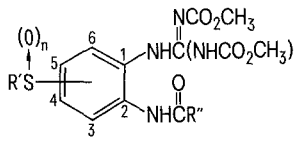
Efficacy on injectable phenylguanidine anthelmintics in naturally infected sheep



Compound No.	R′	R′ position	R″	n	Injection[a]: % reduction in fecal egg count[c] (dose in mg/kg)	Oral[b]: % reduction in fecal egg count (dose in mg/kg)
1	$(CH_3)_2CHCH_2-$	4	H_3C-	1	95 (10)	99 (10)
2	$(CH_3)_2CHCH_2-$	5	H_3C-	1	93 (10)	NT[d]
3	$(CH_3)_2CHCH_2-$	5	CH_3OCH_2-	0	77 (10)	100 (20)
4	$(CH_3)_2CHCH_2-$	5	CH_3OCH_2-	1	99 (10)	99 (10)
5		5	H_3C-	0	23 (17)	100 (10)
6		5	H_3C-	1	65 (17)	100 (10)
7	$(CH_3)_2CHCH_2-$	4	CH_3SCH_2-	1	73 (20)	100 (20)

[a] Compounds administered s.c. as a suspension in water. [b] Compounds administered p.o. in gelatin capsules. [c] The percent reduction in the fecal egg count for a given compound was calculated by the following equation: $\frac{\text{pretreatment egg count - post treatment egg count}}{\text{pretreatment egg count}} \times 100$. [d] NT = not tested.

administered at a single s.c. injection of 10 mg/kg. Likewise, taeniacidal activity in mice (against *Hymenolepis nana*) was limited to but a few analogues in this phenylguanidine series (unpublished data).

Compound **1** administered orally at 20 mg/kg to sheep artificially infected with metacercariae of *Fasciola hepatica* was 100% effective against patent infections. Compound **1** given s.c. at 20 mg/kg for 2 consecutive days was 99% effective against *Fasciola* in sheep.

Preliminary studies indicate that compound **1** was marginally effective (2–33%) against *Ancylostoma caninum* infections in dogs when administered both s.c. as a single injection of 50 mg/kg and orally at 100 mg twice a day for 2 consecutive days. Compound **1** was 100% effective against the dog whipworm *Trichuris vulpis* when administered s.c. at 50 mg/kg. Compound **1** demonstrated partial activity (60%) against experimental *Ascaridia galli* infections in chickens when give orally at 50 mg/kg.

Compound **1** as well as those listed in the table are chemically related to Febantel, a new broad-spectrum anthelmintic, 2′-[2,3-bis(methoxycarbonyl)guanidino]-5′-phenylthio-2-methoxyacetanilide[3].

1 For the previous paper in this series, see R.D. Haugwitz, B.V. Maurer, G.A. Jacobs, V.L. Narayanan, L.R. Cruthers and J. Szanto, J. med. Chem. *22*, 1113 (1979).
2 L.R. Cruthers, R.D. Haugwitz, M. Haslanger, B.V. Maurer, J. Watrous and W.H. Linkenheimer, Experientia *34*, 1574 (1978).
3 W. Wollweber, H. Kolling, A. Widdig, H. Thomas, H.P. Schulz and P. Murrmann, Drug Res. *28*, 2193 (1978).

The reactivation of human interferons by guanidine thiocyanate

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Summary. The addition of 1.5 M guanidine thiocyanate (GuSCN) reactivates inactive human leukocyte interferon. The biological activity of inactivated human fibroblast interferon can be only partially recovered with GuSCN if additional (thermal) energy is supplied.

The interferon proteins have a variety of biological effects, in addition to their well-known antiviral and antineoplastic actions[4]. These proteins also exhibit some unusual stability characteristics. Although most globular proteins become unstable when they are unfolded by conditions that disrupt their non-covalent structures, the interferons produced by mouse L cells[5–8], human fibroblasts[7,9–12], as well as human leukocytes[7,9–11], can be stabilized against thermal denaturation by such conditions (or reagents), including chaotropic salts[6,9], low pH[5,7,11], and sodium dodecyl sulfate (NaDodSO$_4$)[8,10–12]. The current studies show that the biological activity of interferon (especially of the leukocyte type), that has been denatured by heat, can be reactivated by the chaotropic salt, guanidine thiocyanate.

Materials and methods. Sendai-virus-induced human leukocyte interferon (provided by K. Cantell, Helsinki, Finland[13]) contained 3.2 mg/ml of protein and had a sp. act. of $1.5\text{–}3.0 \times 15^5$ units per mg protein. Human fibroblast interferon was prepared by J. Vilcek (New York, NY) using poly(I)·poly(C) in human foreskin cells under conditions

Reactivation of thermally inactivated human interferons in guanidine thiocyanate (GuSCN)

Interferon sample (protein concentration)	Treatment*	Interferon activity units/ml	% control
Leukocyte (0.46 mg/ml)	1 Unheated control (pH 7)	29,000	100
	2 Denatured (pH 7, 100 °C for 2.5 min)	2,600	8.9
	3 Denatured; 1.5 M GuSCN	18,000	62
	4 Denatured; 1.5 M GuSCN; reheated (100 °C for 2.5 min)	25,000	86
	5 Denatured; 1.5 M GuSCN + 1% 2-ME; reheated (100 °C for 2.5 min)	6,250	21.6
Fibroblast (40 mg/ml)	1 Unheated control (pH 7)	600,000	100
	2 Denatured (pH 7, 100 °C for 2.5 min)	680	0.1
	3 Denatured; 1.5 M GuSCN	15,000	2.5
	4 Denatured; 1.5 M GuSCN; reheated (100 °C for 2.5 min)	27,000	4.5

* The interferon preparations were diluted to the desired activity in a 0.1 M phosphate buffer at pH 7 and then dialyzed against the same buffer before the initial heating. All heated and control samples were then diluted with an equal volume of either phosphate buffer or 3.0 M GuSCN. Before assay for interferon activity, the samples were again dialyzed at 4 °C for 4 h against 100 volumes of phosphate buffer.

of superinduction[14]. The protein concentration was 80 mg/ml and the sp. act. 1.2×10^4 units per mg protein.

