APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1996, p. 4206–4209 Vol. 62, No. 11
0099-2240/96/$04.00+0

Degradation of the Fluoroquinolone Enrofloxacin by Wood-Rotting Fungi

RAINER MARTENS,[1] HEINZ-GEORG WETZSTEIN,[2]* FRANTISEK ZADRAZIL,[1] MARINA CAPELARI,[3] PETER HOFFMANN,[4] AND NORBERT SCHMEER[2]

Bundesforschungsanstalt für Landwirtschaft, Institut für Bodenbiologie, D-38116 Braunschweig,[1] Bayer AG, Business Group Animal Health Research, D-51368 Leverkusen,[2] and Deutsche Sammlung von Mikroorganismen und Zellkulturen, D-38124 Braunschweig,[4] Germany, and Instituto de Botânica, 01061-970 São Paulo, Brazil[3]

Received 14 May 1996/Accepted 8 August 1996

The veterinary fluoroquinolone enrofloxacin was degraded in vitro by four species of wood-rotting fungi growing on wetted wheat straw containing carbonyl-^{14}C-labeled drug. A maximum $^{14}CO_2$ production of 17% per week was observed with the brown rot fungus *Gloeophyllum striatum*, resulting in up to 53% after 8 weeks. However, rates reached at most 0.2 and 0.9% per week, if enrofloxacin was preadsorbed to native or gamma ray-sterilized soil, respectively.

Fluoroquinolones (FQs) are synthetic antimicrobial agents which have found wide application in human and veterinary medicine (5, 15). They act against a broad spectrum of gram-negative and -positive pathogenic bacteria by inhibiting the enzymes DNA topoisomerase II (gyrase) and DNA topoisomerase IV (9, 15). Enrofloxacin, the first FQ developed for veterinary medicine, is used to treat various infections in pets and livestock (5, 12). During its passage through the animal, enrofloxacin is degraded only to a small extent, i.e., most of the drug is excreted in urine and feces (17). Additionally, manure from livestock is often disposed of by spreading onto agricultural soils and pastures. However, FQs are tightly bound to feces and soils and are hardly bioavailable which also may contribute to their apparent recalcitrance (11).

The only published evidence on biodegradation of a FQ concerns sarafloxacin which is used for the treatment of poultry. Velagaleti et al. (11) reported the production of ≤0.66% of $^{14}CO_2$ from [^{14}C]sarafloxacin in three soils, but neither the position of the label nor the radiochemical purity of the drug nor the duration of the experiment was indicated. Sarafloxacin persisted in a marine sediment, and its fate was attributed to leaching and redistribution rather than degradation (6).

Wood-rotting basidiomycetes are characterized by their ability to generate highly reactive radicals (4, 7, 16) for the degradation of either lignin (white rot fungi) or cellulose and hemicellulose constituents of wood (brown rot fungi). The nonspecificity of these radical-mediated reactions also enables white rot fungi to degrade recalcitrant xenobiotics like polyaromatic hydrocarbons, polychlorinated biphenyls, chlorinated pesticides (dichloro-diphenyl-trichloroethane [DDT] and lindane), and explosives (trinitrotoluene [TNT]) (2, 3). Radicals are diffusible, and diffusion offers the means to attack molecules apparently unavailable to microbes because of a low water solubility and/or strong adsorption to the surrounding matrix.

The purpose of this study was to assess the degradation potential of selected wood-rotting fungi for [^{14}C]enrofloxacin bound to either wheat straw or agricultural soil.

Organisms and culture conditions. Species of wood-rotting fungi (Table 1), except *Gloeophyllum striatum* DSM 10335, were selected on the basis of screening experiments in which these strains had shown a high potential to degrade lignocellulose and to mineralize [^{14}C]pyrene (8). *Phellinus gilvus* did not degrade [^{14}C]pyrene (8) and was included here as a negative control. All strains were grown on 2% malt extract agar at 20 to 22°C for 7 to 12 days before use. For the inoculation of wheat straw, pieces of agar (6 mm in diameter) were cut out from the margin of actively growing mycelia.

