Antibiotics from the group of anthracyclines occupy a special position in the contemporary tumor chemotherapy because of their wide range of action. They are used both in the treatment of tumor diseases of haemogenic system and in the therapy of solid tumors. However, complete usage of the antitumor activity of anthracyclines is limited by their toxic action on heart muscle on the one hand, and by multidrug resistance of tumor cells, on the other hand.

Cardiomyopathy induced by anthracyclines may lead to death because of heart failure. The pathogenic mechanism of cardiotoxicity of anthracyclines is mainly connected with their generation of free oxygen radicals during metabolic transformations. The heart muscle is particularly sensitive to oxidative stress, considering low concentration of protective enzymes (glutathione peroxidase and superoxide dismutase) preventing from gathering of reactive oxygen species in cells.

The phenomenon of multidrug resistance is connected with an increased efflux of cytotoxic agents from the cells and is a serious problem in the treatment with anthracycline antibiotics. Search for new antitumor drugs from the anthracycline group are focused on the synthesis of derivatives with changes in saccharide and anthracycline parts. The genotoxic activity of selected nine new derivatives of anthracycline was evaluated basing on reference tests: micronucleus test \textit{in vitro} and the Ames test. A correlation was observed between the results obtained in the Ames test and those received in the micronucleus test. In both tests, derivative WP903 demonstrated a strong genotoxic action.

**Keywords:** new anthracycline derivatives; genotoxicity; Ames test; micronucleus test

EXPERIMENTAL

Chemicals
Adriamycin – ADR (adriblastin – Sigma), encoded anthracycline derivatives: WP401, WP744, WP839, WP889, WP890, WP892, WP902, WP903 and WP904 (M.D. Anderson Cancer Center, Houston), cytochalasin B (Sigma), Giemsa stain (P.O.Ch), methyl methanesulfonate, 4-nitrophenyldiamine (Merck), histidine and biotin (Reanal). Medium MEM, fetal calf serum (Gibco), PBS without calcium and magnesium ions and trypsin (L. Hirszfeld Institute of Immunology and Experimental Therapy), antibiotic (Antibiotic Antimycotic – Sigma). All compounds were dissolved in DMSO. All solutions were made immediately before the experiment.

Cells
L929 cell line (cells in two mouse lymphoma C3H/AN) (ATCC), ME18 cell line (cells of human melanoma) (Institute of Oncology, Warsaw). Cells were grown in MEM medium supplemented with serum and antibiotics.

The bacterial tester strains: Salmonella typhimurium strains: TA97, TA98, TA100, TA102.

Each tester strain contains a different type of mutation in the histidine operon (Table 1). In addition to the histidine mutation the standard tester strains contain other mutations that greatly increase their ability to detect mutagens.

Micronucleus test
To evaluate genotoxicity, a micronucleus test in vitro was used with the cytokinesis – block technique through cytochalasin B (10). The 48 h cell cultures were trypsinized, suspended in culture medium MEM enriched in calf serum and spread on 4-chamber glass slides in the amount 0.7–1.0 × 10⁵ cells/chamber and then incubated for 24 h at 37°C. Next, the cells were subjected to the action of the studied compound at various concentrations for 2 h. After that, the cells were twice washed with the me-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation in histidine gene</th>
<th>uvrB</th>
<th>rfa</th>
<th>Plasmid</th>
</tr>
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<tbody>
<tr>
<td>TA97</td>
<td>his D6610, his 01242</td>
<td>–</td>
<td>–</td>
<td>pKM101</td>
</tr>
<tr>
<td>TA98</td>
<td>his D3052</td>
<td>–</td>
<td>–</td>
<td>pKM101</td>
</tr>
<tr>
<td>TA100</td>
<td>his G46</td>
<td>–</td>
<td>–</td>
<td>pKM101</td>
</tr>
<tr>
<td>TA102</td>
<td>his G428*</td>
<td>+</td>
<td>–</td>
<td>pKM101, pAQ1</td>
</tr>
</tbody>
</table>

* The mutated histidine gene is located on a plasmid; the chromosome does not contain this gene.

