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Report

The Metabolism of CGP-291: The Use of Microorganisms as Models of Mammalian Metabolism

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CGP-291 is an investigational antiprotozoal agent with unknown metabolism. Microbial systems were utilized, as a model of mammalian metabolism, to predict the oxidative metabolic pathway of this nitroimidazole. Large-scale fermentation of CGP-291 with *Beauvaria bassiana* produced two major metabolites, IV and V. Structures of both were elucidated by comparing spectral data of metabolites to that of the starting material. The presence of two minor monohydroxylated metabolites was verified using LC-MS.

KEY WORDS: CGP-291; niridazole; metabolism; microbial metabolites.

INTRODUCTION

CGP-291 (I) is an investigational antiprotozoal agent which was synthesized by Ciba-Geigy in an effort to produce a compound combining the structural features of metronidazole (II) (Flagyl) and niridazole (III) (Ambilhar) (Fig. 1) (1,2). While these drugs are potent amoebicides, both suffer from side effects which can limit their usage. CGP-291, developed in hopes of providing an active agent with few side effects, was shown to be at least three times as active as metronidazole against *Entamoeba histolytica* (2).

Before any new drug can be approved for use, its safety and efficacy must be evaluated. Part of this evaluation includes determination of the metabolic fate of the compound. It was predicted that CGP-291, being structurally very similar to niridazole, should be metabolized in a similar manner.

The metabolism of niridazole in humans has been well documented (3,4). Upon oral administration, niridazole is slowly absorbed from the intestinal tract and is rapidly and almost completely metabolized. Anaerobic reduction serves as the principle metabolic pathway in which the nitro group is reduced to an amine, which is the active form of the schistosomacide (5). Reductive cleavage of the nitrothiazole ring produces 1-thiocarbamoyl-2-imidazolidinone (TCI), a metabolite implicated in the immunosuppression exhibited by niridazole (6). The remaining metabolism occurs via oxidation of the imidazolidinone ring, yielding several hydroxylated and dehydrated metabolites (7).

The study of the metabolic fate of CGP-291 was undertaken in our laboratory utilizing microorganisms as models of mammalian metabolism, a technique which has proven valuable in predicting the types of metabolites which might occur in mammals (8,9).

MATERIALS AND METHODS

General

Melting points were determined on a Thomas–Hoover apparatus and are uncorrected. Infrared spectra were run as KBr pellets with a Perkin–Elmer 281B spectrophotometer. UV data were obtained on a Perkin–Elmer Lambda 3B UV/Vis spectrophotometer in 95% ethanol. ^1H (300-MHz) and ^{13}C (75-MHz) nuclear magnetic resonance (NMR) spectra were obtained on a Varian VXR-300 NMR spectrometer with deuteropyridine as the solvent and tetramethylsilane as the internal standard. Two-dimensional NMR data were obtained using the standard pulse sequence of the VXR for heteronuclear correlated spectroscopy (HETCOR). Low-resolution electron-impact mass spectra were obtained on a Finnigan 3221-F200 mass spectrometer (70-eV potential) coupled to a Teknivent Vector One data system. High-resolution mass spectra were obtained in the Department of Chemistry, University of Kansas, Lawrence.

Cultures and Fermentation Screening Procedures

A stock culture of *Beauvaria bassiana* ATCC 7159 was maintained on Mycophil (BBL) agar slants and stored at 4°C. Initial screening was carried out in 125-ml Erlenmeyer flasks containing 25 ml of medium consisting of the following (per liter of distilled water): glucose, 20 g; NaCl, 5 g; K_2HPO_4, 5 g; yeast extract, 5 g; and peptone, 5 g. A two-stage fermentation procedure was carried out as previously described (10). CGP-291 was added to 24-hr-old stage II cultures (0.2 mg/ml) as a solution in dimethylformamide (100 mg CGP-291/ml of dimethylformamide).

