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Studies on the *in vitro* and *in vivo* Chemotherapeutic Properties of the Antibiotic Myxin

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Myxin (6-Methoxy-1-phenazinol 5,10-dioxide) was first isolated from a Sorangium species by PETERSON *et al.* (1) who showed that it possessed marked activity against bacteria and fungi *in vitro*. Its chemical structure was initially described by EDWARDS and GILLESPIE (2) and recently corrected by WEIGELE and LEIMGRUBER (3). The present report summarizes the data obtained from a diverse series of *in vitro* and *in vivo* chemotherapeutic tests with synthetic material prepared by WEIGELE and LEIMGRUBER.

Materials and Methods

A. *In vitro Tests*

Conventional serial two-fold broth dilution tests were performed using trypticase soy broth for bacteria, Sabouraud's broth for fungi, Dubos broth for *M. tuberculosis* H37Rv, Simplified trypticase serum (STS) broth for *T. vaginalis*, cysteine peptone liver maltose (CPLM) broth for *T. foetus* and mixture 199 for adult worms and larvae. The ability of myxin to prevent the cytopathic effect of Coxsackie B1 virus was studied employing rhesus monkey kidney cell cultures fed with mixture 199.

To test for the bactericidal effect of myxin, two-fold serial dilutions were prepared in water from a 1% suspension of myxin in 100% dimethylsulfoxide. The final volume of each dilution was 4.5 ml. One-half ml of an undiluted 24 hr broth culture was added to each dilution. The inoculated tubes were placed in a water bath at 37C. At intervals of 1 and 24 hours after inoculation a standard 4-mm loopful was removed from each tube and inoculated into 10 ml of trypticase soy broth. The subcultures were incubated at 37C and examined for growth after 72 hours. The lowest drug concentration at which growth failed to occur in the subculture was considered to be the minimum bactericidal concentration (MBC).

B. *Local Infection*

1. *Bacteria:* 0.2–0.5 ml of a 24 hour broth culture were injected into the ventral subcutaneous tissue of 18–20 gm mice. The animals were treated immediately afterward by infiltration of myxin into the site of infection. At the end of 24 hours the animals were sacrificed and cultures from the infected and treated areas were made on papain digest bacto beef agar plates supplemented with whole blood in the case of streptococci. The presence of 10 colonies or less indicated successful treatment.

2. *Candida albicans:* 0.5 ml of a saline suspension containing 250,000 cells were injected into the ventral subcutaneous tissue of 18–20 gm mice preconditioned with 50 mg/kg cortisone intramuscularly the morning and afternoon of the day before and the day of infection. The animals were treated 1, 3 and 5 days after infection either by infiltration into the site of infection or orally. Seven days after infection the animals were sacrificed and cultures from the infected and treated areas were made on Sabouraud's agar plates and evaluated as above.

3. *Trichomonas vaginalis:* Albino mice weighing 18–20 gm were infected subcutaneously on the ventral surface with approximately 750,000 cells from a 24 hour STS broth culture. The animals were treated subcutaneously at the site of infection at 1 and 24 hours after infection or orally daily for a total of four treatments. The animals treated subcutaneously were sacrificed 72 hours after infection while those treated orally were sacrificed on the fifth day after infection and examined for the presence or absence of lesions.

C. *Systemic Infections*

1. *Bacteria:* Swiss albino mice weighing 18 to 20 gm were used. The experimental infections employed were: *Streptococcus pyogenes*, *Diplococcus pneumoniae* type I, *Staphylococcus aureus* Smith, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhosa*, *Salmonella schottmuelleri*, *Neisseria meningitidis* Serogroup A and B and *Hemophilus influenzae* Type A. Mice were infected intraperitoneally with 0.5 ml of inoculum containing 100 to 1000 minimal lethal doses. Properly diluted overnight broth cultures of streptococci, pneumococci and Klebsiella or suspensions in 5% gastric mucin of staphylococci, members of the coli-salmonella group, Proteus, Pseudomonas, Neisseria and Hemophilus were employed. For the experiments with *M. tuberculosis* H37Rv, mice were infected intravenously with 0.5 ml of a 10^{-1} dilution of a 7–10 day old Dubos culture in saline.