Interferon activity was determined from graphical plots of the inhibition of yields of encephalomyocarditis viral hemagglutinin in the BUD-8 strain of human skin fibroblasts grown as previously described[15]. Protein was assayed by the method of either Lowry et al.[16] or Sedmak and Grossberg[17].

Inactivation was carried out by boiling for 2.5 min in a water bath 1-ml volumes of interferon distributed in 15×48 mm screw-cap glass vials. Interferon samples heated for purposes of inactivation or reactivation were cooled by keeping them in air at room temperature for 20–30 min, since the manner of cooling influences stabilization[5].

Results and discussion. The addition of GuSCN to a concentration of 1.5 M significantly reactivated leukocyte and fibroblast interferons (table). This reactivation may result from refolding of the inactive interferons as GuSCN is dialyzed away prior to assaying for antiviral activity. Furthermore, the yield of reactivated interferon was even greater if the inactive interferons to which GuSCN was added were reheated. Activity of the leukocyte preparation was essentially completely restored when reheated in the presence of GuSCN, whereas human fibroblast interferon was only partially reactivated by GuSCN and heat. The addition of heat may unfold a greater portion of interferon molecules than does GuSCN alone; and thus a greater portion of the unfolded molecules will refold to biologically active molecules during cooling and the subsequent dialysis needed to remove GuSCN. That leukocyte and fibroblast interferons can be reactivated by GuSCN to different degrees should not be surprising since increasing evidence indicates that they differ biologically, antigenically, and structurally[4,18], having only 29% amino acid homology[19].

Leukocyte interferon was reactivated to a lesser degree by GuSCN when 2-mercaptoethanol (2-ME) was present, similar to the reduced reactivation Stewart et al.[10] obtained for leukocyte interferon with $NaDodSO_4$ and 2-ME. Stewart and Desmyter[20] have found that the major leukocyte species (which accounted for about 80% of the activity of the original sample) was destroyed by reduction in $NaDodSO_4$, whereas the minor species (20% of the original activity) was not. Our leukocyte interferon also probably contains 2 species of interferon, one inactivated by 2-ME and another that is not.

Since GuSCN is readily removed by dialysis, it may be preferred to $NaDodSO_4$ for reactivation of leukocyte interferon intended for experimental or clinical use, inasmuch as it is very difficult to remove $NaDodSO_4$ from proteins[21].

The reactivation schemes described have great potential value for restoring the potency of human leukocyte interferon inactivated during production, processing, and purification. The observed differences in reactivation by GuSCN reported herein provide additional evidence for the structural differences between the fibroblast and leukocyte types of human interferon. They further emphasize the unusual character of interferons compared to other proteins, which may be rendered inactive in high-molarity chaotropic salts such as guanidine thiocyanate.

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4 S. Baron, P.A. Brunell and S.E. Grossberg, in: Antiviral Agents and Viral Diseases of Man, p. 151. Ed. G. Galasso, T.C. Merigan and R. Buchanan. Raven Press, New York 1979.
5 R.J. Jariwalla, S.E. Grossberg and J.J. Sedmak, Archs. Virol. *49*, 261 (1975).
6 R.J. Jariwalla, S.E. Grossberg and J.J. Sedmak, J. gen. Virol. *35*, 45 (1977).
7 L.W. Marshall, P.M. Pitha and W.A. Carter, Virology *48*, 607 (1972).
8 W.E. Stewart, II, P. De Clercq and P. De Somer, Nature, Lond. *249*, 460 (1974).
9 R.J. Jariwalla, J.J. Sedmak and S.E. Grossberg, Abstr. A. Meet. Am. Soc. Microbiol. *75*, 270 (1975).
10 W.E. Stewart, II, P. De Somer, V.G. Edy, K. Paucker, K. Berg and C.A. Ogburn, J. gen. Virol. *26*, 327 (1975).
11 J. Vilcek, E.A. Havell and S. Yamazaki, Ann. N.Y. Acad. Sci. *284*, 703 (1977).
12 E. Knight, Jr, Proc. natl Acad. Sci. USA *73*, 520 (1976).
13 K. Cantell, S. Hirvonen, K.E. Mogensen and L. Pyhala, in: The Production and Use of Interferon for the Treatment and Prevention of Human Virus Infections, p. 35. Ed. C. Waymouth. Tissue Culture Association, Rockville, Maryland, 1974.
14 E.A. Havell and J. Vilcek, Antimicrob. Ag. Chemother. *2*, 276 (1972).
15 P. Jameson, M.A. Dixon and S.E. Grossberg, Proc. Soc. exp. Biol. Med. *155*, 173 (1977).
16 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
17 J.J. Sedmak and S.E. Grossberg, Analyt. Biochem. *79*, 544 (1977).
18 M.J. Otto, J.J. Sedmak and S.E. Grossberg, J. Virol. *35*, 390 (1980).
19 T. Taniguchi, N. Mantei, M. Schwarzstein, S. Nagata, M. Muramatsu and C. Weismann, Nature *285*, 547 (1980).
20 W.E. Stewart, II, and J. Desmyter, Virology *67*, 68 (1975).
21 G.P. Tuszynski and L. Warren, Analyt. Biochem. *67*, 55 (1975).