Culture controls consisted of fermentation blanks in

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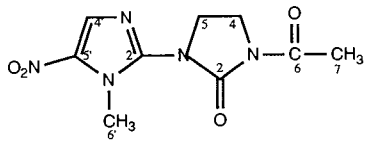
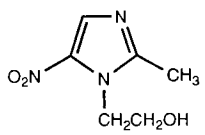
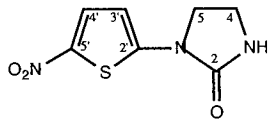


CGP-291 (I)



Metronidazole (II)



Niridazole (III)

Fig. 1. CGP-291 and structurally related drugs.

which the organism was grown under conditions used in the screening process but without substrate. Substrate controls were conducted by adding substrate (1 mg CGP-291/50 μl dimethylformamide) to sterile medium and incubating under conditions used in the screen. Autoclaved substrate controls consisted of cultures, grown under identical conditions, that were autoclaved and then administered substrate (1 mg CGP-291/50 μl dimethylformamide).

Fermentation Sampling and Thin-Layer Chromatographic Analyses

The fermentations were sampled by withdrawing 5 ml of culture, adjusting to pH 7 (if necessary) with 10% NH_4OH, and extracting with 5 ml of ethyl acetate. The ethyl acetate layer was evaporated, and the residue was redissolved in ethyl acetate and spotted on precoated silica gel G plates (0.25 mm; Sil G-25 UV_{254}; Brinkmann Instruments, Inc.). The plates were developed in 10% methanol/chloroform and visualized under UV light. The R_f values were as follows: CGP-291, 0.80; IV, 0.48; and V, 0.47.

Preparation of Metabolites by *B. bassiana*

Preparative-scale fermentation of CGP-291 by *B. bassiana* was carried out in 6 liters of medium held in 1-liter Erlenmeyer flasks (200 ml per flask). A total of 500 mg of CGP-291 (in 6 ml of dimethylformamide) was distributed evenly among the cultures. The cultures were incubated on a rotary shaker (250 rpm) at room temperature for 10 days. Cultures were pooled and extracted three times, each time with 6 liters of ethyl acetate. The ethyl acetate layer was dried (Na_2SO_4) and evaporated *in vacuo* (40°C), to leave a dark brown, oily residue (693 mg).

Purification of Metabolites

A portion of the residue (218 mg) was applied to a column (2-cm I.D.) of silica gel (230–400 mesh, 30 g) as a Celite plug. Elution of the column (under low pressure) with chloroform followed by 10% methanol/chloroform afforded two major fractions: A (82 mg) and B (68 mg). Fraction A was applied as a Celite plug to a column (1.5-cm I.D.) of alumina (for dry column chromatography, 20 g). The column was eluted (under low pressure) with chloroform to yield IV (46 mg) and with 20% methanol/chloroform to give V (18 mg).

IV was recrystallized twice from water as yellow needles (9 mg): mp 201–202°C [203–205°C lit. (1)]; HR-MS, observed M^+ 211.010, calculated for $C_7H_9N_5O_3$, M^+ 211.010; EI-MS, m/z (RA): 211 (M^+, 21%), 181 (32%), 155 (100%); UV (95% EtOH) 216 (ε 9574), 325 (ε 8170) nm; IR (KBr)ν_{max} 3200, 3120, 1745 cm^{-1}; ^1H NMR (Table I); ^{13}C NMR (Table II).

Synthesis of IV. CGP-291 (100 mg, 0.4 mmol) was refluxed in 2 *N* H_2SO_4 for 1 hr (1). The reaction was cooled in ice and 10 *N* NaOH was added until alkaline. The pH was adjusted to 4 with acetic acid and IV was crystallized from water (28 mg, 33% yield). Synthetic IV was identical in all respects to that isolated from *B. bassiana* [no depression in mixed mp, one spot when cochromatographed on TLC, superimposable IR (KBr) and ^1H NMR].

