Marked Activity of Irofulven toward Human Carcinoma Cells: Comparison with Cisplatin and Ecteinascidin

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ABSTRACT

Purpose: To characterize the activities of irofulven, a novel anticancer agent derived from the mushroom natural product illudin S toward human cancer cells.

Experimental Design: We have determined the activity spectrum of irofulven toward a human tumor cell panel comprised of 10 different tumor types in comparison with cisplatin and ET-743. We have also evaluated the influence of major resistance mechanisms, such as expression of multidrug resistance-associated drug efflux pumps, cisplatin resistance, loss of p53 function, and absence of mismatch repair on the cytotoxic activity of irofulven.

Results: The activity spectrum of irofulven is clearly different from that of ET-743 and cisplatin. Irofulven shows excellent cytotoxicity toward the majority of human carcinoma cell lines tested, but lesser activity toward sarcoma and leukemia cell lines. The cytotoxic activity of irofulven was particularly pronounced toward head and neck, non-small cell lung, colon, and ovary carcinoma cells, as well as toward malignant glioma cell lines. In addition, irofulven displayed good activity toward poorly differentiated, androgen-independent prostate cancer cells and cell lines expressing high levels of the detoxifying enzymes glutathione S-transferase and γ-glutamyl cysteine synthetase. The cytotoxicity of irofulven was not affected by loss of p53 or mismatch repair function, and the drug was not a substrate for multidrug transporters, such as the P-glycoprotein and multidrug resistance protein 1.

Conclusions: Irofulven has an unusual activity spectrum with strong activity toward tumor cells of epithelial origin. Furthermore, irofulven is not or only marginally affected by resistance mechanisms limiting the efficacy of other alkylating agents.

INTRODUCTION

Cytotoxic agents remain the backbone of current cancer chemotherapy. To advance further, there is a clear need to identify novel cytotoxic agents with different activity spectra. Irofulven (MGI-114, 6-hydroxymethylacylfulvene) is a novel semisynthetic antitumor compound derived from the mushroom natural product illudin S (for chemical structures, see Fig. 1). Although the mechanism of action of irofulven is not fully elucidated, its capacity to bind covalently to biological macromolecules characterizes it as an alkylating agent (1, 2).

Irofulven display an original cytotoxicity profile in the NCICOMPARE program, suggesting that the mechanism of action and resistance pattern of irofulven differs from classical alkylating agents, such as cisplatin. The COMPARE program also indicates that irofulven is closely related to the marine product ecteinascidin 743 (ET-743), a novel DNA minor groove binding drug. Intriguingly, the CC between irofulven and ET-743 (CC = 0.727, where 1 indicates a perfect positive correlation and 0 no correlation) is even superior to that observed between irofulven and the structurally related compounds acylfulvene (CC = 0.701) and illudin S (CC = 0.553).

Irofulven has shown excellent activity in human tumor xenograft models derived from breast, colon, lung, and gastric carcinomas, as well as from human glioblastoma multiforme (3–5). Importantly, irofulven showed activity toward both parental MV522 lung carcinoma xenograft and sublines overexpressing MDR-associated proteins, including the P-gp and MRP (6, 7). In contrast, irofulven showed no activity toward murine melanoma and leukemia cell lines (3).

To facilitate further clinical development of this original compound, we have explored the time dependence of irofulven cytotoxicity and determined the activity spectrum of irofulven toward a human tumor cell panel comprised of 10 different tumor types in comparison with cisplatin and ET-743. We have also evaluated the influence of major resistance mechanisms, such as expression of MDR-associated drug efflux pumps, cisplatin-resistance, loss of p53 function, and absence of MMR on the cytotoxic activity of irofulven.

MATERIALS AND METHODS

Chemicals. Purified Irofulven was supplied by MGI-Pharma, Inc. (Bloomington, MN). ET-743 was generously pro-

4 The abbreviations used are: NCI, National Cancer Institute; CC, correlation coefficient; MRP, multidrug resistance protein; MMT, mismatch repair; MDR, multidrug resistance; P-gp, P-glycoprotein; TT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
vided by the NCI (Frederick, MD). Cisplatin and vincristine were purchased from Quamed Laboratories (Levallois-Perret, France) and Faulding ASTA Medica (Merginac, France), respectively. Adriamycin was obtained from Sigma (Saint-Quentin Fallavier, France).

