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Study of molecular mechanisms of anti-tumor effect of hemorphin-7 *in vivo*

N. H. Barkhudaryan[1], O.V. Hunanyan[1], F.P. Sarukhanyan[1], H.M. Stepanyan[2], H.H. Zakaryan[1], I. E. Grigoryan[3], E. B. Dalyan[3]

[1]H. Buniatian Institute of Biochemistry NAS RA,
[2]Institute of Fine Organic Chemistry NAS RA,
[3]Department of Molecular Physics, Faculty of Physics
Yerevan State University,
5/1 P. Sevag str., 0014, Yerevan, RA

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Hemorphin-7 (H7, YPWTQRF) is a member of hemorphins family, an endogenous nonclassical opioid peptide derived from haemoglobin (Hb), demonstrating a wide spectrum of biological activities [For review see Ref. 26]. H7 exhibits anti-inflammatory and immunoregulatory properties [30, 17]. It was shown the small, but significant passage of hemorphin-7 across the blood-brain-barrier [26], however α- and β- globin mRNAs were identified in mouse brain, implying the synthesis of globin in the central nervous system (CNS) [27]. Brain catepsin B was shown to participate in the generation of several hemorphins, including hemorphin-7 from LVV-hemorphin-7 (LVV-H7) in vitro [2]. It should be noted that LVV-H7 contains the amino acid sequence of all known hemorphins. By using in vivo microdialysis in combination with electrospray mass spectrometry in vivo processing of hemorphin-7 from LVV-hemorphin-7 in rat brain and blood was shown [31]. It has been shown that H7, like other hemorphins, in vitro modulates Ca_{2+}/calmodulin (CaM)-dependent protein phosphatase calcineurin activity by binding to CaM, exhibiting a concentration-dependent biphasic response on enzyme activity [5, 7, 10, 17]. Calcineurin is known as a key enzyme in the signal transduction cascade leading to T cell activation. This enzyme controls gene expression of several cytokines (interleukin (IL)-2, tumor necrosis factor α, (TNFα), IL-4, IL-5 etc.) and other regulatory proteins via dephosphorylation and nuclear translocation of NFATc (nuclear factor of activated T cell) family members [33]. Importantly,

H7 was shown to regulate DNA-binding activity of NFAT, AP-1 and NF-κB transcription factors in stimulated human Jurkat T cells [6]. Moreover, it has been demonstrated that H7 in nM concentrations negatively regulates IL-2 promoter transcriptional activity [17]. It has been revealed that H-7 in μM concentrations significantly (2,5 fold) inhibits in vitro DNA methylation in pathophysiology of tumor (sarcoma-45) [18]. It is well known that methylation of DNA often correlates with the lack of transcriptional activity, and inhibition of DNA methylation is a specific characteristic of anti-tumor drugs [15, 21]. It has been reported that some of the members of hemorphins family (e.g. LVVYPW (MP-2), VVYPWTQ (valorphin)) exert anti-tumor properties [8, 11, 25]. It has also been shown that μ-opioid receptors (MOR) agonists exert anti-tumor properties [22-23]. Because H7 is the most potent among the hemorphins in MOR binding [37], and, moreover, H7 has a capacity to induce the release of β-endorphin from the pituitary tissue into circulation [26], it is suggested that H-7 may exhibit anti-tumor properties as well. This view is supported by several reports from different laboratories indicating that Ca_{2+}/CaM/calcineurin/NFAT pathway is involved in pathophysiology of cancer [24, 28].

It is to be noted that hemorphins change the sensitivity of CaM to its antagonists (trifluoperazine (TFP), chlorpromazine, W-7, vinblastine and vincristine) [3]. All of these compounds are drugs that are implicated in the treatment of different diseases, including cancer [20, 35]. Therefore in 1992, we predicted that hemorphins had good prospects for applied medicine as drugs without side effect [3].