V was crystallized from acetonitrile as fine yellow needles (6 mg): mp 214–216°C; HR-MS, observed M^+ 209.053, calculated for $C_7H_7N_5O_3$, M^+ 209.055; EI-MS, m/z (RA): 209 (M^+, 58%), 179 (30%), 167 (100%); UV (95% EtOH) 220 (ε, 11,021), 324 (ε, 7426) nm; IR (KBr)ν_{max} 3240, 3120, 1720 cm^{-1}; ^1H NMR (Table I); ^{13}C NMR (Table II).

LC-MS Analyses

Cultures of *B. bassiana* were grown in 25 ml medium/125-ml flask using the procedure discussed earlier. Cultures were incubated with CGP-291 (5 mg/50 μl dimethylformamide) for 14 days at room temperature with shaking (250 rpm). LC-MS data were obtained on a Vestec Model 201 LC-MS with thermospray interface in positive ion mode [filament on (400 μA)], Whatman Partasil 5 ODS-3 column, at a flow rate of 1 ml/min. A linear solvent program was

Table I. Comparative ^1H NMR Data for CGP-291 and Metabolites[a]

No. H	δH		
	CGP-291 (I)	IV	V
3	—	8.66 (1H, ex with D_2O)	12.12 (1H, ex with D_2O)
4	3.97 (2H, m)	3.53 (2H, t, J = 7.6)	6.79 (1H, br d, J = 3)
5	4.04 (2H, m)	4.08 (2H, t, J = 7.6)	6.90 (1H, d, J = 3)
7	2.55 (3H, s)	—	—
4′	8.17 (1H, s)	8.13 (1H, s)	8.15 (1H, s)
6′	3.87 (3H, s)	3.88 (3H, s)	3.94 (3H, s)

[a] Chemical shifts are reported as parts per million (C_5D_5N); J values are as Hertz.

Table II. Comparative ^{13}C NMR Data for CGP-291 and Metabolites[a]

No. C	δC		
	CGP-291 (I)	IV	V
2	153.2 (0)	158.6 (0)	153.3 (0)
4	40.7 (2)	38.3 (2)	112.1 (1)[b]
5	42.6 (2)	45.9 (2)	112.2 (1)[b]
6	169.8 (0)	—	—
7	23.5 (3)	—	—
2′	143.8 (0)	146.3 (0)	142.5 (0)
4′	131.2 (1)	131.5 (1)	131.6 (1)
5′	138.3 (0)	138.0 (0)	138.6 (0)
6′	34.6 (3)	34.8 (3)	34.8 (3)

[a] Chemical shifts are reported as parts per million (C_5D_5N); numbers in parentheses refer to number of attached protons as determined by DEPT and APT.
[b] Assignments are interchangeable.

utilized beginning at 10% methanol/0.1 *M* ammonium acetate and progressing to 30% methanol/0.1 *M* ammonium acetate. Metabolites were eluted from the column as follows: VI, *RT* = 2.4 min, MS 228 ($M+H^+$); VII, *RT* = 3.15 min, MS 228 ($M+H^+$), 210 ($M+H^+$-18); V, *RT* = 3.65 min, MS 210 ($M+H^+$); IV, *RT* = 4.85 min, MS 212 ($M+H^+$).

RESULTS AND DISCUSSION

A total of 54 microorganisms were evaluated for their ability to metabolize CGP-291 using the usual two-stage fermentation procedure (10). Thin-layer chromatographic analyses of the culture extracts indicated that seven microorganisms were capable of metabolizing the compound. Based on TLC analyses, *B. bassiana* ATCC 7159 was chosen for preparative-scale conversion of CGP-291, as it was found to convert CGP-291 almost completely to several more polar metabolites.

