Pharmacokinetics, Metabolism, and Routes of Excretion of Intravenous Irofulven in Patients with Advanced Solid Tumors

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Running title: Pharmacokinetics and metabolism of irofulven

List of abbreviations:

ESI: Electrospray ionization
MS/MS: Tandem Mass Spectrometry
NMR: Nuclear Magnetic Resonance
HPLC: High Performance Liquid Chromatography
AUC: Area Under Curve
MRT: Mean Residence Time
T_{1/2}: half-life
CLp: Total Body Clearance
C_{max}: Maximal Plasma Concentration
T_{max}: Time to reach C_{max}
K_{el}: Terminal phase elimination rate constant
V_{dss}: Volume of distribution at steady-state
AUMC: Area under the first moment curve
EDTA: Ethylenediaminetetraacetic Acid
LSC: Liquid Scintillation Counting
UV: Ultra-Violet
GSH: Glutathione
t_{R}: Retention Time
RBCs: Red Blood Cells
ABSTRACT

Irofulven is currently in Phase 2 clinical trials against a wide variety of solid tumors and has demonstrated activity in ovarian, prostate, gastro-intestinal and non small cell lung cancer. The objectives of this study were to determine its pharmacokinetics and route of excretion and to characterize its metabolites in human plasma and urine samples after a 30-minute intravenous infusion at a dose of 0.55 mg/kg in patients with advanced solid tumors. Three patients were administered intravenously 100 µCi of [14C]-irofulven over a 30-minute infusion on Day 1 of Cycle 1. Serial blood and plasma samples were drawn at: 0 (before irofulven infusion), and up to 144 h after the start of infusion. Urine and fecal samples were collected for up to 144 h after the start of infusion. The mean urinary and fecal excretion of radioactivity up to 144 h were 71.2% and 2.9%, respectively, indicating renal excretion was the major route of elimination of [14C]-irofulven. The Cmax, AUC_{0-∞}, and terminal half-life values for total radioactivity were 1130 ng-Eq/mL, 24400 ng-Eq.h/mL, and 116.5 h, respectively, and the corresponding values for irofulven were 82.7 ng/mL, 65.5 ng.h/mL, and 0.3 h, respectively, suggesting that the total radioactivity in human plasma was due to the metabolites. A total of twelve metabolites of irofulven were detected in human urine and plasma by ESI-MS/MS mass spectra. Among these metabolites, the cyclopropane ring-opened metabolite (M2) of irofulven was found and seven other were proposed as glucuronide and glutathione conjugates.
INTRODUCTION

Irofulven (6-hydroxymethylacylfulvene) is a semisynthetic acylfulvene derivative of illudin S. The sesquiterpenes, illudin S and illudin M, are toxic compounds obtained from the jack o’lantern mushroom *Omphalotus illudens* (McMorris and Anchel, 1965). These natural products possess antibacterial and antitumor properties but have an unfavourable toxicity profile when tested in animals (Kelner et al., 1987). Irofulven has a greater therapeutic index than the parent compound, illudin S (McMorris et al., 1996). Irofulven has strong concentration- and time-dependent cytotoxic effects in a broad variety of human cancer lines with natural and acquired resistance to classical and non-classical alkylating agents (Poindessous et al., 2003). This derivative has been extensively investigated and is currently in Phase 2 clinical trials against a wide variety of solid tumors and has demonstrated activity in ovarian, prostate, gastro-intestinal and non small cell lung cancer.

Irofulven is a derivative of acylfulvene, the stereoselective synthesis of this compound has been described by McMorris et al. (McMorris and al., 2004). The mechanism of action of irofulven is thought to involve enzymatic activation leading to an unstable intermediate that is subject to nucleophilic attack. Nucleophilic compounds such as proteins or DNA quench the reactive intermediate on the cyclopropane ring through opening and the concomitant loss of the tertiary hydroxyl in the C2 position.

Metabolism and excretion studies of irofulven (M) in rats after single intravenous administration of [14C] irofulven (M*) (see Fig. 1) have shown that the main excretion route was fecal and two metabolites (M₁ and M₂) were identified in plasma and urine (Toda et al., 1998). Metabolism of irofulven by rat liver cytosol has produced four other metabolites identified by mass spectrometry and NMR studies (McMorris et al., 1999). In humans, the
pharmacokinetics of irofulven has been characterized following intravenous administration over a wide range of doses from 1 to 17.69 mg/m² (Eckhart et al. 2000 and Alexandre et al., 2004).

The plasma concentrations of irofulven increased dose proportionally during infusion, and rapidly declined in a biexponential manner with a very short half-life of approximately 5 min and high body clearance up to 11 L/min. The mean volume of distribution was approximately 54 L. The intersubject variability in pharmacokinetic parameters was moderate (Urien and al., 2003 and Alexandre and al., 2004).

