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Alkylphosphocholines induce apoptosis in HL-60 and U-937 leukemic cells

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Abstract Alkylphosphocholines (APC) represent a new group of ether-lipid-related compounds with remarkable activity against transformed cells in vitro and good tolerability in vivo. Their mechanism of action remains unknown. The aim of the present study was to investigate the effects of a series of APC on three human leukemic cell lines: K-562, HL-60, and U-937. The tetrazolium dye-reduction (MTT) assay and cell counting were used to determine the cytotoxicity of the APC used. DNA gel electrophoresis and enzyme-linked immunosorbent assay (ELISA) detection of oligonucleosomes were performed to identify and quantify DNA fragmentation. Electron and phase-contrast microscopy were used to detect morphologic changes specific for programmed cell death. HL-60 and U-937 cells were found to be sensitive, but K-562 cells were relatively resistant to APC exposure. APC with long alkyl chains exerted stronger cytotoxicity than did those with short alkyl chains. DNA fragmentation was found after treatment with APC in HL-60 and U-937 cells but not in K-562 cells. In HL-60 cells the increase in mono- and oligonucleosome formation as measured by ELISA was correlated with the length of the alkyl chains at 14 h of exposure to APC but plateaued at 20 h. The morphologic alterations in HL-60 and U-937 cell lines, such as cell shrinkage, chromatin condensation, and formation of apoptotic bodies, confirmed the induction of apoptosis after APC exposure. It is concluded that programmed cell death plays an important role in the cytotoxicity of APC against certain human leukemic cell lines. The antineoplastic profiles of APC with long alkyl chains render them attractive for further therapeutic application.

Key words Alkylphosphocholines · HL-60 cells · U-937 cells · K-562 cells · Apoptosis

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Introduction

Ether lipids and related compounds have been recognized as potential anticancer drugs [4, 16]. The class of alkylphosphocholines (APC) has emerged from ether lipids by structural simplification: APC lack the glycerol backbone of ether lipids and show select anticancer activity and a spectrum of toxicity that differs completely from that of conventional anticancer agents [3, 9]. APC were found to be effective against leukemic cell lines in vitro and methylnitrosourea (MNU)- as well as dimethylbenzanthracene (DMBA)-induced breast carcinoma of the rat [3, 15]. The best-known agent of this group is hexadecylphosphocholine (HPC, Miltefosine). HPC has recently been found effective in controlling human breast-cancer skin metastases [28]. The mode of action of APC remains unknown; inhibition of protein kinase C and modulation of signal-transduction pathways originating from the membrane are thought to play a central role. In line with the latter assumption, induction of differentiation has been observed in target cells [11, 21]. The ether lipid 1-octadecyl-2-methyl-rac-glycero-3-phosphocholine (Et-18-OCH_3, Edelfosine) has been shown to induce apoptosis in the human promyelocytic HL-60 cell line [5, 6]. In the present study we

investigated the cytotoxic effects of APC and the induction of apoptotic changes in three human leukemic cell lines: chronic myelocytic leukemia (CML)-derived erythroleukemia K-562 cells, acute myeloblastic leukemia (AML)-derived promyelocytic HL-60 cells, and diffuse hystiocytic lymphoma-derived U-937 cells.

Materials and methods

Compounds

The structure of the APC under investigation is presented in Fig. 1. They can be grouped according to their structure into those with a typical choline polar head and saturated alkyl chain (dodecylphosphocholine, DPC; tetradecylphosphocholine, TPC; hexadecylphosphocholine, HPC; and octadecylphosphocholine, OPC), those with a cyclic polar head and saturated alkyl chain {octadecyl-[2-(*N*-methylpiperidino)ethyl]-phosphate, OMPEP; and octadecyl-(1,1-dimethyl-piperidino-4-yl)-phosphate, ODPP}, and those with a typical or slightly modified choline polar head and unsaturated alkyl chain (erucylphosphocholine, EPC; and erucylphospho-*N*,*N*,*N*-trimethylpropanolamine, EPC_3).