Preparative-scale conversion of 500 mg of CGP-291 by *B. bassiana* yielded 693 mg of crude extract. Column chromatography on silica gel was utilized to separate the two major metabolites (fraction A) from the rest of the extract (fraction B). These two metabolites were then separated from each other by chromatography of fraction A over alumina to yield IV and V. Structure elucidation of both metabolites was accomplished by comparison of their spectral data to those of the starting material. All spectral data for CGP-291, with the exception of the ^{13}C NMR spectrum, had been previously reported in the literature (1,2). The ^1H NMR spectrum was reported, fully assigned except for H-4 and H-5, which were listed as two multiplets (4H, A_2B_2). Spectral data obtained at 300 MHz resolved these signals into two 2H multiplets which could be tentatively assigned as H-4 (δ3.97) and H-5 (δ4.04) based on chemical shift theory and comparison to ^1H NMR spectral assignments of niridazole (7). ^{13}C NMR spectral data were obtained for CGP-291 and assigned based on multiplicity determinations, chemical shift theory, and HETCOR data (Table II).

The IR spectrum of metabolite IV showed a loss of the absorption at 1680 cm^{-1} for the carbonyl of the *N*-acetyl side chain in CGP-291. The cleavage of the *N*-acetyl group was confirmed by the ^1H NMR spectrum (Table I) in which the absence of the signal at δ2.55 (3H, s) for the methyl of the *N*-acetate was noted and in its place was a new signal at δ8.66 (1H, exchangeable with D_2O). The signal, assigned to H-4 (δ3.97) in CGP-291, shifted upfield to δ3.53 with the loss of the neighboring *N*-acetate, while the H-5 signal (δ4.04) was relatively unaffected, verifying the assignments of these protons in CGP-291. The ^{13}C NMR spectrum (Table II) of IV provided further confirmation that CGP-291 had been deacetylated to yield IV. All carbon signals in the metabolite were relatively unchanged with the exception of the absence of the two signals for C-6 and C-7 of the *N*-acetate. The signals for C-4 and C-5 were assigned unambiguously, based on HETCOR data which allowed correlation of these carbons to their attached protons, H-4 and H-5. Additionally, the signal assigned to C-4 was shifted slightly upfield upon the loss of the acetate group, while C-5 was shifted downfield, consistent with the assignments. Thus, the spectral data indicated that the major metabolite of CGP-291 in *B. bassiana* is *N*-desacetyl CGP-291 (IV) (Fig. 2).

N-Desacetyl CGP-291 is a known compound synthesized previously by acid hydrolysis of CGP-291 (1). The deacetylation of CGP-291 was repeated in our laboratory using the reported procedure and the synthetic compound was found to be identical in all respects to that isolated from *B. bassiana*. Melting points of both the synthetic and microbial products agreed with the literature value (1).

Spectral evaluation of the second metabolite (V) also showed a loss of the *N*-acetyl side chain upon metabolism. The IR spectrum of V showed a carbonyl absorbance at 1720 cm^{-1}, compared to 1745 cm^{-1} for I. The ^1H NMR spectrum (Table I) showed the loss of the methyl of the *N*-acetyl and a new signal at δ12.1 (1H, exchangeable with D_2O). In addition, the two signals for H-4 and H-5 were shifted downfield (δ6.82 and 6.93) into the olefinic region in V and each now resonated as 1H doublets. The most upfield signal (δ6.82) was assigned to H-4, based on its broadness, caused by coupling to H-3. This signal was sharpened to a doublet on exchange with D_2O, while the signal assigned to H-5 (δ6.93) was unchanged. The ^{13}C NMR spectrum showed the absence of the two carbons of the *N*-acetate with all other signals relatively unchanged with the exception of C-4 and C-5, which now resonated at δ112.1 and 112.2 (Table II). Thus, based on the spectroscopic data, the metabolite was identified as dehydro *N*-desacetyl CGP-291 (V) (Fig. 2), similar to the dehydro metabolite reported for niridazole (7).

