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Differentiation between inducers of apoptosis and nonspecific cytotoxic drugs by means of cell analyzer and immunoassay

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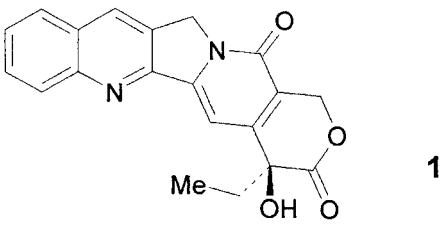
Differentiation between specific and nonspecific cytotoxic drugs by means of cell analyzer and immunoassay is described. The determination of antiproliferative, cytotoxic, and apoptotic effects as well as enlargement of cells in response to toxic compounds can be used for the disclosure of compounds with a specific mode of action. The usefulness of the procedure was demonstrated with camptothecin (**1**) as a cytotoxic drug and established inducer of apoptosis. Aphidicolin (**2**) and the new compound oxoaphidicolin (**3**) were shown to display no comparable effect. Isolation and structural data of **3** are reported.

1. Introduction

Various natural compounds from plants, fungi and bacteria have been shown, to display antiproliferative, cytotoxic and/or apoptotic activities. However, for most of the so far reported compounds little information is available about their mode of action and specific inhibition of cellular functions [1]. Thus, effective prescreening methods will be needed to select interesting compounds such as regulators of the cell cycle and apoptosis from the bulk of compounds showing nonspecific cytotoxicity.

Otherwise, an increasing array of methods has been made available measuring cell proliferation and cytotoxicity by use of well characterized cell lines such as e.g. L-929, K-562, HL-60, and HeLa. Even the automated analysis of suspended and adherent cells is possible using microplate cultures [2–4]. Cellular growth inhibition (GI_{50}), cytotoxic efficacy (CC_{50}), and enlargement of cells in response to toxic compounds can be observed simultaneously. In addition, photometric enzyme-immunoassays are useful to determine qualitatively and quantitatively the appearance of histone-associated DNA fragments as a characteristics of apoptosis.

The aim of this work was to evaluate the usefulness of these methods for differentiation between inducers of apoptosis and drugs showing nonspecific cytotoxicity. Thereby we compared camptothecin (**1**), as an established inducer of apoptosis (5) with aphidicolin (**2**) and the new compound oxoaphidicolin (**3**). Compounds **2** and **3** were isolated from a tropical basidiomyces strain.



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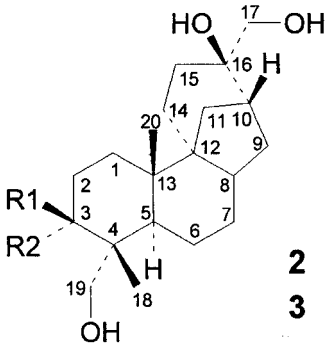

2 $R_1 = OH, R_2 = H$

3 $R_1, R_2 = O$

2. Investigations, results and discussion

The antiproliferative (L-929, K-562, HL-60) and cytotoxic (HeLa) efficacy of compounds **1**–**3** is shown in the Table. The lowest GI_{50} and CC_{50} values were found for camptothecin (**1**). The cytotoxic effects of **1**, **2** and **3** on HeLa cells were clearly distinguishable. Due to the higher cytotoxic efficacy of compounds **1** and **3** any indication for cell synchronization was missing. The dose-response curve of aphidicolin (**2**) and oxoaphidicolin (**3**) on the HL-60 cell proliferation after 3 days of incubation is depicted in Fig. 1.

In another series of experiments the mean cell volume distribution was measured by the CASY cell analyzer system (see Experimental) which is suitable to visualize cell debris, dead and vital cells and cell aggregates as well. The influence of different concentrations of **2** and **3** on K-562 cell enlargement after 3 days is shown by the curves for cell counts vs. control (%) in relation to cell diameters