In the case of the gram-positive and gram-negative bacteria, myxin was administered either as a single dose shortly after infection or in a series of four treatments distributed over 4 days, with the first dose also administered shortly after infection. Mice which died were autopsied, and cultures were taken from heart blood. Survivors were observed for a period of 2 weeks. Animals infected with *M. tuberculosis* were administered the drug in the diet for a period of 21 days at which time they were sacrificed and the lungs examined for the presence or absence of lesions.

2. *Fungi:* Mice were infected by the intravenous route with *Candida albicans* (50,000 organisms/mouse) and yeast phase cells of *Histoplasma capsulatum* (2,500,000 organisms/mouse). Animals were conditioned with cortisone (50 mg/kg intramuscularly) on the day before and on the day of infection. Mice were treated daily or until death for a total of 21 days after which time spleen smears were examined

for the presence of Histoplasma and kidneys cultured for the presence of Candida.

3. *Protozoa:* Mice were infected with *T. foetus* by inoculation of 9,000,000 cells intraperitoneally. To test for the effect of myxin against the *T. foetus* peritonitis, mice were treated 3 times orally at the following intervals after infection: 1, 24 and 48 hours. The number of mice surviving 14 days after infection was the criterion used for determining activity in this experimental model.

For the *Endamoeba histolytica* infection, weanling rats weighing 30–35 gm were anesthetized with 30 mg/kg sodium pentobarbital administered intraperitoneally. Following laparotomy, each rat was infected intracecally with 0.25 ml of a 48 hour egg-albumin slant with Stone's-Locke's overlay culture diluted with an equal volume of Stone's-Locke's solution. The animals were treated once orally on the day following infection. Six days after infection the animals were sacrificed and scrapings taken from the cecum were examined microscopically for presence or absence of amoebae.

4. *Helminths: Hymenolepis nana* – Mice were infected with 1.0 ml of a washed pooled suspension containing approximately 1000 eggs obtained by blending the posterior third of mature tapeworms obtained from the small intestines of mice infected 2 weeks earlier. Two weeks after infection the mice were treated orally once daily for 3 consecutive days. Mice were sacrificed one day after the last treatment and the small intestine examined for adult worms.

Nematospiroides dubius – Mice were infected orally with approximately 50 larvae hatched at room temperature from the feces of previously infected mice. Treatment and evaluation were the same as for *H. nana.*

Syphacia obvelata – Mice harboring a natural infestation, as ascertained by microscopic examination of a "Scotch tape" preparation from the perianal region, were treated once orally for 3 consecutive days and sacrificed one day after the last treatment. The cecum of each animal was then removed and examined for the presence or absence of adult pinworms.

5. *Viruses:* Mice were infected intraperitoneally with 10 LD_{50} of brain homogenates containing Columbia SK and herpes viruses and with tissue culture fluid containing Coxsackie B1 virus. The influenza infection was accomplished by the intranasal administration of chorioallantoic fluid diluted to contain 10 LD_{50}. All virus-infected mice, with the exception of influenza A, were treated 24 hours prior to, immediately after and then 24 hours after infection. In the case of influenza A, the animals received treatments immediately before and then at intervals of 1, 5, 24, 30, 48 and 72 hours after infection.

6. *Tumors: Ehrlich carcinoma* – Solid form—The solid form of this tumor was produced by the subcutaneous injection of 0.5 ml of a saline suspension of 5–10 million Ehrlich carcinoma cells derived from the ascitic fluid of a donor bearing a 7 to 10-day-old ascitic tumor. Treatment was begun immediately after implantation and continued once daily for a total of 8 treatments. The animals were sacrificed 8 days after implantation and the average weight of the tumors from the untreated control group (C) was compared with the average weight of the tumors from the treated group (T); if the C/T ratio was 2.0 or greater (50% or more inhibition) a positive antitumor effect was considered to have occurred.

