NADPH Alkenal/One Oxidoreductase Activity Determines Sensitivity of Cancer Cells to the Chemotherapeutic Alkylation Agent Irofulven

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ABSTRACT

Illudins S and M are extremely cytotoxic products of the fungus Omphalotus illudens. They were evaluated as possible anticancer chemotherapeutic agents but displayed unfavorable therapeutic indices. Irofulven (6-hydroxy-methylacylfulvene), a less toxic, synthetic derivative of illudin S, has proven very effective in many preclinical and clinical studies. It has been postulated that metabolism via hydrogenation of the 8,9-double bonds of these molecules would unmask the electrophilic, and thus, the toxic nature of their cyclopropyl moieties. Illudins S and M were found to be rapidly metabolized by NADPH-dependent alkenal/one oxidoreductase activity. Out of the 60 human tumor cell lines used by the National Cancer Institute to screen potential chemotherapeutic compounds, those that were activated by AOR were trapped with glutathione and identified by high performance liquid chromatography with tandem mass spectrometry. Samples of the 60 human tumor cell line panel used by the National Cancer Institute to evaluate potential chemotherapeutic compounds were assayed for AOR activity, which correlated positively with previously determined growth inhibitory measures for irofulven, but not illudin M or S. Collectively, these data indicate that bioactivation of irofulven by AOR plays a predominant role in its chemotherapeutic activity.

INTRODUCTION

Of the limited number of clinically active anticancer chemotherapeutic compounds, alkylating agents are an invaluable subset (1). They are electrophilic and trigger cell death by covalently binding to cellular nucleophiles such as DNA and proteins (2). Several of these agents, including cyclophosphamide, ifosphamide, and mytomycin C, are prodrugs that require metabolic activation to unmask inherent electrophilicity of certain of their functional groups. Selective toxicity to cancer cells can be achieved by specific uptake of the activated metabolite (cyclophosphamide and ifosphamide) or by activation of the agent by an enzyme or microenvironment that is predominant in these cells (temozolomide and mytomycin C; Ref. 2). Hundreds of alkylating compounds have been tested for anticancer activity; however, most are unable to provide preferential killing of cancer cells. Irofulven has proven to be an exception by performing exceptionally well in multiple human tumor xenograft experiments (3, 4). Several studies have demonstrated complete cures of mice bearing tumors derived from relatively hard to kill HT29 and MV522 cell lines after irofulven administration (5, 6). Such success in preclinical studies has spawned several Phase I and II (and one Phase III) clinical trials (7). However, little is known about the chemical mechanism of toxicity of this molecule.

Irofulven is a semisynthetic derivative of the mycotoxin illudin S (8). The sequiterpenes illudin S and illudin M are unique toxins produced by the bioluminescent jack o’lantern mushroom (Omphalotus illudens) and related species (9). They possess potent antibacterial and antitumor properties. Illudin S exhibits cytotoxic and cytostatic properties at nanomolar concentrations in several human tumor cell lines in vitro (10). It is actively transported into cells where it is thought to form protein crosslinks, causing exceptionally well in multiple human tumor xenograft experiments (3, 4). Several studies have demonstrated complete cures of mice bearing tumors derived from relatively hard to kill HT29 and MV522 cell lines after irofulven administration (5, 6). Such success in preclinical studies has spawned several Phase I and II (and one Phase III) clinical trials (7). However, little is known about the chemical mechanism of toxicity of this molecule.

