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Indoloquinolizidine Derivatives as Novel and Potent Apoptosis Inducers and Cell-Cycle Blockers

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A collection of approximately 11 000 natural-product derived and inspired compounds was screened for potential apoptosis inducers in the human tumour cell lines HepG2 (liver), HeLa (cervix) and MCF-7 (breast) by means of MTT and ATP-luminescence assays, automated cell counting, caspase 3/7 assay as well as by fluorescence activated cell sorting (FACS) analysis. A group of seven indoloquinolizidine derivatives was identified that exhibited IC_{50} values for cell proliferation as low as 2 $\mu mol\,L^{-1}$, with no major necrosis of cells detectable. At the same time, an increase in the rate of apoptosis of up to 600% relative to the reference level was observed. FACS analysis indicated that these effects are related to an arrest of cells in the G_2M phase of the cell cycle.

Introduction

In multicellular organisms, somatic cells are derived by mitosis and almost all eventually die by apoptosis—the so-called programmed cell death.[1,2] Apoptosis is a well defined, physiological process and one of its tasks is to counterbalance proliferation that occurs in an uncontrolled manner, which can otherwise lead to tumourigenesis and the development of cancer.[3] Among novel treatments in cancer therapy, tumour-specific induction of apoptosis, rather than the use of cytotoxic and/or antiproliferative chemicals, is a promising strategy that is supposed to limit the death of normal cells considerably.[4,5]

In recent years, a variety of natural compounds have been tested for potential apoptosis induction. Some compounds appear to be rather promising with IC_{50} values close to 1 $\mu mol\,L^{-1}$ (or even lower), which is a concentration that might well be achieved as a systemically relevant therapeutic dose.[6–10] Many others, however, exhibit IC_{50} values that are clearly higher than 10 $\mu mol\,L^{-1}$ and, in some instances, even approach the millimolar range.[11–14]

In the present study a group of seven indoloquinolizidine derivatives was identified on the basis of functional screening of approximately 11 000 natural-product derived and inspired compounds, which with IC_{50} values close to 2 $\mu mol\,L^{-1}$, inhibited proliferation in different cell lines and increased the amount of apoptotic cells by up to 600% as compared to the control value. The observed effects appear to be related to an arrest of cells in the G_2M phase of the cell cycle.

Results and Discussion

In the present study, an in-house collection of 11 264 natural-product derived and inspired compounds (denoted here as group I) was tested for potential apoptosis inducers in various human tumour cell lines. These compounds were synthesised as described[15,16] or obtained from commercial sources.[17] As the first screen, an MTT "single-shot" assay was performed with HepG2 cells, that is, one single experiment per compound at the fixed concentration of 100 $\mu mol\,L^{-1}$ was carried out. Out of group I, 916 compounds were found to reduce HepG2 cell proliferation by 50% or more relative to the control measurements, and these were assigned as group II.

Group II was examined in more detail by means of automated cell counting, which is a technique that also allows the precise determination of the amount of necrotic cells on the basis of trypan blue staining. Experiments were performed in the 0.3 to 30 $\mu mol\,L^{-1}$ range (Figures 1 A and 2 A) and only those compounds that caused a reduction in HepG2 cell proliferation equal to or higher than 60% and, at the same time, resulted in a percentage of necrotic cells that was less than 20% (in other words, a cell viability better than 80%) were investigated further. This group III of compounds amounted to 120 substances and was very likely to contain actual apoptosis inducers. Among these our interest was particularly aroused by compounds **1**–**7** (Scheme 1). The structures of these indole-derived heterocycles are similar since they were all synthesised over the course of a program aimed at the development of new phosphatase inhibitors;[15] thus raising the possibility that their

