METABOLISM OF ANTITUMOR HYDROXYMETHYLACYLFULVENE BY RAT LIVER CYTOSOL

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ABSTRACT:

Acylfulvenes are a potent class of antitumor agents derived from illudin S, a fungal sesquiterpene. Illudin S possesses antitumor activity but has a poor therapeutic index. Acylfulvene is 100-fold less toxic against human lung adenocarcinoma cells than illudin S, but inhibits tumor growth in human xenografts, opposite to illudin S. An analog of acylfulvene, MGI 114 (hydroxymethylacylfulvene), shows much greater efficacy, producing complete tumor regression in xenograft models. MGI 114 is currently in phase II clinical trials. Cytotoxicity of MGI 114, like that of illudin S, is believed to involve both chemical reaction and enzymatic reduction. Enzymatic reduction by a cytosolic NADPH-dependent enzyme (from rat liver) produced an aromatic metabolite similar to that formed from illudin S. However, the reaction occurred more slowly. In addition, four new metabolites were isolated, two hydroxylated derivatives and two in which the primary allylic hydroxyl was replaced by hydride. All retained the reactive centers of the parent MGI 114.

The sesquiterpenes illudin S (1) and illudin M (2) are toxic compounds obtained from the jack o’lantern mushroom Omphalotus illudens (McMorris and Anchel, 1965; Ammirati et al., 1985; Fig. 1). They possess antibacterial and antitumor properties but have an unfavorable toxicity profile when tested in animals (Kelner et al., 1987). Derivatives of illudins have been prepared that display increased histospecific toxicity toward malignant cells versus normal cells. Among these are dehydroilludin M and acylfulvene (3) formed by the treatment of illudin S with dilute sulfuric acid (McMorris et al., 1996a). The most efficacious derivative is MGI 114 (4, hydroxymethylacylfulvene) prepared by the reaction of acylfulvene with paraformaldehyde in dilute sulfuric acid (McMorris et al., 1996b).

Extensive preclinical studies on MGI 114 have led to phase I trials, which began in December, 1995 (McMorris et al., 1996b). Phase II clinical trials targeting several different solid tumor types are in progress under the sponsorship of MGI Pharma, a pharmaceutical company, and the National Cancer Institute (Murgo et al., 1999).

Studies of the mechanism of toxicity of illudins indicate that they may behave as alkylating agents. Illudin S reacts spontaneously at room temperature with thiols such as cysteine or glutathione at an optimum pH of about 6, and toxicity to HL60 myeloid leukemia cells can be modulated by altering glutathione levels in cells (McMorris et al., 1990). Michael-type addition to the α,β-unsaturated ketone gives a cyclohexadiene intermediate, an extremely reactive alkylating agent, which is rapidly converted to a stable aromatic product (McMorris et al., 1999).

Illudin S undergoes bioreductive activation with NADPH in a rat liver cytosol preparation (Tanaka et al., 1992). Addition of hydride to the α,β-unsaturated ketone presumably occurs, producing a highly unstable intermediate as in the reaction with thiols (cf. McMorris et al., 1999). This intermediate is a potent alkylating agent. Aromatic products were isolated from the enzyme-catalyzed reaction (Tanaka et al., 1994).

Acylfulvene (3) is approximately two orders of magnitude less toxic (to MV522 cells) than illudin S (vide infra) but displays a more selective antitumor effect (Kelner et al., 1995). It reacts very slowly with thiols, compared with the parent compound. Likewise, acylfulvene is metabolized slowly with NADPH in a rat liver cytosol preparation (McMorris et al., 1999). One metabolite was an aromatic product (5) similar to that obtained with illudin S. The other metabolite was a hydroxylated compound (6), which could be further metabolized with NADPH yielding an aromatic product (7).

The similarity in structure of MGI 114 and acylfulvene suggests a similar metabolic fate for the compounds. However, the hydroxymethyl group in MGI 114 may have an important role in its pharmacokinetics or in binding to DNA or protein.

