Studies on the Antitumor Effect of Cucurbitacins*

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SUMMARY

The carcinostatic activity of elatericin A, elatericin B, elaterin, elaterin methylether, dihydroelatericin A, and tetrahydroelatericin A, all cucurbitacins of a triterpene structure, and of trans-4-hydroxy-4-methylpent-2-enolic acid, the isolated side chain of the cucurbitacin molecule, was studied.

Three cucurbitacins, elatericin A, elatericin B, and elaterin produced a moderate inhibition of growth of Sarcoma 180, Sarcoma Black, and Ehrlich ascites carcinoma. Sarcoma 180 proved to be the most sensitive tumor.

Morphological changes of Ehrlich ascites cells, consisting of blistering and thread formation without swelling or vacuolization, were obtained by incubation with low concentrations of elatericin A, elatericin B, elaterin methylether, and elaterin. Comparatively higher concentrations of cucurbitacins were needed to affect the tumor-producing capacity of these cells after in vitro incubation and to depress the rate of respiration measured by Warburg manometry.

The cucurbitacins are a group of highly oxygenated substances which have been isolated from various species of the Cucurbitaceae (6). Some of the species of this plant family, e.g., Ecballium elaterium and Citrullus colocynthis, have been used for their cathartic properties since remote times. In the last few years the cucurbitacins have been the object of more detailed studies, and complete structures have been proposed for elatericin A (I), elatericin B (IIa), and elaterin (IIb) (8) (Chart 1). All are tetracyclic triterpenes, differing in the nature of their oxygenated functions in ring A (e.g., I and IIa), or in the tertiary acetoxy group occurring in the side chain (IIb), as described in previous publications (7–12). When elatericin A is hydrogenated catalytically, 1 mole of hydrogen is absorbed. In the resulting product, dihydroelatericin A, the double bond of the side chain has been reduced (III) (7). The molecule can be further reduced with a second mole of hydrogen to yield tetrahydroelatericin A (IV). In this derivative the α-ketol system of ring A is reduced to an α-glycol. Elaterin methylether (IIc) was prepared by methylation of the enolic hydroxyl of elaterin with methyl iodide (11). During degradation experiments carried out on elatericin A, the side chain was split with periodic acid, and trans-4-hydroxy-4-methylpent-2-enolic acid (4-HMP) was obtained (10).

Some of the above described cucurbitacins had been screened previously for their antitumor activity (13). In the present study their influence both on tumor growth in vivo and on viability of tumor cells after incubation in vitro was investigated.

MATERIALS AND METHODS

Tumors.—The experimental mouse tumors used in this study were: Ehrlich ascites carcinoma (Lettré), Sarcoma 180, and Sarcoma Black (SBL1). Ehrlich ascites carcinoma was obtained from the Israel Institute for Biological Research, Ness Ziona, Sarcoma 180 from the Sloan-Kettering Institute for Cancer Research, New York, and Sarcoma Black (SBL1) (16) from the Hebrew University, Jerusalem.

Hosts.—Inbred strains of RIII, Swiss, and

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1 Although the validity of the structure of ring A has been recently challenged (14).

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Chemotherapeutic studies

Retardation of tumor growth.—The substances were injected intraperitoneally, 24 hours after tumor implantation. Injections were repeated every 48 hours for 8 days (four treatments) in Ehrlich ascites carcinoma-bearing mice and for 2 weeks (six treatments) in the solid tumor-bearing hosts. Simultaneously, control animals were given injections of physiological saline solutions according to the same scheme. All substances were tested on groups of ten mice for each type of tumor, and each test was performed twice. Control experiments were carried out in groups of not less than ten mice.

Animals bearing solid tumors were sacrificed by cervical disarticulation, 1 day after the last injection. The tumors were carefully excised and weighed. The ratio of tumor weight of treated versus control animals (T/C) was established according to CCNSC specifications (4). The Ehrlich ascites-bearing mice were sacrificed by ether, 1 day after the last injection. Total ascites tumor volume (TV) and total packed cell volume (TPCV) of treated versus control animals were also established according to CCNSC specifications.

