Induction of Apoptosis by Alkaloids, Non-Protein Amino Acids, and Cardiac Glycosides in Human Promyelotic HL-60 Cells

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Z. Naturforsch. **62c**, 458–466 (2007); received December 12, 2006/February 16, 2007

The induction of apoptosis by 66 alkaloids of the quinoline, quinolizidine, pyrrolizidine, isoquinoline, indole, terpene, tropane, steroid, purine, and piperidine type, of 9 cardiac glycosides, 11 non-protein amino acids and 10 further secondary metabolites was assayed in HL-60 cell cultures and measured by quantification of the subdiploid DNA content by flow cytometry, detection of DNA fragmentation by gel electrophoresis, and cell morphology. Several alkaloids of the isoquinoline, quinoline, and indole type were active, whereas quinolizidine, tropane, pyrrolizidine, terpene and piperdine alkaloids were mostly inactive. The proapoptotic alkaloids can be characterized by their property to inhibit protein biosynthesis and their intercalation into DNA at the same time, or by their inhibition of microtubule formation. All cardiac glycosides, which are both membrane detergents and Na^+,K^+-ATPase inhibitors, are potent apoptosis inducers. Also proapoptotic were a few non-protein amino acids, podophyllotoxin and the flavonoid quercetin.

Key words: Apoptosis, DNA Intercalation, Protein Biosynthesis Inhibition, Microtubule Inhibitor

Introduction

Nature provides a wide variety of secondary metabolites, which are mainly produced by plants, but also by marine animals and microorganisms. They generally serve as chemical defence substances against herbivores and, to a lesser degree, against microbes, viruses, and competing plants (Harborne, 1993; Rosenthal and Janzen, 1991; Seigler, 1998; Roberts and Wink, 1998; Wink, 1993, 1999a, b).

In order to function as effective defence compounds the structures of alkaloids and other secondary metabolites have been shaped during evolution so that they can interfere with a multitude of molecular targets in animals or microbes. These targets include, among others, DNA (intercalation, alkylation), RNA and related enzymes, protein biosynthesis, biomembranes, cytoskeleton proteins, neuroreceptors, transporter, ion channels, metabolic enzymes, and signal transduction (reviews: Wink, 1993, 2000, 2007; Wink and Schimmer, 1999; Chen *et al.*, 2005).

It has been already observed for some time that several alkaloids, of which more than 20,000 have been described today, have cytotoxic properties (review: Wink, 1993, 2000). In a recent comparative study, a number of alkaloids and other secondary metabolites have been identified which inhibit the growth of blood stream forms of *Trypanosoma brucei* and *T. congolense* and human promyelocytic leukaemia cells (HL-60) (Merschjohann *et al.*, 2001). The HL-60 cell line provides a valid model system for testing antileukaemic or general antitumour compounds.

In most of the studies no distinction has been made between necrotic and apoptotic cell death. Necrosis is a "passive" death resulting in cellular lysis, but apoptosis is a form of physiological cell death that is controlled by intrinsic cellular mechanisms. It can be initiated by many factors, *e.g.* several signalling pathways or chemicals (Kerr *et al.*, 1972; Nagata, 2000; Meier *et al.*, 2000; Debatin, 2004).

However, a number of investigations have already shown, that alkaloids and other secondary metabolites can indeed induce apoptosis in animal cells (review: Wink, 2007). The induction of apoptosis is considered as a new approach to treat tumour cells, and several antitumour compounds, such as *Vinca* alkaloids, taxol, camptothecin, and podophyllotoxin, have these properties. Other