The metabolic profile and the routes of excretion of irofulven in humans are not completely known. Characterization of the pharmacokinetics, metabolism and routes of excretion of irofulven in humans is of potential benefit in understanding the biotransformation of irofulven and aid in further development of this drug. Therefore, the objectives of this study were to determine the pharmacokinetics, metabolism, and the route of excretion of irofulven after an intravenous dose of [14C]-irofulven in patients with advanced solid tumors.

METHODS

Chemicals.

Commercially available chemicals and solvents used were HPLC or analytical grade. Alltima C_{18} HPLC analytical columns were obtained from Alltech (Templemars, France). Instagel®, Hionic-Fluor®, FLO SCINT III scintillation cocktails and Soluène-350®, were supplied by Perkin Elmer Life Science and Analytical Instruments Inc. (Villebon-sur-Yvette, France). Methylene chloride and pentane were purchased from Aldrich (Saint Quentin Fallavier,
France). Acetonitrile and isopropanol were obtained from VWR International Inc. (Fontenay-sous-Bois, France). Methanol was supplied by Carlo Erba (Val de Reuil, France). ß-glucuronidase was purchased from Sigma (St-Quentin Fallavier, France).

**Reference Standards**

[¹⁴C]-irofulven was supplied by Amersham (Cardiff, United Kingdom) and possessed a specific activity of 136.3 µCi/mg. The [¹⁴C]-irofulven is labelled on the C-14 position (Fig. 1). Irofulven (6'-hydroxy-3'-hydroxymethyl-2',4',6'-trimethyl-spiro[cyclopropane-1,5'-5H–indenel]-7'(6H)-one, 6-hydroxymethylacylfulven, HMAF, MGI-114) and its metabolites, the glutathione-conjugate M₁ and the cyclopropane ring-opened derivative M₂, were provided by MGI PHARMA Inc. (Minneapolis, USA) as reference standards.

**Study Design**

1] Radioactive study: this was an open-label Phase 1, single centre, pharmacokinetic study of [¹⁴C]-irofulven. Three patients were administered an intravenous infusion over 30 min with a mixture of 0.55 mg/kg dose of irofulven containing 100 µCi of [¹⁴C]-irofulven on Day 1 of Cycle 1.

Pharmacokinetic parameters were calculated from plasma irofulven concentration-time and plasma radioactivity concentration-time data using noncompartmental methods by WinNonlin version 4.1. The mean maximal plasma concentration of irofulven and total radioactivity (Cmax) and the time to reach Cmax (Tmax) were recorded from individual subject concentration-time curve. The terminal phase elimination rate constant (Kel) was determined from the slope of the terminal portion of the log-concentration versus time curve by linear least square regression analysis of the plasma concentration-time profile. Terminal phase elimination half-life (T₁/₂) was calculated as (ln2)/Kel. The area under the plasma
concentration-time curve from time 0 to the last measurable concentration at time t (AUC\(_{0-t}\)) was calculated using the linear trapezoidal method. For irofulven, AUC\(_{0-\infty}\) was computed as AUC\(_{0-t}\) plus the extrapolation from the last time point to infinity using \(C_t/K_e\), where \(C_t\) is the last quantifiable concentration. For irofulven, the total body clearance (CL\(_p\)) was calculated from the dose/AUC\(_{0-\infty}\). The volume of distribution at steady state (V\(_{dss}\)) was calculated as CL\(_p\) x MRT, where mean residence time (MRT) was calculated as \([AUMC/ AUC_{0-\infty} - \tau/2]\), where \(\tau\) is the duration of infusion. The area under the first moment curve (AUMC) was determined using the linear trapezoidal rule and extrapolated to infinity as: AUMC\(_{0-t}\) + \(t \times C_t/K_e\) + \(C_t/(K_e)^2\).

Plasma concentrations below the assay lower limit of quantitation (BLQ) were treated as 0.0 ng/ml for purposes of calculating pharmacokinetic parameters. Actual times after the start of infusion were used in calculation of pharmacokinetic parameters.

2) Non radioactive study: in order to study the metabolic profile, non-radioactive plasma and urine samples were obtained because the mass spectrometry could only be performed with cold irofulven. Samples were obtained from 3 patients who were enrolled in another Phase 1 clinical and pharmacokinetic study of irofulven in combination with oxaliplatin in patients with advanced solid tumors.

**Subjects**

In the pharmacokinetic study (radioactive), three patients (two males #1 and #3/ one female #2) were enrolled.

In the metabolism study (non radioactive), three patients (A, B and C) were treated with 0.55 mg/kg of cold irofulven.
Inclusion Criteria:

- Histologically or cytologically confirmed malignant solid tumor.
- Disease refractory to anticancer treatment or for which no standard treatment exists.
- Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) ≤ 2 (= in bed < 50% of time).

