Irofulven Cytotoxicity Depends on Transcription-Coupled Nucleotide Excision Repair and Is Correlated with XPG Expression in Solid Tumor Cells

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ABSTRACT

Background: Irofulven is a novel alkylating agent with promising clinical activity, particularly toward ovarian and hormone-refractory prostate cancers. To facilitate additional clinical development, we have aimed to identify biological markers associated with sensitivity to the compound.

Methods: Fibroblasts derived from patients with xeroderma pigmentosum or Cockayne’s syndrome along with a panel of 20 human cancer cell lines (eight different tumor types) were examined to establish the importance of nucleotide excision repair proteins in the sensitivity to irofulven.

Results: Human cells deficient in nucleotide excision repair are up to 30-fold more sensitive to the cytotoxic effects of irofulven compared with repair-proficient controls, clearly indicating that nucleotide excision repair plays a crucial role in the sensitivity to the drug. Interestingly, our results show that irofulven-induced lesions are recognized by transcription-coupled repair but not by global genome repair. Another unique feature is the pronounced sensitivity of XPD and XPB helicase-deficient cells to the drug. Comparison of the IC50 values for irofulven, cisplatin, and ecteinascidin 743 with the expression levels of ERCCI, XPD, and XPG genes in different solid tumor cell lines shows no correlation between the expression levels of any of the three nucleotide excision repair proteins and the sensitivity to ecteinascidin 743. In contrast, expression of the XPG endonuclease was correlated with the cytotoxicity for irofulven and, to a lesser degree, for cisplatin. Importantly, XPG expression was also correlated with cellular nucleotide excision repair activity.

Conclusions: Increasing evidence indicates that compromised nucleotide excision repair activity is frequent in several solid tumor types. The results presented here suggest that XPG expression in such tumors may be a useful marker to predict their sensitivity to irofulven.

INTRODUCTION

Irofulven (MGI-114, 6-hydroxymethylacylfulvene, HMAF) is a novel semisynthetic antitumor agent derived from the mushroom toxin illudin S. Irofulven has shown promising clinical activity in particular toward ovarian and hormone-refractory prostate cancers (1, 2). To facilitate additional clinical development, we have aimed to identify biological markers associated with sensitivity to the compound.

Although the molecular pharmacology of irofulven is not fully elucidated, the ability of the drug to bind covalently to biological macromolecules characterizes it as an alkylating agent (3). In contrast to other alkylators, the cytotoxicity of irofulven is not affected by loss of p53 or DNA mismatch repair function, and the drug is not a substrate for multidrug transporters such as the P-glycoprotein and multidrug resistance protein 1 (4). Furthermore, irofulven displays good activity toward tumor cell lines expressing high levels of the detoxifying enzymes glutathione S-transferase and ɣ-glutamyl cysteine synthetase suggesting that the drug is not deactivated by glutathione-mediated drug metabolism (4).

DNA repair-deficient cells often show altered sensitivity to alkylating agents. For most alkylating agents such as cisplatin, cell lines deficient in proteins needed for nucleotide excision repair show increased sensitivity (5). However, it has been reported that ecteinascidin 743 is less cytotoxic to nucleotide excision repair-deficient cell lines (6–8).

Nucleotide excision repair removes a wide diversity of DNA lesions, including UV-induced lesions, bulky chemical adducts, and some forms of oxidative damage (9–11). Nucleotide excision repair involves the action of >25 polypeptides that are present in dynamic multiprotein complexes. In humans, mutations in any of the complementation group genes, XPA to XPG, that code for different nucleotide excision repair proteins cause the hereditary disease xeroderma pigmentosum, characterized by hypersensitivity to sunlight and increased incidence of skin tumors. Recognition of DNA damage can occur either by
global genome-nucleotide excision repair that removes DNA damage from the entire genome or by transcription-coupled-nucleotide excision repair that preferentially repairs the transcribed strand of actively expressed genes. Both processes are essentially similar except for the initial damage recognition step that is performed by XPC and XPE in global genome-nucleotide excision repair or by the stalled RNA polymerase II complex in transcription-coupled-nucleotide excision repair. Transcription-coupled repair also requires the Cockayne’s syndrome-related factors CSA and CSB that are involved in remodeling the chromatin around the arrested RNA polymerase complex and/or removal of the stalled polymerase probably by ubiquitination (12–14).

