Eur J Cancer Clin Oncol, Vol. 20, No. 3, pp. 421–425, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Comparison of Antitumor Effects of Daunorubicin Covalently Linked to Poly-L-Amino Acid Carriers

FRANCO ZUNINO, GIUSEPPINA SAVI, FERNANDO GIULIANI,† ROMOLO GAMBETTA, ROSANNA SUPINO, STELLA TINELLI and GABRIELLA PEZZONI

Division of Experimental Oncology B, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20133 Milano, Italy

Abstract—*Daunorubicin was covalently linked to poly-L-aspartic and poly-L-lysine of different molecular weights via the methylketone side-chain of the drug by the use of a method that employs the 14-bromo derivative of the antibiotic. During reaction ester and C–N linkages were formed with poly-L-aspartic acid and poly-L-lysine respectively. Whereas a reduction of drug toxicity was observed with both types of conjugate, only the linking to the anionic polymer produced an enhancement of drug activity. In contrast, when drug was covalently attached to poly-L-lysine, cytotoxic activity and in vivo potency and efficacy were markedly reduced. The different therapeutic properties of these conjugates can be explained in terms of the different nature and stability of chemical bonds formed between the drug and the amino groups and carboxyl functions of the polyamino acid carrier.*

INTRODUCTION

WE HAVE recently reported a new method for covalently linking daunorubicin to protein via the methylketone side-chain to avoid reaction of the amino sugar moiety, which is thought to be essential for optimal drug activity [1]. Using this procedure for drug–protein conjugation, both amino and carboxyl groups of the protein can react with the 14-bromo- derivative of daunorubicin. Thus both C–N and ester linkages are formed during reaction. However, the stability of these bonds is quite different [1]. Since severance of bond between carrier and cytotoxic agent is required, the relative stability of the linkage formed between drug and protein may play an important role [2–4]. Therefore it is of interest to compare the biological activity of daunorubicin linked to protein carriers via different bonds. We have recently found that daunorubicin linked to poly-L-aspartic acid (PAA) (essentially through ester linkages) was more effective than the free drug against some experimental tumor systems [5].

In this study we compared the antitumor effectiveness of daunorubicin–PAA and daunorubicin–poly-L-lysine (PLL) conjugates since polypeptides are good candidates as carriers for antitumor agents [3–6]. In the latter polymeric derivative the antitumor drug is linked to the polyamino acid essentially through C–N linkages.

MATERIALS AND METHODS

Drugs

Daunorubicin, doxorubicin and 14-bromo-daunorubicin were supplied by Farmitalia Carlo Erba (Milan, Italy) as hydrochlorides. Drug solutions were freshly prepared immediately before use. PAA (mol. wt 20,000) and PLL (mol. wt 40,000) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PLL (mol. wt 2700–3400) and PAA (mol. wt 5300) were purchased from Miles Laboratories (Elkhart, IN, U.S.A.). Conjugates were prepared essentially according to the previously described procedure [1]. Attachment of daunorubicin to the polyamino acids was achieved by nucleophilic substitution reaction of the 14-bromo- derivative of the drug. The conjugates tested for antitumor activity contained different amounts of dauno-

Accepted 8 September 1983.

*This work was supported in part by a research grant from the Consiglio Nazionale delle Ricerche, Rome (Progetto Finalizzato Controllo della Crescita Neoplastica).

†Present address: Farmitalia Carlo Erba, Laboratori di Ricerca, Nerviano (Milano), Italy.

rubicin, as indicated. The concentration of bound drug in the polymeric derivative was estimated by absorbance at 495 nm. Both conjugates were stable in aqueous solution as checked by thin-layer chromatography, using a mixture of chloroform:methanol:acetic acid (80:20:4) as solvent. No free drug could be detected by thin-layer chromatography or by high-pressure liquid chromatography.

Animals, tumors and antitumor testing

C3H/He and BDF_1 adult mice of both sexes were obtained from Charles River Laboratories (Calco, Como, Italy). After arrival the animals were acclimated for about a week and then randomized into experimental groups. The experiments with P388 leukemia were carried out in BDF_1 mice inoculated i.p. with 1×10^6 cells per mouse. The experiments with Gross leukemia were carried out in C3H/He mice inoculated i.v. with 2×10^6 cells per mouse. Tumor lines were maintained as previously described [5].