Comparison of the ^{13}C NMR spectral data of CGP-291,

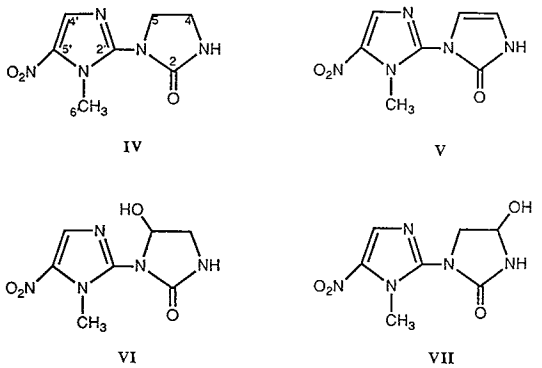


Fig. 2. Metabolites of CGP-291.

IV, and V allowed assignments of C-2′ and C-5′. Upon deacetylation and/or dehydration, one carbon signal shifts, while the other is unaffected. The unaffected signal must be C-5′, while C-2′, being in close proximity to the imidazolidinone ring, is affected directly by changes in that ring. Therefore, both proton and carbon spectral assignments for all compounds have now been made unambiguously and are summarized in Tables I and II.

It was speculated that fraction B, being more polar (by TLC) than fraction A, was composed of a mixture of hydroxylated metabolites of CGP-291, since metabolism studies of niridazole detected monohydroxylation, as well as dihydroxylation, at the 4 and 5 positions of the imidazolidinone ring as the major mammalian oxidative metabolic route (7). The separation of fraction B into pure compounds proved difficult partly because of the ease of dehydration of the hydroxylated metabolites. The formation of the hydroxylated metabolites of niridazole was previously shown to be directly influenced by pH, with the production of the major metabolite, 4-hydroxyniridazole, being optimum at pH 7.5 (7). The pH of the culture broth of *B. bassiana* during fermentation with CGP-291 was approximately 4, which presumably would not favor formation of the hydroxylated metabolites. Therefore, low and varying yield as well as separation difficulties prevented the isolation of useful quantities of hydroxylated CGP-291 metabolites. However, thermospray LC-MS was utilized to determine the nature and total number of hydroxylated metabolites present in the culture broth of *B. bassiana*. The advantage of this method of analysis was that the culture broth could be injected directly into the LC-MS, thereby eliminating the extraction step. This provided an accurate profile of metabolites which was not affected by the extraction characteristics or low quantities of metabolites. Analysis of the LC-MS data showed that CGP-291 was completely consumed during the fermentation. Four metabolites were detected, with IV being the major metabolite, followed by V. Two monohydroxylated metabolites (M^+, 228) (VI and VII) were detected, one of which dehydrated in the MS. Previous experiments with feeding niridazole metabolites to rat liver microsomes showed that only 4-hydroxyniridazole could dehydrate to form dehydroniridazole, while 5-hydroxyniridazole could not (7). Based on mass spectral data, the first compound to elute from the LC column (RT = 2.4 min.) was tentatively assigned to 5-hydroxy-*N*-desacetyl CGP-291 (VI), as it did not undergo any dehydration. The second compound (RT = 3.15 min.), which displayed a M^+-18, was speculated to be 4-hydroxy-*N*-desacetyl CGP-291 (VII), based on its ability to dehydrate. No dihydroxylated CGP-291 compounds were detected in the culture broth of *B. bassiana*, which was not surprising considering that the formation of dihydroxylated niridazole metabolites was shown to be favored at pH 8.3, far above that of the normal fermentation conditions of *B. bassiana* (7).

The detection of the two monohydroxylated compounds in the culture broth of *B. bassiana* indicated that they were produced as metabolites, although in a low yield. In addition, the presence of V in the aqueous culture broth strongly suggests that the dehydro compound is an enzymatically produced metabolite and not an artifact of the isolation procedure.

Our studies show that, after *N*-deacetylation, CGP-291 follows an oxidative metabolic pathway in microorganisms similar to that seen with niridazole in humans (7). These results further document the utility of microorganisms as predictive models for metabolic transformations in mammals.

ACKNOWLEDGMENTS

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