In the present study we have determined the influence of nucleotide excision repair on irofulven-mediated cytotoxicity. We found that cell lines deficient for nucleotide excision repair proteins show marked sensitivity to irofulven, clearly implicating nucleotide excision repair as a major factor in the sensitivity to the drug. Furthermore, irofulven shows an unusual activity spectrum toward the different nucleotide excision repair complementation groups. Most strikingly, irofulven is repaired by transcription-coupled but not by global genome repair. Another unique feature is the pronounced sensitivity of XPD and XPB helicase-deficient cells to the drug. Comparison of IC_{50} values for irofulven, cisplatin, and ecteinascidin 743 with the expression of nucleotide excision repair proteins in 20 tumor cell lines representing eight different solid tumor types shows a correlation between cytotoxicity and expression levels of the XPG endonuclease for irofulven and, to a lesser extent, for cisplatin, but not for ecteinascidin 743. Increasing evidence indicates that compromised nucleotide excision repair activity may be frequent in several types of solid tumors. Our results suggest that XPG expression in such tumors may be a useful marker to predict their sensitivity to irofulven.

**MATERIALS AND METHODS**

**Chemicals.** Purified irofulven was supplied by MGI Pharma, Inc. (Bloomington, MN). Ecteinascidin 743 was generously provided by the National Cancer Institute (Frederick, MD). Cisplatin was purchased from Qualimed Laboratories (Levallois-Perret, France), whereas hydroxyurea was obtained from Sigma (Saint-Quentin Fallavier, France).

**Cells and Culture Media.** Nucleotide excision repair deficient cell lines were derived from unexposed skin biopsies of patients with xeroderma pigmentosum in the laboratory of A. S. and are described in Table 1. NCl-H460, NCI-H522, HT-29, IGROv1, OvCar-3, OvCar-5, SKOV-3, DU-145, SNB-19, and U-251 glioma cells were obtained from Richard Camalier, Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute. A2780 cells were a gift from Robert Brown (University of Glasgow, Bearsten, UK), whereas HCT-116 cells were provided by Bert Vogelstein (The Johns Hopkins University, Baltimore, MD). MCF-7 and SKBr-3 cells were gifts from Nancy Hynes (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland). LnCaP cells were donated by Andrzej Skladanowski (Technical University of Gdansk, Gdansk, Poland). Hep-2 and SCC-61 cells were kindly provided by Eric Deutsch (Institut Gustave-Roussy, Villejuif, France), whereas SaOs-2 and U2-0s were gifts from Annick Harel-Bellan (CNRS Institut André-Lwoff, Villejuif, France). A-549 lung carcinoma cells were donated by Olivier Brison (Institut Gustave-Roussy, Villejuif, France).

The cells were maintained in HAM F10/DMEM 1:1 (XPCS2BASV), MEM (HEP-2 and nucleotide excision repair-deficient cell lines), McCoy’s A (HCT-116 and SaOs-2), RPMI 1640 (A-549, A2780, NCI-H460, NCI-H522, LnCaP, DU-145, IGROv1, OvCar-3, OvCar-5, SKOV-3, SNB-19, and U-251) or in DMEM (HT-29, MCF-7, MDA-MB-231, SKBr-3, and SCC-61) supplemented with 10% fetal calf serum (Invitrogen, Cergy-Pontoise, France) or 15% fetal calf serum (Hep-2 and SCC-61), 2 mmol/l l-glutamine (Eurobio, Les Ulis, France), 100 units/ml penicillin, and 100 µg/ml streptomycin (PanPharma, Fougeres, France). All of the cell lines were tested regularly for Mycoplasma contamination by PCR analysis.

**Growth Inhibition Assays.** The cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (15). Growth inhibition was expressed as the growth of drug-treated cells compared with that of untreated control cells.

