Mutagenicity Experiments on Agroclavines, New Natural Antineoplastic Compounds

Hansruedi Glatt, Eckart Eich, Heinz Pertz, Christoph Becker, and Franz Oesch


ABSTRACT
Agroclavine, an alkaloid produced by some species of fungi and dicotyledon plants, and its 1-alkylated derivatives are potentially useful as antineoplastic drugs, since they exert potent and selective cytostatic effects. In the present study, we have investigated agroclavine and its 1-propyl and 1-pentyl derivatives for mutagenicity. The genetic end point studied was the reversion of strains of Salmonella typhimurium (TA 100, TA 98, TA 1537) and Escherichia coli (WP2 uvrA), auxotrophic for histidine and tryptophan, respectively. The compounds were tested directly and in the presence of a mammalian xenobiotic-metabolizing system. In the direct test, agroclavine and the two alkylated derivatives examined exhibited substantial bacteriotoxicity but no mutagenicity. Addition of NADPH-fortified postmitochondrial supernatant fraction of rat liver homogenate led to a clear-cut decrease in bacteriotoxicity and to the formation of mutagenic products. Each compound was effective in all three strains of S. typhimurium used. In E. coli only spurious effects were seen. 1-Pentylagroclavine, the most hydrophobic compound in the series, was the strongest mutagen. Agroclavine, the least hydrophobic compound, was the weakest. The mutagenic potencies and efficacies of all these test compounds were much weaker than those of the positive controls, which were known mutagens and carcinogens. Moreover, the differential effect of metabolism by liver enzymes demonstrates that the toxicity and mutagenicity of agroclavine and its derivatives are caused by different chemical species. Hence, it may be possible to develop derivatives that are cytotoxic but not mutagenic.

INTRODUCTION
Agroclavine (Fig. 1), an 8-methyl-ergoline type clavine alkaloid, is produced by fungi of the genera Claviceps, Penicillium, and Aspergillus, and by certain species of the dicotyledon family Convolvulaceae (1). It was discovered at the beginning of the fifties by Abe (2, 3). In contrast to the therapeutically used lysergic acid amide type ergot alkaloids (e.g., ergotamine) agroclavine is characterized by a methyl substituent at C-8 and a double bond in position 8,9. Nevertheless, it interacts, like the lysergic acid amide type ergot alkaloids (e.g., ergotamine), with dopamine and serotonin receptors and even with ß-adrenoceptors, although with a lower affinity (4).

Recently we reported that agroclavine possesses antibiotic activity against some pathogenic and apathogenic bacterial species (5, 6) and remarkable cytostatic activity in the L5178Y mouse lymphoma cell system (7). This in vitro activity was 10-fold higher than that for non-tumor cells (murine spleen lymphocytes). Agroclavine was also active against L5178Y in vivo. Substitution at N-1 by an alkyl group (C7-C9) led to highly active compounds; for 1-pentylagroclavine a 50% cytostatic concentration in vitro was observed which was as low as 0.87 μM (8, 9). It was also shown that 1-propylagroclavine inhibited the incorporation of thymidine into DNA. The obvious pre-

Received 3/3/86; revised 12/18/86; accepted 1/7/87.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

MATERIALS AND METHODS
Compounds. Agroclavine was isolated from surface cultures of Claviceps strain 47a by a phase separation procedure. The 1-alkyl derivatives were synthesized from the natural alkaloid by alkylation of the indole nitrogen via a reaction with potassium and the corresponding alkyl iodide in ammonia (9, 10). The purity of the compounds was higher than 98%, as determined by chromatographic methods as well as by elementary analysis. Agroclavine was present as a free base. Due to the instability of the free bases of the alkylated derivatives, 1-propylagroclavine was stored and normally used as hydrogen (2R,3R)-tartrate, and 1-pentylagroclavine as (-)-di-O,O'-p-toluoyl hydrogen (2R,3R)-tartrate. In selected mutagenicity experiments, the salts were converted to the free bases immediately prior to their use.

