Effects of Alkyl-Lysophospholipids on Human Leukemic Cell Lines Measured by Nuclear Magnetic Resonance


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ABSTRACT

Part of the cytotoxic action of alkyl-lysophospholipids (ALP) on leukemic cells is known to result from the lack of an O-alkyl cleavage enzyme and its antimetabolic effect which results in a toxic lysophospholipid buildup. Further, ALP (5 μg/ml) suppresses clonogenicity and tritiated thymidine uptake in HL60 cultures after 24 hr of exposure. The effect of ALP on two leukemic cell lines, HL60 and K562, measured by two nuclear magnetic resonance (NMR) techniques and examined by electron microscopy is reported. 31P-NMR spectroscopy indicates that the adenosine 5′-triphosphate:adenosine 5′-diphosphate ratios are unaffected after 24 hr, as is mitochondrial morphology, judging by electron micrographs. However, cell membrane integrity in HL60 is altered at that time. The earliest ALP effects occur in NMR internal water relaxation at 1 hr after ALP exposure, followed by a small reduction in tritiated thymidine uptake at 4 hr. No effect is observed in K562 cell cultures in morphology or NMR measurements. No new 31P-labeled metabolites were detected in either cell line as a result of drug treatment.

INTRODUCTION

Naturally occurring ether-linked lipids are found in abnormally high levels in neoplastic cells (23, 24); this is due to the absence of an O-alkyl cleavage enzyme activity (3, 22). A synthetic ALP, ET180CH3 (Chart 1), inhibits or retards tumor cell growth in vivo (14) and tumor cell growth in vitro (25). ALP accumulates in tumor cells due to the absence of an O-alkyl cleavage enzyme activity, and the accumulation interferes with normal phospholipid metabolism (13), which is responsible for the continuous renewal of membrane components (26). The accumulation of ALP results in the accumulation of naturally occurring, but at high levels, cytotoxic lysophospholipids (14). The antimetabolic effect of ALP results in a decreased synthesis and increased degradation of cellular phosphatidylcholine.

Tidwell et al. (25) developed recently a model system for investigating the mechanism of action of ALP. HL60, a human promyelocyte cell line (7, 8), and K562, a blast cell line (11), were tested for ET180CH3 effect on clonogenicity in semisolid agar, [3H]TdR uptake, and growth kinetics. The HL60 cell line is sensitive, and the K562 cell line is resistant to the cytotoxicity of ALP, judging by the results of the 3 tests (25).

The potential use of ALP as a chemotherapeutic agent in human solid tumors (4, 20) and leukemia (2, 25) has intensified interest in the investigation of the mechanism of cytotoxic action of the drug. The pertinent question of how ALP may affect membranes and the possible involvement of membrane function in the cytotoxic effect of ALP action stimulated the present study. We used NMR spectroscopy to study the effect of ALP on intracellular water relaxation and energy metabolism in HL60 and K562 cell suspensions. NMR relaxation of internal water was strikingly affected in ALP-treated HL60 cells rather than K562 cells. Further, this effect is much earlier than any reported so far, occurring only 1 or 2 hr after ET180CH3 exposure. In contrast, the ATP:ADP ratio does not change in either cell line after 24 hr of exposure, judging by 31P-NMR spectroscopy. Electron micrographs show that K562 is not morphologically affected by ALP, nor is HL60 at 4 hr after incubation with ALP; however, morphological changes do occur in HL60 but not in K562 after 24 hr of incubation with the drug. The presence of intact mitochondria even in the HL60 (24-hr treatment) case in which cellular damage has occurred is in line with the 31P results which show no change in ATP:ADP ratios after 24 hr of incubation.

The data presented outline the temporal sequence of events leading to the differential action of ALP on HL60 rather than K562, with the earliest reported effect being the change in internal water relaxation behavior.