Hints into the chemistry behind illudin and acylfulvene toxicity have been gleaned from the structures of metabolites isolated from reactions with rat liver cytosol (13, 14). It was postulated (13, 14) that reduction of the carbon-carbon double
bond of the α,β-unsaturated ketone of an illudin or acylfulvene would lead to an extremely unstable, electrophilic cyclohexadiene intermediate (Fig. 1). Attack of the cyclopropyl group by a cellular nucleophile would lead to adduct formation, dysfunction of the adducted macromolecule, and ultimately cell death. Heretofore such a biological produced adduct has not been observed directly. Chlorinated and hydroxylated metabolites have been isolated and are thought to represent reaction of this electrophilic intermediate with chloride anion and water nucleophiles, respectively (13). A second electrophilic site, an α,β-unsaturated ketone, exists in each of the molecules and readily reacts, in the case of the illudins only (15), with reduced glutathione (GSH) and other sulfhydryl-containing species (Fig. 1). An enzyme responsible for reduction of the α,β-double bond of illudin S has been partially characterized from rat liver cytosol (16). Several of its characteristics were identical to those of rat NADPH-dependent alkenal/one oxidoreductase (AOR) including subcellular localization, cofactor preference, and inhibition profile (17). AOR had been characterized previously as an inducible enzyme that catalyzes the detoxication of α,β-unsaturated lipid aldehydes formed during lipid peroxidation (17). We found that recombinant AOR rapidly reduces the α,β-double bonds of illudins S and M, as well as irofulven. Cells engineered to overexpress rat AOR demonstrate increased sensitivity to irofulven but not to illudin M, suggesting that illudin cytotoxicity is not dependent on enzymatic activation, whereas that of irofulven is. Furthermore, activities of AOR in 60 human tumor cell lines used for evaluating potential anticancer agents were positively associated with irofulven sensitivity. These data indicate that the mechanism of irofulven cytotoxicity is distinct from that of the illudins and that the level of expressed AOR activity in cells is a major determinant of sensitivity to irofulven.

MATERIALS AND METHODS

Chemicals and Reagents. Illudins M and S were supplied by the Developmental Therapeutics Program of the National Cancer Institute (NCI). Irofulven was synthesized from illudin S as described previously (8). It was purified using preparative TLC and flash chromatography and analyzed by 1H nuclear magnetic resonance (NMR) and electrospray-mass spectrometry. Purity was estimated to be >95% by TLC. Recombinant rat AOR was expressed and purified as described previously (17). GSH, NADPH, and all of the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Kinetic Measurement of Metabolism of Illudins and Irofulven by AOR. Three similar assays were used to quantify rates of metabolism of illudins M and S, and irofulven. Varied concentrations of illudin M were added to a 200 μl 0.5× PBS solution containing 115 ng of AOR and 43.4 nmol NADPH, and incubated for 1 min at 37°C. Reactions were then extracted with ethyl acetate containing piperine (internal standard), dried under vacuum, resuspended in high-performance liquid chromatography (HPLC) buffer A (60% acetonitrile:40% water), and injected into a Hewlett-Packard 1050 HPLC system equipped with a Luna C18 column (Phenomenex Inc.). Metabolites were separated using buffer A at a flow rate of 1 ml/min and quantitated using diode array detection at 205 nm and previously reported extinction coefficients (13). V_{max} and K_{m} were calculated using the HYPER program. Illudin S was assayed in the same way with the following modifications: HPLC buffer B (60% acetonitrile:40% water), an umbelliferone internal standard, 38.3 ng of AOR, and a 2-min incubation time were used. Irofulven was assayed in a similar fashion but with the following modifications: HPLC buffer C

Fig. 1 Illudins and irofulven have the potential to react with nucleophiles nonenzymatically (NE) through addition to the 8,9-double bond or with the cyclopropyl group after enzymatic reduction (E) of this double bond.
Identification of Metabolites. Large-scale reactions were completed for analysis of illudin M and irofulven metabolites by NMR and/or mass spectrometry. Ten mg of substrate were incubated with 66.7 mg of NADPH and 460 μg of AOR in 50 ml 0.5× PBS overnight at 37°C. Metabolites were extracted with ethyl acetate, dried under vacuum, and purified using either flash chromatography or preparative TLC. ¹H and ¹³C NMR analyses were completed on a Varian UNITY Plus 500-MHz NMR spectrometer. Electrospray-mass spectrometry was completed on a PE-Sciex API 150EX mass spectrometer.

