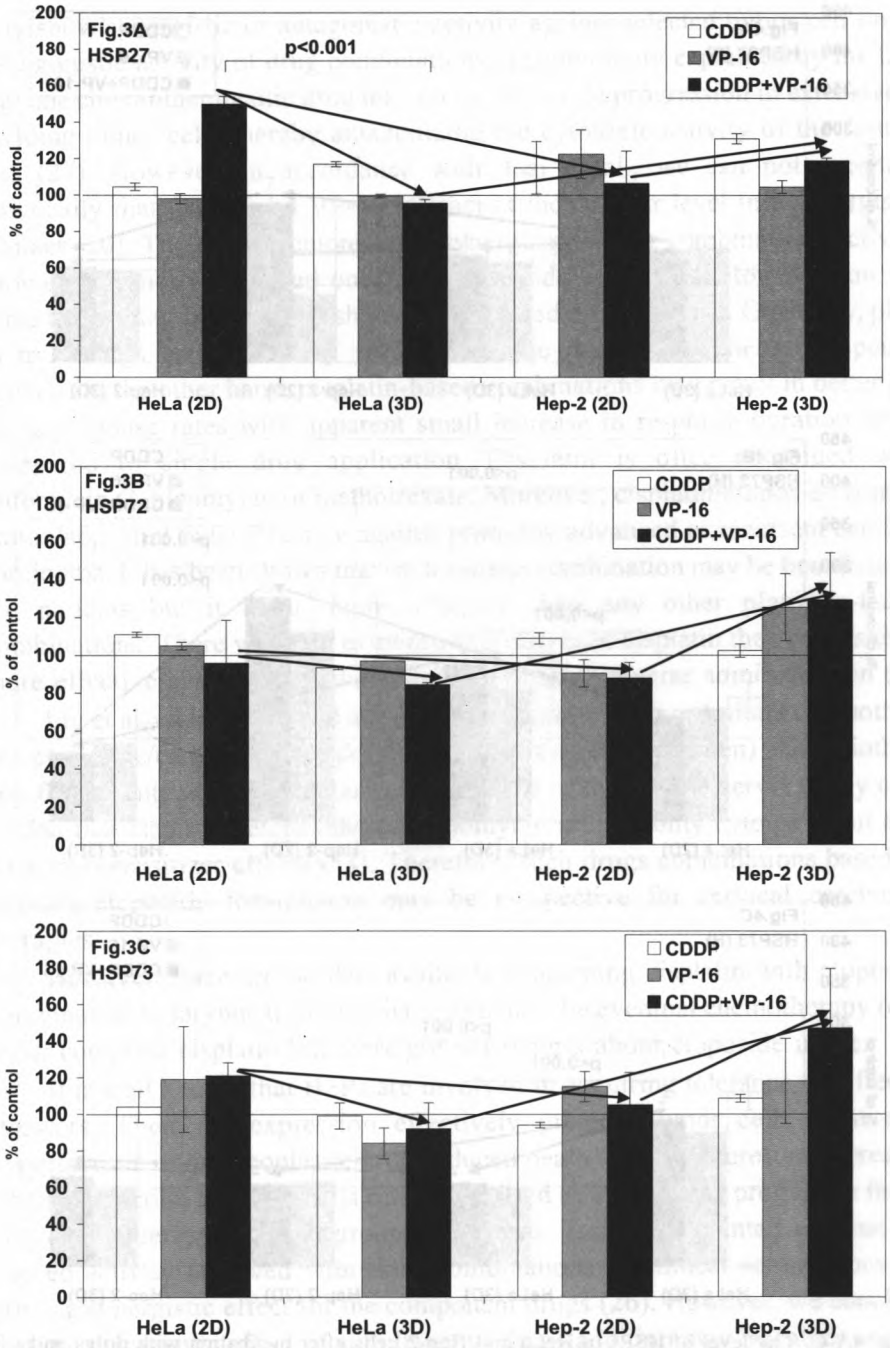

Fig. 3A-C. The level of HSPs in HeLa and Hep-2 cells after incubation with drugs. The horizontal line indicate a basal (100%) level of the control. The arrows indicate decrease or increase of the HSPs levels among values used to significance analysis

