Structure-Apoptotic Potency Evaluations of Novel Sterols Using Human Leukemic Cells

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ABSTRACT: Three oxidized analogs of cholesterol have been characterized for their ability to cause apoptotic cell death in CEM-C7-14 human leukemic cells. In addition to testing 15-ketocholestenol (K15), 15-ketocholestenol hydroxyethyl ether (CK15), and 7-ketocholesterol hydroxyethyl ether (CK7), an oxysterol of known apoptotic response, 25-hydroxycholesterol (25OHC), served as a standard for comparison. Growth studies based on dye exclusion by viable cells while using a sublethal concentration of oxysterols ranked their potency for cell kill as 25OHC > K15 > CK15 > CK7. Both the TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling), which quantifies the amount of DNA nicks caused by a toxic agent, and the MTT assay, which measures cell metabolism and thus reflects cell viability, substantiated the same rank order. An ELISA assay for evaluating release of DNA fragments into the cytosol after treatment gave a similar potency order. The oncogene c-*myc* mRNA was suppressed by all three oxysterols, with 25OHC and K15 being the most potent suppressors. Hoechst and Annexin V staining documented that these oxysterols kill cells by an apoptotic pathway as evidenced by condensation of nuclear chromatin and plasma membrane inversion, respectively. From these *in vitro* studies, we believe that 25OHC, K15, and possibly CK15 have the potential to be chemotherapeutic agents.

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Both glucocorticoids and oxysterols can cause apoptosis of certain cells. For this action, glucocorticoids require a specific cytosolic protein, the glucocorticoid receptor (GR). No oxysterol receptor has been unequivocally identified, although two cellular proteins do specifically bind oxysterols: the oxysterol binding protein (OBP) and LXR, a member of the nuclear receptor family of proteins. As yet it is not certain that either protein is required for oxysterols' apoptotic actions. In our test system, the human leukemic cell line CEM, analysis of the events following addition of either class of steroid shows similarities at several points. These include a delay of about 1 d before the activation of caspases and nucleases, which occurs around the time of overt apoptosis. During this initial phase of cell death when the steroidal effects can be reversed, both types of steroids cause profound suppression of the oncogene product, cMyc (1,2). Because of their apoptotic action, glucocorticoids are used widely as antileukemic drugs. The similarity of effects of oxysterols suggests that they also might have therapeutic usefulness, alone, or in conjunction with glucocorticoids.

Oxysterols are able to elicit changes in cholesterol synthesis, cell growth, and membrane composition, and can cause immunosuppression (3–8). These activities have led to many studies of oxysterols, usually as toxins in the environment and foods (9,10), or as implicated in atherosclerosis (11–14). The relatively greater sensitivity of certain malignant, as opposed to normal, cells offers an opportunity to use oxysterols as chemotherapeutic drugs. A few investigators have recognized their potential as therapeutic agents (15–18).

Two oxysterols that are known to cause apoptosis have been well studied: one with high potency, 3β-hydroxycholest-5-en-25-diol (25-hydroxycholesterol, 25OHC) and the weaker 3β-hydroxycholest-5-en-7-one (7-ketocholesterol, K7) (19–23). Here we present tests of three oxysterols: 3β-hydroxy-5α-cholest-8(14)-en-15-one (15-ketocholestenol, K15), 3β-hydroxy-5α-cholest-8(14)-en-15-one 3β-2′-hydroxyethyl ether (15-ketocholestenol hydroxyethyl ether, CK15), and 3β-hydroxycholest-5-en-7-one 3β-2′-hydroxyethyl ether (7-ketocholesterol hydroxyethyl ether, CK7), comparing their apoptotoic effects and potencies to those caused by the known compound, 25OHC. The structures of these five oxysterols, as well as their parent compound, cholesterol, are shown in Scheme 1.

Two of the oxysterols under investigation were synthesized with bulky substitutions in the 3 position with the original intent of studying the importance of that position for binding to OBP. The third experimental oxysterol tests the effect of a keto substitution at position 15. C15 oxysterols have been evaluated for their synthetic paths, metabolism, occurrence, and effects on 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase; they are quite potent inhibitors of

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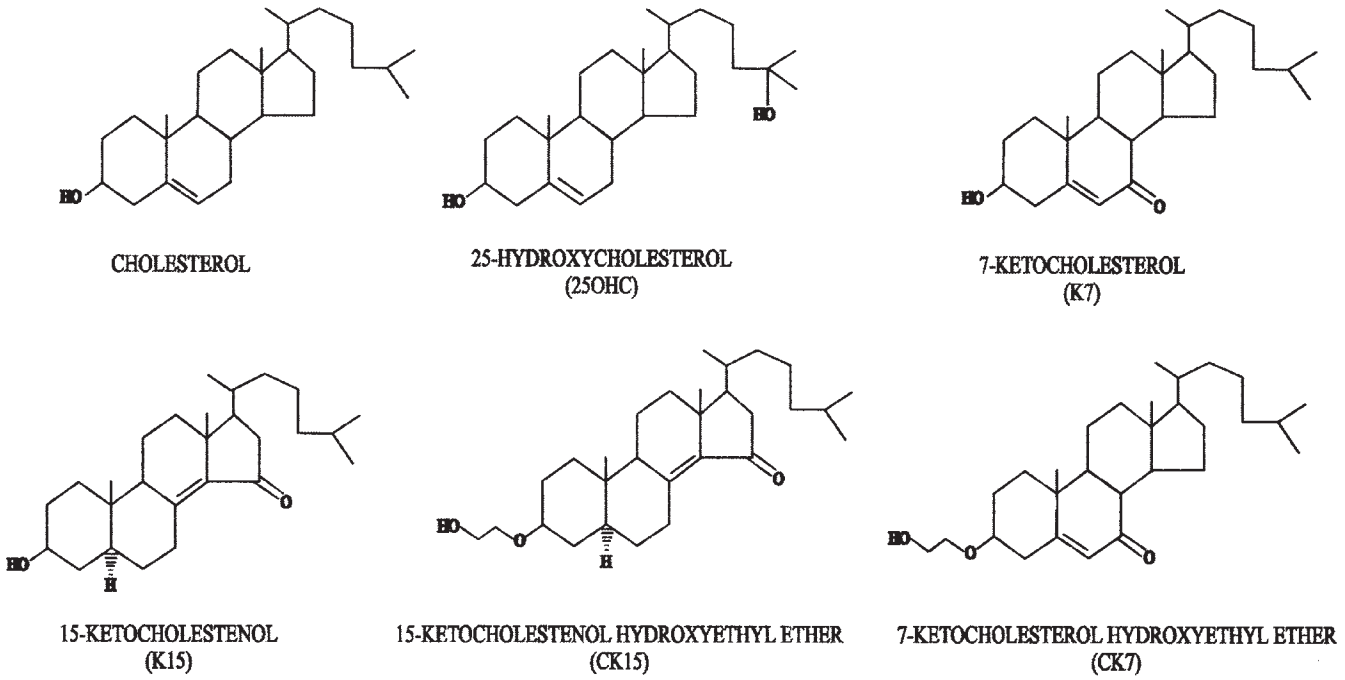
Abbreviations: 7-ADD, 7-amino-actinomycin D; BSA, bovine serum albumin; CK7, 7-ketocholesterol hydroxyethyl ether; CK15, 15-ketocholestenol hydroxyethyl ether; HPLC, high-performance liquid chromatography; K7, 7-ketocholesterol; K15, 15-ketocholestenol; 25OHC, 25-hydroxycholesterol; DFBS, delipidated fetal bovine serum; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GR, glucocorticoid receptor; HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; MTT, dimethyl tetrazolium salt; OBP, oxysterol binding protein; PS, phosphatidylserine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling.