**Cells and Culture Media.** NCI-H460 and NCI-H522 lung carcinoma cells; HT-29, SW-620, and CoLo 205 colon carcinoma cells; IGROV1, OvCar-3, OvCar-5, and SKOV-3 ovarian carcinoma cells; DU-145 and PC3 prostate carcinoma cells; as well as SNB-19 and U-251 glioma cells were kindly provided by Dr. Richard Camalier, Division of Cancer Treatment and Diagnosis tumor repository, NCI. HT-1080 fibrosarcoma and SW-480 colon carcinoma cells were purchased from American Type Culture Collection (Rockville, MD). A2780 and 2008 ovarian carcinoma cells and their cisplatin-resistant variants A2780/CF70 and 2008 C13* were kindly provided by Robert Brown (Glasgow, Scotland) and Jean-Sebastien Hoffmann (Toulouse, France), respectively. Colon HCT-116 colon carcinoma cells and their p53−/− and p21−/− sublines were generously provided by Bert Vogelstein (Baltimore, MD), whereas HCT-116 supplemented with chromosome 3 was a kind gift from David Newell (Newcastle on Tyne, United Kingdom). HL-60, CEM, and their resistant sublines CEM/Vinc, HL-60/ Vinc, and HL-60/Adr were donated by Johann Hofmann (Innsbruck, Austria) and Guy Laurent (Toulouse, France), respectively. MCF-7, MDA-MB-231, and SKBr-3 breast carcinoma cells were a gift from Nancy Hynes (Basel, Switzerland). HEL, K562, and Dami leukemia cell lines were kindly provided by Jacqueline Robert-Lezénes (Villejuif, France), whereas HeLa-S3 and HeLa-M cervical carcinoma cells and LNCaP prostate cancer were gifts from Andrzej Skladanowski (Gdansk, Poland). Hep-2 and SCC-61 head and neck carcinoma cells were kindly provided by Eric Deutch (Villejuif, France), whereas SaOs-2 and U2-Os were gifts from Annick Harel-Bellan (Villejuif, France). A-549 lung carcinoma and KB head and neck carcinoma cells were kindly donated by Olivier Brison (Villejuif, France) and Jacques Robert (Bordeaux, France), respectively.

The cells were maintained in MEM (HT-1080, KB), McCoy’s A (HCT-116, SaOs-2), RPMI 1640 (A-549, A2780, A2780/CF70, 2008, 2008 C13*, NCI-H460, NCI-H522, CoLo 205, LNCaP, DU-145, PC3, IGROV1, OvCar-3, OvCar-5, SKOV-3, HeLa-S3, HeLa-M, SNB-19, U-251, CEM, Dami, HEL, HL-60, K562, MOLT-4,), or in DMEM (HT-29, MCF-7, MDA-MB-231, SKBr-3, SW-480, SW-620, U2-Os) supplemented with 10% FCS (Invitrogen, Cergy-Pontoise, France), 2 mM L-glutamine (Eurobio, Les Ulis, France), 100 units/ml penicillin, and 100 μg/ml streptomycin (PanPharma, Fougères, France). Adherent cells were split twice a week, whereas cells in suspension culture were recultured at 2.5 × 10^4 cells/ml every other day. All cell lines were tested regularly for Mycoplasma contamination by PCR analysis.

**Growth Inhibition Assays.** The cytotoxicity was determined by the MTT assay. Briefly, cells were seeded in 24-well tissue culture plates at 10,000–15,000 cells/well and incubated overnight. The exponentially growing cells were then exposed to different drug concentrations for three to four generation times. Cellular viability was determined by exposing cells to the MTT tetrazolium salt for 4 h at 37°C, and the formation of formazan was measured at 560 nm by a microplate reader. The concentration inhibiting cell growth by 50% compared with untreated controls was determined from the curves plotting survival as a function of dose. All values are average of at least three independent experiments each done in duplicate.

**Immunolocalization of p53.** To determine the localization of p53, immunocytochemistry was carried out as described previously (8). Briefly, HCT-116 cells were attached to glass slides overnight and exposed to isotoxic concentrations of irofulven (400 ng/ml), ET-743 (4 ng/ml), or cisplatin (11 μg/ml) for 6 h. After drug exposure, cells were fixed with 3.7% formaldehyde, permeabilized with 0.25% Triton X-100, and blocked with 1% BSA. Cells were then incubated for 1 h with anti-p53 polyclonal antibodies (Sc-6243; Santa Cruz Biotechnology) followed by secondary antirabbit FITC-conjugated antibodies (Amersham Life Sciences). Coverslips were mounted in Vectashield (Vector Laboratories) and analyzed with an epifluorescence microscope Axiovert 100M equipped with appropriate filters and laser confocal scanning system LSM 510 by using a plan Apochromat ×63 objective (Zeiss).

