Biochemical Pharmacology, Vol. 54, pp. 1371–1376, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00435-8

SHORT COMMUNICATION

High Sensitivity of Leukemic Peripheral Blood Lymphocytes to Triethyllead Action

Eftichia Stiakaki, Christos Stournaras,*‡ Helen Dimitriou† and Maria Kalmanti†*

*Departments of Biochemistry and †Pediatric Hematology-Oncology, School of Medicine, University of Crete and the University General Hospital, GR-71110 Heraklion, Greece

ABSTRACT. In a previous study we reported that triethyllead (Et_3Pb^+) inhibits cell proliferation of normal human lymphocytes. To further characterize this interaction, we studied herein the effects of Et_3Pb^+ on the cell viability of normal and leukemic human lymphocytes and analysed the expression and dynamics of the monomer/polymer equilibrium of tubulin in these cells. Short- and long-term cell culture experiments demonstrated significantly different dose-dependent effects of Et_3Pb^+ on cell viability of leukemic compared to normal lymphocytes. Indeed, in the presence of increasing concentrations of Et_3Pb^+ (10^{-12}–10^{-5} M), primary cultures of chronic lymphocytes (CLL) and acute lumphoblastic (ALL) leukemic human lymphocytes were much more sensitive to Et_3Pb^+ treatment when compared to normal peripheral blood lymphocytes (PBL). The IC_{50} values were approximately 5×10^{-6} M for PBL and 8×10^{-10} M for both CLL and ALL respectively, when cells were preincubated for 3 h with this agent. These experiments revealed a 1000-fold higher responsiveness of leukemic cells to Et_3Pb^+ treatment. Quantitative immunoblot analysis showed that leukemic cells express up to 4-fold higher total tubulin amounts. However, the proportion of polymerized tubulin in leukemic compared to normal lymphocytes increased only slightly (up to 1.4-fold). These findings reveal a significant decrease in the polymeric to total tubulin ratio in leukemic lymphocytes, indicating important modifications in tubulin dynamics and reorganization of the microtubular structures. Our results demonstrate that leukemic cells are much more sensitive than normal lymphocytes to Et_3Pb^+ action. This effect may be due to the altered monomer/polymer dynamic equilibrium of tubulin shown in leukemic cells. It is, therefore, worthwhile exploring future applied uses of Et_3Pb^+ as a potential suppressor of leukemic cell growth. BIOCHEM PHARMACOL **54**;12: 1371–1376, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. triethyllead; tubulin; polymeric/total tubulin ratio; cell viability; normal and leukemic lymphocytes

Cytoskeletal changes have been known to occur during cell transformation, while alterations in cytoskeletal protein organization and morphology have been reported in neoplastic cells. These modifications include the organization, stability and dynamic exchange between soluble and polymerized pools of actin [1–7] as well as tubulin, microtubules and microtubule-associated proteins [8–10]. Based on these reports, several agents which interact with cytoskeletal proteins have been considered as cancer chemotherapeutics. These include: the vinca alkaloids, which bind to the tubulin dimmer inhibiting microtubule assembly and disrupting the microtubular network; the taxols, which bind to microtubules preventing their disassembly and reorganization during mitosis; and finally the cytochalasins, the fungal toxins which interact with actin filaments inducing microfilament shortening.

The organolead compound triethyllead (Et_3Pb^+) is a degradation product of the antiknocking agent tetraethyllead (Et_4Pb)§. Previous studies on the biochemical and cellular level have shown that Et_3Pb^+ interacts with tubulin, inducing depolymerization of microtubules *in vitro* as well as in various mammalian cells [11, 12]. The molecular mechanism of the Et_3Pb^+-tubulin interaction has been elucidated, and involves the interaction of the organolead compound with two cysteine groups of the protein [11]. Further studies based on the inhibition of tubulin polymerization by Et_3Pb^+ succeeded in establishing a specific biological assay for Et_3Pb^+ quantitations in environmental air and water samples [13–15]. In addition to the above action, Et_3Pb^+ was shown to inhibit important molecular systems such as the membrane-bound Na^+-K^+-ATPase [16, 17], the mitochondrial F_0-F_1-ATPase complex and the oxidative phosphorylation [18, 19] and glutathione-*S*-transferase [20]. Moreover, due to its amphiphylic character, Et_3Pb^+ has been shown to easily penetrate biological membranes, and accumulate in cells and tissues [18, 21, 22].

