PREPARATION AND ANTILEUKEMIC EFFECT OF DOXORUBICIN – CHITOSAN CONJUGATE AX_1 AGAINST MOUSE LEUKEMIA P 388

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ABSTRACT

Chitosan is stepwise treated with periodic acid, urea and formaldehyde and then introduced hydroximethyl groups reacted with doxorubicin.The conjugate obtained AX1is fully soluble in dimethylformamide in contrast to the free chitosan. The appearance of new bands at 1540 cm^{-1} and 3440 cm^{-1} of the conjugate leads to the suggestion that the NH_2 group of antibiotic has involved in bond formation.The numbers of doxorubicin residues introduced in chitosan are 402.3 or 25.2%. The antitumor activity has studied against Bacillus subtilis AT6633.Compared to unmodified doxorubicin the activity of the conjugate was 89.4%.The antitumor activity of the conjugate against P 388 mice leukemia was maximaly at 8.0 mg/kg and T/C was 315.6 % in comparation 167.6% at dose 0.25 mg/kg.

Introduction

Anthracycline antibiotics are one of the most wide used high-effective antitumor drugs for healing of the cancer of lactiferous gland, cancer of bladder, cancer of lung.These drugs occupy a basic position too, in a combined therapy of hematological neoplasia of Hodghin's disease, of non-hodgikinic lymphons, of acute myeloleukemia and ect. They intercalate in DNA,suppress the action of topoisomerase II, release free radicals and injure cell membrane.Their therapeutically utilization is confined because of their cardiotoxicity and myelosuppression mainly due to the last two mechanisms. The reveal of these secondary effects to great extend are determined by there pharmacokinetic profile.

The using of biopolymers as carriers of antitumor means can provide opportunity in changes of their kinetics during their release in the body, in antineoplastic spectrum and direct cytotoxic influence of the anthracyclines and in particular of the doxorubicin.The synthesis of antibiotic conjugates with biopolymers has the purpose of prolongating the antibiotic action and decreasing of its toxicity(2, 3).

In present study we show the comparative analysis of the doxorubicin antileukemic effect and new doxorubicin conjugate AX_1 bound to the biopolymer chitosan against mouse leukemia P 388.

Materials and Methods

Activation of chitosan

Chitosan of 1 g has treated with 0.25 M sodium periodate at 20 ºC at pH 3.6 and continuous strirring for 4 hours in the dark. After abundant washing with water by filtration, the chitosan particles have processed with 20 ml of 15% urea solution containing 0.9% sulphuric acid at 65 ºC for 15 hors. The product obtained was washed abundantly with water until the rinsing showed a neutral reaction.

1 g of urea derivative was proceed with 20 ml 0.1M phosphate buffer, pH 7.5, containing 12.5% formaldehyde, with stirring for 7 hours at 45 ºC.

The activated chitosan was used immediately for antibiotic binding.

Activated chitosan particles of 100 mg were suspended in 10 ml phosphate buffer, pH 8.0 and doxorubicin of 1% concentration was added.The reaction was performed at 20 ºC with stirring for 16 hours.

Isolation of conjugate

The conjugate was isolated by filtration and washed abundantly with water; 0.1 M sodium chloride and again with water. Air dried product might be keeping at 4 ºC. The number of residues of doxorubicin in conjugate with chitosan was determined spectrophotometricaly as 0.002% solution in 20% acetic acid. The determination was performed using molecular extinction coefficient of antibiotic.

The amount of doxorubicin binding to the chitosan was determined spectrophotometricaly as a 0.002% solution in 20% acetic acid cording to formula:

$$N = \Delta A_{500}.M/c \cdot \varepsilon .10$$

Where N – denotes the number of doxorubicin residue, binding to the chitosan, ΔA_{500} is absorption difference of the sample at 500 nm, M is the molecular weight of the chitosan, c is the sample concentration and ε - is the molecular extinction coefficient of antibiotic used.