Viability.—Ascites cells were obtained from R III mice, 7–9 days after implantation. They were suspended in a phosphate-buffered solution of 0.9 per cent NaCl in water, pH 7.2, in a concentration of 10–20 million cells/ml. The suspensions were incubated with varying concentrations of the test substances in a shaking water bath at 37°C.
for 1 hour. The cells were centrifuged and washed twice with phosphate-buffered saline. Bioassay for cell viability was performed by intraperitoneal implantation of 4–10 million incubated ascites cells. The percentage of growth inhibition of the tumor and mortality after implantation was determined. Surviving animals were observed for a period of 3 months.

Morphological studies.—Ehrlich ascites carcinoma cells were incubated for 1 hour at 37°C with various concentrations of different cucurbitacins. The cells were then fixed in a 0.5 per cent buffered isotonic NaCl solution of OsO₄, pH 7.2, and morphological changes were examined with a Wild phase contrast microscope. The percentage of dead cells was determined by vital staining of ascites cells, with the use of eosin (0.5 per cent) or trypan blue (0.5 per cent). In another series of experiments elatericin A was injected intraperitoneally into Ehrlich ascites carcinoma-bearing mice, 1 day after tumor implantation. Samples of tumor cells were obtained from the peritoneal cavity of animals sacrificed 1 or 24 hours after the injections.

Determination of respiratory rates in vitro.—Respiration of Ehrlich ascites carcinoma cells was determined in presence of elatericin A by the standard manometric technic of Warburg. The tumor-bearing mice were sacrificed by cervical disarticulation 8–10 days after implantation of the Ehrlich ascites carcinoma. Cells were quickly removed from the peritoneal cavity and kept in an ice-water bath. The cells were centrifuged at 2,500 r.p.m. for 10 minutes, resuspended in 0.9 per cent NaCl solution, and washed twice. The oxygen consumption of 105 mg. (wet weight) of the packed cells, suspended in 1 ml. of medium (Krebs-Ringer phosphate or ascitic fluid), was determined in single side-arm Warburg flasks, containing 0.1–0.6 ml. of elatericin A solutions of different concentrations in the side-arm. Finally, the volume of the reaction mixture in the Warburg flasks was brought to 3 ml. by adding sufficient amounts of medium. The center well contained 0.2 ml. of a 20 per cent KOH solution. The gas phase was air. A homogeneous dispersion of cells in the medium was obtained by stirring in a Potter-Elvehjem homogenizer for a few seconds. Control experiments without elatericin A were run simultaneously for each batch of cells. After 10 minutes of temperature equilibration at 37°C, manometric changes were recorded at 10-minute intervals. Elatericin A was tipped into the main flask after 30 minutes, and manometric changes continued to be recorded for 2 hours.

Results were calculated on a standard “Q” basis of cu mm change/mg dry weight/hour, and they are given as percentage inhibition of control values. Dry weight was established after constant weight of packed cells had been reached at a temperature of 105°C, 1 mg. of dried cells corresponding to 2.5 million cells and to 7 mg. of wet weight. The number of cells was determined in a Neubauer hemocytometer.

RESULTS

Tumor growth retardation.—Since it was shown that the subcutaneous and the intramuscular route of injection often caused necrosis of the skin, of the connective tissues, and of the muscles at the site of injection, the intraperitoneal route was preferred. Furthermore, the antitumor activity of all compounds tested was highest after intraperitoneal administration.

The LD₅₀ (single dose) of the substances in Swiss mice was about 2 µg/gm mouse weight for elatericin A, elatericin B, and elaterin, and 10 µg for elaterin methylether. The repeated administration of ½ LD₅₀ had no marked influence upon the mortality of normal or tumor-bearing mice, as may be seen in Table 1. Lower doses were shown to be less active. Concentrations as high as 800 µg/gm mouse weight of 4-HMP and 1000 µg of di- and tetrahydroelatericin A did not provoke toxic reactions.

Symptoms of poisoning observed after the administration of toxic doses of elatericin A, elatericin B, and elaterin appeared after a prolonged latent period, varying according to the dose and the route of injection. The latent period in mice after the intravenous injection of 1 LD₅₀ was 6 hours. The signs of poisoning were: intermittent tachypnea, diarrhea, severe prostration, coma, and death.

