Synthesis and investigation of the specific activity of the DNA-doxorubicin conjugates

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Abstract. In the present work, the method of obtaining the conjugate of the anticancer chemotherapeutic agent doxorubicin to the exogenous double-stranded DNA of the sturgeons is proposed (the source: commercial drug "Derinat"). The optimal conditions for synthesis of conjugate (pH, temperature and the mass ratio of the components), ensuring the highest degree of binding the chemotherapeutic agent to a carrier, were picked out. Clearing the conjugate from the non-encapsulated chemotherapeutic agent was being made by ultrafiltration method. The investigation of the toxicity and specific antineoplastic activity of the synthesized complex was conducted. The performance of the drug toxicity were established on the intact mice in compliance with the accepted standards. The antineoplastic activity was evaluated upon the Tumor Growth Inhibition Index and Metastasis Inhibition Index on mice with the transplanted Lewis lung carcinoma (LLC). It was demonstrated that the conjugate toxicity is approximately lower that the one of the unconjugated doxorubicin (LD 50 was equal 14.6 mg/kg and 9.9 mg/kg for the conjugate and doxorubicin, respectively). The specific antineoplastic activity was investigated in equitoxic doses of the drug. It was established that the conjugate being administered in equitoxic doses possesses a stronger antineoplastic activity, than the water-soluble drug (maximum 35% more as to the tumor volume and 51% more as to the Tumor Growth Inhibition index).

1. Introduction

Increasing the selectivity of the anticancer chemotherapeutic agents is one of the most crucial tasks of Oncopharmacology. The most popular way of solution thereof is binding the cytostatic chemotherapeutic agent to one or another vector, enabling specific binding to the receptors on the surface of the tumor cell as well as upcoming endocytosis. Our attention was attracted by the possibility to use a native exogenous DNA as such a vector. It is a fact that the exogenous DNA can be phagocytized by the animal cells [1 – 8]. It has been established that the endocytosis of DNA is of receptor-mediated nature [2 – 6]. This mechanism of the intracellular penetration of DNA is established for tumor cells too [7, 8]. These facts became a reason for using the exogenous DNA as a vector for the target-specific delivery of the anticancer drugs [10 – 12]. But in these works, not the

chemotherapeutic agent alone, but its complex with the human albumin was used for the synthesis of the DNA-dox conjugate. In fact, albumin was as a linker in binding the chemotherapeutic agent to DNA. It should be noted that, in aqueous media, DNA forms strong complexes with cationic polymers only at the body temperature, and the conjugate to the native albumin having a comparable number of anionic and cationic groups, would be not stable enough [13 – 15]. As albumin can be taken up by cells, including tumor cells [18], under stress too intensively [16 – 17], it's impossible to establish the role of each vector in delivering the chemotherapeutic agent, so the necessity to use DNA was put in doubt. In this context we developed and approved the method for the synthesis of the DNA-Dox conjugate not demanding albumin using.

Work objective: the development of the method for synthesis of the conjugate of the anticancer drug doxorubicin to DNA; the investigation of the toxicity and specific antineoplastic activity of the synthesized conjugate.

2. Materials and methods

2.1. Materials.
The following reagents were used in the work:

- DNA-Na (the source: commercial drug "Derinat", Closed Joint-Stock Company "Tekhnomedservice", Russia);
- Doxorubicin hydrochloride (Dox), > 98% – "Sigma-Aldrich", USA;
- "Milli-Q" water;
- Phosphate buffer (PBS), "Sigma-Aldrich", USA;
- Hank's balanced salt solution ("BioloT" Ltd., Russia).

2.2. The synthesis of DNA- Dox conjugates.
The following was mixed aseptically: 2 ml of the DNA solution with the content of the substance of 15 mg/ml, 2 ml of the aqueous solution of doxorubicin with the drug content of 7, 5, 10 or 15 mg/ml (depending on the batch) and 2 ml of Phosphate buffer with 7.0 or 8.0 pH (depending on the batch). The batch description is given in the part "Findings". The mixture was incubated within 60 minutes at a constant temperature (4^oC, 20^oC and 37^oC) and a continuous shaking. The obtained conjugate was cleared from the unconjugated doxorubicin by ultrafiltration method on the unique facility [19] using the cellulose membrane Q1210-55 F3 ("Orangescientific", Belgium) with the pore diameter of 12 – 14 kDa, procedure duration was 4 hours. 3 x 3 syntheses were performed, for each variable parameter (pH, temperature, reagents ratio). The degree of the drug encapsulation into the conjugate was being calculated by the formula:

$$\omega = (m_0 - m)/m_0 \cdot 100\%,$$

where m_0 – initial quantity of doxorubicin, m – the quantity of the non-encapsulated doxorubicin. The quantity of the non-encapsulated doxorubicin was calculated due to its concentration in ultrafiltrate. The drug concentration in dialysate was being estimated spectrophotometrically on the UV-2600 Shimadsu spectrophotometer (Japan), at the wave length of 490 nm upon the preliminary plotted calibration schedule.