Exclusion Criteria

- Patients who have had radiation therapy to more than 30% of their bone marrow prior to entry into study.
- Prior chemotherapy with nitrosourea or high dose carboplatin (> 6 AUC), prior mitomycin C cumulative dose ≥ 25 mg/m², prior bone marrow transplant or intensive chemotherapy with stem cell support.

Study drug (preparation, administration):

A mixture of $^{14}$C-irofulven and cold irofulven was dissolved with 150 µL of dehydrated ethyl alcohol and 2.85 mL of 5% dextrose in water and then administrated over 30 min as an intravenous infusion into a 100 mL minibag of 5% dextrose injection, on Day 1 Cycle 1. A total of 100 µCi of $^{14}$C-irofulven (equal 0.45 mg irofulven) combined with cold irofulven to achieve a dose of 0.55 mg /kg, was administered.

Sample Collection for radioactive study.

Blood samples were drawn at: 0 (immediately before initiation of the irofulven infusion), 5, 10, 20, 35, 40, 50, 60, 70, 90 min, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, and 144 h after the start of infusion of $^{14}$C Irofulven. Samples of blood (11 mL) were drawn in EDTA tubes from a peripheral venous access in the arm opposite drug administration. The blood sample
was centrifuged for 5 min at 10 000 rpm at +4°C. The plasma was removed and divided into 3 equal volumes in transfer tubes and immediately stored at –70 to –80°C until analysis.

Urine and fecal samples were collected for up to 144 h post dose at 0 to 6, 6 to 12, 12 to 24, 24 to 48, 48 to 72, 96, 120, and 144 h after the start of infusion. At least 3 mL of saliva samples were collected at each time point up to 72 h after the start of infusion. Tear samples were collected up to 48 h post dose using absorbent paper at 0 (baseline before the start of infusion), 28 min (just prior to the end of infusion) and 30 min, 6 h, 24 h, and 48 h after the end of infusion. Expired air was collected from each patient in scintillation vials at: 0 (just prior to the start of infusion), 25-28 min (just prior to the end of infusion), 30 min, 6 h and 24 h after the end of the infusion. The patient was allowed to blow gently into the pink solution of scintillation vials containing benzethonium hydroxide (approximately 1 M in methanol) with absolute ethanol and 1% phenolphthalein solution through a tubing set equipped with one way valve to prevent aspiration of the solution (the pink color disappeared when approximately 2 mM of CO₂ was captured). All samples were labelled and immediately frozen at –70 to –80°C until analysis.

**Sample collection for metabolite profiling and identification.**

Urine and plasma samples were collected from 3 Patients A, B and C enrolled in another Phase 1 clinical and pharmacokinetic study of irofulven in combination with oxaliplatin.

**Urine samples**

For each patient, A and B, a predose urine sample was collected before the infusion of cold irofulven and urine samples were collected 45 min after the start of infusion. Aliquot of (1.5 mL) each urine sample was extracted with 5 mL of the extraction mixture (methylene chloride/pentane, 50:50, v/v). After centrifugation for 10 min at 4000 rpm, the supernatant
was evaporated. Enzymatic hydrolysis of the same urine sample of patients A and B was performed to detect glucuronide metabolites. To 1.5 mL of each urine sample, 500 units of β-glucuronidase were added. The mixture was incubated at 37°C for 2 h. The reaction was stopped by the addition of the extraction mixture.

**Plasma samples**

Blood samples were drawn on the first dosing day at 0 (before infusion of irofulven), 10 min, prior to the end of infusion (30 min), and 5, 10, 15, and 30 min post infusion into a 7 ml lavender-top (EDTA-K3) evacuated tube, inverted to mix and placed in an ice-bath. The tubes were centrifuged within 30 minutes of collection at 4000 rpm for 10 min at +4°C. The plasma (approximately 2-2.5 ml) from each tube was immediately stored vertically (at –70°C) until analysis.

For each patient, A and C, two extracts of control plasma (i.e. collected before the perfusion of irofulven) and two extracts of plasma (i.e. collected 60 min after the start of infusion of irofulven) were obtained according the same procedure.

**Determination of radioactivity**

Radioactivity in samples was measured by liquid scintillation counting (LSC). Aliquots of each plasma, whole blood, urine, feces, saliva, expired air and tears samples (in duplicate) described above were placed into vials and mixed with a scintillation cocktail and counted in a liquid scintillation counter for five minutes (Packard Tri-Carb 1900TR; Perkin Elmer Life Science and Analytical Instruments Inc.). Aliquots of feces and whole blood were dissolved with a mixture of Soluène-350 (1 mL) and isopropanol (0.5 mL) and decolorized with 30% hydrogen peroxide solution.
The radioactivity in the actual dose was expressed as 100%, and the radioactivity in urine and feces at each sampling time was defined as the percentage of dose excreted in the matrices at that sampling time.