**Inhibition of RNA Synthesis.** Exponentially growing cells in 30-mm Petri dishes were treated with irofulven (200 ng/ml) for 3 h at 37°C followed by postincubation in drug-free medium for 21 h. RNA synthesis was determined for untreated cells, during the last hour of irofulven exposure or during the

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<td>198VI</td>
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<td>MRC5SV</td>
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Abbreviations: XP, xeroderma pigmentosum; CS, Cockayne’s syndrome; GG, global genome; GGR, global genome repair; TCR, transcription-coupled repair.
last hour of postincubation by 1-h pulse-labeling with [3H]uridine (1 mCi/ml). Cells were washed three times with PBS, trypsinized, and treated with 15% trichloroacetic acid for 30 min at 4°C. Cellular residues were filtered on GF/C glass microfiber filters (Whatman, Maidstone, England) and rinsed three times in 5% trichloroacetic acid and three times in ethanol. Filters were dried, and the incorporated radioactivity was determined by scintillation counting.

**TaqMan Analysis.** Total RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extraction yield was quantified spectrophotometrically. The quality of the RNA samples was determined by electrophoresis through denaturing agarose gels and staining with ethidium bromide. The 18 S and 28 S RNAs were visualized under UV illumination. One μg total RNA of each sample was reverse transcribed in a total volume of 20 μl containing 50 units of M-MLV reverse transcriptase, 20 units of RNase inhibitor (Applied Biosystems, Forster City, CA), 1 mmol/l dA/T/C/G (Amersham-Pharmacia, Uppsala, Sweden), 5 mmol/l MgCl₂, 10 mmol/l Tris HCl (pH 8.3), 100 mmol/l KCl, and 50 pmol random hexamers. The resulting cDNA was diluted 20 times with nuclease-free H₂O (Promega Corporation, Madison, WI).

ERCC1 (NM 001983), XPD (X52221), and XPG (NM 000123) primers and probes were designed using Primer Express (Applied Biosystems) and Oligo 4 softwares (National Biosciences, Plymouth, MN) and purchased from MWG Biotech, (Ebersberg, Germany). The sequences are as follows: ERCC1, forward primer starting at nucleotide 919, 5'-TCGGAACAGCTCATGCCG-3', reverse primer starting at nucleotide 1029, 5'-ACTTCAGAAGGCTGTGCG-3', TaqMan probe starting at nucleotide 939, 6-carboxyfluorescein-CATCAAGAAGATCTGGCCTATGCCCA-6-carboxytetramethylrhodamine; XPD, forward primer starting at nucleotide 693, 5'-GGAGGCGCTCTGGTACT-3', reverse primer starting at nucleotide 800, 5'-AGGACTCCTAGACCCTTGCC-3', TaqMan probe starting at nucleotide 736, 6-carboxyfluorescein-CCCCGAGCAGTTCTCCTAATGCCGG-6-carboxytetramethylrhodamine; and XPG, forward primer starting at nucleotide 2477, 5'-CGCTGCTACTGTCAACCGG-3', reverse primer starting at nucleotide 2587, 5'-AGGATGGGCGACTGCG-3', TaqMan probe starting at nucleotide 2532, 6-carboxyfluorescein-TTCCGACATCCATCAGGCTC-6-carboxytetramethylrhodamine. 18S RNA primers and probes (Applied Biosystems) are used according to the manufacturer’s instructions. PCR amplifications were performed using the TaqMan Universal Master Mix in standard conditions according to the manufacturer’s instructions. The reaction was performed in 50 μl containing the cDNA equivalent to 25 ng total RNA, 1X TaqMan Buffer A, 5 mmol/l MgCl₂, glycerol, 200 μmol/l dATP, dCTP, dGTP, and 400 μmol/l dUTP, 125 units of AmpliTaq Gold, 0.5 units of UNG (AmplErase uracil-N-glycosylase), 250 nM of each primer, and 100 nM of the probes. Each reaction was performed in triplicate. ABI 7700 Sequence Detector System was set up according to the manufacturer’s standard thermal cycling conditions. The SDS software was used to analyze the fluorescent signals and to calculate the cycle threshold.