Taking into consideration all of the above mentioned the aims of the present study were:

1) to examine the effects of intraperitoneal (ip) injections of synthetic LVV-H7 and H7 alone and H7 in combination with anti-tumor agent doxorubicin (DOX) on tumor growth in rats bearing S-45;

2) to study the effect of LVV-H7 and H7 on plasma and brain calcineurin activity in sarcoma-45 bearing rats;

3) to determine the in vivo effect of LVV-H7 and H7on thermodynamic parameters of DNAs, isolated from S-45 and S-45 treated with hemorphin (LVV-H7 and H7) and H7+DOX;

4) to examine if LVV-H7 and H7 can directly bind to DNA and form DNA-hemorphin complex.

Materials and Methods

In the experiments the synthetic hemorphins (H7 and LVV-H7) and anticancer drug DOX (anthracycline antibiotic, trade name Adriamycin) were used. The experiments were carried out on Wistar line rats of both sexes weighing 100-120 g at the time of experiment. Rats provided by Animal House of the Institute of Biochemistry NAS RA, were caged in groups of 5 with food and water given ad libitum. Rats were randomly divided into 5 groups: control group (healthy rats, n=6) and 4 experimental groups inoculated with sarcoma-45 (S-45) by the method of Chernov W., 1971 [14]. The first experimental group (n=10) received daily single ip injection of LVV-H7 (1mg/kg) dissolved in saline (0.9% NaCl) (0.5 ml), during 8 days. The second experimental group (n=10) received daily single ip injection of H7 (1mg/kg) dissolved in saline, during 8 days. The third experimental group (n=10) received daily mutual ip injection of H7 (1mg/kg) + DOX (2mg/kg) during 8 days. The fourth experimental group (n=10) received daily 0.5 ml of saline injection. The rats in control group were injected by saline (0.5 ml). On the 14th day of the experiment animals were decapitated. The brains and tumors were rapidly removed, frozen and stored at -70° C until use. The trunk blood was immediately collected into sodium citrate (3.2%)-coated vacutainer tubes. Then, the blood samples were centrifugated at 1500 rpm for 10 min, separated into aliquots and stored at -70° C.

Calcineurin Activity

Calcineurin activity was measured by spectrofluorimetric assay using 4-methylumbelliferyl phosphate (4-MUP) as a substrate [1]. A typical enzyme assay was performed in 1 ml of incubation mixture, containing 50 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 1 mg/ml bovine serum albumin (BSA), 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 1 μM 4-MUP, and necessary amount of enzyme (100 000 x g soluble brain protein fraction or plasma). One unit of enzyme activity is defined as amount of enzyme that caused the formation of 0.1 nM of 4-methylumbelliferon (4-MU) at 32° C for 1 h. The quantity of 4-MU was determined fluorimetrically using a Perkin-Elmer MPF-44A spectrofluorimeter. The fluorescence was measured at 445 nm (Exitation at 365 nm). As control the substrate and enzyme were incubated separately.

Thermodynamic investigation of DNA

Excess heat capacity (ΔC_p) versus temperature (T°C) profiles for the thermally induced transitions of DNAs isolated from S-45 and S-45 treated with hemorphins (LVV-H7 and H7) alone or with H7 in combination with DOX were measured by using a differential scanning microcalorimetry (DSM). Measurements were conducted on a DASM-4 microcalorimeter (Russia). In these experiments, the heating rate was 1°C/min at temperature ranges 10-100°C. Enthalpies (ΔH) were calculated from the areas under the experimental ΔC_p versus T°C curves using Scal Dos software. The DNAs were dissolved in 0.1 M sodium chloride–sodium citrate (SSC) buffer (pH 6.9) containing 15 mM NaCl, 1.5 mM sodium citrate. The concentration of solution of DNAs isolated from S-45 was 288 μg/ml and concentrations of DNAs isolated from S-45 treated with H7, LVV-H7 and H7+DOX were in the range of 300-304 μg/ml respectively.

Detection of DNA-peptide complex

The formation of DNA-H7 and DNA-LVV-H7 complexes *in vitro* was detected by differential absorbance measurements, which were conducted on a SPECORD UV/VIS spectrophotometer (Germany). Absorbance was measured at 200-350 nm. DNA (at concentration of 28 μg/ml), isolated from healthy rats' liver, was reacted with LVV-H7 or H7 in 1 mM NaCl (pH 6.9). Registration of DNA-H7 and DNA-LVV-H7 complexes was detected in μM concentration ranges of peptides.