Ascitic form—The ascitic form of the tumor was produced by the intraperitoneal injection of approximately one million cells from a similar donor. Treatment was begun immediately after implantation and continued once daily

until a total of 8 doses had been administered. The experiment was terminated after 30 days and the average survival time (days) of the treated group (T) was compared with the average survival time (days) of the untreated control group (C); if the T/C ratio was 1.5 or greater (50% or more increase in survival time) a positive antitumor effect was considered to have occurred.

Sarcoma 180: Small pieces of tumor (20–30 mg) were implanted subcutaneously by trocar into the ventro-lateral surface of mice. The fragments were obtained from donors bearing firm subcutaneous tumors implanted 7–10 days previously. Treatment and evaluation procedures were identical with those employed for the solid form of the Ehrlich carcinoma.

Results

The *in vitro* effects of myxin against bacteria and fungi are shown in Tables I and II. Similar data for protozoa, helminths, viruses and rhesus monkey kidney cell cultures are presented in Table III.

Table I. In vitro Activity of Myxin Against Bacteria and Fungi

Bacteria	MIC: mcg/ml
Streptococcus pyogenes	1.25
Streptococcus agalactiae	2.5
Diplococcus pneumoniae Type I	0.31
Staphylococcus aureus ╪209	0.62
Escherichia coli J	0.31
Klebsiella pneumoniae	0.39
Proteus vulgaris 190	0.39
Pseudomonas aeruginosa B	3.12
Salmonella typhosa F	0.31
Salmonella schottmuelleri	0.39
Pasteurella multocida	0.16
Erysipelothrix insidiosa	0.16
Mycobacterium tuberculosis H37Rv	0.04
Mycoplasma gallinarum	0.31
Fungi	
Candida albicans	10
Trichophyton mentagrophytes	1
Microsporum audouini	1
Ustilago Zeae	10
Fusarium oxysporum	1
Bortrytis paeoniae	10
Aspergillus flavus	1
Aspergillus niger	100

Table II. In vitro Bactericidal Activity of Myxin

	MBC: mcg/ml	
	1 h	24 h
Streptococcus pyogenes	250	0.24
Staphylococcus aureus 209	50	25
Escherichia coli J	31	31
Klebsiella pneumoniae	25	3.1
Proteus vulgaris	1000	3.9
Pseudomonas aeruginosa B	>1000	500
Salmonella typhosa F	1000	12.5
Salmonella schottmuelleri	500	500

Table III. In vitro Activity of Myxin against Protozoa, Helminths, Coxsackie B1 Virus and Monkey Kidney Cells

Protozoa	MIC: mcg/ml
Trichomonas vaginalis	250
Trichomonas foetus	250
Helminths	
Hymenolepis nana (adults)	1
Ascaris suum (larvae)	100
Nematospiroides dubius (adults)	>1000
Virus	
Coxsackie B_1	>1
Cytotoxicity	

	Maximum tolerated dose mcg/ml	
	24 hr	7 days
Rhesus monkey kidney cells	10	1

From the data in Tables I-III it can be seen that myxin exerted appreciable activity *in vitro* against gram-positive and gram-negative bacteria, *M. tuberculosis*, *M. gallinarum*, *C. albicans*, filamentous fungi including dermatophytes and certain helminths. It exerted a slight effect against trichomonads. Paper disc assays have also been performed which confirm the high *in vitro* anti-bacterial and anti-fungal activity of myxin. The substance was moderately cytotoxic for monkey kidney cells after 24 hours and markedly cytotoxic after one week. Furthermore, myxin exerted at least at the 24 hour period an appreciable bac-

tericidal effect against all of the gram-positive and gram-negative bacteria tested except *P. aeruginosa* and *S. schottmuelleri.*

The acute toxicity and the effects of myxin against systemic infections of mice with representative gram-positive and gram-negative bacteria are shown in Table IV.

Table IV. Acute Toxicity and *in vivo* Antibacterial Activity of Myxin

I. Acute toxicity for mice	LD_{50}: mg/kg per os	subcutaneous	intraperitoneal
	>2000	>2000*	133

* Drug deposit subcutaneously.

