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# DOSE ENHANCEMENT EFFECT OF ANTICANER DRUGS ASSOCIATED WITH INCREASED TEMPERATURE *IN VITRO*

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***Aim:*** **To evaluate in vitro the influence of elevated temperature (42 °C for 60 min) on the action of anticancer drugs doxorubicin, vinorelbine, carboplatin, ifosfamide, etoposide, oxaliplatin, docetaxel and gemcitabine.** ***Methods:*** **HeLa tumor cell cultures, 24 h after seeding, were incubated for 60 min with different concentrations of chemotherapeutical drugs at a temperature of 37 °C or 42 °C. 48 h later the number of viable cells in the flasks were counted using trypan-blue exclusion on a hemacytometer.** ***Results:*** **Hyperthermia alone caused only 10–20% growth inhibition of cell culture. All the chemotherapeutic drugs used demonstrated a dose enhancement effect at elevated temperature. Thermal enhancement ratio for cell proliferation for oxaliplatin, vinorelbine, carboplatin and ifosfamide exceeded 4, for doxorubicin and gemcitabine exceeded 2. Thermal enhancement ratio for cell death did not exceed 1.4.** ***Conclusion:*** **Synergism of hyperthermia and chemotherapeutical drugs was clearly demonstrated for oxaliplatin, vinorelbine, carboplatin, ifosfamide and to a lesser extent for doxorubicin and gemcitabine. Enhancement of the cytostatic effect of anticancer drugs by hyperthermia was more prominent than their cytotoxic effect.**
***Key words:*** **hyperthermia, anticancer drugs, HeLa cells, thermal enhancement ratio.**

Multiple studies in hyperthermic oncology testify the enhancing efficacy of combination or multimodality therapy of cancer patients by application of high temperature (40–43 °C for whole-body exposure and 42–47 °C for local treatment). Hyperthermia has been shown to be a powerful modifying factor that increases tumor sensitivity to both radio- and chemotherapy [1–6].

Thermal enhancement of cytotoxic effects for many anticancer drugs has been observed in different experimental systems. The mechanisms involved in the interaction of chemotherapy and hyperthermia are depended on the particular chemotherapeutic agent and thought to be related to an increase in drug uptake, expand of DNA damage and inhibition of DNA repair [1–3, 7]. In vitro drug-induced cell killing usually increased with temperature from 40 °C to 45 °C and maximal cytotoxicity occurred when the drug was scheduled simultaneously with hyperthermia. In vivo the therapeutic efficacy of the thermotherapy was maximized at mild temperature (40.5–43 °C) and the greatest potentiation occurred when the two modalities were administered close together [2, 6, 7].

Despite several experimental studies on the interaction of hyperthermia with chemotherapeutic drugs data concerning treatment efficiency are often conflicting. The use of various cell lines, different treatment schemes and differing end points complicates the interpretation of research findings. Drug concentration and drug exposure time are also of great importance. Furthermore, in vitro and vivo results often differ due to physiology factors of tumor tissue such as microcirculation, pH and oxygenation.

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*Abbreviations used:* IC50 (IC90) – the drug concentration causing a 50% (90%) growth inhibition; LC50 – the drug concentration causing a 50% cell killing; TER – thermal enhancement ratio.

Alkylating agents melphalan, cyclophosphamide and ifosfamide were shown to be the most effective drug at moderately elevated temperatures for many types of tumors, effectiveness of cisplatin or oxaliplatin was less pronounced [8, 9, 10]. Hyperthermia was proved to enhance the sensitivity of human tumor cells to doxorubicin [7, 11, 12], vincristine [12] and carboplatin [13, 14], whereas the efficacy of etoposide is only slightly influenced by additional application of heat [15, 16] or decreased [17]. However it was demonstrated that appropriately scheduled hyperthermia and etoposide treatments result in increased etoposide-induced death of human leukemia cells [18]. Simultaneous application of gemcitabine and heat led to decreased cytotoxicity [19, 20], had not the influence on cytotoxicity [12] or augmented cytotoxic effect of drug [10, 21, 22]. There are also contradictory reports as to the interaction of hyperthermia and the taxanes [10, 23–28].

Therefore it is important to compare in equal treatment conditions the impact of hyperthermia on the action of the main chemotherapeutic drugs currently used for cancer treatment. In this study we investigated the differences in HeLa tumor cell culture growth after incubation with different anticancer drugs at a temperature of 37 °C or 42 °C.