SCHEME 1

HMGCoA receptor expression and bind well to OBP (15). However, they have not been tested for their antileukemic effects. Clearly, it is important to determine the structural basis for other oxysterols' potency as apoptotic agents.

These agents were examined in the well-characterized human $CD4^+$ acute lymphoblastic leukemic clone CEM-C7, which was cloned without selective pressure from the original CEM line (24). CEM-C7 and cells of a recently obtained subclone CEM-C7-14 are both sensitive to glucocorticoids and oxysterols, but through independent pathways (20).

MATERIALS AND METHODS

Materials. K15, CK7, and CK15 were synthesized in the Cardiology Research Center (Moscow, Russia). 25OHC and K7 were purchased from Steraloids (Wilton, NH). All oxysterols were dissolved in ethanol and stored at a concentration of 10^{-2} M at –20°C in glass, protected from light. Prior to use each oxysterol was assayed by high-performance liquid chromatography (HPLC) to ascertain purity. RPMI 1640 medium was obtained from Fisher Scientific (Houston, TX), fetal bovine serum (FBS) from Atlanta Biologicals (Norcross, GA), and trypan blue dye from Gibco/BRL (Grand Island, NY). Delipidated FBS (DFBS), Dulbecco's phosphate-buffered saline (PBS), pH 7.4, propidium iodide, Hoechst 3342 stain, bovine serum albumin (BSA), growth supplements (insulin, transferrin, selenium), and the tetrazolium salt 3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphenyl] tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). All the reagents used for RNA extraction were molecular biology grade. Formamide and TRIzol reagent were purchased from Gibco/BRL, formaldehyde from J.T. Baker Chemical (Phillipsburg, NJ), and electrophoresis-grade agarose from FMS Products (Rockland, ME). The blotting membrane was from Schleicher & Schuell (Keene, NH). Radionuclides were purchased from ICN Radiochemicals (Cleveland, OH). The human c-*myc* exon 3 cDNA was obtained from Oncor (Gaithersburg, MD). The TUNEL and Cell Death Detection ELISA^{Plus} kits were purchased from Boehringer Mannheim (Mannheim, Germany). Ribonuclease A was obtained from Worthington Biochemical (Freehold, NJ). The Annexin V/FITC kit was from PharMingen (San Diego, CA).

Cell culture/growth inhibition. CEM-C7-14 cells were cultured in RPMI 1640 medium with 5% heat-inactivated whole FBS at 37°C, maintaining logarithmic growth in a humidified atmosphere of 95% air and 5% CO_2. To avoid any effects from the sterols in whole serum prior to oxysterol addition, cells were acutely transferred to 3% DFBS with 1% growth supplements in medium and incubated overnight. Oxysterols were then added as an ethanolic solution in 1% BSA in PBS; controls received ethanol only plus BSA; ethanol never exceeded 1% of culture volume. The number of viable cells at every time point was determined by using a hemacytometer and the trypan blue vital dye exclusion method (25). Control cells were fully viable for at least 4 d in medium containing delipidated serum and supplements.

MTT. An *in vitro* MTT assay for sensitivity to oxysterols was performed with the following modifications (26). For each concentration of oxysterol, triplicate 80 µL samples containing 2×10^5 cells/mL were plated in a 96-well tissue culture plate. Each well then received 20 µL oxysterol from stock solutions or dilutions thereof; final concentrations were 0.3, 0.5, 0.7, and 1 µM. Controls consisted of CEM-C7-14 cells in medium without oxysterol and medium without cells. After incubation at 37°C in 5% CO_2 for 48 h, 25 µL of MTT stock

solution was added. Following a 4 h incubation, 150 µL lysis buffer (20% sodium dodecyl sulfate, 20% dimethylformaldehyde, 2.5% 1 N HCl, 2.5% 80% acetic acid) was added to each well. After overnight incubation at 37°C, the samples were analyzed colorimetrically on a Biotek plate reader at 600 nm which was zeroed by assay blanks containing no cells.

DNA fragmentation. DNA fragmentation was followed by two methods. The first was a photometric enzyme immunoassay using the Cell Death Detection ELISA^{Plus} according to the protocol of the supplier (Boehringer Mannheim). CEM-C7-14 cells adapted to culture in supplemented medium with delipidated serum were treated with 1 µM oxysterol for 48 h. Triplicate samples (5×10^4 cells) were centrifuged 200 × *g* for 10 min at room temperature, and the supernatant was carefully removed. Two positive controls (DNA-histone complex) and two background samples (incubation buffer) were included. Color intensity was evaluated on a Packard microplate reader at 405 nm against the substrate solution as a blank.

The second DNA fragmentation method employed the terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling (TUNEL) assay performed according to the protocol provided by the manufacturer (Boehringer Mannheim) with some modifications. Before fixation, 3×10^6 cells were washed twice with PBS containing 1% BSA. Cells were then fixed with freshly prepared 4% paraformaldehyde in PBS and incubated at room temperature for 30–60 min. Each assay contained one negative control (no transferase enzyme) and one positive control (cells treated with DNase I + transferase enzyme). After washing, all samples were stained with propidium iodide according to a method described by Van Houten and Budd (27) and incubated overnight in the dark. Samples (2×10^4 cells) were analyzed for DNA content and DNA breaks by flow cytometry using a FACScan (Fluorescence Activated Cell Sorter; Becton Dickinson, Bedford, MA) with Cell Quest 3.1 software (Becton Dickinson). Analysis was done with an argon-ion laser with excitation at 488 nm. Doublets and cells aggregates were excluded, and only the singlet cell population was analyzed. Red (propidium iodide) and green (fluorescein-dUTP) fluorescences were detected using 530- and 548-nm filters, respectively.

