Exp Oncol 2006
28, 4, 303–307

Experimental ONCOLOGY

THE STUDY OF POSSIBILITY TO ELEVATE ANTITUMOR ACTIVITY AND DECREASE OF SYSTEMIC TOXIC EFFECTS OF CISPLATIN BY ITS BINDING WITH DELIGANDED ALBUMIN

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Aim: **To evaluate antitumor and toxic action of cisplatin (CP) in non-bound form and in a complex with deliganded albumin.** ***Methods:*** **To study complex-formation between CP and albumin, differential scanning and isothermic flow microcalorimetry were used. For quantitive evaluation of albumin-bound CP, the method of ultrafiltration was applied. Concentration of platinum in the samples was determined by atomic-absorption spectral analysis. Antitumor and toxic effect of CP and CP-albumin complex was studied in vivo using Guerin carcinoma (GC) model.** ***Results:*** **It has been shown that the second drug-binding site, located in the III domain of albumin molecule is one of the main points of binding of CP. Purification of officinal human serum albumin (HSA) on highly active carbon hemosorbents of HSGD mark allows to obtain deliganded albumin (dHSA) with elevated complex-forming ability toward CP. Administration of CP-dHSA complex provides higher rate of GC growth inhibition, than that of CP, and the content of creatinine in blood plasma of GC-bearing rats increases by 15% versus 40% in the case of CP administration.** ***Conclusion:*** **The data obtained allow recommend application of CP-dHSA to complex for enhancement of antitumor action and decrease of toxic effects of cisplatin.**

Key Words: **cisplatin, human serum albumin, HSGD carbon hemosorbent, Guerin carcinoma.**

Cisplatin (CP) possesses wide spectrum of antitumor action and is effectively used for therapy of patients with ovarian cancer, malignant tumors of testis and for combined chemotherapy of lung cancer and other solid tumors. However, clinical use of CP is limited by its significant systemic toxicity and requires an application of modern means of detoxification therapy [1].

The main idea of present research is an elevation of antitumor action of CP and minimization of its toxicity via binding of CP with high molecular weight protein of blood plasma — albumin. The choice of macromolecular system that includes CP and albumin as a carrier was not occasional. Unique transport functions of human serum albumin (HSA) and its ability to form complexes with numerous medicinal agents, in particular, anticancer ones, are well known. Presently the complexes between HSA and methotrexate (MTX) and its derivatives are generated, and it has been shown that independently on the route of administration to obtain equal antitumor effect there are required 4-5 fold higher doses of nonconjugated MTX than these of MTX-HSA complexes [2]. The differences between antitumor activity of nonconjugated MTX and MTX bound to HSA is determined by the period of existence of the preparations in bloodstream, and by different ways of their penetration into the cells: 24 h after administration of nonconjugated MTX and MTX-HSA complex to the rats bearing Walker's carcinoma, nearly 12% of the introduced dose of MTX-HSA and only 0.05% of nonconjugated MTX was detected in blood plasma, whilst tumor tissue accumulated approximately 14% of complex and 0.04% of nonconjugated MTX [3]. In experiments *in vitro*, the different kinetics of accumulation in the cells of tritium-labeled MTX and MTX-HSA was demonstrated. In contrary, incorporation of ^{125}I-MTX-HSA and ^{125}I-HSA was characterized by similar kinetics [4]. These facts evidence that MTX bound to HSA contrary to nonconjugated agent is penetrating the cells by endocytosis. In all experimental studies, the use of MTX-HSA complexes leads to decrease of toxic effects that are taking place upon the use of nonconjugated MTX [2, 3].

In experimental studies carried on malignant cell lines, tumor-bearing animals and in clinical trials it has been shown that albumin is accumulated in sufficient amount and metabolized by malignant cells. In patients with bronchogenous adenocarcinoma the content of albumin labeled by indium-111 was 2.2-5.4-fold higher in tumor tissue than in surrounding ones [5]. HSA added to culture medium was tightly bound by murine and human cells and could be removed only after treatment with enzymes or by culturing in albumin-free culture medium for period more than 2 days [6]. The authors have isolated albumin-binding protein with molecular weight of 18 kD that is expressed by tumor cells.

Presently there is obtained some clinical evidence that not only CP, but CP bound to albumin possesses antitumor activity [7]. The fact that patients with hypoalbuminemia reveal lower sensitivity for action of CP, serves as indirect evidence on the role of CP-HSA binding in appearance of antitumor activity of the complex [8]. One should note that in patients with malignant tumors, albumin is ligand-hyperloaded, causing decreased ligand-binding capacity of HSA [9, 10].

