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Pharmacokinetic studies of the herbicide and antitumor compound oryzalin in mice

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Abstract

Oryzalin [3,5-dinitro-N,N-di(*n*-propyl)benzensulfanilamide] is a widely used sulfonamide herbicide that selectively inhibits microtubule formation in algae and higher plants. Oryzalin has also been found to be an inhibitor of intracellular free Ca^{2+} signaling in mammalian cells and to have antitumor activity in animals. Despite its widespread use there have been no reports of the pharmacokinetics of oryzalin in animals or man. A reversed-phase high-performance liquid chromatographic (HPLC) method was developed to measure oryzalin in biological fluids. Following repeated daily administration of oryzalin to mice by the i.p. route at 200 mg/kg, or the p.o. route at 300 mg/kg, peak plasma concentrations of up to 25 μg/ml were achieved. The half life for oryzalin in plasma of mice given i.p. oryzalin was 14.3 h with a clearance of 0.07 l/h. A major metabolite of oryzalin, N-depropyloryzalin, was identified in plasma and its structure confirmed by mass spectral analysis ($M+H^+=305$). This metabolite was cleared more rapidly than oryzalin with a half life of 1.15 h and a clearance of 0.17 l/h. N-Depropyloryzalin caused similar inhibition of colony formation by HT-29 colon cancer cells as oryzalin with $IC_{50}=8$ μg/ml. The results suggest that oryzalin and its N-depropyl metabolite can inhibit tumor colony formation at pharmacologically achievable levels.

Keywords: Oryzalin

1. Introduction

Oryzalin [3,5-dinitro-N,N-di(*n*-propyl)benzensulfanilamide] is used under the trade names Surflan and Dirimal as a selective, pre-emergence herbicide for the control of annual grasses and certain broadleaf weeds in private gardening and commercial agriculture. The primary mode of oryzalin action in plants is inhibition of microtubule formation [1]. Oryzalin binds selectively free plant tubulin to form a complex that is incapable of polymerizing into microtubules. However, the drug does not inhibit microtubule function in animal cells [2,3]. Oryzalin is a relatively nontoxic compound to mammals. The LD_{50} of technical grade oryzalin given intravenously to adult male and female rats or gerbils is greater than 10 g/kg. Exposure of rats for 1 h to a mist of formulated oryzalin containing 3.56 mg/l, showed no adverse effects [4].

We identified oryzalin in an extract of the plant *Geranium carolinium* which had been treated with the herbicide, as an inhibitor of growth factor-mediated intracellular Ca^{2+} signaling in mammalian cells [5]. Further work showed that oryzalin and its analogues at low micromolar concentrations inhibited the growth of cancer cell lines in culture and

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that oryzalin had in vivo antitumor activity against B16 melanoma in C57/BL mice [5]. The in vitro potency of oryzalin as an inhibitor of cell proliferation and its in vivo antitumor activity is intriguing in light of its widespread agricultural use and lack of toxicity to animals or humans.

Despite its widespread use, there are no reports on the pharmacokinetics or metabolism of oryzalin in animals or man. We have developed a high-performance liquid chromatographic (HPLC) assay for measuring oryzalin and its metabolites in biological fluids. The goal of the current study was to characterize the plasma disposition and metabolism of oryzalin after administration to mice in relation to its cell growth inhibitory and antitumor activity in vitro. We have found that plasma concentrations close to those required to inhibit HT-29 colon cancer colony formation in soft agar assays can be achieved in mice. A major metabolite of oryzalin, N-depropyloryzalin, was identified in plasma and urine and was shown to have cell growth inhibitory activity similar to oryzalin.

2. Experimental

2.1. Chemicals

Oryzalin [3,5-dinitro-N,N-di(*n*-propyl)sulfanilamide] (Fig. 1) was purified by repeated acetone extraction from the commercially available herbicide Surflan and confirmed to be 98% pure by HPLC. Its structure was confirmed by mass spectral analysis. N-Depropyloryzalin [3,5-dinitro-N-(*n*-propyl)benzensulfanilamide] was provided by Dr. David Hanselman from DowElanco (Indianapolis, IN, USA). Acetonitrile was HPLC grade and was purchased from Burdick and Jackson (Muskegon, MI, USA). All the other chemicals used were analytical grade and were obtained from Sigma (St. Louis, MO, USA).