AOR Overexpression Studies. AOR-overexpressing 293 cells were produced and maintained as described previously (17). Blank vector control, pCEP4, and overexpressing cells, pCEP4/AOR, were plated in 96-well plates at a density of 30,000 cells/well. Twenty-four h later, medium was replaced with serum-free medium containing the appropriate concentration of illudin M, irofulven, or metabolite dissolved in methanol. One to three serial dilutions were used to create the range of concentrations. Dicumarol was dissolved in 0.1× NaOH and did not change the pH of the medium after addition. Cells were incubated for 24 h, then viability was quantitated using a methylthiazoletetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (18). A methanol-only control was used to determine 100% viability.

Nonenzymatic Reactivity of Illudin M and Irofulven with GSH. Reaction of the 8.9-double bond with GSH was followed spectrophotometrically at 330 nm. At this wavelength illudin M and irofulven have significant absorbances due to conjugation of the 8.9 and 4,5-carbon-carbon double bonds with the ketone (9). Rearranged metabolites have no absorbance at this wavelength. GSH (80 μM) was added to 100 μM of either illudin M or irofulven in PBS (pH 7.4), and the reaction was monitored for 1 h at 30°C.

High Performance Liquid Chromatography with Tandem Mass Spectrometry (LC/MS/MS) of GSH Adducts. GSH adducts were prepared by adding 60 nmol of either illudin M or irofulven dissolved in acetonitrile to 200 μl of ammonium bicarbonate (pH 7.2) containing 1.8 μg AOR, 3.25 mM GSH, and 0.6 mM NADPH. These reactions were incubated at 37°C for 1 h. Controls lacking NADPH were performed. Reactions were stopped and deproteinized with addition of 400 μl of ice-cold acetonitrile, spun at 3500 × g for 10 min at 4°C, and the supernatants were evaporated to dryness under vacuum. Residual GSH and NADPH cofactors were removed using an Oasis C18 sample prep column (Waters Chromatography). Adducts were eluted with methanol, evaporated to dryness under vacuum, resuspended in 250 μl of 0.1% acetic acid, and detected using liquid chromatography electrospray ionization tandem mass spectrometry performed in the positive ion mode. Samples were directly injected into a Finnigan MAT HPLC system equipped with a Luna C18 column and coupled to a Finnigan LCQ detector. A flow rate of 0.1 ml/min and gradient of 0% Buffer D [70% acetic acid (0.1%):30% acetonitrile] to 100% Buffer E [20% acetic acid (0.1%):80% acetonitrile] over 30 min were used to resolve the compounds. Adducts were detected by monitoring for selected molecular ions or characteristic molecular fragments after MS/MS. Molecular fragments were obtained with 35% relative collision energy.

Correlation of AOR Activity with Irofulven Sensitivity. Frozen cell pellets of the 60 human tumor cell lines used to screen potential chemotherapeutic agents were obtained from the molecular targeteers program of the NCI. These pellets were thawed and resuspended in 400 μl PBS containing a mixture of mammalian protease inhibitors (Sigma). Cells were lysed by three freeze-thaw cycles, and centrifuged at 10,000 × g for 30 min at 4°C and then 100,000 × g for 1 h. Supernatants were then transferred to clean microfuge tubes and their protein content measured using BCA reagent (Pierce). AOR activities were measured using the illudin M HPLC assay described supra with the following changes: 20 μg of total protein, 60 nmol of illudin M, and 120 nmol of NADPH were dissolved in 200 μl of chloride-free 100 mM potassium phosphate buffer (pH 7.2). Reactions were incubated at 37°C for 1 h and then stopped by addition of 300 μl of cold ethyl acetate containing curcumin as internal standard. Only hydroxylated metabolite peaks were detected and quantified. Activities were determined in triplicate and controls lacking NADPH were performed for each cell line. This assay was validated using a previously described chalcone assay to measure AOR activity in a random set of six cell lines (17). Growth-inhibitory measures of irofulven and illudin sensitivity were obtained from the NCI. Logarithmic values of both sensitivity and AOR activity were plotted, and R² values were calculated using linear regression.