Received: November 15, 2006.

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Abbreviations used: CP – cisplatin; dHSA – deliganded human serum albumin; GC – Guerin carcinoma; HSA – human serum albumin; HSGD – hemosorbent granulated deliganded; MTX – methotrexate; TGIR – inhibition of tumor growth ratio.

So, the abovemmentioned results allow to suppose that albumin possesses the properties which allow to consider it as a perspective carrier for CP. The use of CP as a component of macromolecular complex on the base of albumin make it possible to increase antitumor effect of the cytostatic and to decrease its toxicity. That's why the main tasks of the research are the study of HSA-CP interactions, search for means to increase complex-forming capacity of HSA in relation to CP, and comparative analysis of antitumor activity and toxicity of CP and CP-HSA complex.

MATERIALS AND METHODS

In the study the next reagents were used: sodium octanoate, myristinic acid (Sigma, USA), phenol red, salicylic acid (Reanal, Hungary), fat-free HSA (Sigma, USA), officinal HSA (Biopharm, Ukraine), cisplatin (Veropharm, Russia). Reagents were of "superpure"grade.

Thermograms of melting of albumin preparations were done on differential adiabatic scanning microcalorimeter DASM-4 (Biopribor, Russia).

Thermal effects of complex formation between albumin and marker ligands were registered on flow microcalorimeter TAM-2277 (LKB, Sweden). Working concentration of albumin was 5 mg/ml in 0.05 M phosphate buffer solution, pH = 7.15.

Conditions for binding of CP to HSA were selected according to the laboratory data [11]. To determine the part of albumin-bound CP, the method of ultrafiltration was used (QTY membranes, 1PKG, Millipore, USA).

For purification of officinal solution of albumin, carbon sorbents of HSGD mark with power density of 0.098 g/cm^3 and total volume of sorption pores of 2.2 g/ cm^3 (IEPOR NAS of Ukraine) were used.

Antitumor and toxic activities of CP and CP-dHSA complex were studied on inbred white rats weighting 200 ± 20 g bearing transplanted Guerin carcinoma. Animals were obtained from the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine). All animal procedures were carried out under the rules of Ethic Committee.

Tumor-bearing animals were housed in 4 experimental groups: in groups 1 and 2 animals were injected in the tail vein with CP at the dose of 0.7 mg/kg of body weight (n = 16) or CP-dHSA (n = 15) in an equivalent platinum dose at each 2nd day for 2 weeks starting from the day 5th after GC cells transplantation, when the volume of tumor reached 0.54 ± 0.03 and 0.56 ± 0.13 cm^3, respectively. The animals from groups 3 and 4 received dHSA (n = 15) or physiologic solution (n = 15) by the same scheme. In the beginning of treatment tumor volume reached 0.50 ± 0.07 and 0.49 ± 0.10 cm^3 respectively. Intact rats (n = 6) served as the control group. Tumors size was determined by three orthogonal diameter' (a, b, c) according to Schrek's formula: V = (a x b x c) x 0.52. Coefficient K, presenting the ratio between tumor volume at the given moment and initial tumor volume (at the beginning of administration of preparation) was used as an index characterizing the dynamics of tumor growth. Antitumor effect of CP in a complex with dHSA compared to that of CP was evaluated as tumor growth inhibition ratio (TGIR):

$$TGIR = \frac{V_{CP} - V_{CP\text{-}dHSA}}{V_{CP}} \times 100$$

where V_{CP} and $V_{CP\text{-}dHSA}$ are the volumes of tumors upon administration of non-bound CP and CP in a complex with dHSA, respectively.

In 3 days after the last injection blood was taken from *vena cava inferior* under ether narcosis.

Content of creatinine in blood plasma was determined by the method of Popper [12].

To evaluate "metabolic intoxication", the pool of compounds of low- and medium molecular weight (CLMMW) of blood plasma and erythrocytes was analyzed by the method based on the registration of spectral characteristics (238–310 nm) of aqueous solutions of supernatants obtained by sedimentation of high molecular weight proteins of blood plasma and erythrocytes with 15% TCA solution [13]. CLMMW pool was calculated as an area between the curve of extinction values and abscissa axis, and expressed in relative units.

Intensity of oxidative modification of proteins of blood plasma was evaluated by the method based on the reaction between carboxylic derivatives of protein with 2,4-dinitrophenylhydrazine (2,4-DNP-hydrazine) with the generation of stable derivatives of dinitrophenylhydrazone, optical density of which was registered at the wave length of 370 nm [14]. The content of carboxylic derivatives of proteins in blood plasma was counted using the coefficient of molar absorption that is equal to 21 000 $M^{-1}cm^{-1}$ for DNP-derivatives at the wave length of 370 nm.