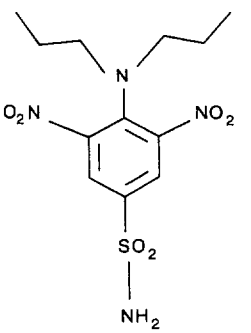


Fig. 1. Structure of oryzalin [3,5-dinitro-N,N-di(*n*-propyl)-sulfanilamide].

2.2. Animal studies

Oryzalin was formulated for oral (p.o.) administration to mice as a 120 mg/ml suspension in 5% dimethylsulfoxide, 4% polysorbate 20 (Tween 20) in 0.9% NaCl; or for intraperitoneal (i.p.) and subcutaneous (s.c.) administration as a 30 mg/ml solution in a depot formulation containing 32% soybean oil, 32% ethanol, 23% 0.9% NaCl, 8% Tween 80 and 5% cetyl alcohol. Female C57BL/6 mice weighing 20 to 25 g were administered oryzalin, 300 to 600 mg/kg p.o. twice daily, or as a single 200 mg/kg i.p. dose for pharmacokinetics studies. Groups of three mice at each time point were anesthetized with diethyl ether and exsanguinated by retro-orbital bleeding and blood collected into heparinized tubes. Plasma was immediately separated and stored frozen at −80°C until assay. Urine was collected by housing the mice in all glass metabolism cages for 24 h.

2.3. HPLC

HPLC was performed using a PE Biocompatible Binary Pump, a Hitachi autosampler (Model AS-2000) and a Hewlett-Packard 1050 UV–Vis detector at a fixed wavelength of 254 nm. Chromatographic peaks were integrated using a PE NelsonTurboChrom 4 program. The column used was Adsorbosphere HS C18 (Alltech Associates, Deerfield, IL, USA), 150 mm×4.6 mm, 5 μm particle size. The mobile phase was water–acetonitrile (47:53) at a flow-rate of 2 ml/min.

2.3.1. Standard solutions

Stock solutions of oryzalin and N-depropyloryzalin (1 mg/ml) were prepared in acetone and could be stored at −20°C for a period of three weeks without any perceptible degradation. Calibration curves were prepared over the concentration range 0.1–20 μg/ml by adding appropriate amounts

of oryzalin or N-depropyloryzalin to control plasma. Blank extracts of plasma were also prepared and analyzed at the same time.

2.3.2. Sample preparation

The plasma samples were initially extracted on C_{18} solid-phase extraction columns (BondElut; Varian Sample Preparation Products, Harbor City, CA, USA). Each column was conditioned with 3 ml acetonitrile followed by 3 ml water. The plasma sample (0.5 ml) was applied and the column was washed with 3 ml water and vacuum dried. The drugs were eluted with three 200 μl rinses of 100% acetonitrile. Aliquots of 10–50 μl were injected onto the chromatograph.

2.4. Microsomal incubation

S9 liver homogenates from rats induced with Aroclor 1254 were purchased from Organon Teknika (Durham, NC, USA). The incubation mixture consisted of S9 rat liver homogenate, 1.6 mg/ml, 33 m*M* KCl, 8 m*M* $MgCl_2$, 0.1 *M* phosphate buffer, pH 7.4, 5 m*M* glucose-6-phosphate, 4 m*M* $NADP^+$ and oryzalin at a final concentration of 100 μg/ml (0.29 m*M*). Incubations were carried out at 37°C for 10 min to 2 h [6]. The mixture was centrifuged at 12 000 *g*, and 0.9 ml of the supernatant was loaded on C_{18} BondElut columns for HPLC analyses, as described in Section 2.3.

