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Research Articles

Effects of Potential Inhibitors on Metabolism of Griseofulvin *In Vitro*

By S. A. KAPLAN, S. RIEGELMAN, and K. H. LEE

The inhibitory effects of *p*-ethoxyacetanilide, *p*-methoxybenzylamine, codeine, and SK&F 525-A on the metabolism of griseofulvin were studied in a Krebs-Ringer bicarbonate liver-slice system. This investigation sought to determine the possibility of prolonging the biological activity of griseofulvin.

GRISEOFULVIN, the first available oral antifungal antibiotic, was originally isolated from the mycelium of *Penicillium griseofulvum* by Oxford in 1939 (1). However, it was not until 1958 that Gentles (2) reported that he was able to eradicate experimental ringworm of guinea pigs as a result of oral treatment with griseofulvin. This was followed by the works of Riehl (3, 4), Blank *et al.* (5), and Williams (6), who obtained favorable results in the treatment of superficial fungi infections of man.

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It has been shown that griseofulvin localizes in the prekeratin cells (7–9), and that the amount of griseofulvin deposited in the prekeratin cells depends upon the blood levels achieved. Therefore, in treatment it is important that the drug be deposited in the keratin of the hair shaft, skin, or matrix of the nails in fungistatic concentration to keep the organism in check pending removal of the infected tissue and replacement by healthy tissue. Since the turnover rate of these tissues is slow, oral therapy can involve periods of treatment up to 6 months or longer.

The high-dosage regimen required for griseofulvin to exert its therapeutic effect can be attributed to its low water solubility of approximately 1 mg. %, its slow rate solubility, and its relatively rapid rate of metabolism. It is difficult, therefore, to maintain a prolonged high concentration of drug in the blood.

Generally, the duration of drug action after absorption depends upon the rate of metabolism and excretion. Griseofulvin is unique in that it is not excreted intact but apparently only after being metabolized. Barnes *et al.* (10) reported that the major metabolite of griseofulvin in man and rabbits is 6-demethylgriseofulvin. *In vitro* studies by Bedford *et al.* (11) also confirm that griseofulvin is metabolized by the liver and results in the formation of 6-demethylgriseofulvin. Aromatic ethers are known to be metabolized by *O*-dealkylation (12). The authors have chosen several potential inhibitors of griseofulvin. These include *p*-ethoxyacetanilide, *p*-methoxybenzylamine, codeine, and SK&F 525-A. The first three mentioned compounds are aromatic ethers metabolized by oxidative *O*-dealkylation as is griseofulvin, and could therefore act in a competitive manner to inhibit the metabolism of griseofulvin and thereby prolong its biological activity. SK&F 525-A is a well-known nonspecific metabolic inhibitor in animals and affects many *O*-dealkylation reactions.

EXPERIMENTAL

Tissue Preparations.—Livers were obtained from young adult New Zealand white male rabbits of 2.0 to 2.5 Kg. The rabbits were stunned and exsanguinated, and the slices were prepared on a McIlwain mechanical tissue chopper (13) at a thickness of approximately 0.5 mm. at 3° in the cold room. Thirty-milliliter samples containing 10 mcg./ml. griseofulvin in Krebs-Ringer bicarbonate buffer (14) were incubated with 1.5 Gm. of liver slices, with and without the addition of the potential inhibitors at 37°, with shaking under an atmosphere of 5% carbon dioxide in oxygen saturated with water vapor. These samples were incubated for varying time intervals, after which the flasks were removed from the incubator, and supernatants were separated from the tissue slices by decanting. An aliquot of the supernatant was then immediately extracted twice with ethyl ether, evaporated, and reconstituted in ethanol. The griseofulvin was determined by both the spectrophotometric and spectrophotofluorometric methods of analysis. In the ultraviolet the samples were determined in ethanol at 292 mμ, while in the spectrophotofluorometric analysis the samples were activated at 315 mμ, and the maximum fluorescence determined at 450 mμ. SK&F 525-A did not interfere with the ultraviolet analysis of griseofulvin and the samples could be determined directly. However, codeine, *p*-ethoxyacetanilide, and *p*-methoxybenzylamine interfered with the ultraviolet analysis of griseofulvin as they were partially extracted by the ethyl ether along with the griseofulvin. Codeine and *p*-methoxybenzylamine were completely removed by washing the ether extracts with two 25-ml. portions of 1 *N* hydrochloric acid, allowing for the direct ultraviolet analysis of griseofulvin. *p*-Ethoxyacetanilide could not be separated from griseofulvin by extraction and was therefore analyzed by the spectrophotofluorometric method.