0939–5075/2007/0500–0458 $ 06.00

apoptotic alkaloids found already include cepharanthine, tetrandrine, noscapine, harmine, homoharringtonine, harringtonine, solamargine, usambarensine, stauroporine, berberine, sanguinarine, and emetine (Ye *et al.*, 1998; Kuo *et al.*, 1995; Li *et al.*, 1994; Meijermann *et al.*, 1999; Bicknell *et al.*, 1994; Dassonneville *et al.*, 1999; Weerasinghe *et al.*, 2001; Chen *et al.*, 2005; Wink, 2007). Apoptotic properties have also been detected for some flavonoids and isoflavones, such as apigenin and genistein (Alhasan *et al.*, 1999; Zheng *et al.*, 2005), cardiac glycosides (McConkey *et al.*, 2000), hyperforin (Hostanska *et al.*, 2003), lectins (Hostanska *et al.*, 1997), curcumin (Pan *et al.*, 2001), ajoene (Dirsch *et al.*, 1998), podophyllotoxin (Tseng *et al.*, 2002), a number of sesquiterpene lactones (Dirsch *et al.*, 2001), and antibiotics (daunorubicin, bleomycin, mitomycin).

Since alkaloids and cardiac glycosides are both important for plant defence and several of them are being used in medicine, it is important to know whether the induction of apoptosis is a widely distributed property of secondary metabolites or whether it is restricted to compounds with defined interactions with particular molecular targets, such as DNA intercalation or inhibition of protein biosynthesis (Meijermann *et al.*, 1999; Bicknell *et al.*, 1994; Kochi and Collier, 1993; Wink, 1993, 2000, 2007; Chen *et al.*, 2005).

In this communication we report whether a series of 66 alkaloids representing major alkaloidal groups, 9 cardiac glycosides, 11 non-protein amino acids, and 10 further metabolites (antibiotics, polyphenols) can induce apoptosis in HL-60 cells, which are widely used in apoptosis research. The alkaloids employed have already been analyzed for their interaction with other molecular targets (DNA, protein biosynthesis, neurotransmission) (Schmeller *et al.*, 1994, 1995, 1997a; Wink *et al.*, 1998; Wink, 2000). Apoptosis was assessed by morphological analysis of cells as well as characterization and quantification of DNA fragmentation by flow cytometry and gel electrophoresis.

Materials and Methods

Cell line and culture conditions

The human promyelotic leukaemia HL-60 cell line (DSMZ GmbH, Braunschweig, Germany) was maintained in RPMI 1640 medium without phenol red and L-glutamine (Gibco BRL, Life Technologies GmbH, Karlsruhe, Germany). The medium was supplemented with 10% inactivated foetal calf serum (Seromed®, Biochrom KG, Berlin, Germany), 100 units/ml penicillin, 100 μg/ml streptomycin (BioWhittaker, Walkersville, MD, USA) and 1% L-glutamine.

Suspension-cultured cells were grown in 25 cm^2 or 75 cm^2 culture flasks (Cellstar, Greiner Labortechnik GmbH, Frickenhausen, Germany), incubated at 37 °C with 5% CO_2 and diluted every 2–3 d to a final concentration of ca. 1×10^5 cells/ml. Cell counts were performed with a Neubauer-count chamber, and general viability was assessed by Trypan blue exclusion.

Tested substances

The origin of the tested compounds has been documented in Schmeller *et al.* (1995, 1997a), Minas (1999), Wink *et al.* (1998), and Merschjohann *et al.* (2001). Compounds were dissolved in aqua bidest, DMSO or ethanol to final concentrations of 10^{-1} to 10^{-3} M. After a 1:10-dilution with RPMI medium the mixture was sterilized by filtration (0.2 μm diameter). Further 1:10-dilutions were made with sterile medium containing 10% of the solvent agent to ensure an equal amount of the solvent in the tests, about 1%.

Induction of apoptosis

For experiments cells were seeded into 24-well plates (Gibco BRL), each well was filled with 1 ml cell culture medium. The cell number used in each experiment depended on the incubation time. With a 24 h incubation time HL-60 cells were seeded into the wells with a concentration of 5×10^5 cells/ml. Compounds were given to the cells in various concentrations, from 25 to 5 μl/well. Untreated cultures and cultures treated with medium containing 10% solvent were used for control measurements.

