Cytotoxicity of Some Azines of Acetophenone Derived Mono-Mannich Bases against Jurkat Cells

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Acetophenone derived mono-Mannich bases (Ig1—Ig4), 1-aryl-3-amino-1-propanone hydrochlorides, which are known to have cytotoxicity in Jurkat cells, were synthesized. Then, they were converted to corresponding azine derivatives (D1—D4), N,N'-bis(3-amino-1-aryl-propylidene)hydrazine dihydrochlorides, which are bifunctional agents. The aryl part was replaced by phenyl in Ig1, Ig2, Ig3, D1, D2, and D3, and by p-hydroxyphenyl in Ig4 and D4. The amine part was replaced by dimethylamine in Ig1, D1, Ig4 and D4, by piperidine in Ig2 and D2, and by morpholine in Ig3 and D3. The aim of this study was to investigate whether the modification in chemical structure, converting the mono-Mannich base to a corresponding azine derivative, improves the cytotoxicity. In addition, the effect of the representative compound, D3, N,N'-bis(3-morpholine-4-yl-1-phenylpropylidene)hydrazine dihydrochloride, on cellular glutathione level after 1 h exposure in phosphate buffer at 37 °C was also determined to provide information on a possible mechanism of cytotoxic action. Compounds D2—D4 are reported for the first time in this study. Except for Ig2 and D2, the cytotoxicity of mono-Mannich bases, Ig1, Ig3 and Ig4 and corresponding azine derivatives, D1, D3 and D4 were higher than the reference compound 5-FU. Azine derivatives D1 and D4 had almost equal cytotoxic potency with corresponding mono-Mannich bases Ig1 and Ig4, respectively. On the other hand, azine derivatives D2 and D3 had 1.28 and 1.90-times less cytotoxicity in Jurkat cells compared with the mono-Mannich bases, Ig2 and Ig3, respectively, from which they are derived. Azine derivative D3 dose-dependently decreased the total cellular glutathione level, suggesting that azine derivatives may exert cytotoxicity by thiol alkylation. Azine derivatives with equal or less cytotoxic potency compared to the mono-Mannich bases are derived from seemed to be less suitable derivatives for the development of new cytotoxic compounds.

Key words Mannich base; azine; cytotoxicity; Jurkat cell; glutathione; thiol alkylation

Mannich bases have several biological activities such as antimicrobial,1—5) cytotoxic,6—9) antitumor,10) analgesic,11) anti-inflammatory,12,13) diuretic14,15) and anticonvulsant16—19) activities. An amino ketone, Mannich base, containing at least one activated hydrogen atom at the β position to an amino function, can undergo deamination in in vivo20) or under simulated in vitro conditions1,3,6) to liberate the corresponding α,β-unsaturated ketones. The α,β-unsaturated ketone is an active center for nucleophilic attack. The biological activities of Mannich bases, such as antimicrobial,1—3,21,22) cytotoxic and antitumor,9,23) activities, have been attributed to these liberated α,β-unsaturated ketones which can alkylate nucleophiles, especially thiol groups,1,6,24,25) rather than amino and hydroxyl groups,20) by Michael type addition reactions of nucleophiles to α,β-unsaturated ketones.27) In addition, enzyme-catalyzed alkylation of the thiol group of cysteine with α,β-unsaturated carbonyl compounds has been observed.28) The antimicrobial activity of α,β-unsaturated ketones is due to their reactions with essential thiol groups in bacteria and fungi, resulting in β-ketothioethers.29) Electron densities on β-carbons are lower30) than on α-carbons in various unsaturated ketones, and thus the attack of the nucleophiles should occur at the β-carbon atoms. It was also shown that increased antimicrobial activity was associated with an increased breakdown of the Mannich bases.29) Similarly, a number of α,β-unsaturated ketones have alkylating ability against biologically important nucleophiles to produce cytotoxicity.21,23) The amino group in Mannich bases would increase the water solubilizing property of the molecule, which may assist in transportation of the compound to the site of action.

Production of thiol adducts in the stability studies,1,3,6) carried out under in vitro physiological conditions (in phosphate buffer with pH 7.4, at 37 °C), with 2-mercaptoethanol, various unsaturated ketones or Mannich bases, suggests that α,β-unsaturated ketones and Mannich bases producing unsaturated ketones by deamination to exert their antimicrobial activities by thiol alkylation of unsaturated ketones.