Tissue Preparations. Male Sprague-Dawley rats (200–300 g) were treated with Aroclor 1254 in order to induce xenobiotic-metabolizing enzymes (11). Aroclor 1254 was diluted with sunflower oil (1:5, v/v) and i.p. injected at a dose of 500 mg/kg body weight on the sixth day before the rats were killed. The livers were homogenized in 3 volumes of sterile, cold solution of 150 mM KCl in 10 mM sodium phosphate buffer, pH 7.4. The homogenate was centrifuged at 10,000 × g for 10 min. One volume of the resulting supernatant was mixed with two volumes of a solution which contained 12 mM MgCl2, 50 mM KCl, 6 mM NADP, and 7.5 mM glucose 6-phosphate in 75 mM sodium phosphate buffer, pH 7.4. This preparation is termed S9 mix. Liver homogenate was always prepared on the day of the experiment and used immediately.

Bacteria. The histidine-auxotrophic Salmonella typhimurium strains TA 98 and TA 1537 are derived from prototrophic bacteria by frame-shift mutations (12). The histidine auxotrophy of S. typhimurium TA 100 (12) and the tryptophan auxotrophy of Escherichia coli WP2 uvrA (13) are due to substitution mutations. The bacteria were grown overnight in nutrient broth (25 g Oxoid nutrient broth No. 2/liter). For inoculation, stock cultures which were stored at −70°C were used. Before the assay, bacteria were centrifuged, resuspended in medium B (1.6 Bacto nutrient broth plus 5 g NaCl), and adjusted nephelometrically to a titer of 1.5–2 × 108 bacteria (colony-forming units)/ml.

Mutagenicity Experiments. The mutagenicity experiments with his- S. typhimurium strains were performed as described by Ames et al. (12) with minor modifications. The test compound (in 10–30 μl dimethyl sulfoxide), 500 μl 59 mix (or 500 μl 150 mM KCl), 100 μl of the bacterial suspension, and 2 ml of 45°C warm top agar (0.55% agar-0.55% NaCl-50 μM histidine-50 μM tryptophan-50 μM biotin-25 mM sodium phosphate buffer, pH 7.4) were mixed in a test tube and poured onto a Petri dish with minimal agar (1.5% agar-Vogel-Bonner E medium with 2% glucose). Experiments with the trp+ E. coli strain (13) were conducted the same way. Where indicated, the epoxide hydrolase inhibitor (14) and glutathione depletor (15), 1,1,1-trichloropropane 2,3-oxide, was added (0.3 μl/plate) in 10 μl dimethyl sulfoxide. Correspond-

Downloaded from cancerres.aacrjournals.org on April 15, 2017. © 1987 American Association for Cancer Research.
TTAC 1537, except for the solvent controls, for which four and six ing plates without 1,1,1-trichloropropene 2,3-oxide received 10 nM di

TTAC 1537 were not toxic (surviving fraction, >0.9). At a dose of 12 μmol, (-)-(2A,3A)-tartrate, respectively. (2A,3A)-Tartaric acid used up to a dose of 100

MuMTicity Experiments. To estimate toxicity under the incubation conditions of the mutagenicity experiments, his* bacteria (about 600 colony-forming units) were added as an internal standard to otherwise normal mutagenicity plates. The his* bacteria used were spontaneous revertants from TA 1537. They were added to plates together with the strain that gives the lowest numbers of revertant colonies, TA 1537. The difference in the number of colonies on plates with and without added his* bacteria, in the presence of test compound, is compared to the value obtained with solvent controls. The ratio of these two values gives the surviving fraction.

RESULTS

Bacterial Cytotoxicity. Agroclavine and the two 1-alkylated derivatives tested showed bacteriotoxic effects (Fig. 2). The bacteriotoxic potency paralleled the hydrophobicity of the three compounds in that 1-pentylagroclavine was most toxic and agroclavine was least toxic. Addition of a mammalian xenobiotic-metabolizing system, liver S9 mix, drastically reduced the bacteriotoxicity of all three test compounds.