The results were analyzed using Origin version 6.1 software. Statistical significance – $p<0.05$. All data were expressed as means ± SEM.

Results and Disscussion

The effects of LVV-H7 and H7 alone, as well as the effect of H7 in combination with DOX have been studied on tumor growth after 8 days of treatment of rats bearing S-45. As one can see (fig.1), treatment of rats with H7 or H7+DOX induced statistically significant inhibition of tumor growth, by 44, 9 % and 57, 3% respectively in comparison with S-45 bearing rats, which received daily single injection of 0,9% NaCl during 8 days. It is suggested that H7 and DOX act synergistically. It should be noted that DOX alone induces the inhibition of tumor growth on 52% (data not shown). We did not observe statistically significant inhibition of tumor growth by treatment of rats with LVV-H7. The mechanism of DOX action has been linked to DNA damage, topoisomerase II inhibition, and irons sequestration with subsequent free radical generation, rendering cells more vulnerable to apoptosis [19, 32, 36]. In addition, it has been reported that DOX pretreatment sensitize prostate cancer cell lines to TRAIL (TNF-related apoptosis inducing ligand)-induced apoptosis, which correlates with the loss of anti-apoptotic protein cFLIP (cellular FLICE inhibitory protein) expression. Moreover, DOX generates a pro-apoptotic phenotype by phosphorylation of EF-2 (elongation factor-2), which in turn also degrades cFLIPs [36,19]. Importantly, CaM antagonist TFP modulates cytotoxicity of DOX in vitro and anti-tumor effect of DOX in vivo and this effect is dependent on the level of DOX resistance [16]. Another CaM antagonist vincristine attenuates DOX cardiotoxicity. Treatment with DOX+vincristine activated pro-survival signal Akt (protein kinase B) and diminished cytochrome C release. It is suggested that these findings may have clinical implication [13]. As one can see (Fig.1) H7 potentiates the anti-tumor effect

of DOX. Because hemorphins are suggested to be allosteric regulators of CaM, and have a capacity to change the sensitivity of CaM to its antagonists (e.g. TFP, vinctistine and etc.), it is suggested, that H7 may make the action of DOX less toxic, acting by binding to CaM. Using the ICPL (isotope coded

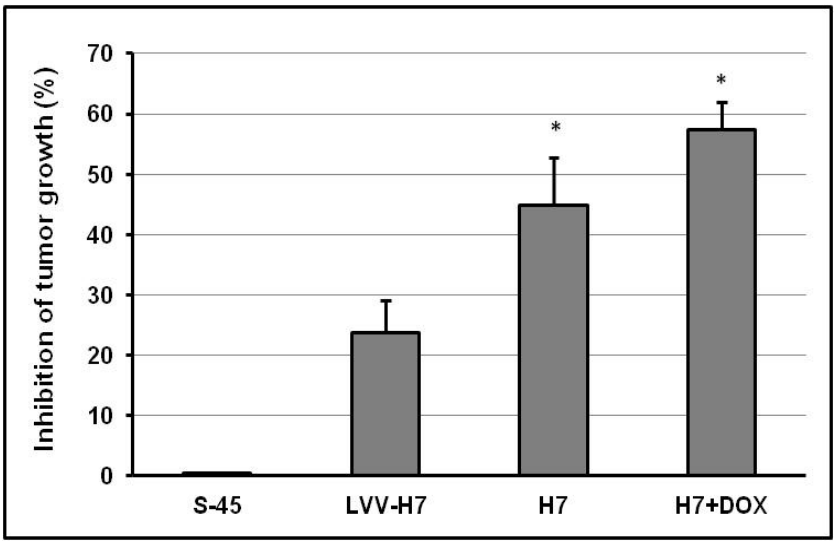

Fig.1. Effect of hemorphin (LVV-H7 or H7) alone and H7 in combination with DOX on tumor growth in rats bearing S-45, after 8 days of in vivo treatment. * $p<0.05$.

