THE PHOTODEACTIVATION OF HEDAMYCIN, AN ANTITUMOR ANTIBIOTIC OF THE PLURAMYCIN TYPE

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The cytotoxicities of hedamycin and photohedamycin A as well as of kidamycin and isokidamycin were determined using HeLa cell cultures. Photohedamycin A proved to be 15 times less cytotoxic than hedamycin thus explaining the loss of biological activity observed for solutions of hedamycin left in daylight. The fact that photohedamycin A is less active than hedamycin, and isokidamycin less than kidamycin points to the important role the rings E and F play in the biological activity of hedamycin and kidamycin.

Some years ago, biochemists reported that solutions of the antitumor antibiotic hedamycin (1) lost their biological activity rapidly when allowed to stand in the daylight\(^2\). We recently investigated the products that were formed when hedamycin or kidamycin were irradiated with light\(^3\). When the photolysis was carried out in the presence of oxygen — thus imitating the laboratory conditions under which the photodeactivation was first observed by WHITE and WHITE — one major product was formed, photohedamycin A (2). We now wish to report on the cytotoxicity of this photoprodut as compared with the parent antibiotic, hedamycin.

Materials and Methods

Hedamycin (1) was a gift of Dr. W. T. BRADNER, Bristol Laboratories, Syracuse. Kidamycin (3) and isokidamycin (4) were a gift of Dr. M. FURUKAWA, Daiichi Seiyaku, Tokyo. Photohedamycin A (2) was obtained by photolysis of hedamycin\(^3\).

Phosphate Buffer Solution

Na\(_2\)HPO\(_4\) • 2H\(_2\)O (1.9 g), KCl (0.2 g), KH\(_2\)PO\(_4\) (0.2 g) and NaCl (8 g) were dissolved in H\(_2\)O to make 1,000 ml of solution (pH 7.4), which then was sterilized by filtration.

Trypsin/EDTA Solution

Trypsin (200 ml of 0.2% solution, Gibco, Madison, WI), NaCl (8 g), KCl (0.2 g), KH\(_2\)PO\(_4\) (0.2 g), Na\(_2\)HPO\(_4\) (1.44 g) and EDTA (0.2 g) were dissolved in H\(_2\)O to make 1,000 ml of solution, which was sterilized by filtration.

Culture Medium

CaCl\(_2\) • 2H\(_2\)O (376 mg), Fe(NO\(_3\))\(_3\) • 9H\(_2\)O (1 mg), KCl (0.8 g), MgSO\(_4\) • 7H\(_2\)O (0.4 g), NaCl (12.8 g), NaHCO\(_3\) (8.4 g), NaH\(_2\)PO\(_4\) • H\(_2\)O (250 mg), glucose (9 g), arginine (420 mg), cystine (96 mg), glutamine (870 mg), histidine (124 mg), isoleucine (210 mg), leucine (209.6 mg), lysine (232 mg), methionine (60 mg), phenylalanine (128 mg), threonine (192 mg), tryptophan (40 mg), tyrosine (144 mg), valine (181 mg), Ca D-pantothenate (4 mg), choline chloride (4 mg), folic acid (4 mg), L-inositol (8 mg), nicotinamide (4 mg), pyridoxal • HCl (4 mg), riboflavin (0.4 mg), thiamine • HCl (4 mg), and phenol red (20 mg) were dissolved in H\(_2\)O to make 3,000 ml of solution,

\(^1\) From the dissertation of A. F.
which was sterilized by filtration through a 0.2 μm Millipore filter. Then, 220 ml of heat deactivated (56°C/30 minutes) fetal calf serum (Gibco) were added.

Antibiotic stock solutions and dilution series: the appropriate antibiotic (0.5 mg) was dissolved in 10 ml of 0.1% aq ascorbic acid solution and sterilized by filtration. This stock solution proved to retain its activity for at least 20 days when stored in the dark at 4°C. Immediately before use, it was diluted stepwise by factors of 10 with the ascorbic acid solution to give the desired concentrations. Three ml of the resulting solutions were diluted further with 7 ml of ascorbic acid solution to give the intermediate concentration steps.

HeLa Cell Suspension

Stock cultures of HeLa cells were freed from the culture medium by decantation, and the cells, which adhered to the walls of the flask, were washed once with 5 ml of phosphate buffer solution. The cells were then detached by wetting them briefly with 2.5 ml of trypsin/EDTA solution, which was immediately removed as completely as possible. After about 15 minutes the cells no longer adhered to the walls of the flask. They were suspended in 5 ml of culture medium, counted under the microscope, and the suspension was diluted with culture medium to contain 2×10^6 cells/0.5 ml.

To 4-ml portions of culture medium in tissue culture flasks (25 cm² surface, Falcon, Oxnard, CA) were added 0.5 ml aliquots of the HeLa cell suspension. The cells were then incubated in a CO₂ incubator (Forma Scientific, Marietta, OH) at 37°C, 90% relative humidity and 5% CO₂ atmosphere overnight. The caps of the culture flasks were only loosely put on the necks. After this first incubation period, the cells again adhered to the walls of the flasks indicating normal growth. The antibiotic solution (0.5 ml) of the appropriate concentration was now added to the cells (in the blank tests, 0.5 ml of the 0.1% ascorbic acid solution was used) and incubation was carried on as stated above for 3 days. Then the cells were detached from the walls of the flasks using the trypsin/EDTA solution as detailed above.

Fig. 1. Survival of HeLa cells after 3-day incubation with hedamycin (1), photohedamycin A (2), kidamycin (3), or isokidamycin (4).