Mutagenicity Experiments. The number of mutants on solvent control plates and on plates containing known mutagens (Table 1) were within the range previously observed in our laboratory (16–18). Detailed mutagenicity results with agroclavine and its derivatives are presented in Figs. 3 and 4. The results are summarized in Table 2. None of the compounds was directly mutagenic with any of the four bacterial strains used. However, when liver S9 mix was added as a metabolizing system, all three compounds exhibited mutagenicity in each strain. The effects in E. coli WP2 were extremely weak (data not shown) and quantification was therefore not possible. At high concentrations, the compounds were toxic in E. coli, as was seen from a decrease in the trp* background lawn. With the other substitution-mutated strain used, S. typhimurium TA 100, each test compound showed about 3-fold increases in the number of revertants per plate above solvent control (Fig. 3). The dose required for eliciting this effect was lowest when 1-pentylagroclavine was used and was highest when agroclavine was used. This was true independently of whether the alkylated agroclavines were tested as the salts (Fig. 3, left), the form in which they can be stored as stable compounds, or as the free bases (Fig. 3, right). The differences (about 2-fold) in the concentrations of the salts and the bases leading to equal effects were within the variation of the assay, but could also have their reason in a partial decomposition of the free bases during their handling prior to the mutagenicity experiments. The effects caused in the frameshift-mutated strains TA 1537 and TA 98 usually were weak (Fig. 4). However, potent effects (up to 13-fold increases in the number of revertants) were seen in strain TA 1537 with 1-pentylagroclavine as the test compound.

Table 1 Solvent and positive controls of mutagenicity experiments

<table>
<thead>
<tr>
<th>Compound, dose/plate</th>
<th>TA 100*</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>TA 98</th>
<th>Experiment 1</th>
<th>TA 1537</th>
<th>Experiment 1</th>
<th>TA 1537</th>
<th>Experiment 2</th>
<th>WP2 uvrA</th>
<th>Experiment 1</th>
<th>WP2 uvrA</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent blank (dimethyl sulfoxide, 10 μl)</td>
<td>–</td>
<td>120</td>
<td>100</td>
<td>–</td>
<td>19</td>
<td>12</td>
<td>NT</td>
<td>7</td>
<td>12</td>
<td>72</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzo(a)pyrene 4.5-oxide (1 μg)</td>
<td>–</td>
<td>2,700</td>
<td>2,400</td>
<td>–</td>
<td>2,800</td>
<td>2,300</td>
<td>NT</td>
<td>420</td>
<td>460</td>
<td>250</td>
<td>160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Methyl-N'-nitro-N-nitrosoguanidine (10 μg)</td>
<td>–</td>
<td>27,000</td>
<td>27,000</td>
<td>–</td>
<td>39</td>
<td>35</td>
<td>NT</td>
<td>110</td>
<td>96</td>
<td>430</td>
<td>280</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Ethyl-N'-nitro-N-nitrosoguanidine (15 μg)</td>
<td>–</td>
<td>19,000</td>
<td>18,000</td>
<td>–</td>
<td>320</td>
<td>360</td>
<td>NT</td>
<td>23</td>
<td>42</td>
<td>6,000</td>
<td>6,300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent blank (dimethyl sulfoxide, 10 μl)</td>
<td>+</td>
<td>130</td>
<td>110</td>
<td>+</td>
<td>52</td>
<td>28</td>
<td>34</td>
<td>21</td>
<td>10</td>
<td>79</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzo(a)pyrene (10 μg)</td>
<td>+</td>
<td>1,500</td>
<td>1,500</td>
<td>+</td>
<td>540</td>
<td>460</td>
<td>440</td>
<td>210</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzo(a)pyrene (50 μg)</td>
<td>+</td>
<td>240</td>
<td>300</td>
<td>+</td>
<td>86</td>
<td>61</td>
<td>290</td>
<td>NT</td>
<td>24</td>
<td>86</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Aminonaphthalene (10 μg)</td>
<td>+</td>
<td>4,200</td>
<td>4,200</td>
<td>+</td>
<td>2,500</td>
<td>2,700</td>
<td>2,200</td>
<td>310</td>
<td>260</td>
<td>440*</td>
<td>410*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methylcholanthrene (90 μg)</td>
<td>+</td>
<td>3,500</td>
<td>3,500</td>
<td>+</td>
<td>1,400</td>
<td>1,700</td>
<td>1,600</td>
<td>150</td>
<td>130</td>
<td>87</td>
<td>72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data of a third experiment (Fig. 3, right) are not shown. Values deviated by factors of less than 1.5 from those shown here.
* TCO, 1,1,1-trichloropropene 2,3-oxide (0.3 μl/plate) was added in order to inhibit microsomal epoxide hydrolase and to deplete glutathione; NT, not tested.
* Mean of two to six replicate incubations.
* The dose in the experiments with E. coli WP2 uvrA was 50 μg.

