Macedonian Journal of Chemistry and Chemical Engineering, Vol. **26**, No. 2, pp. 147–150 (2007)
GHTMDD – 510 ISSN 1857 – 5552
Received: March 12, 2007 UDC: 615.277
Accepted: October 19, 2007

Short communication

ANTITUMOR EFFECT OF NEW CONJUGATES OF ANTHRACYCLINE ANTIBIOTIC CARMINOMYCIN BOUND TO CHITOSAN

Nedyalka Todorova[1], Maya Ilarionova[2], Dimiter Todorov[2]

[1]*Department of Biotechnology, University of Chemical Technology and Metallurgy, Sofia 1756, 8, Kl. Ohridski blv., Bulgaria*
[2]*National Centre of Oncology, Sofia 1756, 6, Plovdivsko pole blv., Bulgaria*
todorova-nelly@abv.bg

The antileukemic activities of two conjugates of the anthracycline antibiotic carminomycin – CX_1 and CX_2, were investigated. The direct cytotoxic efficacies of the two conjugates and free carminomycin (C), were measured by an MTT-assay which provides excellent reproducibility and statistical significance. The treatment of HL-60 cells with the three compounds for 48 and 96 hours showed considerable sensitivity to the free carminimycin, as well as to its conjugates CX_1 and CX_2. The ascetic form of leukemia P388 and leukemia L1210 (transplantation dose of $1 \cdot 10^6$ tumor cells) in hybrid mice BDF_1 was used as leukemic models. All compounds investigated showed a maximal cytotoxic response and strong concentration dependence. By comparing the cytotoxic profile of carminomycin to those of CX_1 and CX_2, it can be assumed that a slower release of the active drug carminomycin have occurred. The criterion T/C showed a considerable antileukemic effect from 1.0 to 18.0 mg/kg. In conclusion, the conjugates CX_1 and CX_2 represent biologically active compounds with a putative depot-effect of slow release of the active intercalating agent carminomycin.

Key words: carminomycin; pharmacokinetics; HL-60 cells; leukemia P388; leukemia L1210; chitosan; anthracycline antibiotics

АНТИТУМОРЕН ЕФЕКТ НА НОВИ КОНЈУГАТИ ОД АНТРАЦИКЛИНСКИОТ АНТИБИОТИК КАРМИНОМИЦИН И ХИТОЗАН

Два конјугата на антрациклинскиот антибиотик карминомицин, CX_1 и CX_2, се испитувани во однос на нивната антилеукемиска активност. Ефикасноста на конјугатите и слободниот карминомицин (C) се определувани со постапка MTT, којашто претставува погоден експериментален пристап и се одликува со одлична репродуцибилност и статистичка значајност. Кога клетките на леукемија, HL-60, биле третирани со карминомицинот и со двата конјугата во текот на 48 и 96 часа, покажаа значителна сензитивност, Како модел за леукемија беа користени формите P388 и L1210 (доза на трансплантација од $1 \cdot 10^5$ туморни клетки) на хибридни глувци BDF_1. Сите испитани соединенија покажаа максимален цитотоксичен одговор кој особено зависел од нивната концентрација. Споредувајќи го цитотоксичниот профил на C со CX_1 и CX_2, се забележува побавно ослободување на активната компонента на карминомицинот. Критериумот T/C покажа значителен антилеукемиски ефект од 1,0 до 18,0 mg/kg. Како заклучок: и двата испитувани конјугати претставуваат биолошки активни соединенија со претпоставен депо-ефект на бавно ослободување на внесената активна супстанција, карминомицинот.

Клучни зборови: карминомицин; фармакокинетика; HL-60 клетки; леукемија P388; леукемија L1210; хитозан; антрациклински антибиотици

INTRODUCTION

Improving the effectiveness and decreasing the toxicity of anthracycline antibiotics are the two basic objectives in the development of new antineoplastic tools. Utilization of polymer carrier for the cytotoxic substances is one of the contemporary approaches for successful modulation of the pharmacokinetics and pharmacodynamics of the antitumor substances. The physical and chemical

properties of the carrier are important decision parameters in this relation. The binding of the cytotoxic compound to the macromolecular biopolymer carrier decreases its fast penetration in the cells, which leads to localization of the impact in specific tissues with increased metabolism and good vascualization (e.g., tumor tissues). Such carrier system is convenient when the cytotoxic drug remains bound to the carrier in the central pharmacokinetic compartment, and it is released gradually in tumor and weaker in another normal tissue [7, 8]. If a drug maintains its activity after release from the carrier, the entire complex could be considered a macromolecular prodrug analog. The biopolymer chitosan is one such potential carrier of the conventional cytostatics and antracyclic antibiotics in particular [1–3].