Northern blots. Total RNA was isolated using the TRIzol reagent according to the method provided by the manufacturer, Gibco/BRL. Total RNA was subjected to electrophoresis under denaturing conditions using a 1% agarose/6% formaldehyde gel and electroblotted on Nytran Plus nylon membranes. Hybridization was carried out for 18 h at 60°C with a ^{32}P-labeled random-primed c-*myc* cDNA fragment with specific activities of 1 to 8×10^9 dpm/µg of DNA. Normalization was accomplished by using the ethidium bromide stained ribosomal RNA that was transferred to the filter. The membranes were exposed for 24 h in a PhosphorImager cassette and analyzed using a PhosphorImager 425 (Molecular Dynamics, Sunnyvale, CA). The densities of the mRNA bands obtained from the PhosphorImager screen were quantified using the MD ImageQuant software (version 3.3) from Molecular Dynamics. The relative amounts of c-*myc* mRNA on the filters were compared by normalizing the quantified values to 18S ribosomal RNA bands on the same filters. At the exposure time selected for quantification, RNA signals were linear.

Flow cytometry. The staining for the flow cytometric evaluation of apoptotic changes was performed with the AnnexinV-FITC-7-AAD kit from Boehringer Mannheim. After CEM-C7-14 cells had been treated with 1 µM oxysterol for 4, 6, 24, 30, or 52 h, 2×10^5 cells were treated with Annexin V and 7-amino-actinomycin D (7-AAD) according to the supplier's protocol. CEM-C7-14 cells treated with 1 µM dexamethasone for 48 h were used as a positive control. Cells (2×10^4) were analyzed for Annexin V- and 7-AAD-positive cells by flow cytometry using a 488-nm excitation and a 515-nm bandpass filter for fluorescein detection and a filter >600 nm for 7-AAD detection on a FACScan (Becton-Dickinson). For photographs of stained cells, 3×10^5 CEM-C7-14 cells ± 1

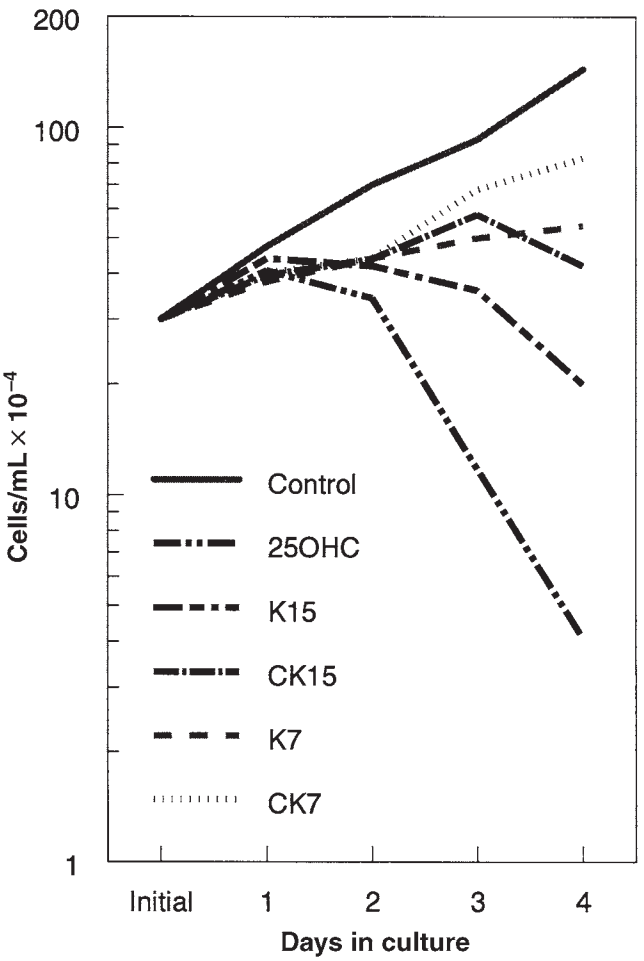


FIG. 1. Effect of 300 nM oxysterols on the growth and viability of CEM-C7-14 cells. Cells in logarithmic growth were exposed to 300 nM of each oxysterol, as described in the Materials and Methods section. Viable cells were counted daily for 4 d. The legend identifying each oxysterol is located to the left of the graph. Each time point represents the mean of 3–5 independent experiments; error bars were omitted for clarity of presentation, but the SD of each data point was less than or equal to 5%. 25OHC, 25-hydroxycholesterol; K15, 15-ketocholestenol; CK15, 15-ketocholestenol hydroxyethyl ether; K7, 7-ketocholesterol; CK7, 7-ketocholestenol hydroxyethyl ether.

μM oxysterols were treated with Annexin V/7-AAD as above and fixed with 4% fresh paraformaldehyde solution in PBS. Cells pellets were washed twice in cold PBS/BSA and resuspended in 100 μL permeabilization buffer (0.1% Triton-X 100 in 0.1% sodium citrate), washed twice in cold PBS/BSA, and resuspended in 800 μL PBS/BSA. Microscope slides that had been precoated with poly-L-lysine solution and dried were loaded with 2×10^4 cells and cytospun at 600 rpm for 5 min. The slides were stained for 10 min Hoechst 3342 stain (0.1 mg/mL water). Slides were mounted with Crystal Mount (Biomeda, Foster City, CA) and stored in the dark at 4°C. Photographs of the cytospun, stained cells were taken on a Nikon Eclipse E600 microscope (Melville, NY) with a Nikon FDX-35 camera at 100× magnification with illumination by white polarized light, 360/500-nm filter for the Hoechst stain and 488/515-nm for the Annexin V/7AAD stain.

RESULTS

Potency of oxysterol as judged by growth inhibition. The growth responses of cells from the freshly isolated CEM-C7-14 clone to the three experimental oxysterols were compared to those caused by the reaction of these cells to the standards 25OHC and K7. Sublethal concentrations of each oxysterol (300 nM) were added to cultures growing logarithmically in supplemented, delipidated RPMI 1640 medium. Total viable cells were counted initially and every 24 h for 4 d. Figure 1 shows the results of such a study with little to no cell death occurring in any oxysterol treatment during the first 24 h, though all slowed cell growth. Even by 48 h, there was little difference in the effect caused by these agents. However, beyond 72 h, 25OHC exhibited the greatest cell kill, followed by K15, K7 and CK15 continued to retard growth, and CK7 had the least effect. All of the treated cultures did contain trypan blue-positive cells, indicating that some cell death had occurred.