Clark and co-workers31) found that the thiol adduct had the greatest fungitoxicity, which was probably due to the same reaction. Dimmock et al. (1994) have shown that Mannich bases of conjugated styril ketones inhibit one or more of the following enzymes in the glutathione metabolic pathway: namely, glutathione S-transferases, glutathione reductase, gamma-glutamyl transpeptidase and glutathione peroxidise in Candida albicans.32) They concluded that the antifungal activity of the Mannich bases of conjugated styril ketones may be due at least in part, to interference with the GSH metabolic pathway. In one of our previous studies, we observed that mono-Mannich bases and bis Mannich bases decrease the total glutathione level in a dose-dependent manner when Jurkat cells are exposed to the compounds in phosphate buffered saline for 1 h at 37 °C with gentle shaking.23) However, in our subsequent study, where the Jurkat cells were exposed to the Mannich bases in culture conditions, the glutathione level was increased, possibly because of the stimulation of feed-back mechanisms regulating the cellular glutathione level.24) These findings also suggested that thiol alkylation may be an important mechanism responsible for

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the cytotoxic activity of Mannich bases. A number of bifunctional agents display antitumor properties and are often formulated as prodrugs, e.g. the administration of clinically useful nitrogen mustards produces aziridines, which are bioactive species alkylating various cellular constituents.\(^{33}\)

The objectives of the present investigation were as follows: First, to synthesize a small number of prototype molecules, mono-Mannich bases which are known cytotoxic activities against Jurkat cells,\(^{34}\) and then to convert them to the corresponding azine derivatives, which have the potential for liberating bifunctional alkylating species in living tissues. Second, to investigate how the cytotoxic activity is affected by the conversion of mono-Mannich bases to the corresponding azine derivatives. The effect of the representative compound, D3, on the most abundant cellular thiol, glutathione,\(^{34}\) in Jurkat cells was determined to provide information about their mechanism of action.

**EXPERIMENTAL PROCEDURES**

**Materials** All chemicals used in syntheses were purchased from Aldrich Chemical Co. (Munich, Germany). Melting points were determined on a Thomas Hoover apparatus (Philadelphia, PA, U.S.A.) and were uncorrected. UV spectra were recorded in H\(_2\)O by a Shimadzu double-beam spectrometer UV-150-02. (Tokyo, Japan). \(^1\)H-NMR (400 MHz) and \(^1\)C-NMR (100.6 MHz) spectra were obtained on a Bruker AM 400 WB Instrument (Karlsruhe, Germany) using tetramethylsilane (TMS) as an internal standard (chemical shift in \(\delta\), ppm). Electrospray ionization (ESI) mass spectra were recorded using a LCQ quadruple ion trap mass spectrometer (Finnigan, San Jose, CA, U.S.A.). The spray needle was set at 5.5 kV in the positive ion mode. The spray was stabilized by a nitrogen sheath flow and the value was set at 5.5 kV in the positive ion mode. The spray was stabilized by a nitrogen sheath flow and the value was set at 100. The inlet capillary temperature was 200 °C. The samples were dissolved in 50% methanol in water (10 mg/μl), and 5 μl samples were injected. The eluent consisted of 50% methanol in water, and the flow was set to 20 μl/min. Elemental analyses were performed with a CHN-rapid elemental analyzer (Perkin Elmer Instruments of Norwalk, CT, U.S.A.). The purity of the compounds was assessed by TLC on silica gel HF254-366 (E. Merck, Darmstadt, Germany). Developing solvents for TLC were chloroform–methanol (8 : 2) for the compounds.

Jurkat cells (American Type Culture Collection, TIB-152, Jurkat, clone E6-1, T cell leukemia, Human, Rockville, Maryland) were kindly provided by Reitu Agrawal, Ph.D., A.I. Virtanen Institute, Kuopio, Finland. RPMI 1640 medium (Gibco BRL, Life Technologies (Paisley, Scotland). Multidish 6 and other culture dishes used were obtained from Nunc, Nunc (Roskilde, Denmark). Trypan blue, GSH, GSSG reductase, NADPH, and 5,5’-dithiobis (2-nitrobenzoic acid) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals and reagents used were of the available analytical grade.