Relative quantification of steady state levels of mRNAs in the different samples was performed using the comparative cycle threshold method according to the manufacturer's instructions. With this method, one of the samples is used as the calibrator (here, the OvCar-3 cells), and all of the steady state levels in the other samples are expressed as an x-fold ratio relative to the calibrator.

**Unscheduled DNA Synthesis.** Unscheduled DNA synthesis was determined as described previously (16). Cells were seeded on glass coverslips and grown for 24 h. The concentration of fetal calf serum was reduced to 0.5% in the presence of 10 mmol/l hydroyxurea and 100 μmol/l fluordeoxouridine. The following day, cells were labeled with [3H]thymidine (10 μCi/ml) for 1 h and irradiated with the indicated doses of UV irradiation. Cells were then labeled with [3H]thymidine (10 μCi/ml) for 3 h, postincubated with cold thymidine for 1 h, and subjected to autoradiography. After 1 week, the average number of grains was determined for at least 30 nuclei/sample. Results are the average of a minimum of two independent experiments.

**Statistical Analysis.** All of the statistical analyses were performed using the R language. 6 The relationship among ERCC1, XPD, and XPG mRNA expression and the sensitivity to irofulven, cisplatin, and ecteinascidin 743 was analyzed by Pearson correlation. Values of P < 0.05 were considered statistically significant. When appropriate, data are presented as the mean ± SD.

**RESULTS**

**XPA Is Required for Repair of Irofulven Damage.** To determine the influence of nucleotide excision repair on the cellular response to irofulven lesions, we compared the cytotoxicity of irofulven toward XPA cells that are totally deficient in nucleotide excision repair and repair-proficient normal or SV40 transformed human fibroblasts. The results show comparable sensitivity to irofulven for the normal and the SV40-transformed human fibroblasts (Fig. 1A). In contrast, XPA cells were 30-fold more sensitive to irofulven compared with the repair-proficient control cells clearly indicating that nucleotide excision repair plays a crucial role in the cellular sensitivity to the drug.

**Cells Deficient in the XPD and XPB Helicases Are Highly Sensitive to Irofulven.** An interesting feature of illudin S and acylfulvene, two compounds structurally related to irofulven, is their potent cytotoxic activities toward XPD- and XPB-deficient cells (17–19). We show here that this feature is shared by irofulven, because cells with compromised XPD and XPB functions are up to 20-fold more sensitive to irofulven compared with repair-proficient control cells (Fig. 1B).

**Cells Deficient in the XPG and XPF Endonucleases Show Increased Sensitivity to Irofulven.** Cells deficient in XPG and XPF endonuclease activities are up to 17-fold more sensitive to irofulven compared with repair-proficient controls (Fig. 1C). Interestingly, the sensitivity for the XPG-deficient XPCS1LV cells was much more pronounced compared with the XPG-deficient XP440VI cells. A major difference between the two cell lines is that XPCS1LV cells in addition to the endo-
Irofulven and Cisplatin Show Different Activity Profiles Toward Nucleotide Excision Repair-Deficient Cells. Comparison of the activity spectrum toward the different nucleotide excision repair complementation groups reveals a number of important differences between irofulven and cisplatin (Fig. 4). Deficiency in nucleotide excision repair proteins was associated with up to 30-fold increased sensitivity to irofulven compared with 8-fold increased sensitivity to cisplatin. These results suggest that nucleotide excision repair is more crucial for the sensitivity to irofulven than to cisplatin. This is consistent with our previous findings showing that other factors such as p53 and DNA mismatch repair status that are known to influence the sensitivity to cisplatin play no role for irofulven (4).

Another difference between the two agents is that cisplatin- but not irofulven-mediated DNA lesions inhibit transcription and require functional transcription-coupled nucleotide excision repair to overcome this effect.

