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CYTOTOXICITY OF LAZAROID U-75412E IN HUMAN EPITHELIAL CELL LINE (WISH)

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Abstract—The 21-aminosteroids (or lazaroids) are a recently synthesized class of compounds demonstrated to protect tissue against damage induced by trauma and/or ischemia. Currently, very little is known about the biological effects of lazaroids. In this work the action of lazaroid U-75412E on a human epithelial cell line (Wish) was evaluated. The data obtained showed an inhibition of cell growth and a dose- and time-dependent decrease of cell viability. Furthermore, a dose- and time-dependent increase of cells in the G2/M phase with the appearence of apoptotic cells was observed by flow cytometric analysis. Nuclear fragmentation was also evident. Lactate dehydrogenase release and scanning electron microscopy experiments suggested that plasma membrane integrity was altered by this compound. The immunofluorescence technique and transmission electron microscopy images also showed intracellular damage, such as alteration of microtubular arrangement, mitochondrial swelling and the presence of vacuoles. This study demonstrated that 1 μM U-75412E was unable to modify these parameters, while higher concentrations (6–75 μM) had a cytotoxic effect on Wish cells.

Key words: cytotoxicity; lazaroids; wish cells; apoptosis

Lipid peroxides and oxygen reactive species are thought to be involved in major physiological or pathological events, such as inflammation, radiation damage, mutagenesis, cellular ageing and reperfusion damage [1]. Evidence of the potential role of oxidants in the pathogenesis of many diseases suggests that antioxidants may be used in the therapy or prevention of these diseases. The 21-aminosteroids, or lazaroids, are a novel series of compounds specifically designed to localize within cell membranes and to inhibit lipid peroxidation reactions [2–4]. These molecules have shown activity in *in vivo* models of experimental central nervous system trauma and ischemia [5–7]. In cell-free systems they are potent inhibitors of lipid peroxidation, having an IC_{50} of 2–60 μM in rat brain homogenate [2]. Lazaroids seem to inhibit lipid peroxidation by a mechanism similar to vitamin E; in addition, the group of these drugs which contain a NC=CN fragment, like U74500, also possess the ability to interact with ferrous ions [4].

Very little is known about other biological effects of lazaroids, except that they improve endothelial cell viability at 4°, U-74500 being the most effective [8]. Furthermore, they inhibit growth of cultured Balb/c 3T3 clone A31 fibroblasts [9]. Therefore, it was of interest to evaluate the action of lazaroid U-75412E on an epithelial cell line (Wish) derived from human amnions.

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¶ Abbreviations: LDH, lactate dehydrogenase; SEM, scanning electron microscopy; TEM, transmission electron microscopy; FCS, fetal calf serum; DAPI, 4,6-diamidinophenylindole; MT, microtubules.

MATERIALS AND METHODS

Chemicals. Lazaroid U-75412E (Lot 0295-DJH-7A) was a kind gift of Upjohn Laboratories (Kalamazoo, MI, U.S.A.); it was dissolved directly in FCS¶ (Flow, Irvine, U.K.).

Wish cells were grown in 25 cm^2 plastic flasks using RPMI-1640 (Gibco, Paisley, U.K.) medium supplemented with 10% heat-inactivated FCS, 100 U penicillin g/mL and 100 μg streptomycin sulphate/mL at 37° in a 5% CO_2 atmosphere. Cells were passaged at near confluency with 0.25% trypsin–EDTA (Aldrich, Steinheim, Germany). Viability was evaluated by the nigrosin exclusion test in a Nageotte chamber.