**Synthesis of Mono-Mannich Bases (Ig1—4, Table 1)**

Synthesis of the mono-Mannich bases, Ig1—3, was reported previously,\(^9\) Ig4 was synthesized as described.\(^{35}\)

**Synthesis of the Corresponding Azine Derivatives (D1—D4, Table 1)**

A solution of hydrazine hydrate (1.75 g, 0.035 mol) in ethanol (15 ml) was added to a solution of 3-amino-1-aryl-1-propanone hydrochloride type of mono-Mannich base (0.07 mol) in ethanolic acetic acid (3% w/v, 70 ml). 3-Dimethylamino-1-phenyl-1-propanone hydrochloride for D1, 3-piperidino-1-phenyl-1-propanone hydrochloride for D2, 3-morpholino-1-phenyl-1-propanone hydrochloride for D3, and 3-dimethylamino-1-(p-hydroxyphenyl)-1-propanone hydrochloride for D4 were used as mono-Mannich bases in the reactions to synthesize suitable azine derivatives. The mixtures were stirred at room temperature for 24 h. After removal of the solvent, the residue was washed with chloroform three times (3 x 20 ml), dried and crystallized from ethanol to give the corresponding azine derivatives. The compounds were dried by heating under vacuum for 24 h prior to submission for elemental analysis. Melting points of the compounds and yields of reactions were as follows, respectively:

- **D2**: (172.5—175 °C (dec.), 81.96%) , **D3** (173—175 °C, 57.27%), **D4** (185—186 °C, 57.56%). Elemental analyses (C, H, N) results of **D2** (C\(_{26}\)H\(_{36}\)Cl\(_2\)N\(_4\)O\(_2\)), and **D3** (C\(_{25}\)H\(_{46}\)Cl\(_2\)N\(_2\)O\(_5\)), and **D4** (C\(_{22}\)H\(_{35}\)Cl\(_2\)N\(_2\)O\(_2\)) were within 0.4% of the calculated values.

**Spectral Data of New Compounds:** **D2**: \(\lambda_{\text{max}}\) (H\(_2\)O) nm (log \(e\) max) : 290 (4.03). \(^1\)H-NMR (D\(_2\)O/CD\(_3\)OD; 60/40) \(\delta\) : 1.70 (2H, m), 1.74—1.86 (10H, m), 3.12 (4H, m), 3.24 (4H, m), 3.36 (4H, bs), 3.50 (4H, m), 7.57—7.65 (6H, m), 7.98 (4H, m). Signal 3.36 ppm was very broad and hardly detected from the baseline. This is due to chair-boat tautomers of the piperidine ring. \(^1\)C-NMR (D\(_2\)O/CD\(_3\)OD; 60/40) : 23.9 (t), 25.6 (t), 26.7 (t), 47.4 (t), 56.1 (t), 130.1 (d), 132.0 (d), 134.4 (s), 138.2 (s), 166.4 (s). ESI-MS m/z : 431.2 (M\(^+\)+H).

**D3**: \(\lambda_{\text{max}}\) (H\(_2\)O) nm (log \(e\) max) : 285 (4.19). \(^1\)H-NMR (D\(_2\)O/CD\(_3\)OD; 60/40) : 23.9 (t), 25.6 (t), 26.7 (t), 47.4 (t), 56.1 (t), 130.1 (d), 132.0 (d), 134.4 (s), 138.2 (s), 166.4 (s). ESI-MS m/z : 431.2 (M\(^+\)+H).

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Table 1. Mono-Mannich Bases and Corresponding Azine Derivatives Synthesized

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Formula</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig1</td>
<td>C(<em>{26})H(</em>{36})Cl(_2)N(_4)O(_2)</td>
<td>Mono-Mannich base</td>
</tr>
<tr>
<td>Ig2</td>
<td>C(<em>{25})H(</em>{46})Cl(_2)N(_2)O(_5)</td>
<td>Corresponding azine derivative of Ig1</td>
</tr>
<tr>
<td>Ig3</td>
<td>C(<em>{22})H(</em>{35})Cl(_2)N(_2)O(_2)</td>
<td>Corresponding azine derivative of Ig2</td>
</tr>
<tr>
<td>Ig4</td>
<td>C(<em>{21})H(</em>{35})Cl(_2)N(_2)O(_2)</td>
<td>Corresponding azine derivative of Ig3</td>
</tr>
</tbody>
</table>
(CD3OD) δ: 3.22—3.40 (12H, m), 3.56 (4H, m), 3.86 (8H, bs), 7.53 (6H, m), 8.03 (4H, m). 13C-NMR (CD3OD) δ: 24.7 (t), 53.2 (t), 55.0 (t), 65.1 (t), 128.6 (d), 132.4 (d), 137.2 (s), 164.9 (s). ESI-MS m/z: 435.2 (M^+ + H^+).