Concentration of platinum in the samples was determined using atomic absorptional spectrophotometer C-115M1 (Ukraine) at the wave length of 265.9 nm and standard platinum solution (Fluka, Switzerland).

RESULTS AND DISCUSSION

The first task was to examine complex-forming ability of HSA toward CP and to study the possibility to increase this index via sorptional activation of the protein.

Using differential scanning microcalorimetry approach, alterations of thermodynamic characteristics of fat-free HSA preparation after its interaction with CP were analyzed. Upon titration of HSA samples with CP at molar ratio of 1 : 1, 1 : 2, 1 : 3, 1 : 5, 1 : 7 and 1 : 10, gradual shift of high temperature maximum from 61.9 ± 0.2 °C to 64.0 ± 0.5 °C without change in the form of thermodynamic curve has been shown. Insignificant increase of melting temperature upon the molar ratio of albumin to cisplatin of 1 : 10 evidences on low constant of association of cytostatic with albumin molecule ($< 10^4$ M^{-1}).

By the method of isothermic flow microcalorimetry, temperature effects of interaction of HSA-CP complexes obtained at the molar ratio of albumin to cisplatin of 1 : 10, with marker ligands for second and

third HSA domains have been studied. After loading of albumin samples with CP, enthalpy of binding with sodium octanoate (domain III) statistically significantly decreased from 19.1 ± 0.7 kJ/mole to 15.8 ± 0.2 kJ/mole. In the remaining sites insignificant decrease of thermal effects of reaction of interaction with phenol red (domain II), salicylic and myristinic acids (domains II and III) were registered; possibly, it is determined by cytostatic-induced conformational transition. So, the second site of binding of drugs (marker ligand — sodium octanoate) located in domain III of albumin molecule, is one of the main sites for CP binding. The obtained result is of certain importance, because there are studies that have shown that free thiol group of Cys-34 binds only low amount of CP [9], and these that proved the presence of the other CP-binding site (apart from Cys-34) in albumin molecule, the origin of which was not identified so far [15].

On the base of the studies carried in our Department for many years, it has been shown that sorptional purification of officinal HSA on highly active carbon adsorbents of HSGD mark allows obtain HSA preparation free from the large part of hydrophobic ligands that are present in blood serum of the donors, and from thermostabilizer — exactly, sodium octanoate [16], thus making grounds to expect some elevation of complex-forming ability of HSA toward CP, too.

Binding of CP to HSA and dHSA has been studied in bench-top experiments by the method of ultrafiltration. Upon the molar ratio of CP and officinal HSA of 1 : 1, 2 : 1, 5 : 1 and 10 : 1, non-bound part of CP yielded 9.5, 18.5, 24.5 and 42.2%, respectively (Fig. 1). Upon mentioned CP/dHSA ratio non-bound part of cytostatic was 4.6, 8.3, 18.9 та 22.4%, respectively.

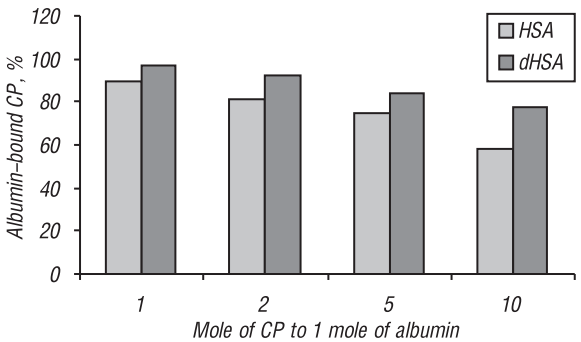

Fig. 1. Binding of cisplatin with HSA and dHSA in dependence on their molar ratio. The data represent the mean values of three separate experiments

So, sorptional deliganding of HSA on highly active carbon HSGD pyropolymers is an effective way to elevate its complex-forming ability toward cisplatin: deliganded HSA binds *ex tempore* 80–96% of cisplatin in dependence on their molar ratio, that is 5–25% higher than the part of cytostatic bound to officinal HSA.

The second task of the research was to study antitumor and toxic activity of CP and CP-dHSA complex in the model *in vivo*.