**High-performance liquid chromatography with radioactivity detection.**

Plasma and urine samples obtained during this study were assayed for irofulven by a validated HPLC method with a β-counter detection. The analytical HPLC system (Waters, St Quentin en Yvelines, France) consisted of a 717 plus autosampler, a 515 pump and controlled by Millenium® software for data handling and a radioactive detector (β-RAM; IN/US system). Chromatography was performed on a reversed phase column C$_{18}$ 5 µm Alltima®, 250 mm x 4.6 mm i.d. (Alltech, Templemars, France) with a mobile phase containing a mixture of methanol, acetonitrile and water (15:25:60, v/v/v) at 1 mL/min flow-rate. Standard curve was prepared by isotopic dilution and linear over 2.5 to 2500 ng/mL concentrations of irofulven.

**High-performance liquid chromatography with UV detection (HPLC/UV)**

The HPLC system (Waters, St Quentin en Yvelines, France) consisted of two 510 pumps, a 710 autosampler and a 486 UV detector, controlled by Millennium® software. The standards and the extracted urine and plasma samples were analyzed on a reversed phase column C$_{18}$ 5µm Alltima®, 250 mm x 4.6 mm i.d. (Alltech, Templemars, France) and detected at 260 nm UV wavelength. Isocratic elution (methanol/acetonitrile/water, 10:20:70, v/v/v) was carried out at 1 mL/min flow-rate. The extracts were dissolved in 100 µL of solvent (acetonitrile/water, 50:50, v/v) before analysis of an aliquot by HPLC.
High-performance liquid chromatography with mass spectrometry (HPLC/ESI-MS/MS)

The 1100 series HPLC system (Agilent Technologies, Massy, France) consisted of a binary pump, an autosampler fitted with a microbore column C18 5µm Uptisphere®, 150 mm x 1 mm i.d. (Interchim, Montluçon, France). The flow-rate of 50 µL/min was achieved with an elution gradient composed of solvent A (acetonitrile/water with 0.1 % formic acid, 20:80, v/v) and of solvent B (acetonitrile/water with 0.1% formic acid, 80:20, v/v). After an initial 5-min step with 100 % of solvent A, elution gradient was running from 0 % to 70 % solvent B up to 18 min, then back again to initial conditions up to 30 min. The standards and extracted samples were diluted in solvent A, then analyzed with the triple quadrupole mass spectrometer Quattro® LCZ (Micromass-Waters, Manchester, UK) equipped with the Z-spray electrospray source. The HPLC-ESI-MS/MS chromatograms were obtained by scanning in positive-ion mode over the m/z 150-600 range and processed with Masslynx® NT software. Electrospray mass spectra (ESI-MS/MS) were performed with collision induced dissociation of selected parent ions. The capillary and cone voltages were set at 3500V and 30V respectively. The collision energy was set at 10 eV, using argon as collision gas.

RESULTS

Mass balance.

Mass balance after i.v. infusion of 0.55 mg/kg dose of irofulven containing 100 µCi of [14 C] irofulven to human subjects, was achieved with a 74.1% mean recovery of the administered dose in urine and feces. As shown in Table 1, the administrated radioactivity was eliminated predominantly in the urine. The mean urinary excretion amounted to 71.2% with the majority (88%) of radioactivity excreted in the first 24 h. The radioactivity in the urine was excreted up
to 144 h after the start of infusion. The mean fecal recovery was 2.9%. Radioactivity was also measured in the expired air, tears and saliva samples (data not shown). Urinary excretion was a major route of elimination of [14C]-irofulven from the body.

Pharmacokinetics.

Mean radioactivity and irofulven concentration time profiles are shown in Fig. 2. Plasma concentrations of irofulven increased during the 30-min infusion period and rapidly declined biexponential manner following discontinuation of infusion. A summary of plasma pharmacokinetic parameters is presented in Table 2. The mean Cmax was 1130 ng/mL and 82.7 ng/mL for total radioactivity and irofulven respectively. The mean total radioactivity AUC$_{0-\infty}$ was 24400 ng·h/mL and mean irofulven AUC$_{0-\infty}$ was 65.5 ng·h/mL. In plasma, irofulven was a minor circulating moiety representing approximately 0.27% of total radioactivity AUC. The plasma radioactivity disappeared with an initial distribution phase followed by a slow elimination phase and was measurable up to 144 h. The mean terminal phase elimination half-life was 116.5 h and 0.3 h for total radioactivity and irofulven, respectively. The mean total body clearance and the apparent volume of distribution for irofulven were 8.48 L/h/kg and 3.12 L/kg, respectively. The blood to plasma distribution ratio of radioactivity was 1.6. Plasma irofulven pharmacokinetic profile and parameters are consistent with previous Phase 1 studies (Eckhardt and al., 2000; Urien et al., 2003).