Irofulven Lesions Are Exclusively Removed by Transcription-Coupled Repair. To additionally establish the importance of transcription-coupled repair in the recognition of irofulven-induced DNA lesions, the influence of irofulven toward XPC-deficient XP202VI cells that are only deficient in global genome repair were compared with that of CS539VI cells, which are only deficient in transcription-coupled repair. The results show no difference in the cytotoxic effects of irofulven toward XPC-deficient cells and the repair-proficient controls suggesting that irofulven-induced lesions are not processed by global genome repair (Fig. 1D). In clear contrast, the CS539VI cells, which are derived from a patient with Cockayne’s syndrome and are only deficient in transcription-coupled repair, showed >10-fold increased sensitivity to irofulven.

Brief Irofulven Exposure Is Associated with Prolonged Transcriptional Inhibition in Nucleotide Excision Repair-Deficient Cells. Transcription-coupled repair is initiated when the transcription fork is stalled by an unrepaired DNA lesion thereby preventing ongoing RNA synthesis (Fig. 2A). Subsequent removal of the lesion will allow progression of the RNA polymerase complex and transcriptional recovery (12–14). Three-hour exposure of human fibroblasts to irofulven (200 ng/ml) was associated with transcriptional inhibition in both nucleotide excision repair-deficient and repair-proficient control cells (Fig. 2B). Subsequent postincubation in drug-free medium was accompanied by transcriptional recovery of XPC and control cells. In contrast, the RNA synthesis remained depressed in both XPD- and Cockayne’s syndrome-deficient cells even after 21 h postincubation in drug-free medium. These results demonstrate that irofulven-mediated DNA lesions inhibit transcription and require functional transcription-coupled nucleotide excision repair to overcome this effect.

Irofulven Sensitivity Is Common for XPD Cells Lines and Can Be Overcome by Complementation with Functional XPD. To establish if the unusual high degree of sensitivity to irofulven observed for the XPD cells is a common feature of XPD-deficient cells, a second XPD cell line that was derived from a different patient was examined. The XP22VI is homozygote for the R683W mutation, whereas the XP6BE is mutated on this site for one allele and carries a 36–61 amino acid deletion in the second allele. The results show that the two XPD-deficient cell lines have comparable sensitivity to irofulven with IC50 values of 7 and 9 ng/ml (Fig. 3A). To additionally establish the role of XPD activity in the sensitivity to irofulven, XPD-deficient XP22VI cells were complemented with cDNA of the wild-type XPD gene as described previously (20). The results show that re-expression of functional XPD restored the drug resistance of XP22VI cells to the same levels as the repair-proficient control cells (Fig. 3B).
not irofulven-induced lesions are recognized by global genome repair. However, even for cisplatin, the role of XPC is less pronounced than for transcription-coupled-nucleotide excision repair-deficient cells, in agreement with the findings of others (21). Finally, XPD deficiency is associated with 20-fold increased sensitivity to irofulven but only 2.5-fold increased sensitivity to cisplatin. Together, these findings clearly indicate that irofulven-induced DNA lesions are differently recognized and repaired compared with the lesions induced by cisplatin, a classical alkylating agent.

Influence of Nucleotide Excision Repair Protein Expression on the Sensitivity of Solid Tumor Cells to Irofulven, Cisplatin, and Ecteinascidin 743. Increasing evidence suggests that decreased nucleotide excision repair may be frequent in several tumor types including carcinomas of the lung, ovary, and head and neck, as well as in gliomas (22–30). Determination of the cytotoxicity of irofulven toward 20 different human tumor cell lines representing eight different tumor types suggests that irofulven is consistently active toward the above-mentioned tumor types (Fig. 5). In comparison, cisplatin and ecteinascidin 743 give a more heterologous activity pattern. Interestingly, only irofulven is active toward both head and neck cancer cell lines, whereas only ecteinascidin 743 is active toward the two sarcoma cell lines.

Expression of three nucleotide excision repair proteins, the XPD helicase, the ERCC1 endonuclease, and the XPG endonuclease, were quantified by real-time reverse transcription-PCR (TaqMan), and their expression levels compared with the IC_{50} values of irofulven, cisplatin, or ecteinascidin 743 in the same tumor cell lines (Fig. 6). The r value corresponds with the correlation coefficient calculated by linear regression analysis. The results show that there is a correlation between cytotoxicity and nucleotide excision repair expression levels for the XPG endonuclease. The correlation was greatest for irofulven and was seen to a lesser degree for cisplatin. In contrast, no correlation was observed between the expression levels of any of the three nucleotide excision repair proteins and the sensitivity to ecteinascidin 743.