Experiment A •; experiment B ○.
Table 1. Percentage of surviving HeLa cells at different antibiotic concentrations.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Experiment</th>
<th>Antibiotic concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.5x 10^{-5}</td>
</tr>
<tr>
<td>Hedamycin (1)</td>
<td>A</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Photohedamycin A (2)</td>
<td>A</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>Kidamycin (3)</td>
<td>A</td>
<td>(125)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62</td>
</tr>
<tr>
<td>Isokidamycin (4)</td>
<td>A</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Values in parentheses are extraneous values and were rejected.

Phosphate buffer solution (3 ml) containing 10% fetal calf serum and 7 drops of a 0.5% aq trypan blue solution were added. After about 1 hour the dead cells had been stained and the surviving cells could be counted under the microscope. The number obtained was divided by the cell count in the corresponding blank tests to give the percentage of surviving cells (see Table 1 and Fig. 1). Two independent series of experiments (labeled A and B) were carried out, and for most antibiotic concentrations duplicates were run.

Results and Discussion

Two antibiotics of the pluramycin type, hedamycin (1)\textsuperscript{4)} and kidamycin (3)\textsuperscript{5)} as well as a derivative of each were tested for cytotoxicity with HeLa cell cultures. The results are given in the Table 1 and summarized in the Fig. 1. From the plot of the percentage of surviving cells against antibiotic concentration, ID\textsubscript{50} values for the four substances were determined to be 3 x 10\textsuperscript{-8} mg/ml for hedamycin (1), 4 x 10\textsuperscript{-7} mg/ml for its derivative, photohedamycin A (2), 4.5 x 10\textsuperscript{-6} mg/ml for kidamycin (3), and 1.4 x 10\textsuperscript{-4} mg/ml for isokidamycin (4). We assume these values to be exact by a factor of ca. two. As can be seen from the Fig. 1, the differences between the two independent series of experiments (A and B) is quite large. Furthermore, some extraneous values can be seen in the Table 1: they had to be rejected. The main sources of errors are probably incomplete detachment of the cells from the walls of the flasks and fluctuations in the number of cells at the beginning of the experiments. These latter fluctuations became particularly important, since the cells lived for about four generations during the three day incubation period.

The ID\textsubscript{50} value determined for hedamycin is in reasonable accord with the value published earlier by Bradner et al.: 1.3 x 10\textsuperscript{-7}.\textsuperscript{6)} The difference must be due to the fact, that the experimental procedure
of Bradner et al. was different from ours. They started with a smaller number of cells and did not count the surviving cells at the end of the incubation period, but rather determined the amount of protein corresponding to the cells still attached to the walls of the flask (and thus still living) using the Lowry test. We observed, however, that not only the surviving cells, but also many dead cells still adhered to the flask. These would be included in a Lowry test and thus lead to a lower ID₅₀ value.

Kidamycin proved to be less cytotoxic than hedamycin by about two orders of magnitude, as could be expected from a comparison of the antimicrobial tests and the LD₅₀ for mice.

The fact, that solutions of hedamycin (1) lose their activity when allowed to stand in the light, can now be explained—at least in part—from a comparison of the cytotoxicities of hedamycin and of photohedamycin A (2). Compound 2, being the first and major photoproduct formed from 1 in the presence of oxygen, is indeed less cytotoxic than the parent antibiotic by a factor of ca. 15. However, the deactivation noted is certainly also due to further photodegradation of 2 and to the formation of the many minor photoproducts observed. The comparison of the biological activities of hedamycin (1) and photohedamycin A (2) on the one hand, and of kidamycin (3) and isokidamycin (4) on the other hand gives some indication about the role of the amino sugars (rings E and F). It is well known that the indomycinones (4H-anthra[1,2-b]pyran-
4,7,12-triones related to the pluramycin antibiotics but lacking rings E and F) are biologically inactive\(^9\). Our results now show that even slight alterations of the sugars lower the cytotoxicity of a pluramycin antibiotic quite distinctly. In photohedamycin A (2) ring E is an enol ether attached to the anthraquinone chromophore through a planar \(sp^2\)-carbon atom, whereas in the parent hedamycin this sugar is linked to the chromophore by a \(sp^3\)-hybridized carbon. This change lowered the cytotoxicity by a factor of ca. 15. Isokidamycin (4), an artifact formed by acid treatment of kidamycin\(^5\), is epimeric at C(6") with respect to kidamycin. Here again, the geometry at the carbon atom through which one of the sugars (in this case ring F) is attached to the aromatic nucleus was altered with respect to the parent kidamycin; as a consequence, the cytotoxicity was reduced ca. 30-fold. Thus, the cytotoxic activity of hedamycin, kidamycin and presumably all antibiotics of the pluramycin type seems to be quite sensitive to the steric environment around rings E and F, especially at their point of attachment to the anthraquinone. It should be noted, however, that acetylation also affects the biological activity of kidamycin: its tri-\(O\)-acetyl derivative, acetylkidamycin, has a distinctly lower LD\(_{50}\) than the parent compound, whereas the antimicrobial and antitumor activities are quite similar to those of kidamycin\(^10\). In acetylkidamycin, however, not only the two sugar rings are altered by acetylation of their respective hydroxyl groups, but also the phenolic HO-group is esterified. In this compound it is therefore difficult to ascribe differences in biological activity to specific structural changes. In contrast, the 3"-\(O\)-acetyl derivative of kidamycin, neopluramycin, is similar to kidamycin in toxicity, but much less active against microorganisms.\(^11\) It is not clear, in which way the amino sugars of the pluramycin antibiotics are involved in the mode of action of these compounds; this mechanism has not been fully investigated so far\(^12\) and is not well understood.

Acknowledgments

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