Analyses of standards by HPLC/UV and by HPLC/ESI-MS/MS.

Irofulven and metabolite standards M1 (glutathione conjugate) and M2 (the cyclopropane ring-opened product) were analyzed by HPLC/UV at 260 nm and by HPLC/ESI-MS/MS. The HPLC/UV retention times ($t_R$) for irofulven, M1 and M2 were 15.7 min, 10.6 min, and 9.2 min, respectively.
Electrospray mass spectra of standards (Fig. 3) provided the following molecular masses of metabolites (M₁ = 535.2) and (M₂ = 248.2) and of irofulven (M = 246.1). The product ion mass spectrum (Fig. 3a) of irofulven-glutathione conjugate M₁ (m/z 536) showed a characteristic fragment ion m/z 308 corresponding to the [GSH+H]+ ion of glutathione and another fragment ion (m/z 229) indicating the linkage between irofulven and glutathione (GSH). The mass spectrum (Fig. 3b) of M₂ standard showed a molecular ion at m/z 249 and an intense m/z 231 fragment ion (249-18), corresponding to the water loss likely from the allylic hydroxyl at carbon 14 (Fig. 1) of metabolite M₂, and an other ion (m/z 213) indicating an additional water loss from the hydroxyl on the opened cyclopropane. The mass spectrum (Fig. 3c) of irofulven showed the molecular ion at m/z 247 and that two hydroxyls were present on the molecule by two successive losses of water (m/z 229 and 211). The HPLC/ESI-MS/MS analyses of the standards mixture were obtained by reconstructed ions mass chromatograms (m/z 536, 249 and 247) indicated that M₁ and M₂ compounds eluted with a close polarity at acidic pH (t_R:M₁ = 15.4 min; t_R:M₂ = 15.5 min) and that the retention time of irofulven was 16.6 min.

Characterization of metabolites in urine samples

The HPLC/UV analysis (Fig. 4a) of patient A urine extract, collected at 45 min after the start of infusion, showed the presence of several chromatographic peaks corresponding to irofulven (t_R = 15.3 min), M₁ (t_R = 9.8 min) and M₂ (t_R = 9.3 min) and to other unknown products, that were different from endogenous peaks detected in control urine (Fig. 4b).

The HPLC/ESI-MS/MS analyses (Fig. 5) of patient A urine (collected after and before the perfusion) confirmed these observations and detected several significant products. Seven metabolites of irofulven were characterized in the urine of patient A by their retention times,
their molecular masses, and their fragment ions indicated by their ESI-MS/MS mass spectra corresponding to the selective ions m/z 536 (M₁), 538 (M'₁), 249 (M₂), 263 (M₃, M₄), 231 (M₆), and 247 (M₅) (see Fig. 5 and Table 3).

The mass spectra of peaks (m/z 536; t_R = 14.7 min) and (m/z 249; t_R = 14.9 min) were identical to those of M₁ and M₂ standards, respectively (Fig. 3a and 3b). Based on these data, the metabolite (M₁ = 535) was characterized as the glutathione conjugate of irofulven (246+307–18 = 535). The metabolite (M₂ = 248) corresponded to the known (cyclopropane ring-opened) derivative of irofulven.

A polar compound M₀ (t_R =10 min) has a molecular mass of 553. Its mass spectrum showed the fragment ions m/z 536 ([M₀+H-H₂O]⁺) and m/z 518 (536-18) indicating the presence of two hydroxyls. The fragment ion m/z 308 ([GSH+H]⁺) suggested that the structure could be a glutathione conjugate resulting from the addition on the C-8,9 double bond of irofulven as demonstrated by Dick (Dick et al., 2004). The mass spectrum of another compound (t_R =14.4 min) showed the molecular ion m/z 538 (M'₁ = 537) and the fragment ion m/z 308 corresponding to a glutathione conjugate of reduced irofulven M₂ (248+307-18 = 537) as described in vitro by the same authors (Dick et al., 2004).

The M₃ and M₄ metabolites have identical molecular mass of 262 (m/z 263) and the same fragment ions (m/z 247 and 227). They had respective retention times of 14.8 min and 16.3 min, close to that of irofulven (16.1 min). The mass increase of 16 compared to parent drug, irofulven, shows that these metabolites could be hydroxylated derivatives of irofulven. Hydroxylation could occur either at C-13 for metabolite M₃ or at C-15 for metabolite M₄.