2.3. Experiments on animals.
All the animals were received from the "Stolbovaya" nursery and were kept under the standard vivarium conditions. Experiments on animals were performed subject to bioethical principles upon the approval by the local Ethics Committee at the Federal State-Funded Educational Institution of Higher Professional Education "Mordovia State University named after N. P. Ogaryov".

2.3.1. The investigation of the acute toxicity. The toxicity investigation was performed on 240 BALB/cWt mice. The animals were divided into 4 groups (a control and 3 experimental ones). In the control group, the animals were being administered with 0.2 ml of isotonic sodium chloride solution intravenously. In the 1st experimental group, doxorubicin was administered intravenously; in the 2nd experimental – doxorubicin conjugated to DNA; in the 3rd experimental – 1.5% DNA solution. In each experimental group, the 6 subgroups were divided (males as well as females in fives in each), in which the drugs were administered in the dosage of 2, 4, 8, 16, 24 and 32 mg/kg in equivalent of pure doxorubicin). The animals were observed within 30 days; the lethality was being registered daily. Establishing the toxicity performance was being executed by Probit Analysis. The LD 10, LD 50, and LD 100 of the substances studied as well as the 95% confidence intervals thereof were calculated.

2.3.2. The investigation of the antineoplastic activity. The investigation of the antineoplastic activity was performed on 60 C57Bl/6 mice of both sexes. The antineoplastic activity was being studied on the syngeneic tumor system from the bank of tumor strains from the Russian Cancer Research Center named after N. N. Blokhin of the Russian Academy of Medical Sciences – Lewis lung carcinoma (LLC).

The tumor tissue of LLC was transplanted in animals intramuscularly into the femur of the hind paw on the left in amount of 1×10^6 cells in the Hank's balanced salt solution. On the 22nd day upon the transplantation of the tumor cells, the animals discontinued the experiment.

The antineoplastic as well as antimetastatic activity were evaluated due to the guideline for studying the specific activity of the anticancer drugs being in force in the Russian Federation [20, 21].

The volume of the primary tumor node was being determined in the course of the trial, as well as the weight thereof – at the end of the trial. The number of the lung metastases was counted upon the fixation thereof in Carnoy's solution with using MBS-9 binocular loupe (16x magnification). The size of the tumors on the transplantation site was being established by a beam-compass and calculated their volume by the formula for ellipsoid volume:

$$V = 0{,}131\times L\times(D1+D2)^2,$$

where L – tumor length, $D1$ and $D2$ – two other mutually perpendicular diameters.

The drug entry was made intravenously threefold 72 hours apart, starting the treatment from the 7th day after the LLC strain transplantation. The drug dosage in the groups is shown in the Table 1. The dosage selection was made on the basis of the earlier toxicology studies (the equitoxic doses were compared). In the 1st and in the 3rd experimental group, the drug doses made a half of LD_{10}, in the 2nd and in the 4th ones – 1 LD_{10}. The DNA-dose in the 5th group was equal to the one in the group with the maximum dose of the conjugate.

Table 1. The schemes of the administered therapy**.**

Group	No.	Drug	Dose	
			% of LD	mg/kg
The control one	10	-	-	-
The 1st experimental	10	Water-soluble doxorubicin	0.5 of LD_{10}	2 mg/kg
The 2nd experimental	10	Water-soluble doxorubicin	LD_{10}	4 mg/kg
The 3rd experimental	10	Doxorubicin-DNA conjugate	0.5 of LD_{10}	6 mg/kg (on DOX)
The 4th experimental	10	Doxorubicin-DNA conjugate	LD_{10}	12 mg/kg (on DOX)
The 5th experimental	10	DNA	-	7.5 mg/kg (on DNA)

The antineoplastic activity was being evaluated due to the Tumor Growth Inhibition Index (TGII), which was calculated by the formula:

$$TGII = (V_k - V_0)/V_k \cdot 100,$$

where $V_к$ and V_o – average tumor volume in the control as well as in the experimental groups, respectively.

