BIOLOGICAL ACTIVITY OF A NEW CLASS OF RIFAMYCINS SPIRO-PIPERIDYL-RIFAMYCINS

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The biological properties of spiro-piperidyl-rifamycins, a new class of rifamycin antibiotics, are described. In these derivatives the positions 3 and 4 have been incorporated into an imidazolyl ring bearing a spiro-piperidyl group N substituted with linear and branched aliphatic chains. The *in vitro* antibacterial activity against *Staphylococcus aureus* and *Mycobacterium tuberculosis* increases with the number of the carbon atoms in the linear side chain, whereas the inhibitory effect on *Escherichia coli* is lowered. The antibacterial activity is only marginally affected by branching of the side chain. *In vivo* (experimental infections of mice) the optimal therapeutic activity againt *M. tuberculosis* is shown by compounds bearing 3~5 carbon atoms as a linear or branched side chain; in comparison with rifampicin, the potency of these derivatives is 2~3 times higher. The finding is in a good agreement with the exceptional tissue tropism, which seems to be a favourable property of this group of derivatives.

Rifamycins are members of a group of naturally occuring antibiotics[1] that has been subjected to an intensive program of semisynthetic modifications[2,3,4]. They are, therefore, a good example of a system where the biological properties of a molecule have been modified by chemical manipulation.

The synthetic program on new rifamycin derivatives carried out in our laboratories led to some new classes of rifamycins, using 3-amino-4-iminorifamycin S as starting material[5]. One of these new classes is the 3-amino-4-deoxo-4-imino-3,4-imidazolinyl-(1H)-[2-spiro(4-piperidyl)]-rifamycin S[6] (abbreviated name: spiro-piperidyl-rifamycins) in which positions 3 and 4 have been incorporated into an imidazoline ring bearing a spiro-piperidyl group N substituted with different linear and branched aliphatic chains with increasing number of carbon atoms.

In this paper we report the *in vitro* and *in vivo* antibacterial activity and the plasma and tissue levels of some of these derivatives.

Materials and Methods

Thin-layer chromatography (TLC)

TLC was performed on silica gel plates Merck F 254. The eluants were: benzene - ethyl acetate - methanol, 13: 2: 2 (eluant A); chloroform - methanol, 9: 1 (eluant B); chloroform - methanol, 9: 2 (eluant C).

In vitro activity

Minimal inhibitory concentrations (MIC) were determined by the serial twofold dilution technique in Difco Antibiotic Medium No. 3 with 15‰ of Difco Agar for *Staphylococcus aureus* FDA 209 P, *Escherichia coli* B, *E. coli* C1 (mutant strain resistant to 200 μg/ml of rifampicin), and *E. coli* ginetta strain (parent strain of C1) and in Difco Bacto Dubos Medium for *Mycobacterium tuberculosis* H37Rv.

The MICs were the lowest concentrations of antibiotic which prevented any visible growth after 1 day (*S. aureus* and *E. coli*) or 7 days (*M. tuberculosis*) of incubation at 37°C.

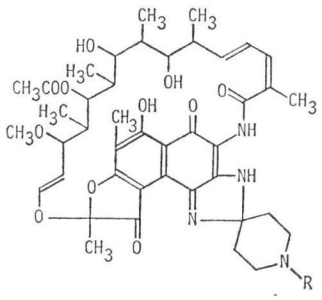
In vivo activity

(a) Groups of 8 female CDl Cobs mice were infected by i.p. route with 3 LD_{50} of *Staphylococcus aureus* PV_1 and *Salmonella abortivoequina* isolated in our laboratories. One or two hours after infection, the animals were treated by oral or s.c. route with solutions of the compounds to be tested in phosphate buffer, pH 7, +5% of dimethylformamide (0.1 ml/10 g body weight). Deaths were recorded and the median protective dose (PD_{50}) was calculated after 7 days[7].