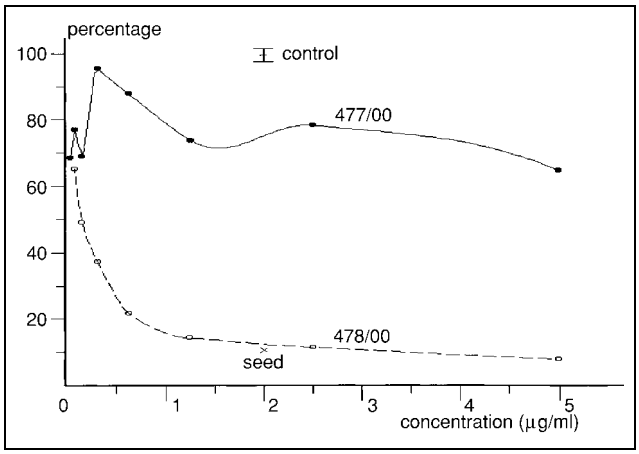

Fig. 1: Concentration-response curve of HL-60 cells after incubation for 72 h with aphidicolin (478/00) and oxoaphidicolin (477/00)

Table: Antiproliferative and cytotoxic efficacy of camptothecin (1), aphidicolin (2), and oxoaphidicolin (3)

Compd.	Antiproliferative effect			Cytotoxic effect
	L-929 GI_{50} (μg/ml)	K-562 GI_{50} (μg/ml)	HL-60 GI_{50} (μg/ml)	HeLa CC_{50} [μg/ml]
1	0.018	0.0002	0.0003	0.2
2	0.840	0.083	0.1	96.8
3	0.920	0.110	>5	8.0

(Figs. 2 and 3). The various hatched curves describe the distribution of cell diameters. The area of living cells is marked by the vertical broken lines. The control curve shows the optimal cell proliferation in absence of inhibitors.

As could be exptected, camptothecin (**1**) displayed the typical characteristics of an inducer of apoptosis [5]. The influence of different concentrations of aphidicolin (**2**) and oxoaphidicolin (**3**) on K-562 cell enlargement was investigated after 3 days using the cell analyzer system CASY, too. Similar to the DNA-binding agents [6] the cell size

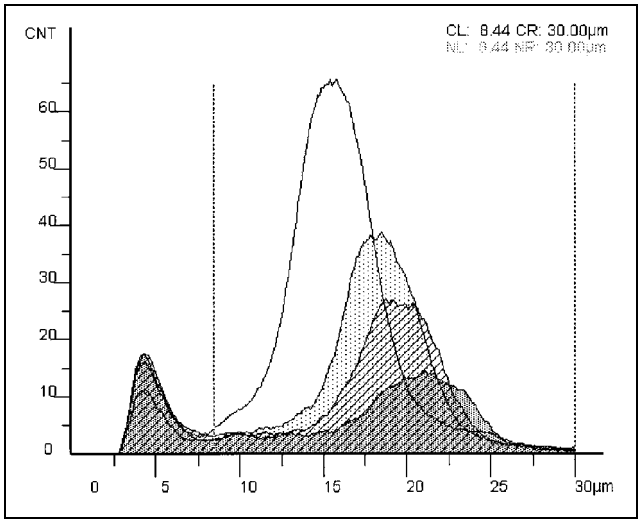

Fig. 2: Distribution curves of counts (CNT) of K-562 cell diameters after incubation with aphidicolin in concentrations of 0.2 μg/ml, 0.1 μg/ml and 0.05 μg/ml for 72 h

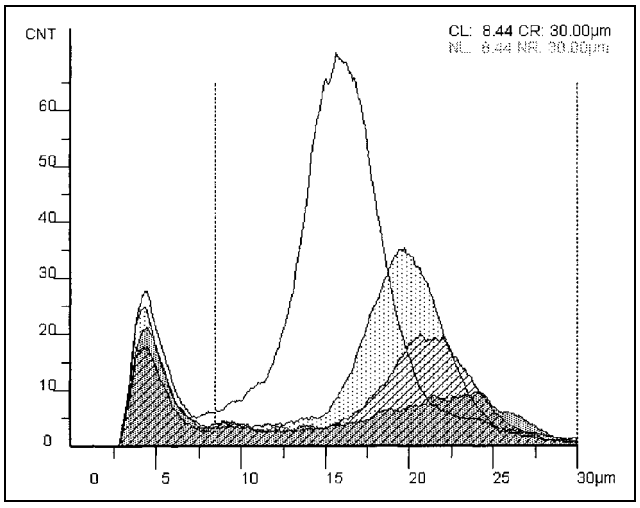

Fig. 3: Distribution curves of counts (CNT) of K-562 cell diameters after incubation with oxoaphidicolin in concentrations of 0.4 μg/ml, 0.2 μg/ml and 0.1 μg/ml for 72 h