Experimental Procedures

Materials. Illudin S was isolated by extraction of the culture medium of Omphalotus illudens. Acylfulvene was obtained by treatment of illudin S with 1 M H2SO4 at room temperature. MGI 114 was obtained by reaction of illudin S with paraformaldehyde in 1 N H2SO4 for 2 to 4 days at room temperature (McMorris et al., 1996a). Stock solutions of MGI 114 and MgCl2 were made in water. Cytosolic and microsomal rat liver fractions were obtained from ABS al., 1990).
ethyl acetate. Extracts were pooled, dried over MgSO₄, and the solvent was removed in vacuo. The residue was analyzed by thin-layer chromatography (TLC), which was performed on Whatman 4410 222 silica gel plates using a mixture of ethyl acetate:hexanes (3:2). Compounds were detected under UV light (254 nm) and by a (1%) vanillin/H₂SO₄ spray, followed by heating at 120°C. Column chromatography was carried out with silica gel (Davisil 230–425 mesh; Fisher Scientific Co., Fairlawn, NJ) and gave unchanged MGI 114 as well as metabolites 8, 9, 10, and 11. Metabolite 12 was detected only by HPLC and was isolated by semipreparative HPLC using a linear gradient starting at 100% H₂O with 0.05% trifluoroacetic acid and increasing to 40% CH₃CN with 0.02% trifluoroacetic acid over a 60-min period. When a 5-h incubation was performed, acetone was added to the samples (50% final concentration) to precipitate cellular proteins. The samples were centrifuged at 36,000 g for 10 min. The supernatant was concentrated to remove the acetone and eluted through a Fisher Sep-Pak C₁₈ column. The first elution (2 ml) was in water, the second elution (2 ml) in MeOH. Both fractions (aqueous and MeOH) were lyophilized, dissolved in water, and analyzed and purified by HPLC.

MS and ¹H NMR data for MGI 114 and metabolites are given in Table 1.

**Synthesis of 10.** To compound 6 (structure established as reported in McMorris et al., 1999; 922.5 mg, 3.75 mmol), paraformaldehyde (36.3 g), 300 ml of acetone and 275 ml of 1.5 M H₂SO₄ were added. The mixture was stirred at room temperature for 24 h, then washed with NaHCO₃ solution and saline. The organic layer was dried over MgSO₄. After filtration, the crude product afforded a diol (2.6 mg, 20%) as a yellow gum. A solution of diol (1.2 mg, 0.02% trifluoroacetic acid over a 60-min period. When a 5-h incubation was performed, acetone was added to the samples (50% final concentration) to precipitate cellular proteins. The samples were centrifuged at 36,000 g for 10 min. The supernatant was concentrated to remove the acetone and eluted through a Fisher Sep-Pak C₁₈ column. The first elution (2 ml) was in water, the second elution (2 ml) in MeOH. Both fractions (aqueous and MeOH) were lyophilized, dissolved in water, and analyzed and purified by HPLC.

**Assessment of Cytotoxicity.** Compounds were dissolved in dimethyl sulfoxide (DMSO) (1 mg/ml stock solution) and the solutions were diluted in 20% DMSO/PBS just before addition to the culture of MV522 lung carcinoma cells. Control cells received equal amounts of DMSO/PBS. After incubation for 48 h, the cells were washed, trypan blue was added, and the cells counted.

**Results**

TLC analysis of the incubation mixture showed 8, 9, and 10 as more polar compounds compared with MGI 114 and 11 as less polar.

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MS Data</th>
<th>¹H NMR Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGI 114 (4)</td>
<td>C₁₅H₁₈O₃</td>
<td>246</td>
</tr>
<tr>
<td>8</td>
<td>C₁₅H₁₈O₃</td>
<td>246</td>
</tr>
<tr>
<td>9</td>
<td>C₁₅H₁₈O₄</td>
<td>262.1216</td>
</tr>
<tr>
<td>10</td>
<td>C₁₅H₁₈O₄</td>
<td>262.1205</td>
</tr>
<tr>
<td>11</td>
<td>C₁₅H₂₀O₃ Na⁺</td>
<td>230.1307</td>
</tr>
<tr>
<td>12</td>
<td>248.3502</td>
<td></td>
</tr>
</tbody>
</table>

Spectra were measured in CDCl₃ or CD₃OD solutions. Data are ppm from tetramethylsilane. (d = doublet, m = multiplet, t = triplet)
Although they are more water soluble than their precursors, they turn blue on development with vanillin reagent. HPLC analysis also showed that 12 is more polar than MGI 114. These compounds, 8, 9, 10, 11, and 12, were clearly metabolites of MGI 114 because they were not produced in the incubation mixture in the absence of substrate or NADPH. The metabolites were also not observed when the cytosolic or microsomal fraction was boiled for 15 min before addition of substrate and cofactor. With the cytosol, about 5% of MGI 114 was metabolized to 10, with trace amounts of 8, 9, and 11 (less than 1% metabolized MGI 114) detected by TLC. A trace amount of 12 was detected by HPLC analysis and 5% of MGI 114 was recovered unchanged. Trace amounts of metabolites were detected in the microsomal fraction incubation mixture. The remainder of MGI 114 is presumably bound to macromolecules as suggested for metabolism of illudin S. (Tanaka et al., 1994).