Extent of apoptosis as judged by MTT, cytosolic DNA fragmentation, and TUNEL assays. Although there are numerous assays available to evaluate cell death, we selected three diverse methods to quantify an apoptotic process: the MTT, cytosolic DNA fragmentation, and TUNEL assays. The MTT assay evaluates mitochondrial reduction of the yellow MTT tetrazolium salt dye to a highly colored blue formazan prod-

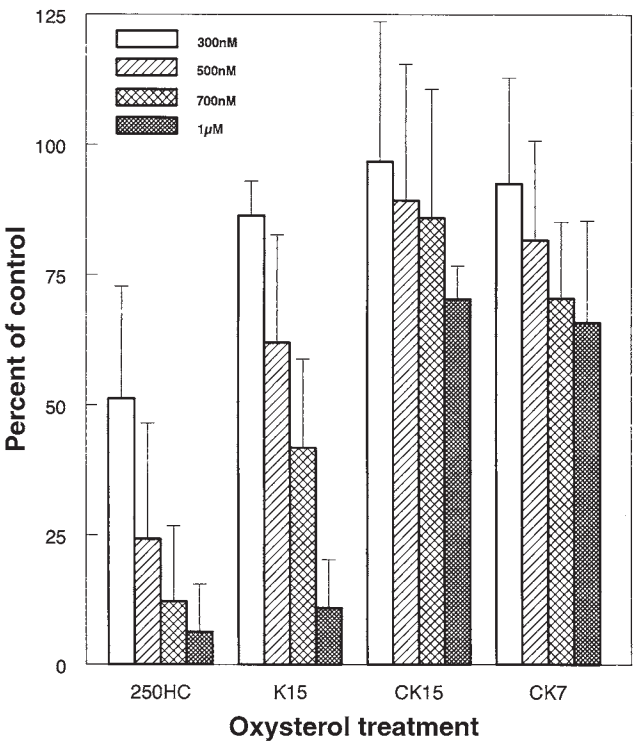


FIG. 2. Effects of varying concentrations (300 nM, 500 nM, 700 nM, and 1 μM) of oxysterols on the mitochrondrial reductive capacities of CEM-C7-14 cells. Growth conditions and delivery of oxysterols were the same as in Figure 1. After 48 h of incubation, the dimethyl tetrazolium salt MTT reagent was added, and cells were cultured for an additional 4 h before being lysed and then analyzed on a Biotek plate reader at 600 nm. The results were an average percentage of absorbance values by oxysterol-treated wells vs. the average of control wells and expressed as percentage of control. Each bar represents the mean from five independent experiments; error bars = 1 SD. See Figure 1 for abbreviations.

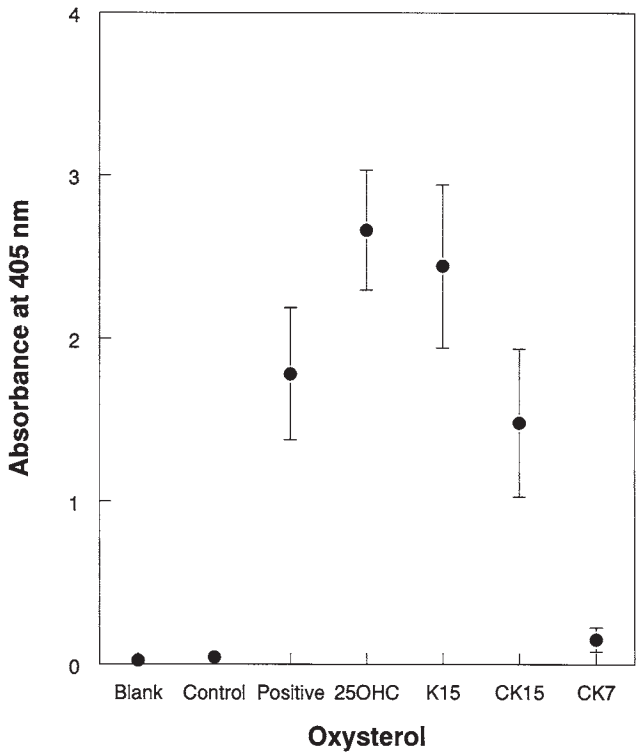


FIG. 3. Effect of 1 μM oxysterols treatment on internucleosomal DNA fragmentation in CEM-C7-14 cells. Growth conditions and delivery of oxysterols were the same as in Figure 1. After 48 h of treatment with each oxysterol, cells were collected, lysed, and the cytosolic fraction was prepared. Of this, triplicate samples were complexed with biotin-labeled antihistone and peroxidase-conjugated anti-DNA antibodies. The absorbance was read on a microplate reader at 405 nm against the substrate solution as blank. The blank averaged 25 mU, CEM-C7-14 control 44 mU, and the positive control (DNA histone complex) 1759 mU. Each dot with its error bar represents the mean ± 1 SD from three independent experiments. See Figure 1 for abbreviations.