The antineoplastic activity of the drugs were estimated due to the following performances:

1. The metastasis frequency of the tumor – percentage of the animals with metastases towards the total amount of the animals in the group;
2. The mean number of the metastases per one animal in each group;
3. Metastasis Inhibition Index (MII) was being calculated by the formula:

$$MII = ((A_k \times B_k) - (A \times B))/A_k \times B_k \times 100\%,$$

where $A_к$ and A – the frequency of lung metastasizing in mice of the control and experimental groups; $B_к$ and B – the mean number of the lung metastases per one animal in the control and experimental groups.

Statistical data manipulation was being performed with using the Student's t-test and Chi-square. The critical level of the difference significance constituted as equal 5% ($p < 0.05$).

3. The findings and the discussion thereof.

3.1. The development of the method for synthesis of DNA- Dox conjugate.

The influence of the concentration ratio of DNA-doxorubicin, pH and temperature on the efficacy of binding DNA to dox was investigated. The findings are shown in the Table 2.

Table 2. The efficacy of binding the doxorubicin by DNA at various parameters of synthesis.

pH	Mass ratio of DNA/ Dox	Temperature, ^{o}C	Conjugation rate	Dox content (mg per 1 mg of DNA)
7.0	2:1	4	74 ± 3	0.37 ± 0.02
		21	64 ± 3	0.32 ± 0.01
		37	48 ± 4	0.24 ± 0.01
	3:2	**4**	**71 ± 5**	**0.47 ± 0.02**
		21	66 ± 4	0.44 ± 0.02
		37	61 ± 6	0.41 ± 0.03
	1:1	4	The complex is instable.	
		21	42 ± 3	0.42 ± 0.01
		37	38 ± 4	0.38 ± 0.02
8.0	2:1	4	43 ± 5	0.22 ± 0.03
		21	39 ± 5	0.20 ± 0.03
		37	34 ± 6	0.17 ± 0.03
	3:2	4	44 ± 3	0.29 ± 0.01
		21	42 ± 4	0.28 ± 0.02
		37	38 ± 6	0.25 ± 0.03
	1:1	4	The complex is instable.	
		21	32 ± 6	0.32 ± 0.03
		37	29 ± 5	0.29 ± 0.02

The colloidal system was instable in acidic media, therefore the efficacy of the synthesis was investigated at the neutral pH as well as at weakly alkaline one. We can see that the neutral pH is the most optimal for the synthesis of the conjugate, whereby the degree of Dox encapsulation into the conjugate was increasing with the temperature decrease. The maximum content of the chemotherapeutic agent per a mass unit of the carrier was to be registered at the mass ratio of DNA

:Dox = 3:2. Therefore, the following conditions were chosen for the synthesis of the conjugate used in the present work: pH = 7.0, temperature = 4 ^{o}C, mass ratio DNA/ Dox = 3/2. The sequence of the synthesis operations is described above.

It is a fact that one of the main mechanisms of the antitumor effect of doxorubicin is the intercalation between the nucleotides during the DNA synthesis, what discontinues the latter. In this regard, the question arises as to whether the drug activity not to be lost during the interaction with DNA in vitro? But the findings of the earlier studies [10 – 12] and the findings obtained in the present work demonstrate that doxorubicin bound to exogenous DNA, preserves its antineoplastic activity. This phenomenon appears to be explained due to the fact that the irreversible binding of doxorubicin to the nucleotides doesn't take place under the used synthesis conditions. The links of Dox with nucleotides as part of DNA are not of covalent, but of hydrogen nature (mean bond energy = 4.9 kcal/mol) [22 – 23]. The phagocytized complexes DNA- Dox are being disintegrated by endonucleases, what is accompanied by release of the chemotherapeutic agent within the cell.

3.2. The investigation of the acute toxicity of the DNA- Dox conjugate.
The findings of the acute toxicity of doxorubicin are given in the Tables 3 and 4.

Table 3. The lethality rate of the animals at administering of various dosage forms of DOX.

