SPECIFIC INHIBITION OF DNA-POLYMERASES FROM RNA TUMOR VIRUSES BY SOME NEW DAUNOMYCIN DERIVATIVES

P. CHANDRA*, F. ZUNINO**, A. GÖTZ, D. GERICKE and R. THORBECK
Institut für Therapeutische Biochemie der Universität, Frankfurt/M., W. Germany

and

A. DI MARCO
Istituto Nazionale per lo Studio e la cura dei Tumori, Milan, Italy

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1. Introduction

Daunomycin is an antibiotic of the anthracycline group isolated from cultures of *Streptomyces peucetius* [1,2]. This antibiotic consists of a pigmented aglycone (daunomycinone) bound by a glycosidic linkage to an amino sugar (daunosamine, fig. 1)[3,4]. The biological activity of daunomycin is dependent on its ability to interact with primer DNA [5]. The inhibition of DNA-polymerase activity in RNA tumor viruses by daunomycin has been recently studied [6,7]. Whereas, Müller et al. [6] got 50% inhibition of the DNA-polymerase reaction at 2 μg/ml of daunomycin, Hirschman [7] reported only 30% inhibition at 100 μg/ml. The present communication describes the effect of daunomycin and its structural analogues on the DNA-polymerase activity of Friend Leukemia virions (FLV), Murine Sarcoma virus-Moloney (MSV-M) and Rous Sarcoma virus (RSV). These studies were carried out using various templates,e.g. poly (dA–dT), poly (dI.dC) and poly rA.$(dT)_{12}$. The results show that the inhibitory action of daunomycin and its derivatives is a template specific one. The substitutions in the amino sugar moiety lead to an inactivation of the daunomycin molecule.

*Correspondance should be sent to: Professor P. Chandra, Institut für Therapeutische Biochemie der Universität, D-6 Frankfurt/M., Ludwig-Rehn Str. 14, W. Germany.
**Permanent address: Istituto Nazionale per lo Studio e la cura dei Tumori, Milan, Italy.

2. Materials and methods

^{3}H-labeled deoxynucleoside triphosphates were obtained from NEN-Chemicals GmbH, Germany. Unlabeled deoxynucleoside triphosphates were purchased from Nutritional Biochemical Corp., Ohio, USA. Poly (dA – dT) and poly rA were supplied by Miles Chemical Laboratories, Elkhart, Indiana, USA. Poly (dI.dC) and poly rA.$(dT)_{12}$ were provided by Dr. G. Weimann of Boehringer Mannheim, Tutzing.

MSV-M, kindly supplied by Dr. J.B. Moloney (NCI, Bethesda, Md.) was passed by intramuscular injection into suckling Swiss mice, and extracted from the tumor tissue. FLV was isolated from infected spleens (AKR mice) and purified by sucrose-density gradient centrifugation. RSV was isolated from tumors grown on chickens and purified by sucrose-density gradient centrifugation. The viral extracts used to assay for DNA-polymerase activity were stored at –70° before use.

DNA-polymerase assay: DNA-polymerase activity was assayed essentially by the method of Ross et al. [8]. The reaction mixture, regardless of template, was similar to that of Ross et al. [8], except that we used 0.04 M Tris and the end concentration of Nonidet P-40 (Shell Chemie, Hamburg) was 0.2%. The reaction mixture contained 0.25 μg of the template used. The reaction mixture (0.25 ml) containing virion (28 μg of protein) was incubated at 37° for 90 min. Acid precipitable material was counted

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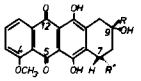
CHEMICAL STRUCTURES OF DAUNOMYCIN DERIVATIVES

General structure  Structure of daunosamine 

SUBSTITUTIONS

	R	R′
Daunomycin	$-CO-CH_3$	Daunosamine
Adriamycin	$-CO-CH_2OH$	Daunosamine
13-Dihydro-daunomycin	$-CHOH-CH_3$	Daunosamine
N-guanidine-acetamide-daunomycin	$-CO-CH_3$	N-guanidine-acetamide-daunosamine
N-acetyl-daunomycin	$-CO-CH_3$	N-acetyl-daunosamine

Fig. 1. Chemical structures of daunomycin derivatives.

on "Millipore" filters (HAWP 02500) in a liquid scintillation counter. Protein was estimated by the method of Lowry et al. [9].