## MATERIALS AND METHODS

***Chemotherapeutic drugs.*** The drugs investigated were ifosfamide (holoxan, Baxter Oncology Gmbx, Germany), doxorubicin (doxorubicin hydrochloride, RUE "Belmedpreparaty", Belarus), carboplatin (carboplatin-LENS, Veropharm, Russia), oxaliplatin (eloxatin, Sanofi-Synthelabo, France), vinorelbin (neocitec, Labinca, Argentina), etoposide (etoposide-LENS, Veropharm, Russia), docetaxel (docetal, Dabur Pharma Ltd., India), gemcitabine (gemzar, "Eli Lilly", USA). Just before use, the drugs were diluted with culture medium.

***Cell culture.*** HeLa cells (human cervical carcinoma) were obtained from the cell culture collection of Research Institute of Epidemiology and Microbiology (RIEM, Minsk, Belarus). The cells were cultured as a monolayer in culture flasks covered with 199 medium (RIEM) supplemented with 10% fetal calf serum (RIEM) and 100 μg/ml kanamycin.

***Cell treatment.*** One day before the drug treatment, cell monolayer was detached from the culture flasks with 0.02% versen (DIALEK, Belarus) and plated at a concentration of $2 \times 10^5$ cells/flask. All the experiments were carried out on exponentially growing cells. On the day of the experiment, 100 μl of each drug dilutions were added into the flasks with cell monolayer and then the cells were incubated in water bath at 37.0 °C ± 0.1 °C or 42.0 °C ± 0.1 °C for 60 min. Treated and control cells were cultured for additional 48 h, then cell monolayers were versenized and viable cells were counted using trypan-blue exclusion on a haemocytometer. The mean ± standard deviation was calculated from three experiments.

To differentiate between cytostatic and cytotoxic effects, the number of viable cells after the treatment was compared with the initial cell number before any treatment. Percentage of viable cells above the initial cell number denoted a cytostatic effect, whereas those below showed cytotoxicity [23]. The percentages of viable cells for each treatment were calculated from $100 \times [(N_t - N_o) / (N_k - N_o)]$, if $N_t$ was greater than or equal $N_o$, or $100 \times [(N_t - N_o) / N_o]$, if $N_t$ was less than $N_o$ ($N_t$ — cell number in treated cultures with chemotherapeutic drug and/or hyperthermia; $N_k$ — cell number in control cultures that had not been exposed to chemotherapeutic drugs or hyperthermia; $N_o$ — initial cell number before the treatment).

The values of $IC_{50}$ and $IC_{90}$, concentrations of each drug required for a 50% and 90% reduction in cell number relative to control value, and $LC_{50}$, concentration of drug required for a 50% reduction in cell number relative to initial value before the treatment, were determined for each experiment using regression analysis of the data received. Thermal enhancement ratios for inhibition of cell proliferation and cell killing for each chemotherapeutic drug were calculated as $IC_{50}$, $IC_{90}$, or $LC_{50}$ for drug alone divided by $IC_{50}$, $IC_{90}$, or $LC_{50}$ for drug combined with hyperthermia.

## RESULTS AND DISCUSSION

The survivals of exponentially growing HeLa cells exposed to various concentration of ifosfamide doxorubicin, carboplatin, docetaxel, vinorelbine, etoposide, oxaliplatin or gemcitabine at 37 °C or 42 °C are shown in Figure. These data demonstrate that cell number reduced with increasing concentrations of chemotherapeutic agents. All used drugs demonstrated the dose-dependent enhancement of cytotoxicity at elevated temperature — the decrease of cell number at 42 °C was more pronounced than at 37 °C. It must be noted the heat treatment alone did not alter significantly cell number causing only 10–20% growth inhibition.