XPG Expression Is Correlated with Sensitivity to UV Radiation and Cellular Nucleotide Excision Repair Activity. Because XPG has several cellular functions in addition to its role as a repair endonuclease, we wanted to determine whether

Fig. 2 Influence of irofulven on RNA synthesis. A, model illustrating the stalling of transcription forks by DNA lesions. The elongating RNA polymerase is shown in gray. CTD, COOH-terminal domain of RNA polymerase II. B, transcriptional recovery after irofulven exposure. Cells were exposed to irofulven (200 ng/ml) for 3 h followed by postincubation in drug-free medium. RNA synthesis was determined by pulse-labeling with [H]uridine for 1 h before irofulven exposure, after 2-h irofulven exposure, or after 20-h postincubation in drug-free medium. Relative rates of RNA synthesis are expressed in percentage compared with untreated control cells. ● 198VI (normal cells), ○ XP202VI (XPC), △ CS539VI (CS), □ XP6BE (XPD). Bars, ±SE and are indicated when they exceed the symbol size.

Fig. 3 Influence of the XPD helicase activity on irofulven cytotoxicity. Cellular viability was determined by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay after continuous exposure to irofulven for four doubling times. A, cytotoxicity of irofulven toward XPD cells derived from different patients. Symbols used: ● 198VI (normal cells), △ XP22VI (XPD). B, cytotoxicity of irofulven toward XPD cells and XPD cells complemented with functional XPD. Symbols used: ● 198VI (normal cells), ○ XP22VI (XPD), △ XP22VI complemented with the wild-type cDNA of XPD. Bars, ±SE, and are indicated when they exceed the symbol size.
the cellular level of XPG reflects its role in nucleotide excision repair or if the correlation between irofulven cytotoxicity and XPG expression may be due to other activities. To establish this point, three solid tumor cell lines, OvCar-3 ovarian, HT-29 colon, and SkBr-3 breast cancer cells, which exhibit a wide range of XPG expression, were selected (Fig. 7A). The three cell lines also exhibit a wide range of sensitivities to irofulven as well as to UV radiation, the classical example of an agent that is repaired by nucleotide excision repair (Fig. 7B). To determine whether XPG levels were correlated with in vivo nucleotide excision repair activity, the cell lines were irradiated by UV light and the nucleotide excision repair activity determined by unscheduled DNA synthesis. The results show very low DNA repair in the OvCar-3 compared with HT-29 and SkBr-3 cells. Interestingly, the dose response differed among the three cell lines. DNA repair in the very sensitive OvCar-3 cells reaches a maximum around 7 J/m² and decreases at higher doses. DNA repair in the moderately sensitive HT-29 cells reaches a maximum around 10 J/m² and remains stable at higher doses, whereas the highly resistant SkBr-3 cells show a linear dose response over the entire range of UV doses tested (Fig. 7C). These results strongly suggest that in these cell lines, the expression of the XPG endonuclease reflects the levels of cellular nucleotide excision repair activity. In comparison, no dose response was apparent between ERCC1 expression and unscheduled DNA synthesis activity, whereas a weak dose response was observed for XPD.

DISCUSSION

DNA repair-deficient cells often show altered sensitivity to alkylating agents such as cisplatin or ecteinascidin 743. We
report here that nucleotide excision repair-deficient human cells are up to 30-fold more sensitive to the cytotoxic effects of irofulven compared with repair-proficient controls clearly implicating nucleotide excision repair as a major factor in the sensitivity to the drug.

The exact structure of the irofulven adducts has not yet been elucidated. However, current evidence suggests that the adducts are most likely monofunctional, because irofulven exposure is not associated with the appearance of DNA interstrand cross-links or in the formation of covalent DNA-protein complexes (31). We now show that irofulven-induced DNA lesions are repaired by transcription-coupled repair but not by global genome repair. The absence of global genome repair suggest that the irofulven-DNA adducts do not result in major perturbations of local DNA architecture.

