Alkaloids of Cynanchum vincetoxicum: Efficacy against MDA-MB-231 Mammary Carcinoma Cells

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Alkaloids 1-4 from Cynanchum vincetoxicum (asclepiadaceae) (Scheme 1) do not have affinity to the oestrogen receptor but they inhibit the growth of the hormone-independent mammary carcinoma cells MDA-MB-231 (Fig. 1) and bind to nucleosides and nucleotides (Table 1). Intercalation was not observed.

In former times Cynanchum vincetoxicum had been used against mammary carcinoma\(^1\). First citations of this therapy are already found in the 6th century\(^2\). Also in medieval times preparations of Cynanchum vincetoxicum (ointments, cataplasms, lotions) had been used for the treatment of external breast cancer\(^3,4\).

We wanted to know whether the alkaloids 1, 2, 3, and 4 (Scheme 1) of this plant might contribute to the claimed cytostatic activity of this plant. Therefore, we tested whether these alkaloids\(^5\) show affinity to the oestrogen receptor of hormone dependent mammary carcinoma cells\(^6,7,8\) in comparison with \(^3\)H-oestradiol: according to our experiments none of these alkaloids nor the natural mixture of alkaloids from C. vincetoxicum show affinity. On the other hand these alkaloids inhibit growth of the hormone independent breast cancer cells MDA-MB 231\(^9\) (Fig. 1): Whilst Alkaloid A shows only weak activity at 1 \(\mu\)M concentration, Alkaloid B and Alkaloid C are most effective: within the biological scattering (0.8% and 1.1%, respectively) no cell growth was observed, whilst Alkaloid D shows T/C\(_{corr}\) = 6.7% (biological scattering: 2.5%).

In order to test whether this growth inhibition is caused by interaction (intercalation, e.g.) with DNA we used UV-difference spectroscopy\(^10,11,12\): all the alkaloids interact with

Scheme 1

\(^*\)Dedicated to Prof. Dr. J. Slavík, Brno, on the occasion of his 70th birthday.
Cytostatic effect on the growth of MDA-MB-231 cells
Concentration 0.001 mM/1

Figure 1

Alkaloids

Difference spectra for 1: Ethidium bromide with native DNA;
2: Ethidium bromide with sonicated DNA;
R=[Ethidium bromide]/[DNA]=1.33.

Difference spectra for 1: Alkaloid D with sonicated DNA;
2: Alkaloid D with native DNA;
R=[Alkaloid D]/[DNA]=1.33.

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native as well as with sonicated DNA (Fig. 2). Therefore, intercalation cannot contribute much to this efficacy. This is corroborated by the negative results of ethidium bromide displacement (Fig. 3).

The degree of interaction of the alkaloids with the respective DNA-nucleosides (Fig. 4) and DNA-nucleotides (Fig. 5), however, is very different and points towards an unspecific effect (Table 1).

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Experimental Part

**UV-spectroscopy:** Uvikon 810 (Kontron), tandem quartz cuvettes. - Fluorescence spectroscopy: Hitachi F 3000, fluorescence cuvettes. - Chromatography: TLC: SiO_2_, Merck 5554, TLC Al-foils, silica 60 F_254_. Detection: Dragendorff reagent (Munier/Machboef). - Column chromatography: SiO_2_, Merck 7734 silica 60 (70-230 mesh). - Drying of org. phases: Na_2SO_4_. - All temp. in °C.

**Extraction of Alkaloids**

4.6 kg of coarsely milled, dried overground parts of *C. vincetoxicum* from the surroundings of Regensburg were macerated 4 times with a total of 40 l of MeOH for 24 h at room temp. - After squeezing the pertinent liquid was combined with the extract, evaporated in vacuo, acidified by dil. HCl to pH 2-3, and filtered after 12 h at 4°. In order to remove non-

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*We thank Iso-Werk, Regensburg, for kind assistance.*

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**Table 1: UV-difference spectroscopy of alkaloids A, B, C, D and natural mixture.**