Growth of Wish cells. This was evaluated after plating ~4.5 × 10^5 cells on sterile plastic Petri dishes, either in the absence or presence of 1, 6, 12.5, 25, 50 or 75 μM of lazaroid U-75412E. The cells were counted at 24, 48, 72 and 96 hr with a Nageotte chamber, taking into account both adherent and non-adherent cells, after trypsinization of monolayers. The viability was measured as described above. For the cell-cycle analysis performed by flow cytometry, the cells were exposed to lazaroid (1, 6 and 12.5 μM) for 10, 17, 24 and 48 hr, harvested by centrifugation (after trypsinization of the adherent cells), washed in PBS and were then fixed in 70% ethanol at 0°. Before staining with 1 μg/mL of DAPI (Sigma, St Louis, MO, U.S.A.) they were centrifuged and rehydrated in Tris–HCl buffer (0.1 M Tris, 0.1 M

NaCl, pH 7.5). The cellular DNA content was measured by a PARTEC PAS II flow cytometer. Calculations of the percentage of cells in the various cell-cycle phases were based on the cumulative frequency curves of the appropriate DNA histograms. Cells growing on coverslips, under the same experimental conditions, were also examined by microscope after DAPI staining, to visualize nuclei.

SEM. Cells ($\sim 4 \times 10^5$) were seeded onto sterile glass coverslips into wells of 24-well polystyrene plates and grown at 37° under 5% CO_2. After 2 and 24 hr of incubation in the presence and absence of lazaroid, the cells were processed as described by Mattana *et al.* [10] and examined using an I.S.I. DS 130 scanning electron microscope operating at 20 kW.

TEM. Cells were washed twice with PBS for 10 min before fixation for 40 min in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) containing 7.5% sucrose. After fixation the cells were processed as described by Mattana *et al.* [10].

Immunofluorescence experiments. Cells growing on glass coverslips, after several washings with PBS, were permeabilized with 0.25% glutaraldehyde, 0.5% Triton X-100 in Cytoskeleton buffer (pH 6.1) [11] for 2 min and then fixed with 1% glutaraldehyde in the same buffer for 10 min. They were then processed three times with sodium borohydride (0.5 mg/mL) freshly prepared in TBS (10 mM Tris–HCl, 155 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, pH 7.6) and the residue removed by further washing in the same buffer. To visualize tubulin and vimentin, an indirect immunofluorescence was performed with monoclonal anti-tubulin antibody TU-01, a kind gift of Dr Viklicky [12], diluted 1:20 in TBS and monoclonal anti-vimentin antibody (Sigma) (1:40 in TBS), respectively. Anti-mouse IgG Rhodamine conjugated (Sigma) (TRITC-Ab, 1:40 in TBS) was used as the secondary antibody. Actin was visualized using rhodamine-conjugated phalloidin (Organon Teknika Corp., Oss, Holland) diluted 1:500 in TBS. After washing several times with TBS, coverslips were mounted in Gelvatol (Monsanto Corp., Springfield, MA, U.S.A.) and examined with a Nikon Optiphot microscope. LDH activity was measured spectrophotometrically in the supernatants of cultured cell, either in absence or in the presence of lazaroid, according to Wrobleski and La Due [13].

Statistical analysis. Statistical differences between groups was determined using a two-tailed Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

As shown in Fig. 1, U-75412E caused inhibition of cellular growth that was both concentration- and time-dependent. Cell viability was also decreased by U-75412E in a dose- and time-dependent manner (Fig. 2); with 6 μM lazaroid the effect on viability

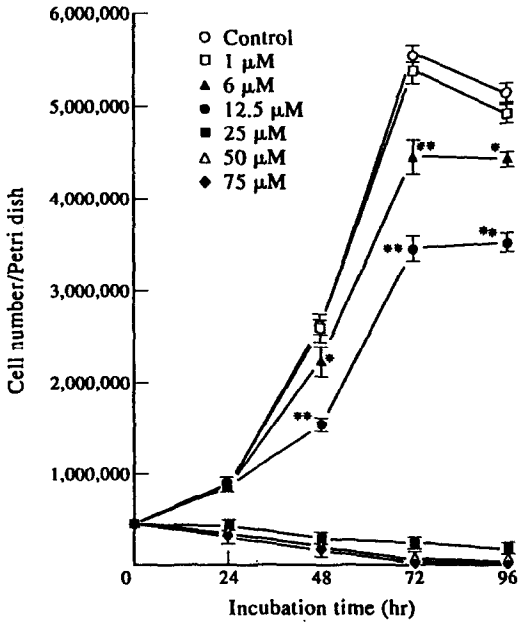

Fig. 1. Effect of U-75412E on proliferation of Wish epithelial cells. Cell growth in the absence or presence of different lazaroid concentrations for 4 days. Results are means ± SE of three experiments; * = $P < 0.05$; ** = $P < 0.01$ vs controls.