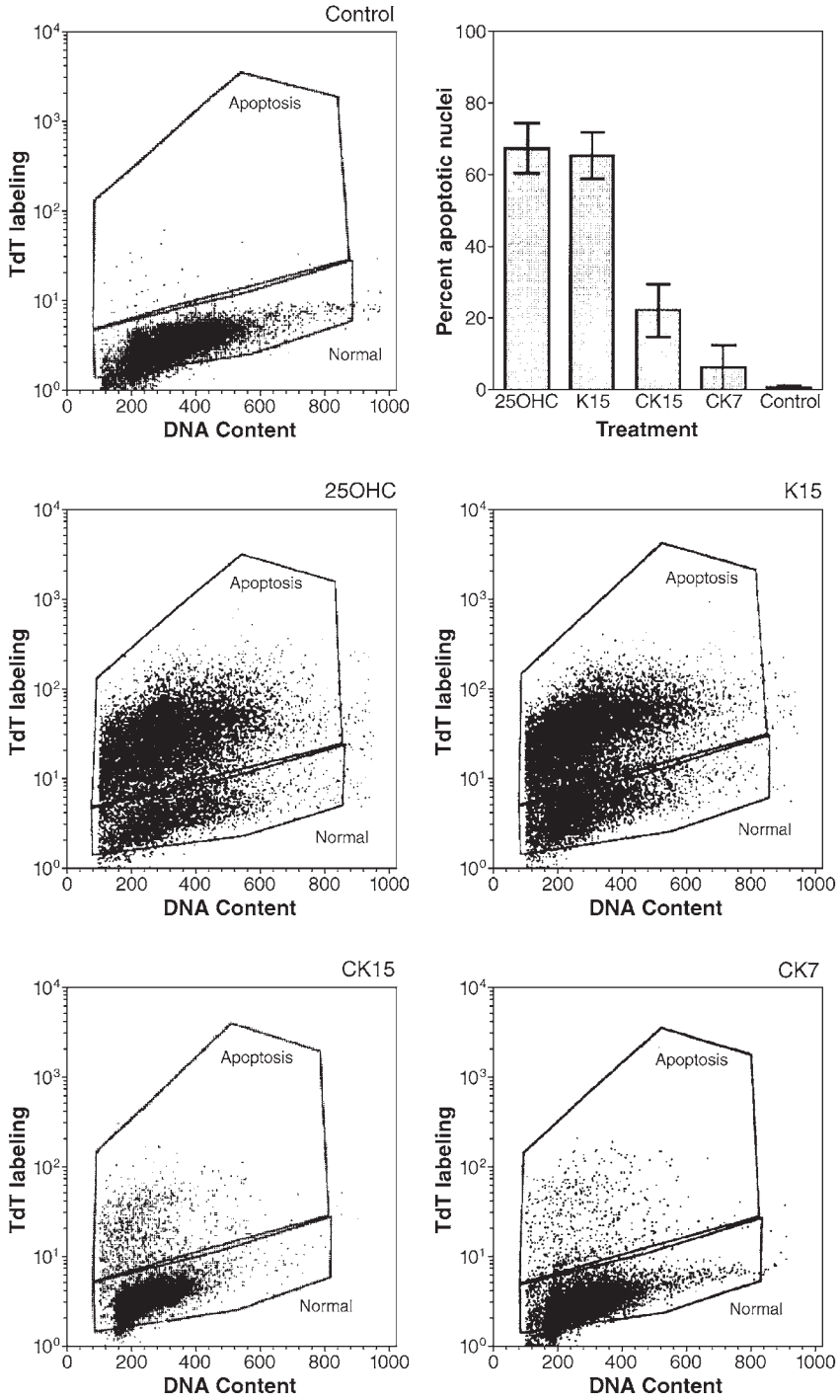


FIG. 4. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) evaluation of single-stranded nicks and double-stranded breaks in DNA caused by the treatment of CEM-C7-14 cells with 1 μM oxysterols for 48 h. Growth conditions and delivery of oxysterols were the same as in Figure 1. Vehicle-treated control cells were used to establish the gates for normal and apoptotic cells. Twenty thousand cells were counted for controls and each oxysterol treatment, and the data were expressed as "percent apoptotic nuclei," i.e., percentage TUNEL-positive nuclei. The upper right-hand graph shows the mean values from four separate experiments with standard deviations. The remaining five panels are typical data obtained by FACS (Fluorescence Activated Cell Sorter; Becton Dickinson, Bedford, MA) analyses. See Figure 1 for abbreviations.

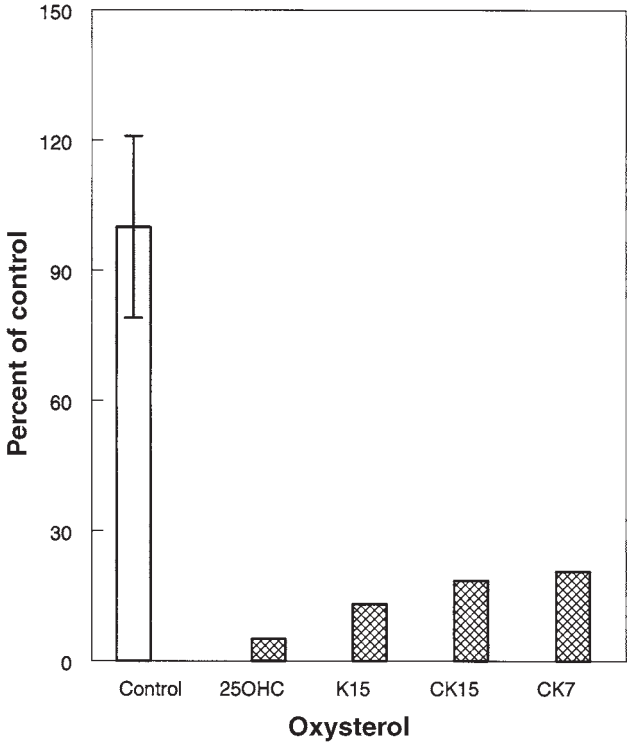


FIG. 5. Effect of 1 µM oxysterols on the level of c-myc mRNA in CEM-C7-14. Growth conditions and delivery of oxysterols were the same as in Figure 1. After 48 h in oxysterol, total RNA was extracted from the cells and 20 µg was electrophoresed, transferred to Nytran Plus® nylon membrane, and hybridized with a human c-myc exon 3 cDNA. Ethidium bromide-stained 18S ribosomal RNA was transferred to the same membrane to estimate the efficiency of RNA loading and transfer. Each oxysterol treatment included an individual control of vehicle-treated CEM-C7-14 cells. The error bar indicates the mean ± SD for the four control samples of constitutive c-myc mRNA levels. See Figure 1 for abbreviations.

uct. This assay has shown a high correlation with cell viability (28). Figure 2 demonstrates that at each of four sterol concentrations K15 was slightly less potent than 25OHC. At 1 µM, the two were not statistically different. Both CK15 and CK7 were much less able to suppress cell reductive capacity.

The "cytosolic" DNA fragmentation assay evaluates internucleosomal DNA cleavage, assayed by measuring the accumulation of mono- and oligonucleosomes in the cytoplasm of apoptotic cells, which occurs before plasma membrane breakdown. In Figure 3, a pattern of potency similar to that seen in Figures 1 and 2 for the four oxysterols is observed: 25OHC and K15 were the most potent apoptotic agents with almost the same ability to cause extensive appearance of DNA/histones in the cytoplasm. In this assay, CK15 clearly caused some DNA cleavage in CEM-C7-14 cells and CK7 showed little effect.

A flow cytometric TUNEL assay combined with a secondary stain for total DNA was used to detect both single-stranded nicks and double-stranded breaks in DNA. In Figure 4 (upper right-hand panel) the percentage of TUNEL-positive "apoptotic nuclei" in CEM-C7-14 cells after 48 h of treatment with 1 µM oxysterol showed the potency pattern: 25OHC ≥ K15 > CK15 > CK7 ≥ control cells. The remaining panels in Figure 4 present the data as directly obtained by FACS analyses. In each panel, the lower gated area reflects the normally cycling cells, while the upper gated area shows the TUNEL-positive cells.

Suppression of c-myc *mRNA by oxysterols.* In 1993, the suppression of the c-*myc* protooncogene, which plays an important role in the control of normal cell growth and differentiation, was shown to be a critical step in the glucocorticoid-induced lysis of CEM-C7 cells (1). More recently, Ayala-Torres *et al.* found that c-*myc* also is suppressed by 25OHC in apoptotic CEM-C7 cells (2). Figure 5 documents that in CEM-C7-14 cells treated with 1 µM 25OHC or K15 for 48 h, c-*myc* mRNA levels were also suppressed to 5 and 13%, respectively, of constitutive levels. The 1 µM concentrations of the CK15 and CK7 also depressed c-*myc* mRNA significantly (19% of control for CK15 and 21% for CK7). Thus, the rank order of potency for c-*myc* suppression is 25OHC > K15 > CK15 > CK7, although all oxysterols depressed c-*myc* significantly.