The compounds were synthesized as previously described [8]. OMPEP and ODPP were obtained from Asta Pharma (Frankfurt, Germany). Cytosine arbinoside (Ara C) was obtained from Mack (Illertissen, Germany) in a quality sufficient for clinical use. All compounds were more than 99% pure.

Cells

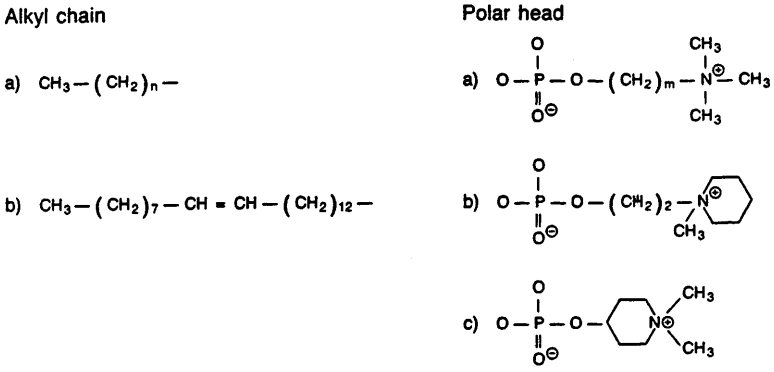
K-562 human chronic myelogenous leukemia, multipotential haematopoietic cells (ATCC CCL 243), HL-60 promyelocytic (ATCC CCL 240) and U-937 histiocytic lymphoma promonocytic cells (ATCC CRL 1593) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), streptomycin (100 µg/ml), penicillin (100 IU/ml), and L-glutamine (4 µmol/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. To keep the cells in the log phase they were passaged two to three times weekly. Some experiments were performed using HL-60 cells grown under serum-free conditions (RPMI-1640 medium supplemented with Nutridoma HU; Boehringer Mannheim, Mannheim, Germany).

Cytotoxicity determination

Tetrazolium dye-reduction assay

Cells were seeded in 96-well plates (100 µl/well at a density of 1×10^5 cells/ml) and exposed to various concentrations of APC for 48 and 96 h as indicated below. The cell survival fraction was

Fig. 1 Structure of the APC used



Chemical name:	Abbreviation:	Structure: Alkyl chain:	Polar head:
dodecylphosphocholine	DPC	a(n=11)	a(m=2)
tetradecylphosphocholine	TPC	a(n=13)	a(m=2)
hexadecylphosphocholine	HPC	a(n=15)	a(m=2)
octadecylphosphocholine	OPC	a(n=17)	a(m=2)
octadecyl-[2-(N-methylpiperidino) ethyl]-phosphate	OMPEP	a(n=17)	b
octadecyl-(1,1-dimethyl-piperidino-4-yl)-phosphate	ODPP	a(n=17)	c
erucylphosphocholine	EPC	b	a(m=2)
erucylphospho-N.N.N-trimethylpropanolamine	EPC_3	b	a(m=3)

determined with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide] dye-reduction assay as described by Mosmann [23], with some modifications. In brief, after incubation with the test compound, MTT solution [2 mg/ml in phosphate-buffered saline (PBS)] was added (10 µl/well). Plates were further incubated for 4 h at 37 °C, and the formazan crystals formed were dissolved by the addition of 0.04 *N* HCL in 2-propanol at 100 µl/well. Absorption was measured by an enzyme-linked immunosorbent assay (ELISA) reader (Anthos 2001) at 540 nm (reference filter 690 nm). For each concentration at least eight wells were used. The blank solution comprised 100 µl RPMI 1640 medium with 10 µl MTT stock and 100 µl 0.04 *N* HCl in 2-propanol.

Cell number determination

Cell number was determined after treatment using a Coulter Counter ZM (Coulter Electronics, Krefeld, Germany). At least three measurements were performed.