For antitumor testing daunorubicin and doxorubicin were dissolved in distilled water to a concentration that allowed the dose to be given in a volume of 0.1 ml/10 g body weight and administered i.v. or i.p., as indicated. Polymeric derivatives were dissolved in distilled water or physiologic saline for i.p. or i.v. administration respectively. Animals were observed daily. Comparative antitumor effects of various dosages and schedules were determined from the median survival time (MST) in days for treated (T) vs control (C) groups and expressed as T/C (%).

Cell survival determinations

HeLa cells were exposed to the drugs for 24 hr. Survival was determined by the ability of treated cells to form colonies. After treatment the medium was removed and cells were washed and suspended in Eagle's minimal essential medium containing 10% fetal calf serum, and plated colonies were counted after 8 days of incubation.

RESULTS

Antitumor activity

Antitumor effects of daunorubicin and its conjugates against P388 leukemia are compared in Fig. 1. A dose-dependent antitumor activity was observed for both free and polyamino acid-bound daunorubicin. However, at high doses (>4 mg/kg) the toxicity of free daunorubicin exceeded the therapeutic activity, resulting in a reduction of survival. At the doses tested (up to 50–60 mg/kg) this was not observed with daunorubicin conjugates, thus indicating an appreciable reduction of drug toxicity in both conjugates. Whereas no appreciable reduction in

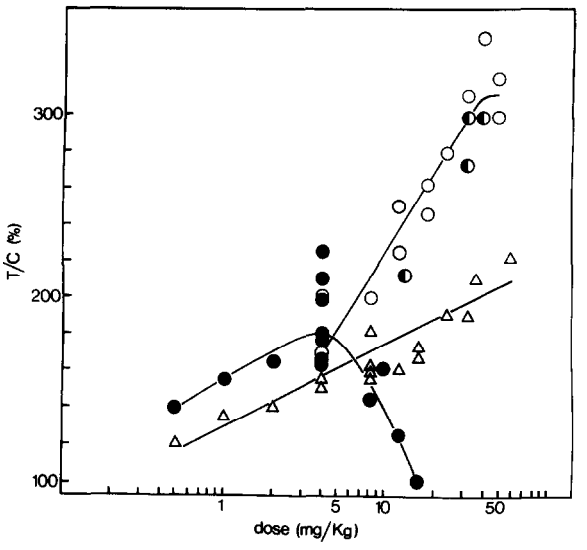

Fig. 1. Dose-response curve for the effect of daunorubicin (●) and its polymeric derivatives against P388 leukemia. Single treatment i.p. on day 1 after tumor transplantation. Dose refers to actual amount of daunorubicin in the conjugate. Different preparations of daunorubicin–PAA (○) (range of drug:PAA molar ratio, 0.2–35.5; most experiments were carried out with preparation with high drug:PAA ratios) and daunorubicin–PLL (△) (range of drugs:PLL molar ratio, 1.6–4.8). The mol. wt of PAA was 20,000, except for preparations denoted by the symbol ◐, where a polymer of mol. wt 5300 was used.

drug potency was observed with the daunorubicin –PAA conjugate, daunorubicin–PLL at low doses was four times less potent than free drug. In both conjugates the decrease in drug toxicity allowed an increase in drug dose (with reference to the actual amount of daunorubicin) with a consequent enhancement in the therapeutic effect assayed against P388 leukemia. Whereas the daunorubicin–PLL conjugate provided only somewhat greater efficacy at the highest doses tested (60 mg/kg), linking to PAA caused efficacy to improve substantially over that of free daunorubicin–PLL conjugate provided essentially similar efficacy at the highest doses tested (60 mg/kg), linking to PAA caused efficacy to improve substantially over that of free daunorubicin, as expected by previous observations [5]. The therapeutic properties of free and bound drug in this experimental system are summarized in Table 1. Only a marginal influence of the molecular weight of PAA on the conjugate antitumor activity was noted in these experiments. Polymers alone were devoid of activity at doses similar to those used for conjugates, as already observed for PAA [5]. Mixtures of drug plus polymers produced effects similar to those of drug alone, except for mixtures containing high doses of PLL that were very toxic (not shown).

this experimental model the daunorubicin–PLL conjugate was devoid of significant activity. In

contrast, the daunorubicin–PAA conjugate provided clearly superior efficacy compared to free drug at its optimal dose. This observation is in keeping with the results obtained with a different treatment schedule [5]. Relevant to this point is the observation that using different treatment schedules, drug linked to carrier was consistently more effective than free daunorubicin and doxorubicin (Table 3).