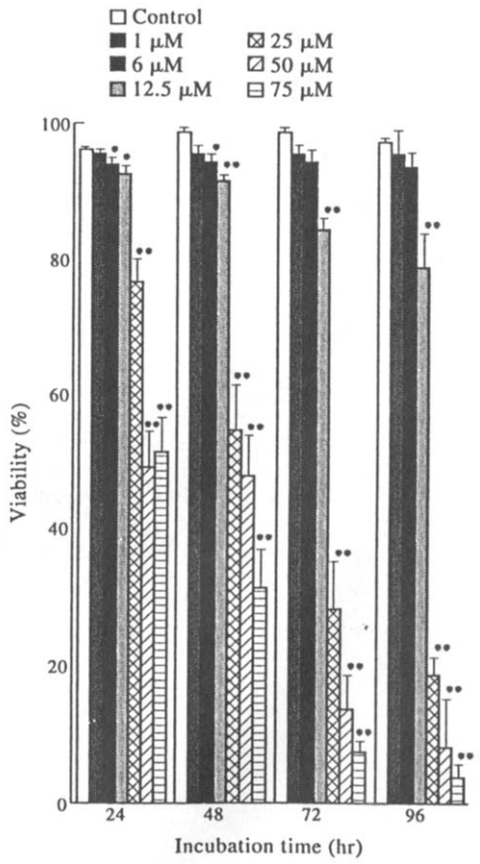

Fig. 2. Effect of U-75412E on viability (%) of Wish cells. Viability in the absence or presence of different lazaroid concentrations for 4 days. Results are means ± SE of three experiments; * = $P < 0.05$; ** = $P < 0.01$ vs controls.