Intravenous administration of dHSA to the rats in the amount equal to that in CP-dHSA complex, practically did not influence the dynamics of growth of Guerin's carcinoma (Fig. 2). Starting from the day 7 after initiation of administration of CP and CP-dHSA, the pronounced inhibition of tumor growth has been observed. Upon introduction of CP at the days 7, 9 and 11, the increase of tumor volume (K) was 2.5, 4.7 and 7.2 fold lower than in the case of administration of physiologic solution, respectively, whilst in the group of animals injected with CP-dHSA comlex this index was 7.1, 20.9 and 19.1 fold lower, respectively. TGIR value for CP-dHSA (compared to CP) was 14.5, 26.1, 40.4 and 34.2% at the days 4, 7, 9 and 11 respectively, evidencing on higher inhibitory action of cytostatic bound with dHSA. After introduction of non-bound CP or CP-dHSA, regression of tumors registered during 14 days has been observed in 15 and 40% of animals, respectively. Potentiation of antitumor action of CP after its binding with dHSA could be caused by prolonged existence of cytostatic in blood stream and its gradual accumulation in tumor tissue. Pharmacological analysis is under way.

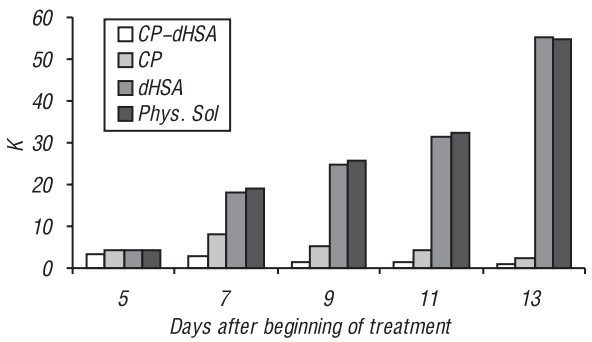

Fig. 2. Dynamics of growth of Guerin carcinoma in rats upon the treatment. The data represent the averages of two separate experiments. K values for animals treated with CP or CP-dHSA were statistically different (Student's t-test, $p < 0.05$) at days 7, 9 and 11

High nephrotoxicity of cisplatin (due to the fact that excretion by kidneys is the main route for elimination of CP) is well-known negative pattern of this agent [1]. In blood plasma of rats three days after termination of administration of non-bound CP statistically significant increase of creatinine content was observed (100.3 ± 15.9 μM/l) compared to intact animals (70.5 ± 10.4 μM/l), whilst in animals treated with CP-dHSA this index was 77.4 ± 5.9 μM/l and didn't differ significantly from its level in intact animals.

Upon the influence of antitumor agents, significant elevation of endogenous intoxication is observed, for evaluation of which the pool of compounds of low- and medium molecular weight (CLMMW) in blood plasma and erythrocytes of rats has been analyzed. CLMMW pool in supernatants of blood plasma increased in average by 35, 40, and 25% in animals that were injected by physiologic solution, non-bound CP and CP-dHSA complex respectively and compared with that in intact control (18.6 ± 3.8 a.u.). CLMMW pool of erythrocytes that yielded 59.8 ± 4.7 a.u. in intact animals, was significantly elevated only in rats treated with non-bound CP (73.2 ± 6.3 a.u.). In animals treated with CP-dHSA complex this index was equal t 63.7 ± 5.6 a.u.

It is known that intense chemotherapy promotes the production of reactive oxygen species causing oxidative modification of proteins of tissues and blood plasma with generation of aldehyde and ketone derivatives [17]. In our study, on the background on insignificant differences of the level of total protein, the content of carboxylic derivatives of proteins in blood plasma of rats after administration of non-bound CP was in average 1.5 fold higher than that in animals that received CP-dHSA complex (Fig. 3). At the same time in animals with retarded dynamics of tumor growth inhibition higher level of carboxylic derivatives was observed (3.82 and 3.94 mMole/g protein), whilst in animals with tumor regression — lower values (2.15 and 2.34 mMole/g protein).

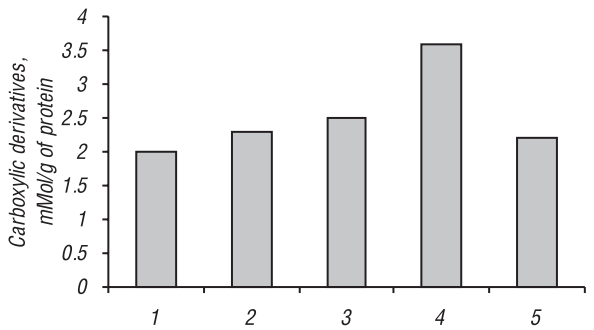

Fig. 3. Content of carboxylic derivatives of blood plasma of rats: intact (*1*), bearing GC after administration of physiologic solution (*2*), dHSA (*3*), CP (*4*) and CP-dHSA (*5*). The data represent the averages of two separate experiments

In conclusion, the study has shown that only in animals that received therapy with non-bound CP, significant elevation of the level of blood plasma creatinine and intensity of oxidative modification of proteins was registered on the background of the significant increase of the pool of compounds of low- and medium molecular weight in blood plasma and erythrocytes.