<table>
<thead>
<tr>
<th>Compound studied</th>
<th>Concentration of studied compound (µg/plate)</th>
<th>0</th>
<th>0.1</th>
<th>1.0</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>148 ± 33</td>
<td>143 ± 27</td>
<td>174 ± 38</td>
<td>216 ± 41</td>
</tr>
<tr>
<td>ADR</td>
<td></td>
<td>122 ± 17</td>
<td>89 ± 17</td>
<td>158 ± 44</td>
<td>Toxic</td>
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<tr>
<td>WP401</td>
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<td>108 ± 7</td>
<td>267 ± 14</td>
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<tr>
<td>WP744</td>
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<td>144 ± 37</td>
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<td>309 ± 44</td>
<td>Toxic</td>
</tr>
<tr>
<td>WP839</td>
<td></td>
<td>167 ± 5</td>
<td>149 ± 25</td>
<td>156 ± 34</td>
<td>255 ± 85</td>
</tr>
<tr>
<td>WP889</td>
<td></td>
<td>143 ± 7</td>
<td>133 ± 1</td>
<td>130 ± 13</td>
<td>162 ± 34</td>
</tr>
<tr>
<td>WP890</td>
<td></td>
<td>137 ± 2</td>
<td>174 ± 34</td>
<td>282 ± 14</td>
<td>211 ± 19</td>
</tr>
<tr>
<td>WP902</td>
<td></td>
<td>148 ± 43</td>
<td>182 ± 9</td>
<td>206 ± 21</td>
<td>412 ± 67</td>
</tr>
<tr>
<td>WP903</td>
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<td>149 ± 8</td>
<td>177 ± 6</td>
<td>391 ± 40</td>
<td>Toxic</td>
</tr>
<tr>
<td>WP904</td>
<td></td>
<td>114 ± 8</td>
<td>129 ± 5</td>
<td>256 ± 12</td>
<td>Toxic</td>
</tr>
</tbody>
</table>

The number of revertants per plate represents means for there experiments ± SD.
dium and incubated in the culture medium supplemented with cytochalasin B at a final concentration of 3 µg/mL. Then, the culture cells were twice washed with PBS, dried, preserved with methanol for 10 min and stained for 20 min with 10% Giemsa stain in 0.05 M phosphate buffer at pH = 6.8. The dried preparations were evaluated under a microscope. As a measure of genotoxic action, the percent of cells with micronuclei in the population of binucleate cells (not less than 1000 binucleate cells) was assumed.

**The Ames test**

The Ames test was carried out according to the procedure described by Maron and Ames (11).

The test strains *Salmonella typhimurium* were grown in Oxoid nutrient broth for 18 h in a water bath at 37°C with shaking. 0.1 mL of the bacterial culture and 0.1 mL of the studied compound were added to 2 mL of top agar held at 45°C containing 0.05 mM biotin and 0.05 mM histidine and poured out on a nutrient agar plates. After 48 h incubation at 37°C, the colonies of revertants *his* were counted on the plates. The following positive controls was used: 20 µg/plate of 4-nitrophenyldiamine for the strains TA97 and TA98 and 0.5 µg/plate of methyl methanesulfonate for TA100 and TA102. In accordance with the commonly accepted procedures concerning this test as a mutagenic action was defined causing duplication of the number of revertants *his* compared with control (12).

**RESULTS**

Potential mutagenic properties of ten derivatives of anthracyclines were examined using the Ames test. In the study, four strains of *Salmonella*

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<table>
<thead>
<tr>
<th>Compound studied</th>
<th>Concentration of studied compound (µg/plate)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>ADR</td>
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<tr>
<td>WP401</td>
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</tr>
<tr>
<td>WP744</td>
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<td>WP889</td>
<td>19 ± 7</td>
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<tr>
<td>WP890</td>
<td>23 ± 2</td>
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<td>WP892</td>
<td>20 ± 7</td>
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<td>23 ± 6</td>
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<td>WP903</td>
<td>20 ± 3</td>
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<tr>
<td>WP904</td>
<td>20 ± 4</td>
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</table>

<table>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>ADR</td>
<td>128 ± 29</td>
</tr>
<tr>
<td>WP401</td>
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<tr>
<td>WP744</td>
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<td>WP839</td>
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<td>WP889</td>
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<td>WP892</td>
<td>157 ± 52</td>
</tr>
<tr>
<td>WP902</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>WP903</td>
<td>162 ± 21</td>
</tr>
<tr>
<td>WP904</td>
<td>140 ± 8</td>
</tr>
</tbody>
</table>
Salmonella typhimurium, differ in the mutations type in histidine gene (Table 1), therefore have various sensitivity towards the action of different chemical compounds.

Results of the Ames test for ADR and nine new derivatives of anthracycline, denoted as WP401, WP744, WP839, WP889, WP890, WP892, WP902, WP903, WP904 are presented in Tables 2–5.

None of the studied compounds increased the number of revertants in strain TA97 at a concentration of 0.1 µg/plate; however, at a concentration of 1.0 µg/plate five compounds (WP744, WP892, WP902, WP903, WP904) have shown mutagenic action for this strain, at 10 µg/plate as much as five compounds were toxic (WP744, WP903, WP401, WP839, WP904) and only derivative WP902 manifested mutagenic action. The highest number of revertants his+ observed in strain TA97, at still non-toxic concentration, was induced by derivative WP903.