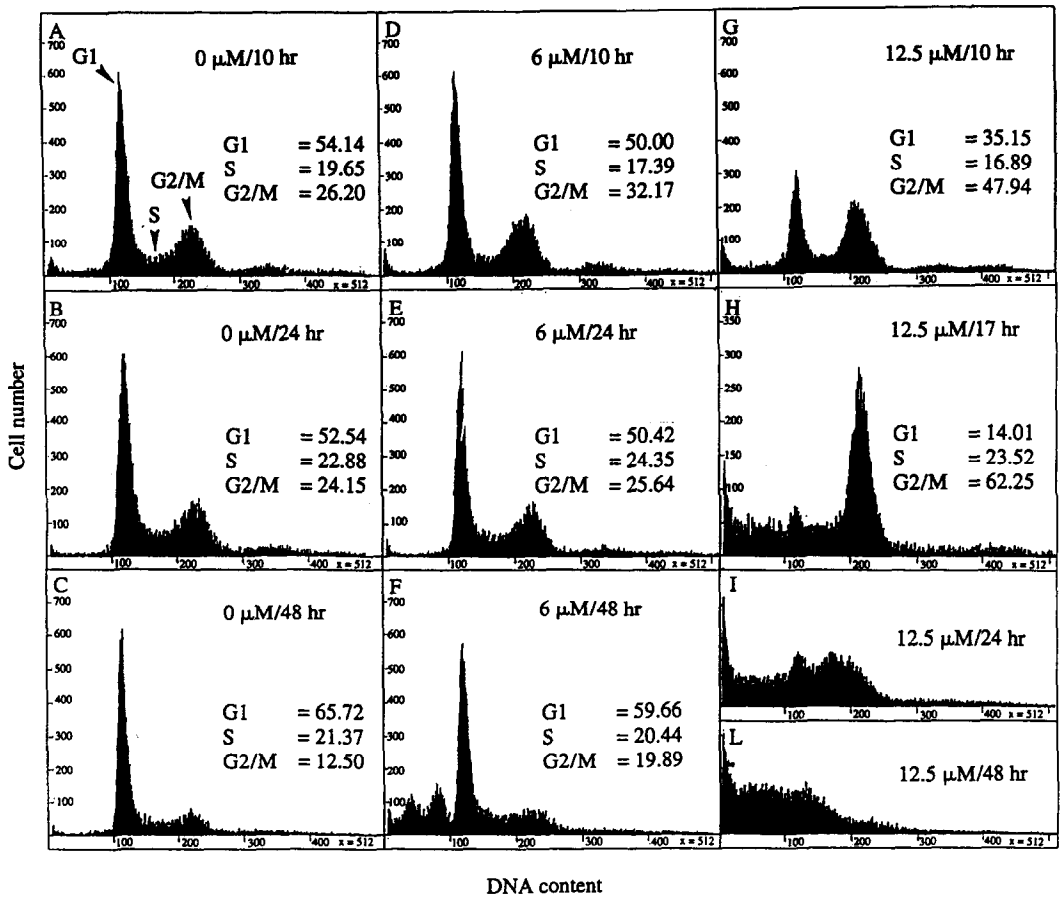

Fig. 3. DNA content frequency histograms (after staining with DAPI) of Wish cells, untreated for 10 hr (A), 24 hr (B), 48 hr (C); treated with 6 μM U-75412E for 10 hr (D), 24 hr (E), 48 hr (F); treated with 12.5 μM lazaroid for 10 hr (G), 17 hr (H), 24 hr (I) and 48 hr (L). G1 = postmitotic cells; S = cells in S phase; G2/M = cells in G2 phase and mitosis.

was significant only at 24 and 48 hr. One micromolar of U-75412E did not induce any significant modification to the cell cycle (data not shown); while with 6 μM U-75412E after 48 hr of incubation two hypo 2c peaks appeared, indicating degenerative DNA phenomena (Fig. 3F). Moreover, a time-dependent alteration of the cell cycle was obtained with 12.5 μM U-75412E: after 10 and 17 hr, cells with 4c DNA content were increased, indicating that they were arrested in the G2/M phase (Fig. 3G and H). The fluorescent microscopy of nuclei, performed under the same experimental conditions, showed different levels of nuclei fragmentation (Fig. 4).

By SEM it was determined that control cells showed a polygonal shape and were well flattened and adherent to the substrate; many thin microvilli were present on their surface, which were regularly distributed (Fig. 5). After 2 hr incubation with 25–75 μM U-75412E cells appeared more rounded and showed "blobs" whose dimensions increased in a dose-dependent way (Fig. 5), however, those treated with 1 μM U-75412E were similar to control cells (data not shown). After 24 hr incubation at 25–75 μM the cell surface exhibited remarkable alterations: the number of microvilli decreased and dose-related damage of the cell surface was evident (Fig. 6).

Intracellular damage caused by incubation with 75 μM lazaroid for 2 hr was investigated by TEM. Drug-treated cells showed a remarkable number of vacuoles. Mitochondria had an ovoidal shape in control cells, whereas they were smaller, rounded and showed a widening of the intercrystal spaces in treated cells (Fig. 7).

The control cells examined by immunofluorescence techniques showed a complex highly-organized network of MT, radially distributed or arranged in parallel to the major axis in the polygonal and the spindle-shaped cells, respectively (Fig. 8). In Wish cells treated for 2 hr with 25–75 μM lazaroid, MT appeared thickened and their spatial organization was modified, in fact they were arranged around the nucleus (Fig. 8). No alterations were seen with 1, 6 and 12.5 μM lazaroid after 2 hr incubation (data not shown), while after 24 hr, 6 and 12.5 μM produced MT modifications (data not shown). On the other hand, no alteration was observed in actin and vimentin (data not shown).