2.5. Mass spectral analysis

Atmospheric pressure chemical ionization (APCI) mass spectrometry analyses were performed on a TSQ tandem mass spectrometer (Finnigan Instruments, San Jose, CA, USA), equipped with an APCI source coupled to a Model 1050 HPLC (Hewlett-Packard, San Jose, CA, USA). The effluent from the variable wavelength detector of the HPLC was coupled directly to the APCI source so that both UV and MS data could be obtained from a single injection. The APCI source was operated with a vaporizer temperature of 450°C and a corona discharge current of 5 μA. The mass spectrum was scanned from 50 to 1500 u/s for conventional mass spectral analysis. Product ion spectra of the metabolite were obtained by subjecting the precursor ion of interest to collision-induced dissociation (CID) with neutral Argon at −31 eV energy. These product scans spanned the mass range of 50 to 325 u in 3 s.

2.6. Cytotoxicity

Inhibition of colony formation by HT-29 human colon cancer cells in soft agarose in the continuous presence of oryzalin or N-depropyloryzalin for seven days was measured as previously described [7].

2.7. Pharmacokinetics

Pharmacokinetic analyses of oryzalin and the polar metabolite in mouse plasma used a compartmental method (WIN-NONLIN, Scientific Consulting, Apex, NC, USA). There was uniform weighting of all data points for the pharmacokinetic analyses. Pharmacokinetic parameters measured were the elimination half life, area under the curve (AUC) from zero to infinity, volume of distribution and clearance, calculated as dose/AUC.

3. Results

3.1. Chromatography

A sensitive HPLC assay was developed for the measurement of oryzalin in biological fluids based on a previously reported method for detecting impurities in oryzalin herbicide preparations [8]. Final conditions were selected to give optimum peak shape and the separation of oryzalin from endogenous substances in plasma or urine. The extraction efficiency of oryzalin from plasma was 78%. The best separation of oryzalin was achieved on a C_{18} reversed-phase column eluted with a mobile phase of acetonitrile–water (53:47, v/v). The overall chromatographic analysis time was 10 min with oryzalin eluting as a symmetrical peak at a retention time of 4.7 min. The limit of detection of the assay (3× baseline variation) was 0.1 μg/ml for oryzalin and 0.01 μg/ml for N-depropyloryzalin. The detector response was linear up to concentrations of 20 μg/ml. The within-day coefficient of variation of the assay for oryzalin was ±5%. Fig. 2A shows a typical oryzalin chromatogram of a plasma extract from a