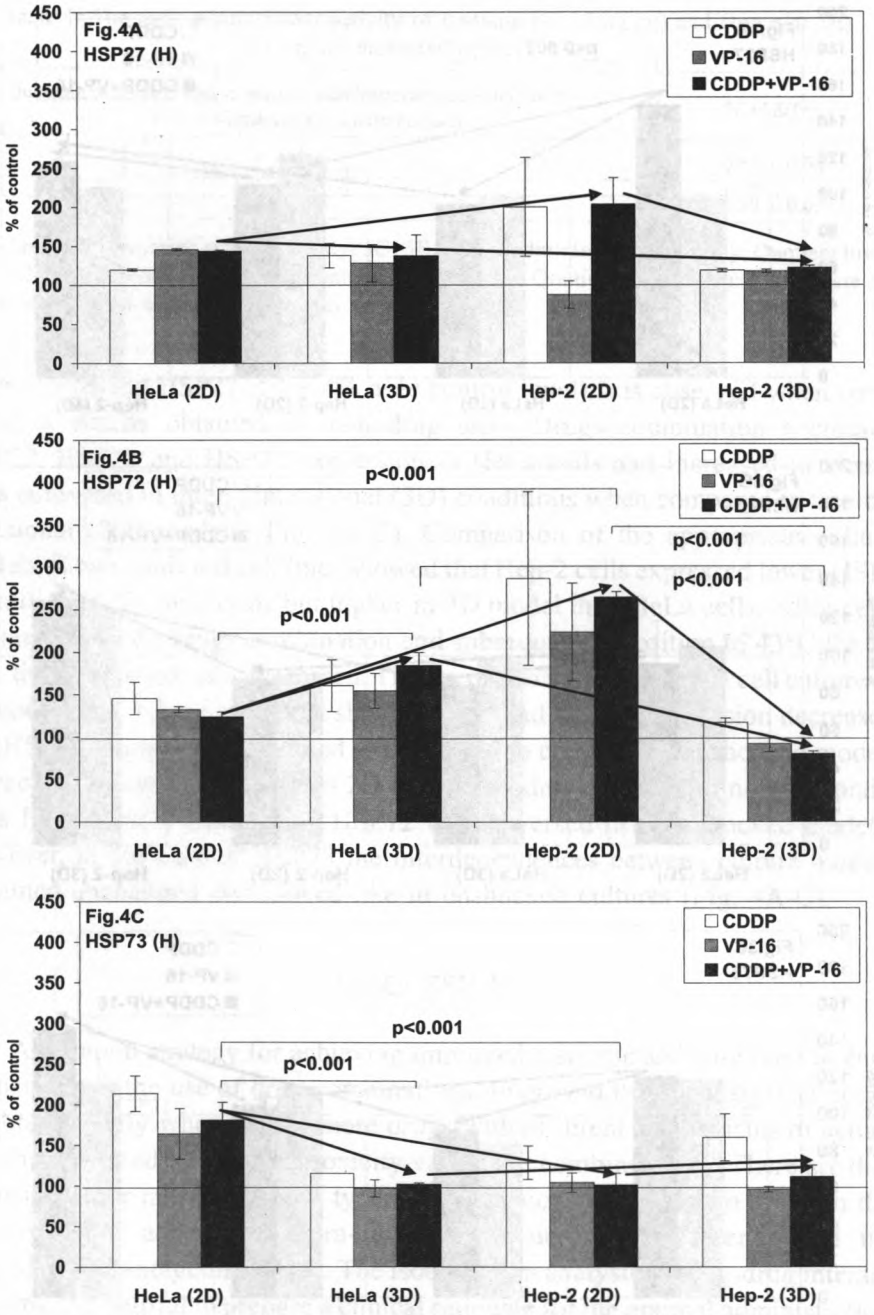

Fig. 4A-C. The level of HSPs in HeLa and Hep-2 cells after incubation with drugs and subsequent exposition to 43°C for 30 min. The horizontal line indicate a basal (100%) level of the control. The arrows indicate decrease or increase of the HSPs levels among values used to significance analysis