II. Chemotherapeutic Activity *In vivo*

	CD_{50}: mg/kg No. of treatments per os		sc		ip	
Bacteria	1	4	1	4	1	4
Streptococcus pyogenes	>500	>500	>500	>500	~50	>25
Diplococcus pneumoniae Type I	—	>500	—	>500	9.3	>25
Staphylococcus aureus Smith	>500	>500	>500	>500	35	~25
Escherichia coli	—	>500	—	>500	9.7	>25
Klebsiella pneumoniae	—	>500	—	>500	>50	>25
Proteus vulgaris	>500	>500	>100	>500	>50	>25
Pseudomonas aeruginosa	>500	>500	>100	>500	>50	>25
Salmonella typhosa	—	>500	—	>500	>50	>25
Salmonella schottmuelleri	—	>500	—	>500	>50	>25
Neisseria meningitidis Serogroup A	>500	—	>500	—	5	—
Neisseria meningitidis Serogroup B*	—	—	—	—	3.2	—
Hemophilus influenza Type A	—	>500**	—	—	—	>12.5**

* Resistant to 5 mg% sulfadiazine.

** 6 treatments-twice on the day of infection and the day after infection and then daily for 2 days.

The toxicity data in Table IV indicate that myxin is poorly absorbed when administered by the oral or subcutaneous route to the mouse. In agreement with this finding, chemotherapeutic activity was only detected when the substance was administered intraperitoneally, as can

be seen for infections with *S. pyogenes*, *D. pneumoniae* Type I, *S. aureus* Smith, *E. coli* and *N. meningitidis* Serogroup A and B.

The substance was inactive against *M. tuberculosis* H37Rv at a dose of 125 mg/kg diet.

Myxin was also tested for its local chemotherapeutic activity against subcutaneous bacterial infections. These data are shown in Table V.

Table V. Activity of Myxin Against Local Subcutaneous Bacterial Infections

Bacteria	mg/kg per os	mcg/ml sc
Streptococcus pyogenes B	>500	0.24
Staphylococcus aureus 503/288	>500	0.92
Escherichia coli J	>500	250
Proteus vulgaris 190	>500	20
Pseudomonas aeruginosa B	>500	2657

From the data in Table V one can conclude that myxin exerted a potent local *in vivo* antibacterial effect against *S. pyogenes* B, *S. aureus* 503/288 and *P. vulgaris*, a moderate effect against *E. coli* J and slight activity against *P. aeruginosa* B.

Table VI summarizes the *in vivo* effects of myxin against Candida and Histoplasma. Except for slight local activity against *C. albicans*, the substance was inactive.

Table VI. In vivo Activity of Myxin Against Pathogenic Fungi

Fungi	CD_{50} mcg/ml sc	mg/kg per os	ip
Candida albicans (local infection)	378	>500	—
Candida albicans (systemic infection)	—	—	>20
Histoplasma capsulatum	—	>250	>20

Table VII summarizes the *in vivo* effects of myxin against protozoa and helminths.

Table VII. In vivo Activity of Myxin Against Protozoa and Helminths

Protozoa	CD_{50} mcg/ml sc	mg/kg per os
Trichomonas vaginalis (local infection)	3.5	>500
Trichomonas foetus	—	>500
Endamoeba histolytica	—	>1200
Helminths		
Syphacia obvelata		586
Hymenolepis nana		138
Nematospiroides dubius		>1000
Ascaris suum		>500

The data in Table VII show that myxin was capable of exerting a marked chemotherapeutic effect against the local *T. vaginalis* infection when administered subcutaneously but not against the same infection when administered orally. Slight to moderate anthelmintic activity was seen in the cases of *S. obvelata* and *H. nana* respectively. Otherwise myxin was inactive against *Endamoeba histolytica*, *Nematospiroides dubius* and *Ascaris suum*. It should be pointed out that the high level of local *in vivo* activity against *T. vaginalis* stands in sharp contrast to its low activity *in vitro* against the organism.