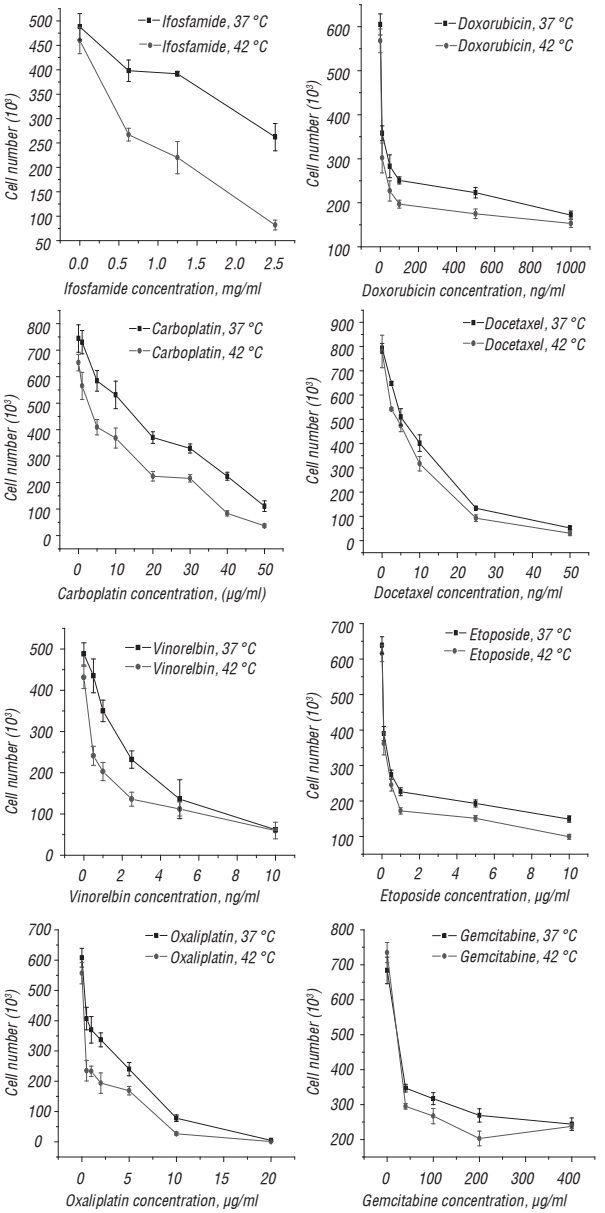


**Figure.** Dose-response curves for HeLa cells treated with ifosfamide, doxorubicin, carboplatin, oxaliplatin, vinorelbine, etoposide, docetaxel or gemcitabine at 37 °C or 42 °C. The cells were incubated with different concentration of anticancer drug for 1 h at 37 °C or 42 °C and counted 48 h later. Each data point represents the mean of three separate experiments. Bars are standard errors

The parameters of cytostatic ($IC_{50}$ and $IC_{90}$) and cytotoxic ($LC_{50}$) effects of chemotherapeutic drugs at 37 °C and 42 °C are presented in Table. The data obtained testify the enhanced efficiency at elevated temperature to a greater or lesser extent of all the drugs under investigation. So, by $IC_{50}$ and $IC_{90}$ criteria, hyperthermia caused significant enhancement of cell culture growth inhibition induced by ifosfamide, carboplatin, vinorelbine, oxaliplatin, with thermal enhancement ratio exceeded 4. The values of $IC_{50}$ and $IC_{90}$ decreased actually for vinorelbine 5.5-fold and for oxaliplatin 7.2-fold after combination with heat. To a lesser degree hyperthermia enhanced cytostatic effect of doxorubicin and gemcitabine, with thermal enhancement ratio exceeded 2. The differences in $IC_{50}$ and $IC_{90}$ for docetaxel and etoposide were not significant statistically. By $LC_{50}$ criterion, statistically

**Table.** The values of $IC_{50}$, $IC_{90}$, and $LC_{50}$ of dose-response curves for HeLa cells treated with different chemotherapeutic drugs at a temperature of 37 °C or 42 °C

| Drug | IC50 | | | IC90 | | | LC50 | | |
|---|---|---|---|---|---|---|---|---|---|
| | 37 °C | 42 °C | TER | 37 °C | 42 °C | ER | 37 °C | 42 °C | TER |
| Ifosfamide (mg/ml) | 1.7 ± 0.1 | 0.4 ± 0.1* | 4.3 | > 2.5 | 1.1 ± 0.1* | > 2.3 | > 2.5 | 2.3 ± 0.1 | > 1.1 |
| Doxorubicin (ng/ml) | 8.1 ± 1.8 | 8.1 ± 1.6 | 1 | 106.2 ± 1.8 | 37.7 ± 1.6* | 2.8 | > 1000 | > 1000 | – |
| Carboplatin (μg/ml) | 11.7 ± 1.7 | 2.7 ± 1.9* | 4.3 | 32.1 ± 1.7 | 14.1 ± 1.9* | 2.3 | 48.9 ± 1.7 | 36.8 ± 1.9* | 1.3 |
| Vinorelbine (ng/ml) | 1.1 ± 0.3 | 0.2 ± 0.3* | 5.5 | 2.5 ± 0.3 | 0.6 ± 0.3* | 4.2 | 7.4 ± 0.3 | 6.0 ± 0.3* | 1.2 |
| Docetaxel (ng/ml) | 5.6 ± 1.9 | 4.2 ± 2.3 | 1.3 | 18.0 ± 1.9 | 13.9 ± 2.3 | 1.3 | 35.2 ± 1.9 | 24.6 ± 2.3* | 1.4 |
| Etoposide (μg/ml) | 0.1 ± 0.1 | 0.1 ± 0.1 | 1.0 | 0.7 ± 0.1 | 0.5 ± 0.1 | 1.4 | > 10 | 9.2 ± 0.1 | > 1.1 |
| Oxaliplatin (μg/ml) | 0.4 ± 0.6 | 0.2 ± 0.2 | 2.0 | 3.6 ± 0.6 | 0.5 ± 0.2* | 7.2 | 8.6 ± 0.6 | 6.5 ± 0.2* | 1.3 |
| Gemcitabine (μg/ml) | 26.8 ± 20.2 | 25.3 ± 12.8 | 1.1 | 187.7 ± 20.2 | 84.4 ± 12.8* | 2.2 | > 400 | > 400 | – |