The obtained results create the grounds to recommend the use of CP in the complex with officinal albumin solution, that should be reasonable to purify prior to binding with the use of highly active carbon sorbents of HSGD mark. These procedures are not requiring complex technological approaches and could be carried out immediately before administration of cytostatic (Fig. 4).

We present the formula by which the volume of deliganded HSA could be counted for preparation of CP-containing solution with molar ratio C : dHSA = 2 : 1 (1 mg CP : 0.11 g albumin) and 5 : 1 (1 mg CP : 0.04 g albumin), when *ex tempore* 95% or more than 80% of cytostatic is bound:

$$V = \frac{0.11\ (0.04) \times D \times 100 \times 1.15}{C},$$

where V — volume of dHSA, ml;

D — the dose of CP calculated for administration to patient, mg;

C — concentration of officinal HSA, %;

1.15 — coefficient accounting the decrease of albumin concentration after sorptional purification.

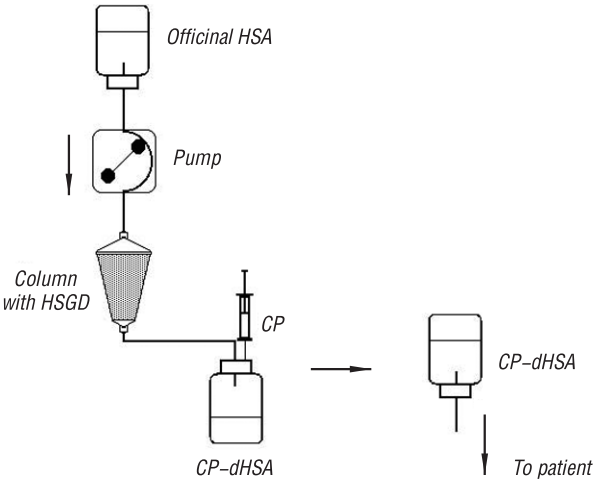

Fig. 4. Principal scheme for generation of CP-dHSA preparation for clinical use

ACKNOWLEDGEMENT

This study was supported by the Program of NAS of Ukraine "Peculiarities of Oncogenome Functioning" (№ 0102U003228).

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ИЗУЧЕНИЕ ВОЗМОЖНОСТИ ПОВЫШЕНИЯ ПРОТИВООПУХОЛЕВОЙ АКТИВНОСТИ И СНИЖЕНИЯ СИСТЕМНЫХ ТОКСИЧЕСКИХ ЭФФЕКТОВ ЦИСПЛАТИНА ПУТЕМ ЕГО СВЯЗЫВАНИЯ С ДЕЛИГАНДИЗИРОВАННЫМ АЛЬБУМИНОМ

Цель: **оценить противоопухолевое и токсическое действие цисплатина (ЦП) в свободной форме и в комплексе с делигандизированным альбумином.** ***Методы:*** **дифференциальную сканирующую и изотермичную проточную микрокалориметрию использовали для исследования комплексообразования ЦП с альбумином. Для количественной оценки связанного с альбумином цитостатика использовали метод ультрафильтрации. Концентрацию платины в образцах определяли методом атомно-абсорбционного спектрального анализа. Противоопухолевый и токсический эффекты ЦП и ЦП в комплексе с делигандизированным альбумином изучали на крысах с карциномой Герена.** ***Результаты:*** **показано, что одним из основных мест связывания ЦП является второй лекарственный сайт, расположенный в III домене молекулы альбумина. Очистка фармакопейного сывороточного альбумина (ЧСА) на высокоактивных углеродных гемосорбентах марки ГСГД позволяет получить делигандизированный альбумин (дЧСА) с повышенной комплексообразующей способностью в отношении ЦП. ЦП в связанной с дЧСА форме обеспечивает повышение ингибирующего влияния цитостатика на рост карциномы Герена, при этом содержание креатинина в плазме крови повышается на 15% по сравнению с 40% при использовании несвязанного ЦП.** ***Выводы:*** **результаты проведенных исследований дают основания рекомендовать использование ЦП в комплексе с альбумином для усиления его противоопухолевого действия и снижения токсических эффектов.**

Ключевые слова: **цисплатин, человеческий сывороточный альбумин, углеродный гемосорбент ГСГД, карцинома Герена.**