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Coumermycin inhibition of murine retrovirus replication in cultured cells

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The effect of coumermycin A_1 activity on the infection and replication of murine type C retroviruses was studied *in vitro*. The infectivity of five prototype ecotropic retroviruses was reduced by 50 to 94%, with viral titres decreased up to seven-fold. These values were substantiated by progeny production studies. Similar results were obtained with five strains of xenotropic retroviruses. Delayed inhibition of growth kinetics in mouse SC-1 cells was observed with 7·5 and 10 mg/l of coumermycin A_1. This effect was markedly reduced after three cycles of freezing and thawing of the drug. Changes in the absorption spectra of coumermycin A_1 were observed after eight cycles of freezing and thawing.

Introduction

Coumermycin A_1 is a carbohydrate and coumarin-containing antibiotic produced by *Streptomyces richiriensis*, which acts as a specific inhibitor of the β subunit of DNA gyrase in *Escherichia coli* (Sugino *et al.*, 1978). The drug has also been shown to inhibit Herpes simplex (HSV-1) and SV40 virus replication, while being cytotoxic for hamster BHK and CV-1 cells, which were used for growing these viruses (Franke & Margolin, 1981; Edenberg, 1980). The relative effectiveness in reducing viral production parallels the effect on cell growth, indicating a not strictly specific antiviral activity.

Murine type C retroviruses replicate in cultured cells after their integration into the cellular DNA by a reverse transcription of the viral genome by a RNA-dependent DNA-polymerase, which is normally not present in eukaryotic cells (Temin & Mizutani, 1970). We have previously shown that the murine retrovirus experimental model can be used for studying the effect of steroid hormones (Varnier & Levy, 1979) and the antiviral drug phosphonoformate (Varnier *et al.*, 1982) on different phases of the viral replication cycle.

In order to give more insight into the mechanism of the antiviral activity of the drug, we have investigated the effect of coumermycin A_1 on the infection and replication of murine type C retroviruses *in vitro*.

Material and methods

Cells and viruses

The SC-1 cell line of wild mouse embryo and the XC cell line of Rous sarcoma virus-transformed rat cells were provided by J. A. Levy, San Francisco, California

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Mink S+L− cells were a gift of P. Allen, Frederick, Maryland. Cell lines were cloned, propagated and selected for their sensitivity to virus infection in our laboratories, as previously described (Varnier & Levy, 1979). The ecotropic AKR, Gross passage A, Friend, Moloney and Rauscher strains of murine retroviruses were originally obtained from Levy and then propagated in our laboratories (Levy, 1978). The xenotropic AKR (X-AKR/T-Mlc) and NFS (X-NFS/T-Mlc) viruses were obtained from J. H. Hartley, Bethesda, Maryland. The C57L (X-C57L/T-Mlc) and NZB isolates were derived and cloned in Levy's laboratory by cocultivation of thymus, spleen (X-NZB/S-Huc) and kidney (X-NZB/K-Mlc) cell cultures with heterologous cell lines (Varnier *et al.*, 1983*a*, 1984).

Media and chemicals

The cultures were maintained in Dulbecco's modified minimal essential medium (DEM, Flow, Italy) containing 5% fetal bovine serum (FBS), 1% glutamine (2 mM) and gentamicin (10 mg/l). Coumermycin A_1 was dissolved in dimethylsulphoxide (DMSO) (2 mg/l) and stored at −20°C in aliquots of 0·5 ml. Cells used for virus assays were pretreated with diethyl-aminoethyl-dextran (DEAE-D, 25 mg/l, 30 min) to increase their sensitivity to virus infection (Duc-Nguyen, 1968).

Virus assays

Detection and quantitation of ecotropic retroviruses were determined by the XC plaque assay, according to Rowe, Pugh & Hartley (1979), with slight modifications (Varnier *et al.*, in preparation). Briefly, infected and control SC-1 cultures were UV-irradiated three days after infection, overlayed with 1×10^6 XC cells and then fixed two days later. Xenotropic retroviruses were measured by the focus formation assay in mink S+L− cells (Peebles, 1975).

Cell growth kinetics

About 2×10^5 cells were seeded in 4 ml of complete growth medium in 60 mm Petri dishes. Twenty-four hours after plating cells were replenished and given a medium change on the third day. Triplicate control and treated cultures were trypsinized and counted with a Coulter Counter daily for six days.

Absorption spectra of coumermycin A_1

A stock aliquot coumermycin A_1 (2 mg/ml in DMSO) was submitted to different cycles of freezing and thawing; temperature varied from −20°C to +20°C. Samples for spectroscopic measurements were taken each time after thawing the antibiotic solution so as to have the compound in an aqueous solvent (99·5% H_2O–0·5% DMSO) at a concentration of 9 μM (10 mg/l). The absorption spectra were recorded on a Perkin-Elmer 124 spectrophotometer. All operations were carried out at a temperature of 20° ± 1°C in commercial quartz cells (Hellman) with a path length of 1 cm.