**Western Blot Analysis.** Western blot analysis was performed as described previously (8, 9) with minor modifications. Whole cell lysates were prepared from cells treated with isotoxic concentrations of irofulven (400 ng/ml), ET-743 (4 ng/ml), or cisplatin (11 μg/ml) for 6 h. Proteins (50 μg/lane) were separated on a 4–12% polyacrylamide SDS gel and transferred to PolyScreen membranes (NEN, Le Blanc Mesnil, France). The presence of p53, p21, and β-actin was revealed by anti-p53 antibodies (Sc-6243; Santa Cruz Biotechnology), anti-p21 antibodies (Sc-3976; Santa Cruz Biotechnology), and anti-actin antibodies (Sc-1616; Santa Cruz Biotechnology), respectively, followed by incubation with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) and detection by enhanced chemiluminescence (New England Nuclear).

**RESULTS**

**Influence of Irofulven on the Viability of Human Tumor Cell Lines.** The influence of irofulven on the viability of 10 different types of human tumor cells, including carcinomas of the lung, head and neck, colon, breast, ovary, cervix, prostate, sarcomas, gliomas, and leukemias, was determined after continuous exposure to irofulven for three doubling times (Fig. 2). The cytotoxicity differs ≥100-fold between the different cell lines ranging from 6 ng/ml for CoLo 205 colon carcinoma cells to 640 ng/ml for U2-Os osteosarcoma cells. Generally, irofulven has potent activity toward human tumor cells of epithelial origin. The cytotoxic effect of irofulven was most
pronounced toward non-small cell lung and ovarian carcinomas with IC$_{50}$s ranging from 11 to 68 ng/ml. Interestingly, irofulven also showed potent activity toward malignant glioma cells (IC$_{50}$s $\approx$ 30 ng/ml). In contrast, limited activity was observed against sarcoma and leukemia cell lines (IC$_{50}$ > 100 ng/ml).

**Time Dependence of Irofulven Cytotoxicity.** To determine the influence of exposure time on the cytotoxic effects of irofulven, DU-145, HCT-116, or HT-29 carcinoma cells were exposed to different concentrations of irofulven for 5, 10, 20, 30, or 45 min or 1, 2, 6, 24, or 120 h. Clear time-dependent cytotoxic effects of irofulven were observed for all three cell lines with longer exposure times being associated with increased cytotoxicity (Fig. 3). The time dependence was particularly dramatic for exposure times $\leq$ 30–45 min. In contrast, extending the drug exposure time beyond 24 h had no influence on the cytotoxicity.

**Activity Spectra for Irofulven, Cisplatin, and ET-743.** Comparison of the activity spectra of irofulven, cisplatin, and ET-743 toward 10 different types of human tumor cells shows clear differences between the three drugs (Fig. 4). The activity of irofulven was more marked than that of cisplatin toward lung, head and neck, breast, ovary, colon, and cervix cell lines and more pronounced than that of ET-743 toward head and neck, ovary, prostate, and colon cancer cells. Interestingly, irofulven showed activity toward all cell lines, whereas cisplatin was active toward one of the three cell lines. Surprisingly, irofulven has only very limited activity toward leukemias, which is unusual for alkylating agents, and different from what is observed for both cisplatin and ET-743.

**Activity toward Multidrug Resistant Cells.** Most natural products are recognized by membrane-associated drug efflux pumps, such as the P-gp and members of the MRP superfamily. The influence of MDR was determined by comparing the cytotoxicity of irofulven on HL-60/Vinc cells, which overexpress functional P-gp (10), and HL-60/Adr cells, which overexpress functional MRP1 (11) toward that of parental HL-60 cells (Table 1). P-gp overexpression in the HL-60/Vinc cells was accompanied by $\approx$ 1400-fold resistance to vincristine and $\approx$ 30-fold cross-resistance to ET-743. In contrast, no changes were observed for irofulven or cisplatin. Overexpression of MRP1 in the HL-60/Adr cells was associated with $\approx$ 220-fold resistance to Adriamycin. In contrast, only very modest cross-resistance ($\approx$ 4-fold) was observed for ET-743, and little, if any, cross-resistance was observed for irofulven and cisplatin.