MATERIALS AND METHODS

The source and maintenance procedures for the cell lines HL60 and K562 and drug incubation procedures were carried out as described by Tidwell, et al. (25). The ET180CH3 was kindly supplied by Dr. Paul Munder of the Max-Planck-Institut für Immunobiologie, Freiburg, Germany. Immediately after incubation of 1 to 3 million HL60 and K562 log-phase cells with 5 μg/ml ET180CH3, the cells were transferred to Wilmad Model 572 7-inch NMR tubes. After centrifugation at 400 x g, the supernatant was removed from the packed cells (50 μl), and the sample was kept at ice temperature until NMR measurements were made. The samples were equilibrated at probe temperature. No provisions to aerate the cells were made. Measurements were compared between treated and control samples for each experiment. The time required for the T2 measurements is msec. The Carr–Purcell Mei-boom–Gill (6, 12) technique for the measurement of the transverse relaxation time (T2) of intracellular water was used as described previously. The experiments were conducted at 22° with a modified Spin Lock CPS-2 pulsed spectrometer (Spin-Lock, Ltd., Credit, Ontario, Canada) and interface, which was constructed here (1).

The data were analyzed during both a nonlinear regression program NLE* and an eigenfunction expansion program DISCRETE (18, 19). The latter allows the determination of the number of exponentials necessary to fit the data without initial biased estimates of parameters. Results obtained with the respective programs were identical. Experi-

* B. Blumenstein, personal communication.

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to Sr,--glycerol-3-phosphocholine at the SN-1 position in ether linkage, and a

The pellets were fixed with cold 2.5% glutaraldehyde in Millonig's
creatine phosphate to perchloric acid extracts of the cells.

was identified on the basis of chemical shift and by the addition of
temperature of 10 ~ The techniques for resonance assignment follow
aglas. Thin sections were stained with uranyl acetate and lead citrate
and examined in a Phillips EM-201 transmission electron microscope
(5).

for ultrastructural examination, ALP-treated and control HL60 and
K562 cells were chilled rapidly to 4 ~ and centrifuged gently into pellets.

(17). 3~p-NMR spectra at a frequency of 121.488 MHz were obtained on a Bruker CXP-300 spectrometer
(Bruker Instruments, Billerica, Mass.) operating in the fourier transform
mode. In the results reported, 3600 scans were acquired at a sweep
width of 10 kHz and 16,384 data points using quadrature detection with a repetition rate of 1 sec. The temperature of the sample was
maintained at 10° by passing a stream of thermostated nitrogen gas
over the sample, the temperature of which was controlled by a BVT-
1000 controller. Two ml of a 50% cell suspension (v/v) in cell culture
medium Roswell Park Memorial Institute Tissue Culture Medium 1640
were placed in 10-mm NMR tubes. Chemical shifts were measured
with respect to external 85% phosphoric acid. Cell cultures for 31P-
NMR experiments were harvested by centrifugation at 400 x g and
maintained on ice until NMR experiments were begun at a preset
temperature of 10°. The techniques for resonance assignment follow
closely those of Navon et al. (15, 16). In K562 cells, creatine phosphate
was identified on the basis of chemical shift and by the addition of
creatine phosphate to perchloric acid extracts of the cells.

For ultrastructural examination, ALP-treated and control HL60 and
K562 cells were chilled rapidly to 4° and centrifuged gently into pellets.
The pellets were fixed with cold 2.5% glutaraldehyde in Millonig's
buffer, postfixed in 1.25% OsO4, dehydrated, and embedded in Mar-
aglas. Thin sections were stained with uranyl acetate and lead citrate
and examined in a Phillips EM-201 transmission electron microscope
(5).

RESULTS

Water Transverse Relaxation Studies on HL60 and K562
Cell Lines. HL60 and K562 cell cultures were incubated for
appropriate times, and doses of ALP and intracellular water
relaxation (T2) measurements were made. Chart 2 shows a
typical magnetization versus time curve for packed HL60 cells
using the Carr, Purcell, Meiboom, and Gill pulse sequence.
Both the experimental data and the fitted curve for a model
composed of a biexponential decay are shown. The 2 decay
times for the curve are 138 and 76 msec. The fraction of the
total magnetization contributed by each exponential is 0.50
(Table 1). The effects of ALP with time on Fraction 1 (expressed as the change in the amount of Fraction 1 present
in controls compared to experimental). The concentration of
ALP was 5 µg/ml, and the time course extends from 0 to 24
hr. The error bars indicate the standard error of the mean. The
chart shows significant changes in Fraction 1 with time. Fur-
thermore, the relaxation effects occur early, at 1 hr after
incubation.

In K562, 2 components are also detected. One was over-
whelmingly large, consisting of about 95% of the total with a T2
of 134 msec and a very small fraction, about 5% of the total,
with a T2 of 28 msec. For K562, both water populations and T2
values remain essentially identical in the untreated and drug-
treated cases. Fluctuations have been observed in the T2 values for the small (5%) population (ranging from 10 to 50 msec).