protein label) technology with MALDI-MS-MS mass spectrometric analysis, ferritin H chain is identified as a potential target for hemorphin in mouse brain [4]. This protein has already been reported to be involved in the pathophysiology of cancer [29]. In addition, melanoma-derived ferritin H chain isoform is able to stimulate up regulation of p53 andmediate apoptosis involving Fas (CD95) [12]. It is thought that H7 by up regulation of ferritin expression may mediate the process of apoptosis in cancer cells. It should be noted that β-endorphin has been recorded to increase apoptosis in lung cancer cells [23]. H7 may affect apoptotic processes by inducing the release of β-endorphin as well. Thus, synergistic action of H7 and DOX in inhibition of tumor growth may be based on different mechanisms (e.g. increase in apoptosis, release of β-endorphin, regulation of ferritin expression etc.).

In the present work we have studied the influence of ip administration of hemorphins (H7 and LVV-H7) on calcineurin activity in pathophysiology of S-45. We have observed reduction of calcineurin activity in brain by 58,16% and in plasma by 63, 44% respectively, in comparison with

calcineurin activity in brain and plasma of healthy rats, treated with saline (Fig.2 A and B). These data correlate with literature data, indicating to down-regulation of calcineurin activity in pathophysiology of cancer [28]. It has been demonstrated for the first time that both H7 and LVV-H7 are able to recover in vivo calcineurin activity in the brain and plasma of S-45 bearing rats. Both H7 and LVV-H7 after 8 days of treatment of S-45 rats, induce increase in brain (by 20,1% and 36,83% respectively) and plasma (by 47,44% and 44,14% respectively) calcineurin

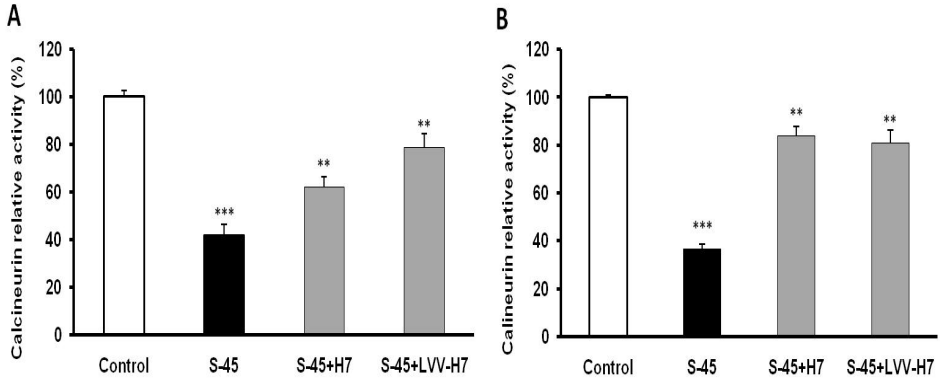

Fig. 2. The effect of ip administration of LVV-H7 and H7 on calcineurin activity in the brain (A) and plasma(B).

** p< 0.01; *** p<0.001.

activity in comparison with brain and plasma calcineurin activity of S-45 untreated rats. The data obtained confirm our previous findings that calcineurin is involved in the molecular mechanisms of hemorphins action in physiology and pathophysiology [5, 8-10]. It is well known that calcineurin participates in gene expression and production of IL-2 via calcineurin/NFAT pathway, so now it is clear why anti-cancer effect of LVVYPW (MP-2) is bound with restoration of IL-2 synthesis suppressed in tumor bearing organism [16]. LVVYPW induce the increase of calcineurin activity and calcineurin, in turn, stimulates IL-2 synthesis on gene transcription level. The data obtained give as reason to suggest that anti-tumor effect of hemorphins is associated with the regulation of calcineurin activity by hemorphins. However in the present study we have shown, that though LVV-H7 demonstrates the inhibition of tumor growth (by 23,8%), the data obtained are not statistically significant. At the same time it has been shown that LVV-H7, likewise H7, in vivo recovered calcineurin activity. To clear up these results, there is need for further investigations.