<table>
<thead>
<tr>
<th>Nucleosid</th>
<th>Alkaloid A</th>
<th>Alkaloid B</th>
<th>Alkaloid C</th>
<th>Alkaloid D</th>
<th>natural mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'-Deoxy-adenosine</td>
<td>R^1=0.07</td>
<td>R^2=0.113</td>
<td>R^1=0.059</td>
<td>R^2=0.063</td>
<td>R^1=0.146</td>
</tr>
<tr>
<td>2'-Deoxy-cytidine</td>
<td>R^1=0.15</td>
<td>R^2=0.080</td>
<td>R^1=0.089</td>
<td>R^2=0.089</td>
<td>R^1=0.089</td>
</tr>
<tr>
<td>2'-Deoxy-guanosine</td>
<td>R^1=0.064</td>
<td>R^2=0.018</td>
<td>R^1=0.153</td>
<td>R^2=0.000</td>
<td>R^1=0.031</td>
</tr>
<tr>
<td>2'-Deoxy-thymidine</td>
<td>R^1=0.100</td>
<td>R^2=0.050</td>
<td>R^1=0.063</td>
<td>R^2=0.131</td>
<td>R^1=0.075</td>
</tr>
<tr>
<td>2'-Deoxy-uridine</td>
<td>R^1=0.14</td>
<td>R^2=0.025</td>
<td>R^1=0.074</td>
<td>R^2=0.061</td>
<td>R^1=0.002</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleotid</th>
<th>Alkaloid A</th>
<th>Alkaloid B</th>
<th>Alkaloid C</th>
<th>Alkaloid D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'-Deoxyadenosine-5'-phosphate</td>
<td>R^1=0.032</td>
<td>R^2=0.037</td>
<td>R^1=0.075</td>
<td>R^2=0.100</td>
</tr>
<tr>
<td>2'-Deoxy-cytidine-5'-phosphate</td>
<td>R^1=0.018</td>
<td>R^2=0.084</td>
<td>R^1=0.254</td>
<td>R^2=0.244</td>
</tr>
<tr>
<td>2'-Deoxy-guanosine-5'-phosphate</td>
<td>R^1=0.065</td>
<td>R^2=0.020</td>
<td>R^1=0.020</td>
<td>R^2=0.025</td>
</tr>
<tr>
<td>2'-Deoxy-thymidine-5'-phosphate</td>
<td>R^1=0.020</td>
<td>R^2=0.040</td>
<td>R^1=0.044</td>
<td>R^2=0.050</td>
</tr>
<tr>
<td>2'-Deoxy-uridine-5'-phosphate</td>
<td>R^1=0.005</td>
<td>R^2=0.050</td>
<td>R^1=0.080</td>
<td>R^2=0.180</td>
</tr>
</tbody>
</table>

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Figure 3
Difference spectra for 1: natural alkaloid mixture; \( R = \frac{[\text{nat. mixture}]}{[2'-\text{Deoxyguanosine}]} = 0.5; \)
2: Alkaloid B; \( R = \frac{[\text{Alkaloid B}]}{[2'-\text{Deoxyguanosine}]} = 0.5; \)
3: Alkaloid D; \( R = \frac{[\text{Alkaloid D}]}{[2'-\text{Deoxyguanosine}]} = 0.5; \)
4: Alkaloid A; \( R = \frac{[\text{Alkaloid A}]}{[2'-\text{Deoxyguanosine}]} = 0.5; \)
5: Alkaloid C; \( R = \frac{[\text{Alkaloid C}]}{[2'-\text{Deoxyguanosine}]} = 0.5; \)

Figure 4

Difference spectra for 1: Alkaloid A; \( R = \frac{[\text{Alkaloid A}]}{[2'-\text{Deoxyguanosine}-5'-phosphate]} = 1; \)
2: Alkaloid A; \( R = \frac{[\text{Alkaloid A}]}{[2'-\text{Deoxyguanosine}-5'-phosphate]} = 0.5; \)