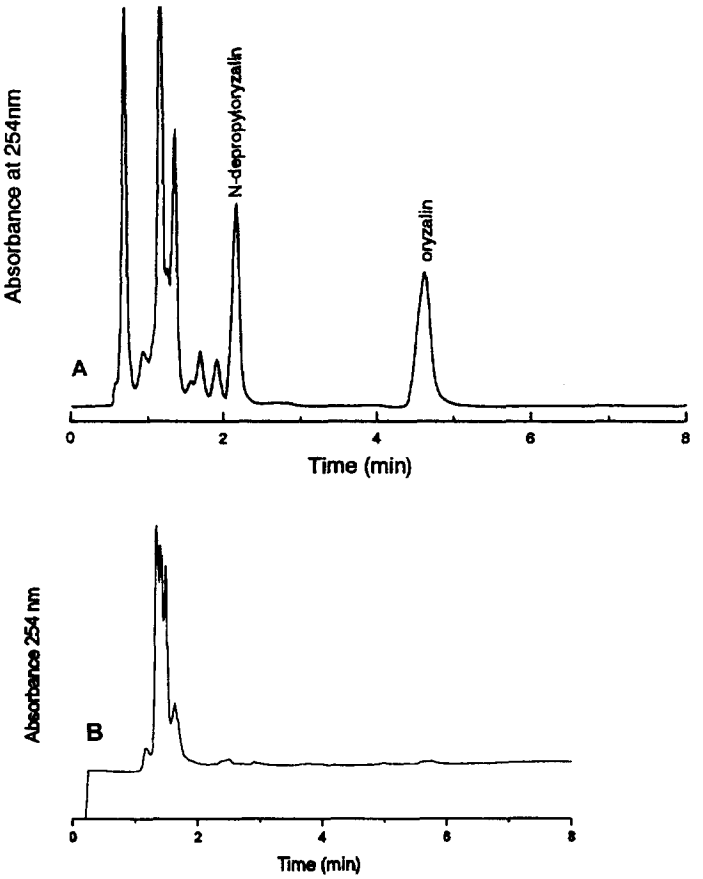


Fig. 2. (A) Chromatogram of plasma extract from a mouse administered oryzalin i.p. at a dose of 200 mg/kg 45 min previously. (B) Chromatogram of blank mouse plasma extract.

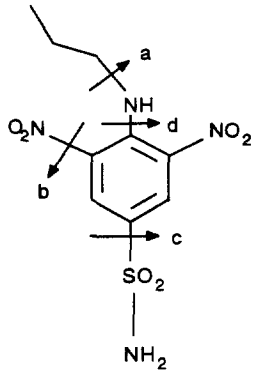
mouse administered oryzalin. A putative metabolite peak was also observed in the plasma of mice administered oryzalin at a retention time of 2.2 min. An extract from control plasma showed no interfering peaks at the retention time of oryzalin (Fig. 2B).

3.2. Metabolite identification

A fraction containing the putative metabolite was analyzed by flow injection APCI mass spectrometry. The resulting mass spectrum contained a strong signal at m/z 305 which corresponded to, and was tentatively assigned as, the [M+H] ion for N-depropyloryzalin. Further structural evidence for the tentative assignment was obtained by CID analysis of the m/z 305 ion. The resulting product-ion mass spectrum contained signals that corresponded to single and multiple losses of exocyclic functional groups as detailed in Fig. 3.

3.3. Microsomal incubation

The HPLC profile of supernatants from an incubation mixture of oryzalin with rat liver microsomes in the presence of NADPH-generating system showed oryzalin and a single peak at a retention time of 2.2 min. This peak was undetectable when the NADPH-generating system was omitted from the reaction mixture. The peak eluting at 4.7 min corresponded to parent compound oryzalin and the retention time of the metabolite corresponds exactly to that of N-depropyloryzalin. There was a time dependent de-



m/z of ion signals	Assigned structure
305	[M+H]
287	loss of water
263	loss of a
246	loss of d
217	loss of b and a
198	loss of b and d
182	loss of a and c
133	loss of a, b and c

Fig. 3. Structure of N-depropyloryzalin [3,5-dinitro-N-(*n*-propyl)sulfanilamide] and *m/z* of ion signals observed in the CID.

crease in oryzalin as the concentration of N-depropyloryzalin increased up to 1 h (Fig. 4). After this time there was a decrease in the concentration of N-depropyloryzalin which may indicate further metabolism.

3.4. Pharmacokinetics

Due to its hydrophobicity oryzalin could not be formulated in a vehicle suitable for i.v. administration. In order to study the pharmacokinetics of a single bolus administration, mice were administered oryzalin 200 mg/kg i.p.. Postmortem analysis after blood recovery showed no detectable oryzalin left in the abdomen after 12 h. A peak plasma concentration of oryzalin of 25 μg/ml was achieved within 15 min of administration. Plasma levels then declined in a biphasic manner with half-lives of 2.1 and 14.3 h. The apparent volume of distribution (V_d) was 150 ml, the area under the plasma concentration×time curve (AUC) was 74.1 μg/ml/h, and the clearance 70 ml/h (Fig. 5). A metabolite peak was also observed within 15 min of administration (Fig. 2). The metabolite was identified by mass-spectral analysis as N-depropyloryzalin. The peak concentration of the