DNA fragmentation

DNA gel electrophoresis

RNA-free DNA was isolated from about 10^7 treated or untreated cells according to the protocol of the QUIAmp Blood Kit (Qiagen Inc., Hilden, Germany). At least 10 µg/probe was analyzed by gel electrophoresis in a 1% agarose gel and then stained with ethidium bromide. DNA was visualized and photographed using a UV transilluminator and a camera connected to a computer-aided analysis system and videocopy processor (Herolab E.A.S.Y., Germany).

ELISA detection of oligonucleosomes

DNA fragmentation was quantitated using a cell-death detection ELISA. The cytosolic fraction (13,000-g supernatant) of 10^6 treated or untreated cells was used as an antigen source in a sandwich ELISA with a primary anti-histone antibody-coated microplate and a secondary anti-DNA antibody conjugated to peroxidase. The photometric immunoassay for cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) was performed according to the manufacturer's recommendations (Boehringer Mannheim, Mannheim, Germany). The specific enrichment factor of mono- and oligonucleosomes released into the cytoplasm (ER) was calculated using the following formula:

$$\mathrm{ER} = \frac{\text{absorbance of the sample (treated cells)} - \text{absorbance blank}}{\text{untreated control (viable cells)} - \text{absorbance blank}}$$

Electron and light microscopy

For ultrastructural examination, cell samples were analyzed by electron microscopy. Cells were chilled rapidly to 4 °C and centrifuged gently into pellets. The pellets were fixed with cold 2.5% glutaraldehyde in PBS for 30 min, washed again, and postfixed in 1% OsO_4 + 0.8% $K_4[Fe(CN)_6]$ in 50 m*M* cacodylate buffer (pH 7.2) for 60 min. After three washes in the buffer, specimens were dehydrated in ethanol (20%, 30%, 50%, 70%) and embedded in LR White (London Resins, Woking, UK). Thin sections were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope (EM 400; Philips; Eindhoven, The Netherlands). Electron microscopy was performed by Prof. Dr. E. Spiess, Department of Cancerogenesis and Differentiation, Biomedical Structure Research, German Cancer Research Center [26].

Statistical analysis

Models were fitted to experimental data according to the following formula:

$$E(y) = (100 - \alpha) + \alpha \cdot 2^{-c/\theta},$$

where c is the concentration of APC and y is the cytotoxic effect obtained as a percentage of the untreated control value. IC_{50} values and 95% confidence limits were calculated using S-PLUS 3.3 software [31]. For experimental curves that could not be fitted with the described model a linear approximation was used. The analysis was performed by Dr. Axel Benner, Department of Biostatistics, German Cancer Research Center.

Results

Cytotoxic effects

Data obtained by MTT assay and cell counts were processed and IC_{50} values were calculated (Table 1). All APC tested showed significant cytotoxicity against cell lines HL-60 and U-937. There was a good correlation between MTT viability and cell number determinations. HPC, OPC, OMPEP, and ODPP exerted comparably strong cytotoxic effects. DPC, TPC, EPC, and EPC_3 were found to be less effective. In Fig. 2 the concentration-dependent cytotoxicity of HPC on K-562, HL-60, and U-937 cells is presented. The erythroleukemia cell line K-562 was found to be relatively resistant as compared with the sensitive promyelocytic HL-60 and promonocytic U-937 cells.