In the present study the direct cytotoxic effects of two conjugates of the antracyclinic antibiotic carminomycin (C) covalently bound to the biopolymer chitosan (CX_1 and CX_2) have been investigated. The synthesis, physico-chemical properties of the conjugates, as well as their antitumor activity on transplanted mouse's tumors have been described earlier – Fig. 1 [4, 5].

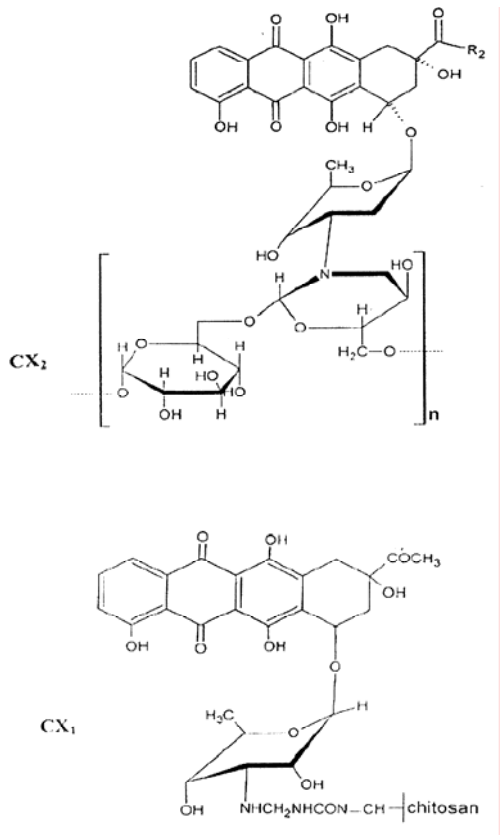

Fig. 1. Conjugates of carminomycin with chitosan

MATERIALS AND METHODS

The cell line HL-60 obtained from a patient with acute promyelocitic leukemia was used. Cells were maintained as a suspension culture. The steady suspension growth in logarithmic phase was attained each week by a three-fold transfer. The synthetic medium RPMI-1640 was used, enriched additively with 10% fetal calf serum and 2 mM L-glutamine. The cells were dispersed in 96-well flat plate, with 100 μl /well and with an initial density of $1 \cdot 10^5$ cells/ml. The plates were incubated at standard conditions (maximum atmosphere's humidity, 5% CO_2, 37°C). After 24 hours from the inoculation in the wells, solutions of the investigated compounds were added at their corresponding concentrations. A minimum of 8 wells were used for each of the tested concentrations. After the treatment time expired, a solution of 3-(4,5-dimetyltiasol-2-il)-2-5-diphenyltetrasolium bromide (MTT) in sterile phosphate buffer (10 mg/ml) was added in each well (10 μl per well). The plates were incubated for four hours at 37 °C. The yellow tetrasolic salt is reduced to purple formasan under the influence of mitochondrial dehydrogenases of the vital cells. In each well, 100 μl of 5%-solution of formic acid diluted in 2-propanol was added. After careful stirring, the reaction was measured photometrically at 580 nm by an ELISA-reader UNISCAN. The method has been previously described by Mosman [6]. The results obtained were statistically evaluated and presented as the percentage of the untreated control by the computer software Slide3.0 Plus.

The anthracyclines were evaluated for antitumor activity against the implanted murine P 388 lymphotic leukemia and L 1210 leukemia. Mice received an i.p. inoculum of 10^6 cells on day 0 and treatment with the anthracycline was initiated 24 hours later. Anthracyclines were administrated on days 2, 4 and 8 after tumor transplantation. Five doses of each anthracycline covering a 2–18 mg/kg dosase 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

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

A T/C value of 125 % is considered necessary to demonstrate activity, whereas a T/C value of 85% indicated toxicity. An acceptable median survival time range for control animals was 9–13 days.

RESULTS AND DISCUSSION

The results obtained with the human promyelocitic leukemia cell line HL-60 demonstrated the sensitivity to free carminomycin as well as to the de novo synthesized conjugates of the antibiotic with chitosan – CX_1 and CX_2. All three compounds reached the maximal cytotoxic response in the investigated range of concentrations. A strong dependence of the effect on the concentrations tested was found. The MTT test used is convenient and precise enough experimental protocol for the assessment of the cytotoxic response, with excellent reproducibility, statistical significance, and it is a competitive alternative to the radioisotope methods [6].

The free carminomycin shows significantly steeper slope of the concentration-effect curve, with intensification of the cytotoxicity after 96 hours incubation period in contrast to the period of 48 hours (Fig. 2). The conjugates with chitosan CX_1 and CX_2 are distinguished by their flatter slope of the curve in contrast of the free carminomycin (Fig. 3a and Fig. 4a). These differences are especially apparent after 96 hours treatment with CX_1 and CX_2 (Fig. 3b and Fig. 4b).