Microsomal Preparations.—Twenty-five per cent homogenates of liver were prepared in 1.15% potassium chloride solution by use of a Dounce homogenizer (15) in the cold room at 3°. The tissues were homogenized first with the loose-, then the tight-fitting pestle, and centrifuged at 9000 × *g* for 20 min. The supernatants were decanted and used in the subsequent experiments where each 5.1-ml. sample contained glucose-6-phosphate, 20 μm.; NADP, 0.4 μm.; nicotinamide, 50 μm.; magnesium chloride, 75 μm.; potassium chloride, 1 *M*, 0.1 ml.; griseofulvin in 0.1 *M* phosphate buffer or buffer alone, 1.5 ml.; and enzyme preparation, 1.5 ml. The above samples were incubated at 37° with shaking (110 oscillations per minute) in a Dubnoff metabolic shaker for varying time intervals at which time 2.0-ml. aliquots were removed from the flasks and added to 7.0 ml. of freshly distilled anhydrous ether and analyzed by means of the spectrophotofluorometric assay. The co-factors did not interfere with the analysis of griseofulvin on the Aminco Bowman spectrophotofluorometer.

Standardization of the Microsomal Activity.—*p*-Ethoxyacetanilide has been shown to be metabolized by the microsomal enzyme fraction (12). Therefore, with each determination samples containing *p*-ethoxyacetanilide without the addition of griseofulvin were run to determine the relative activity of the enzyme preparation.

Analysis of 6-Demethylgriseofulvin.—This compound is characterized by its ultraviolet spectrum which has a maximum peak at 293 mμ in acidic ethanolic solution, which shifts to 328 mμ in neutral to alkaline solution. 6-Demethylgriseofulvin is recoverable by extracting the acidified supernatant (pH 1) after removal of the griseofulvin, with two 25-ml. portions of ethyl ether. Other supernatant samples after removal of the griseofulvin were divided into two equal portions. One portion was analyzed for 6-demethylgriseofulvin as described above. The other portion was acidified to pH 4.5 with acetic acid, and incubated with β-glucuronidase at 37°. This system was then extracted as described to determine the amount of free 6-demethylgriseofulvin present in solution.

RESULTS AND DISCUSSION

The per cent of griseofulvin remaining unmetabolized in solution as a result of being incubated with

the Krebs-Ringer bicarbonate rabbit liver slice system has been calculated from 17 individual experiments. The metabolism of griseofulvin appears to follow pseudo first-order kinetics as is determined from the linearity of the semilogarithmic plot seen in Fig. 1. The half-life for the disappearance of griseofulvin has been calculated by the method of least squares to be 53.5 ± 3.8 min. (p = 0.025). Since the metabolism of griseofulvin in this system occurs within defined limits, a control was established to which the inhibitory studies with the potential inhibitors could be compared.

In each of the following experiments rabbit liver slices were incubated with and without the addition of a 100 to 1 molar ratio of inhibitor to griseofulvin. The experiments were repeated at three different levels of griseofulvin concentration, but the system is limited by the low solubility of griseofulvin in water. Figure 2 includes three curves, each of which is from the mean data of a minimum of four experiments and illustrates the pseudo first-order metabolism of griseofulvin and p-methoxybenzylamine with griseofulvin. Figure 3 shows similar experiments utilizing codeine as the potential inhibitor. The compound, SK&F 525-A, is not added to the *in vitro* system but is administered prior to sacrifice and preparation of liver slices. Figure 4 shows the results obtained from animals pretreated 2 hr. prior to sacrifice with 75 mg./Kg. of SK&F 525-A as compared to control animals.