Fig. 2. Cytotoxicity of agroclavine (O, ◦), 1-propylagroclavine (Δ, △), and 1-pentylagroclavines (□, □) to S. typhimurium. Toxicity was determined directly (O, Δ, □) and with addition of rat liver S9 mix as a mammalian metabolic system (●, △, ◦). Toxicity was determined by adding his* mutants as an internal standard to mutagenicity plates and measuring the surviving fraction of these mutants, as compared to solvent control plates. 1-Propylagroclavine and 1-pentylagroclavagines were tested as hydrogen (2R,3R)-tartrate and (−)-di-O,O'-p-toluoyl hydrogen (2R,3R)-tartrate, respectively. (2R,3R)-Tartaric acid used up to a dose of 100 μmol/plate and (−)-di-O,O'-p-toluoyl (2R,3R)-tartaric acid used up to a dose of 4 μmol/plate were not toxic (surviving fraction, >0.9). At a dose of 12 μmol, (−)-di-O,O'-p-toluoyl (2R,3R)-tartaric acid reduced the surviving fraction to 0.46 in the presence of S9 mix.

Fig. 1. Structures of the investigated compounds.
Fig. 3. Mutagenicity of agroclavine (○, ●), 1-propylagroclavine (△, ▲), and 1-pentylagroclavine (□, △) in S. typhimurium TA 100 (V, ♦; solvent controls). Mutagenicity was determined directly (○, △, V, □) and with addition of rat liver S9 mix as a mammalian metabolic system (●, ▲, ♦, △). Values are means from duplicate determinations. The individual values deviated by less than 10% from the means. a, experiments in which 1-propyl- and 1-pentylagroclavine were used as hydrogen (2R,3R)-tartrate and (−)-di-O,O′-p-toluoyl hydrogen (2R,3R)-tartrate, respectively. (2R,3R)-Tartaric acid (30 nmol–100 μmol/plate) and (−)-di-O,O′-p-toluoyl (2R,3R)-tartrate acid (10 nmol–10 μmol/plate) were not mutagenic in either the absence or presence of S9 mix. (The increases in the number of colonies per plate above the solvent control values were less than 30.) b, a repeat experiment, in which 1-propyl- and 1-pentylagroclavine were converted from their salts to the free bases and immediately tested for mutagenicity.

Table 2. Mutagenic potency of agroclavine and derivatives

<table>
<thead>
<tr>
<th>Test compound</th>
<th>TA 100 Direct</th>
<th>WA With S9 mix</th>
<th>TA 1537 Direct</th>
<th>With S9 mix</th>
<th>WP2 uvrA Direct</th>
<th>With S9 mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agroclavine</td>
<td>&lt;50°</td>
<td>100</td>
<td>&lt;30°</td>
<td>60</td>
<td>&lt;30°</td>
<td>10</td>
</tr>
<tr>
<td>1-Propylagroclavine</td>
<td>&lt;400°</td>
<td>300</td>
<td>&lt;200°</td>
<td>50</td>
<td>&lt;200°</td>
<td>30</td>
</tr>
<tr>
<td>1-Pentylagroclavine</td>
<td>&lt;1000°</td>
<td>780</td>
<td>&lt;500°</td>
<td>140</td>
<td>&lt;500°</td>
<td>850</td>
</tr>
</tbody>
</table>

* There was no mutagenicity, but there was, however, strong toxicity with all three test compounds when they were tested in the absence of liver S9 mix. The values are the estimated detection limits of the mutagenicity experiments.

In the liver S9 mix-mediated experiments with strain WP2 uvrA, statistically significant increases in the number of revertants above solvent control were seen, but the effects were too weak to allow quantification.