**Activity toward Cisplatin-resistant Cells.** The development of resistance to cisplatin is associated with treatment failure and disease progression in several tumor types, such as ovarian cancer. We therefore compared the activities of irofulven and ET-743 toward two well-characterized, cisplatin-resistant ovarian carcinoma cell lines, A2780/CP70 and 2008 C13*. The results show that A2780/CP70 is $\approx$ 2-fold resistant to both irofulven and ET-743 compared with the 7-fold resistance to cisplatin (Fig. 5A). Unexpectedly, 2008 C13* cells were three times more sensitive to irofulven than the parental cells, whereas
no changes were observed with respect to ET-743 efficacy, and cisplatin displayed ~2-fold resistance in this cell line (Fig. 5B).

**Influence of MMR.** Lack of MMR is associated with low-level resistance to several anticancer drugs, including cisplatin, carboplatin, and etoposide (12). To determine the influence of MMR on the cytotoxicity produced by irofulven, cisplatin, and ET-743, we compared their activity against HCT-116 colon carcinoma cells, which are deficient in DNA MMR because of inactivating hMLH1 mutations and a HCT-116 subline (HCT-116/H11001 Chr 3) where the MMR deficiency has been corrected by transfer of chromosome 3, which contains a wild-type copy of the hMLH1 gene (13). As reported previously, absence of MMR was associated with ~2-fold resistance to cisplatin (Fig. 5C). In contrast, MMR status had no influence on the sensitivity to neither irofulven nor ET-743.

**Influence of p53 and p21 Status.** Increasing evidence suggests that loss of p53 function is accompanied by increased resistance to alkylating agents, such as cisplatin (14, 15). We have compared the influence of irofulven toward parental HCT-116 human colon adenocarcinoma cells and the HCT-116 p53/−/− subline, where the p53 gene has been deleted by homogenous recombination (16). The results (Fig. 6A) show that loss of p53 function has only marginal effect on the cytotoxicity of both irofulven and ET-743. In contrast, p53 deficiency is associated with ~4-fold resistance to cisplatin compared with parental cells expressing p53. p53 is a transcription factor that is expressed at low levels in the absence of cellular stress, and its expression is induced by a variety of stimuli, usually including DNA damage. Therefore, the independence of p53 status with respect to irofulven-mediated cytotoxicity could be caused by lack of p53 induction. Alternatively, p53 might be induced by irofulven but not playing an important role in irofulven-mediated cell death. To distinguish between these two possibilities, HCT-116 cells were treated with an isotoxic dose of cisplatin, irofulven, or ET-743 followed by immunocytochemistry with a p53-directed antibody. The results show that not only cisplatin but also irofulven and ET-743 were able to induce the accumulation of nuclear p53 (Fig. 6B). The induction of p53 was further confirmed by Western blot analysis (Fig. 6C). Among the many p53 target genes, the cyclin-dependent kinase inhibitor p21cip-1/waf-1 is the most universally expressed in tumor cell lines (17). Western blot analysis of p21 expression in cells treated with an isotoxic dose of irofulven, cisplatin, and ET-743 indicated that all three agents were able to induce p21, thus suggesting that the drug-induced p53 is transcriptionally active. It should be noted that untreated HCT-116 control cells express constitutive levels of p21, which may explain the relatively modest induction of p21 after drug treatment.

We then compared the cytotoxic effect of irofulven, ET-743, and cisplatin toward HCT-116 and HCT-116 p21/−/− cells where the p21 gene has been deleted by homologous recombination (16). The results show comparable cytotoxicity toward parental and p21-null cells for all three drugs (Fig. 6A). Together, these findings clearly show that neither p53 nor p21 status play an important role in the cellular sensitivity to irofulven.

**DISCUSSION**

In this study, we have shown that irofulven has a broad activity spectrum toward many different human tumor cell lines. To identify cell types of potential interest, we have used 100 ng/ml irofulven, which corresponds to the average IC50 as a cutoff point classifying cells with IC50 < 100 ng/ml as sensitive and those with
IC_{50} > 100 ng/ml as resistant. Our results show that the majority of carcinoma cell lines tested are sensitive to irofulven.