Cytotoxicity activity

Figure 2 compares the effects of daunorubicin and its polymeric derivatives on survival of HeLa cells after exposure to the drugs for 24 hr. The covalent linking to the polyamino acids reduced the cytotoxic activity of the drug. This effect was more marked for the daunorubicin–PLL conjugate. In addition, the results of these cytotoxicity studies indicated no appreciable

Table 1. *Comparison of antitumor activity of doxorubicin, daunorubicin and its polymeric derivatives against P388 leukemia**

Drug†	Molar ratio drug:polymer	Optimal dose (mg/kg)‡	T/C (%)§
Doxorubicin		10	277 (242–312)
Daunorubicin		4	189 (163–225)
Daunorubicin–PAA (20,000)	35.5	32	312
Daunorubicin–PAA (5000)	6.3	32	287 (273–300)
Daunorubicin–PLL (2700)	2.8	60‖	222

*Mice were implanted i.p. with 10^6 tumor cells. The range of median survival time (MST) of control mice was 8–9.5 days in 7 experiments.

†PAA, poly-L-aspartic acid; PLL, poly-L-lysine. In parentheses, molecular weight of the unconjugated polymer.

‡Treatment i.p. on day 1 after tumor transplantation. In the conjugate the dose is expressed as dose of drug component.

§MST of treated mice/MST of control mice × 100. In parentheses, range.

‖Maximal dose tested. For the *in vivo* experiments only low-molecular-weight polymers were used, because they possess limited toxicity.

Table 2. *Effect of doxorubicin, daunorubicin and polymeric derivatives against Gross leukemia**

Drug†	Drug:polymer molar ratio	Optimal dose (mg/kg)‡	T/C (%)§
Doxorubicin		7	224 (216–233)
Daunorubicin		8	175
Daunorubicin–PAA	27.3	12	433 (433–433)
Daunorubicin–PLL	42	25‖	125

*C3H/He mice received 2×10^6 leukemia cells i.v. on day 0. Median survival time (MST) of control mice was 6 days.

†PAA, poly-L-aspartic acid; mol. wt of PAA, 20,000. PLL, poly-L-lysine; mol. wt of PLL, 2700.

‡Treatment i.v. on days 1, 3 and 5 after tumor transplantation. In the conjugate the dose is expressed as dose of drug component.

§MST of treated mice/MST of control mice × 100. In parentheses, range.

‖Maximal dose tested. Higher doses were not tested because of the difficulty of i.v. administration. This dose represents approximately the maximally tolerated dose.

Table 3. *Influence of treatment schedule on the activity of anthracycline derivatives against Gross leukemia**

Days of i.v. treatment	T/C (%)† Daunorubicin	Doxorubicin	DR–PAA‡
1, 3, 5	175	224 (216–233)	433 (433–433)
1, 4, 7	325 (230–406)	358 (279–509)	456 (320–679)
1, 5, 9	140	224	303

*See Table 2 for experimental details.

†Average date of different experiments. In parentheses, range. Data obtained at the optimal doses for each drug are compared.

‡DR–PAA, daunorubicin–poly-L-aspartic acid conjugate.

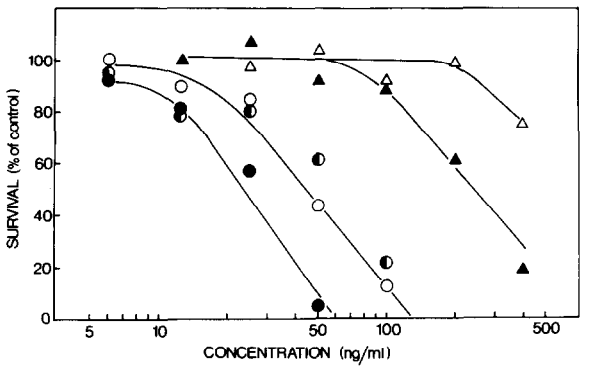

Fig. 2 Effect of daunorubicin and its polymeric derivatives on survival of HeLa cells. (●) Daunorubicin; (○) daunorubicin–PAA conjugate (mol. wt 20,000); (◐) daunorubicin–PAA conjugate (mol. wt 5300); (▲) daunorubicin–PLL conjugate (mol. wt 40,000); (△) daunorubicin–PLL conjugate (mol. wt 2700).

molecular weight dependence for the daunorubicin–PAA conjugate. In contrast, the survival curves for daunorubicin linked to PLL indicated some influence of molecular weight on lethal activity of these polymeric derivatives, as expected by cytotoxic effects of PLL alone [7]. However, both drug–PLL conjugates tested showed low potency compared to the drug–PAA conjugate.