Documentation of apoptosis by phosphatidylserine (PS) and DNA staining. One of the earliest features of cells undergoing apopotsis is the translocation of the phospholipid PS from the inner to the outer side of the plasma membrane. This externalization of PS precedes the nuclear changes associated with apoptosis. The phospholipid-binding protein Annexin V, when conjugated to a fluorochrome, such as fluorescein isothiocyanate (FITC), can be used to identify cells that have translocated their PS to the outer plasma membrane. The nucleic acid dye 7-AAD enters and binds to DNA in dead cells. We followed its entry with FITC-labeled monoclonal antibodies. Two-color analysis, with minimal spectral overlap between the 7-AAD and FITC fluorescence emissions, allowed simultaneous assay of the two reactions. Chromatin condensation, another hallmark of apoptosis, was followed by staining the same cells with Hoechst 33342 dye.

Figure 6 presents the density plots from a two-color FACS analysis of CEM-C7-14 cells treated for 52 h and stained with Annexin V and 7-ADD. The upper panel shows the reactivity of control cells and defines the areas containing cells with specific fluorochrome reactions. The lower panels illustrate the effects of treatment with the various oxysterols. Note the dense numbers of dead cells after 25OHC and K15 treatment (quadrant 3). Virtually no cells reacted with 7-AAD unless they were also positive for Annexin V binding, but some cells were Annexin V positive without being 7-ADD positive (quadrant 2). Table 1 provides the percentages of cells for these five conditions over the time course of 4, 6, 24, 30, and 52 h. The increase in apoptotic cells in the 1 µM 25OHC is seen by 24 h, in K15 by 6 h, in CK15 by 52 h, and little increase is seen in CK7 treated cells at any time of treatment. By 52 h, both 1 µM 25OHC and K15 had killed >90% of the CEM-C7-14 cells.

The upper photographs in Figure 7 were taken at 30 h for control CEM-C7-14 cells and 1 µM 25OHC- and K15-treated cells, while the lower two sets of photographs were taken at 52 h for 1 µM CK15- and CK7-treated cells. The photographs using polarized white light clearly identify the field of cells. Hoechst dye staining of the same cells reveals the onset of nu-

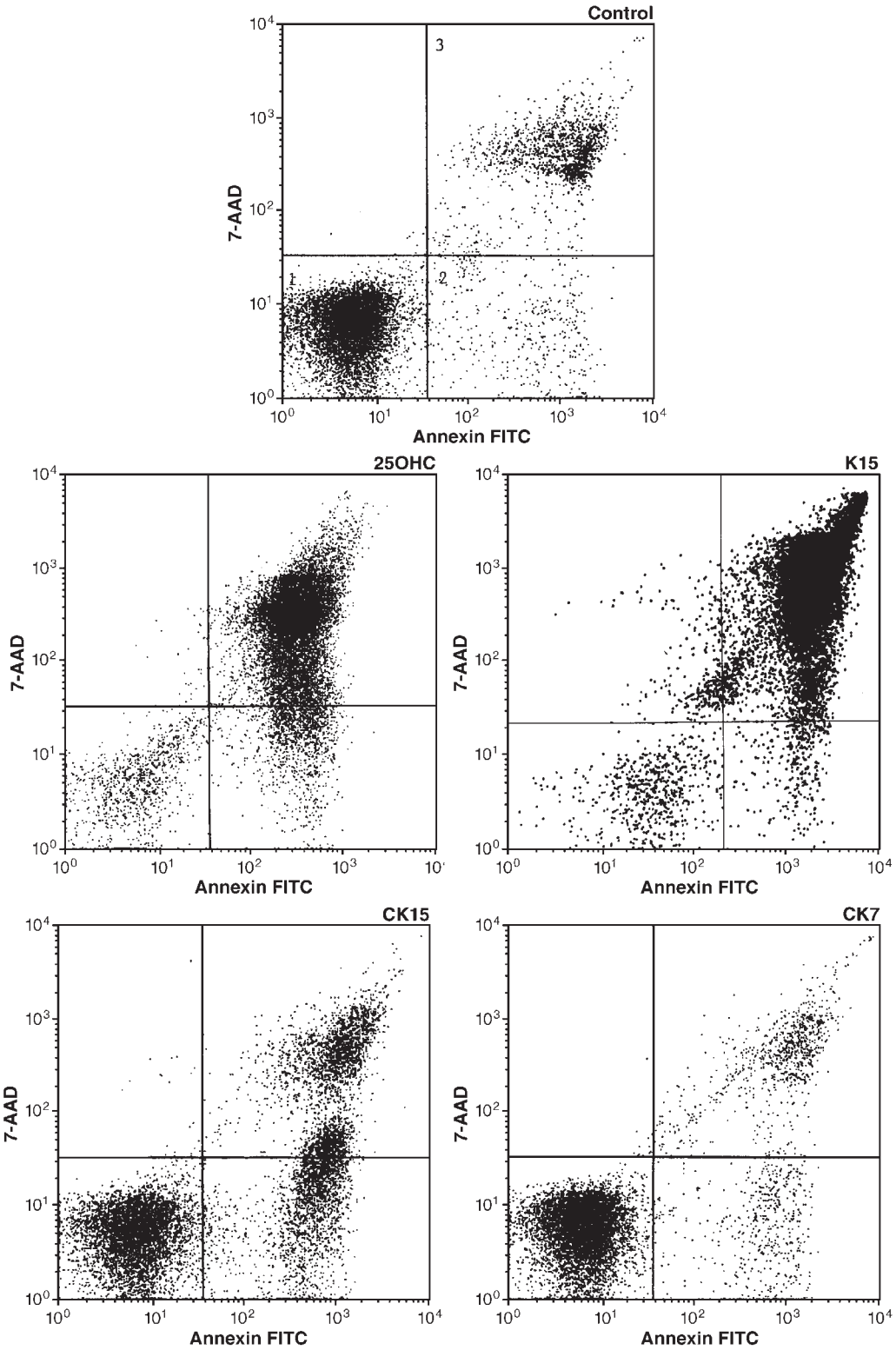


FIG. 6. Two-color FACS analysis of cells reacted with Annexin V for evidence of externalized membrane phosphatidylserine (PS) and 7-amino-actinomycin D (7-AAD) for DNA staining due to loss of membrane integrity. CEM-C7-14 cells were treated with 1 μM of each oxysterol for 52 h. Growth conditions and delivery of oxysterols were the same as in Figure 1. The five panels represent FACS analyses of CEM-C7-14 cells treated with oxysterol or vehicle. Upper panel: vehicle-treated control cells were used to establish the gates for unstained, viable cells in the lower left quadrant [1]; Annexin V positive/7-AAD negative, apoptotic cells in the lower right quadrant [2]; and Annexin V/7-AAD positive, dead cells in the upper right quadrant [3]. Other panels: cells that had been exposed to the indicated sterol. FITC, fluorescein isothiocyanate; for other abbreviations see Figure 1.