Fig. 4. Liver S9 mix-mediated mutagenicity of agroclavine (○, ●), 1-propylagroclavine (△, ▲), 1-pentylagroclavine (□, △) and solvent controls (V, ♦) in S. typhimurium TA 1537 (○, △, V, □) and TA 98 (●, ▲, ♦, △). Values are means from duplicate (TA 98) or triplicate determinations (TA 1537). The individual values deviated by less than 10% and/or less than 5 colonies from the means. 1-Propyl- and 1-pentylagroclavine were used as hydrogen (2R,3R)-tartrate and (−)-di-O,O′-p-toluoyl hydrogen (2R,3R)-tartrate, respectively. (2R,3R)-Tartaric acid (30 nmol–100 μmol/plate) and (−)-di-O,O′-p-toluoyl (2R,3R)-tartrate acid (10 nmol–10 μmol/plate) were not mutagenic in either bacterial strain. (The increases in the number of colonies per plate above the solvent control value were less than 10.)

DISCUSSION

Agroclavine and the investigated alkylated derivatives by themselves were cytotoxic but not mutagenic to bacteria. Addition of a subcellular rat liver preparation resulted in a substantial decrease of cytotoxicity and in the formation of mutagens. The differential effect of the mammalian metabolic system demonstrates that cytotoxicity and mutagenicity of the agro-
MUTAGENICITY OF AGROCLAVINES

agroclavines are caused by different chemical species. Hence, it may be possible to develop derivatives that are cytotoxic but not (proximately nor ultimately) mutagenic.

Our discovery that cytostatically active agroclavines also show antibiotic properties (5, 6) has been demonstrated once more by the bacteriotoxicity results of this study. Cytostatic and antibiotic activities seem to be mediated by the same mechanism of action because we could observe a strongly marked parallelism of activity against L5178Y mouse lymphoma cells and bacteria species (5–9). The fact that on the one hand agroclavines are not directly mutagenic whereas on the other hand their mutagenic metabolites show only drastically reduced bacteriotoxicity could be considered as evidence for the assumption that the remarkable cytostatic/antibiotic activities of agroclavines are not mediated by mutations.

The mutagens formed in the presence of the liver preparation were able to cause both frameshift and substitution mutations in S. typhimurium, whereas in E. coli only spurious effects were noted. Whether this result reflects a true species difference or not is unknown. It is possible that the particular mutations required for reversion of the E. coli strain used are less efficiently induced by the metabolites of the agroclavines than those mutations which lead to reversion of the Salmonella strains used. In Salmonella, among the three compounds tested, agroclavine was the weakest and 1-pentylagroclavine the strongest mutagen. Compared to the positive controls used (Table 1) and cytostatic drugs for which mutagenicity data have been published (19), metabolized agroclavine and its metabolized 1-alkyl derivatives were weak mutagens in the Ames test. Furthermore, in some experiments with 1-pentylagroclavine, effects did not occur below a threshold dose (or, at least, were much less than expected by linear interpolation from the effects at higher doses). Saturable metabolic detoxification or DNA repair processes are potential explanations for this threshold effect.

The nature of the mutagenic metabolites is unknown. The molecules of agroclavine and its derivatives offer a number of possible sites for oxidative transformations by liver preparations. Wilson et al. (20) showed that rat liver microsomes demethylated agroclavine at N-6 to 6-nor-agroclavine (main intact ergoline metabolite, 5.6%) and oxidized the C-8 methyl to a hydroxymethyl group (elymoclavine, 1.8%). Furthermore, agroclavine was transformed to setoclavine, isosetoclavine, and penniclavine. Finally, the paper also mentions that phenolic agroclavine was transformed to setoclavine, isosetoclavine, and to a hydroxymethyl group (elymoclavine, 1.8%). Furthermore, demethylated agroclavine at N-6 to 6-nor-agroclavine (main effect). Repair processes are potential explanations for this threshold dose (or, at least, were much less than expected by linear interpolation from the effects at higher doses). Saturable metabolic detoxification or DNA repair processes are potential explanations for this threshold effect.

ACKNOWLEDGMENTS

We thank K. Pauly for her excellent technical assistance.

REFERENCES

1. Floss, H. G. Biosynthesis of ergot alkaloids and related compounds. Tetrahe- 
12. Ames, B. N., McCann, J., and Yamasaki, E. Methods for detecting carcino-
gens and mutagens with the Salmonella/mammalian-microsome mutagenic-
Mutagenicity Experiments on Agroclavines, New Natural Antineoplastic Compounds


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/7/1811

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.