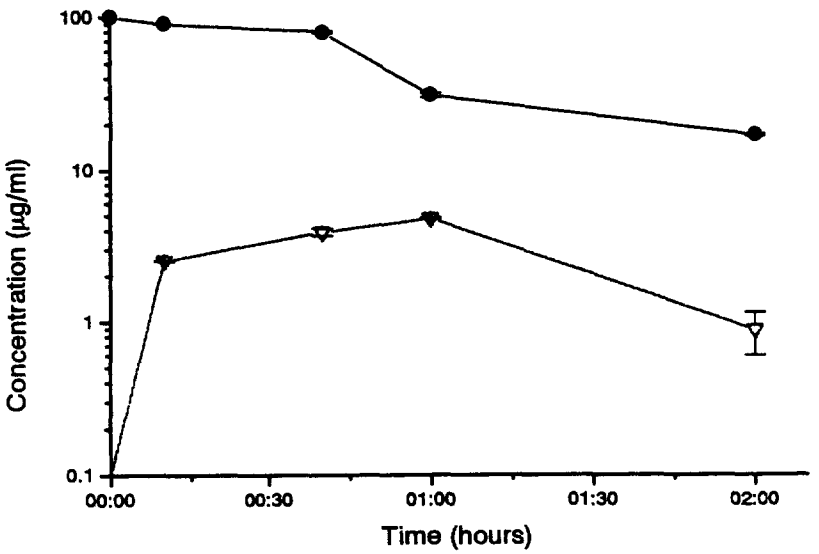


Fig. 4. Concentrations of (●) oryzalin and (▽) N-depropyloryzalin during incubation with rat microsomal preparation in the presence of NADPH. Values are the mean of three determinations and bars are S.D..

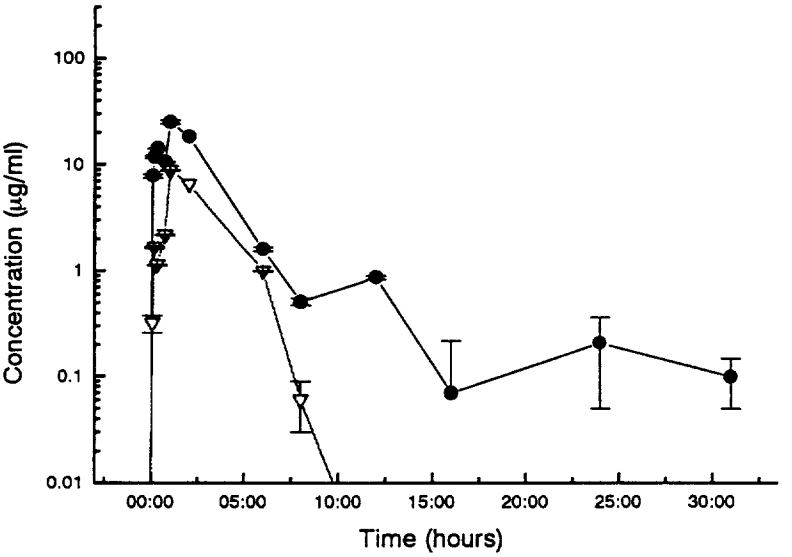


Fig. 5. Plasma concentration of (●) oryzalin and (▽) N-depropyloryzalin following i.p. administration of oryzalin to mice at 200 mg/kg. There were three mice at each time point. Bars are S.D..

metabolite was 10 μg/ml and its levels declined more rapidly than oryzalin, with a half-life of 1.1 h and a clearance of 170 ml/h, a V_d of 290 ml and an AUC of 28.4 μg/ml/h. The peak concentration was approximately 10 μg/ml. The amount of oryzalin in urine from mice treated similarly was below the limit of detectability of the assay but N-depropyloryzalin was detected at concentration of approximately 0.02 μg/ml.

Plasma concentrations of oryzalin following repeated oral administration as previously used for antitumor studies [5] were also measured. Mean peak and trough oryzalin plasma concentrations following 5 days of twice daily p.o. administration of oryzalin at 300 mg/kg were 4.0 and 0.8 μg/ml (data not shown). There was a dose dependent increase in peak oryzalin plasma concentrations with increasing oral doses up to 600 mg/kg with no further increase at 1200 mg/kg.