Enrofloxacin. Enrofloxacin, 1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid · HCl, ^{14}C labeled at the carbonyl position (Fig. 1), was provided by R. Thomas, Bayer AG, Wuppertal, Germany. It had a radiochemical purity of >98% and a specific activity of 4.98 MBq mg^{-1}. All experiments were conducted in the dark to prevent photodegradation of the molecule.

Degradation of [^{14}C]enrofloxacin adsorbed to straw. Five grams of wheat straw meal (particle size, <1 mm) in 250-ml conical flasks was wetted with 5 ml of distilled water and autoclaved at 121°C for 30 min. Then, 10 ml of a sterile aqueous solution of [^{14}C]enrofloxacin hydrochloride was added to the straw substrate, resulting in 50 μg of enrofloxacin per flask and a radioactivity of 37 kBq per flask. Each flask was inoculated with two agar plugs containing mycelium and sealed with a silicone stopper holding two glass tubes to allow continuous aeration at 4 to 6 ml min^{-1} with sterile, CO_2-free, humidified air. Produced CO_2 was absorbed in 25 ml of 2 M NaOH, and $^{14}CO_2$ was quantified weekly by liquid scintillation counting (LSC 1801 [Beckman, Fullerton, Calif.]; Rotiszint eco plus [Roth GmbH, Karlsruhe, Germany] was employed as liquid scintillation fluid). To test whether the collected radioactivity accounted specifically for $^{14}CO_2$, and not for other volatile metabolites, 1 ml of the trapping solution in an LSC vial was acidified with 600 μl of 20% H_2SO_4. The vial was left open for 24 h. Thereafter, it was checked for remaining radioactivity. At the end of the experiment, after 8 weeks, the loss of straw organic matter was estimated by dry weight determination. All assays were run in quadruplicate at 25°C.

The amounts of $^{14}CO_2$ liberated from [*carbonyl*-^{14}C]enrofloxacin by seven strains, representing five different species of wood-rotting fungi, are presented in Fig. 2. Three strains of *G. striatum* showed the highest rates of degradation, reaching up to 17.3% ± 2.1% (mean ± standard deviation) of $^{14}CO_2$

* Corresponding author. Phone: 49 2173 38 4882. Fax: 49 2173 38 3766.

TABLE 1. Wood-rotting fungi used in this study

Species	Strain[a]	Original designation[b]	Type of wood rot
Gloeophyllum striatum	DSM 10335	NCWRF 204A	Brown
	DSM 9594	CCB 188	Brown
	DSM 9592	CCB 177	Brown
Stropharia rugosoannulata	DSM 9616	FAL Z 10	White
Phanerochaete chrysosporium	DSM 9620	CCB 478	White
Irpex lacteus	DSM 9595	CCB 196	White
Phellinus gilvus	DSM 9602	CCB 254	White

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.
[b] NCWRF, National Collection of Wood Rotting Fungi, Garston, United Kingdom; CCB, Culture Collection of Basidiomycetes, São Paulo, Brazil; FAL, Bundesforschungsanstalt für Landwirtschaft, Braunschweig, Germany.

per week and liberating a total of 53.3% ± 1.2% (DSM 9594), 49.1% ± 0.6% (DSM 10335), and 42.8% ± 1.8% (DSM 9592) of the initially applied radioactivity within 8 weeks. *Phanerochaete chrysosporium* degraded 25.6% ± 0.6% of the [^{14}C]enrofloxacin, attaining a maximum rate of 5.1% ± 0.2% per week, while *Irpex lacteus* and *Stropharia rugosoannulata* showed lower rates and a total degradation of 13.7% ± 0.8% and 5.1% ± 0.6%, respectively. As expected, *Phellinus gilvus* formed only 0.19% ± 0.02% of $^{14}CO_2$. In no instance was residual radioactivity found in the acidified trapping solution, indicating that measured radioactivity was specifically due to $^{14}CO_2$. Losses of organic matter from the straw substrate were between 74 and 82%.