(b) Groups of 10~12 mice, as above, were infected by the i.v. route with 3 LD_{50} of *M. tuberculosis* H37Rv. The animals were treated orally as described in (a) starting 3 days after infection. The treat-

Table 1. Chemical structure and Rf values on thin-layer chromatography (silica gel plates) of spiro-piperidyl-rifamycins.

Eluant A: benzene - ethyl acetate - methanol (13:2:2)
Eluant B: chloroform - methanol (9:1)
Eluant C: chloroform - methanol (9:2)



Compound No.	R—	Rf		
		Eluant A	Eluant B	Eluant C
1	H—	0.06	0.16	0.48
2	CH_3—	0.19	0.43	0.59
3	CH_3—CH_2—	0.21	0.45	0.60
4	CH_3—$(CH_2)_2$—	0.21	0.57	0.68
5	CH_3—$(CH_2)_3$—	0.37	0.67	0.77
6	CH_3—$(CH_2)_4$—	0.34	0.57	0.78
7	CH_3—$(CH_2)_5$—	0.35	0.56	0.87
8	CH_3—$(CH_2)_6$—	0.48	0.71	0.85
9	CH_3—CH(—CH_3)—	0.23	0.46	0.60
10	CH_3—CH_2—CH(—CH_3)—	0.31	0.48	0.72
11	CH_3—CH(—CH_3)—CH_2—	0.41	0.81	0.84
12	CH_3—CH(—CH_3)—CH(—CH_3)—	0.37	0.56	0.86
13	$(CH_3$—$CH_2)_2$CH—	0.34	0.56	0.78
14	CH_3—CH(—CH_3)—$(CH_2)_2$—	0.34	0.56	0.78

ment was repeated for 6 weeks (5-day treatment and 2-day interval weekly). The PD_{50} was determined 8 weeks after the infection.

Plasma levels

Groups of 5 female CDl Cobs mice were treated orally or subcutaneously with 50 mg/kg of the compounds prepared as described in (a). After 30, 60, 120, 240, 420 minutes 3 mice/group were sacrificed, the heparinated blood was collected, pooled, and centrifuged. The separated plasma was stored at −20°C before microbiological assay on *Sarcina lutea* ATCC 9341[8].

Tissue levels

Groups of 5 male CD rats weighing about 200 g, treated orally with 50 mg/kg of the compounds prepared as described in (a), were sacrificed 4 hours after treatment. Plasma, lung, liver and spleen were removed, homogenized in pH 7 phosphate buffer and used for microbiological assay on *Sarcina lutea* ATCC 9341[8].

Results

Table 1 reports the chemical structure and the Rf values on thin-layer chromatography of the new derivatives. Rf values increase with increasing the number of carbon atoms of the radical (R) but do not appear to be substantially affected by branching.

The MICs of the compounds on five representative strains of *Schizomycetes* are reported in Table 2.

Table 2. Antibacterial activity *in vitro* of spiro-piperidyl-rifamycins.

Compound No.	MIC (μg/ml)				
	S. aureus FDA 209 P	*E. coli* B	*E. coli* C_1*	*E. coli* ginetta**	*M. tuberculosis* H 37 Rv
1	0.01	0.78	6.25	3.12	0.01
2	0.0025	1.56	6.25	3.12	0.0025
3	0.005	3.12	12.50	3.12	0.005
4	0.01	3.12	12.50	3.12	0.005
5	0.005	1.56	25	3.12	0.0012
6	0.0025	6.25	>100	6.25	0.0012
7	0.005	12.50	>100	25	0.0012
8	0.005	12.50	>100	>50	0.0012
4	0.01	3.12	12.50	3.12	0.005
9	0.0012	3.12	6.25	3.12	0.005
5	0.005	1.56	25	6.25	0.0025
10	0.01	3.12	50	3.12	0.0025
11	0.005	1.56	50	3.12	0.005
6	0.0025	6.25	>100	6.25	0.0025
12	0.005	3.12	>100	12.5	0.0025
13	0.005	3.12	>100	6.25	0.0006
14	0.005	3.12	50	6.25	0.00015
Rifampicin	0.005	3.12	>100	6.25	0.005