The results indicate that at a molar ratio of 100 to 1, p-ethoxyacetanilide to griseofulvin, the rate of metabolism of griseofulvin is decreased approximately fourfold to give a half-life of 200.6 ± 9.2 min., indicating the rate of metabolism is significantly different in the presence of p-ethoxyacetanilide ($p < 0.01$). p-Methoxybenzylamine decreased the rate of metabolism approximately twofold to 116.5 ± 8.8 min., again indicating a significant difference in the rate of metabolism ($p < 0.001$), codeine with a half-life of 57.4 min. ($p > 0.60$), and SK & F 525-A with a mean half-life of 49.6 min. ($p > 0.80$) showed no inhibitory effects on the metabolism of griseofulvin.

In studying the metabolism of griseofulvin it has been inferred that the metabolism of the drug probably occurs in the microsomal enzyme fraction of the liver. The results in Table I clearly indicate that the microsomal enzymes in the presence of the co-factors of the soluble fraction are responsible for the metabolism of griseofulvin.

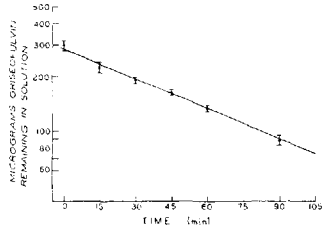

Fig. 1.—Disappearance of griseofulvin from the Krebs-Ringer bicarbonate liver-slice system reported with the 95% confidence intervals.

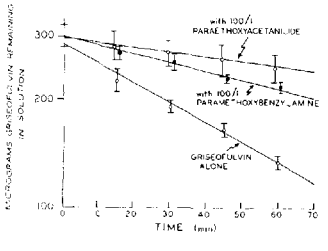

Fig. 2.—Disappearance of griseofulvin in the presence of a 100 M ratio of p-ethoxyacetanilide or p-methoxybenzylamine to griseofulvin with its 95% confidence interval.

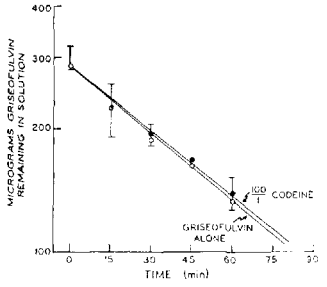

Fig. 3.—Disappearance of griseofulvin in the presence of a 100 M ratio of codeine phosphate to griseofulvin with its 95% confidence interval.

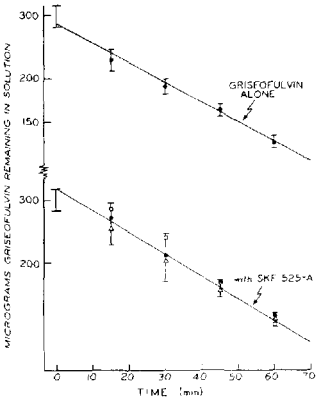

Fig. 4.—Disappearance of griseofulvin as a result of incubating the drug with liver slices pretreated with 75 mg./Kg. of SKF 525-A, reported with its 95% confidence intervals, as compared to that of griseofulvin alone.

TABLE I.—METABOLISM OF GRISEOFULVIN IN THE MICROSOMAL ENZYME SYSTEM

Expt.	Half-Life of Metabolism, min.
1	57.5
2	50.3
3	101.4
4	40.35
5	40.25
6[a]	44.0
7[a]	30.9

[a] Animals starved prior to experimentation.

The results indicate the mean half-lives of experiments *1* through *5* to be 57.9 ± 39.7 min. (p = 0.025).

Starvation is known to lower glycogen levels markedly. Therefore, rabbits were starved for 24 and 38 hr. prior to sacrifice and preparation of the microsomal and soluble fractions of the liver. When the metabolism of griseofulvin was followed as a result of incubation with these glycogen-depleted microsomal enzymes, the results in Table I indicate that starvation did not interfere with or affect the ability of the microsomal enzymes to metabolize griseofulvin. A comparison of the results of the metabolism studies of griseofulvin, to those previously reported by Axelrod for p-ethoxyacetanilide (12) are recorded in Table II.

TABLE II.—COMPARISON OF RESULTS OF METABOLISM STUDIES OF GRISEOFULVIN

	Griseofulvin	*p*-Ethoxyacetanilide
Metabolized by the microsomal system	+	+
Inhibited by SK&F 525–A	−	−
Inhibited by *p*-methoxybenzylamine	+	+
Inhibited by codeine	−	−
Inhibited by *p*-ethoxyacetanilide	+	

Based on the criteria established by Axelrod for differentiating between the two possible *O*-dealkylating enzyme systems, it would appear that griseofulvin is metabolized by the same enzymes that metabolize *p*-ethoxyacetanilide and *p*-methoxybenzylamine rather than by the *O*-dealkylating enzyme system shown to metabolize codeine.