Figure 5

basic materials the aqueous phase was extracted 5-6 times with half its
volume of \( \text{Et}_2\text{O}, \) basified with \( \text{NH}_3 \) to \( \text{pH} \ 9, \) and the bases were extracted 6
times by about 1/3 of its volume with \( \text{Et}_2\text{O}. \) After drying ether was removed
in vacuo: 0.06% of the dry plant material were obtained as partially
crystalline mixture of crude alkaloids.- For CC the crude alkaloids were
dissolved in \( \text{CHCl}_3 \) and adsorbed at 4 times its weight of \( \text{SiO}_2 \) in vacuo.
Alkaloids 1-4 were separated using \( \text{SiO}_2 \) and \( \text{MeOH}/\text{acetone}/\text{benzene} 5/30/65. \) The separation process was controlled by TLC.

Specific Rotations

Alkaloid A:
\( c = 0.10 \text{ g/100 ml MeOH; } T = 24^\circ. \)

<table>
<thead>
<tr>
<th>Wavelength ( \lambda ) (nm)</th>
<th>( \alpha ) (°)</th>
<th>( [\alpha] ) (°·ml/g·dm)</th>
<th>( \Delta[\alpha] ) (°·ml/g·dm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>589</td>
<td>-0.037</td>
<td>37.9</td>
<td>-4.0</td>
</tr>
<tr>
<td>578</td>
<td>-0.039</td>
<td>40.0</td>
<td>-4.1</td>
</tr>
<tr>
<td>546</td>
<td>-0.047</td>
<td>48.2</td>
<td>-4.5</td>
</tr>
<tr>
<td>436</td>
<td>-0.095</td>
<td>97.4</td>
<td>-7.0</td>
</tr>
</tbody>
</table>

Alkaloid B:
\( c = 0.10 \text{ g/100 ml MeOH; } T = 24^\circ. \)

<table>
<thead>
<tr>
<th>Wavelength ( \lambda ) (nm)</th>
<th>( \alpha ) (°)</th>
<th>( [\alpha] ) (°·ml/g·dm)</th>
<th>( \Delta[\alpha] ) (°·ml/g·dm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>589</td>
<td>-0.099</td>
<td>94.8</td>
<td>-6.5</td>
</tr>
<tr>
<td>578</td>
<td>-0.104</td>
<td>99.0</td>
<td>-6.7</td>
</tr>
<tr>
<td>546</td>
<td>-0.120</td>
<td>114.3</td>
<td>-7.5</td>
</tr>
<tr>
<td>436</td>
<td>-0.224</td>
<td>213.3</td>
<td>-12.3</td>
</tr>
</tbody>
</table>

Alkaloid C:
\( c = 0.10 \text{ g/100 ml MeOH; } T = 24^\circ. \)

<table>
<thead>
<tr>
<th>Wavelength ( \lambda ) (nm)</th>
<th>( \alpha ) (°)</th>
<th>( [\alpha] ) (°·ml/g·dm)</th>
<th>( \Delta[\alpha] ) (°·ml/g·dm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>589</td>
<td>-0.102</td>
<td>103.0</td>
<td>-7.2</td>
</tr>
<tr>
<td>578</td>
<td>-0.108</td>
<td>109.1</td>
<td>-7.5</td>
</tr>
<tr>
<td>546</td>
<td>-0.125</td>
<td>126.3</td>
<td>-8.4</td>
</tr>
<tr>
<td>436</td>
<td>-0.236</td>
<td>238.4</td>
<td>-14.0</td>
</tr>
</tbody>
</table>

Alkaloid D:
\( c = 0.11 \text{ g/100 ml MeOH; } T = 24^\circ. \)

<table>
<thead>
<tr>
<th>Wavelength ( \lambda ) (nm)</th>
<th>( \alpha ) (°)</th>
<th>( [\alpha] ) (°·ml/g·dm)</th>
<th>( \Delta[\alpha] ) (°·ml/g·dm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>589</td>
<td>-0.021</td>
<td>19.4</td>
<td>-2.8</td>
</tr>
<tr>
<td>578</td>
<td>-0.022</td>
<td>20.4</td>
<td>-2.8</td>
</tr>
<tr>
<td>546</td>
<td>-0.025</td>
<td>23.1</td>
<td>-3.0</td>
</tr>
</tbody>
</table>