RESULTS

Identification of Illudin and Irofulven Metabolites. Incubations of illudins M and S (Fig. 2; 1) with recombinant rat AOR in 0.5× PBS supplemented with NADPH lead to the formation of two metabolites each, as was reported after incubation with rat liver cytosol (13). Illudin M metabolites were purified from a larger scale reaction, subjected to analyses by ¹H NMR and electrospray-mass spectrometry, and determined to be identical to the previously reported metabolites (3 and 4 from 1, and 5 and 6 from 2). Incubations of irofulven, 7, with AOR lead to formation of only one metabolite, 8 (14). Similar analyses were used to confirm its identity.

Kinetic Analysis of Illudin and Irofulven Metabolism by AOR. Three HPLC assays (as outlined in “Materials and Methods”) were used to measure the Vmax and Km of metabolism of illudins S and M, and irofulven. Rates were quantitated from metabolite peak areas using previously reported extinction coefficients (13, 14). Illudins S and M are rapidly metabolized by AOR at rates exceeding those of any other substrate identified (Table 1; Ref. 17). UV/visible wavelength spectra of all of the illudin metabolites were identical. Hydroxylation at the 15-position of illudin S appears to negatively effect binding to AOR, as evidenced by a higher Km than illudin M, but allows a slightly higher maximal rate. The aromaticity of the cyclopentane ring of irofulven is likely the cause of the 100-fold decrease in the rate of metabolism by AOR. However, this modification

3 Internet address: http://www.dtp.nci.nih.gov.
does not seem to effect binding, as its $K_m$ is comparable with that of illudin M.

**Effect of AOR Overexpression on Sensitivity of 293 Cells to Agents.** Human embryonic kidney cells were transfected with either an episomal AOR overexpression vector (pCEP4/AOR) or a control vector (pCEP4). Transfected cells were then selected by addition of hygromycin to the growth medium, producing a 25-fold increase in AOR activity over control cells. Percentage of viability was measured in cells seeded in 96-well plates using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 24 h after treatment with irofulven, illudin M, or vehicle. Overexpression of AOR did not significantly affect illudin M cytotoxicity over many concentrations (Fig. 3A). The concentration required to produce 50% lethality of this compound was measured to be $\sim 1 \mu M$, which is 2 orders of magnitude less than its $K_m$. Thus, illudin cytotoxicity is likely not dependent on reduction of the $\alpha,\beta$-double bond by AOR. Overexpression of AOR dramatically (>100-fold) decreased the concentration required to produce 50% lethality of irofulven (Fig. 3B). This drastic change is likely due to the unmasking of the electrophilicity of the cyclopropyl group through metabolism by AOR. This activation was attenuated partially with addition of 100 $\mu M$ dicumarol, an AOR inhibitor. This concentration of dicumarol alone was found to have no effect on cell growth. Inhibition of metabolically mediated cell death was not complete, presumably because of poor dicumarol solubility coupled with meager penetration of the cell membrane. The irofulven metabolite, 8, displayed no toxicity at concentrations up to 40 $\mu M$, ruling out the possibility that a combination of the metabolite and increased AOR levels is responsible for the change in cytotoxicity (data not shown).

**Nonenzymatic Reductions of the 8,9-Double Bond with GSH.** The sulfhydryl group of GSH is a potent nucleophile that rapidly reacts with most electrophilic $\alpha,\beta$-unsaturated carbonyl groups via a Michael-addition mechanism. The reactivity of this moiety in the illudins and irofulven can be monitored spectrophotometrically by following a change in absorbance at 330 nm (9). On reduction of the $\alpha,\beta$-bond and intramolecular rearrangement, this absorbance is lost, as evidenced by spectra of the metabolites. Incubations at physiologically relevant GSH concentrations and pH suggest that the $\alpha,\beta$-unsaturated ketone of irofulven is relatively resistant to nucleophilic attack, whereas that of illudin M is not (Fig. 4). These data, in conjunction with those gathered in the AOR overexpression experiments, support the hypothesis that illudin cytotoxicity is mediated by nonenzymatic attack of the $\alpha,\beta$-double bond by cellular nucleophiles and subsequent macromolecular dysfunction. However, it is possible that another enzyme, with a much higher affinity for illudins, is responsible for their activation.