Irofulven cytotoxicity was strongly time dependent with higher cytotoxic activities associated with longer exposure times. This was particularly evident within the first 30–45 min, where even a modest increase in exposure time was accompanied by a greatly increased cytotoxicity. The strongly time-dependent cytotoxicity of irofulven toward different carcinoma cell lines as well as the short plasma half-life of irofulven in human suggested that better antitumor effects might be obtained in patients with longer i.v. infusions than the initial administration schedule of 5 min daily for 5 days every 3 weeks. We have therefore proposed to modify the duration of irofulven exposure in clinical trials to ≥30 min. Although it is too early to evaluate the full impact of this schedule modification on the clinical activity of irofulven, preliminary data show that 30-min infusion every other week is well tolerated in patients with advanced cancer (18, 19), resulting in improved body exposure and objective clinical response.

A comparison between the cytotoxic activities of irofulven, ET-743, and cisplatin reveals numerous differences between the three agents. Most importantly, irofulven shows potent cytotoxic activity toward the majority of human carcinoma cell lines tested but modest activity toward sarcoma and limited activity toward leukemia cell lines. Cisplatin shows activity toward leukemias and many carcinoma cell lines but no activity toward

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**Table 1** Influence of irofulven, ET-743, or cisplatin on the viability of multidrug resistant cells

<table>
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<th>HL-60</th>
<th>HL-60/Vinc (P-gp)</th>
<th>HL-60/Adr (MRP)</th>
<th>Resistance level (P-gp)</th>
<th>Resistance level (MRP)</th>
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<td>Vincristine (ng/ml)</td>
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<td>900</td>
<td>ND</td>
<td>1385</td>
<td>ND</td>
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<tr>
<td>Adriamycin (ng/ml)</td>
<td>5.4</td>
<td>ND</td>
<td>1184</td>
<td>ND</td>
<td>221</td>
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<tr>
<td>Irofulven (ng/ml)</td>
<td>305</td>
<td>385</td>
<td>503</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Cisplatin (ng/ml)</td>
<td>310</td>
<td>400</td>
<td>305</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>ET-743 (pg/ml)</td>
<td>400</td>
<td>10700</td>
<td>1700</td>
<td>27</td>
<td>4.3</td>
</tr>
</tbody>
</table>

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sarcomas. Finally, ET-743 is active toward leukemia cell lines as well as toward many carcinoma and sarcoma cell lines.

There are several important differences between our tumor cell panel and the NCI 60 panel both with respect to methodology and cell types studied. The NCI 60 panel includes renal cancer and melanoma cell lines, whereas ours includes head and neck, cervix, and sarcoma cell lines. Furthermore, our results are based on long-term viability testing by MTT, whereas the NCI results are based on a short-term assay measuring the concentration of biological macromolecules by sulforhodamine B. Nevertheless, if we only consider cell lines which are common between the two tumor cell panels, the correlation in sensitivity to irofulven (NCI entry S683863) between our cell panel and the NCI 60 panel was unexpectedly good (CC = 0.733), considering the differences between the two assays.

However, irofulven and ET-743 were only weakly correlated (CC = 0.398) in our cell panel in marked contrast to the NCI results. There are two major reasons for this discrepancy. First, the correlation is influenced by the cell types included in the panel. This is illustrated by the fact that if only the cell lines which are common between the two tumor cell panels are taken into account, the correlation in sensitivity to irofulven (NCI entry S683863) between our cell panel and the NCI 60 panel was unexpectedly good (CC = 0.733), considering the differences between the two assays.

A closer analysis of the influence of irofulven on the different tumor cell lines reveals several interesting features. Irofulven showed good activity toward the poorly differentiated DU-145 and PC3 prostate cancer cell lines that are both androgen receptor negative (20) with IC50s of 25 and 75 ng/ml, respectively. In contrast, the more differentiated prostate adenocarcinoma cell line LNCaP, which is androgen receptor positive, was more resistant to irofulven (IC50 = 180 ng/ml). The activity toward hormone-independent prostate cancer cells warrants additional studies considering the need for new active compounds toward this disease.