Affinity to the oestrogen receptor

The uteri of freshly slaughtered calves were stored in ice cold physiological
NaCl solution until preparation of the cytosol. All preparations were
done at 4°; the longitudinally cut horns of the uteri were freed from fat,
parametrium, and perimetrium. Remainings of blood and mucus were
removed by washing with physiological NaCl solution. After adding of
tris-buffer (10 mM tris, 1.5 mM EDTA, 3 mM NaNO\(_3\), pH 7.5) the horns
were cut by a scissors. 10 g of tissue were suspended in 10 ml of tris-buf-
fer and homogenized (Ultraturrax, 3 times 10 s, then glass homogenizer).
Then the homogenate was centrifuged (10 500 x g, 60 min, +4°). The clear
supematant was taken off by a pipette without the fatty material.- Protein
content: 10-20 mg/ml cytosol.
Binding affinity to the oestrogen receptor was determined indirectly because no radioactive inhibitor was available: According to the recommendations of EORTC\(^3\) we used the charcoal adsorption technique. The interaction of \(^{3}H\)-oestradiol, reduced by an inhibitor (run B), is compared with the control (no inhibitor, run A), and the unspecifically bound amount of \(^{3}H\)-oestradiol is determined in run C:

<table>
<thead>
<tr>
<th>solution (µl)</th>
<th>run A</th>
<th>run B</th>
<th>run C</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{3}H)-oestradiol</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>inhibitor</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>17ß-oestradiol</td>
<td>300</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>tris-buffer</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>cytosol</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Runs A, B, and C were incubated at +4° for 16 h under gentle shaking. The non-bound amounts of \(^{3}H\)-oestradiol and of inhibitor were removed by incubation with 0.5 ml of charcoal suspension (0.8% activated charcoal Norit, 0.008% dextrane in tris-buffer pH 7.5) for 1.5 h at 4°. Adsorbing material was removed by centrifugation (700 x g, 10 min). The determination was done in minivials: 100 µl of the supernatant were mixed with 2 ml of scintillation liquid. The rate of bound \(^{3}H\)-oestradiol was determined as the average of three measurements. The percental rate of \(^{3}H\)-oestradiol is plotted against the logarithm of the molar concentration of the inhibitor. We have chosen six concentrations of inhibitor in order to cover the range of 10-90% of bound \(^{3}H\)-oestradiol. From the diagram, that concentration of inhibitor is determined which inhibits the binding of \(^{3}H\)-oestradiol to the receptor for 50%. The RBA-value is calculated as follows: \(RBA = [\text{oestradiol}] \cdot 100/[\text{inhibitor}]; \ RBA_{\text{oestradiol}} \approx 100\%.

**Efficacy against MDA-MB 231 cells**\(^9,14\)

These cells have been obtained from ATCC and were cultivated as monolayers in dishes (Costar) in a water steam saturated atmosphere containing 5% of CO\(_2\). We used McCoy medium, enriched with 10% of newborn calf serum, gentamycine (40 µg/ml) and NaHCO\(_3\) (2.2 g/l, pH 7.35). Just before confluence the cells were harvested with 0.05% trypsin in a water steam saturated atmosphere containing 5% of CO\(_2\). We used McCoy medium, enriched with 10% of newborn calf serum, gentamycine (40 µg/ml) and NaHCO\(_3\) (2.2 g/l, pH 7.35). Just before confluence the cells were harvested with 0.05% trypsin in a water steam saturated atmosphere containing 5% of CO\(_2\). We used McCoy medium, enriched with 10% of newborn calf serum, gentamycine (40 µg/ml) and NaHCO\(_3\) (2.2 g/l, pH 7.35). Just before confluence the cells were harvested with 0.05% trypsin in a water steam saturated atmosphere containing 5% of CO\(_2\). We used McCoy medium, enriched with 10% of newborn calf serum, gentamycine (40 µg/ml) and NaHCO\(_3\) (2.2 g/l, pH 7.35). Just before confluence the cells were harvested with 0.05% trypsin in a water steam saturated atmosphere containing 5% of CO\(_2\). We used McCoy medium, enriched with 10% of newborn calf serum, gentamycine (40 µg/ml) and NaHCO\(_3\) (2.2 g/l, pH 7.35). Just before confluence the cells were harvested with 0.05% trypsin