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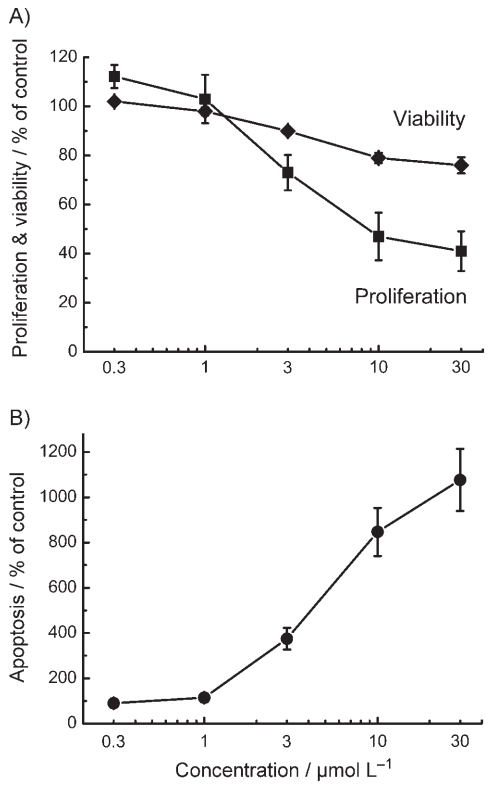


Figure 1. Effects of compound **1** on A) proliferation and viability as determined by automated cell counting, and B) on apoptosis as monitored by using caspase 3/7 activity in human hepatocarcinoma cell line HepG2 ($n=7$ to 12); incubation time: 24 h.

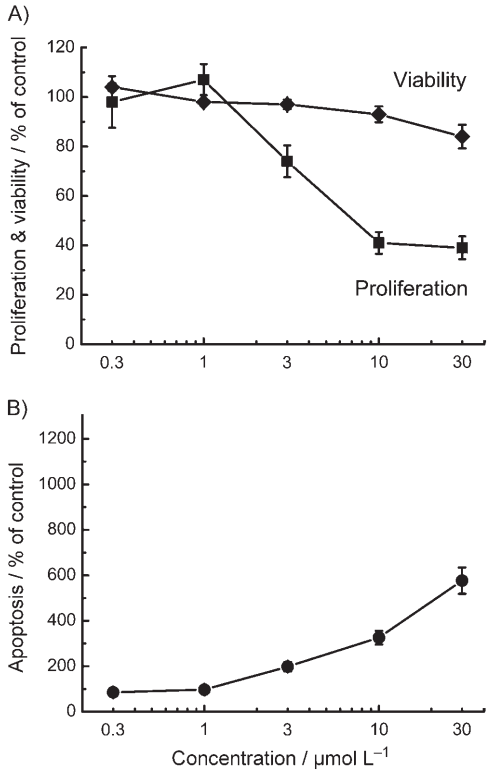


Figure 2. Effects of **7** on A) proliferation and viability as determined by automated cell counting, and B) apoptosis as determined by caspase 3/7 activity in HepG2 cells ($n=7$ to 12); incubation time: 24 h.

mode of action might be similar. In fact, compounds **1**–**4** are diastereomers of a given indoloquinolizidine and compounds **5**–**7** are closely related indoloquinolizidinones.

Compounds **6**, **7** and **1** were most potent in reducing HepG2 cell proliferation, and yielded IC_{50} values of (6.6 ± 1.2), (7.5 ± 0.4) and (8.3 ± 0.2) μmol L^{-1}, respectively (Table 1); these compounds were investigated further.

Table 1. Inhibition of proliferation in different cell lines.

Compound	IC_{50} [μmol L^{-1}] HepG2[a]	HeLa[a]	MCF-7[a]
1	8.3 ± 0.2	2.4 ± 0.2	2.6 ± 0.9
2	24.7 ± 5.3	2.0 ± 0.5	3.5 ± 1.6
3	30.0 ± 8.1	2.1 ± 0.3	3.2 ± 1.1
4	22.8 ± 3.5	4.9 ± 0.2	5.4 ± 0.1
5	23.0 ± 1.9	6.1 ± 0.1	10.4 ± 1.9
6	6.6 ± 1.2	5.1 ± 0.2	3.2 ± 1.5
7	7.5 ± 0.4	0.8 ± 0.1	2.7 ± 0.6
aristoforin	9.0 ± 1.5	2.1 ± 0.4	1.4 ± 0.1

[a] Experiments with HepG2 cells were carried out by means of automated cell counting ($n=7$–12), whereas for HeLa and MCF-7 cells the ATP-luminescence assay was employed ($n=3$); incubation time: 24 h.