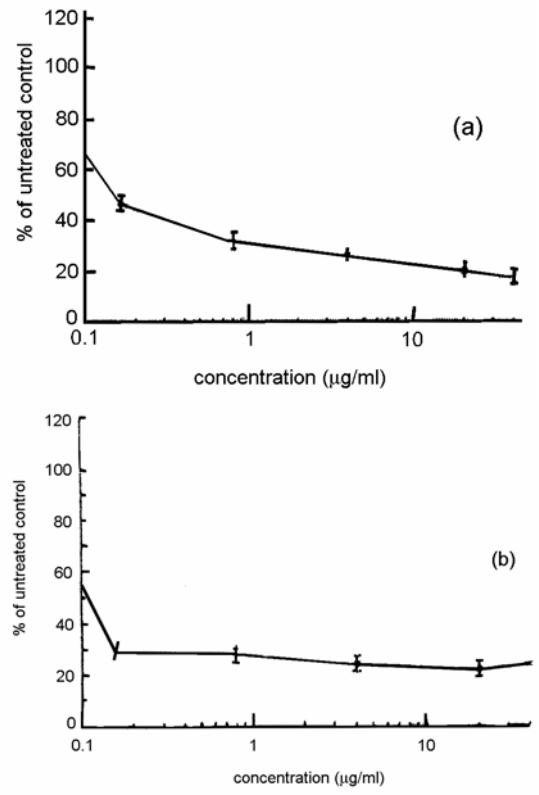

Fig. 2. Cytotoxic effect of carminomycin on the human promyclocytic leukemic cell tine HL-60 after 48 hours (a) and 96 hours (b)

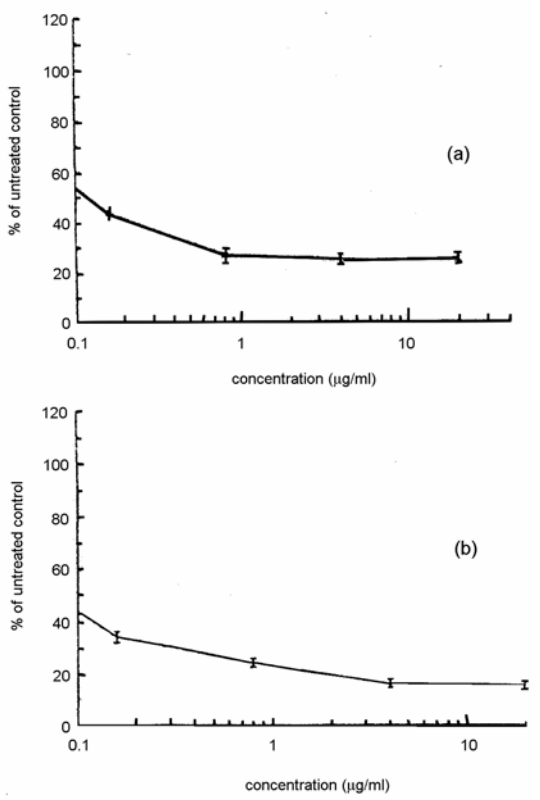

Fig. 3. Cytotoxic effect of carminomycin-chitosan conjugate CX_1 on promyclocytic leukemic cell line HL-60 after 48 hours (a) and 96 hours (b)

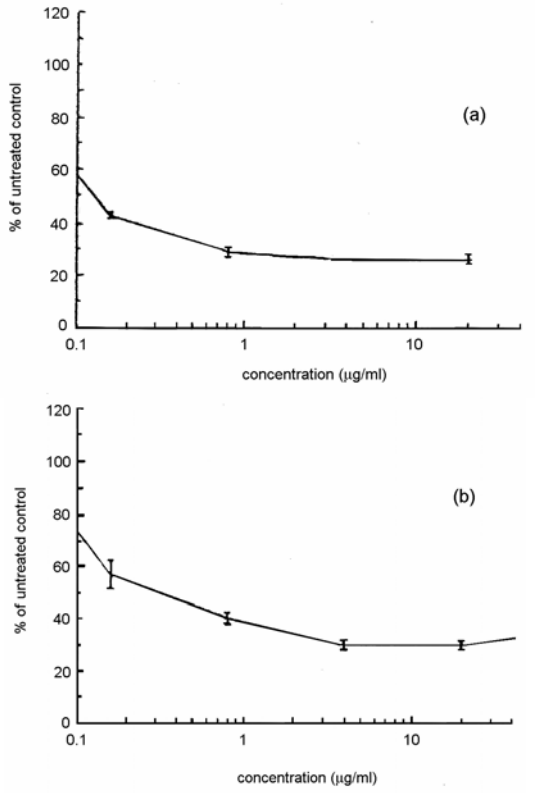

Fig. 4. Cytotoxic effect of carminomycin conjugate with chitosan CX_2 on human promyclocytic leukemia cell line HL-60 after 48 hours (a) and 96 hours (b)