**Interaction of the alkaloids with DNA (UV-difference spectroscopy)**

Solutions: tris buffer pH 7.4; 12.1 g tris and 5.85 g NaCl in 700 ml water, N-HCl was added for adjusting to pH 7.4, then filled up to 1 l by water.- DNA-solution: 3.7 mg of calf thymus DNA type I (Sigma) were dissolved in 100 ml of tris buffer pH 7.4 by gentle stirring for 12 h at 4°.- Stock solutions of test compounds: 10\(^{-2}\) M in DMSO.- Concentration of DNA was determined by measurements at 260 and 280 nm, respectively (content of phosphorus).- For \(E_{260}/E_{280} < 1\), Lambert-Beer’s law is valid: 

\[
\frac{c_{\text{DNA}}}{E_{260}} = 6600 \cdot M \cdot \lambda = 260 \text{ nm}; \ d = 1 \text{ cm}; E_{260} = \text{extinction of DNA solution at 260 nm.}
\]

We used tandem cuvettes. For determination of the base line, 1 ml of DNA solution was pipetted into the sample cuvette, 1 ml of tris-buffer was given into the reference cuvette. For getting the difference spectrum 1 µl of alkaloid solution was added to the DNA and to the blank, respectively. The volume differences in both cuvettes were balanced by 1 µl of DMSO. After gentle mixing extinction differences were determined between 550 and 230 nm. The quotient [alkaloid]/[DNA] is plotted against the extinction. A linear increase points towards a dependence on the concentration.

**Interaction with sonicated DNA**

In order to destroy the DNA structure, the DNA solutions (vide supra) were sonicated for 10 min at room temp. The UV-difference spectra of native and sonicated DNA were compared.

**Interactions with phosphorylated and non-phosphorylated DNA-increments**

Instead of DNA solutions stock solutions of the nucleosides 2'-deoxyadenosine, 2'-deoxyctytidine, 2'-deoxyguanosine, 2'-deoxymethylidine, and 2'-deoxyuridine or of the corresponding nucleotides were used. The base line was determined analogously (vide supra) using 5 µl of nucleoside- or nucleotide stock solution, respectively. Consequently 5 µl of DMSO were needed for volume balance. The difference spectra were determined using 2.5 and 5 µl of test solutions added to the nucleosides or nucleotides, respectively.- Determination parameters: scan: 550-230 nm; speed: 100 cm/min.

**Ethidium bromide displacement**

The methodology of LePecq\(^13\) was used.- Excitation: 546 nm, emission: 590 nm.- Tris buffer pH 7.4 and DNA-solution: vide supra.- Concentration of DNA-solution (determination at 260 nm): 7.56 • 10\(^{-3}\) M (P).- Ethidium bromide stock solution: 10\(^{-2}\) M in DMSO.- Control: actinomycin D, 10\(^{-2}\) M in DMSO.- Test substances: alkaloids 1-4.

1 ml of ethidium bromide solution - [DNA][Ethidium bromide] = 1.5 - was pipetted into the fluorescence cuvette and the maximal fluorescence (100%) was determined. After addition of varying amounts of alkaloid solution (1-5 µl) or actinomycin D, respectively, the solutions were mixed gently and the fluorescence was determined (mean value of three determinations). For graphic evaluation the molar ratio [substance][DNA] or the added quantity of test substance [µl], respectively, is plotted against the fluorescence intensity [%].- Parameters: excitement wavelength: 546 nm; emission wavelength: 590 nm; excitation bandpass: 5 nm; emission bandpass: 5 nm.

**References**

1 J. Hartwell, Lloydia 30, 379 (1967).

[Ph25]