Analysis of cell death

Apoptosis was examined by cell morphology (fluorescence microscopy), DNA gel electrophoresis, but mainly by flow cytometry. Also the activity of caspase 3 (including the caspase 3 inhibitor z-VAD-fmk) and membrane potential were determined using actinomycin D, CCCP, staurosporin, and valinomycin as positive controls.

Fluorescence microscopy: Cells from each well (1 ml) were harvested after treatment with alkaloids, centrifuged (10 min at 4 °C, 400 × *g*), washed

with HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (0.1 M, pH 7.4), centrifuged again, and resuspended in 500 μl HEPES buffer, 500 μl fixing solution (4% paraformaldehyde and 0.4% glutardialdehyde in 0.1 M HEPES, pH 7.4) and 1 μg/ml Hoe33342 (Sigma-Aldrich). Incubation was for 30 min in the dark. Fixed and stained cells were mounted on pre-treated (with poly-L-lysine hydrobromide; Sigma-Aldrich) glass slides with Mowiol® 40–88 (Aldrich-Chemie, Steinheim, Germany) and DABCO (1,4-diazabicyclo-[2.2.2]octane, 25 mg/ml Mowiol; Merck-Schuchardt, Hohenbrunn, Germany). They were investigated with a fluorescence microscope (Zeiss Axioskop, Zeiss, Oberkochen, Germany) and the filter block 02 (Zeiss), using a tenfold ocular and a 100× objective (Plan-Neofluar, Zeiss) with immersion oil (518 C, Zeiss). Pictures were taken with a microscope camera by Zeiss (MC 100 Spot), using a Kodak Elite Chrome slide film with ISO 400/27°.

Agarose gel electrophoresis and DNA fragmentation: 3 ml of treated or untreated cells were harvested from the 24-well plates, centrifuged at 4 °C and 800 × *g* for 10 min, and incubated with 1 ml lysis buffer (NaCl 100 mM, Tris 10 mM, EDTA 25 mM, SDS 0.5%) and proteinase K (Carl Roth GmbH and Co., Karlsruhe, Germany; 0.2 mg/ml) for 1 h at 50 °C. After application of 2 μl RNAse (Roth; 10 mg/ml RNAse buffer, Tris [tris(hydroxymethyl)-aminomethane]/HCl 10 mM, NaCl 15 mM, pH 7.5, boiled for 15 min) and incubation for another hour at 50 °C, samples were divided into two fractions of 500 μl each and extracted twice with the equivalent volume of phenol/chloroform/isoamylalcohol (25:24:1). DNA was precipitated from 400 μl of the supernatant by addition of 800 μl ice-cold ethanol and 40 μl 3 M Na-acetate, pH 5, over night at −20 °C. DNA was precipitated by centrifugation at 4 °C and 12,000 rpm for 20 min, then washed with 500 μl 70% ethanol and dried afterwards. The DNA was resuspended in aqua bidest and stored at −20 °C. DNA content of the samples was determined with a spectrophotometer (Beckmann DU 640, Beckmann Instruments Inc., Fullerton, CA, USA) at a wavelength of 260 nm.

A 2% agarose gel was prepared: 2% agarose (Qualex Gold Agarose, Hybaid GmbH, Heidelberg, Germany) was dissolved in TAE buffer (Tris acetate 0.04 M, EDTA 1 mM, pH 8, acetic acid 1.15%, pH 8.5), containing 0.5 μg/ml ethidium bromide.

Gel pockets were loaded with at least 2 μg DNA, and the gel ran with 90 V for ca. 1.5 h. Gels were documented with UV-light and a camera-printer-system (LTF-Labortechnik, Wasserburg, Germany).

Flow cytometry analysis: 1 ml treated or untreated cell culture was harvested from the 24-well plates and centrifuged for 10 min at 4 °C and 400 × *g*. After a washing step with 1 ml HBSS (Hank's balanced salt solution without Mg^{2+}/Ca^{2+}; Gibco BRL) cells were incubated with 900 μl HBSS and 300 μl PC buffer (Na_2HPO_4 0.2 M, citric acid 0.1 M, pH 7.8) for 5 min, then centrifuged as described above. Cells were fixed with 1 ml ice-cold 70% ethanol and incubated at −20 °C for at least 2 h.