Since, in many instances, caspase 3/7 activation was not saturating in the concentration range tested (Figure 2B) apoptosis induction is summarised here as the actual percentage of control values determined at 3 and 10 μmol L^{-1} of each compound. As shown in Table 2, the group of indoloquinolizidines

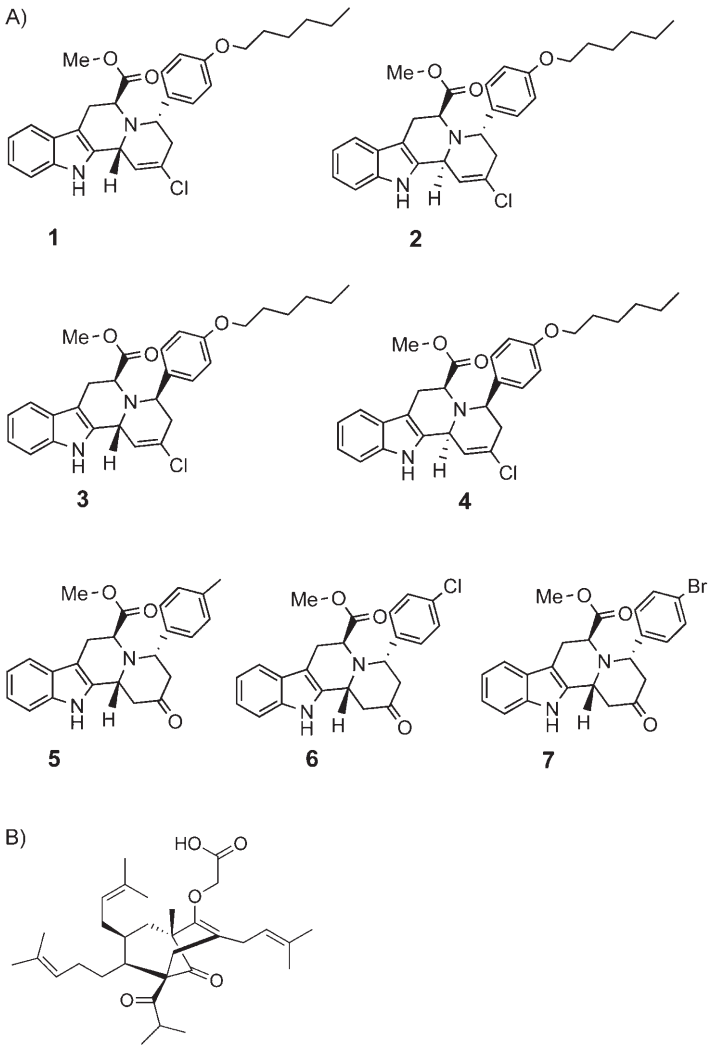
Table 2. Induction of apoptosis in HepG2 cells.[a]

Compound	3 μmol L^{-1}	10 μmol L^{-1}
1	374.9 ± 48.2	847.0 ± 106.0
2	607.7 ± 50.1	853.9 ± 82.9
3	161.8 ± 18.7	996.0 ± 132.2
4	345.6 ± 77.8	977.4 ± 181.1
5	100.3 ± 2.2	125.8 ± 8.3
6	107.7 ± 3.0	378.3 ± 55.7
7	198.0 ± 20.6	326.0 ± 30.2
aristoforin	202.0 ± 19.0	789.7 ± 32.3

[a] Apoptosis was determined on the basis of caspase 3/7 activity and with reference to control recordings (DMSO), which were set as 100% ($n=4$ to 5); incubation time: 24 h.