Table 1 IC_{50} values of APC for HL-60 and U-937 cells (*ND* Not done)

Cell line	Compound	MTT assay (48 h)	Cell counts (48 h)
HL-60	DPC	35.7[a] (33.3–38.1)[b]	21.4 (15.7–27.0)
	TPC	14.0 (12.7–15.3)	5.2 (4.4–6.0)
	HPC	3.6[c] (3.1–4.1)[c]	3.1 (2.6–3.7)
	OPC	2.6 (2.4–2.9)	1.9 (1.7–2.0)
	OMPEP	4.1 (3.8–4.4)	3.0 (2.7–3.2)
	ODPP	7.2 (6.8–7.6)	ND
	EPC	19.9 (15.8–24.1)	ND
	EPC_3	22.4 (17.7–27.2)	ND
U-937	DPC	56[c] (55.1–56.8)	56[c] (55.1–56.9)
	TPC	30.9 (28.9–32.3)	22.4 (18.8–26.1)
	HPC	21.2 (19.1–23.3)	7.7 (5.8–9.6)
	OPC	9.0 (8.1–9.8)	5.0 (4.1–5.9)
	OMPEP	15.6 (13.5–17.6)	8.5 (7.8–9.2)

[a] IC_{50} as derived by the exponential model
[b] 95% confidence limits of IC_{50}
[c] Linear model was used

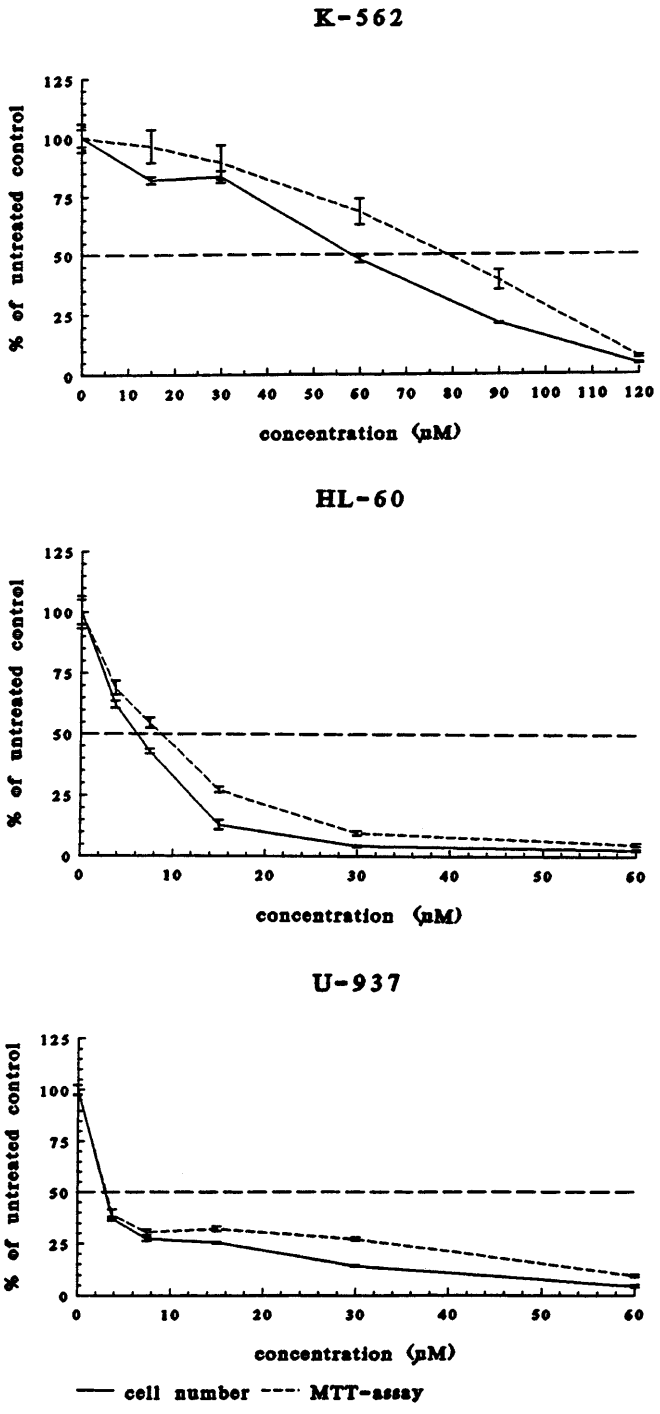


Fig. 2 Cell survival following exposure to HPC for 96 h as measured by MTT assay and cell counts. Comparison of K-562 cells (*top*), HL-60 cells (*middle*), and U-937 cells (*bottom*)

DNA fragmentation

For investigations on DNA fragmentation a concentration was used that was moderately active and allowed comparison of all derivatives at an equimolar level.