As shown in Table 1, the lazaroid (25–75 μM) induced a dose-dependent release of LDH from

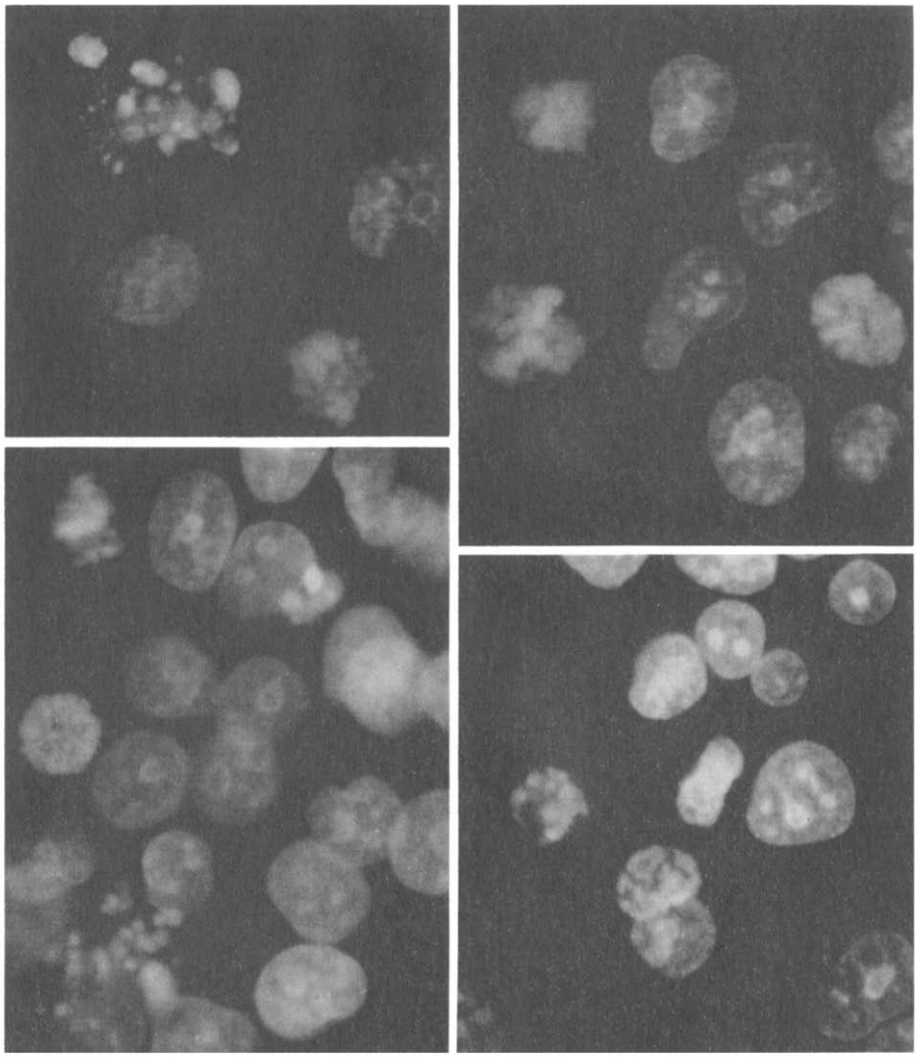

Fig. 4. Nuclear fragmentation (apoptosis) in Wish cells incubated with 6 μM U-75412E for 48 hr after DAPI staining.

Wish cells after 24 hr of incubation, whereas 1 μM was not able to modify LDH outflow. At the highest concentration (75 μM) LDH release increased significantly from 5.05 ± 0.88 to 13.88 ± 1.62 U/mL ($N = 3$; $P < 0.01$) after 90 min incubation.

DISCUSSION

Previous observations showed that many lazaroids inhibited cell growth in cultured mouse 3T3 fibroblasts, without producing a cytotoxic effect [9]. These data show that lazaroid U-75412E inhibited growth of human cultured epithelial cells (Wish). The flow cytometry analysis confirmed this anti-proliferative effect, in fact, after 10 hr of treatment with 12.5 μM U-75412E a large number of cells were blocked in the G2/M phase, without apparently degenerative phenomena.