Growth retardation produced by elatericin A, elatericin B, and elaterin was tested in mice of different strains bearing S-180, SBL1, and Ehrlich ascites tumor, whereas the effect produced by 4-HMP, elaterin methylether, and the hydrogenated elatericins is still under investigation. As may be seen from Table 1 strongest growth retardation was obtained for S-180. A moderate response was observed for Ehrlich ascites carcinoma if measured according to the total volume criterion, whereas total packed cell volume showed weak growth retardation only.

Viability of in vitro-treated Ehrlich ascites cells.—Viability of Ehrlich ascites cells incubated with various cucurbitacins is summarized in Table 2.

Elatericin B and elaterin methylether produced complete inhibition of viability in a concentration of 10 µg/1 million cells, since no signs of tumor
growth were found after their implantation. Elatericin A in a concentration of 10–30 μg produced only a partial inhibition of tumor growth (20–30 per cent). A complete inhibition was obtained by a concentration of 60 μg. Complete inhibition was produced by 4-HMP in a concentration of 50 μg, whereas no inhibition could be obtained by incubation with the other substances or with 6-MP.

Morphological changes of Ehrlich ascites carcinoma cells induced by the cucurbitacins.—Damage was caused to the membrane of Ehrlich ascites cells by elatericin A, elatericin B, elaterin, and elaterin methylether (Table 2). Incubation of the cells with these substances caused blistering (see Fig. 1). The blisters were of different sizes, surrounding the cell and adhering to it. They had a cytoplasmic structure, proved by staining with Giemsa and Feulgen’s light green. Furthermore, threadlike structures, up to about one cell diameter in length, protruded from the cucurbitacin-treated cells. No swelling or vacuolization was observed in cucurbitacin-treated cells. Dihydroelatericin A produced a deformation of the cell shape, without formation of clear blisters or threads. No morphological changes could be found after incubation with tetrahydroelatericin A, 4-HMP, or 6-MP.

Although 1 μg of elatericin A or B per one million cells in vitro produced blistering in almost 90 per cent of the cells, it did not change their permeability to vital stains, such as eosin or trypan blue. Only higher concentrations, producing a gradual decrease of viability of the Ehrlich ascites cells (Table 2), increased permeability to vital stains.

The intraperitoneal injection of 1 μg/gm of elatericin A into Swiss mice, 1 day after implantation of 2 million Ehrlich ascites tumor cells, produced morphological changes in vivo in 80–90 per cent of the cells, obtained 1 hour after the injection. The percentage of damaged cells found 24 hours after this injection did not differ from that observed in untreated control mice.

Respiration of Ehrlich ascites cells in the presence of elatericin A.—Whereas 0.1 μg of elaterin per one million tumor cells did not produce any measurable reduction of oxygen uptake, 1 μg produced an inhibition of 29 per cent after 2 hours (Chart 2). It may be seen that concentrations

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tumor</th>
<th>Dose (μg/gm)</th>
<th>No. treatments</th>
<th>Morality* (gm.)</th>
<th>Av. change of body weight (gm.)</th>
<th>Tumor wt. (mg.) ± S.D.</th>
<th>Total volume (ml.) ± S.D.</th>
<th>Total packed cell volume (ml.) ± S.D.</th>
<th>Inhibition % (1-T/C)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elatericin A</td>
<td>S-180</td>
<td>1</td>
<td>6</td>
<td>0/20</td>
<td>-1.3</td>
<td>241 ± 132</td>
<td>494 ± 340</td>
<td>2.2 ± 1.8</td>
<td>86 ± 35</td>
<td>53, 37 S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>6</td>
<td>0/20</td>
<td>0.2</td>
<td>494 ± 340</td>
<td>4.0 ± 1.2</td>
<td>2.2 ± 1.8</td>
<td>135 ± 49</td>
<td>44, 34 S</td>
</tr>
<tr>
<td>Ehrlich (in Swiss)</td>
<td>1</td>
<td>4</td>
<td>0/20</td>
<td>2/20</td>
<td>+2.2</td>
<td>2.2 ± 1.8</td>
<td>138 ± 30</td>
<td>2.2 ± 0.8</td>
<td>180 ± 40</td>
<td>34, 24 S</td>
</tr>
<tr>
<td>Ehrlich (in R III)</td>
<td>1</td>
<td>4</td>
<td>0/20</td>
<td>0/30</td>
<td>0.0</td>
<td>2.2 ± 1.8</td>
<td>144 ± 31</td>
<td>4.0 ± 1.3</td>
<td>163 ± 48</td>
<td>30, 12 N.S.</td>
</tr>
</tbody>
</table>