Apoptotic cells were detected using propidium iodide (PI) staining (Nicoletti *et al.,* 1991). Cells in 70% ethanol were centrifuged for 10 min at 4 °C and 400 × *g*, washed once with 1 ml HBSS, and incubated with 200 μl PI staining solution (containing 0.05 mg PI/ml HBSS and 0.5 mg RNAse/ml HBSS) for at least 30 min before measurement with the FACScan analyzer. Experiments were performed in triplicate and repeated at least three times.

Data analysis

A total of 10,000 cells per sample was analyzed by FACScan and Cell Quest software (Becton Dickinson, Heidelberg, Germany) and with WinMDI software (version 2.7, Microsoft Corp.).

Results and Discussion

Apoptosis was identified by fragmentation of HL-60 DNA; a quantitative documentation of the DNA content in apoptotic cells induced by varying amounts of emetine (as an example of an active alkaloid) is shown in Fig. 1. The effect is dose-dependent; first effects are seen with 0.25 μM emetine and a 100% induction is achieved with 2.5 nM (Fig. 1). Variation of incubation time indicated that a complete induction of apoptosis is reached for all concentrations after 40 h of incubation.

About 66 alkaloids of the quinoline, quinolizidine, pyrrolizidine, isoquinoline, indole, terpene, tropane, steroid, purine, and piperidine type were assayed in concentrations ranging from 1 nM to 1 mM in HL-60 cell cultures. As can be seen from Table I, only a selected number of alkaloids are