**Mass Spectrometry of GSH Adducts.** LC/MS/MS was used to further characterize pathways and consequences of enzymatic (E) and nonenzymatic (NE) reduction of the 8,9-double

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**Table 1** Kinetic constants for metabolism by alkenal/one oxidoreductase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>$V_{max}$ (nmol/min/mg)</th>
<th>$K_m$ ($\mu M$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irofulven</td>
<td>8</td>
<td>275</td>
<td>145</td>
</tr>
<tr>
<td>Illudin M</td>
<td>3</td>
<td>29,200</td>
<td>109</td>
</tr>
<tr>
<td>Illudin M</td>
<td>4</td>
<td>14,900</td>
<td>113</td>
</tr>
<tr>
<td>Illudin S</td>
<td>5</td>
<td>65,400</td>
<td>486</td>
</tr>
<tr>
<td>Illudin S</td>
<td>6</td>
<td>50,500</td>
<td>308</td>
</tr>
</tbody>
</table>

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**Fig. 2** Molecular structures of illudin S, M, irofulven, and metabolites.
bonds of irofulven and illudin M (Fig. 1). Measuring GSH adduction is a commonly used technique for estimating covalent protein binding, and thus cytotoxicity, of a drug (19). The nucleophilic thiol moiety of GSH effectively scavenges electrophilic molecules, and its large size and charge prevent it from entering the active site of most enzymes. In the representative NE reaction (Fig. 1) illudin M is reduced by GSH at the \(/H9251, /H9252/-double bond and a hydroxide ion adds to and opens the cyclopropyl ring. Thus, the net change to the molecular weight of the molecule is 307 \(m/z\) (\(\text{GSH} - \text{OH}\)).

GSH adducts formed after enzymatically catalyzed hydride transfer (E) experience a net molecular weight change of 291 \(m/z\) (\(\text{GSH} - \text{H}^+ - \text{OH}^-\)) and are, thus, easily distinguishable from the NE adducts. Daughter ions of adducts, which represent the loss of water (\(-18 m/z\)) or glutamine (\(-129 m/z\)), were common and monitored for in these experiments after MS/MS.

Irofulven and illudin GSH adducts were formed \textit{in vitro} in incubations that favored enzymatic or nonenzymatic reduction of their 8,9-double bonds (Fig. 1). Enzymatic reduction occurred in incubations containing recombinant AOR, NADPH, and GSH, whereas companion nonenzymatic reductions lacked NADPH. After a 1-h incubation at 37°C, samples were deproteinized and subjected to preliminary purification with Oasis C18 sample prep columns. With addition of NADPH, the enzymatically reduced irofulven-GSH adduct (Fig. 5A) was easily detected when samples were monitored for the parent ion (\(M^+ = 538 m/z\)) or a specific daughter ion (\(M^+ - \text{H}_2\text{O} = 520 m/z\)). However, the NE irofulven-GSH adduct (Fig. 5B) was detected only after monitoring for a daughter ion (\(M^+ - \text{H}_2\text{O} = 536 m/z\)) and was 42-fold less abundant than the E adduct. Similar amounts of NE irofulven-GSH adduct, and as expected no E adduct, were detected in NADPH-deficient samples. Surpris-
were plotted; bars, which varied by several orders of magnitude in this panel of cell of illudin M and S sensitivity (data not shown). AOR activities, ((14-ingly, the E illudin M-GSH adduct (Fig. 5C) was vastly (14-fold) predominant over the NE adduct (Fig. 5D) when incubations contained NADPH. This result indicates that AOR is capable of activating the illudins through metabolism; however, the AOR overexpression data suggest that this route of activation is pharmacologically unimportant. Contrary to findings with irofulven, a great deal of NE illudin M-GSH adduct was detected in NADPH-deficient incubations.