Irofulven showed similar activity toward MCF-7 and MDA-MB-231 breast cancer cells with an IC50 of ~50 ng/ml. This suggests that the cytotoxic activity of irofulven is not noticeably influenced by estrogen receptor status, because MCF-7 is estrogen receptor positive, whereas MDA-MB-231 is estrogen receptor negative (21). More surprisingly, MDA-MB-231 cells overexpress the epidermal growth factor receptor 1 (HER-1) in contrast to MCF-7 cells that express low levels of HER-1 (22). Overexpression of HER-1 has been associated with poor prognosis, enhanced metastatic potential, and both chemoresistance and radioresistance (23). HER-1 is overexpressed in a majority of non-small cell lung

Fig. 5 The influence of irofulven, ET-743, and cisplatin on the viability of different tumor cell lines was measured by the MTT assay after continuous exposure to the indicated drugs for three doubling times. All values represent means ± SD (error bars). A, influence of the indicated drugs on parental A2780 ovarian carcinoma cells (shaded columns) and cisplatin-resistant A2780/CP70 cells (hatched columns). B, influence of the indicated drugs on parental 2008 ovarian carcinoma cells (shaded columns) and cisplatin-resistant 2008 C13* cells (hatched columns). C, influence of the indicated drugs on parental HCT-116 colon carcinoma cells (shaded columns) and HCT-116 + Chr3 cells (hatched columns) where the MMR deficiency has been corrected by transfer of chromosome 3. The indicated IC50s are in nanograms per milliliter for irofulven, picograms per milliliter for ET-743, and micrograms per milliliter for cisplatin.

sarcomas. Finally, ET-743 is active toward leukemia cell lines as well as toward many carcinoma and sarcoma cell lines.

January 2003. It is readily appreciable that the different IC50s for ET-743 are remarkably similar. This is because ET-743 is so potent that the lowest concentrations tested (10^{-10} M) is used to approximate all of the IC50s. As a result, COMPARE has only a limited number of values to correlate. In striking contrast, marked differences in sensitivity to ET-743 are observed between different tumor cell lines under our experimental conditions with ~30 times difference between the most sensitive and most resistant cell line.
cancers and virtually all squamous cell carcinomas of the head and neck (24, 25). Interestingly, irofulven was also active toward all three non-small cell lung cancer cell lines as well as toward the three head and neck carcinoma cell lines. Recently, we have confirmed these observations for the epidermoid A431 cell line, which is considered as the classical model for HER-1 overexpression.6 These results suggest that irofulven may be potentially active toward HER-1-overexpressing tumors in contrast to many other treatment modalities, such as irradiation, thereby providing a rational basis for combinations of irofulven and irradiation. Although the molecular mechanisms underlying this phenomenon are unknown, it is interesting to note that another alkylating agent, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (carmustine), has been reported to block the early activation of S6 kinase by acidic growth factor or insulin-like growth factor 1 (26). Moreover, it has been shown that cisplatin is able to activate the epidermal growth factor receptor in various types of cells that overexpress the receptor (27).

These results suggest that alkylating agents may not only activate nuclear and cytoplasmic signaling pathways but also influence the activity of membrane-integrated proteins.

The primary hydroxyl group in irofulven is readily replaced by thiols such as N-acetylcysteine and glutathione under experimental conditions (28). This suggests that irofulven is likely to be metabolized by glutathione conjugation in tumor cells, which often have very high concentrations of glutathione-associated enzymes (29). A-549 and NCI-H460 lung cancer cells have been shown to express elevated levels of glutathione S-transferase, whereas SKOv-3 ovarian carcinoma cells express high levels of γ-glutamyl cysteine synthetase (29). A-549 and NCI-H460, and SKOv-3 are among the most sensitive cell lines with IC50s ranging from 11 to 30 ng/ml irofulven (Fig. 2). Therefore, it is unlikely that glutathione conjugation is an important determinant in the natural resistance to irofulven.

Natural products are frequently recognized by membrane-associated drug efflux pumps, such as the P-gp and MRP. Expression of these proteins is associated with reduced cellular drug accumulation and cytoplasmic drug sequestration (for re-

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6 V. Poindessous, unpublished results.