‡ Corresponding author: Prof. Dr. Christos Stournaras, Dept. of Biochemistry, School of Medicine, University of Crete, GR-71110 Heraklion, Greece. TEL: 30-81-3945 63; FAX: 30-81-3945 30.

Received 24 February 1997; accepted 20 June 1997.

§ *Abbreviations:* ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; Et_3Pb^+, triethyllead; PBL, peripheral blood lymphocytes.

In a previous study, we reported that Et_3Pb^+ may also affect the immune system, since it was shown to inhibit proliferation of normal human lymphocytes by decreasing the expression of interleukin 2 receptor Tac-chain [17]. Taking into account that the microtubule cytoskeleton is an important cellular target of Et_3Pb^+ [11, 12] and based on the reports which showed alterations of the microtubular network in neoplastic cells [8–10], we hypothesized differential Et_3Pb^+ effects in normal and malignant human lymphocytes. For this purpose, we studied the dose- and time-dependent effects of Et_3Pb^+ on cell viability of normal (PBL) and leukemic (ALL and CLL) human lymphocytes in primary cell culture experiments. In addition, using quantitative immunoblot analysis, we studied the expression levels of total tubulin amounts and the ratio of polymerized tubulin in normal and malignant lymphocytes.

MATERIALS AND METHODS

Materials

Culture media, Hank's balanced salt solution (HBSS), fetal calf serum and the antibiotic-antimycotic mixtures were from Life Technologies Inc. Ficoll-Hypaque was from Pharmacia Biotech AB. PMSF, EGTA and the monoclonal antibody against β-tubulin were obtained from Sigma. Et_3Pb^+ was from Ventron and was further purified as previously described [13]. The ECL Western blotting kit was purchased from Amersham Corp. Tubulin from porcine brain was prepared as described previously [11].

Human Lymphocyte Cultures

Peripheral blood lymphocytes (PBL) were isolated from freshly obtained heparinized venous blood of healthy volunteers, from patients with chronic lymphocytic leukemia (B-CLL), or from children with acute lymphoblastic leukemia (ALL, cALLA+). Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. Interface cells, washed twice in HBSS, were resuspended (10^6 cells per mL) in complete medium RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 g/L sodium bicarbonate, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL). For the short-term cell viability experiments, cells were preincubated for 3 hr in the absence or presence of increasing concentrations (10^{-12}–10^{-5} M) of Et_3Pb^+. Then cells were extensively washed to remove Et_3Pb^+ from the culture medium and resuspended in fresh complete medium without Et_3Pb^+. Cultures were followed for 48 to 72 hr in 5% CO_2–95% air at 37°. For the long-term experiments, cells were cultured in the absence or presence of increasing concentrations of Et_3Pb^+ (10^{-12}–10^{-5} M) for 48 and 72 hr, respectively. Cell viability was determined by counting intact cells in the presence of trypan blue dye.

Total Cellular Tubulin and Microtubule Polymer Levels

Total cellular tubulin and microtubule polymer levels were determined by immunoblot analysis of cell fractions prepared as described previously [23]. Equal amounts of protein (5 μg or 10 μg) were subjected to SDS electrophoresis and the resulting protein-bands were transferred to nitrocellulose membranes, using an LKB electroblot apparatus (LKB). Nitrocellulose blots were incubated with monoclonal mouse antitubulin antibodies, followed by incubation with the appropriate labeled second antibody, using the ECL Western blotting kit. Nitrocellulose blots were exposed to Kodak X-omat AR films for variable lenghts of time. Band intensities were quantitated by PC-based Image Analysis (Image Analysis Inc.). Tubulin content in the samples was calculated by reference to a standard curve of pig brain tubulin.

Statistical Analysis

Statistical analysis of the total and polymerized tubulin content in normal and malignant cells was performed by one-way analysis of variance (ANOVA) and by unpaired Student's test. In the cell viability experiments, values were normalized as percentage of control. Thus, these data were statistically evaluated by two nonparametric methods (Kruskal–Wallis and Mann–Whitney tests). In all experiments, n represents the number of persons (volunteers or patients) from whom cells were isolated.