More prolonged treatment showed a further increase of cells in the G2/M phase; moreover, DNA degradation was also evident, which suggested the presence of degenerative processes (Fig. 3I and L). These last aspects were dramatically evident at 24 and 48 hr treatments. Different phases of nuclear fragmentation (apoptosis) were also evident when the cells were incubated with 6 μM U-75412E for 48 hr. These results confirmed the findings of flow cytometric analysis. Reduced DNA stainability observed in apoptotic cells was a consequence of a partial loss of DNA due to activation of endogenous endonuclease [14, 15]. Cell growth inhibition by lazaroid was probably due to a cytotoxic action of the compound; in fact, in treated cells, viability significantly decreased. Release of the intracellular enzyme LDH, used as an indicator of cytotoxicity, confirmed these data. The primary event was most likely damage of the plasma membrane; in fact, LDH release at the highest concentration of the drug became statistically significant even after 90 min of incubation, when all the cells were viable,

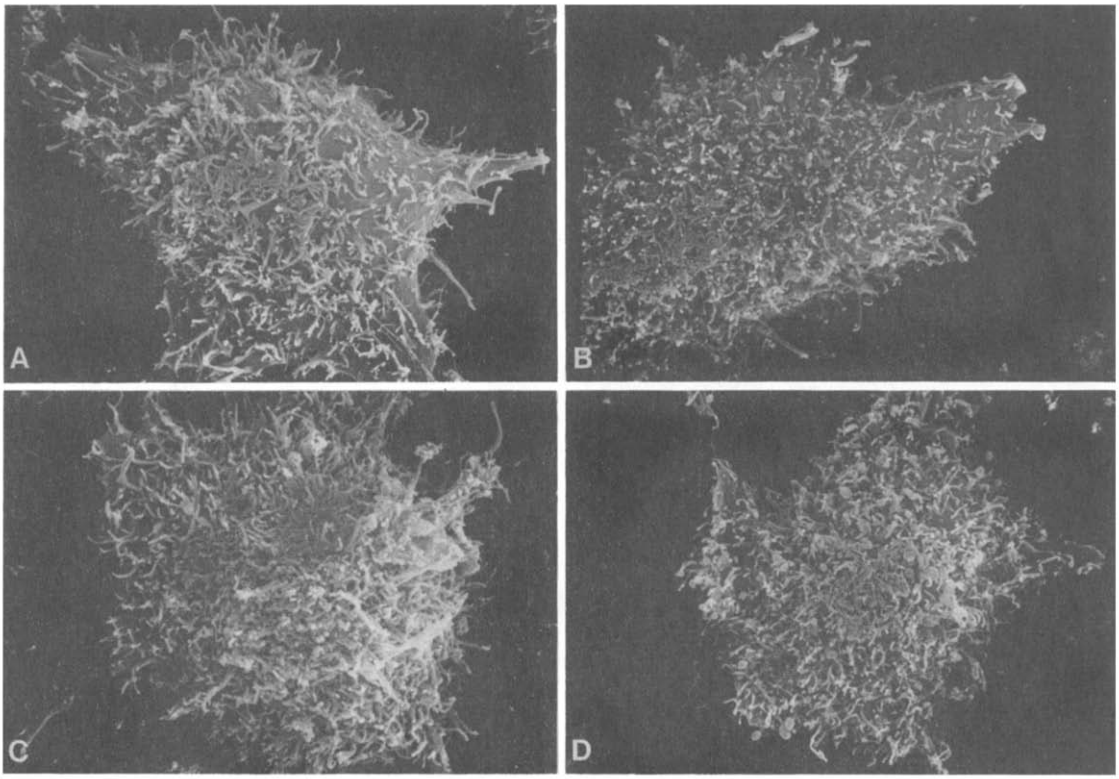

Fig. 5. SEM of Wish cells exposed to U-75412E for 2 hr. (A) Control; (B) 25 μM; (C) 50 μM; (D) 75 μM. Magnification, 4000×.