The biological activity of the conjugate AX_1 obteined was tested against *Bacillus subtilis* AT6633 by diffusion in agar [5].

The free doxorubicin and conjugate AX_1 were evaluated for antitumor activity against the i.p. implanted murine leukemia P 388. Mice received an i.p. inoculum of 10^6 cells on day 0 and i.p. treatment with the anthracycline was initiated at least 24 hours later.Anthracyclines were administrated on dey 2.4 and 8 after tumor transplantation.Five doses of AX_1 and doxorubicin – A covering an 0.25-16.0 mg/kg dosage range were evaluated.Median survival times of treated mice and non-treated controls were determined and the results expressed as a percent T/C where

$$T/C, \% = \frac{\text{Median survival time of test animals}}{\text{Median survival time of control animals}} .100.$$

A T/Cvalue > 125% is considered necessary to demonstrate activity, whwreas a T/C value 85% indicate toxicity.

Accetable median survival time range for control animals was 9 – 13 days.

Results and Discussion

The conjugate was produced by a reaction of activated chitosan and doxorubicin. The matrix was activated by successive treatment with periodic acid, carbamide and formaldehyde. The resulting hydroxymethyl groups could react with the amino group of the carbohydrate part of the antibiotic(10).As a result of this reaction, a chemical analogue was produced of doxorubicin with chitosan. The most probable scheme of the binding is showed on **Figure**.

The antibacterial activity of the conjugate was studied against *Bacillus subtilis* ATCC 6633. Compared to unmodified doxorubicin the activity of conjugate was 88.1%.

The citotoxic activity of the conjugate was studied against sensitive and resistant K 562 cancer cells(6). In this assay the doxorubicin-chitosan comjugate and the free doxorubicin were used in 5, 10 ,25 and 50 mg/ml concentrations. The results show that in these concentrations doxorubicin inhibits the growth of sensitive K 562 cells and not of the resistant ones. The doxorubicin-chitosan conjugate has no inhibiting effect neither on resistant, nor on sensitive cancer cells, when used in the above concentrations. This is probably due to the binding with chitosan, which, after hydrolysis in the organism, can be separated from the active antibiotic. This hypothesis was contirmed in the *in vivo* studies, where the doxorubicin-chitosan conjugate showed its activity.

The *in vivo* studies were performed in hybrid BDF_1 mice.The antileucotic activity was studied on P 388 leucisis with a transplantation dose of $1x10^6$ tumor cells.