These identifications are supported by McMorris (McMorris et al., 1999) with structural NMR analysis of hydroxymethylacylfulvene metabolites in rat.
The mass spectrum of metabolite M₆ (tᵢ = 18.8 min) showed a protonated molecular ion ([M₆+H]⁺) at m/z 231 and only one fragment ion (m/z 213) corresponding to water loss [M₆+H-H₂O]⁺. The combined HPLC-ESI-MS/MS analyses of this metabolite (M₆=M-16) suggested the presence of only one hydroxyl for M₆. This hydrophobic compound compared to irofulven most likely could correspond to 6-methylacylfulvene as characterized by McMorris (McMorris et al., 1999). These authors described that the formation of M₆ results from displacement of the primary allylic hydroxyl of irofulven by hydride from NADPH. The M₅ metabolite has the same molecular mass of 246 (m/z 247) and the identical mass spectrum as irofulven, but it has a shorter retention time (15.2 min). The M₅ metabolite is an isomer of irofulven and its structure could be a hydroxylated derivative of the M₆ compound with the hydroxyl either at C-13 or at C-15. According to NMR analysis achieved by McMorris (1999), the expected position could be at C-15.

Characterization of glucuronide conjugates metabolites in urine.

The HPLC/UV analyses of the urine sample, incubated with β-glucuronidase for patient B (Fig. 6a and 6b), showed a significant increase in metabolite M₂ (tᵢ = 9.6 min) and metabolite M₅ (tᵢ = 19.1 min). Furthermore, in the case of the urine sample of patient B, peaks corresponding to irofulven (tᵢ = 15.0 min) and to its hydroxylated metabolite, M₄ (tᵢ = 5.6 min), seemed to be higher. Thus, the HPLC/ESI-MS/MS analyses of urine sample of patient B allowed the detection of four glucuronide conjugates by their respective mass spectra (Fig. 7). M₂-glucuronide conjugate (M’₂) has a molecular mass of 424 (m/z 425 = 249+176), M₅-glucuronide (M’₅) has a molecular mass of 422 (m/z 423 = 247+176), M-glucuronide (M’) has a molecular mass of 422 (m/z 423 = 247+176), and M₄-glucuronide (M’₄) has a molecular mass of 438 (m/z 439 = 263+176). Moreover, these HPLC/ESI-MS/MS analyses (Fig. 5a and 5b) were used for peak area comparison for each compound. They showed a large increase (x
50) in $M_2$ metabolite (m/z 249) and a relative slightly increase (x 5) of irofulven (m/z 247) and its metabolites $M_4$ (m/z 263) and $M_5$ (m/z 247) in urine of patient B treated with $\beta$-glucuronidase. Furthermore, the ions $[M+H]^+$ and $[M+H-H_2O]^+$ confirmed the O-glucuronide linkage.

**Characterization of metabolites in plasma samples**

The HPLC/ESI-MS/MS analyses of plasma samples from two patients (A and C) detected irofulven (m/z 247; M = 246), its metabolite (m/z 249; $M_2 = 248$) and its glutathione conjugate ($M_1 = 535$). Mass spectra of these metabolites in the collected plasma of patient C (Fig. 8) were identical to those obtained for standards (Fig. 3).

The characterization of these metabolites ($M_1$, $M_2$, $M_3$, $M_4$, $M_5$ and $M_6$) observed in human urine and plasma samples and glucuronide conjugates in human urine by HPLC/ESI-MS/MS suggests the proposed *in vivo* metabolic pathways of irofulven in human as shown in Fig. 9.

**DISCUSSION**

The pharmacokinetics, metabolism and excretion of irofulven were investigated in patients with advanced solid tumors after a 30-min i.v. infusion of 0.55 mg/kg dose of irofulven containing $[^{14}C]$-irofulven. The majority of the administrated radioactive dose was excreted in urine (71%), suggesting that urinary excretion was the primary route of elimination of irofulven-associated radioactivity in human as compared to excretion in rats where fecal excretion was predominant. Unchanged irofulven was not detected in the urine sample possibly due to low renal elimination of irofulven and dilution of sample due to collection.
over the larger interval. The pharmacokinetic profile of irofulven after the administration of a single dose was characterized by a short mean half-life of 0.3 h and high total body clearance, which is similar to the finding of the prior Phase 1 trial of irofulven (Eckhardt et al., 2000). As shown in Fig. 2, the higher blood radioactivity concentrations for the last time points resulted in higher AUC in blood with a blood to plasma ratio of 1.6 for AUC but only 0.8 for Cmax. This blood to plasma distribution ratio of radioactivity, indicated a slight partitioning of irofulven derived radioactivity into the red blood cells (RBCs). Plasma levels of total radioactivity were higher than those for the unchanged irofulven and were measurable up to 144 h after infusion, suggesting that the majority of the circulating radioactivity was due to the presence of metabolites.