| Elatericin B | S-180     | 1            | 6              | 5/28            | -2.3                           | 350 ± 201              | 749 ± 392                 | 2.8 ± 1.5                             | 144 ± 31             | 30, 12 N.S. |
| Ehrlich (in R III) | 1        | 4            | 0/20           | 4/35             | +0.2                           | 350 ± 201              | 4.0 ± 1.3                 | 144 ± 31                             | 163 ± 48            | 30, 12 N.S. |
| Elaterin     | S-180     | 1            | 6              | 3/20            | -1.0                           | 685 ± 418              | 638 ± 445                 | 2.4 ± 0.7                             | 129 ± 36             | 32, 23 S    |
| Ehrlich (in R III) | 1        | 4            | 0/20           | 1/30             | +1.0                           | 2.4 ± 0.7              | 3.5 ± 0.8                  | 129 ± 36                             | 167 ± 36            | 32, 23 S    |

* Number of dead animals/total number of animals treated.
† S.D. calculated according to procedure described in Cancer Chemotherapy Reports 1959, No. 1, p. 37.
‡ Significance of differences of tumor weight was calculated by the “t” test, P ≤ 0.05.
S. = significant.
N.S. = not significant.
exceeding 4 μg. produced partial inhibition only, if the time of incubation had been limited to 1 hour; progressive respiratory inhibition was obtained by a more prolonged incubation: a 100 per cent inhibition was produced by elatericin A in a concentration of 60 μg. per one million cells after an incubation of 2 hours.

**Sensitivity of the different methods used.**—A comparison of the effects of elatericin A on respiration, viability, and morphology of Ehrlich ascites carcinoma cells shows that remarkable changes of tumor cell morphology were obtained with concentrations as low as 0.1 μg. of elatericin A (see Table 2), whereas respiration was only partially inhibited by concentrations at least 10 times higher (1 μg. producing slight inhibition). Viability of the tumor cells was influenced by concentrations 100 times higher (10 μg. producing partial inhibition of viability).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration of cucurbitacins (μg./one million cells)</th>
<th>Percentage of damaged cells</th>
<th>Mortality after implantation†</th>
<th>Percentage of growth inhibition in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elatericin A</td>
<td>0.01, 0.05, 0.1, 1, 2, 5, 10, 15, 30, 60</td>
<td>10, 91, 88, 87, 90, 91, 90, 96, 96</td>
<td>10/10, 10/10, 10/10, 10/10, 7/10, 8/10, 7/10, 0/10</td>
<td>0, 0, 0, 0, 20, 30, 30, 100</td>
</tr>
<tr>
<td>Elaterin B</td>
<td>0.1, 1, 2, 5, 10, 10</td>
<td>92, 87, 88, 91, 93, 89</td>
<td>10/10, 10/10, 10/10, 5/10, 0/16, 10/10</td>
<td>0, 0, 0, 0, 100, 100</td>
</tr>
<tr>
<td>Elaterin methy-</td>
<td>10, 40</td>
<td>89, 87</td>
<td>0/14, 0/12</td>
<td>0, 100</td>
</tr>
<tr>
<td>ether</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydroelate-</td>
<td>1, 10, 50, 100</td>
<td>9, 15, 46, 87</td>
<td>10/10, 10/10, 10/10, 10/10</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>ricin A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetrahydro</td>
<td>100, 300, 800</td>
<td>17, 18, 34</td>
<td>10/10, 15/15, 15/15</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>elatericin A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-HMP</td>
<td>10, 50, 200</td>
<td>10, 10, 10</td>
<td>10/10, 0/10, 0/10</td>
<td>0, 100, 100</td>
</tr>
<tr>
<td>6-MP</td>
<td>10, 200</td>
<td>14, 16</td>
<td>10/10, 10/10</td>
<td>0, 100</td>
</tr>
<tr>
<td>Control†</td>
<td>0</td>
<td>10</td>
<td>150/150</td>
<td>0</td>
</tr>
</tbody>
</table>