RESULTS

Dose-Dependent Effect of Et_3Pb^+ on the Viability of PBL and Leukemic Cells in Culture

SHORT-TERM INCUBATION EXPERIMENTS. A 3-hr cell incubation with various concentrations of Et_3Pb^+ (10^{-12}–10^{-5} M) revealed differential effects of the organolead compound on the cell viability of normal and leukemic cells (Fig. 1A,B). In the range between 10^{-8} and 10^{-7} M, cell viability of both CLL and ALL cultured for 48 hr was lowered, compared to normal PBL (Fig. 1A). Even more prominent were the effects when cells were cultured for 72 hr (Fig. 1B). A significant 60% decrease in cell viability was evident in leukemic cells pretreated for 3 hr with 10^{-9} M Et_3Pb^+, while PBL were not affected under these conditions. The calculated IC_{50} values from these experiments (Fig. 1B) were approximately 5×10^{-6} M for PBL and 8×10^{-10} M for both CLL and ALL. These results indicated that 1000-fold lower Et_3Pb^+ concentrations are sufficient to induce a decline of cell viability in CLL and ALL lymphocyte cultures, similar to that observed in normal human lymphocytes. It is concluded that leukemic human lymphocytes are much more sensitive to the short-term action of Et_3Pb^+ compared to normal PBL.

LONG-TERM INCUBATION EXPERIMENTS. In a second step, we studied cell viability of normal and leukemic cells

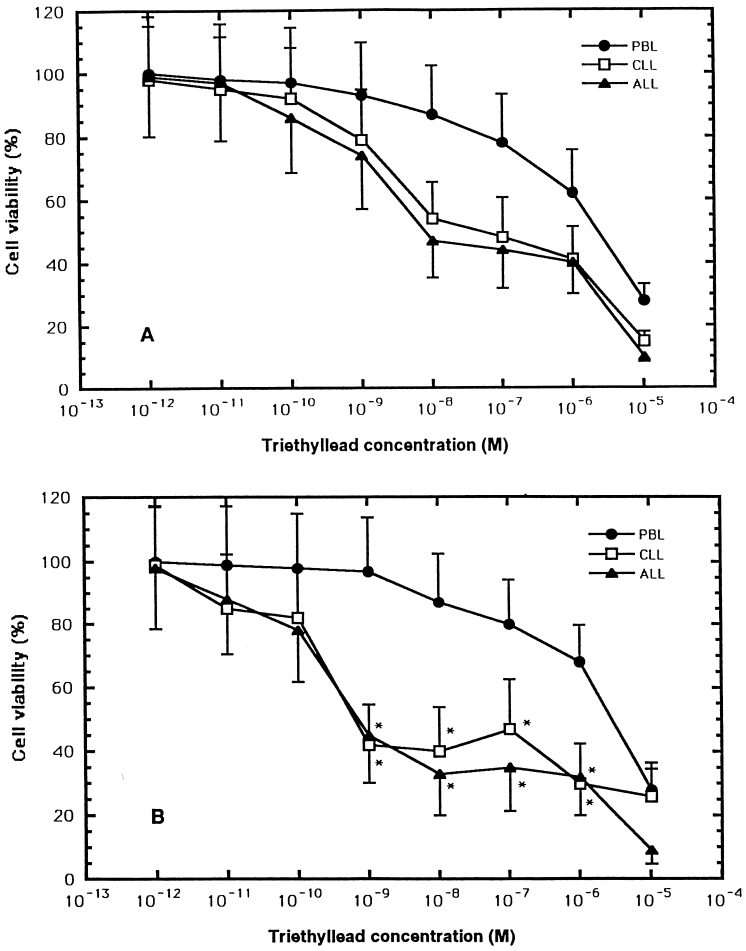

FIG. 1. Short-term, dose-dependent effects of Et_3Pb^+ on cell viability of normal and leukemic cells. Cells, pretreated for 3 hr with increasing Et_3Pb^+ concentrations, were cultured in complete normal medium for 48 hr (A) and 72 hr (B), respectively. Cell viability, assessed by trypan blue dye exclusion, is expressed as percentage of control (cells not exposed to Et_3Pb^+) for PBL (●——●, n = 20 distinct experiments), CLL (□——□, n = 16 distinct experiments) and ALL (▲——▲, n = 12 distinct experiments). Percentage of decrease in cell viability is expressed as mean values ± SE. * Denotes $p < 0.05$ for statistically significant differences between equally treated PBL and leukemic cells.