1–**4** exhibited a strong stimulation of HepG2 apoptosis, which at 3 μmol L^{-1} was already in the range of 160 to 610% relative to the control value (see also Figure 1B). At 10 μmol L^{-1}, apoptosis was further increased to approximately 850 to 1000% of the reference level, that is, by a factor of up to 10. When compared to these data, apoptosis induction by the indoloquinolizidinones was less pronounced with a three- to fourfold stimulation of apoptosis at most, at 10 μmol L^{-1} (Figure 2B and Table 2).

In order to visualise apoptosis induction and to obtain further evidence for the absence of necrosis in the inhibition of



Scheme 1. A) Structures of indole-derived hits identified in the screens and B) aristoforin.

HepG2 cell proliferation, an annexin-V–fluorescein/propidium iodide assay was performed. As exemplified in Figure 3, at 3 μmol L^{-1} both **1** and **7** elicited a distinct increase in apoptosis-induced cell fluorescence and necrosis was not detectable at all. In contrast, one of the compounds from group II that by means of automated cell counting had been identified to induce approximately 80% necrosis at the same concentration (data not shown), did in fact elicit strong necrosis as judged from the marked propidium iodide staining of the nuclei (Figure 3D).

Next, we tested the effects of the indole derivatives on cell growth in the HeLa and MCF-7 tumour models. As summarized in Table 1, both cell lines were significantly more sensitive to all compounds tested when compared to HepG2 cells. In HeLa and MCF-7 cells, the IC_{50} values for **1**–**3** were in the range of 2.0 to 2.4 μmol L^{-1} and 2.6 to 3.5 μmol L^{-1}, respectively, which is very close to the IC_{50} values for aristoforin, namely 2.1 and 1.4 μmol L^{-1}. The latter substance, which is chemically unrelated to the group of indoloquinolizidine derivatives analysed in the present study (Scheme 1B), was employed here as an additional standard for apoptosis induction. Aristoforin is a hyperforin derivative known to interfere with the sirtuins SIRT1 and SIRT2, which are class III histone deacetylases in charge of the regulation of the tumour suppressor protein p53, as well as the formation of microtubules.[6] Compound **4** was found to be slightly less effective in inhibiting HeLa and MCF-7 cell growth than its congeners, and the same was true for **5** and **6**. For compound **7**, on the other hand, IC_{50} values of 0.8 and 2.7 μmol L^{-1} were obtained, which rendered this substance comparably as effective as **1**–**3** (Table 1).

Finally, possible effects of the compounds on HeLa cell-cycle distribution was analysed by fluorescence activated cell sorting (FACS). As shown in Figure 4 and summarised in Table 3, cells in G_1 and G_2M phase equalled 51 and 24% under control (DMSO) conditions. This ratio (of 2.14) dramatically changed with the various indoloquinolizidine derivatives tested (at 5 μmol L^{-1}), and the maximal effect with the two groups was achieved with **3** and **5**, which yielded G_1 and G_2M phases of 30 vs. 48% and 11 vs. 62%, respectively; this is equivalent to actual ratios of 0.62 and 0.17 (Table 3). Consequently, the inhibition of cell growth and induction of apoptosis by the compounds in the present study appear to be related to an arrest of the cell cycle in the G_2M phase.

It is noteworthy, however, that in the FACS analysis the effects of the indole-derived ketones were significantly more pronounced than those of the vinyl chlorides, whereas with regard to apoptosis induction the potency of both groups was opposite.