DISCUSSION

The results presented in this study indicate that the covalent binding of daunorubicin to polyamino acids results in reduction of drug toxicity in both PAA and PLL conjugates. The attachment of the drug to PLL also produced a reduction in the toxicity of the polymer itself (results not shown). In contrast to PAA, which is a non-toxic polyamino acid [5], a molecular-weight-dependent toxicity of PLL has been reported [7].

The reduction in overall toxicity of toxic agents linked to carrier molecules is not unique for these polymeric derivatives, since this phenomenon has been already observed [8]. The reduction in drug toxicity after conjugation could allow the therapeutic use of very toxic drugs characterized by a low therapeutic index [9].

The reduced toxicity of daunorubicin–polyamino acid conjugates was also accompanied by a reduction of drug potency and a reduced cytotoxicity to HeLa cells (Fig. 2). However, the low potency is a disadvantage only for the daunorubicin–PLL conjugate, since a lack of efficacy against Gross leukemia was found at the maximally tolerated dose (25 mg/kg). The difficulty of i.v. administration of higher doses precluded further studies in this tumor model. The drug activity against P388 leukemia was not lost by conjugation to PLL; however, both potency and efficacy were markedly reduced compared to those of the daunorubicin–PAA conjugate. The partial loss of drug activity in the PLL conjugate is surprising, since PLL has been used as the drug carrier for methotrexate [3, 10] and PLL itself has cytotoxic properties [7].

It is unlikely that the different antitumor activity of the drug in these conjugates is attributable to the difference in molecular weight, since cytotoxic properties of daunorubicin–PAA (Fig. 2) indicated no appreciable molecular-weight-dependence. Similarly, the *in vivo* activity of the drug linked to PAA did not change appreciably with a decrease in the molecular size of the polyamino acid used (Fig. 1).

The mechanism(s) of the increased effectiveness of daunorubicin linked to PAA is still unclear, since no tumor specificity of this carrier is known. The improved therapeutic effect of the polymeric derivative of the drug could be explained by different events: (1) a slow release of doxorubicin from the conjugate could produce prolonged drug exposure of tumor cells. Anthracycline cytotoxicity is dependent on duration of drug exposure [11]. Indeed, prolonged continuous infusion of doxorubicin has been reported to increase the therapeutic index of the drug [12]; (2) alternatively, a preferential uptake of drug–macromolecular conjugate by tumor cells, as a result of their preferential endocytotic activity [13], could facilitate the delivery of drug to target sites; and (3) finally, since a macromolecular drug derivative is expected to be partially removed by the reticuloendothelial system, drug storage by macrophages [14] and possible activation of macrophage function [14–16] could play a relevant role in the therapeutic effectiveness of the conjugate. Activated macrophages are recognized as an important component of natural resistance against malignant cells [17].

In any event, the different effectiveness of these conjugates suggests that the specific nature of the chemical linkage plays an important role in the activity of polymeric carrier-bound drugs. Indeed, the attachment of daunorubicin to polyamino acid by an easily hydrolyzable bond (i.e. ester linkages) potentiated its antitumor efficacy. In contrast, when the drug was attached by a more stable linkage (i.e. a C–N bond present in the daunorubicin–PLL conjugate), the activity was markedly reduced. The critical influence of the stability of the chemical linkage on activity has already been noted [2–4, 18]. Polyamino acids have been used as multifunctional polymeric carriers of antitumor drugs [5, 10, 19]. When conjugation by reaction involving the bromo-derivative of daunorubicin [1] is used, only anionic polymers appear suitable to achieve optimal drug activity.

Finally, the marked reduction in cytotoxic activity of daunorubicin covalently linked to PLL, a membrane-interacting polymer [7], does not support the hypothesis that anthracycline antibiotics produce cytotoxic effects through alternative mechanisms involving cell surface interaction [20].

Acknowledgments—We thank Miss L. Locati and V. Pelis for technical assistance, Mrs G. Barp and Ms B. Johnston for editorial assistance and manuscript preparation, and Dr G. Pratesi for helpful comments.