Influence of native soil on the degradation process. To assess the effect of native soil microflora on the degradation of [^{14}C]enrofloxacin bound to straw, two strains of *G. striatum* (DSM 9594 and DSM 10335), which had shown highest activity before, were grown on straw as described above. After 7 days of preculturing, the straw was covered with 30 g (dry weight) of soil. This soil had been sampled from the upper 20 cm of an agricultural field at the Bundesforschungsanstalt für Landwirtschaft, Braunschweig, Germany. It was composed of 47.1% sand, 46.2% silt, and 6.7% clay and contained 1.26% organic carbon and 0.12% nitrogen; the pH was 5.4. After the soil was sieved (particle size now <2 mm), the water content was adjusted to 13.2%, corresponding to 50% of its maximum water-holding capacity. The production of $^{14}CO_2$ was recorded as described above. Both strains were studied in quadruplicate at 25°C for 8 weeks.

Maximum degradation rates of 11.0% ± 1.8% and 16.8% ± 0.3% were obtained by *G. striatum* DSM 9594 and *G. striatum* DSM 10335 during the second and third week, respectively. These rates were abruptly decreased to 1 to 2% in the following week and thereafter. Simultaneously, the fungal mycelium visible on the soil surface disappeared. Apparently, microbes originating from the soil had gained the upper hand over *G. striatum*. After 8 weeks, 27.9% ± 3.2% and 32.6% ± 2.4% of the initially applied radioactivity were liberated as $^{14}CO_2$ under these conditions.

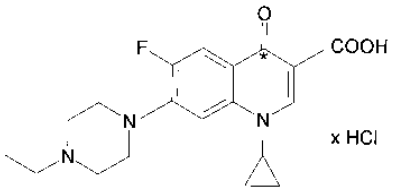

FIG. 1. Molecular structure of enrofloxacin. The position of ^{14}C label (*) is indicated.

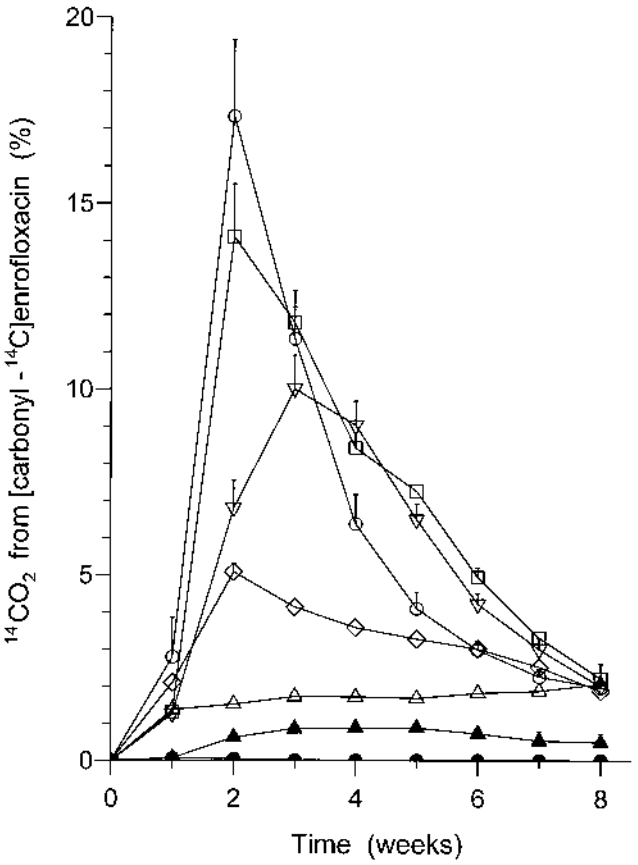

FIG. 2. Weekly rates of degradation of [*carbonyl*-^{14}C]enrofloxacin to $^{14}CO_2$ by seven strains of wood-rotting fungi. The seven strains were *G. striatum* DSM 10335 (○), *G. striatum* DSM 9594 (□), *G. striatum* DSM 9592 (▽), *Phanerochaete chrysosporium* DSM 9620 (◇), *I. lacteus* DSM 9595 (△), *S. rugosoannulata* DSM 9616 (▲), and *Phellinus gilvus* DSM 9602 (●). Values shown are the means ± standard deviations of quadruplicate assays.