Chart 3 also presents data for the difference in Fraction 1
(T2 ~ 134 msec) for ALP-treated and untreated K562 cells (5
µg/ml). Sufficient data have not been acquired to estimate the
S.E. for each individual time for K562, but it is clear that no
significant variation occurs for K562.

31P-NMR Studies of High-Energy Phosphate Metabolites.
The metabolic status of phosphate metabolites (sugar phos-
phates, ATP, and ADP) in intact, functioning HL60 and K562
cell lines has been monitored by 31P-NMR spectroscopy. Both
treated and untreated samples were studied. Chart 4A shows
the 121.488-MHz phosphorus spectrum of untreated HL60
cells. The spectrum represents 3600 free induction decays (1
hr total time for data acquisition). The areas of each peak are
proportional to the amount of the species present averaged
over the accumulation time. Peak positions in ppm relative to
external 85% phosphoric acid are given in the charts. Assign-
ment of each species to the proper chemical shift has been
made on the basis of literature values (21). In K562 cells,
creatine phosphate was further identified by the addition to
perchloric acid extracts and monitoring the intensity increase of
the appropriate peak. The region of the spectrum from ~-4.8
to ~-4.0 ppm is attributable to overlapping peaks of sugar
phosphates. The remaining important peaks are P1 (~-2.8 ppm),
ATP, (5.9 ppm), ATP, (9.9 ppm), and ATP, (18.7 ppm). Addi-
tional peaks at 10.5 and 12.2 ppm are due to a combination of

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fraction present</th>
<th>T2 (msec)</th>
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<tbody>
<tr>
<td>1</td>
<td>0.508 ± 0.092</td>
<td>138 ± 15</td>
</tr>
<tr>
<td>2</td>
<td>0.492 ± 0.092</td>
<td>76 ± 6</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
NAD\(^+\) and a diphosphodiester compound observed by Navon et al. (15, 16) in HeLa cells. Chart 4B shows the spectrum 3 hr later. Very little change occurs in ATP levels. Similar time courses for K562 controls are given in Chart 5, A and B. In addition to the compounds identified for HL60, creatine phosphate in K562 is observed along with an unidentified resonance at -0.7 ppm. After 2 hr, the concentration of creatine phosphate had diminished, and considerable buildup of ADP had occurred unlike HL60. It is clear from the above results that K562 cells are more metabolically active than HL60 cells at 10\(^{+}\). Under the conditions of sample preparation, K562 starts at higher ATP levels, and the rate of ATP depletion is more rapid. Table 2 presents the ATP depletion results. The ratios shown are area ratios of the \(^{31}\)P peaks for the species. The ratios in the table should become larger on ATP depletion. Comparison of the data of control and drug-treated cells for the first hr shows no significant effect of drug treatment for both HL60 and K562. Thus, after treatment of the cells with 5 \(\mu\)g/ml of ALP for 24 hr, the ATP-generating capacity of both cell lines is functioning normally, judging by initial ATP levels.

Viability of ALP-treated HL60 Cultures. Chart 6 demonstrates the changes in viability, judging by trypan blue exclusion and cloning ability in semisolid agar, that occur after a preliminary short ALP exposure followed by continued incubation. Cells were incubated with ALP and washed free of the drug.
and incubation continued for 48 hr. At 5 μg of ALP per ml, there is no decrease in trypan blue exclusion nor clonogenicity; however, after drug exposure of 24 hr, there is a decrease in clonogenicity. By contrast, high levels of ALP, 20 μg/ml, a concentration that may be surface active (13, 20), do affect trypan blue exclusion after a short drug exposure.

**Ultrastructure of Treated and Control HL60 and K562 Cells.** Untreated HL60 cells demonstrated ultrastructural features typical of myeloblasts and promyelocytes (Fig. 1), but a small number of cells showed maturation to the myelocyte and band stages of development (9). The cells at the myeloblast-promyelocyte stage were characterized by large, round to slightly indented nuclei containing euchromatic chromatin, and one to 3 large nucleoli. Occasionally, nuclear blebs were seen. The cytoplasm was characterized by variable numbers of well-developed primary azurophilic granules and small numbers of specific granules. Mitochondria, strands of rough endoplasmic reticulum, and centrioles were similar to those found in bone marrow specimens from patients with acute myelogenous leukemia (10). Electron micrographs of control K562 cultures (Fig. 1) demonstrated highly differentiated blasts with variable degrees of nuclear lobulation. The nuclei contained large amounts of euchromatin and showed envelope-associated heterochromatin. Most nuclei contained one or occasionally 2 prominent nucleoli. Mitochondria, ribosomes, and small deposits of glycogen were scattered throughout the cytoplasm.