The XPD helicase is a multifunctional protein that is needed for both nucleotide excision repair and transcriptional initiation (32). Whereas the helicase activity is required during nucleotide excision repair for the formation of both the 5′ and the 3′ incision around a site of DNA damage, the catalytic activity is not absolutely necessary for transcriptional initiation (33). The importance of functional XPD suggests that it is difficult to gain access to and open the regions with irofulven-induced DNA lesions. This notion is also consistent with the importance of XPA that together with RPA play a key role in damage recognition and repair (34–36).

The heterodimeric ERCC1/XPF endonuclease plays a role in nucleotide excision repair as well as in the repair of bifunctional alkylating agents, where it is needed to release one arm of the cross-link or to remove them by recombination (37–39). The modest influence of XPF in the sensitivity to irofulven suggests that irofulven acts as a monofunctional DNA damaging agent in agreement with previous findings (31).

XPG is a multifunctional protein with different roles in nucleotide excision repair. Besides its catalytic endonuclease activity that is required to make the incision 3′ to the DNA lesion, XPG needs to be present to permit the 5′ incision by ERCC1/XPF (40–42). XPG is also able to recruit proliferating cell nuclear antigen, an essential component of repair DNA synthesis (43). Finally, XPG has a, thus far poorly understood, activity that is needed for the initial step of transcription-coupled repair and most likely is based protein-protein in-
XPD. These results indicate fundamental differences between cisplatin and irofulven, in particular with regard to XPC and sion repair complementation groups is clearly different for the irofulven adducts by transcription-coupled repair. To sum are in full agreement with the preferential recognition of XPG cells derived from a patient with mild xeroderma pigmentosum are much more sensitive to the cytotoxic effects of irofulven than from a patient with xeroderma pigmentosum/Cockayne are phenotypes (46, 47). Our observation that XPG cells derived rise to both the xeroderma pigmentosum and the Cockayne mutations leading to decreased endonuclease activity are associated with xeroderma pigmentosum, whereas protein truncations associated with loss of transcription-coupled repair give XPD levels were not related to the cytotoxicity for any of the three drugs. ERCC1 expression has been correlated consistently with tumor sensitivity to platinum chemotherapy in ovarian cancer (48, 49). However, ERCC1 levels showed no obvious correlation with drug sensitivity when multiple different tumor types were compared (Ref. 50 and this study). A lack of correlation was also observed for XPD. These findings are in apparent contrast with a recent study reporting that XPD protein levels are inversely associated with the sensitivity to alkylating agents in the National Cancer Institute tumor cell panel (50). However, the same study also showed that XPD levels were neither correlated with nucleotide excision repair activity of cellular extracts nor with the sensitivity to UV light, strongly suggesting that XPD might contribute to other repair functions besides nucleotide excision repair (50).

In contrast to ERCC1 and XPD, XPG levels were correlated with the cytotoxic effects of irofulven and cisplatin. The correlation was greatest for irofulven ($r = 0.65; P = 0.002$) and was seen to a lesser extent for cisplatin ($r = 0.59; P = 0.006$). Importantly, XPG levels were also associated with the sensitivity to UV light as well as with cellular nucleotide excision repair activity as determined by unscheduled DNA synthesis. These results suggest that in many tumor cells, XPG might be the rate-limiting step for nucleotide excision repair consistent with its generally very low cellular protein levels compared with other nucleotide excision repair factors.

Figure 7: The influence of XPG expression on cellular nucleotide excision repair activity. A, OvCar-3, HT-29, and SKBr-3 cells express different levels of XPG. Bars, ±SE. B, sensitivity of OvCar-3, HT-29, and SKBr-3 toward UV irradiation, a classical DNA damaging agent predominantly repaired by nucleotide excision repair, and toward irofulven. Open columns, irofulven-treated cells; hatched columns, UV-treated cells. Bars, ±SE. C, cellular nucleotide excision repair activity as measured by unscheduled DNA synthesis (UDS) after exposure to the indicated doses of UV-radiation. Bars, ±SE and are indicated when they exceed the symbol size.