* 400 cells counted.
† All experiments were carried out with 4-10 million cells per mouse.
‡ In phosphate-buffered 0.9 per cent NaCl solution, pH 7.2.

**DISCUSSION**

Antitumor activity of the cucurbitacins in vivo was best observed on S-180, in which there was a 50-60 per cent tumor growth inhibition. For Ehrlich ascites tumor growth, inhibition of 53 per cent was obtained in Swiss mice, whereas the tumors were more resistant in the other host. In vitro, however, a complete inhibition of viability of Ehrlich ascites cells could be obtained by using higher concentrations of cucurbitacins, exceeding one lethal dose for mice. Obviously, such doses...
FIGS. 1-3.—Lettré-Ehrlich ascites tumor cells, suspended 1 hour in saline buffer at 37° C., ×400. (Wild phase contrast.)

FIGS. 4-6.—Lettré-Ehrlich ascites tumor cells after incubation for 1 hour with elatericine A in a concentration of 2 μg per one million cells, ×400. (Wild phase contrast.)

FIGS. 7-8.—Lettré-Ehrlich ascites tumor cells after incubation for 1 hour with elatericine A (2 μg. per one million cells). The photographs were taken in different forms—focus on threads and on the membrane of the cells, respectively, ×400. (Wild phase contrast.)

FIGS. 9-10.—Lettré-Ehrlich ascites tumor cells after incubation for 1 hour with elatericine B, 10 μg. per one million cells, ×400. (Wild phase contrast.)
could not be tested in vivo for tumor growth-inhibitory activity.

Inhibition of respiration of Ehrlich ascites cells by elatericin A progressed gradually with increasing doses and with time of incubation. The morphological changes of these cells, incubated in vitro with elatericin A, did not show a gradual increase with higher doses, as a maximum of uniformly changed cells was reached abruptly by incubation with very low concentrations. It is noteworthy that morphological changes were also found in vivo, 1 hour after intraperitoneal injection of elatericin A into Ehrlich ascites carcinomabearing mice. In samples drawn 24 hours after the injection, however, no deformed or necrotized tumor cells were found. It seems, therefore, that the cell damage produced by elatericin A might be of a reversible character. This is now being studied.

The type of alteration of Ehrlich cells produced by cucurbitacin B differed from that described by Belkin et al. (1) for plant polysaccharides in that no vacuolization and swelling were produced. The blisters surrounding the cells did, however, bear resemblance to the changes produced by specific antiserum (3) and by certain lytic agents (4), both of which produce protrusion of cellular material of cytoplasmic structure. The possibility that the thread-like structures observed in elatericin A-, elaterin B-, or elaterin-treated Ehrlich ascites cells could be spicula of thrombocytes adhering to these cells was ruled out, because these threads were observed even in tumor cells carefully separated from blood elements by previous centrifugation. Furthermore, Giemsa-stained preparation showed the blue cytoplasmic nature of these threads.

The decreased viability and respiratory activity of Ehrlich ascites cells, produced by cucurbitacin B, accompanied by increased permeability to vital stains, are in accordance with the observations of Eaton et al. (5). It is noteworthy, however, that the cucurbitacin-induced morphological cell damage occurred with concentrations too low to produce changes of viability and of respiration of the tumor cells or of their permeability to vital stains.

It seems that the antitumor activity of the cucurbitacins, and especially of elatericin A, differs qualitatively from that of 5-diazouracil, nitrogen mustards, or azaserine (15), since the latter substances, similar to 6-MP in our experiments, neither damage the cell membrane nor affect its permeability to vital stains.

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