* Resistant to 200 μg/ml of rifampicin

** Parent strain of *E. coli* C1

The parent compound (**1**) displays a broad-spectrum antibacterial activity which is slightly modified in the series of homologous compounds bearing linear alkyl chains from 1 to 7 carbons. With respect to the compound **1** there is a tendency towards an increased potency on *S. aureus* and even more on *M. tuberculosis*, whereas there is a lowered efficacy against strains of *E. coli*, both rifampicin-sensitive (*E. coli* B, *E. coli* ginetta strain) and rifampicin-resistant (*E. coli* C1). It is to be noted that this latter strain maintains a good degree of sensitivity to the lower homologues of the series of spiro-piperidyl-rifamycins in spite of being rifampicin resistant.

The pattern observed is in accordance with the increasing lipophilic nature of the compounds within the series, as expressed by the Rf values reported in Table 1.

When the isomers with 3, 4 and 5 carbons in the side chain are considered, the only relevant observations are the high activity of compound **9** against *S. aureus* and the increased activity of compounds **13** and **14** over that of the unbranched isomers.

The plasma levels in mice within 7 hours and the therapeutic efficacy are summarized in Tables 3 and 4.

In Table 3 the areas under the plasma curves (AUC), the time of appearance (*t* max) and the values

Table 3. Kinetic parameters in plasma of mice after oral and subcutaneous administration of 50 mg/kg.

Compound No.	p.o.			s.c.			RC**
	AUC*	*t* max (mins)	*C* max (μg/ml)	AUC	*t* max (mins)	*C* max (μg/ml)	
1	3.9	30→120	0.8	43	60	7.8	0.091
2	31	30→120	7.5	46	60	8.5	0.67
3	24	30	4.3	27	60	5.5	0.89
4	30	60	3.7	38	60	4.3	0.79
5	35	60	5.0	26	60→420	2.0	1.35
6	30	60	3.0	25	240	3.0	1.2
7	80	30→240	6.0	47	420	4.7	1.7
8	15.7	240→420	1.5	5.6	240→420	0.5	2.8
4	30	60	3.7	38	60	4.3	0.79
9	20	120	2.8	30	60	4.5	0.67
5	35	60	5.0	26	60→420	2.0	1.35
10	50	60	7.0	84	60→420	7.5	0.6
11	67	240	7.9	57	240→420	6.7	1.18
6	30	60	3.0	25	240	3.0	1.2
12	63	60→420	4.0	38	240→420	3.0	1.66
13	107	60→240	9.0	79	420	8.5	1.35
14	34	60→240	3.0	26	240→420	2.5	1.3
Rifampicin	420	60	70.0	480	30→60	65	1.08

* AUC=Area under the plasma curve within 7 hours.

** $RC = \frac{\text{AUC p.o.}}{\text{AUC s.c.}}$

of the peaks (*C* max) in the plasma are reported after oral and subcutaneous administration.

The AUC after oral administration is negligible for the parent compound (**1**), whereas the homologous derivatives with longer linear aliphatic chains show increased AUCs up to the compound **7**, after which the AUC considerably decreases again (compound **8**). This break point is confirmed by s.c. administration. The ratio AUC p.o./AUC s.c. increases gradually along the series of the homologous compounds suggesting the importance of lipid solubility for gastrointestinal absorption. The *t* maxs are delayed along the series of unbranched derivatives, both after oral and subcutaneous administration, whereas the *C* maxs are rather homogeneous, with the exception of compounds **1** and **8**.

Within the isomers, the branched compounds frequently show better bioavailability, delayed *t* maxs and/or longer persistance of the *C* max compared with the linear analogues (compounds **10**, **11**, **12**, **13**).