TER – thermal enhancement ratio calculated as $IC_{50}$, $IC_{90}$, or $LC_{50}$ for drug alone divided by $IC_{50}$, $IC_{90}$, or $LC_{50}$ for drug combined with hyperthermia.
*The difference between the values for 37 °C and 42 °C are statistically significant, with $p < 0,01$.

significant enhancement of drug-mediated cytotoxicity under elevated temperature was observed only for carboplatin, vinorelbine, docetaxel and oxaliplatin, with thermal enhancement ratio for cell death did not exceed 1.4. Thus, thermal enhancement for drug-mediated inhibition of cell proliferation by combination of chemotherapeutic drugs with hyperthermia was more pronounced than for drug-mediated cell killing. The most efficient chemotherapeutic drugs in HeLa cells at elevated temperature 42 °C were ifosfamide, carboplatin, vinorelbine and oxaliplatin.

In this study we investigated in cell culture the action of eight anticancer drugs in broad range of concentrations to describe their both cytostatic and cytotoxic effects. To quantitatively evaluate the impact of the hyperthermia on the chemosensitivity of tumor cells we compared the values of $IC_{50}$, $IC_{90}$, and $LC_{50}$ for dose-response curves of HeLa cell culture treated with chemotherapeutic drugs alone or in combination with heat. It was found that interaction of hyperthermia and simultaneous chemotherapy depends on the drug concentration. At low doses of chemotherapeutic agents that exerted mainly cytostatic effect it was shown synergistic interaction with heat for all the drugs under investigation except for docetaxel and etoposide, whereas at high doses of drugs that exerted predominantly cytotoxic action their interaction with heat was only additive.

Thermal enhancement of the cytostatic effect we have observed in this study in vitro may be of importance under in vivo conditions where events inducing the delay of tumor growth are expected to be cytostatic rather than cytotoxic [10]. We believe the data present in this study have clinical implication having recently begun animal tumor experiments to evaluate efficiency of the same chemotherapy in protocols with whole-body hyperthermia.

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# ЭФФЕКТ УСИЛЕНИЯ АКТИВНОСТИ ПРОТИВООПУХОЛЕВЫХ ПРЕПАРАТОВ ПРИ ПОВЫШЕНИИ ТЕМПЕРАТУРЫ *IN VITRO*

***Цель:*** **изучить *in vitro* влияние повышенной температуры (42 °С в течение 60 мин) на действие противоопухолевых препаратов: доксорубицина, винорельбина, карбоплатина, ифосфамида, этопозида, оксалиплатина, доцетаксела и гемцитабина.** ***Методы:*** **культуру опухолевых клеток HeLa через 24 ч после рассева инкубировали в течение 60 мин с различными концентрациями химиотерапевтических препаратов при температуре 37 °С или 42 °С. Спустя 48 ч подсчитывали количество живых клеток во флаконах, используя гемоцитометр и метод исключения красителя трипанового синего.** ***Результаты:*** **гипертермия сама по себе вызывала 10–20% угнетение роста культуры клеток. У всех исследованных химиотерапевтических препаратов отмечали эффект усиления при повышенной температуре. Коэффициент теплового усиления в отношении клеточной пролиферации для оксалиплатина, винорельбина, карбоплатина и ифосфамида превысил 4,0, для доксорубицина и гемцитабина — 2,0. Коэффициент теплового усиления в отношении гибели клеток не превышал 1,4.** ***Выводы:*** **синергизм гипертермии и химиотерапевтических препаратов продемонстрирован для оксалиплатина, винорельбина, карбоплатина, ифосфамида, в меньшей степени — для доксорубицина и гемцитабина. Усиление цитостатического эффекта противоопухолевых препаратов под действием гипертермии было более выраженным, чем их цитотоксического эффекта.**

***Ключевые слова:*** **гипертермия, противоопухолевые препараты, клетки HeLa, коэффициент теплового усиления.**