cultured in the presence of graded concentrations of Et_3Pb^+ (10^{-12}–10^{-5} M) for 48 and 72 hr, respectively. Interestingly, the Et_3Pb^+ effect was less extensive in CLL cell cultures, while cell viability of ALL was significantly affected by this drug when cells were cultured for 48 or 72 hr (Fig. 2A,B). The calculated IC_{50} values from these experiments (72 hr cultures) were approximately 8×10^{-9} M for ALL, 5×10^{-7} M for CLL and 10^{-6} M for PBL. This was an interesting finding, indicating that CLL cells, which seem to be highly sensitive to the short-term action of Et_3Pb^+ (Fig. 1B), showed similar cell viability curves with PBL in the presence of graded Et_3Pb^+ concentrations during 48 or 72 hr cultures. On the contrary, ALL cells were highly sensitive to both short- and long-term action of Et_3Pb^+. Although the biological relevance of this experimental finding remains unclear, it may indicate that ALL and CLL cells interact differentially with antimitotic agents, including Et_3Pb^+.

Effect of Leukemic Cells on Tubulin Amounts and Polymer/Total Tubulin Ratio

We further raised the question whether the differential sensitivity of normal and leukemic cells to Et_3Pb^+ action may be associated with modifications in the dynamic equilibrium between monomer and polymer tubulin in these cells. Indeed, quantitation of tubulin content by reference to a standard curve prepared with purified pig brain tubulin (Fig. 3, lanes 1–5, $r = 0.978$) showed that ALL cells express *ca.* 2.7- and 4-fold higher tubulin amounts compared to CLL and PBL, respectively (Table 1). On the other hand, the amounts of polymerized tubulin changed only slightly (Fig. 3, lanes 6, 8, 10, Table 1). From these experimental data, the calculated ratio of polymerized to total tubulin was found to decrease significantly in leukemic cells (0.36 ± 0.02 for CLL, $P < 0.01$ and 0.15 ± 0.01 for ALL, $p < 0.01$, respectively) compared to PBL (0.47 ± 0.02, Table 1). Moreover, the values for polymer and total tubulin and the polymer to total tubulin ratio between CLL and ALL cells were also significantly different. These experimental findings indicate that the polymer/total tubulin ratio is significantly decreased in leukemic cells, implying differential organization of microtubular structures in malignant cells.

DISCUSSION

The results reported in this study clearly indicate that leukemic human lymphocytes are more sensitive to Et_3Pb^+