may show synergistic or antagonistic activity against selected tumor cell lines. Antagonistic activity of drug combinations are commonly explained by the fact that one chemiotherapeutic drug may block cell-cycle progression in extensively dividing tumor cells thereby antagonizing the cytotoxic activity of the former one (27). However, in accordance with Tsai et al., we can not ascertain univocally that CDDP and VP-16 interact at the cellular level in a synergistic manner (26). Therefore, demonstrated synergism in drug combinations activity should be rather recognized only as a supra-additive effect. However, on the same basis antagonistic effect should be so-called infra-additive. Generally, platinum analogs are commonly regarded as the best single chemotherapeutic agents. On the other hand, cisplatin-based combinations may result in better patients response rates with apparent small increase in response duration when compared to single drug application. Cisplatin is often combined with mitomycin C, bleomycin or methotrexate. Moreover, cisplatin/etoposide combination appeared to be effective against primarily advanced or recurrent cervical carcinoma. It has been shown that such a drugs combination may be beneficial to the patients but it is no more effective than any other platinum-based combinations. There was also evidence that sequence cisplatin then etoposide is more effective and less hematologically toxic than reverse administration (3).

Liu et al., also performed a prospective study with concomitant chemotherapy etoposide/cisplatin with additional bleomycin (PEB regimen) and radiotherapy for patients with squamous cell carcinoma of the uterine cervix. They concluded that cisplatin, etoposide and bleomycin are not only cytotoxic but also have radiosensitizer effects (13). Therefore, such drugs combinations based on cisplatin/etoposide formulation may be perspective for cervical carcinoma therapies.

However, there are no data available concerning cisplatin with etoposide combination in laryngeal carcinoma treatment. The eventual chemotherapy regimens comprise cisplatin but there are not reports about etoposide usage.

It is well known that HSPs are involved in acquiring tolerance to different stressors. Their overexpression effectively protects tumor cells from e.g., hyperthermia or antineoplastic drug-induced death (7, 10). Therefore decreased HSPs production in tumor cells can be admitted as a beneficial prognostic factor in a chemotherapy exposed group of patients. Tsai et al. pointed out that enhanced activity achieved with drug combinations in clinical setting does not prove a synergistic effect for the component drugs (26). However, we conclude that the therapeutic effect of simultaneous drugs addition may be rather associated with inhibition of HSPs expression in tumor cells, decreased drug resistance and in consequence synergistic-like toxicity of CDDP with VP-16 combination.

HSPs analysis revealed that after addition of drug combination the protein expression in HeLa cells decreased or was inhibited and increased in Hep-2 cell line incubated in three-dimensional (3D) culture conditions when compared to two-dimensional (2D) models. The results may explain why some authors working on different cell lines incubated in classical culture models cannot find any statistical evidence that CDDP and VP-16 interact at the cellular level in a synergistic manner while clinical synergy using drug combinations is widely accepted. We suppose that it may be a result of differences in cytoskeletal architecture and intercellular interactions in tested culture models that may influence *hsp* gene expression. However, the influence of cellular origin and the original pre-stress level of HSPs can not be excluded. We also showed that heat stress plays important role not only in inducing HSPs expression but also in modulating their production in drug combination environment, depending on cells origin and cell culture model. The above-mentioned modulation effect refers only to inducible HSP27 and HSP72 proteins but not to constitutively expressed HSP73. It may be partially explained on the basis of hypothesis which assumes that during stress the pre-existing levels of HSPs may be sufficient to create e.g. thermotolerance in the absence of new inducible HSPs synthesis. Therefore, the key should be redistribution of constitutively expressed HSPs, rather than overall amounts (17). On the other hand, when inducible HSPs expression was increased after heat shock it may indicate that hyperthermia induced cellular resistance against applied drugs. HSP27 and HSP72 are currently supposed as a survival proteins capable to protect cells against a variety, potentially lethal stressors. However, drug–heat interaction should be interpreted with a great caution. It is a fact that drugs which are effective in normal temperature do not always expose their activity at higher temperatures. It may be one of the reasons of modulating HSPs production in our models. Moreover, hyperthermia affects fluidity and stability of cellular membranes that may vary between different cell types in a given experimental system. Therefore, such changes in cells cultivated in 2D and 3D models may strongly influence inducible HSPs expression after thermal shock.

In conclusion, CDDP and VP-16 combination may influence tumor cell toxicity by change of HSPs expression. The effect may be supposed as synergism but we can not ascertain it univocally. It is difficult to transfer the results obtained *in vitro* to the clinical observations. It is due to different reactivity of tumor cells and simplicity of 2D classical and even 3D models of cell culture *in vitro* when compared to the whole human body. However, 3D models are close to *in vivo* conditions than 2D ones and therefore may to a greater degree reflect the real therapeutic effects.

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