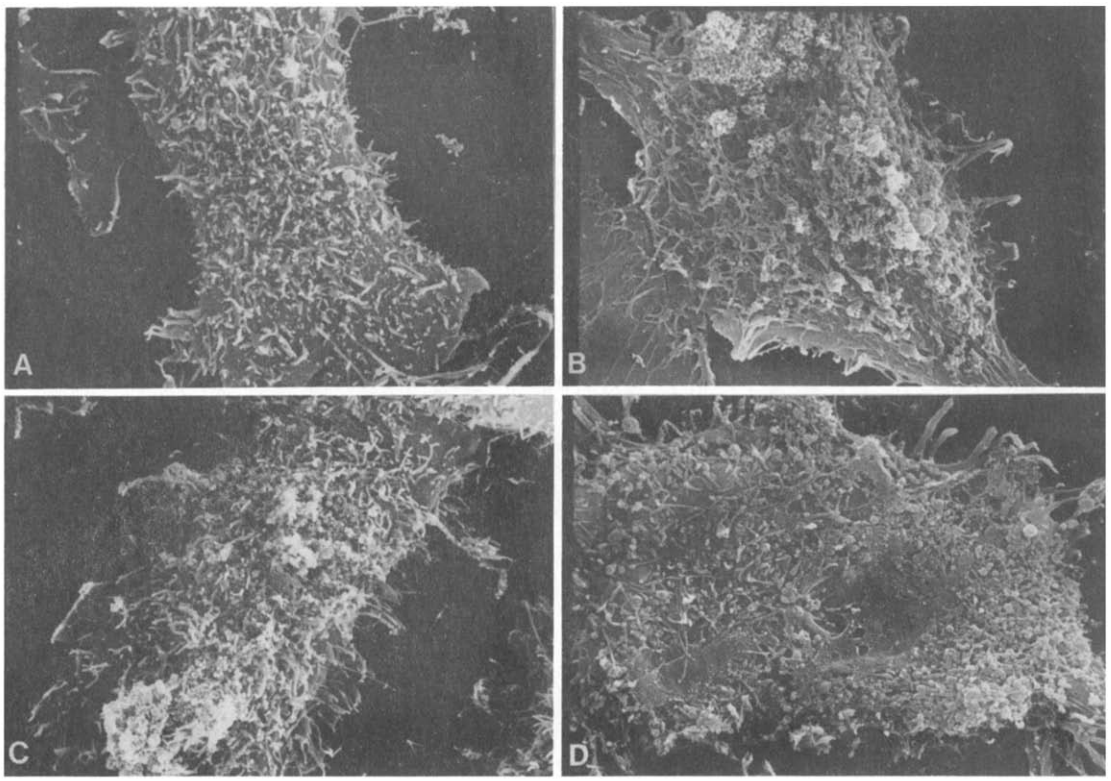

Fig. 6. SEM of Wish cells exposed to U-75412E for 24 hr. (A) 1 μM; (B) 25 μM; (C) 50 μM; (D) 75 μM. Magnification 4000×.

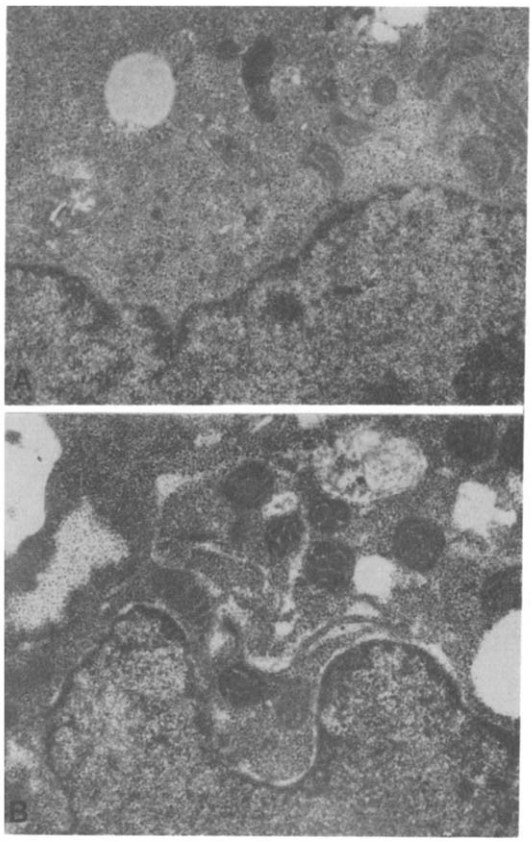

Table 1. LDH release in the culture medium of Wish cells exposed for 24 hr to U-75412E