REFERENCES

1. ZUNINO F, GAMBETTA R, DI MARCO A, ZACCARA A. Biologic activity of daunorubicin linked to proteins via the methyl-ketone side chain. *Tumori* 1981, **67**, 521–524.
2. HURWITZ E, WILCHEK M, PITHA J. Soluble macromolecules as carriers for daunorubicin. *J Appl Biochem* 1980, **2**, 25–35.
3. SHEN WC, RYSER HJ. Poly (L-lysine) and poly (D-lysine) conjugates of methotrexate: different inhibitory effect on drug resistant cells. *Mol Pharmacol* 1979, **16**, 614–622.
4. SHEN WC, RYSER HJ-P. *Cis*-aconityl spacer between daunomycin and macromolecular carriers: a model of pH-sensitive linkage releasing drug from a lysosomotropic conjugate. *Biochem Biophys Res Commun* 1981, **102**, 1048–1054.
5. ZUNINO F, GIULIANI F, SAVI G, DASDIA T, GAMBETTA R. Anti-tumor activity of daunorubicin linked to poly-L-aspartic acid. *Int J Cancer* 1982, **30**, 465–470.
6. TROUET A, MASQUELIER M, BAURAIN R, DEPREZ-DE CAMPENEERE D. A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate: *in vitro* and *in vivo* studies. *Proc Natl Acad Sci USA* 1982, **79**, 626–629.
7. ARNOLD LJ, DAGAN A, GUTHEIL J, KAPLAN NO. Antineoplastic activity of poly(L-lysine) with some ascites tumor cells. *Proc Natl Acad Sci USA* 1979, **76**, 3246–3250.
8. LEE FH, HWANG KM. Antibodies as specific carriers for chemotherapeutic agents. *Cancer Chemother Pharmacol* 1979, **3**, 17–24.
9. GREGORIADIS G. Targeting of drugs: implications in medicine. *Lancet* 1981, **ii**, 241–246.
10. RYSER HJP, SHEN WC. Conjugation of methotrexate to poly(L-lysine) as a potential way to overcome drug resistance. *Cancer* 1980, **45**, 1207–1211.
11. RITCH PS, OCCHIPINTI SJ, SKRAMSTAD KS, SHACKNEY SE. Increased relative effectiveness of doxorubicin against slowly proliferating sarcoma 180 cells after prolonged drug exposure. *Cancer Treat Rep* 1982, **66**, 1159–1168.
12. LEGHA SS, BENJAMIN RS, MACKAY B *et al.* Reduction of doxorubicin cardiotoxicity by prolonged continuous intravenous infusion. *Ann Intern Med* 1982, **96**, 113–139.
13. SILVERSTEIN SC, STEINMAN RM, COHN ZA. Endocytosis. *Annu Rev Biochem* 1977, **46**, 669–722.
14. HASKILL JS. Adriamycin-activated macrophages as tumor growth inhibitors. *Cancer Res* 1981, **41**, 3852–3856.
15. MARTIN F, GAIGNARD A, OLSSON O, JEANNIN JF, LECLERC A. Tumorcidal effect of macrophages exposed to adriamycin *in vivo* or *in vitro*. *Cancer Res* 1982, **42**, 3851–3857.
16. KLEINERMAN ES, ZWELLING LA, SCHWARTZ R, MUCHMORE AV. Effect of L-phenylalanine mustard, adriamycin, actinomycin D, and 4′-(9-acridinylamino) methanesulfon-*m*-anisidine on naturally occurring human spontaneous monocyte-mediated cytotoxicity. *Cancer Res* 1982, **42**, 1692–1695.
17. ALEXANDER P. The functions of the macrophages in malignant disease. *Annu Rev Med* 1976, **27**, 208–228.
18. WILLIAMSON J, SCOTT-FINNIGAN TJ, HARDMAN MA, BROWN JR. Trypanocidal activity of daunorubicin and related compounds. *Nature* 1981, **292**, 466–467.
19. ROWLAND GF, O'NEILL GJ, DAVIES DAL. Suppression of tumour growth in mice by a drug-antibody conjugate using a novel approach to linkage. *Nature* 1975, **255**, 487–488.
20. TRITTON TR, YEE G. The anticancer agent adriamycin can be actively cytotoxic without entering cells. *Science* 1982, **217**, 248–250.