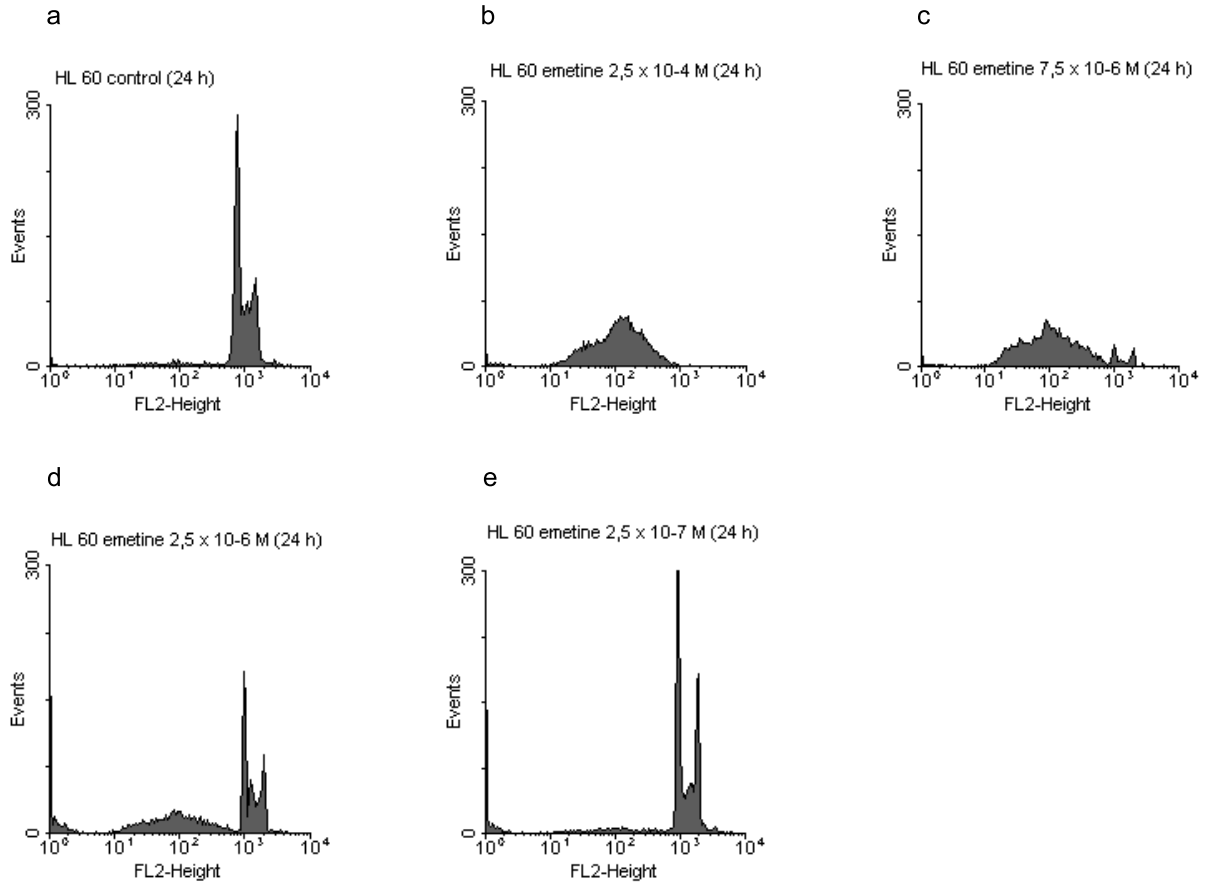

Fig. 1. Illustration of FACScan data analysis of HL-60 cells treated with emetine for 24 h. (a) Control; (b) 2.5×10^{-4} м; (c) 7.5×10^{-6} м; (d) 2.5×10^{-6} м; (e) 2.5×10^{-7} м.

cytotoxic and can induce apoptosis. Active alkaloids (ordered with decreasing induction capacity) were vincristine, vinblastine, homoharringtonine, emetine, cephaeline, colchicine, chelerythrine, sanguinarine, chelidonine, ellipticine, noscapine, protopine, ajmalicine, ergotamine, berberine, harmine, cinchonidine, quinine, arecoline, and piperine. Other alkaloids of the quinolizidine, pyrrolizidine, tropane, purine, isoquinoline, indole, terpene and piperidine type (Table I) did not induce apoptosis in our experiments.

What are then the biochemical characteristics of apoptosis-inducing alkaloids? Several potential interactions of alkaloids with molecular targets have already been analyzed in a comparative study (Schmeller *et al.*, 1994, 1995, 1997a, b; Wink *et al.*, 1998; Wink, 2000). Nearly all alkaloids studied interfere with neuroreceptors and signal transduction, whereas a smaller number intercalates DNA, inhibits DNA-related enzymes, protein biosynthesis, disturbs membrane integrity or interacts with microtubules (Table I).

DNA intercalation, modulation of DNA-related enzymes (*e.g.,* DNA topoisomerase I and II; telomerase), inhibition of protein biosynthesis have already been discussed as activities, which can induce apoptosis for the alkaloids emetine, berberine, camptothecine, chelerythrine, and sanguinarine (Kuo *et al.*, 1995; Dassonneville *et al.,* 1999). These activities can lead to a cell-cycle arrest, as does the inhibition of tubulin polymerization to mitotic spindles and the perturbation of kinetochore-microtubule attachment by the *Vinca* alkaloids taxol, chelidonine, podophyllotoxin, and noscapine. Other mechanisms could include inhibition of protein kinase C or kinases, which phosphorylate p53 (Ye *et al.*, 1998; Chen *et al.*, 2005; Wink, 2007).

Table I. Induction of apoptosis by alkaloids, cardiac glycosides, non-protein amino acids and other metabolites in comparison with their interactions with other molecular targets (according to Wink *et al.*, 1998; Wink, 1999b, 2000, 2007).