[U-75412E] (μM)	LDH ΔU(mL ± SE)	P	N
1	1.83 ± 1.29	n.s.	3
25	33.72 ± 0.59	<0.01	3
50	53.51 ± 0.92	<0.01	3
75	66.22 ± 1.54	<0.01	3

The data are expressed as the difference between LDH samples release and controls. Results are means ± SE of three experiments. Statistic evaluation was done vs control values.

indicating a change in cell permeability. Furthermore, SEM experiments showed that treated cells exhibited evident damage to the cell surface that could be ascribed to the high lipophylia of the molecule. In addition, U-75412E caused ultrastructural damage, as shown by TEM. MT appeared radically modified at concentrations ranging from 25 to 75 μM, while actin and vimentin were not altered (data not shown), indicating that tubulin could be quite a specific target for the lazaroid toxicity. The cell

Fig. 7. TEM of Wish cells exposed to 75 μM U-75412E for 2 hr. (A) Control; (B) drug-treated cells. Magnification 29,900×.

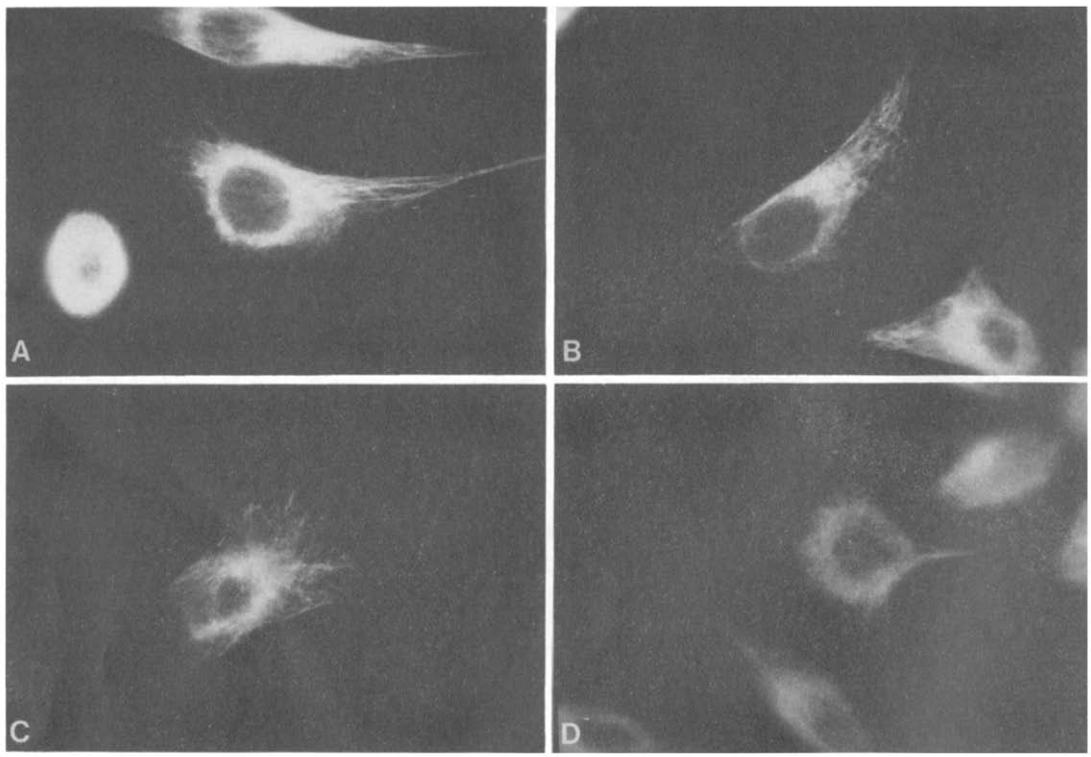

Fig. 8. Immunofluorescence staining of MT in Wish cells exposed to U-75412E for 2 hr. (A) Control; (B) 25 μM; (C) 50 μM; (D) 75 μM.

shape alterations observed by light microscopy may correlate with the MT damage.

In conclusion, these data suggest that lazaroid U-75412E has a cytotoxic effect at concentrations above 1 μM in Wish. Therefore, further studies are needed to determine the actions of lazaroids in different cell lines.

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