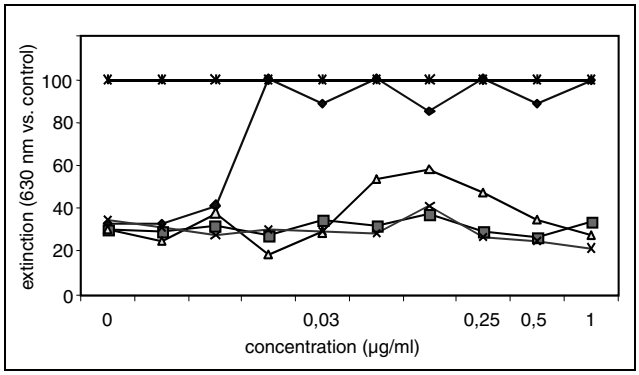

Fig. 4: Induction of the apoptosis of HL-60-cells by camptothecin, aphidicolin, and oxoaphidicolin
◆ Camptothecin; ■ Oxoaphidicolin; △ Aphidicolin; ✳ negative control; ✳ positive control

was increased by compounds **2** and **3**. Distribution curves of K-562 cell diameters were similar (Figs. 2 and 3), suggesting that both are arrested at the transition from G1 to S phase.

From Figs. 1–4 the conclusion can be drawn that inducers of apoptosis such as camptothecin (**1**) are clearly distinguishable from non-inducers such as aphidicolin (**2**) and its oxo derivative (**3**) which both show different inhibition pattern (Fig. 4). This results suggests that the combined use of a cell analyzer and an Elisa assay may be a suitable way for the characterization of apoptotic effects of drugs and the discovery of new inducers of the programmed death of cells.

3. Experimental

3.1. Microorganism and cultivation

The strain HKI 0193 was isolated in a tropical rain forest of South America and identified as a basidiomycete. It was deposited in the strain collection of the Hans-Knöll-Institute for Natural Products Research, Jena. It was disclosed in a screening for cytotoxic compounds using methanolic extracts. For submerged cultivation small pieces (1–2 cm^2) of a slant agar culture of the strain HKI 0193 grown on malt agar (composed of g/l: malt extract 40%, yeast extract 4%, deionized water, pH 6.0) were inoculated into 500 ml Erlenmeyer flasks containing 100 ml of a culture medium composed of (g/l): glucose 10, maltose 20, soytone 2, yeast extract 1, KH_2PO_4 1, $MgSO_4 \cdot 7\ H_2O$ 0.5, $ZnSO_4 \cdot 7\ H_2O$ 0.008 (pH 6.0). Cultivation occurred as surface culture for 14 days whereby a dense lawn of mycelium developed. Thereafter, 20 l of the culture broth were extracted twice overnight by 20 l of ethyl acetate. The dried extracts were evaporated in vacuo.

3.2. Isolation of compounds 2 and 3

The residue of the culture extract (2.2 g) was chromatographed on silica gel 60 (column 100 cm × 5 cm, $CHCl_3$/MeOH, 95 : 5). Fractions were collected displaying cytotoxic activity and/or staining greyish with 1% vanillin in conc. H_2SO_4. Final purification was achieved by repeated preparative TLC (silica gel aluminium sheets Merck, $CHCl_3$, 3 times run, and, respectively, RP_{18} silica gel sheets Merck, acetonitrile/H_2O, 83 : 17, v/v), whereby the yield was 30 mg of **2** and 25 mg of **3**. Compounds **2** and **3** were subjected to optical spectroscopy (IR, UV) mass spectrometry (EI-MS, ESI-MS), and 1D and 2D NMR spectroscopy (^1H, ^{13}C, COSY, HSQC, HMBC, NOESY, TOCSY). According to these data compound **2** was unambiguously identified as aphidicolin which was reported previously as an antiviral, antimitotic antibiotic and apoptose inducing agent [5].