In order to reveal if H7 and LVV-H7, likewise LVVYPW [8], may in vivo directly affect DNA structure, we have studied the thermodynamic characteristics of DNAs, isolated from S-45 bearing rats and S-45 bearing rats treated with H7, LVV-H7 and H7 + DOX.

Using differential scanning microcalorimetry (DSM) we revealed the changes in heat capacity (ΔC_p) of DNAs, isolated from S-45 and S-45 treated with LVV-H7, H7 and H7+ DOX (Fig.3). Using automated data processing software Scal Dos the values of enthalpies (ΔH) of studied DNAs was determined. Data obtained give us reason to suggest that as a result of in vivo treatment of S-45 with LVV-H7, H7 and H7+DOX, the interaction of DNA with peptides takes place. The values of enthalpy for DNAs, isolated S-45, S-45 treated with H7, H7 +DOX and LVV-H7, were 9, 23 kcal/mol, 10,17 kcal/mol,

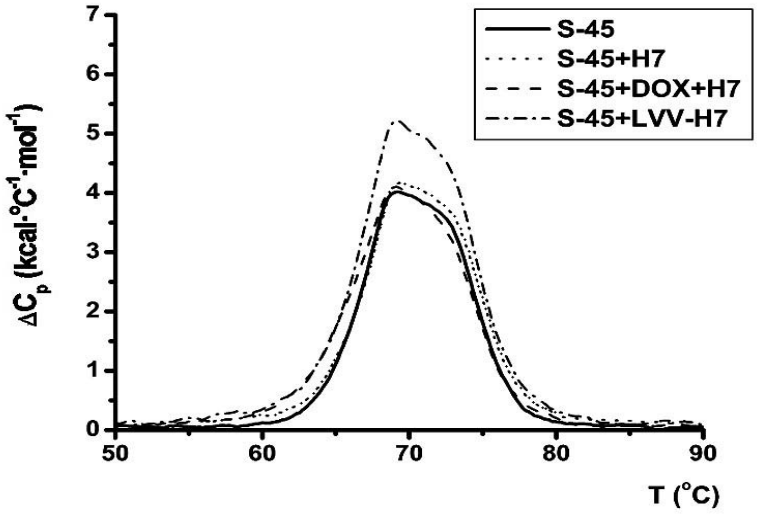

Fig. 3.DSM thermograms for DNAs isolated from S-45 and S-45 treated with LVV-H7, H7, H7+DOX. Enthalpies (ΔH) were calculated from the areas under the experimental

ΔC_p versus T°C curves.

10,1 kcal/mol and 12,8 kcal/mol respectively. It should be noted that the value of enthalpy for DNA isolated from S-45 treated by LVVYPW (LVV-H3) was found out to be 16,2 kcal/mol [8]. The data obtained give us reason to suggest that as a result of in vivo treatment of S-45 with LVV-H7 the hydrophobic interaction of DNA with peptide, due to N-terminal LVV amino acid sequence, takes place, which induces the stabilization of DNA by increasing value of enthalpy (12,88 kcal/mol) in comparison with enthalpy

of S-45 DNA (9,23 kcal/mol). The values of enthalpy for DNAs, isolated from S-45, treated with H7 (10,17 kcal/mol) or H7+DOX (10,1 kcal/mol), have small differences in comparison with that for DNA, isolated from untreated S-45 (9, 23 kcal/mol). It is suggested that H-7 induces weeker interaction with DNA than LVV-H7 or LVV-H3. It seems very likely that anti-tumor action of H-7 is bound with the ability of this peptide to induce the cancer cells apoptosis. This view is supported by finding that the values of enthalpy for DNAs isolated from S-45 treated with H7 or H7+DOX are almost equal (10,17 kcal/mol and 10,1 kcal/mol, respectively). One can also speculate that the differences in values of enthalpy between DNAs isolated from S-45, treated with H7 and treated with LVV-H7 may, possibly, explain lack of anti-tumor effect for LVV-H7.