Results

Optimal concentration of coumermycin A_1

To determine the optimal concentration of coumermycin A_1 inhibiting viral infectivity, SC-1 cell cultures, infected with various inocula of the Friend strain of

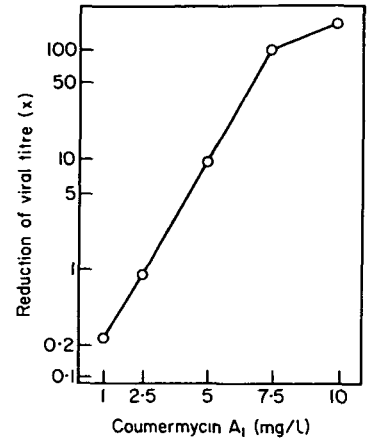

Figure 1. The effect of coumermycin A_1 on Friend virus infectivity. Viral titres were measured by the XC plaque assay on SC-1 cells, as described in the text.

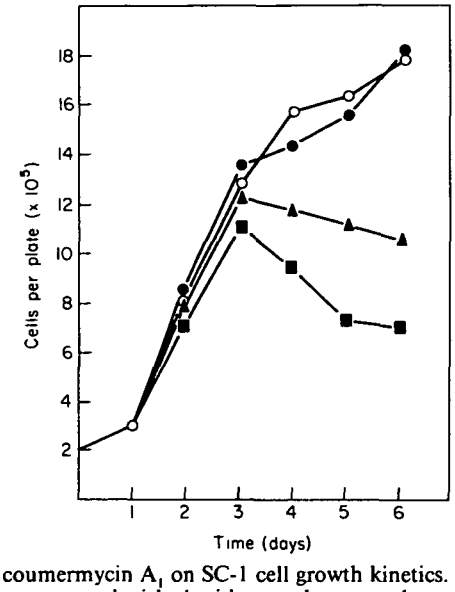

Figure 2. The effect of coumermycin A_1 on SC-1 cell growth kinetics. Twenty-four hours after plating (day 0), SC-1 cell cultures were replenished with complete growth medium supplemented with 5 (●), 7·5 (▲) and 10 (■) mg/l of coumermycin A_1. Treated and control (○) triplicate cell cultures were trypsinized and counted daily for six days.

ecotropic retroviruses, were maintained in growth medium supplemented with the drug at various concentrations (from 0·1–100 mg/l). No antiviral activity was detected at concentrations below 1 mg/l, and the drug showed a macroscopic cytotoxic effect at levels exceeding 25 mg/l. The dose-response curve, measuring the effect of the drug on the infectivity of Friend virus, showed a slight inhibitory effect at a drug concentration of 1 mg/l (Figure 1). The reduction of viral titre increased up to nine-fold at 5 mg/l, reaching the maximum level (169-fold) at 10 mg/l.

Since coumermycin A_1 is a potent inhibitory of cell growth (Franke & Margolin, 1981), we analysed the effect of various concentrations of the drug on the growth kinetics of SC-1 cells (Figure 2). No cytotoxic effect was noted in the first three days

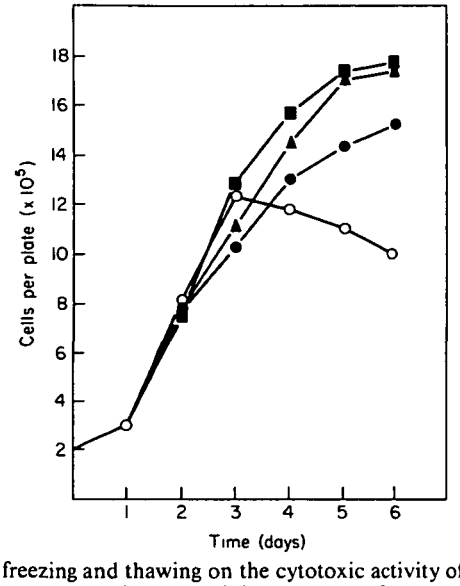

Figure 3. The effect of freezing and thawing on the cytotoxic activity of coumermycin A_1. SC-1 mouse cells were maintained in growth medium containing 7·5 mg/l of coumermycin A_1, thawed 1 (○), 3 (●), 5 (▲) and 8 (■) times, with a medium change on the third day. Triplicate cell cultures were counted daily.

with all the three dilutions employed. Concentrations of 7·5 and 10 mg/l affected SC-1 cell growth, inhibiting the formation of a confluent monolayer with a marked reduction (over 50%) of the viable cells on the sixth day. The values presented in Figure 1 with cytotoxic doses of the drug were obtained in a three-day XC plaque assay, a time interval in which no cytotoxic effect of the drug was observed or counted (Figure 2).