Compound **3** was readily identified as the new diterpenoid metabolite oxoaphidicolin (3-dehydroaphidicolin) due to the physico-chemical data which are given subsequently:

Appearance: wax; molecular weight 336 (ESI-MS: m/z 337.3 ($[M+H]^+$); TLC (silica gel sheets Merck; R_f 0.8 ($ClHl_3$/MeOH 9 : 1); formula $C_{20}H_{32}O_4$ (HREI-MS: m/z 305.2118 $[M-CH_2OH]^+$ (calcd.: 305.2119 for $C_{19}H_{29}O_3$). ^1H NMR (500 MHz; in $CDCl_3$, δ in ppm, TMS as internal standard): 1.6 (H_A-1, m, 1 H), 1.96 (H_B-1, m, 1 H), 2.32 (H_A-2, m, 1 H), 2.68 (H_B-2, m, 1 H), 2.1 (H-5, dd, br, 1 H), 1.40 (H_A-6, m, 1 H), 1.58 (H_B-6, m, 1 H), 1.34 (H_A-7, m, 1 H), 1.45 (H_B-7, m, 1 H), 2.38 (H-8, m, 1 H),

1.25 (H-9, m, 2 H), 2.20 (H-10, m, 1 H), 1.3 (H_A-11, 16.5 d, 7.5 d, 1 H), 1.71 (H-11, 16.5 d, 4.5 d, 1 H), 1.30 (H_A-14, m, 1 H), 1.71 (H_B-14, m, 1 H), 0.98 (H_A-15, m, 1 H), 1.82 (H_B-15, m, 1 H), 3.38 (H_A-17, 11.5 d, 1 H), 3.47 (H_B-17, 11.5 d, 1 H), 1.02 (H-18, s, 3 H), 3.36 (H_A-19, 12.0 d, 1 H), 3.65 (H_B-19, 12.0 d, 1 H), 1.18 (H-20, s, 3 H). ^{13}C NMR (125 MHz; in $CDCl_3$, δ in ppm, TMS as internal standard): 32.6 (C-1), 36.4 (C-2), 218.7 (C-3), 52.9 (C-4), 40.2 (C-5), 24.4 (C-6), 28.1 (C-7), 40.1 (C-8), 29.7 (C-9), 40.9 (C-10), 26.2 (C-11), 48.7 (C-12), 39.3 (C-13), 26.2 (C-14), 31.0 (C-15), 74.4 (C-16), 66.9 (C-17), 17.3 (C-18), 67.3 (C-19), 11.9 (C-20). NOE was observable between H-18/H-19 but not between H-5/H-20, H-8/H-14 and H-10/H-17.

3.3. Cells and culture conditions

Cells of established suspended cell lines K-562 (DSM ACC 10), HL-60 (DSM ACC 3) and adherent L-929 (DSM ACC 2) were cultured in RPMI medium (GIBCO BRL 42402-016), supplemented with 100 U/ml penicillin G-sodium salt/100 µg/ml streptomycin-sulfate (GIBCO BRL 15140-114), 10% heat inactivated fetal bovine serum (GIBCO BRL 10500-064), and L-glutamine (GIBCO BRL 25030-024) at 37 °C in high density polyethylene flasks (NUNC 156340). HeLa (DSM ACC 57) cells were grown in RPMI 1640 culture medium (GIBCO BRL 21875-034) supplemented with 100 U/ml penicillin G-sodium salt/100 µg/ml streptomycin-sulfate (GIBCO BRL 15140-114), 10% heat inactivated fetal bovine serum (GIBCO BRL 10500-064), and 10 ml/l non-essential amino acid (GIBCO BRL 11140-035) at 37 °C in high density polyethylene flasks (NUNC 156340). The adherent cells of L-929 and HeLa were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in HBSS containing 0.038% EDTA (GIBCO BRL 25200-056).

3.4. Antiproliferative and cytotoxicity assays

The target compounds were assayed against cell lines K-562, HL-60, and L-929 for their antiproliferative effects, and against HeLa for their cytotoxic effects. The cells were incubated with ten concentrations of the test compounds.