The cytotoxic profiles of CX_1 and CX_2, as well as the values of the IC50, are not statistically different. After 48 hours of treatment with free carminomycin, the maximal cytotoxic effect is observed at 20 μg/ml, but after 96 hours incubation – at 0.16 μg/ml. The maximal cytotoxic effects of CX_1 and CX_2 are observed at 0.8 μg/ml. Comparing the cytotoxic profile of the free carminomycin with the same of the chitosan's conjugates, a likely impediment in the release of the active carminomycin can be suggested. This corresponds well to previous toxic-therapeutically investigations performed by our group with the same compounds during *in vivo* experiments on the lymphocytes leukemia P388 and leukemia L 1210 (Table 1).

Table 1

Antitumor effect of carminomycin, chitosan and conjugates of carminomycin with chitosan on lymphoid leucosis L 1210 and lymphocytic leucosis P 388 in hybrid mice BDF_1

Substance	Dose mg/kg	L1210 (T/C)	P 388 (T/C)
Carminomycin	0.25	177.9	179.5
Carminomycin	0.50	145.4	147.9
Carminomycin	1.0	72.0	74.6
Carminomycin	2.0	62.8	59.3
Chitosan	125	-	88.9
Chitosan	250	-	89.8
Chitosan	500	-	89.8
CX_1	1.0	117.2	122.9
CX_1	2.0	132.7	150.0
CX_1	6.0	205.4	199.1
CX_1	18.0	217.1	220.3
CX_1	36.0	77.9	86.0
CX_2	1.0	122.9	158.2
CX_2	2.0	150.0	159.6
CX_2	6.0	199.1	203.4
CX_2	18.0	220.3	242.9
CX_2	36.0	86.0	69.2

Intramuscular injection of preparation on days 1, 4 and 8 after tumor transplantation.

T/C is ratio between the median survival time of treated animals (T) and median survival time of tumor-bearing controls (C)x100. Activity criterion T/C > 125%.

In these experiments we found out that both conjugates CX_1 and CX_2 show lower toxicity, higher antitumor effect, and significantly greater therapeutic spectrum.

The results obtained indicate that de novo synthesized covalently binding conjugates represent biologically active compounds with putative depot-effect of releasing of the active interpolator. The investigations on the effect of both original compounds on another sensitive and resistant human malignant transformed cell lines will continue with the aim to further the utility of the investigated antibiotic conjugates.

REFERENCES

[1] J. M. Terwogt, Clinical pharmacology of anticancer agents in relation to formulation and administration routs, *Cancer Treatment Reviews*, **25** (1), 83–101 (1999).

[2] M. Fiallo, A. Laigle, M.N. Borrel, Accumulation of degradation products of doxorubicin and pirarubicin formed in cell culture medium with sensitive and resistant cells, *Biochem. Pharm.* **45** (3), 659–665 (1993).

[3] P. S. Huang, A. Oliff, Drug-targeting strategies in cancer therapy, *Curr. Opin. Genet. Dev*., **11**, 104–110 (2001).

[4] T. N. Suratori, N. Hiroki, U. Kenichi, Conjugate of mitomycin C with N-succinylchitosan, *J. Pharm.*, **1**,120–130 (1993).

[5] H. O. Pae, W. G. Seo, N. Y. Kim, G. S. Oh, G. E. Kim, Y. H. Kim, H. J. Kwak, Y. G. Yun, C. D., Jun, H. T. Chung. Induction of granulocytic differention in acute promyelocytic leukemia cells (HL-60) by water-soluble chitosan oligomer; *Leukemia Research,* **25**, 339–346 (2001).

[6] M. Krysteva, N. Todorova, K. Maneva, D. Todorov, Studies on the synthesis of antibiotic conjugates with chitosan, *Biotech. Biotechnol. Eq*., **11** (2), 30–33 (1997).

[7] N. Todorova, M. Krysteva, K. Maneva, D. Todorov, Carminomycin-chitosan: a conjugated antitumor antibiotics, *J. Bioactive Comp. Polym.*, **14** (1), 178–184 (1999).

[8] H. K. Schmoll, K. Höffken, K. Possinger, *Kompendium internistische Oncologie,* Springer, Berlin, 1997, p. 2031.