Positive Association of Irofulven Sensitivity with AOR Activity Levels. To determine the significance of irofulven and illudin metabolism in a range of human cancers, a resource of the Developmental Therapeutics Program of the NCI was used. The NCI uses a panel of 60 human cancer cell lines to preliminarily evaluate potential chemotherapeutic agents. Sensitivity of cancers arising from nine different organs is estimated using five logarithmic concentrations of an agent and reported by concentration required to produce 50% lethality, total growth inhibition, or growth inhibition of 50%.3 Frozen samples of each of the 58 cell lines originally used to evaluate irofulven, and illudins M and S as chemotherapeutic agents were obtained. AOR activities were measured in cytosolic fractions prepared from each of the cell lines using illudin M as substrate and analyzed by HLPC. AOR-specific activity was subsequently calculated and correlated to irofulven or illudin sensitivity using linear regression. This method of measuring AOR activity was verified in two ways. First a random subset of cell lines was assayed using another AOR substrate, chalcone. Activity levels correlated nearly perfectly with those measured with illudin M. Second, a Western blot of four cell lines that displayed a range of AOR activities was made using an anti-rat AOR primary antibody. Bands of the expected molecular weight were detected and their intensities correlated well with activities (data not shown).

As predicted from the overexpression experiments, AOR activity was not associated (R² < 0.1) with either measurement of illudin M and S sensitivity (data not shown). AOR activities, which varied by several orders of magnitude in this panel of cell lines, were positively associated with irofulven bioassays in terms of total growth inhibition (R² = 0.4929, P < 0.001; Fig. 6) and growth inhibition of 50% (R² = 0.4625); however, maximal concentrations of irofulven used in the NCI assays were not sufficient for accurate estimation of the concentration required to produce 50% lethality. Leukemic cell lines were relatively insensitive to irofulven and displayed the lowest AOR activities. Non-small cell lung cancer cell lines, on average, contained the highest levels of AOR activity and were the most sensitive to irofulven. In previous experiments AOR was detected at moderate levels in rat and human kidney (20); thus, the high sensitivity of renal cancer cells is not unexpected. The highest levels of basal AOR expression are commonly found in the liver; however, cell lines arising from this organ are not present in the panel. AOR has also proven very inducible by monofunctional inducers (e.g., 3H-1,2-dithiole-3-thione) in the rat and, as preliminary evidence suggests, bifunctional inducers (e.g., 3-methylcholanthrene) in the human. Thus, AOR levels and consequently irofulven sensitivity could be greatly influenced by diet and/or other drugs.

DISCUSSION

It has been well documented that rat liver cytosol has the capacity to reduce the 8,9-double bond of the illudins and acylfulvenes (see Fig. 1E; Refs. 13, 16); however, the identity of the responsible enzyme(s) and the role of metabolism in the toxicities of these compounds remained unknown. On the basis of several characteristics of this activity, including cofactor preference, cytosolic localization, inhibitor, and pH profile (16), we surmised that the responsible enzyme was likely NADPH-dependent AOR. Recombinant rat liver AOR indeed demonstrates robust illudin and irofulven reductase activities, and the metabolites of illudin M and irofulven formed were determined to be identical to those reported previously (13, 14). Once AOR was identified as an 8,9-reductase, its role in the mechanism of toxicity of these compounds required elucidation. It has been assumed that since illudins M and S, and irofulven share a core molecular structure they act in similar ways to kill cells. This core structure is notably composed of an α,β-unsaturated ketone that straddles a bicyclic ring system, and a cyclopropyl group adjacent to a vinyl moiety and alcohol bearing carbon (Fig. 2). Upon reduction of the α,β-double bond, intramolecular rearrangement generates an unstable cyclohexadiene intermediate that requires only opening of the strained cyclopropyl ring and expulsion of water to form a stable aromatic product. Several nucleophiles, including those found on DNA presumably, could attack and open this ring (Fig. 1). Depending on the kinetic stability of the intermediate, metabolism could prove to be either a detoxifying or activating process. If the intermediate were rapidly quenched by nucleophiles, such as hydroxide or chloride anions, in the enzyme active site before it had the opportunity to react with important cellular nucleophiles (DNA or protein), metabolism by AOR would result in detoxication. Alternatively, if the intermediate were stable enough to exist outside of the hydrophobic active site and reach important cellular nucleophiles, metabolism would serve to activate the molecule. However, reactivity of the 8,9-double bond adds another possible route for adduct formation and toxicity (15).
Oxidized carbonyl compounds are reactive electrophiles that spontaneously react with strong nucleophiles, including sulphydryls and amines, by a Michael-addition mechanism. If the 8,9-double bond proved reactive enough to form adducts with important cellular nucleophiles, then a nonenzymatic route of toxicity may predominate.