D4: \( \lambda_{\text{max}} \) (H2O) nm (log e_{\text{max}}): 280 (4.43). \(^1\)H-NMR \((\text{D}_2\text{O}/\text{CD}_3\text{OD}; 80/20)\) δ: 2.96 (12H, s), 3.58 (8H, m), 6.99 (4H, d), 7.98 (4H, d, \( J_{\text{HH}} = 9.0 \text{ Hz} \)). \(^13\)C-NMR \((\text{D}_2\text{O}/\text{CD}_3\text{OD}; 80/20)\) δ: 33.9 (t), 44.4 (q), 54.5 (t), 129.3 (s), 132.6 (d), 163.1, 167.0 (d), 199.7 (s). Large \( \delta \) peak, C 14H10O, M: 80/20) nm (log e\(_{\text{max}}\)) 280 (4.43) ppm.

Cytotoxicity of Compounds against Jurkat Cells  
Jurkat cells were maintained as a suspension culture in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) with penicillin (100 U/ml) and streptomycin (100 \( \mu \)g/ml) at 37°C under a humidified atmosphere of 95% air and 5% CO\(_2\). To test the cytotoxicity of the compounds with Jurkat cells, Phillips et al.‘s method was used with slight modifications. A stock solution of Jurkat cells was prepared in culture medium, and the cells were counted with a Model DN Coulter Counter. The test compound was dissolved in double distilled water to give 10 mg/ml concentration. This stock solution of the test compound was sterilized by filtration. The Cells

RESULTS

Table 2. Cytotoxic Activities of the Compounds Synthesized against Jurkat Cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular weight (mol/l)</th>
<th>LC50 (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig1</td>
<td>213.5</td>
<td>0.027</td>
</tr>
<tr>
<td>Ig2</td>
<td>253.5</td>
<td>0.036</td>
</tr>
<tr>
<td>Ig3</td>
<td>255.5</td>
<td>0.010</td>
</tr>
<tr>
<td>Ig4</td>
<td>229.5</td>
<td>0.014</td>
</tr>
<tr>
<td>D1</td>
<td>503</td>
<td>0.046</td>
</tr>
<tr>
<td>D2</td>
<td>507</td>
<td>0.019</td>
</tr>
<tr>
<td>D3</td>
<td>455</td>
<td>0.014</td>
</tr>
<tr>
<td>5-FU</td>
<td>130</td>
<td>0.033</td>
</tr>
</tbody>
</table>

The protein amount of the cell supernatant was determined by Bio-Rad protein assay (Bio-Rad Lab., Richmond, CA, U.S.A.) using bovine serum albumin as a standard according to the method described by Bradford. A Shimadzu UV-240 double-beam spectrophotometer (Kyoto, Japan) was used for protein and total glutathione measurements.

RESULTS

Compounds D2—D4 have been reported for the first time by this study. Cytotoxic activities of the compounds are shown in Table 2. Of them, the cytotoxic activities of Ig1—3 were also reported previously.

Except for Ig2 and D2, the cytotoxicity of mono-Mannich bases, Ig1, Ig3 and Ig4 and corresponding azine derivatives, D1, D3 and D4 were higher than the reference compound 5-FU. Conversion of mono-Mannich bases Ig1 and Ig4 to their corresponding azine derivatives D1 and D4 did not affect the cytotoxicity. However, conversion of mono-Mannich bases Ig2 and Ig3 to their corresponding azine derivatives D2 and D3 decreased the cytotoxicity 1.28 and 1.90 times, respectively.

The effect of the representative compound D3, on the most abundant cellular thiol, glutathione, has been shown in Fig. 1.

DISCUSSION

Mono-Mannich bases (Ig1—4) undergo deamination to give \( \alpha,\beta \)-unsaturated ketone in vivo, or in simulated physiological conditions in vitro. Thus, it is possible to generate an active center for nucleophilic attack for thiol alkylation (Fig. 4).
2). Cytotoxicity increases by an increase in the deamination ratio. The higher the deamination ratio, the higher the cytotoxicity of the compounds.