In both the liver slice and the microsomal studies the disappearance of griseofulvin was compared to the appearance of 6-demethylgriseofulvin. Table III presents an analysis of the total recovery of griseofulvin and 6-demethylgriseofulvin.

TABLE III.—PER CENT RECOVERY OF THE TOTAL DOSE OF GRISEOFULVIN AS GRISEOFULVIN AND 6-DEMETHYLGRISEOFULVIN

Time of Incubation, min.[a]	% Recovered as Griseofulvin	% Recovered as 6-Demethylgriseofulvin	Total % Recovery
0	100	0	80.0
15	75.0	2.16	77.16
30	62.7	15.1	77.8
45	54.6	17.4	72.0
60	44.4	31.9	76.3
75	40.0	33.0	73.0

[a] Extrapolated zero-time value.

In no instance was there a material balance since only 75.3 ± 3.8% of the original dose of griseofulvin could be accounted for. The remainder of the dose was sought as a possible glucuronide conjugate of 6-demethylgriseofulvin which would not be extracted prior to hydrolysis. Supernatants were incubated with β-glucuronidase to determine the possibility of glucuronide conjugation.

No real increase in free 6-demethylgriseofulvin was noted as compared to samples which had not been incubated with β-glucuronidase. These results would tend to indicate that 6-demethylgriseofulvin does not form a glucuronide in the rabbit.

It is possible that the glucuronide conjugate does not form due to properties inherent in the 6-demethylgriseofulvin molecule since the 6 pKa has been found to be 4.3. The accepted mechanism for glucuronide conjugation has been postulated as follows (16, 17):

uridine diphosphoglucuronic acid + R − OH

$$\xrightarrow{\text{Walden inversion}} \text{glucuronosyl} - \text{OR} + \text{uridine diphosphate}$$

If this were to occur with 6-demethylgriseofulvin in the body at a pH of 7.3, the metabolite would be in the form of a phenolate anion.

The Walden inversion is a S_N2 displacement reaction. The nucleophile in a S_N2 reaction attacks by using a pair of its own electrons. Therefore, the most effective nucleophile would be one whose attacking atom has the valence electrons most available for coordination. Since this is the criterion for basic strength, it would be reasonable to assume that the strongest base might make the most effective nucleophilic reagent. The phenolate anion is known to be a nucleophilic reagent with reasonable activity. However, basic strength, resonance characteristics, and steric effects may interfere with the relative nucleophilicity of an agent. Perhaps, owing to some adverse nucleophilic properties of 6-demethylgriseofulvin, it might be possible for the metabolite to form a glucuronide conjugate on a purely mechanistic basis.

Dodgson and co-workers (18) have suggested that the ability of a hydroxyl compound to form an ethereal sulfate may depend on its ionization constant. A survey of phenols which are known to conjugate with sulfate has shown that the pKa's of the hydroxyl groups varied from 7 to 10. Anderton *et al.* (19) compared the amount of ethereal sulfate produced with the pKa of the hydroxyl group for a number of compounds. They concluded that the hydroxyl group must have a pKa between 7 and 10 in order to be able to form an ethereal sulfate. Since the pKa of 6-demethylgriseofulvin is 4.3, this factor alone might disqualify possible sulfate conjugation.

It is clear from the above that an explanation for the lack of a material balance cannot be accounted for by presuming conversion of the metabolite to conjugates such as glucuronide or sulfate, and the possibility of the formation of a second metabolite is being investigated.

Further reports will follow on studies of the effect of varying concentration of *p*-ethoxyacetanilide and *p*-methoxybenzylamine to that of griseofulvin and of *in vivo* effects of these inhibitors.

SUMMARY

1. The metabolism of griseofulvin has been demonstrated *in vitro*, using rabbit liver slices respiring in a Krebs-Ringer bicarbonate buffer medium. The metabolism appears to follow pseudo first-order kinetics with a half-life of 53 min.

2. Griseofulvin has been shown to be metabolized also by the microsomal enzymes of the liver in the presence of the co-factors of the soluble reaction, and the metabolism is independent of the glycogen content of the liver.