For each antiproliferative assay with L-929, K-562, and HL-60 approximately 10,000 cells were seeded with 0.1 ml RPMI 1640 culture medium (GIBCO BRL 21875-034), containing 10% heat inactivated fetal bovine serum (GIBCO BRL 10500-064), 100 U/ml penicillin G-sodium salt/100 µg/ml streptomycin-sulfate (GIBCO BRL 15140-114), but without HEPES, into 96-well microplates. The plates were previously prepared with ten dilutions of test substances in 0.1 ml RPMI 1640 medium.

For cytotoxic assay with HeLa approximately 10,000 cells were seeded with 0.1 ml RPMI 1640 culture medium per well of the 96-well microplates. HeLa cells were preincubated for 48 h without the test substances. The dilution of the compounds were carried out carefully on the monolayers of HeLa cells after the preincubation time. The adherent cells of L-929 and HeLa were seeded into microplates NUNC 167008, and the suspended cells of K-562 and HL-60 were seeded into microplates NUNC 163320.

Cells of L-929, K-562, HL-60, and HeLa were incubated for 72 h at 37 °C in a humidified atmosphere and 5% CO_2.

Suspension cultures of K-562 and HL-60 in microplates were analyzed by an electronic cell analyzer system CASY 1 (Schärfe, Reutlingen, Germany) using an aperture of 150 µm. The software for data evaluation CASYSTAT (Schärfe) offers a fast graphical evaluation of the measurement parameters, for example, as diagrams of cell diameter distributions, overlays of different curves, and cell volume distributions. The principles of measurement and evaluation of data were described [7–9]. The 0.2 ml content of each well in the microplate was diluted 1 : 50 with CASYTON (NaCl: 7.93 g/l; Na_2EDTA: 0.38 g/l; KCl: 0.4 g/l; $NaH_2PO_4 \times 1$ H_2O: 0.22 g/l; $NaH_2PO_4 \times 2$ H_2O: 2.45 g/l; NaF: 0.3 g/l; Schärfe). Every count/ml was automatically calcucated from the arithmetic mean of three successive counts of 0.4 ml each. From the dose response curves the Gl_{50} values (concentration which inhibited cell growth by 50%) were calculated with CASYSTAT. The Gl_{50} value (Fig. 1) was defined as being where the concentration-response curve intersected the 50% line, determined by means of the cell counts/ml, compared to control. The essential parameters for the estimation of growth inhibition and for changes in diameter distribution curves are expressed as diagrams.

The monolayer of the adherent L-929 and HeLa cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gently washing the stain was eluted by 0.2 ml of 0.33 N HCl in the wells. The optical densities were measured at 630 nm in a Dynatech MR 7000 microplate reader. Comparisons of the different values were performed with Microsoft Excel.

Cell Death Detection ELISA^{PLUS} assay (Roche Diagnostics GmbH, Germany) was based on a quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates.

Cell lysate was placed into a streptavidin-coated microplate. A mixture of anti-histone-biotin and anti-DNA-POD (monoclonal antibody from mouse (clone MCA-33), peroxidase conjugated, for the binding of the DNA components of the nucleosomes and the color reaction with ABTS) was added and incubated. During the incubation period, the anti-histone antibody binds to the histone-component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated microplate via its biotinylation. After removal of non-bounded components (antibodies) by a washing step the amount of nucleosomes by the POD retained in the immunocomplex was quantitatively determined, and the apoptosis was measured photometrically at 405 nm against ABTS solution as a blank (reference wavelength 490 nm).

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References

1 Gräfe, U.; Dornberger, K.; Saluz, H. P.: Biochemical drugs as antitumour agents, vol. 7, p. 641, VCH Verlagsges. Weinheim 1997
2 Kozlovsky, A. G. et al.: J. Nat. Prod. **63**, 698 (2000)
3 Schlitzer, M.; Sattler, I.; Dahse, H.-M.: Anticancer Res. **19**, 2117 (1999)
4 Ritzau, M. et al.: J. Nat. Prod. **61**, 1337 (1998)
5 Onishi, Y. et al.: Biochem. Biophys. Acta **1175**, 147 (1993)
6 Gutsche, W.; Stelzner, A.: ATLA **25**, 45 (1997)
7 Glauner, B.; Lindl, T.: BIOforum **16**, 370 (1993)
8 Winkelmeyer, P.; Glauner, B.; Lindl, T.: ATLA **21**, 269 (1993)
9 Glauner, B.: BioTec **5**, 58 (1991)

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