Group	The substance under study	Lethality rate (abs./%) depending on the DOX dosage, mg/kg					
		2	4	8	16	24	32
1	Water-soluble doxorubicin	0	1	3	7	10	10
2	DNA- Dox conjugate	0	0	2	5	8	10
3	DNA	0	0	0	0	0	0

Table 4. The performance of the acute toxicity of the various dosage forms of DOX.

Performance	Water solution of DOX		DOX- Dox conjugate	
	Dose, mg	U_{95}*	Dose, mg	U_{95}*
LD-10	4.4	2.1 – 6.4	6.2	2.7 – 9.0
LD -50	9.9	7.1 – 13.4	14.6	10.5 – 19.9
LD -100	43.9	27.7 – 118.2	69.5	41.1 – 256.8

Note: U_{95} – 95% confidence interval.

We can see that toxic doses of the conjugate are on the average 1.5 more to compare with the water-soluble doxorubicin. The cause of the toxicity decreasing can be incomplete dissociating of the conjugate in somatic cells, a less concentration in myocard as well as inhibiting the pro-oxidant effect of doxorubicin by nucleic acid. Taking into consideration the fact that the toxicity decrease may be accompanied by the decrease of the specific antineoplastic activity of the chemotherapeutic agent, the equitoxic doses of the chemotherapeutic agent were used for further investigating.

3.3. The investigation of the antineoplastic activity.
The evaluation results for the tumor volume are presented on the Figure 1, the Tumor Growth Inhibition Index – in the Table 5.

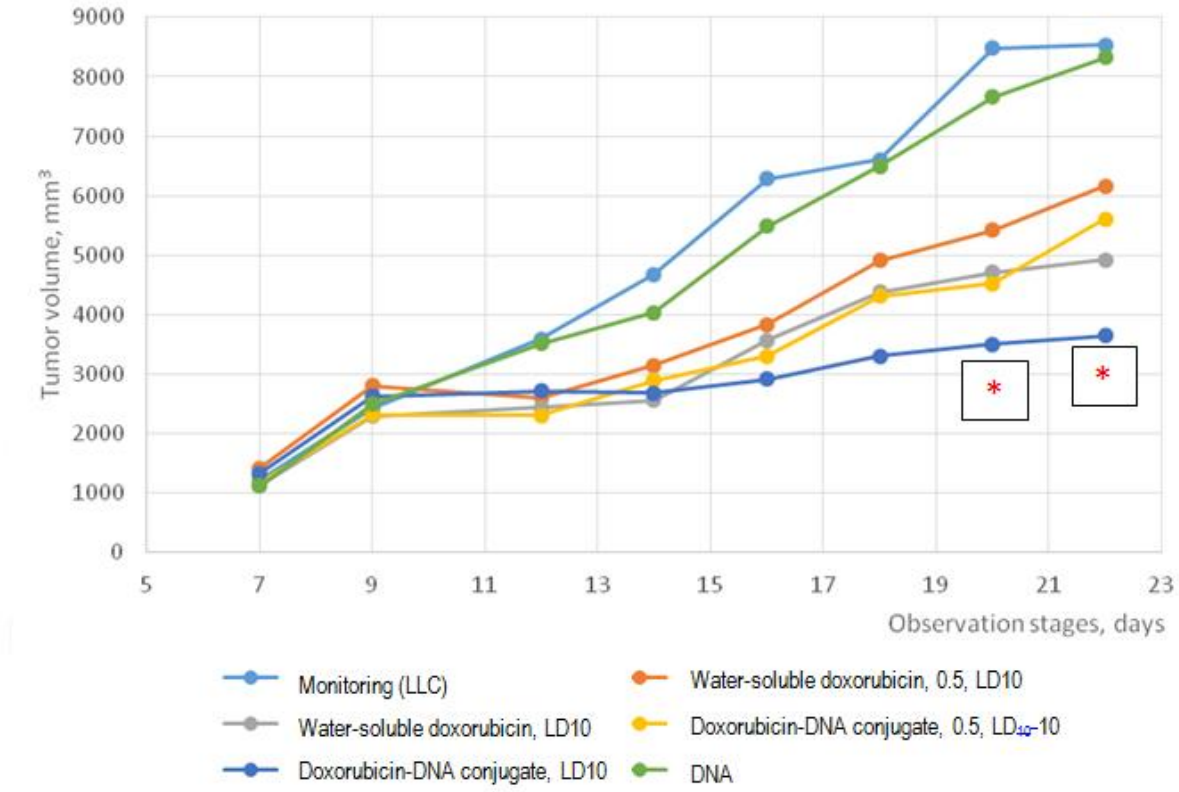

Figure 1. The changes of the tumor volume in the groups. In all the groups, except the group, being treated with DNA, the tumor volume differs significantly from the one in the control group, starting from the 14th day.