Substance	Apoptosis ED50 [mol/l]	Apoptosis (MAC[a]) [mol/l]	Other targets or activities[b]
Isoquinoline alkaloids			
α-Allocryptopine	–	–	neuroreceptors, PDE inhibitor
Apomorphine	–	–	neuroreceptors, PDE inhibitor, PK inhibitor
Berberine	n.d.	10^{-4}	DNA intercalation, neuroreceptors, enzyme inhibitor
Boldine	–	–	DNA intercalation, neuroreceptors, enzyme inhibitor
Chelerythrine	n.d.	10^{-6}	enzyme inhibitor
Chelidonine	n.d.	5×10^{-6}	DNA intercalation, neuroreceptors, enzyme inhibitor
Cephaeline	n.d.	10^{-7}	DNA intercalation, protein biosynthesis inhibitor
Colchicine	n.d.	10^{-7}	inhibition of microtuble formation, neuroreceptors, enzyme inhibitor
Coralyne	–	–	DNA intercalation, enzyme inhibitor
Emetine	4.7×10^{-6}	5×10^{-7}	DNA intercalation, protein biosynthesis inhibitor, enzyme inhibitor, neuroreceptors
Homoharringtonine	0.6×10^{-6}	0.5×10^{-6}	protein biosynthesis inhibitor
Laudanosine	–	–	neuroreceptors
Noscapine	10^{-4}	5×10^{-5}	inhibition of microtuble formation, neuroreceptors
Papaverine	–	–	neuroreceptors, PDE inhibitor, PK inhibitor
Protopine	n.d.	5×10^{-5}	neuroreceptors, enzyme inhibitor
Salsoline	–	–	neuroreceptors, enzyme inhibitor
Sanguinarine	n.d.	5×10^{-6}	DNA intercalation, neuroreceptors, enzyme inhibitor
Indole alkaloids			
Ajmalicine	n.d.	5×10^{-5}	DNA intercalation, neuroreceptors
Ajmaline	–	–	DNA intercalation, ion channel inihibitor
Brucine	–	–	neuroreceptors
Ellipticine	n.d.	5×10^{-6}	DNA intercalation, enzyme inhibitor
Ergotamine	n.d.	5×10^{-5}	neuroreceptors, enzyme inhibitor
Gramine	–	–	neuroreceptors, inihibitor of respiratory chain
Harmaline	–	–	DNA intercalation, neuroreceptors, enzyme inhibitor
Harman	–	–	DNA intercalation, neuroreceptors, enzyme inhibitor
Harmine	–	10^{-4}	DNA intercalation, neuroreceptors, enzyme inhibitor
Norharman	–	–	DNA intercalation, neuroreceptors, enzyme inhibitor
Physostigmine	–	–	neuroreceptors, enzyme inhibitor
Staurosporine	4×10^{-7}	5×10^{-8}	kinase inhibitor
Strychnine	–	–	ion channels, neuroreceptors
Vinblastine	10^{-8}	5×10^{-9}	inhibition of microtuble formation, DNA intercalation, neuroreceptors, enzyme inhibitor
Vincamine	–	–	ion channel inhibitor
Vincristine	10^{-8}	5×10^{-9}	inhibition of microtuble formation, DNA intercalation, neuroreceptors, enzyme inhibitor
Yohimbine	–	–	ion channel inhibitor, neuroreceptors
Quinoline alkaloids			
Quinidine	–	–	DNA intercalation, neuroreceptors, enzyme inhibitor, ion channel inhibitor
Quinine	9×10^{-4}	5×10^{-4}	DNA intercalation, neuroreceptors, enzyme inhibitor
Cinchonidine	10^{-3}	10^{-4}	DNA intercalation, neuroreceptors, enzyme inhibitor
Cinchonine	–	–	DNA intercalation, neuroreceptors, enzyme inhibitor
Purine alkaloids			
Caffeine	–	–	neuroreceptors, enzyme inhibitor
Theobromine	–	–	neuroreceptors, enzyme inhibitor
Theophylline	–	–	neuroreceptors, enzyme inhibitor
Piperidine alkaloids			
Arecoline	n.d.	5×10^{-4}	neuroreceptors, DNA strand breaks
Coniine	–	–	neuroreceptors
Lobeline	–	–	DNA intercalation, neuroreceptors
Piperine	n.d.	10^{-4}	neuroreceptors, enzyme inhibitor
Pseudopelletierine	–	–	neuroreceptors

Table I (continued).