The structure of each metabolite was established by spectral analysis. $^1$H NMR and mass spectrometry data for all metabolites are given in Table 1. Cytotoxicity data for metabolite 10 is given in Table 2.

### Discussion

Metabolism of MGI 114 by rat liver cytosol in the presence of NADPH was found to be similar to that of acylfulvene. The metabolism of MGI 114 was slow compared with that of illudin S and the major metabolite detected was 10, analogous to 6 produced from metabolism of acylfulvene. Compound 12 was also analogous to 5, which is produced from acylfulvene and indicates that bioreductive activation occurs, although less readily, with fulvenes than with illudin S.

Formation of 8 and 11 must result from displacement of the primary hydroxyl of MGI 114 by hydride. (In the case of 8, hydroxylation at C-14 occurs also). The highly reactive primary allylic hydroxyl is known to be displaced by a variety of nucleophiles (McMorris et al., 1997). The most reasonable source of hydride is NADPH, although to our knowledge, such a reaction is without precedent. The presumed enzymatic reaction can be simulated by treatment of MGI 114 with sodium cyanoborohydride (vide supra) giving, after oxidation with Dess-Martin reagent, compound 11.

Hydroxylation of xenobiotics is a well known detoxification pathway. The question arises whether metabolites such as 9 and 10 from MGI 114 and 6 from acylfulvene represent detoxified metabolites. Although they are more water soluble than their precursors, they retain a structure capable of bioreductive activation. Because there were sufficient amounts of 6 and 10 (from synthesis), the toxicity against MV522 metastatic lung adenocarcinoma cells was determined.

Compound 6 was less cytotoxic than its precursor 3 (Table 2). Likewise, 10 was less cytotoxic than MGI 114. Fulvenes 6 and 10 can therefore be regarded as partially detoxified although they still retain the potential to behave as alkylating agents.

Compounds 8 and 11 may result from a detoxification process that involves reduction in the case of 11 and reduction plus oxidation for 8. This requires substantiation by cytotoxicity tests (The amounts of metabolites 8 and 11 were insufficient for testing). It is tempting to predict that loss of the highly reactive primary allylic hydroxyl should lead to less toxic compounds.

A more important reduction pathway leads to metabolite 12. This aromatic compound is expected to be relatively nontoxic, like analogous derivatives obtained from illudin S and acylfulvene (McMorris et al., 1997). However, 12 is formed from MGI 114 via a highly reactive intermediate capable of alkylating macromolecules and this intermediate may well be the species which leads to DNA damage in cells.

Greater efficacy of acylfulvene compared with illudin S may result partly from slower and more selective reaction with cellular nucleophiles. The substantial increase in efficacy with MGI 114 compared with acylfulvene is not fully understood. Increased hydrophilic character may be a factor, but the susceptibility of the allylic hydroxyl to attack by thiols or other nucleophiles could also be involved. Structure-activity studies are in progress to determine the importance of the allylic hydroxyl for antitumor efficacy of MGI 114.

### Acknowledgments

We thank Steve Smith and Hans Erickson (in Dr. Kyte’s laboratory) for the use of the HPLC.

### References


### Table 2

**Drug cytotoxicity (IC$_{50}$) in MV522 cells (48-h trypan blue exclusion assay)**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>IC$_{50}$</th>
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</thead>
<tbody>
<tr>
<td>Illudin S (1)</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Acylfulvene (3)</td>
<td>350 ± 20</td>
</tr>
<tr>
<td>MGI 114 (4)</td>
<td>73 ± 8</td>
</tr>
<tr>
<td>6</td>
<td>3300 ± 900</td>
</tr>
<tr>
<td>10</td>
<td>2200 ± 900</td>
</tr>
</tbody>
</table>

The IC$_{50}$ is reported as a mean ± S.D. for three to seven experiments.