Degradation of [^{14}C]enrofloxacin preadsorbed to soil. The two strains of *G. striatum* mentioned above, *G. striatum* DSM 9594 and DSM 10335, were also used to investigate the degradability of [^{14}C]enrofloxacin preadsorbed to soil. Samples of 30 g (dry weight) of native soil were labeled by the addition of 1.5 ml of an aqueous solution of the drug in drops, resulting in 300 μg of enrofloxacin (37 kBq) in each sample. The water content of the soil was thereby adjusted to 13.2%. After homogenization, the soil was placed on top of 7-day-old fungal cultures actively growing on sterile wheat straw as described above. Similar cultures received 30 g of gamma ray-sterilized soil (treated with 30 to 50 kGy) labeled with [^{14}C]enrofloxacin as described above for native soil. Two sets of controls were included. One consisted of native soil with bound [^{14}C]enrofloxacin, which was placed on sterile wheat straw. The other was made of native soil containing [^{14}C]enrofloxacin and no other additions. The flasks were kept under aeration at 25°C in the dark for 8 weeks, except for those which had received gamma-sterilized soil: those were observed over a period of 15 weeks. All assays were run in quadruplicate.

Degradation rates were low if [^{14}C]enrofloxacin was preadsorbed to soil. Representative results are shown in Fig. 3 for *G. striatum* DSM 9594. Similar amounts of $^{14}CO_2$, 0.09% ± 0.02%, were found after 8 weeks in both controls, native soil and soil in contact with sterile straw as additional substrate. Slightly more $^{14}CO_2$, 0.39% ± 0.03% (0.58% ± 0.13% for

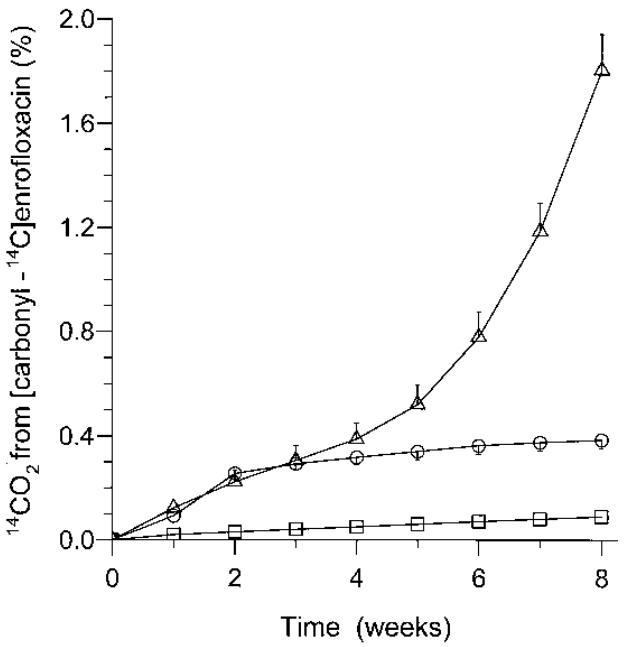

FIG. 3. Total $^{14}CO_2$ production from [*carbonyl*-^{14}C]enrofloxacin bound to native and gamma-sterilized soil by *G. striatum* DSM 9594. Native (○) or gamma-sterilized soil (△) containing 10 ppm of enrofloxacin was placed on fungal cultures which had been grown on straw for 7 days. Native soil with bound enrofloxacin (in the presence or absence of straw) served as control (□). Values shown are the means ± standard deviations of quadruplicate assays.