Two pathways may be suggested for the cytotoxicity of the azines: 1. Azine compounds (D1—D4), which are bifunctional agents, undergo deamination to give compound 3, which contains biologically active unsaturated groups. Then, intermediate 3 produces the thiol adduct, 4. This mechanism could be responsible for the cytotoxic activity of series D (Fig. 3). 2. Azine derivatives generate mono-Mannich bases by undergoing hydrolysis (1 mole azine gives two moles of corresponding mono-Mannich bases). These mono-Mannich bases undergo an elimination-addition reaction, as mentioned above, to give thiol adducts, 2 (Fig. 4). Both pathways mentioned suggest that azines will probably show higher cytotoxicity than mono-Mannich bases.

This controversy observed in this study for the azine compounds, especially for compounds D2 and D3, may be explained by another pathway, as shown in Fig. 5. It is possible that a great amount of compound 8, which does not have a suitable center for alkylation, was formed, while small amounts of compounds 3 and 1, which have suitable centers for alkylation, were also formed in this pathway. It is reported that a small amount of compound 4 type adduct along with a great amount of compound 8, were formed in a stability study using compound D1 in phosphate buffer at pH 7.4, at 37 °C with 2-mercaptoethanol.

While the theoretically calculated enthalpy difference (ΔH, calculated using ChemOffice 2002 software, U.S.A.) for the reaction to form a mono-Mannich base by splitting a hydrazine group from compound 5 is +3.62 kcal/mol (Fig. 6), it is +10.12 kcal/mol for the reaction to form compound 6 by splitting an amine group from compound 5 (Fig. 7a). However, the calculated enthalpy difference for the formation

![Fig. 1. The Effect of Compound D3 on the Cellular Glutathione Level of Jurkat Cells after 1 h Exposure in Phosphate Buffered Saline in a 37 °C Room with Slight Shaking.](image)

Cellular glutathione level of control cells was 36.98 ± 1.99 nmol/mg protein. The results are mean ± S.D. of the two experiments in duplicate.

![Fig. 2. Deamination of Mono-Mannich Bases, Ig1—4, to Produce α,β-Unsaturated Ketones, Which Have Suitable Centers for Thiol Alkylation.](image)

![Fig. 3. Deamination of Azines to Produce α,β-Unsaturated Ketones, Which Have Suitable Centers for Thiol Alkylation.](image)
of compound 7 by cyclization of compound 6 is lower, +2.4 kcal/mole (Fig. 7b). This path has lower energy than the path shown in Fig. 6, and shows that the reaction is less endothermic. On the other hand, compound 8 is more stable compared with its precursors, compounds 7 and 1, from the point of thermodynamics. The enthalpy for the formation of compound 8 is $-14.19$ kcal/mol, and it is more stable (Fig. 7c). Theoretically calculated thermodynamic enthalpy results seem to support the formation of compound 8 rather than compounds 1 and 3, where the cytotoxicity was either not affected or decreased in azine derivatives compared to the mono-Mannich bases they are derived from, contrary to the expectation. Equal cytotoxic potency in mono-Mannich bases Ig1 and Ig4 and corresponding azine derivatives suggests that the same amount of $\alpha,\beta$-unsaturated ketones, 1 or 3, are being formed, which are responsible for the cytotoxicity. However, unchanged or decreased cytotoxicity in azine derivatives, contrary to our expectation (increase in cytotoxicity), may be attributed to the production of compound 8 (Fig. 5) by more or less all azine derivatives.

In this study, the representative azine compound D3 decreased the total cellular glutathione level dose-dependently, when Jurkat cells were exposed to the compound in phosphate buffer for 1 h at 37 °C (Fig. 1). We have previously shown that the mono-Mannich base of D3 also decreases the cellular glutathione level.25) These findings support the thiol alkylation mechanism for the cytotoxicity of the compounds studied.

The cytotoxic activity of the new azine compounds D2—
D4 is reported for the first time in this study. The cytotoxicity of azines may result from the formation of great amount of compound 8, along with the formation of some intermediate compounds 1 or 3, with a center suitable for alkylation. In conclusion, azine derivatives with equal or less cytotoxic potency compared to the mono-Mannich bases they are derived from seemed to be less suitable derivatives for the development of new cytotoxic compounds.

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REFERENCES