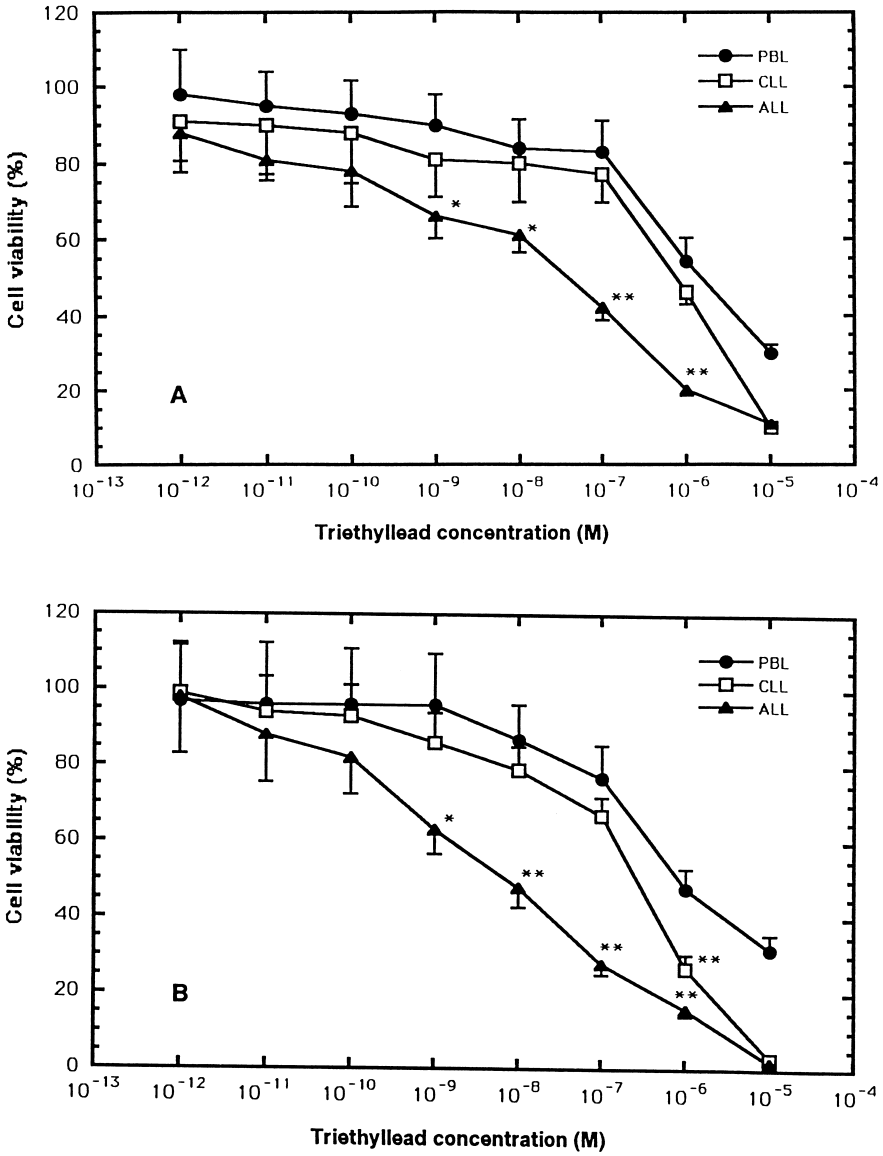

FIG. 2. Long-term, dose-dependent effects of Et_3Pb^+ on cell viability of normal and leukemic cells. Cells were cultured for 48 hr (A) and 72 hr (B), respectively in the presence of graded Et_3Pb^+ concentrations. Cell viability, assessed by trypan blue dye exclusion, is expressed as percentage of control (cells not exposed to Et_3Pb^+) for PBL (●——●, n = 20 distinct experiments), CLL (□——□, n = 16 distinct experiments) and ALL (▲——▲, n = 12 distinct experiments). Percentage of decrease in cell viability is expressed as mean values ± SE. * Denotes $p < 0.05$ and ** $p < 0.01$ for statistically significant differences between equally treated PBL and leukemic cells.

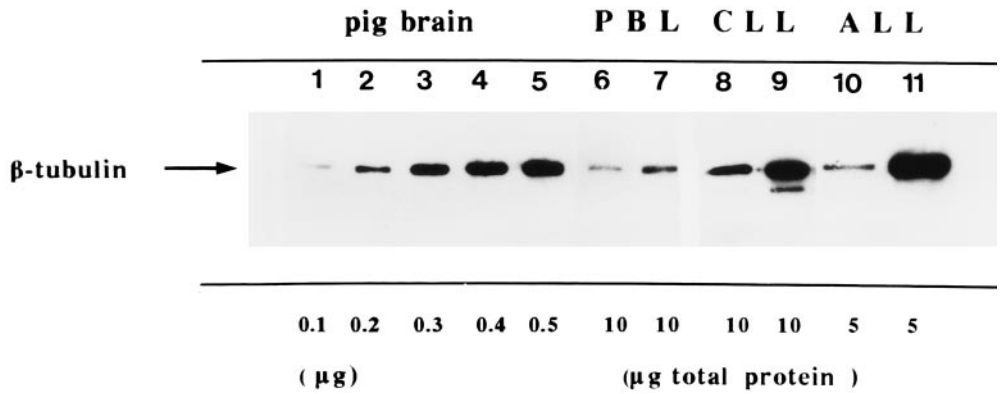

FIG. 3. Quantitative Western blot analysis from a representative experiment, depicting polymeric tubulin amounts (lanes 6, 8, 10) and the corresponding total tubulin contents (lanes 7, 9, 11) in normal and leukemic human lymphocytes. Lanes 1–5: Increasing amounts of isolated pure pig brain tubulin (0.1–0.5 μg). Lanes 6, 7: Polymer and total tubulin amounts in 10 μg cellular protein aliquots, respectively, from PBL cell extracts. Lanes 8, 9: Polymer and total tubulin amounts in 10 μg cellular protein aliquots, respectively, from CLL cell extracts. Lanes 10, 11: Polymer and total tubulin amounts in 5 μg cellular protein aliquots, respectively, from ALL cell extracts.