* - the significant difference of the tumor volume in the group of the water-soluble DOX from such in the group of DNA–conjugated Dox in a dose of LD 10.

Table 5. The values of the Tumor Growth Inhibition Index in the groups.

The groups of animals	TGII values at the observation stages						
	The 9th day	*The 12th day*	*The 14th day*	*The 16th day*	*The 18th day*	*The 20th day*	*The 22nd day*
Water-soluble doxorubicin, 0.5 of LD 10	-15.9	27.5	32.7	38.9	25.7	36.0	27.9
DOX-DNA conjugate, 0.5 of LD 10	4.1	35.7	38.1	47.4	34.7	46.6	34.3
Water-soluble doxorubicin, LD 10	5.0	32.1	45.4	43.2	33.7	42.4	44.1
DOX-DNA conjugate, LD 10	-8.7	24.3	42.7	53.6	50.0	64.0	67.4
DNA	-3.4	2.1	13.8	12.8	1.5	9.6	2.6

It is apparent that the inhibition of the tumor growth occurred in all the groups of the animals in the course of the chemotherapy. The statistically significant differences in the tumor volume between the control and all the experimental groups occurred on the 14th day of the observation. The significant difference as to the mentioned performance among the experimental groups was not registered earlier than on 20th day of the observation. On the 20th and 22nd days, the tumor volume in the group, being treated with the DNA-conjugated dox in a dose of LD_{10}, was 34.3% and 35.0% less than a similar performance in the group, treated with water-soluble doxorubicin ($p < 0.05$ for both points). As to mentioned dose, the difference in TGII between the group of DNA- Dox and the group of the water-soluble doxorubicin made 14.1 and 15.0 percentage points (or 50.9 and 52.8%), respectively, on the 20th and 22nd days.

The similar trend was to be registered as well for the groups of the animals, being treated with the drug in a dose of 0.5 LD 10 but these differences weren't confirmed statistically.

Table 6. The findings of the antimetastatic activity evaluation.

Groups of the animals	Tumor weight on the 22nd day, g (M ± sigma)	Frequency of metastases, %	The mean number of the surficial metastases, (M ± sigma)	Metastasis Inhibition Index
Control (with no treatment)	8.8 ± 2.9	100	94.7 ± 4.2	-
Water-soluble doxorubicin, 0.5 of LD 10	6.4 ± 2.1	100	**71.2 ± 6.9**	24.8
DOX-DNA conjugate, 0.5 of LD 10	**5.8 ± 2.4**	100	**65.3±11.2**	31.0
Water-soluble doxorubicin, LD 10	**5.1 ± 2.2**	90	**44.5±12.**	57.7
DOX-DNA conjugate, LD 10	**4.1 ± 1.8**	80	**35.6±9.8**	69.9
DNA	8.6 ± 3.1	100	90.2±8.5	4.8

Note: Significant differences from the control group are typed in bold.

In estimating the antimetastatic activity, it was established that all the studied except for the water-soluble doxorubicin in a dose of 0.5 LD 10, reduced the number of the lung metastases significantly. The number of metastases in the groups having been administered by the DNA-conjugated drug was less, than one in the groups being treated with aquatic DOX, but those difference wasn't statistically significant. The most probable mechanism of increasing the antineoplastic activity of DOX during its conjugation to DNA is a selective accumulation of the chemotherapeutic agent in the tumor tissue, caused by the endocytosis of the DNA- Dox complex.

4. Conclusion

The method of obtaining the conjugate of the antineoplastic chemotherapeutic agent doxorubicin to the exogenous double-stranded DNA is proposed. The investigation of the toxicity and specific antineoplastic activity of the synthesized complex was conducted. It was demonstrated that the conjugate toxicity is lower than the one of the unconjugated doxorubicin (LD50 was equal 14.6 mg/kg and 9.9 mg/kg for the conjugate and doxorubicin, respectively). The administration of the conjugate in equitoxic doses possesses a stronger antineoplastic activity, than the water-soluble drug (maximum 35% more as to tumor volume and 51% – as to the Tumor Growth Inhibition index).

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