Since an unusual degree of oxidation was noted in the STS and CPLM media during the course of assessing the *in vitro* antitrichomonad activity of myxin and since the media employed to grow the trichomonads contained cysteine, the effect of this and sodium thiogly-

Table VIII. Effect of Sulfhydryl Compounds on the *in vitro* Antibacterial Activity of Myxin

Broth Medium	MIC: mcg/ml S. pyogenes	S. aureus 209	E. coli J	P. aeruginosa B	K. pneumoniae
Fluid Thioglycollate	500.0	31.0	500.0	1000	500.0
Trypticase Soy	0.4	0.2	0.4	12	0.8
Trypticase Soy with 1.0 mg/ml cysteine added	0.4	0.2	0.2	31	0.8
Trypticase Soy with 10.0 mg/ml cysteine added	125.0	62.0	125.0	250	62.0
Adams and Roe	0.8	0.01	0.1	3	0.2
Adams and Roe with 1.0 mg/ml cysteine added	62.0	16.0	250.0	1000	>1000.0

collate on the antibacterial activity of myxin was studied. These data are summarized in Table VIII. It is apparent that the presence of reducing agents such as thioglycollic acid or cysteine caused marked inactivation of the biological effects of myxin when tested against both gram-positive and gram-negative bacteria.

The results of *in vivo* tests with tumors and viruses are summarized in Table IX.

Table IX. Antiviral and Antitumor Properties of Myxin

Viruses			CD_{50}: mg/kg ip	
Columbia SK			>25	
Herpes			>25	
Influenza A (PR8)			>12.5	
Coxsackie B_1			>25	
Tumor	mg/kg	C/T Index	mg/kg	T/C Index
Sarcoma 180	40 ip	4.25 (toxic)	—	—
	20 ip	1.67	—	—
Ehrlich solid	50 ip	toxic	—	—
	25 ip	0.93	—	—
	10 ip	0.75	—	—
Ehrlich ascites	—	—	25 ip	1.19
	—	—	500 po	0.91

It is apparent that myxin had no antiviral activity. The substance was also inactive against Ehrlich carcinoma but did show an effect against sarcoma 180 at a toxic dose.

Since myxin displayed a broad *in vitro* and local antibacterial spectrum and was poorly, if at all, absorbed by the oral route, it seemed of interest to determine what effect it would have on the bacterial intestinal flora of mice after repeated oral dosing. Mice were administered 200 mg/kg orally once daily for 6 consecutive days and then the fecal flora quantitatively examined. The results of this experiment, summarized in Table X, indicated that myxin did not significantly reduce the bacterial flora of the feces.

Table X. Effect of Myxin on the Bacterial Flora of Mice

	Cell count/fecal pellet*
Myxin 200 mg/kg per os once daily for six days	7.2×10^8
Control	9.1×10^8

* Average count from 4 mice. Counts done shortly after last treatment.

Summary

Myxin (6-methoxy-1-phenazinol 5,10-dioxide) is an antibiotic that displays a broad *in vitro* spectrum including activity against gram-positive and gram-negative bacteria, *M. tuberculosis*, *M. gallinarum*, *C. albicans*, filamentous fungi, including dermatophytes, helminths and protozoa. The antimicrobial effect was bactericidal in nature against certain gram-positive and gram-negative bacteria. The *in vitro* antibacterial effect could be partially overcome by the addition of cysteine or sodium thioglycollate to the growth medium. Myxin was cytotoxic for monkey kidney cells.

Myxin was not absorbed when administered by the oral or subcutaneous routes to mice. The substance was active when administered intraperitoneally to mice infected systemically with *S. pyogenes*, *D. pneumoniae*, *S. aureus*, *E. coli* and *N. meningitidis* as well as against mice implanted with sarcoma 180 but was without effect when tested by this same route against fungi, viruses and Ehrlich carcinoma.

When tested for local chemotherapeutic effects against subcutaneous bacterial infections, myxin exerted marked activity against *S. pyogenes*, *S. aureus* and *P. vulgaris*, moderate activity against *E. coli* and a slight effect in the case of *P. aeruginosa*. The antibiotic also exerted a marked effect against the subcutaneous *T. vaginalis* infection of mice when administered by infiltration as well as a slight effect against the subcutaneous *C. albicans* infection in a similar experimental model.

Myxin showed on oral administration slight to moderate anthelmintic activity against *S. obvelata* and *H. nana*.

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