We used the spectrophotometric approach to confirm the formation of DNA-H7 and DNA-LVV-H7 complexes *in vitro* by measuring differential absorbance spectra (Fig.4). Registration of complexes with changed conformation was detected in μM concentration ranges of peptides.

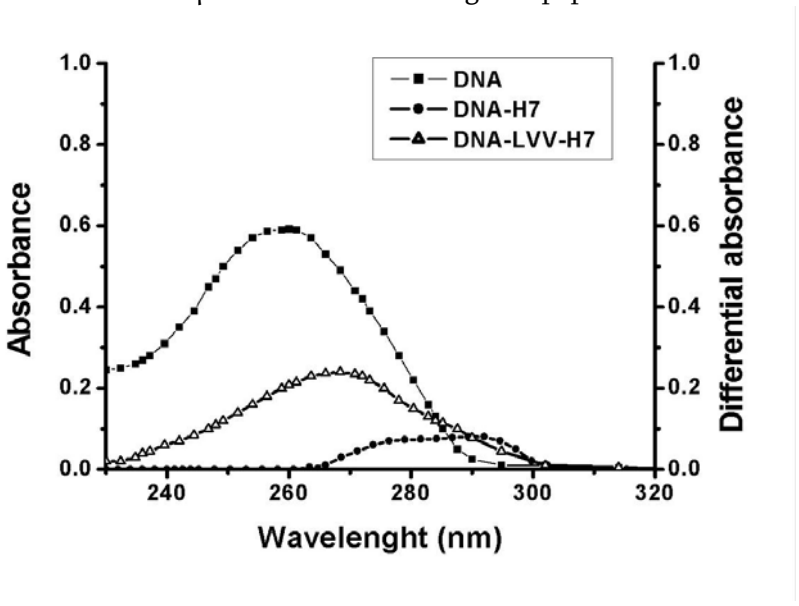

Fig. 4. Differential absorbance spectra for DNA-LVV-H7 and DNA-H7 complexes in comparison with absorbance spectrum for DNA from healthy rats liver.

Thus, the present study demonstrates that Ca_{2+}/CaM/calcineurin/NFAT pathway is involved in the molecular mechanisms of anti-tumor effect of H7 in vivo. One can speculate that H7 via modulation of Ca_{2+}/CaM/calcineurin/ NFAT pathways may affect different genes expression, including the expression of tumor suppressing genes. This view is supported by our recent findings indicating that H7 is able to regulate DNA-binding activity of

NFAT, AP-1 and NFκB transcription factors in stimulated Jurkat T cells [6]. The ability of H7 to induce the inhibition of DNA methylation and to make changes in thermodynamic parameters of DNA may also affect transcriptional activity.

Taking into account all above mentioned, we propose that anti-tumor effect of H7 is based on implication of different mechanisms directed to recover the homeostatic disturbance in the organism in pathophysiology of tumor.

Поступила 12.12.11

Հեմորֆին-7-ի հակաուռուցքային ազդեցության մոլեկուլային մեխանիզմների ուսումնասիրությունը *in vivo*

Ն.Հ. Բարխուդարյան, Օ.Վ. Հունանյան, Ֆ.Պ.Սարուխանյան, Հ.Մ. Ստեփանյան, Հ.Հ. Զաքարյան, Ի.Է. Գրիգորյան, Ե.Բ.Դալյան

Ցույց է տրված, որ ինչպես հեմորֆին-7-ի (H7), այնպես էլ H7+դոքսոռուբիցինի (DOX) ներորովայնային ներարկումները առաջացնում է ուռուցքի աճի վիճակագրորեն հավաստի արգելակում 44.9 և 57.3 %-ով, համապատասխանաբար, այն դեպքում, երբ LVV-հեմորֆին-7-ի (LVV-H7) ներարկումը չի բերում ուռուցքի աճի վիճակագրորեն հավաստի արգելակման: Երկու հեմորֆիններն էլ վերականգնում են S-45-ի պաթոֆիզիոլոգիայում ընկճված կալցինեյրինի ակտիվությունը:

Միկրոկալորիմետրիայի մեթոդով որոշելով ԴՆԹ-ների էնթալպիայի արժեքները, որոնք անջատվել է LVV-H7-ի, H7-ի և H7+DOX-ի ներորովայնային ներարկումներ ստացած S-45-ով հիվանդ առնետներից, բացահայտվել է, որ էնթալպիայի արժեքը մեծանում է տարբեր կերպ (12,8 կկալ/մոլ, 10,17 կկալ/մոլ և 10,1 կկալ/մոլ համապատասխանաբար) ի համեմատ S-45-ից անջատված ԴՆԹ-ի էնթալպիայի արժեքի (9,23 կկալ/մոլ): Ենթադրվում է, որ տեղի է ունենում ԴՆԹ-պեպտիդ կոմպլեքսի առաջացում: Այդ տեսակետը հաստատվեց առողջ առնետների լյարդից անջատված ԴՆԹ-ի հետ պեպտիդների կլանման դիֆերենցիալ սպեկտրների in vitro գրանցման միջոցով:

Կարծում ենք, որ ԴՆԹ-ի էնթալպիայի արժեքների միջև տարբերությունը, որը դիտվում է S-45-ի վրա LVV-H7-ի և H7-ի *in vivo* ազդեցությամբ, կարող է բացատրել LVV-H7-ի ներարկման դեպքում ուռուցքի աճի վիճակագրորեն հավաստի արգելակման բացակայությունը: S-45-ի վրա H7-ի և H7+DOX-ի *in vivo* ազդեցության դեպքում ԴՆԹ-ի էնթալպիայի գրեթե միանման արժեքները հիմք են տալիս ենթադրելու,

որ H7-ի հակաուռուցքային ազդեցությունը DOX-ի նման ևս պայմանավորված է ուռուցքային բջիջների ապոպտոզի խթանմամբ:

Ենթադրվում է, որ H7-ը *in vivo* ազդում է տարբեր մոլեկուլային մեխանիզմներով վերականգնելու համար S-45-ի պաթոֆիզիոլոգիայում խաթարված օրգանիզմի հոմեոստազը:

Изучение молекулярных механизмов противоопухолевого эффекта геморфина-7 *in vivo*

Н.А. Бархударян, О.В. Унанян, Ф.П. Саруханян, Г.М. Степанян, Э.А. Закарян, И.Э. Григорян, Е.Б. Далян

Показано, что внутрибрюшинные инъекции как геморфина-7 (H7), так и H7+доксорубицина (DOX) вызывают статистически достоверное ингибирование роста опухоли на 44.9 % и 57,3% соответственно, в то время как инъекции LVV-геморфина-7 (LVV- H7) не вызывают статистически достоверного ингибирования роста опухоли. Оба геморфина восстанавливали активность кальцинейрина, подавленную при патофизиологии S-45. Использование микрокалориметрического метода для определения величин энтальпии ДНК, выделенной из S-45 крыс, получивших внутрибрюшинные инъекции LVV-H7, H7 и H7+DOX, выявило различное увеличение величин энтальпии (12,8; 10,17 и 10,1 ккал/моль соответственно), по сравнению с величиной энтальпии ДНК, выделенной из S-45 (9,23 ккал/моль). Мы предположили, что имеет место образование комплекса ДНК–пептид. Это мнение подтвердилось *in vitro* измерением дифференциальных спектров поглощения пептидов с ДНК, выделенной из печени здоровых крыс. Думается, что разница в величинах энтальпии ДНК, в случае *in vivo* воздействия LVV-H7 и H7 на S-45, может объяснить отсутствие статистически достоверного ингибирования роста опухоли при инъекции LVV-H7. Почти одинаковые величины энтальпии ДНК, в случае *in vivo* воздействия H7 и H7+DOX на S-45, дают основание предположить, что противоопухолевый эффект H7, подобно DOX, также основан на способности H7 стимулировать процесс апоптоза опухолевых клеток. Предполагается, что H7 действует *in vivo* различными молекулярными механизмами для восстановления нарушенного гомеостаза при

патофизиологии
S-45.

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