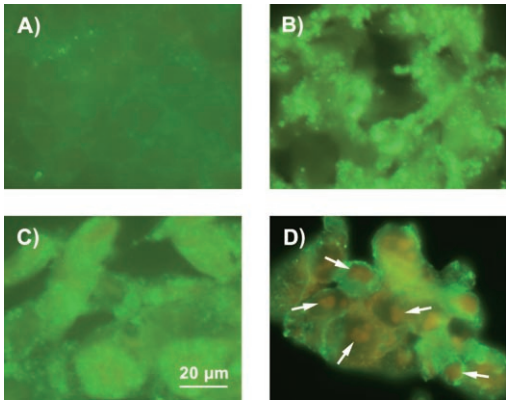


Figure 3. Fluorescence microscopy of HepG2 apoptosis (and necrosis) as determined by use of the annexin-V–fluorescein/propidium iodide assay (see text for details). A) Untreated control cells did not exhibit any detectable apoptosis. B) and C) Cells treated with **1** or **7** (3 μmol L^{-1}) for about 20 h, respectively. Note: the pronounced increase of overall fluorescence reflects the induction of apoptosis. D) Cells were exposed to 3 μmol L^{-1} of one of the compounds from group II that was found to lead to 50% necrosis of cells (determined by means of automated cell counting). This is shown here as a positive control for propidium iodide staining of nuclei (see arrows); incubation time: 24 h.

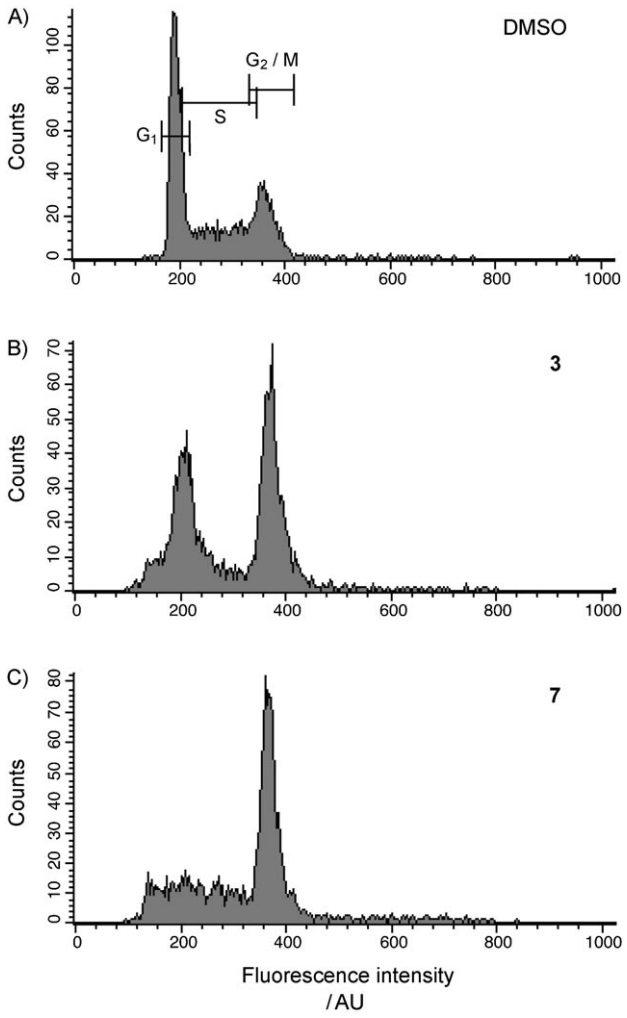


Figure 4. Cell-cycle distribution in HeLa cells. A) Control (DMSO). Effects of B) **3** (5 μmol L^{-1}), and C) **7** (5 μmol L^{-1}); see text for details; incubation time: 24 h.

Table 3. Cell-cycle distribution in HeLa cells.[a]

Compound	G_1	S	G_2M
1	42.8	47.2	29.5
2	38.2	45.7	30.4
3	29.8	29.4	48.1
4	45.0	42.7	29.0
5	10.6	25.8	61.7
6	14.2	32.1	64.4
7	14.8	27.4	57.0
DMSO	51.3	34.3	24.0

[a] Single set of FACS analyses with cell nuclei stained with propidium iodide; incubation time: 24 h.

This suggests a certain variance in the actual interrelation between G_2M arrest and apoptosis induction and, possibly, further intrinsic effects of at least one of the two groups of compounds tested here. Clearly, the actual mechanism of apoptosis induction observed remains to be elucidated.