DNA gel electrophoresis

Our gel electrophoretic observations (summarized in Fig. 3) show that all APC with the exception of DPC induced DNA fragmentation in HL-60 cells at a concentration of 20 μ*M* for 20 h. HPC and OMPEP induced DNA fragmentation in U-937 cells as well. HL-60 and K-562 cells kept under serum-free conditions (medium supplemented with Nutridoma HU) were found to be more sensitive against APC (over 50-fold more than when they were grown with 10% FCS). Under such conditions, even DPC, the APC with the shortest alkyl chain, was capable of inducing DNA fragmentation in HL-60 cells. In K-562 cells, no detectable DNA fragmentation could be found, even at concentrations as high as 100 μ*M* HPC. Exposure for 20 h to 100 μ*M* OMPEP, which was more cytotoxic than HPC on a molar basis, caused intensive DNA destruction in K-562 cells (cf. lanes 4 and 5, Fig. 3D).

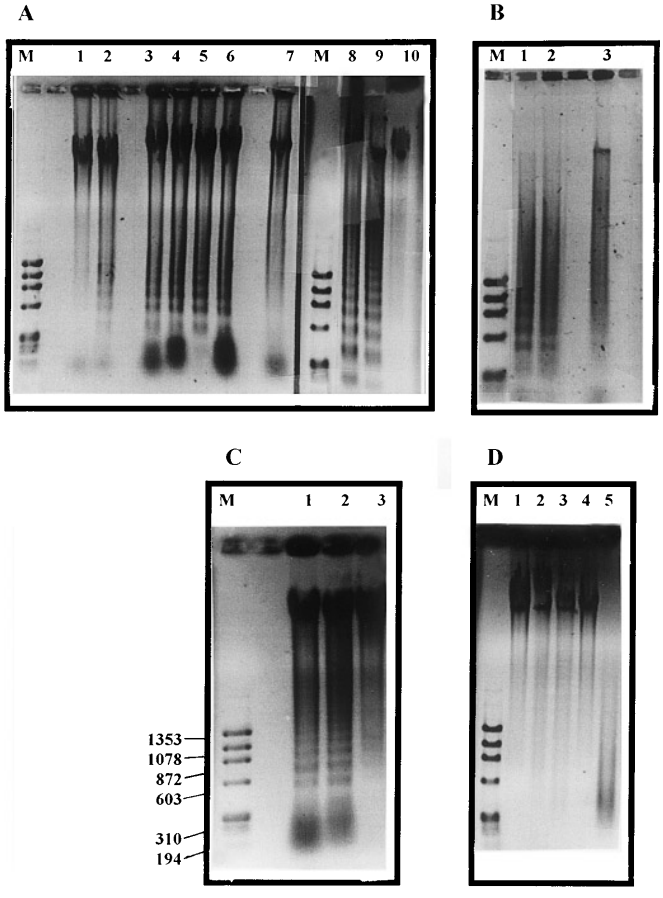


Fig. 3A–D Separation of DNA from HL-60, U-937, and K-562 cells by gel electrophoresis in 1% agarose. **A** APC exposure of HL-60 cells for 20 h (*M* Markers, *1* DPC 20 μ*M*, *2* TPC 20 μ*M*, *3* HPC 20 μ*M*, *4* OPC 20 μ*M*, *5* OMPEP 20 μ*M*, *6* EPC_3 20 μ*M*, *7 10*, untreated cells, *8* ODPP 20 μ*M*, *9* EPC 20 μ*M*). **B** DPC-treated HL-60 cells grown under serum-free conditions (*M* Markers, *1* 5 μ*M*, *2* 10 μ*M*, *3* untreated cells). **C** U-937 cells exposed to 20 μ*M* HPC or OMPEP (*M* markers, *1* HPC, *2* OMPEP, *3* untreated cells). **D** K-562 cells exposed to HPC or OMPEP for 20 h (*M* Markers, *1* untreated cells, *2* HPC 50 μ*M*, *3* HPC 100 μ*M*, *4* OMPEP 50 μ*M*, *5* OMPEP 100 μ*M*)