TABLE 1. Polymeric and total tubulin content and the polymer/total-tubulin ratio, determined by quantitative immunoblot analysis, in primary cultures of PBL, CLL and ALL

Treatment	Polymerized tubulin	Total tubulin	Polymer/total tubulin ratio
PBL ($n = 3$)	27.7 ± 2.3	59.4 ± 3.9	0.47 ± 0.02
CLL ($n = 3$)	33.4 ± 1.7[a]	91.2 ± 6.3[a]	0.36 ± 0.02[a]
ALL ($n = 3$)	38.7 ± 0.7[a]	261.5 ± 7.2[a]	0.15 ± 0.01[a]

The results are expressed in μg tubulin/mg total cellular protein (mean values ± SE from n = number of cell preparations).

[a] Denotes statistically significant differences in polymerized- and total-tubulin amounts and in polymer/total-tubulin ratios of CLL and ALL compared to PBL cells, as well as between CLL and ALL ($P < 0.01$).

action when compared to normal PBL. The samples of both ALL and CLL were fully infiltrated by cells which were all clonal, as shown by immunophenotypical tests. Accordingly, the action of Et_3Pb^+ was directed toward a homogenous cell population. It remains unclear which is the molecular mechanism implicated in the observed Et_3Pb^+ effects on cell viability. A possible explanation may involve differential uptake rates of Et_3Pb^+ in normal and malignant cells. However, several reports in the literature provide strong experimental evidence, countering such a hypothesis. Indeed, Et_3Pb^+ has been repeatedly described to easily penetrate biological membranes and accumulate in various cell types, intracellular organelles, or tissues [18, 21, 22]. Moreover, the permeability of Et_3Pb^+ has also been reported to be very similar in normal and transformed cells [24]. These data indicate that most probably other mechanisms, independent from the cellular Et_3Pb^+ uptake, are involved in the differential action of this agent in malignant lymphocytes.

Another possible mechanism which could be implicated in the differential action of Et_3Pb^+ in normal and leukemic cells may involve altered organization of the microtubule cytoskeleton in leukemic cells. Indeed, the significant differences in the dynamic equilibrium of the monomer/polymerized tubulin ratio and the total tubulin levels observed between normal and leukemic cells (Fig. 3, Table 1) suggest important reorganization of microtubular structures. Moreover, Et_3Pb^+ is known to interact with tubulin and microtubules, inducing their depolymerization and thus transforming the cellular monomer/polymer equilibrium of this protein [11, 12]. Accordingly, our findings are consistent with the hypothesis that the differential responsiveness of normal and leukemic cells to Et_3Pb^+ treatment may be due to the significant decrease in the polymer/total tubulin ratio observed in malignant lymphocytes. Moreover, this hypothesis is additionally strengthened by our experimental findings showing that ALL and CLL cells, which express significantly different ratios of polymerized to total tubulin (Table 1), interact differentially with Et_3Pb^+ in long-term incubation experiments (Fig. 2).

Further studies are still needed to evaluate whether the altered monomer/polymer tubulin equilibrium and/or differential expression of tubulin isoforms may be involved in the observed Et_3Pb^+ effects on normal and leukemic lymphocyte viability. However, it seems worthwhile to explore whether the higher sensitivity of malignant lymphocytes to Et_3Pb^+ can be observed in other types of malignant cells and whether it may provide valuable approaches to the manipulation of malignant lymphocytes.

We thank Prof. V. Georgoulias for fruitful discussions and Dr. A. Moustakas for helpful comments on the manuscript. This work was supported by grants from the Greek Anticancer Society and the **ΕΠΕΤ** *II - Program from the European Union to C.S.*