**DISCUSSION**

This study was undertaken for the purpose of characterizing more clearly the differences in response of HL60 and K562 cell lines to low concentrations of ALP. For this study, both morphological and nuclear magnetic resonance techniques have been used, the latter providing an essentially noninvasive method of observation. The time sequence of events so established after drug treatment should assist in ultimately elucidating the mechanism by which these drugs act. After exposure of HL60 cell cultures to a 5-μg/ml concentration of ET180CH3, the following occur (in order): a change in internal water relaxation; a decrease in [3H]Thd uptake (a decrease of 18% at 4 hr after ALP treatment); a decrease in clonogenicity; and a change in ultrastructural morphology. All events listed occurred between 0 and 24 hr after ALP exposure, a time at which the ATP:ADP ratio is unchanged. The earliest measurable effect of ALP on HL60 is at 1 hr after exposure, when the internal water relaxation changes must represent true changes in either the fractions, the chemical species present averaged over the accumulation time, which is 1 hr. Time in the table represents total time after equilibration to 10%. An increase in a given ratio represents a decrease in ATP.

**Table 2**

<table>
<thead>
<tr>
<th>Cells Time (hr)</th>
<th>P&lt;sub&gt;a&lt;/sub&gt;:ATP&lt;sub&gt;p&lt;/sub&gt;</th>
<th>(ATP + ADP&lt;sub&gt;p&lt;/sub&gt;):ATP&lt;sub&gt;p&lt;/sub&gt;</th>
<th>P&lt;sub&gt;a&lt;/sub&gt;:ADP&lt;sub&gt;p&lt;/sub&gt;:ATP&lt;sub&gt;p&lt;/sub&gt;</th>
</tr>
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<tbody>
<tr>
<td>HL60 Untreated</td>
<td>1 1.8 (1.7)&lt;sup&gt;b&lt;/sup&gt; 1.0 (0.7) 1.2 1.3</td>
<td>3.7 2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 1.8 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K562 Treated&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 1.8 1.2</td>
<td>1 1.1</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>2 2.2 1.3</td>
<td></td>
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</table>

<sup>b</sup> Numbers in parentheses, duplicate samples.

<sup>c</sup> Incubated for 24 hr with ALP in the culture medium at a dose of 5 μg/ml.

The time course of 31P-NMR intensities for treated and untreated HL60 and K562 cells is shown in Chart 6. Effect of 1- to 4-hr incubation of HL60 cultures with ALP on continued incubation after cells were washed free of the drug. Left, 20-ml cultures of HL60 cells pulsed for either 1, 2, or 4 hr with ALP. Cells were washed, and incubation was continued for 24 or 48 hr at which time trypan blue exclusion was measured. Untreated controls; ○, 1 hr, ALP (5 μg/ml); □, 2 hr, ALP (5 μg/ml); ◇, 4 hr, ALP (5 μg/ml); Δ, 1 hr, ALP (20 μg/ml); ■, 2 hr, ALP (20 μg/ml); ◆, 4 hr, ALP (20 μg/ml). Right, HL60 cultures pulsed with ALP, washed, and tested for clonogenicity in semisolid agar. Control; ○, control; □, ALP (5 μg/ml); ■, ALP (20 μg/ml).

The significance of the water relaxation experiments lies in the fact that these detect early changes in the relaxation curves for drug-treated HL60 cell cultures. The data in Chart 3 represent differences in Fraction 1 after drug treatment. The complexity of the cell interior makes difficult the assignment of the water fractions to specific regions of the cell. Furthermore, conditions of exchange rates of water molecules between idealized compartments determine the interpretation placed on the fractions and relaxation times. Only in the case of slow exchange between compartments do the fractions represent true measures of the populations of water sites. In the case of intermediate exchange, the fractions are functions of the true populations of the sites and the exchange rates between the compartments. The observation of 2 components does indicate the absence of fast exchange between sites, since in this case, a single exponential would be observed. Such results are observed in the RBC, which is represented by and conforms to 2 idealized compartments, inside and outside. The intracellular water of the RBC relaxes according to a single exponential (17).