ELISA detection of DNA fragmentation

The formation of cytoplasmatic mono- and oligonucleosomes in HL-60 cells was measured after 14 and 20 h of exposure to APC using a sandwich ELISA (Fig. 4). After 14 h, 20-μ*M* concentrations of OPC, OMPEP, and EPC_3 caused a 2-fold increase in oligonucleosome formation in the cytosolic fraction. TPC and HPC showed similar but weaker effects. DPC showed no effect after exposure for 14 h (Fig. 4a). The longer incubation period (20 h) caused a 2-fold increase in the enrichment factor at 10 μ*M* APC, which was in the same range determined for 10 μ*M* Ara C.

Morphologic changes

The electron microscopy pictures presented in Fig. 5 show that APC caused typical chromatin condensation, cell shrinkage, and vacuolization in treated human leukemic HL-60 cells after exposure for 14 h, similar to the effect of Ara C, which was used as the positive control.

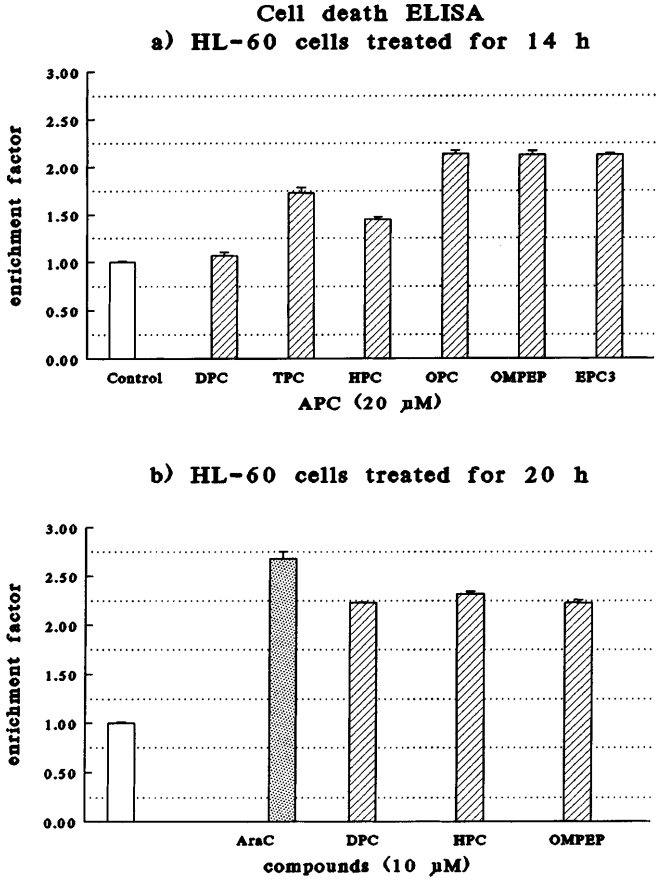


Fig. 4a,b Formation of mono- and oligonucleosomes in the cytosolic fraction of HL-60 cells following exposure to APC and AraC for **a** 14 h and **b** 20 h. The extent of formation (enrichment factor) was assessed as described in Materials and methods

Discussion

HPC has been found to be useful clinically for topical treatment of cutaneous breast-cancer metastases [9, 28]. Systemic administration of HPC failed to show activity in several phase II studies, probably because active dose levels could not be reached due to gastrointestinal toxicity [1, 29]. Therefore, the development of new derivatives concentrates on structures with increased activity and/or reduced toxicity. This aim is hampered, however, in that the mechanism of action remains unknown.