Data gathered in this study contradict the assumption that irofulven is simply a less toxic analogue of illudin S and that their reaction pathways are identical. Metabolic reduction of the \( \beta \)-double bond of irofulven, but not illudin M or S, by AOR is an important determinant of toxicity. Human 293 cells engineered to overexpress AOR are at least 100-fold more sensitive to irofulven than control cells, whereas cells challenged with illudin M display no differential toxicity (Fig. 3). Furthermore, using the measurements of drug sensitivity made by the NCI in a comprehensive panel of 58 human tumor cell lines, a strong positive association was observed between AOR activity and growth inhibition with irofulven, but not illudin.

Because each of these molecules is metabolized by AOR at reasonable rates and concentrations, the potential for metabolic activation in vivo exists. In fact, evidence for illudin M activation by AOR has been collected in this study using GSH

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**Fig. 5** Illudin M and irofulven were incubated with reduced glutathione (GSH) in the presence or absence of NADPH. Nonenzymatic (NE) and enzymatic (E) GSH adducts were detected and identified using high performance liquid chromatography with tandem mass spectrometry: A, irofulven E; B, irofulven NE; C, illudin M E; and D, illudin M NE.

**Fig. 6** Alkenal/one oxidoreductase (AOR) activity levels positively associate with irofulven sensitivity in human tumors. Samples of the cell lines used to evaluate potential anticancer agents were obtained from the National Cancer Institute, and AOR activity levels were measured in triplicate as described in “Materials and Methods.” Linear regression analysis gives an \( R^2 = 0.4929 \) (\( P < 0.001 \)).
trapping and LC/MS/MS detection (Fig. 5). Unlike irofulven, illudin M also readily reacts about its α,β-double bond with thiol nucleophiles (Fig. 4). In conjunction with the observation that most cells are sensitive to illudins at concentrations orders of magnitude below the Kor values, it is likely that cytotoxicity is mediated primarily by reaction through the 8,9-double bond. However, it is possible that another enzyme with characteristics similar to those of AOR, and a much lower Kor, exists and is responsible for illudin activation.

A complete definition of the mechanism of toxicity of an anticancer drug is required to understand the basis by which it preferentially kills tumor cells. With evidence collected in this study we have determined that the disparity between efficacies of the illudins and irofulven in preclinical xenograft experiments is likely due to differential reactivities of their 8,9-double bonds. With an electrophilic α,β-unsaturated ketone, an illudin kills cells indiscriminately, at very low concentrations. Irofulven lacks this characteristic and, therefore, cytotoxicity resulting from metabolic activation by AOR can be detected in vivo. Activated irofulven likely forms adducts with a completely different set of macromolecules, which seem to preferentially trigger apoptosis in cancer cells. A full understanding of the role of metabolism in the chemotherapeutic action of irofulven allows for the design of more potent drugs and identification of candidate tumor types for clinical evaluation.

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