References

1. Kopelovich L, Lipkin M, Blattner WA, Fraumeri JF, Lynch MT and Pollack R, Organization of actin-containing cells in cultured skin fibroblasts from people at high risk of colon cancer. *Int J Cancer* **26:** 302–308, 1980.
2. Carley WW, Barak LS and Webb WW, F-actin aggregates in transformed cells. *J Cell Biol* **90:** 797–802, 1981.
3. Friedman E, Verderame M, Winawer S and Pollack R, Actin cytoskeletal organization loss in the benign-to-malignant tumor transition in cultured human colonic epithelial cells. *Cancer Res* **44:** 3040–3050, 1984.
4. Ben-Ze'ev A, The cytoskeleton in cancer cells. *Biochim Biophys Acta* **780:** 197–212, 1985.
5. Decloitre F, Cassingena R, Estrafe E and Martin M, Concomitant alterations of microfilaments and microtubules in human epithelial cells (HBL-100) in relation to their malignant conversion. *Tumour Biol* **12:** 111–119, 1991.
6. Katsantonis J, Tosca A, Koukouritaki SB, Theodoropoulos PA, Gravanis A and Stournaras C, Differential G/total actin ratio and microfilament stabilities in normal and malignant human keratinocytes. *Cell Biochem Funct* **12:** 267–274, 1994.
7. Stournaras C, Stiakaki E, Koukouritaki SB, Theodoropoulos PA, Kalmanti M, Fostinis Y and Gravanis A, Altered actin polymerization dynamics in various malignant cell types: evidence for differential sensitivity to cytochalasin B. *Biochem Pharmacol* **52:** 1339–1346, 1996.
8. Leung M-F, Sokoloski JA and Sartorelli C, Changes in microtubules, microtubule-associated proteins and intermediate filaments during the differentiation of HL-60 leukemia cells. *Cancer Res* **52:** 949–954, 1992.
9. Anand B and Chou IN, Microtubules and microtubule-associated proteins in resting and mitogenically activated normal peripheral blood human T-cells. *J Biol Chem* **267:** 10716–10722, 1992.
10. Anand B and Chou IN, Microtubule network and microtubule-associated proteins in leukemic T lymphocytes. *Leukemia* **7:** 51–57, 1993.
11. Faulstich H, Stournaras C, Doenges KH and Zimmermann H-P, The molecular mechanism of interaction of Et_3Pb^+ with tubulin. *FEBS Lett* **174:** 128–131, 1984.
12. Zimmermann H-P, Faulstich H, Hansch GM, Doenges KH and Stournaras C, The interaction of triethyllead with tubulin and microtubules. *Mutation Res* **201:** 293–302, 1988.
13. Faulstich H and Stournaras C, Potentially toxic concentrations of triethyllead in Black Forest rainwater samples. *Nature* **317:** 714–715, 1985.
14. Faulstich H and Stournaras C, Trialkyl lead in rain in the Black Forest. *Nature* **319:** 17–17, 1986.
15. Faulstich H, Stournaras C and Endres P, Trialkyllead: occurrence, biological interactions and possible impact of forest decline. *Experientia* **43:** 115–127, 1987.

16. Haeffner EW, Zimmermann H-P and Hoffmann CIK, Influence of triethyllead on the activity of enzymes of the ascites tumor cell plasma membrane and its microviscosity. *Toxicol Lett* **23:** 183–188, 1984.
17. Stournaras C, Georgoulias V, Spanakis E, Perraki M, Athanasiou M and Thanos D, Triethyllead-induced inhibition of proliferation of normal human lymphocytes through decreased expression of the Tac-chain of Interleukin 2 Receptor. *Int J Immunopharmac* **12:** 349–358, 1990.
18. Münter K, Athanasiou M and Stournaras C, Inhibition of cellular activities by triethyllead: role of glutathione and accumulation of triethyllead *in vitro*. *Biochem Pharmacol* **38:** 3941–3945, 1989.
19. Aldridge WN, Effects on mitochondria and on other enzyme systems. In: *Biological Effects of Organolead Compounds*. Boca Raton, CRC-Press, pp. 137–144, 1984.
20. Henry RA and Byington KH, Inhibition of glutathione *S*-aryltransferase from rat liver by organogermanium-lead and tin compounds. *Biochem Pharmacol* **25:** 2291–2295, 1976.
21. Nielsen T, Jausen KA and Grandjean P, Organic lead in normal human brains. *Nature* **274:** 604–605, 1978.
22. Stournaras C, Weber G, Zimmermann H-P, Doenges KH and Faulstich H, High cytotoxicity and membrane permeability of Et_3Pb^+ in mammalian and plant cells. *Cell Biochem Function* **2:** 213–216, 1984.
23. Papakonstanti EA, Emmanouel DS, Gravanis A and Stournaras C, Na^+/Pi cotransport alters rapidly cytoskeletal protein polymerization dynamics in opossum kidney cells. *Biochem J* **315:** 241–247, 1996.
24. Theodoropoulos PA, Gravanis A, Saridakis I and Stournaras C, Normal and *Ha-ras1* oncogene-transformed BRL cells show differential resistance to cytoskeletal protein inhibitors. *Cell Biochem Funct* **10:** 281–288, 1992.