3. The *in vitro* inhibition of griseofulvin metabolism by *p*-ethoxyacetanilide and *p*-methoxybenzylamine is reported. Evidence is presented for the microsomal enzyme fraction responsible for the *O*-demethylation of griseofulvin.

4. The metabolite, 6-demethylgriseofulvin, as the free phenol has been isolated and quantitated. A glucuronide conjugate has not been found, and reasons for this lack of conjugation are presented.

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Interaction of 8-Hydroxyquinoline Sulfate with Components Present in a Tuberculin PPD Solution I

Binding of 8-Hydroxyquinoline by Polysorbate 80

By S. LANDI and H. R. HELD

This article deals with the interaction between the preservative 8-hydroxyquinoline sulfate (8-HQS) and the surface-active agent polysorbate 80. In buffered solution (pH 7.38) 8-HQS is dissociated to 8-hydroxyquinoline (8-HQ) and H_2SO_4, and it is the base 8-HQ that forms a reversible association with polysorbate 80. The degree of binding of 8-HQ to polysorbate 80 was shown to be a function of the concentration of the nonionic surface-active agent. Polysorbate 80 at low concentration (about 0.005 per cent) has practically no effect on the concentration of 8-HQ in a buffered solution (pH 7.38) as used for preparing dilutions of tuberculin PPD for the intracutaneous method (Mantoux test). 8-HQS, in a buffered solution of pH 3, does not interact with polysorbate 80.

8-HYDROXYQUINOLINE sulfate (8-HQS)[1] is added to tuberculin PPD solutions as an antimicrobial agent. In a previous report (1) the authors described how 8-HQS disappears from these solutions when dispensed in glass vials stoppered with rubber closures and showed that most of the loss of 8-HQS from the solution was caused by sorption of 8-HQ by the rubber closures.

Numerous investigators have shown, by using solubility studies and equilibrium dialysis, that binding of preservatives (3–9) or pharmaceuticals (10–14) with polysorbate 80[2] or other macromolecules takes place.

Tuberculin PPD solutions are used intracutaneously for diagnostic purposes in tuberculosis-prevention programs. Such solutions contain 0.01% 8-HQS added as a preservative and 0.005% polysorbate 80 as a stabilizing agent (2). It was, therefore, of interest to find out if some binding between 8-HQS and polysorbate 80 takes place in the buffer used to prepare these solutions.

The solubility method (3) and the equilibrium dialysis method (3) were used by the authors to determine the compatibility of polysorbate 80 and 8-HQS.

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[1] Marketed as Chinosol.

[2] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.

MATERIALS AND METHODS

Reagents.—8-Hydroxyquinoline sulfate (8-HQS)[1] (Eastman Organic Chemicals, 1776), 8-hydroxyquinoline (8-HQ) (Fisher Scientific Co., 0-261), polysorbate 80 (polyoxyethylene 20 sorbitan monooleate).[2]

Buffer Solution (pH 7.38).—Isotonic phosphate buffered solution (2), pH 7.38 (1.45 Gm. of KH_2PO_4, 7.60 Gm. of Na_2HPO_4, and 4.8 Gm. of NaCl in 1050 ml.). This buffer is also used as diluent for the preparation of tuberculin PPD solutions (Mantoux).

Buffer Solution (pH 3.0).—McIlvaine's buffered solution (15) was prepared by mixing 79.5 ml. of 0.1 *M* citric acid solution and 20.5 ml. of 0.2 *M* Na_2HPO_4 solution.

Dialysis Membranes.—Thin nylon membrane,[3] as recommended by Patel and Kostenbauder (3), was used for dialysis of 8-HQ. A seamless regenerated cellulose tubing, size identity 36/32 (obtained from the Visking Division of Union Carbide, Canada, Ltd.) was used for the dialysis of 8-HQS and of 8-HQ.

Determination of 8-HQ and 8-HQS.—8-HQS, dissolved in buffered solution (pH 7.38), is dissociated in 8-HQ and H_2SO_4. The distinct absorption maximum of 8-HQ in the ultraviolet region at 240 mμ lends itself well to the quantitative deter-

[3] Supplied through the courtesy of Youngs Rubber Corp., New York, N. Y.