Next, we examined the effect of freezing and thawing on the cytotoxic activity of coumermycin A_1. Stock aliquots, after one, three, five and eight thawings, were added to SC-1 cell cultures in growth kinetics experiments. In Figure 3, a typical experiment of SC-1 growth kinetics in the presence of 7·5 mg/l, thawed one, three, five and eight times, is represented. No relevant difference in the number of viable cells was observed in the first three days. Once-thawed coumermycin A_1 had a marked effect on SC-1 cell growth. This inhibitory activity was markedly reduced after three freezings and thawings of the drug. SC-1 cultures, maintained in the presence of five or eight times thawed drug, showed the same number of cells per plate as controls. Similar results were obtained with 10 mg/l of the drug, while 5 mg/l of fresh coumermycin A_1 did not affect SC-1 growth kinetics (data not shown).

Absorption spectra experiments showed an absorption maximum at 280 and 347 nm with an E_{280} of 6×10^4 (M cm^{-1}). No significant difference was observed after one and three cycles of freezing and thawing. However, eight successive treatments produce a 4·9% hypochromism of the band at 280 nm together with a more marked reduction (15% hypochromism) of the band centered at 347 nm (data not shown). We therefore used 5 mg/l of once or twice thawed drug in all our experiments.

Effect of coumermycin on ecotropic retroviruses

To study the antiviral activity of coumermycin A_1, we tested five strains of ecotropic retroviruses (Moloney, AKR, Gross, Rauscher and Friend). The experiments were

repeated at least three times and representative results from one study are given in Table I. These experiments were performed in SC-1 cells with the XC plaque assay for virus quantitation in the presence and absence of coumermycin A_1. The drug inhibited the formation of XC plaques by 50 to 94% at various inocula. The Moloney and the AKR strains were the least sensitive to the antiviral effect of the drug, while the Friend virus was the most sensitive. We also estimated the extent of antiviral action by evaluating the reduction in viral titres produced at increasing viral input. Coumermycin A_1 inhibited Moloney, AKR, Gross, Rauscher and Friend viral titres 1·14-, 1·69-, 3·46-, 4·13- and 6·76-fold, respectively. This decrease in the infectivity of the ecotropic retroviruses was reflected in the progeny production. Supernatant fluids at 24 h from plates read for XC plaques were harvested before UV-treatment, filtered and tested for infectious viral particles. Coumermycin A_1 inhibition of replication of ecotropic viruses substantiated previous observations. The 8·77-fold decrease in the titre of viral progeny, noted in SC-1 cells infected with Rauscher virus, emphasizes the difference observed in the drug's effect on the infectivity of this ecotropic strain.

Effect of coumermycin on xenotropic retroviruses

In parallel with preceding experiments we tested five strains of xenotropic retroviruses (AKR, NFS, C57L and two NZBs) for focus formation in mink S+L− cells in the presence and absence of coumermycin A_1 (Table II). The number of transformation foci in S+L− cells correlates with the amount of xenotropic retrovirus present (Peebles, 1975). Inhibition of focus formation ranged from 43 to 90% (X-NFS/T-Mlc and X-NZB/S-Huc, respectively) at different viral inocula. The compound reduced viral titres from 1·34- to 9·47-fold.

Progeny production by the S+L− mink cells infected with the different xenotropic virus strains was also measured. The antiviral activity of the drug was confirmed by an up to 7·71-fold reduction of viral progeny titres.

Discussion

In this report we have shown a consistent effect of coumermycin A_1 on murine retroviruses. The drug inhibited the formation of XC plaques, induced by five prototype ecotropic retroviruses, by 50 and 94% with an up to seven-fold reduction in viral titres. The results with the viral progeny of these viruses support our observations of an antiviral effect on ecotropic retroviruses. Our studies have also indicated that infectivity and replication of several different xenotropic retroviruses were reduced in S+L− mink cells to a similar extent. The variation in inhibition of the viruses tested may indicate differences in the retrovirus strains, which we have observed in previous studies on the effects of dexamethasone and phosphonoformate on viral replication (Varnier & Levy, 1979; Varnier *et al.*, 1982).