Substance	Apoptosis ED50 [mol/l]	Apoptosis (MAC[a]) [mol/l]	Other targets or activities
Pyrrolizidine alkaloids			
Echimidine	–	–	neuroreceptors, DNA alkylation (after enzymatic activation)
Heliosupine *N*-oxide	–	–	neuroreceptors, DNA alkylation (after enzymatic activation)
Heliotrine	–	–	neuroreceptors, DNA alkylation (after enzymatic activation)
Retronecine	–	–	neuroreceptors, DNA alkylation (after enzymatic activation)
Riddeline	–	–	neuroreceptors, DNA alkylation (after enzymatic activation)
Senecionine	–	–	neuroreceptors, DNA alkylation (after enzymatic activation)
Seneciphylline	–	–	neuroreceptors, DNA alkylation (after enzymatic activation)
Quinolizidine alkaloids			
Cytisine	–	–	neuroreceptors
Lupanine	–	–	neuroreceptors, ion channel inhibition
Sparteine	–	–	neuroreceptors, ion channel inhibition
Tropane alkaloids			
Hyoscyamine	–	–	neuroreceptors
Scopolamine	–	–	neuroreceptors
Terpene alkaloids			
Aconitine	–	–	neuroreceptors, ion channel inhibition
Steroid alkaloids			
α-Chaconine	–	–	enzyme inhibitor, membrane permeability
α-Solanine	–	–	enzyme inhibitor, membrane permeability
Tomatine	–	–	enzyme inhibitor, membrane permeability
Veratridine	–	–	ion channel activator
Miscellaneous alkaloids			
Capsaicine	–	–	ion channel inhibition, enzyme inhibitor
Ephedrine	–	–	neuroreceptors
Nicotine	–	–	neuroreceptors, electron chain
Cardiac glycosides			
Convallatoxin	5×10^{-8}	10^{-8}	Na^+,K^+-ATPase inhibitor
Digitoxigenin	5×10^{-7}	5×10^{-7}	Na^+,K^+-ATPase inhibitor
Digitoxin	5×10^{-8}	5×10^{-8}	Na^+,K^+-ATPase inhibitor
Digoxigenin	1.5×10^{-6}	10^{-6}	Na^+,K^+-ATPase inhibitor
Digoxin	10^{-7}	10^{-7}	Na^+,K^+-ATPase inhibitor, inhibition of protein synthesis
Oleandrin	5×10^{-8}	5×10^{-8}	Na^+,K^+-ATPase inhibitor, NF$\varkappa$B inhibition
Ouabagenin	1.6×10^{-6}	10^{-6}	Na^+,K^+-ATPase inhibitor
Ouabain	2×10^{-7}	10^{-7}	Na^+,K^+-ATPase inhibitor
Proscillaridin	1.3×10^{-8}	10^{-8}	Na^+,K^+-ATPase inhibitor
Non-protein amino acids			
Albizziin	–	–	asparagine mimic
Aminoethylcysteine	2.7×10^{-4}	5×10^{-5}	lysine mimic
β-Aminoproprionitril	–	–	collagen modification
Azaserine	–	5×10^{-6}	
Azetidine carboxylic acid	–	–	proline mimic
Canaline	–	–	ornithine mimic
Canavanine	–	–	arginine mimic
β-Cyanoalanine	–	–	NMDA agonist
3,4-DH-proline	–	10^{-4}	proline mimic
Mimosine	–	5×10^{-4}	tyrosine mimic
Quisqualic acid	–	–	NMDA agonist
Antibiotics			
Cycloheximide	6×10^{-5}	10^{-5}	eukaryotic protein biosynthesis inhibitor
Daunomycin	2.4×10^{-7}	10^{-6}	DNA intercalator; enzyme inhibitor
Kanamycin	–	–	prokaryotic protein biosynthesis inhibitor
Penicillin G	–	–	prokaryotic protein biosynthesis inhibitor
Tetracyclin	–	–	prokaryotic protein biosynthesis inhibitor
Valinomycin	–	10^{-8}	ion channel formation

Table I (continued).