Recently, Et-18-OCH_3, an APC-related compound, was shown to induce apoptosis in HL-60 cells but not in K-562 cells [5, 6]. Therefore, our finding that APC induced programmed cell death in HL-60 and U-937 cells is not very surprising. HL-60 cells are known to undergo apoptosis when exposed to various inhibitors of protein kinase C [17]. We identified programmed cell death by gel electrophoresis, by ELISA detection of mono- and oligonucleosomes, and by specific morphologic changes (cell shrinkage, chromatin condensation, and formation of apoptotic bodies; Figs. 3–5). The quantity of DNA fragmentation indicates that induction of apoptosis constitutes an important element of the cytotoxicity of APC against certain human leukemic cell lines. The range of active concentrations is low enough to be reached in vivo [18, 19]. With regard to structure-activity relationships, it is evident that the length of the alkyl chain is related to the cytotoxic and apoptotic effects. Compounds with long, saturated alkyl chains such as HPC, OPC, OMPEP, and ODPP were distinctly more active than DPC and TPC (Table 1, Figs. 3, 4). These data agree with the results of previous in vivo and in vitro studies concerning the chain length of APC [3, 13, 25]; those with long, unsaturated alkyl chains (EPC and EPC_3) were found to be 2- to 6-fold less effective against HL-60 cells (Table 1). However, this contrasts with their in vivo efficacy, where they showed a 3-fold higher activity than HPC [2]. Obviously, modification of the polar head did not contribute significantly to increased cytotoxicity and induction of apoptosis in vitro. When comparing OPC, OMPEP, and ODPP, one has to bear in mind that the advantage of the latter two agents over, e.g., OPC lies in a reduced emetic potential in vivo [27].

The cytotoxicity of APC depends strongly on the protein content of the culture media (data not presented). Other authors have also reported an enhanced cytotoxicity of HPC in the absence of serum [14]. Under serum-free conditions we observed that even DPC – which is considered to be a negative control of APC [12] – was capable of inducing DNA fragmentation in HL-60 cells. This indicates that protein binding is an important property of APC.

Interestingly, the promyelocytic HL-60 cells and the promonocytic U-937 cells could undergo apoptosis under the influence of APC, but the CML-derived multipotential K-562 cell line could not. K-562, HL-60, and