In all our experiments we used 5 mg/l of coumermycin A_1, a concentration which was not cytotoxic for mouse SC-1 cells (Figure 2). Since the viability of the cultured cells in the presence of cytotoxic doses of the compound was affected only after three days of culture (Figures 2 and 3), short-time assays would give false indications of the antiviral activity of the drug. In fact, 10 mg/l gave an almost 200-fold reduction of Friend virus titre in the absence of any detectable cytotoxic alteration of the monolayer (Figure 1). Parallel cultures replenished daily for one week with medium

Table I. Effect of coumermycin on the infectivity and replication of ecotropic retroviruses

Retrovirus strain	Coumermycin A_1 5 mg/l	Inoculum pfu/plate	Number of XC plaque per plate	Inhibition %	Viral titre log_{10}pfu/ml	Reduction value	Viral progeny log_{10}pfu/ml	Reduction value
Moloney	–	110	106	—	5·63	—	5·77	—
	–	11	8	—				
	+	110	50	53	5·30	1·14	5·37	1·51
	+	11	4	50				
AKR	–	350	TNC	—	6·09	—	5·70	—
	–	35	31	—				
	+	350	100	—	5·66	1·69	5·44	0·82
	+	35	11·5	63				
Gross	–	200	TNC	—	4·79	—	4·03	—
	–	20	15·5	—				
	+	200	76·5	—	4·14	3·46	3·40	3·26
	+	20	3·5	77				
Rauscher	–	100	97	—	4·59	—	2·73	—
	–	10	9·5	—				
	+	100	19	79	3·88	4·13	1·74	8·77
	+	10	2	79				
Friend	–	50	50	—	4·30	—	ND	
	–	5	2·5	—				
	+	50	6·5	87	3·41	6·76	ND	
	+	5	0·5	80				

Titres are expressed as Log_{10} of plaque forming units (pfu) per ml of viral preparation. Reduction values were calculated as follows: reduction = [pfu/ml(−coumermycin A_1) − pfu/ml(+coumermycin A_1)]/pfu/ml(+coumermycin A_1). TNC, Too numerous to count; ND, not done.

Table II. Effect of coumermycin on the infectivity and replication of xenotropic retroviruses

Retrovirus strain	Coumermycin A_1 5 mg/l	Inoculum ffu/ml	Number of foci per plate	Inhibition %	Viral titre log_{10}ffu/ml	Reduction value	Viral progeny log_{10}ffu/ml	Reduction value
X-NFS/T-Mlc	–	50	44	—	3·25	—	2·79	—
	–	5	3·5	—				
	+	50	19	57	2·88	1·34	2·26	2·89
	+	5	2	43				
X-NZB/K-Mlc	–	250	TNC	—	5·94	—	5·70	—
	–	25	22	—				
	+	250	TNC	—	5·45	2·09	5·20	2·16
	+	25	7	68				
X-C57L/T-Mlc	–	50	50	—	4·20	—	ND	
	–	5	4	—				
	+	50	13	74	3·60	2·98	ND	
	+	5	1	75				
X-AKR/T-Mlc	–	60	51	—	4·31	—	3·19	—
	–	6	6	—				
	+	60	8	84	3·50	5·45	2·25	7·71
	+	6	1	82				
X-NZB/S-Huc	–	300	TNC	—	5·02	—	4·08	—
	–	30	26	—				
	+	300	22	—	4·00	9·47	3·15	7·51
	+	30	2·5	90				

Titres are indicated as focus forming unit (log_{10}ffu/ml). TNC, Too numerous to count; ND, not done.

containing 5 mg/l of fresh coumermycin A_1 showed no cytotoxic effect, suggesting that intracellular drug accumulation does not take place (data not shown).

The spectral modifications, occurring after three or more freezings and thawings, may be due to an alteration of the cromophor portion of the antibiotic and not to sampling errors. The sensitivity of the compound to physical inactivation may reside in the complexity of its structure and in the weakness and/or the particular exposure of the amidic bond which combines the two moieties of coumarin and sugar (Ryan, 1976).

Evidence from several laboratories has indicated that coumermycin A_1 inhibits Herpes simplex (HSV-1) and SV40 virus replication *in vitro* (Franke & Margolin, 1981; Edenberg, 1980). Nevertheless the antiviral activity may not reside in a specific inhibition of viral polymerase (Palù, Cusinato & Meloni, 1983*a*), since the drug has been shown to be a potent inhibitor of cell growth (Franke *et al.*, 1981).

It cannot be excluded that the inhibition of viral replication merely reflects the inhibition of the host cell metabolism. We have preliminary evidence indicating that a non-cytotoxic dose of coumermycin A_1, (5 mg/l), is still able to depress DNA synthesis to some extent. We have recently shown (Palù *et al.*, 1983*b*), in an eukaryotic model, that coumermycin A_1 interacts with structurally and functionally unrelated cellular components and that the toxicity of the compound stems primarily from its non-specific binding to nucleic acid, histones and enzymatic protein. In this regard the preferential antiviral toxicity of the compound may indicate its complexing with nascent viral macromolecules which have a higher turnover than the cellular ones. However the possibility of a more specific antiviral effect cannot be totally ruled out.

To understand the mechanism of the antiviral action of coumermycin A_1 better we are currently trying to isolate strains of retroviruses that are resistant to the antibiotic.

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