The sensitivity profile of the different nucleotide excision repair complementation groups is clearly different for cisplatin and irofulven, in particular with regard to XPC and XPD. These results indicate fundamental differences between the two drugs not only with respect to the nature of the DNA adducts formed but also with regard to their influence on local chromatin architecture and subsequent recognition by repair proteins. Interestingly, these findings are consistent with our previous drug combination studies reporting that simultaneous exposure to irofulven and cisplatin is associated with at least additive cytotoxic effects toward most tumor cell lines tested (15).

Decreased DNA repair capacity is not limited to patients with xeroderma pigmentosum or Cockayne’s syndrome but has also been described for cancer patients with solid tumors. Decreased DNA repair, as determined by host-cell reactivation of a damaged reporter plasmid, has been observed for cells from lung cancer patients as well as for cells from head and neck cancer patients (22–24). Furthermore, loss of heterozygosity of genes coding for nucleotide excision repair proteins is common in both ovarian tumors and in squamous cell carcinoma of the head and neck (25–28). Decreased copy numbers of genes coding for nucleotide excision repair proteins can also occur in malignant gliomas, as well as in colon and lung tumors (26, 29, 30). Intriguingly, our previous results indicate that irofulven is particularly potent toward glioma cells as well as toward head and neck, ovarian, and lung carcinoma cells (4) suggesting a role for nucleotide excision repair factors in the natural sensitivity to the drug.

To establish if irofulven cytotoxicity is correlated with the expression of specific nucleotide excision repair factors, cellular levels of ERCC1, XPD, and XPG gene expression were determined by reverse transcription-PCR and compared with the cytotoxic effects of irofulven in eight different solid tumor types. For comparison, cisplatin and eteinoscinidin 743 were also included.

The results show that the expression levels of the ERCC1 or XPD genes were not related to the cytotoxicity for any of the three drugs. ERCC1 expression has been correlated consistently with tumor sensitivity to platinum chemotherapy in ovarian cancer (48, 49). However, ERCC1 levels showed no obvious correlation with drug sensitivity when multiple different tumor types were compared (Ref. 50 and this study). A lack of correlation was also observed for XPD. These findings are in apparent contrast with a recent study reporting that XPD protein levels are inversely associated with the sensitivity to alkylating agents in the National Cancer Institute tumor cell panel (50). However, the same study also showed that XPD levels were neither correlated with nucleotide excision repair activity of cellular extracts nor with the sensitivity to UV light, strongly suggesting that XPD might contribute to other repair functions besides nucleotide excision repair (50).
In the current study, no correlation was observed between ecteinascidin 743 cytotoxicity and the expression of any of the nucleotide excision repair factors examined including XPG. Another study has reported down-regulation of XPG for two different colon carcinoma cells selected for resistance to ecteinascidin 743 (7). Therefore, although XPG can contribute to the acquired resistance to ecteinascidin 743, the protein does apparently not influence the natural resistance to the drug.

Taken together, our results show that nucleotide excision repair-deficient cells are markedly sensitive to irofulven clearly implicating nucleotide excision repair as a crucial factor in the cellular sensitivity to the drug. Comparison of the IC50 values for irofulven with the expression levels of three nucleotide excision repair proteins, ERCC1, XPD, and XPG, in 20 human solid tumor lines representing eight different tumor types shows a clear correlation between cytotoxicity and expression of the XPG endonuclease. Increasing evidence indicates that compromised nucleotide excision repair activity is frequent in several solid tumor types. The results presented here suggest that XPG expression in such tumors may be useful to predict their sensitivity to irofulven.

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REFERENCES


8. Zewail-Foote M, Li VS, Kohn H, Bearss D, Guzman M, Hurley LH. The inefficiency of incisions of eteineascidin 743-DNA adducts by the UvrABC nuclease and the unique structural feature of the DNA adducts can be used to explain the repair-dependent toxicities of this antitumor agent. Chem Biol 2001;8:1033–49.


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