Metabolite profiling using HPLC/UV and HPLC/MS in human plasma and urine was performed for 3 patients treated with irofulven in a separate Phase 1 clinical and pharmacokinetic study. In these samples, several peaks corresponding to the potential metabolites were detected. Several of these peaks were characterized as irofulven metabolites by HPLC/ESI-MS/MS in human urine and plasma samples. The structures of the observed metabolites in human are proposed (Fig.9), and seem to be in concert with the published results on metabolism of irofulven from in vitro and in vivo studies in rat (McMorris, 1999, Toda et al., 1998) and a study using NMR method by McMorris (McMorris et al., 1999). These authors have shown that the stereochemistry of the tertiary hydroxyl does play a role in the enzyme-catalyzed activation step. The mechanism of action of its antineoplastic compound is thought to involve an activation step with a nucleophilic attack by thiols or NADPH leading to a highly reactive intermediate. This latter reacts with nucleophilic compounds such as proteins or DNA on the cyclopropane ring with its opening and the concomitant loss of the tertiary hydroxyl in the C-2 position. The glutathione conjugate (M₁) of irofulven still retains
the α,β-unsaturated ketone and cyclopropylmethylcarbinol moieties that can react with nucleophiles (McMorris et al. 2000 and 2001). Therefore, M₁ might still possess alkylating ability and hence antitumor activity (McMorris et al., 2004).

Twelve metabolites of irofulven were detected in human urine based on their retention times and their molecular masses. The known cyclopropane ring-opened metabolite (M₂) and glutathione conjugate metabolite (M₁) of irofulven identified in rat plasma, (Tanaka et al., 1994), have also been found in human urine and plasma. Four metabolites (M₃, M₄, M₅ and M₆) found in human urine in the present study were characterized by mass spectrometry and their structures have been previously determined by NMR in the metabolism study of irofulven in rat liver cytosol (McMorris et al., 1999). The metabolites M₃ and M₄ are hydroxylated metabolites from irofulven formed via oxidation while the metabolite M₆ could be produced from irofulven via a presumed reduction. The M₅ metabolite could be produced from M₆ via oxidation on C-15 position (see Fig. 1). The glucuronide conjugates, detected in urine samples by their ESI-MS/MS mass spectra, probably resulted from association with irofulven and its primary metabolites (M₃, M₄, and M₅). Thus, four new glucuronide conjugates (M¹, M², M³ and M⁵) were found in the human urine. Finally, the formation of primary metabolites, glutathione conjugates and glucuronide conjugates in humans suggests the proposed biotransformation pathways in vivo of irofulven (Fig. 9). Further investigation on metabolism is ongoing to identify the enzymes involved in the formation of metabolites of irofulven.

AKNOWLEDGMENTS

The authors would like to thank the help of pharmacology team at Centre René Huguenin hospital.
REFERENCES


DMD # 10512

FOOTNOTE

**A. PACI and K. REZAI contribute equally to this work.**
LEGENDS FOR FIGURES

Fig. 1. Structure of irofulven (M) and ¹⁴C-irofulven (Carbon-15 is radiolabelled).

Fig. 2. Mean radioactivity concentrations in blood (◊) and plasma (Δ) and mean irofulven in plasma-concentration (□) -time profiles following 0.55 mg/kg dose containing 100 µCi [¹⁴C]-irofulven administered over 30 min in patients with solid tumors. Mean (±SD) plasma concentration-time profile of irofulven is shown in inset.

Fig. 3. Electrospray mass spectra of (a) metabolite M₁, (b) metabolite M₂ and (c) irofulven standards.

Fig. 4. HPLC/UV chromatograms of patient A urine samples collected (a) after 45 min and (b) before the infusion of irofulven.

Fig. 5. HPLC/ESI-MS/MS chromatograms of patient A urine samples collected after 45 min infusion of irofulven.

Fig. 6. HPLC/UV chromatograms of patient B urine sample treated (a) with and (b) without β-glucuronidase.

Fig. 7. Electrospray mass spectra of glucuronide conjugates in treated urine of patient B with respective masses: (a) M'₂ = 424, (b) M'₅ = 422, (c) M' = 422, and (d) M'₄ = 438.

Fig 8. HPLC/ESI-MS/MS chromatograms of irofulven (c) and its metabolites, (a) glutathione conjugate and (b) cyclopropane opened-ring metabolite, in plasma of patient C.

Fig 9. Proposed biotransformation pathways of irofulven in human.
Table 1. Cumulative excretion of total radioactivity in patients.