It is possible that the relaxation curve represents a more complex distribution of sites, which can be satisfactorily represented by a biexponential decay. Nevertheless, the observed changes must represent true changes in either the fractions, exchange rates, or distributions of the former as a result of drug perturbation. The complexity of the problem is illustrated by comparison of the fractions of different relaxation rates in
HL60 and K562. Morphologically, large differences in the size of major organelles are not observed; however, the fractions of the long relaxing component are drastically different, 0.5 to 0.95. T2 values show small variations and no systematic variation with drug treatment, whereas the fractions obtained show a drug effect in HL60; this suggests that the fractions are true water populations.

The results obtained from the 31P-NMR experiments indicate several important points concerning the interaction of ALP with both HL60 and K562 cell lines. (a) The phosphorus measurements of energy metabolism (levels of ATP) show that ATP levels are not depressed significantly even after 24 hr of drug treatment. Thus, high-energy phosphate function is not impaired significantly in either cell line. ATP production continues in ALP-treated HL60 cells despite evidence of changes in internal water relaxation and [3H]dThd uptake at early times after drug exposure. The initial levels of sugar phosphates are somewhat variable. In addition, these levels change during NMR measurements. Therefore, such alterations are most likely due to slight variations in the metabolic status of the cells and not due to drug treatment. Creatine phosphate was detected in K562 but not in HL60 cells. The relevance of these differences to the action of ALP is not clear. Investigation of both whole-cell suspensions and perchloric acid extracts reveals no new phosphorus species as a result of drug treatment.

The time course for a change in [3H]dThd uptake, clonogenicity, and cell growth kinetics has been reported (25). The earliest time measured was 24 hr after drug exposure when HL60 was affected rather than K562. Five μg of ALP per ml are the lowest fully effective dose in suppressing [3H]dThd maximally by 24 hr. This dose results in the death of 98% of the HL60 cells by 72 hr of exposure. The experiment graphed in Chart 6 represents the lack of change at early times in viability or clonogenicity due to the effects of 5 μg ALP per ml. However, [3H]dThd uptake is suppressed slightly (18%) at 4 hr after drug exposure in HL60 but not in K562 cell cultures.

Exposure of HL60 and K562 cell cultures to 5 μg ALP per ml for 1, 2, and 4 hr produced no recognizable morphological changes. In contrast, HL60 rather than K562 cells exposed to ALP for 24 hr showed an apparent loss of integrity of the cyttoplasmic membrane despite normal appearing nuclei, mitochondria, and other cytoplasmic structures.

Thus, ALP affects the internal water relaxation in HL60 but not K562 cell lines at 1 hr after exposure; later, there is a change in [3H]dThd uptake, clonogenicity, and morphology. 31P-NMR spectroscopy indicates that the ATP:ADP ratios remain constant during the events occurring before 24 hr. Electron micrographs show normal appearing mitochondria at 24 hr, which supports the NMR data. The abnormal cell morphology at 24 hr, however, is correlated with a decrease in clonogenicity and reduction in cell numbers.

REFERENCES
Fig. 1. A, HL60 control specimen shows 2 early promyelocytes. Primary granules and mitochondrial profiles are scattered throughout the cytoplasm of both cells. × 6500. B, C, cells from an HL60 cell culture incubated with ALP (5 μg/ml) for 1 (B) and 4 (C) hr, similar in appearance to the control specimen. B, × 4800; C, × 6800. D, HL60 cells incubated with ALP (5 μg/ml) for 24 hr, showing a loss of continuity of cell membranes, (arrows). However, mitochondria appear intact. A portion of a normal-appearing metamyelocyte is seen to the right. × 6800. E, a poorly differentiated blast from a K562 cell culture, demonstrating a highly lobulated nucleus and scattered mitochondria in its cytoplasm. × 4750. F to H, K562 cells incubated in ALP (5 μg/ml) for 2 (F), 4 (G), and 24 hr (H), showing ultrastructural features similar to those seen in the control cells. F, × 6800; G, × 5100; H, × 4750.
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