3. Results and discussion

The inhibitory effect of different daunomycin derivatives (fig. 1) on the viral oncogenesis by FLV and RSV is shown in table 1. FLV suspensions were prepared by filtering the homogenates from infected spleens (AKR mice) through Seitz EK-filters (Seitz Company, Bad Kreuznach, Germany). This suspension was diluted 1:20 with Hank's solution. The diluted suspension was incubated with daunomycin or its derivatives (50 μg/ ml) for 1 hr at 37°. The control suspensions were incubated under similar conditions, without the antibiotic. 0.1 ml of this suspension (ID_{90}) was injected intraperitoneally. Each experimental group contained 6 animals. As follows from table 1 5 of the 6 control animals died after 13 days of infection. At this time the animals injected with daunomycin treated viral suspension were all alive. Adriamycin also has a very beneficial effect, whereas dihydro daunomycin shows a moderate activity. The derivatives substituted in the amino sugar moiety are ineffective. The oncogenesis in chicken by RSV is also inhibited by daunomycin and its analogues. Thus, the mean survival time is prolonged from 12.3 days to 28.3 days by adriamycin. Daunomycin is even more effective, whereas the other derivatives have no significant effect.

Table 1
Effect of daunomycin and its derivatives on the viral oncogenesis in mice and chicken.

Antibiotic used	Oncogenesis in mice by FLV No. of animals survived* / No. of animals infected	Oncogenesis in Chicken by RSV** Mean survival time (days)
None	1/6	12.3
Daunomycin	6/6	30***
Adriamycin	5/6	28.3
Dihydro daunomycin	3/6	14.2
N-guanidine-daunomycin	1/6	13.6
N-acetyl daunomycin	0/6	12.0

*13 days after infection; for details see text.

**Each experimental group contained 6 chickens. The tumor suspension (1:10) was incubated with 50 μg/ml of the antibiotic at 37° for 1 hr. Control suspension was incubated without the antibiotic. 0.1. ml of this suspension was injected intraperitoneally.

***One animal was still alive at the time of writing this paper.

Table 2
Inhibition of reverse-transcriptase activity of RNA tumor viruses by daunomycin derivatives.

System	Antibiotic used*	^{3}H-TMP incorporation into DNA (cpm/reaction mixt.)		
		MSV(Moloney)	FLV	RSV
Without virions	–	7 (3.4)	7 (3.7)	7 (2.9)
Virions + RNase**	–	26 (13)	25 (13.4)	40 (16.8)
Complete	None	202 (100)	187 (100)	237 (100)
	Daunomycin	65 (32.1)	68 (36.3)	80 (33.7)
	Adriamycin	66 (33.1)	83 (44.4)	86 (36.3)
	Dihydro daunomycin	86 (42.5)	87 (46.5)	113 (47.7)
	N-guanidine daunomycin	106 (52.4)	97 (51.8)	117 (49.3)
	N-acetyl daunomycin	196 (97)	192 (102.6)	136 (57.3)

*Antibiotic concentration = 10 μg/reaction mixt. (0.25 ml).
**Virions containing Nonidet P-40 were preincubated at room temp for 25 min with 50 μg/ml pf pancreatic RNase. Figures in parentheses are the percent of control. The reaciton conditions are described under Materials and methods.

The inhibitory activity of daunomycin and its structural analogues in viral oncogenesis suggests that the virus-associated enzymatic activities are sensitive to these antibiotics. The RNA-dependent DNA polymerase of virions is responsible for the synthesis of DNA chains on the RNA template, giving rise to a hybrid molecule (RNA–DNA). These DNA chains are released from the RNA template as single stranded DNA molecules and serve as templates for the synthesis of double stranded DNA. These synthetic processes could be affected by the daunomycin derivatives. The inhibition of reverse transcriptase activity of MSV-M, FLV and RSV by various daunomycin derivatives is shown in table 2. Daunomycin

Table 3
Inhibition of DNA-polymerase activity of FL-virions by daunomycin and its derivatives in the presence of various templates.

Antibiotic used*	^{3}H-TMP incorporation into DNA poly (dA–dT) (cpm/reaction mixt.)	^{3}H-dGMP incorporation into DNA poly (dI.dC) (cpm/reaction mixt.)	^{3}H-TMP incorporation into DNA poly rA.$(dT)_{12}$ (cpm/reaction mixt.)
None	1223 (100)	1006 (100)	723 (100)
Daunomycin	127 (10.3)	1057 (105)	159 (22)
Adriamycin	106 (8.7)	1127 (112)	231 (31.9)
Dihydro daunomycin	151 (12.3)	1654 (164.2)	327 (45.2)
N-guanidine daunomycin	322 (26.3)	941 (93.5)	457 (63.2)
N-acetyl daunomycin	1412 (115.6)	587 (58.3)	673 (93)

*Antibiotic concentration = 5 μg/reaction mixt. (0.25 ml). The figures in parentheses indicate the percent of control. The reaction conditions are described under Materials and methods.