TABLE 1
Apoptotic Response of CEM-C7-14 Cells (control and 1 μM oxysterol-treated)

	Time (h)				
Treatment	4	6	24	30	52
Control					
Annexin V positive (%)	5.6	5.6	4.4	5.6	5.8[b]
+ 7-AAD positive (%)	10.0	7.1	11.1	13.6	8.1[c]
25OHC					
Annexin V positive (%)	4.7	5.9	5.4	26.5	7.9
+ 7-AAD positive (%)	10.6	8.7	9.0	48.8	86.5
K15					
Annexin V positive (%)	6.1	6.7	12.9	15.0	1.7
+ 7-AAD positive (%)	10.8	11.9	18.8	17.6	93.2
CK15					
Annexin V positive (%)	5.2	5.6	4.1	6.1	19.3
+ 7-AAD positive (%)	8.1	11.6	9.5	4.5	24.6
CK7					
Annexin V positive (%)	5.1	5.9	5.3	7.0	7.1
+ 7-AAD positive (%)	7.9	5.9	7.9	11.4	8.5

[a]25OHC, 25-hydroxycholesterol; K15, 15-ketocholestenol; CK15, 15-ketocholestenol hydroxyethyl ether; CK7, 7-ketocholesterol hydroxyethyl ether; 7-AAD, 7-amino-actinomycin D.
[b]Mean for controls = 5.4 ± 0.6.
[c]Mean for controls = 10.0 ± 2.5.

clear condensation and fragmentation as seen in the second panel. The intensity of the fluorescent dyes is sometimes faint, making it difficult to identify every cell in the second and third panels. In viewing the same field, the third panel shows the progressive stages of apoptosis as monitored by Annexin V/7-AAD. Those cells in the early stage of apoptosis contain only the green stain of Annexin V-FITC. The strong yellow to orange signal is due to the movement of 7-AAD across the plasma membrane, which becomes increasingly permeable during the later stages of apoptosis. In order to demonstrate apoptosing cells in the CK15 and CK7 cultures, it was necessary to photograph increased times (52 h), whereas late-stage apoptotic cells could be seen in 30-h cultures of 25OHC- and K15-treated cells.

DISCUSSION

The focus of this study has been threefold: (i) to examine three oxysterols for their ability to kill human leukemic cells; (ii) to determine if the pathway of death was an apoptotic one; and (iii) to assess their relative cell-kill potency. We designed and implemented a battery of tests that were useful in the evaluation of such agents for their apoptotic potential, which could lead to future drug discovery and development. In each of the assays utilized, 25OHC served as a standard for comparison of cell-kill potency because there have been several studies of oxysterol-dependent apoptosis that have used this agent.

To avoid the confounding effects of sterols in whole serum, we carried out this study on cells cultured in medium supplemented with DFBS. We also added BSA as an oxysterol carrier to prevent the loss of drug because of adhesion to the culture flask wall. The *in vivo* environment may be quite different. Human cells express cell-surface receptors for low density lipoprotein (LDL) (29). Since LDL is the major cholesterol-carrying lipoprotein in human plasma, oxysterols may enter cells through this mechanism as well as directly. Previous *in vitro* studies have shown that LDL receptor activity can be enhanced by incubating cells in a lipoprotein-deficient medium (30). However, for this systematic evaluation of the effects of oxysterols on these leukemic cells, a defined culture medium was deemed desirable.

The compounds studied here examined the effects of a keto group on C15 and the addition of a 3β-2′-hydroxyethyl ether on C15 or C7. The initial growth studies demonstrated that all three test oxysterols did inhibit cell growth in treated cultures as compared to control cells under conditions in which the untreated cells maintained logarithmic growth. The most potent agent of the experimental oxysterols to diminish proliferating cells was K15, which was nearly as potent as 25OHC. Adding the 3β-2′-hydroxyethyl ether group reduced the potency of both K15 and K7; in fact, the already weak K7 was rendered nearly nonapoptotic.

Having established that all three test oxysterols could decrease the number of leukemic cells in culture relative to controls, the remainder of our study evaluated the nature of this reduction. Were the cells killed or merely slowed in growth? By examining the reductive metabolism of the treated cells' mitochrondria in the MTT assay, it was demonstrated that these three oxysterols did diminish CEM-C7-14 cells' ability to reduce the MTT dye, with K15 again being the most potent. CK15 and CK7, at the highest concentration tested, only reduced this mitochrondrial function by ~25%.

The Hoechst chromatin staining, TUNEL, and cytosolic DNA fragmentation assays all indicated that the test sterols did cause apoptotic cell death, as determined by DNA condensation, nicks, and fragmentation. Again, K15 created far more DNA damage than CK15 or CK7. Finally, this apoptotic cell death was associated with the classic alterations of the cellular plasma membrane as well as the suppression of an important growth controlling gene, c-*myc*. Fluorescence staining using Annexin V and 7-AAD demonstrated that in K15-treated CEM-C7-14 cells, the plasma membrane's integrity was compromised earlier than with CK15 or CK7.

Northern blot analyses showed a decrease in the level of c-*myc* mRNA for all four oxysterols tested. CK15 and CK7 were much more effective in reducing c-*myc* mRNA than they were in causing apoptosis. This may account for their ability to slow cell growth (Fig. 1) since progression through the cell cycle demands an adequate level of cMyc. Our studies so far have only examined c-*myc* mRNA levels after treatment with the test sterols. Since cMyc protein can be regulated independently from its mRNA, it will be necessary to determine the levels of the protein before drawing further conclusions.