The median protective doses (PD_{50}) in mice either against *S. aureus* or *S. abortivoequina* infections (Table 4) are in a good agreement with the potency and plasma level characteristics of the substances for the extreme members of the series only (compounds **1** and **8**), although some correlation may be seen also within the whole class. For the *M. tuberculosis* infection, it is difficult, and probably faulty, to seek an analogous correlation because of the characteristics of the disease and of the extended treatment regimen. Nevertheless, the very good therapeutic effectiveness on *M. tuberculosis* infections of this class of compounds, particularly of those with higher molecular weight, can be stressed. This property may be related to a favourable distribution of the products in tissues.

Table 4. *In vivo* efficacy of spiro-piperidyl-rifamycins after oral (p.o.) and subcutaneous (s.c.) treatment in experimentally infected mice.

Compound No.	PD_{50} mg/kg			
	S. aureus PV 1		*S. abortivoequina*	*M. tuberculosis* H37Rv
	p.o.	s.c.	s.c.	p.o.
1	>1.6	0.1	6.2	>10
2	0.1	0.1	7	5
3	0.8	0.12	8	7
4	0.8	0.12	10	2.3
5	0.6	0.5	16	2
6	0.9	0.4	6.2	2
7	0.8	0.8	8	3.5
8	1.13	>1.6	>20	3.5
4	0.8	0.12	10	2.3
9	0.8	0.1	10	2.8
5	0.6	0.5	16	2
10	0.8	0.8	12	2.4
11	0.4	0.25	21	1.7
6	0.97	0.4	6.2	2
12	0.24	0.15	6.2	2.3
13	0.4	0.2	10	1.7
14	0.8	0.35	14	2.5
Rifampicin	0.2	0.15	6.2	4.2

Preliminary tests carried out by microbiological assay of tissue homogenates of rats treated with some of the new products (**2**, **5**, **11**), demonstrated very high tissues concentrations, particularly in lung, liver and kidney. The ratio of tissue/plasma levels ranged from 7~10 to 40~50 μg/ml depending on the compound and on the tissue (Table 5).

Table 5. Plasma (μg/ml) and tissue (μg/g) levels 4 hours after oral treatment in rats (50 mg/kg).

	Compound, No.			
	2	**5**	**11**	Rifampicin
Plasma	3.3	3	2	7
Liver	30	80	60	140
Lung	54	120	85	18
Spleen	20	100	48	15
Kidney	20	50	40	35

Discussion

The spiro-piperidyl-rifamycins, a new class

of rifamycin S derivatives obtained by chemical manipulation, show interesting biological properties. Some of these, such as the changes of *in vitro* activity, can be related to their lipid solubility. Other differences, observed in the groups of isomeric compounds with similar lipophilic nature, deserve more attention and may be related to other characteristics of the molecules, such as steric hindrance.

In the context of *in vitro* activity, we want to emphasize that, at least for *E. coli* C1, there is no complete cross-resistance between rifampicin and some members of the class of spiro-piperidyl-rifamycins.

The *in vivo* results support the following conclusions:

(1) Manipulation of the rifamycin S structure furnishes not only compounds with better gastrointestinal absorption, which is already known, but also with different pharmacokinetic properties such as lower plasma levels; early or late plasmatic peaks persisting for shorter or longer times compared with rifampicin; exceptionally high tissue levels not only in relation to plasma levels but also in absolute value.

Modulation of the plasma concentration pattern depends not only on the molecular weight of the compounds, *i.e.* on the number of carbon atoms in the side chain, but also on their branching structure, as shown in the groups of C4 and, particularly, of C5 isomers.

(2) The peculiar characteristics of the spiro-piperidyl-rifamycins allow them to display high therapeutic activity, especially against experimental tuberculosis, where intracellular pathogens are involved and tissue damage is prevalent. The lower effectiveness of the new homologues on the animal models of acute infection that we used (*S. aureus*, *S. abortivoequina*) can be explained taking into account the characteristics of these induced diseases which are rapidly lethal septicemias more susceptible to high blood levels than to high tissue concentrations.

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