Strain TA98 was characterized by a greater sensitivity towards the studied compounds. Three derivatives of anthracycline: WP744, WP892 and WP903 were mutagenic already at a concentration of 0.1 µg/plate. At 1.0 µg/plate, all the derivatives of anthracycline manifested mutagenic action except for WP890. At the highest concentration used, i.e. 10 µg/plate, five derivatives (WP744, WP892, WP902, WP903, WP904) manifested toxic action, while three (WP899, WP401, WP839) were mutagenic. Derivative WP890 did not reveal mutagenic action for this strain at any concentration used.

Strain TA100 manifested sensitivity similar to that of strain TA98 and at a concentration of 0.1 µg/plate none of the studied compounds showed

<table>
<thead>
<tr>
<th>Compound studied</th>
<th>Concentration of studied compound (µg/plate)</th>
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<tbody>
<tr>
<td></td>
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<td></td>
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<td></td>
<td>10</td>
</tr>
<tr>
<td>ADR</td>
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<tr>
<td>WP401</td>
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<tr>
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<tr>
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<td>257 ± 19</td>
</tr>
<tr>
<td>WP904</td>
<td>249 ± 68</td>
</tr>
</tbody>
</table>

Table 5. Effect of the studied compounds on the number of revertants his+ of bacteria Salmonella typhimurium TA102

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Figure 1. Induction of micronuclei in ME18 cells by new anthracycline derivatives.
Evaluation of mutagenic and genotoxic activities of new derivatives...

At a concentration of 0.1 µg/plate, all the studied derivatives were mutagenic except for WP839, WP890 and ADR. The highest number of revertants at this concentration induced derivative WP903. The concentration 10.0 µg/plate was toxic in the case of two derivatives: WP903 and WP904.

In case of strain TA102, also none of the studied compounds, at a concentration of 0.1 µg/plate, manifested mutagenic action. At 1.0 µg/plate only four derivatives revealed mutagenic action (WP744, WP892, WP903, WP904), however at a concentration of 10 µg/plate, the mutagenic activity manifested all the derivatives, except for ADR, WP899 and WP890. In all the concentration used, the highest number of revertants was induced by derivative WP903.

Results obtained in the micronuclei test in vitro are presented in Figures 1–2. The range of concentrations of the studied compounds was limited by their cytotoxic and cytostatic properties. Studies were carried out using two cell lines: mouse cells L929, which, considering their sensitivity towards cytostatic action, are recommended for this type of investigations and on human melanoma cells ME18 sensitive to the action of ADR.

ADR at concentrations 0.001 and 0.005 µg/plate did not induce micronuclei in any of the cell lines used. Micronuclei occurred only at concentrations 0.01 and 0.1 µg/mL, at the same time their increase was statistically significant only at the highest concentration used. The observed genotoxic effect was stronger in the case of mouse cell lines. Derivatives of anthracycline were studied in the concentration range from 0.001 to 0.01 µg/mL because higher concentrations revealed cytostatic action which was manifested as the lack of binucleate cells in the culture. All derivatives of anthracycline have induced micronuclei in both cell lines and the quantity of micronuclei was proportional to the concentration of the studied compound.

The least genotoxic active were derivatives WP889, WP890 and WP892, the most genotoxic for both cell lines were derivatives WP744 and WP903.

CONCLUSIONS

Based on the results given in Tables 2–5, it may be said that the strongest mutagenic effect occurred in strain TA98 in which three of the studied compounds (WP744, WP903, WP892) were mutagenic already at a concentration of 0.1 µg/plate. For strains TA97, TA100 and TA102, the mutagenic effect was observed only at a concentration of 1 µg/plate or higher.

It should be emphasized that ADR increased the number of revertants his+ only in two bacterial strains in TA98 at a concentration of 1 mg/plate and 10 µg/plate, and in strain TA100 at 10 µg/plate.

Comparing the mutagenic activity of ADR and studied derivatives of anthracyclines, it may be concluded that compounds WP744 and WP903 appeared to be the strongest mutagens.

Derivative WP903 deserves special attention. It manifested a mutagenic action already at a concentration of 0.1 µg/plate in strain TA98, at 1.0 µg/plate it was mutagenic for all the strains used in this study and has induced the highest number of revertants in the following strains: TA97, TA100 and TA102. A correlation was observed between the re-
sults obtained in the Ames test and those received in the micronuclei test. In both tests, derivative WP903 demonstrated a strong genotoxic action. The results obtained indicate that the structural modifications introduced in the saccharide part and in glucone, both present in derivative WP903 (methoxyl group in position C4 was removed in glucone and a halogen substituent was introduced to the saccharide part in position C2), distinctly enhanced genotoxic activity of this derivative compared to ADR.

Analysis of the obtained results allows to suggest the justness for further studies aimed at the evaluation of potential possibilities of derivative WP903 in the therapy of tumor diseases.

REFERENCES


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