Substance	Apoptosis ED50 [mol/l]	Apoptosis (MAC[a]) [mol/l]	Other targets or activities
Other secondary metabolites			
Asaron	–	–	DNA alkylation after activation
Cynarin	–	–	enzyme inhibitor
Podophyllotoxin	4×10^{-8}	10^{-8}	inhibition of microtuble formation; DNA Top II inhibitor
Quercetin	5×10^{-5}	10^{-5}	enzyme inhibitor

[a] MAC, minimal apoptotic activity. [b] PDE, phosphodiesterase; PK, protein kinase.
–, No apoptosis; n.d., not determined.

Inhibition of protein biosynthesis alone appears to be not sufficient for apoptosis induction, as shown by the cyclic depsipeptide didemnin B (Beidler *et al.*, 1999). Emetine, a typical protein biosynthesis inhibitor, is a significant DNA-intercalating agent at the same time (Wink *et al.*, 1998). We suggest that the high induction capacity of emetine is due to both, inhibition of protein biosynthesis and DNA intercalation which cause frame-shift mutations and inhibition of several DNA-related enzymes (Wink *et al.*, 1998; Wink and Schimmer, 1999; Möller and Wink, 2007). The quinoline alkaloids also intercalate DNA to a similar degree as emetine; but they are much weaker protein biosynthesis inhibitors. It is thus plausible that they induce apoptosis at mM concentration only. In consequence, the combination of DNA intercalation and potent inhibition of protein biosynthesis appears to be one characteristic of powerful apoptosis-inducing alkaloids, such as emetine and cephaeline.

All nine cardiac glycosides, both of cardenolide and bufadienolide type, are potent inducers of apoptosis with IC_{50} values in the range of 10–100 nM (Table I). The cardiac glycosides are strong inhibitors of membrane Na^+,K^+-ATPase that generate the ion gradients necessary for several transport mechanisms and neuronal signal transmission. One of the consequences is an increase in cellular Ca^{2+} concentration. In addition, they are amphiphilic compounds with detergent properties (Seigler, 1998; Wink, 1999a, b). Apparently, the combination of both activities produce cytotoxic effects and can induce apoptosis, as shown already for some cardenolides in an earlier paper (McConkey *et al.*, 2000).

Only few of the 11 non-protein amino acids were cytotoxic and induced apoptosis; among them were aminoethylcysteine, azaserine, mimosine, and quisqualic acid. Non-protein amino acids are mimics of proteinogenic amino acids and can thus interfere with their uptake and metabolism. If incorporated into proteins, they will regularly change protein conformation and are therefore often toxic. It is thus surprising that only 4 out of 11 compounds were active in our study.

Among the phenolics, podophyllotoxin, and quercetin were active. The positive activity of the flavonoid quercetin is in agreement with apoptotic properties of apigenin and genistein (Zheng *et al.*, 2005; Alhasan *et al.,* 1999). Among antibiotics, only those that are cytotoxic to eukaryotic cells, such as cycloheximide, daunomycin and valinomycin, have apoptotic properties whereas those, that are specific for prokaryotes, were not.

In conclusion, secondary metabolites, which inhibit protein biosynthesis and are DNA-intercalating compounds at the same time, or metabolites, which inhibit microtubule formation and attachment, appear to be potent apoptosis inducers (Wink, 2007). Energy metabolism, membrane integrity and ion gradients are apparently also targets for apoptotic compounds. Some of the secondary metabolites, which were active in this study, are used as chemotherapeutic agents for the treatment of cancer or parasitic infections. Thus, screening for natural products with apoptotic properties is an interesting strategy to identify new chemotherapeutic agents.

Acknowledgements

We thank Klaus Hexel and the people from the Tumor Immunology Department at the Deutsches Krebsforschungszentrum (DKFZ), Heidelberg for their generous support to use their FACScan and the Deutsche Forschungsgemeinschaft for a fellowship (to V. R.) (Graduiertenkolleg 388: Biotechnologie). Thanks are also due to Dr. Dietmar Steverding and his laboratory.

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