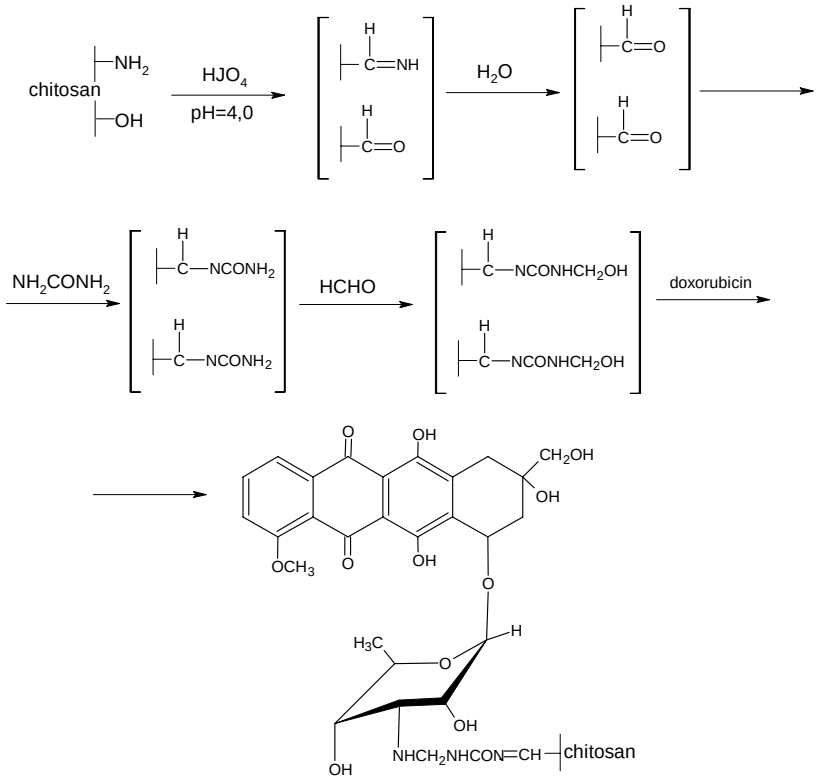

Figure. Probable chemical way of chitosan activation and binding of doxorubicin.

TABLE

Antitumor effect of doxorubicin and conjugate of doxorubicin with chitosan AX_1

Compound	Dose[mg/kg]	T/C[%]
Doxorubicin	0.25	167.6
Doxorubicin	0.50	159.2
Doxorubicin	1.0	112.7
Doxorubicin	2.0	89.7
AX_1	0.50	124.3
AX_1	1.0	129.8
AX_1	2.0	202.6
AX_1	4.0	234.9
AX_1	8.0	345.5
AX_1	16.0	86.1

Doxorubicin and its conjugate with chitosan were introduced intraperitoneally on the first, tourth and eighth day after tumor transplantation.The results for the effect of the anthracycline antibiotic doxorubicin its conjugate with chitosan are shown in **Table**.

As seen in Table, in concentration of 8 mg/ml the conjugate had an antitumor effect.The main results of the binding of the doxorubicin with chitosan is decrease in toxicity and the prolongation of the antitumor activity.The conjugate AX_1 represent biologically active compound with potative depot-effect of releasing of the active doxorubicin.

REFERENCES

1. **Nislida H., Tone H., Okamoto H.** (1994) J. Antibiotics, 1025-1029.

2. **Zunino F., Pezzoni G.** (1987) Cancer Treat. Rep., **71,** 367-373.

3. **Canfarini F., Vittorio A., Arcamone F.** (1993) Tetrahedron Lett, **34(29),** 4697-46700.
4. **Calcabrini A., Molinari A., Mescini S., Arancia G.** (1995) J. Exp. Clin. Cancer Res., **14(1),** 80-92.
5. **Clark A.M., El-Feraley, Lu V.S.** (1981) J. Pharm. Sci., **70,** 951-952.
6. **Fiallo M., Laigle A., Ballini** (1993) Biochimica et Biophysica Acta, **1177,** 236-244.
7. **Hiroshi T., Kaichiro et al.** (1994) J. Antibiotics, **47,** 1025-1029.
8. **Hyun-Ock Pae, Won-Gil Seo, Na-Young Kim et al.** (2001) Leukemia Res., **25,** 339-346.
9. **Karachi A., Pasqui F., Pesfellini V., Aramone F.** (1994) Pure and Appl. Chem., **66(10),** 2319-2322.
10. **Krysteva M., Sokolov T.** (1984) J. Appl. Biochem., **10,** 367-373.
11. **Larsen D., O'Shea M.** (1993) Tetrahedron Lett., **34(23),** 3769-3772.
12. **Leng F., Savkur R., Fokt I., Priebe W.** (1996) J. Am. Chem. Soc., **118,** 4731-4738.
13. **Liu L.F. et al.** (1999) Annu. Rev. Biochem., **58,** 351-375.
14. **Lombardi P., Animati F., Cipallone M., Arcamone F.** (1995) Acta Biochim. Pol., **421(4),** 433-44.
15. **Muzzarelli R., Baldassare F., Conti P.** (1988) Biomaterials, **91,** p. 247.
16. **Schweitzer B., Koch T.** (1993) J. Am. Chem. Soc., **115(13),** 5440-5445.
17. **Tarasiuk J., Garnier-Suillerot A.** (1992) J. Biochem., **204,** 693-698.