3.5. Cell growth inhibition

Oryzalin and N-depropyloryzalin were compared for their ability to inhibit the colony formation by HT-29 human colon cancer cells in soft agar. The IC_{50} values were 8 μg/ml for oryzalin and 4 μg/ml for N-depropyloryzalin (Fig. 6). Inhibition of colony formation was complete with both agents at a concentration of 20 μg/ml and negligible at 1 μg/ml.

4. Discussion

Oryzalin and related analogues are of interest as antitumor compounds because of their low toxicity and unique mechanism of action as inhibitors of Ca^{2+} signaling [5]. We have found that oryzalin administered to mice by the p.o. or s.c. routes a day after tumor inoculation gives up to 65% inhibition of B16 melanoma tumor growth, although other established tumors are less responsive [5]. It is surprising to find that, despite having been in general agricultural use for over 25 years [4] there are no published reports on the absorption, metabolism or elimination of oryzalin in either animals or humans. We therefore addressed the question whether the plasma concentrations of oryzalin achievable in mice following i.p. or s.c. administration are sufficient to kill tumor cells, based on the in vitro cytotoxicity data.

The maximum sustained peak plasma concentration of oryzalin achieved after p.o. administration of daily doses of 600 mg/kg or 1200 mg/kg was about 13 μg/ml (37 μ*M*). These concentrations of oryzalin

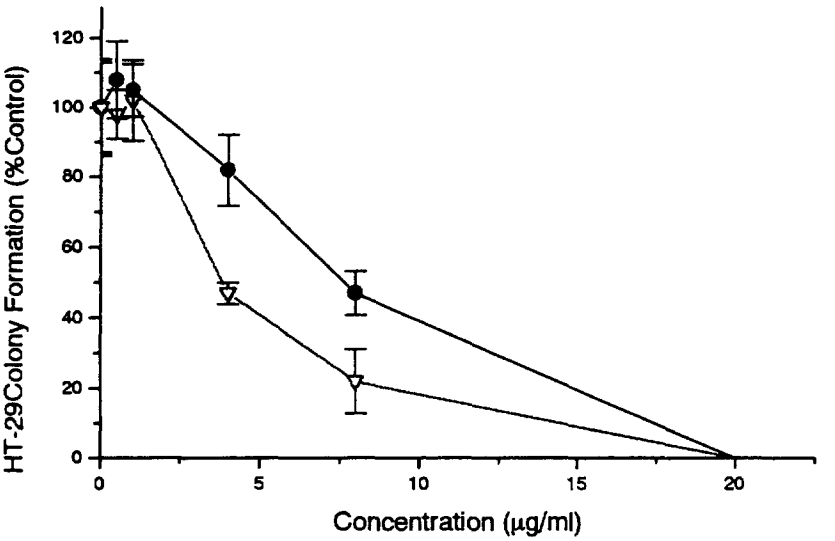


Fig. 6. Inhibition of HT-29 human colon cancer cell colony formation by (●) oryzalin and (▽) N-depropyloryzalin. Each point is the mean of three determinations and bars are S.D..

have previously been shown to inhibit the growth of a variety of tumor cells in culture with IC_{50}s in the range of 2 to 25 μ*M* [5]. However, plasma concentrations after p.o. oryzalin administration were lower than might be expected based on the single i.p. administration data, suggesting that there might be poor absorption or first pass metabolism.

A major metabolite of oryzalin detected in mouse plasma was identified as N-depropyloryzalin. This metabolite is probably formed in the liver since i.p. or p.o. administration of oryzalin gave higher concentrations of metabolite than did s.c. administration. Rat hepatic microsomal preparations showed rapid formation of the metabolite from oryzalin. N-Depropyloryzalin caused similar inhibition of cell colony forming as oryzalin. Thus, it appears that the concentrations of oryzalin and its major metabolite, N-depropyloryzalin, achieved in mouse plasma are sufficient to inhibit the growth of tumor cells based on in vitro cytotoxicity data.

Acknowledgments

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