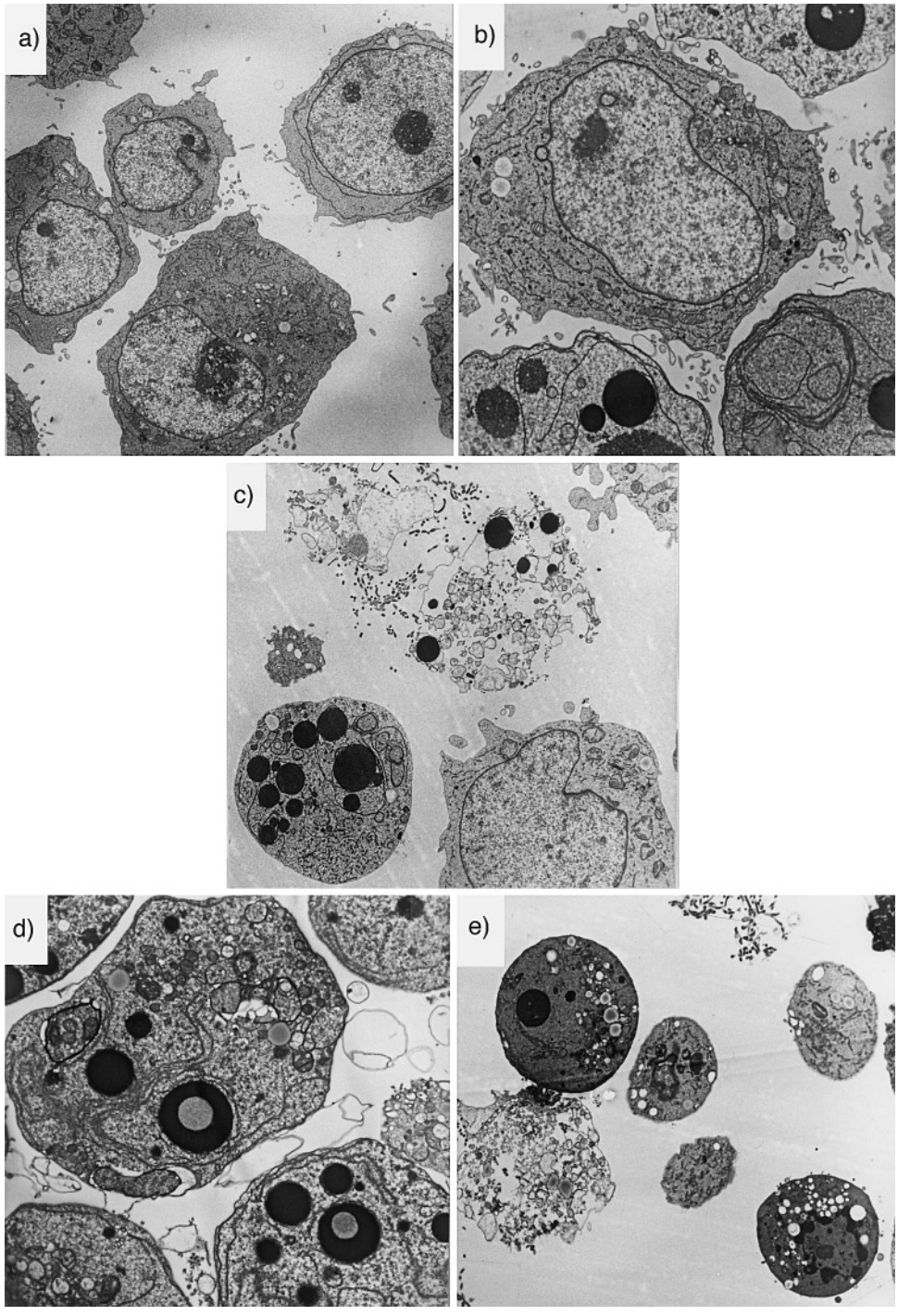


Fig. 5a–e Electron microscopy of HL-60 cells exposed to APC for 14 h (magnification ca. × 7,500). **a** Untreated HL-60 cells. **b** AraC 50 μ*M*. **c** HPC 50 μ*M*. **d** DPC 50 μ*M*. **e** OM-PEP 50 μ*M*

U-937 lines do not express normal p53 protein [7, 34]. Previous studies have shown that various chemotherapeutic agents fail to induce apoptosis in K-562 cells, and this phenomenon is related to the expression of a *bcr*/*abl* chimeric protein, since down-regulation of this protein renders K-562 cells susceptible to induction of apoptosis [20, 22]. We cannot exclude that APC induce atypical apoptosis in K-562 cells, since these cells were reported to produce predominantly DNA fragments in the range of 50–300 kb when undergoing apoptosis. Such large fragments cannot be resolved by conventional agarose gel electrophoresis [33].

Surprisingly, our data on APC-induced apoptosis are similar to those obtained for the new anti-cancer drug Taxol, which has been studied in the same cell lines [10]. However, taxol is known to be highly myelotoxic, whereas APC are bone-marrow-stimulating agents [32]. The missing or very low hematopoietic toxicity of APC [3, 24, 30] makes them very appropriate for bone-marrow-purging therapy and, probably, for systemic

therapy of hematologic malignancies. In conclusion, APC represent an interesting class of antineoplastic agents with remarkable cytotoxic activity against human leukemic HL-60 and U-937 cells. Induction of apoptosis plays an important role in their mode of action.

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