Fig. 6 The influence of irofulven, ET-743, and cisplatin on HCT-116 colon carcinoma cells with respect to p53 and p21. In A, the influence of p53 and p21 status on cellular survival was determined by comparing the cytotoxicity of the three agents toward parental HCT-116 colon carcinoma cells and the HCT-116 p53 −/− and HCT-116 p21 −/− sublines, where the p53 or p21 genes have been deleted by homologous recombination. The indicated values correspond to the IC50 measured by the MTT assay after 6 h drug exposure followed by postincubation in drug-free media for three doubling times. Shaded columns, HCT-116; hatched columns, HCT-116 p53 −/−; unfilled columns, HCT-116 p21 −/−. B, immunolocalization of p53 in untreated HCT-116 colon carcinoma cells and cells treated with isotoxic doses of irofulven, ET-743, or cisplatin for 6 h. The cellular localization of p53 was determined by immunocytochemistry with anti-p53 antibodies followed by FITC-conjugated secondary antibodies. C, Western blot analysis of cellular extracts from untreated HCT-116 colon carcinoma cells and cells treated with isotoxic doses of irofulven, ET-743, or cisplatin for 6 h followed by a chase in drug-free medium for an additional 21 h. Cellular extracts were subject to Western blot analysis with antibodies directed toward p53, p21, or β actin, respectively.
cent review, see Ref. 30). The present results with vincristine- and Adriamycin-selected HL-60 cells overexpressing functional P-gp and MRP1, respectively, clearly show that irofulven is not a substrate for any of these efflux pumps. In contrast, overexpression of P-gp was associated with resistance to ET-743 in agreement with previous findings (31).

Clinical development of cisplatin resistance is associated with treatment failure and disease progression. Therefore, it is important to establish if cisplatin-resistant cells are cross-resistant to irofulven. Unexpectedly, one cisplatin-resistant ovarian cell line, A2780/CP70, was 2-fold cross-resistant to irofulven, whereas the 2008 C13* cell line was 3-fold more sensitive. Previous studies have shown that the two cisplatin-resistant ovarian cell lines share some, but not all, resistance mechanisms. Both cell lines show reduced cisplatin accumulation and DNA-cisplatin adduct formation (32–34). However, A2780/CP70 also shows increased transcription-coupled nucleotide excision repair (TC-NER) as well as altered survival signaling by the X-linked inhibitor of apoptosis protein and Akt/protein kinase B survival pathway (32, 35, 36). We have reported previously that irofulven is preferentially repaired by transcription-coupled nucleotide excision repair, which might explain the cross-resistance of A2780/CP70 cells to irofulven (37). However, it should be noted that A2780/CP70 cells are also cross-resistant to other anticancer agents, which are not recognized by TC-NER, such as 5-fluorouracil, camptothecin, and Taxol, suggesting that the altered survival signaling may render these cells resistant to many apoptotic stimuli.

The hypersensitivity of 2008 C13* cells to irofulven is more difficult to interpret. It has been reported previously that 2008 C13* cells show increased sensitivity to three other compounds, including 6-thioguanine, Taxol, and rhodamine 123. The sensitivity to 6-thioguanine is associated with increased incorporation of this nucleoside analogue into DNA because of a very high expression of the low fidelity polymerase DNA polymerase β (38). The collateral sensitivity to Taxol is not well understood but may be a result of altered expression of cytoskeleton proteins (39, 40). 2008 C13* cells also show an increased mitochondrial membrane potential leading to increased accumulation of Rhodamine 123 and subsequently increased sensitivity to this compound (41, 42). Finally, recent results show that 2008 C13* cells overexpress the α-ketoreductase dihydrodiol dehydrogenase and that this is directly associated with resistance to cisplatin (43). As of now, it is not known which, if any, of these cellular alterations may explain the increased sensitivity of 2008 C13* cells to irofulven. Nevertheless, these results demonstrate that some cisplatin-resistant ovarian tumor cells are sensitive to irofulven and are also consistent with the observed clinical activity of irofulven in patients with platinum-resistant ovarian cancer (44, 45).

Other cellular mechanisms implicated in cisplatin resistance include loss of p53 function and MMR (12, 14, 15). The results presented here suggest that none of these features are likely to play an important role in the cellular sensitivity to irofulven, similar to what has been reported for ET-743 (46, 47).

Taken together, our results show that irofulven has a broad activity spectrum that is different from that of both cisplatin and ET-743. In particular, irofulven shows potent cytotoxic activity toward the majority of carcinoma cell lines tested but no or borderline activity toward sarcoma and leukemia cell lines. Furthermore, irofulven is not or only marginally affected by the major cellular mechanisms associated with resistance to alkylating agents, including overexpression of P-gp or MRP1 transporters, as well as loss of p53 and MMR function. The results presented here should be of considerable importance for the future development of this unusual compound in experimental and clinical oncology.

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