<table>
<thead>
<tr>
<th></th>
<th>% cumulative excretion (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Urine (0-144 h)</td>
<td>71.2</td>
</tr>
<tr>
<td>Feces (0-96 h)</td>
<td>2.9</td>
</tr>
<tr>
<td>Urine + Feces</td>
<td>74.1</td>
</tr>
</tbody>
</table>
Table 2. Pharmacokinetic parameters of irofulven and radioactivity in human (Mean ± SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total Radioactivity</th>
<th>Irofulven</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (ng/mL)</td>
<td>1130 ± 174</td>
<td>82.7 ± 46.0</td>
</tr>
<tr>
<td></td>
<td>1120 (^a)</td>
<td>71.4 (^a)</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.72 ± 0.12</td>
<td>0.29 ± 0.10</td>
</tr>
<tr>
<td>AUC(0-t) (ng(\times)h/mL)</td>
<td>15000 ± 634</td>
<td>30.6 ± 25.8</td>
</tr>
<tr>
<td></td>
<td>15000 (^a)</td>
<td>17.7 (^a)</td>
</tr>
<tr>
<td>AUC(0-(\infty)) (ng(\times)h/mL)</td>
<td>24400 ± 1780</td>
<td>65.5 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>24300 (^a)</td>
<td>65.4 (^a)</td>
</tr>
<tr>
<td>T1/2 (h) terminal half life</td>
<td>116.5 ± 27.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>CL(p) (L/h/kg)</td>
<td>8.48 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Vdss (L/kg)</td>
<td>3.12 ± 1.99</td>
<td></td>
</tr>
<tr>
<td>AUC(0-t) Ratio (^b)</td>
<td>0.00209 ± 0.00180</td>
<td></td>
</tr>
<tr>
<td>(^{14})C-AUC(0-t) Blood to plasma ratio</td>
<td>1.58 ± 0.737</td>
<td></td>
</tr>
<tr>
<td>(^{14})C-Cmax Blood to plasma ratio</td>
<td>0.820 ± 0.205</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) geometric mean

\(^b\) Ratio of irofulven to radioactivity
Table 3. Characterization of irofulven and its human urinary metabolites by mass spectrometry (HPLC/ESI-MS/MS).

<table>
<thead>
<tr>
<th>Irofulven and metabolites</th>
<th>Mass</th>
<th>ESI-MS/MS mass spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irofulven (M)</td>
<td>246</td>
<td>247, 229 (-H₂O), 211 (-2H₂O).</td>
</tr>
<tr>
<td>M-GSH conjugate (M₁)</td>
<td>535</td>
<td>536, 518 (-H₂O), 308, 229.</td>
</tr>
<tr>
<td>M₂-GSH conjugate (M₂₁)</td>
<td>537</td>
<td>538, 520 (-H₂O), 308.</td>
</tr>
<tr>
<td>M₂-GSH conjugate (M₀)</td>
<td>553</td>
<td>554, 536 (-H₂O), 518 (-2H₂O).</td>
</tr>
<tr>
<td>M-glucuronide conjugate (M')</td>
<td>422</td>
<td>423, 405 (-H₂O).</td>
</tr>
<tr>
<td>Cyclopropane ring opened metabolite (M₂)</td>
<td>248</td>
<td>249, 231 (-H₂O), 213, (-2H₂O).</td>
</tr>
<tr>
<td>M₂-glucuronide conjugate (M₂')</td>
<td>424</td>
<td>425, 407 (-H₂O), 249, 231.</td>
</tr>
<tr>
<td>Hydroxylated metabolite (M₃)</td>
<td>262</td>
<td>263, 245 (-H₂O), 227 (-2H₂O).</td>
</tr>
<tr>
<td>Hydroxylated metabolite (M₄)</td>
<td>262</td>
<td>263, 245 (-H₂O), 227 (-2H₂O).</td>
</tr>
<tr>
<td>M₄-glucuronide conjugate (M₄')</td>
<td>438</td>
<td>439, 421 (-H₂O), 403 (-2H₂O), 263.</td>
</tr>
<tr>
<td>Reduced metabolite (M₆)</td>
<td>230</td>
<td>231, 213 (-H₂O), 203.</td>
</tr>
<tr>
<td>Reduced and hydroxylated metabolite (M₅)</td>
<td>246</td>
<td>247, 229 (-H₂O), 219, 211 (-2H₂O).</td>
</tr>
<tr>
<td>M₅-glucuronide conjugate (M₅')</td>
<td>422</td>
<td>423, 405 (-H₂O)</td>
</tr>
</tbody>
</table>
Figure 1

* $[^{14}\text{C}]$
Figure 3

(a) [M₁+H]+

(b) [M₂+H]+

(c) [M+H]+
Figure 5
Figure 6
Figure 7

(a) \([M'_2+H-OGlc]^+\)

(b) \([M'_5+H-OGlc]^+\)

(c) \([M'_6+H-OGlc]^+\)

(d) \([M'_4+H-Glc]^+\)
Figure 8

(a) 
(m/z 536)

(b) 
(m/z 249)

(c) 
(m/z 247)

(a) 
([M1+H]+)

(b) 
([M2+H]+)
Figure 9