In sum, oxysterols continue to show potential as chemotherapeutic agents. Some reports have even suggested that oxysterols could increase the sensitivity of tumor cells to other cancer drugs (31,32). The ability of oxysterols to kill cells could result from the simple regulation of sterol *de novo* synthesis or from the inhibition of the many biologically impor-

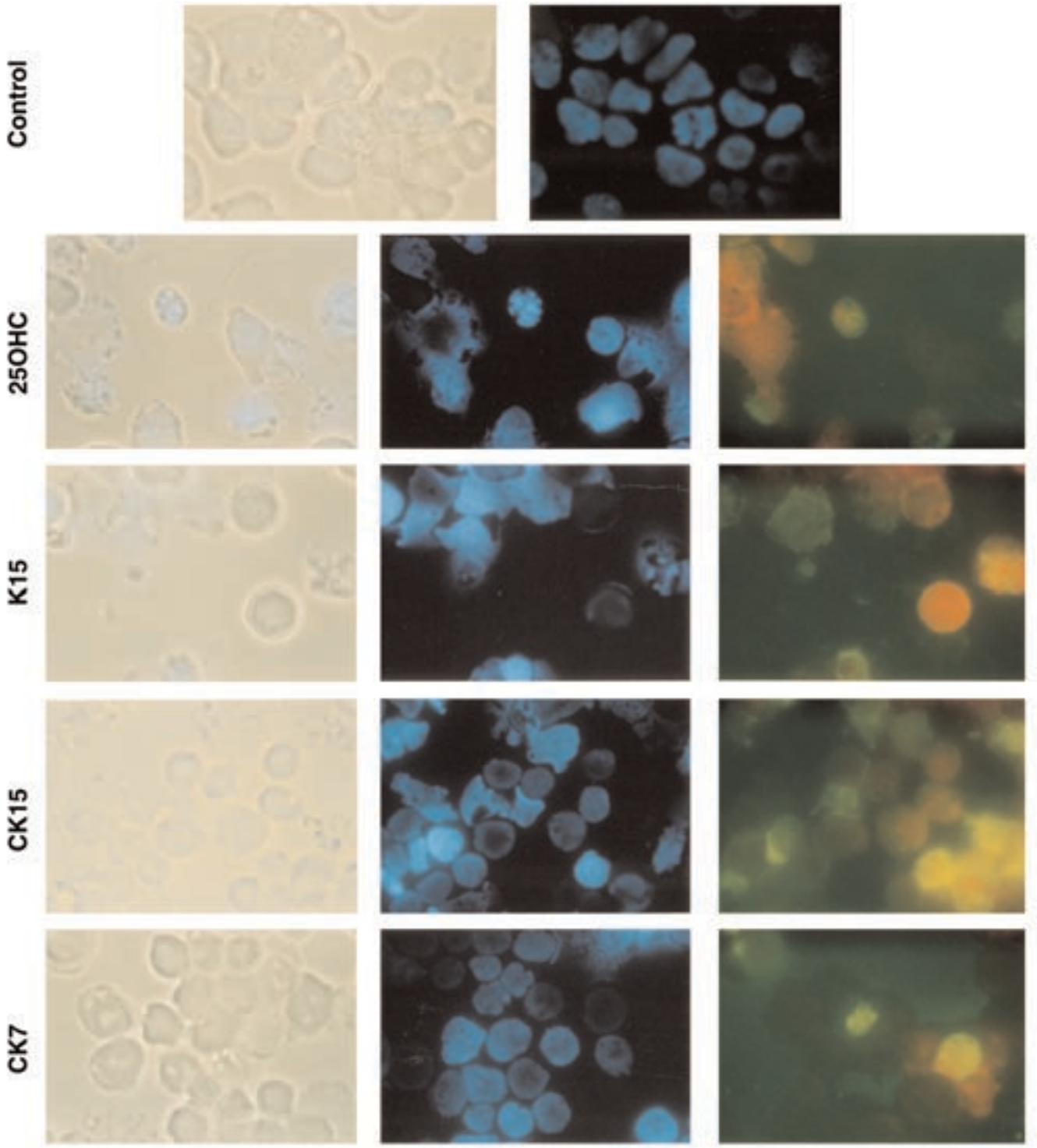


FIG. 7. Fluorescent microscopy of apoptotic events in CEM-C7-14 cells treated ± 1 μM oxysterol. Growth conditions and delivery of oxysterols were the same as in Figure 1. CEM-C7-14 cells were stained with Hoechst 33342, Annexin V, and 7-AAD at the various time points, 30 h for 25OHC and K15; 52 h for CK15 and CK7. Photographs of stained cells were taken on a Nikon Eclipse E600 microscope with a Nikon FDX-35 camera at 100× magnification: first, viewed with polarized light to demonstrate all the cells in the field, second viewed at 488 and at 515 nm to detect the initial loss of membrane integrity (Annexin V, green/yellow) followed by the death of the cell (7-AAD, red); and third, at 355 and at 460 nm to reveal condensed chromatin found in apoptotic cells. See Figures 1 and 6 for abbreviations.

tant mevalonate-derived products such as dolichol, ubiquinones, or isopentyl/farnesyl moieties or some other unidentified pathway. Some years ago it was shown that the relative binding affinity of many oxysterols to OBP correlated with their ability to suppress the rate-limiting step in *de novo* cholesterol synthesis, which is essential for cell homeostasis and

proliferation (33). Taylor and Kandutsch (34) reported that the 3β-hydroxy position of an oxysterol was essential for high OBP binding affinity and that substitution of a hydroxyl or keto function at positions C15, 20, 25, or 26 of the parent cholesterol molecule also produced high OBP affinity ligands. In 1993, our laboratory reported that oxysterol-induced cell death correlated with OBP occupancy with 25OHC demonstrating ~30 nM and K7 ~450 nM OBP-binding affinity in serum-free medium (20). Of the test oxysterols, K15 was the strongest ligand for OBP and in one competition assay was a slightly better competitor than 25OHC for OBP; 50% competition of 25OHC by 8.8 pM for K15, whereas unlabeled 25OHC required 12.5 pM. CK15 required 40 pM for 50% competition and CK7 competed only 10% at 40 pM (Alexander S. Krylov, unpublished data). While there have been studies evaluating the effects of different oxysterols on cholesterol biosynthesis (35), Sinensky *et al*. have challenged the correlation between the regulation of HMG-CoA reductase and the effectiveness of those agents as anticancer drugs (36).

The effectiveness of oxysterols *in vivo* might be diminished by the availability of cholesterol from LDL. For cells in the circulation, this could be protective. In tissues or malignant cells outside the vascular compartment, LDL-cholesterol is not as available. Certainly, higher concentrations of 25OHC are required to kill CEM cells when serum is present. Even so, the protection is not absolute, and no amount of added cholesterol can protect if sufficient 25OHC is present (20). Therefore, the question will be whether *in vivo* oxysterols can be administered to levels sufficient to overcome whatever protection LDL-cholesterol gives.

As to structure–function relations, Zhang *et al*. have reported that 7α-hydroxylation and 3-dehydrogenation abolish the ability of 25OHC and 27-hydroxycholesterol to induce apoptosis in thymocytes (37). For the present study we find that the sterols tested can cause apoptosis. We conclude that a keto group at position 15 produces an oxysterol with high apoptotic potency. The 3β-2′-hydroethyl ethers greatly reduce potency. Our data show the usefulness of applying a battery of tests for cell changes correlated with apoptosis, and they add to the body of relevant structure–function knowledge.

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