DSM 10335), was liberated if native soil was placed on top of fungal cultures growing on straw. *G. striatum* visibly colonized the soil during the second week, the period during which the highest increment of $^{14}CO_2$ formation, 0.16% ± 0.02% (0.22% ± 0.06% for DSM 10335), could be measured. Thereafter, the aerial mycelium disappeared from the soil surface, and $^{14}CO_2$ formation was reduced to ≤0.05% per week (Fig. 3). Obviously, other microbes had gained the upper hand over *G. striatum* again.

In contrast, both strains of *G. striatum* permanently colonized gamma-sterilized soil. Following a lag phase of about 3 weeks, an increasing rate of $^{14}CO_2$ production was found. After 8 weeks, the total activity liberated amounted to 1.8% for both strains (Fig. 3). During the ninth week, the formation of $^{14}CO_2$ reached its maximum rate of 0.87% ± 0.04% with *G. striatum* DSM 9594, remained constant for another 3 weeks and then declined slowly. After 15 weeks, a total of 6.32% ± 0.90% of the applied radioactivity was liberated as $^{14}CO_2$. With *G. striatum* DSM 10335, a maximum rate of 0.66% ± 0.02% was reached after 13 weeks, which remained constant until the end of the experiment after 15 weeks, when a total of 5.87% ± 0.30% $^{14}CO_2$ was attained.

The results of this investigation demonstrate that wood-rotting fungi in pure culture are able to mineralize the carbonyl group of enrofloxacin. It is remarkable that all three strains of the brown rot fungus *G. striatum* showed degradation rates which were much higher than those of the white rot fungi tested. *P. chrysosporium* especially has often been described as a potent degrader of lignin, model compounds of lignin, and a wide range of xenobiotics (2–4, 10). Several agents are known to be involved in these degradation processes, including hydrogen peroxide, lignin peroxidases, manganese peroxidases, and the hydroxyl radical (4, 16). Recently, the generation of hydroxyl radicals has been confirmed by a chemiluminescence method during growth of the brown rot fungus *Coniophora puteana* on various substrates, including wood (1). Although brown rot fungi produce the hydroxyl radical, they are apparently unable to degrade lignin (4, 7). It may be of ecological significance that members of both groups of fungi degraded enrofloxacin. Elimination of the carbonyl group, i.e., cleavage of the heterocyclic ring, gives rise to aniline derivatives which should be considerably more susceptible to degradation.

[^{14}C]enrofloxacin preadsorbed to native soil could be mineralized at a small but constant rate. This can be explained by the reported low bioavailability of quinolones (11), a lack of indigenous microorganisms with sufficient degradation potential in this specific soil, population dynamics effects as described above, or other factors. The small initial burst of $^{14}CO_2$ production during the colonization of native soil by *G. striatum* and the time course of this activity on gamma-irradiated soil indicate that a low bioavailability might slow down degradation but is not prohibitive. A slow onset of degradation activity might reflect the time needed by wood-rotting fungi to establish the specific conditions required for such radical-mediated reactions outside of the hyphae. Even a heavy colonization of gamma-sterilized soil did not result in mineralization rates comparable with those on sterile straw. As straw was degraded by up to 80%, enrofloxacin probably became directly available to the fungi.

On the basis of our results, we conclude that the degradation of enrofloxacin in soil is hampered considerably by adsorption to soil constituents, probably soil organic matter. However, soil fungi still have access to bound enrofloxacin. In practice, most of the enrofloxacin applied during therapy will be released into the environment undegraded but bound to feces. The fate of enrofloxacin in animal dung may well be expected to be catalyzed by lignin-degrading basidiomycetes, which are part of the coprophilous microflora. Wicklow and coworkers isolated various fungal colonists from aging cattle dung and proved that an unidentified basidiomycete as well as a strain of *Cyathus stercoreus* possessed a high potential to degrade lignocellulose (13, 14). In the light of our results, the fundamental findings of Wicklow and colleagues should stimulate further research on the degradation of drug residues in such fecal matrices.