Table 4
Inhibition of DNA-polymerase activity of MSV(M) by daunomycin and its derivatives in the presence of various templates.

Antibiotic used*	^{3}H-TMP incorporation into DNA poly (dA–dT) (cpm/reaction mixt.)	^{3}H-dGMP incorporation into DNA poly (dI.dC) (cpm/reaction mixt.)	^{3}H-TMP incorporation into DNA poly rA.$(dT)_{12}$ (cpm/reaction mixt.)
None	5446 (100)	3757 (100)	1196 (100)
Daunomycin	126 (2.3)	4123 (109)	467 (39)
Adriamycin	106 (1.9)	4252 (113)	472 (39.4)
Dihydro daunomycin	162 (3)	6446 (171.5)	552 (46.1)
N-guanidine daunomycin	781 (14.3)	4897 (130.3)	572 (47.8)
N-acetyl daunomycin	5607 (102.9)	4086 (108.7)	1160 (97.0)

*Antibiotic concentration = 5 μg/reaction mixt. (0.25 ml). The figures in parentheses indicate the percent of control. The reaction conditions are described under Materials and methods.

and adriamycin inhibit the reverse-transcriptase reaction to 60–70% at 10 μg/reaction mixture (0.25 ml). The dihydro derivative is also quite effective whereas the N-guanidine derivative has a moderate activity. However, the N-acetyl derivative is completely ineffective in MSV-M and FLV systems. The RSV system was moderately inhibited by the N-acetyl derivative.

Synthetic polymers containing either deoxyribonucleotide or ribonucleotide strands are known to stimulate the *in vitro* DNA synthesis by RNA tumor viruses [10,11]. Some inhibitors of the DNA-polymerase reaction in RNA tumor viruses are known to exhibit a template-primer specificity [12 – 14]. Table 3 shows the inhibition of template-dependent DNA-polymerase activity of FLV by various daunomycin derivatives.

The reactions catalyzed by poly (dA–dT) and poly rA.$(dT)_{12}$ are highly sensitive to the action of daunomycin and its derivatives. Even under these conditions daunomycin and adriamycin are most effective, and the *N*-acetyl derivative is completely inactive. It is interesting to note that poly (dA–dT) and poly rA.$(dT)_{12}$-dependent reactions are more sensitive to antibiotics than the endogenous reaction (see table 2). The DNA-polymerase reaction catalyzed by poly (dI.dC) is completely insensitive to these antibiotics. As a matter of fact, the most active derivatives (daunomycin, adriamycin and dihydrodaunomycin) exhibit a slight stimulation of ^{3}H-dGMP incorporation catalyzed by poly (dI.dC). This stimulation is particularly noticeable in the case of dihydro daunomycin. Surprisingly, the *N*-acetyl derivative was found to inhibit this reaction. The mechanism of this inhibition is not understood.

Table 4 shows the inhibition of template-dependent DNA-polymerase activity of MSV-M by various daunomycin derivatives. Compared to FLV-system the poly (dA–dT)-dependent reaction in this case is extremely sensitive to daunomycin derivatives. The poly rA.$(dT)_{12}$-dependent reaction shows a similar sensitivity towards the daunomycin derivatives as observed in the FLV system. In both these cases the *N*-acetyl derivative is completely ineffective. Very similar to FLV system we find here that the poly (dI.dC) catalyzed incorporation of ^{3}H-dGMP is not inhibited by any of the derivatives. Contrary to the FLV system the *N*-acetyl derivative in this system did not inhibit the ^{3}H-dGMP incorporation into DNA. Dihydro derivative exhibits here also a very high stimulatory activity.

The present results show that the inhibition exerted by daunomycin derivatives against DNA polymerases from RNA tumor viruses is selectively dependent on the type of primer-template used in the assay system. The inhibitor activity of daunomycin requires specific structural parameters. Thus substitutions in the amino sugar moiety, especially *N*-acetylation, inhibit its antitumor activity and influence its inhibitory action on the DNA polymerases of RNA tumor viruses.

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