Conclusions

In conclusion, a collection of approximately 11 000 natural-product inspired and derived compounds was tested for potential apoptosis inducers in three human tumour cell lines. A group of seven indoloquinolizidine derivatives was identified that with IC_{50} values close to 2 μmol L^{-1} inhibited proliferation in HeLa and MCF-7 cell lines and (with slightly lower efficiency) also in HepG2 cells. The effect was due to a significant increase in the rate of apoptosis that at 3 μmol L^{-1} amounted to 600% of the control value, and necrosis was not detectable at all. It is very likely that apoptosis induction occurred by an arrest in the G_2M phase of the cell cycle.

Experimental Section

Chemistry: For the synthesis of indolo[2,3-*a*]quinolizidines and -quinolizidinones, two preparative routes on polymer carriers were employed as described.[15]

Cell culture: Human HepG2 (hepatocarcinoma), HeLa (derived from cervix carcinoma) as well as MCF-7 (mammary carcinoma) cells were seeded in 96-well plates at a density of 10^4 per 100 μL per well, and cultured in RPMI medium at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Compounds were added after 24 h of incubation, and all assays were conducted after 48 h incubation. For visualisation of apoptosis/necrosis, HepG2 cells were grown on glass coverslips.

Application and use of compounds: In the first screen (of the total library), each compound was tested at a single dose (100 μmol L^{-1}) for its effects on HepG2 cell proliferation as monitored by using the MTT assay (see below). Those substances that exhibited an effect of 50% or higher were then subjected to automated cell counting (at 0.3, 1, 3, 10 and 30 μmol L^{-1}); this procedure also yielded the amount of necrotic cells. Finally, the compounds that with this technique had given a decrease in HepG2 cell proliferation of more than 50% and at the same time a cell viability that was better than 80%, were tested for their apoptotic potency (again in the range of 0.3 to 30 μmol L^{-1}), which was determined by use of a caspase 3/7 assay (see below). In addition, the latter batch of chemicals was tested for their effects on HeLa and MCF-7 cell growth, which was monitored by using an ATP-luminescence kit. The same compounds were also checked for their influence on HeLa cell-cycle distribution (see below). All substances were taken from DMSO stock solutions and in every instance the final detergent concentration was kept constant at 1 mmol L^{-1}.

MTT and ATP-luminescence assays, automated cell counting: With the MTT assay, the mitochondrial activity of cells is quantified on the basis of the enzymatic transformation of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) to MTT formazan (excitation/emission at 550/690 nm). The ATP-luminescence assay employs the reaction:

$$\text{ATP} + \text{D-luciferin} + O_2 \rightarrow \text{oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light}$$

which is catalyzed by the firefly enzyme luciferase and is proportional to the number of metabolically active cells. Both assays were performed by following the manufacturers' protocols (Thiazolyl Blue, Sigma, Taufkirchen, Germany; ATPlite 1step, PerkinElmer, Rodgau, Germany). Automated cell counting was performed by using a Vi-Cell XR cell-viability analyser (Beckmann Coulter, Krefeld, Germany); this yielded the total number of trypsinized cells in sus-

pension as well as the percentage of necrotic cells (on the basis of the accessibility of cells to trypan blue staining).

Apoptosis assays: Apoptosis of HepG2 cells was first quantitatively determined by means of the Apo-ONE homogeneous caspase 3/7 assay (Promega, Mannheim, Germany) in which a rhodamine substrate is caspase activated; this leads to a green fluorescence product (excitation/emission at 500/520 nm). Second, apoptosis was visualised by use of the Annexin-V–FLUOS staining kit (Roche, Mannheim, Germany), which is based on the staining of phospatidylserine, which is flipped to the outer leaflet of the plasma membrane only under apoptotic conditions, and with propidium iodide staining of nuclei as a highly sensitive negative control for necrotic cells.

FACS analysis: HeLa cell-cycle analysis was performed by using a FACScan (Becton–Dickinson, Heidelberg, Germany) with propidium iodide as a DNA marker (and cell permeabilization in 70% ethanol for 1 h).

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Keywords: antitumor agents · apoptosis · cell cycle · indoloquinolizidine derivatives · natural products

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