We thank A. de Jong, Bayer AG, for helpful comments on the manuscript.

REFERENCES

1. **Backa, S., J. Gierer, T. Reitberger, and T. Nilsson.** 1992. Hydroxyl radical activity in brown-rot fungi by a new chemiluminescence method. Holzforschung **46:**61–67.
2. **Barr, D. P., and S. D. Aust.** 1994. Pollutant degradation by white-rot fungi, p. 49–72. *In* G. W. Ware (ed.), Reviews of environmental contamination and toxicology. Springer, New York.
3. **Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust.** 1985. Oxidation of persistent environmental pollutants by a white-rot fungus. Science **228:**1434–1436.
4. **Evans, C. S., M. V. Dutton, F. Guillen, and R. G. Veness.** 1994. Enzymes and small molecular mass agents involved with lignocellulose degradation. FEMS Microbiol. Rev. **13:**235–240.
5. **Greene, C. E., and S. C. Budsberg.** 1993. Veterinary use of quinolones, p. 473–488. *In* D. C. Hooper and J. S. Wolfson (ed.), Quinolone antimicrobial agents, 2nd ed. American Society for Microbiology, Washington, D.C.
6. **Hektoen, H., J. A. Berge, V. Hormazabal, and M. Yndestad.** 1995. Persistence of antibacterial agents in marine sediments. Aquaculture **133:**175–184.
7. **Koenigs, J. W.** 1974. Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown-rot basidiomycetes. Wood Fiber **6:**66–80.
8. **Martens, R., and F. Zadrazil.** Unpublished data.
9. **Ng, E. Y., M. Trucksis, and D. C. Hooper.** 1996. Quinolone resistance mutations in topoisomerase IV: relationship to the *flqA* locus and genetic evidence that topoisomerase IV is the primary target and DNA gyrase is the secondary target of fluoroquinolones in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**1881–1888.

10. **Orth, A. B., and M. Tien.** 1995. Biotechnology of lignin degradation, p. 287–302. *In* K. Esser and P. A. Lemke (ed.), The mycota, vol. II. Springer, Berlin.
11. **Velagaleti, R. R., M. L. Davis, G. K. O'Brien, and J. M. Stamm.** 1993. The bioavailability of ^{14}C-sarafloxacin hydrochloride in three soils and a marine sediment as determined by biodegradation and sorption/desorption parameters, abstr. 92. *In* Abstracts of the 205th National Meeting of the American Chemical Society, Division of Agrochemicals, 1993. American Chemical Society, Washington, D.C.
12. **Wetzstein, H.-G., and A. de Jong.** 1996. *In vitro* bactericidal activity and postantibiotic effect of fluoroquinolones used in veterinary medicine. Compend. Contin. Educ. Pract. Vet. **18**(Suppl.)**:**S22–S29.
13. **Wicklow, D. T., R. W. Detroy, and S. Adams.** 1980. Differential modification of the lignin and cellulose components in wheat straw by fungal colonists of ruminant dung: ecological implications. Mycologia **72:**1065–1076.
14. **Wicklow, D. T., R. W. Detroy, and B. A. Jessee.** 1980. Decomposition of lignocellulose by *Cyathus stercoreus* (Schw.) de Toni NRRL 6473, a "white rot" fungus from cattle dung. Appl. Environ. Microbiol. **40:**169–170.
15. **Wolfson, J. S., and D. C. Hooper.** 1989. Fluoroquinolone antimicrobial agents. Clin. Microbiol. Rev. **2:**378–424.
16. **Wood, P. M.** 1994. Pathways for production of Fenton's reagent by wood-rotting fungi. FEMS Microbiol. Rev. **13:**313–320.
17. **World Health Organization.** 1995. Forty-third report of the Joint FAO/WHO Expert Committee on Food Additives. Evaluation of certain veterinary drug residues in food. WHO Tech. Rep. Ser. **855:**17–24.