# NEW ASPECTS OF THE RELATIONSHIP BETWEEN ACETYLCHOLINESTERASE ACTIVITY AND CANCER I: POLY-APS EXPERIMENTS

ZOVKO A., SEPCIC K., TURK T.
Institute of Microbiology and Immunology, Medical Faculty,
University of Ljubljana, Ljubljana
SLOVENIA

FAIMALI M., GARAVENTA F.,CHELOSSI E.
Institute for Marine Research (ISMAR) ,
National Council of Researches Genova,
ITALY

PALEARI L.
National Institute for Cancer Research (IST, Genova)
ITALY

FALUGI C., ALUIGI M.G., ANGELINI C., TROMBINO S., GALLUS L., FERRANDO S.
Department of Biology (DIBIO),
University of Genova
Viale Benedetto XV, 5, I-16132 Genova
ITALY
falugi@unige.it; http://www.unige.it

*Abstract:* - Since the first '80s, we found that some tumor types, and in particular lung tumors present increase, or generally change in acetylcholinesterase activity. Acetylcholinesterase is an enzyme associated to the cholinergic signal system, whose classic role is to remove acetylcholine from the receptors. Nevertheless, it is also involved in cell-to-cell communication driving embryonic development and in the regulation of several cellular features, such as apoptosis and cell movements. The presence of molecules related to the cholinergic signal system in the healthy and carcinogenic lung tissues, raised the hypothesis that substances inhibiting or affecting the cholinergic signaling system could exert an anti-cancer action at least in these tissues. Cytotoxicity tests on immortalized and primary cell lines derived from lung tumor (NSCLC) showed an AChE inhibition-dependent selective reduction of cell viability, statistically significant. The same cells, exposed to non toxic AChE inhibitors exhibited a loss in the mitochondrial potential, characteristic of the early apoptotic events and showed positive response to the annexin V assay, and to the T-terminal assay, that are specific features of the apoptotic event. Moreover, three-dimensional cell cultures (spheroids) of tumor cells, on exposure to AChE inhibitors show a decrease in the membrane-linked oligosaccharides, that are responsible for the adhesivity of the metastatic cells. In this promising picture, the need emerges of further studies aimed at understanding the effects of AChE inhibition on the regulation of acetylcholine release and the effects of AChE inhibitors on the functioning of acetylcholine receptors.

*Key words:* - Anti-cancer; natural drug; lung cancer, acetylcholinesterase; apoptosis; cell proliferation; *Reniera sarai* sponge.

## 1 Introduction

Nature has supplied several active anticancer agents (vinca alkaloids, anthracyclines, epipodophyllotoxin, and taxanes), which have significantly improved the management of many types of human cancer [1,2,3]. Marine organisms are a rich source of chemically novel products with a broad spectrum of bioactivity and many compounds that are derived from these organisms have generated interest for their cytotoxicities [4].

Among the marine invertebrates, the sponges are considered as chemical factories. Along with

biologically active compounds that have been isolated from marine sponges, there are several pyridinium derivatives [5]. Biologically active pyridinium derivatives, so far isolated from marine sponges, exert different biological activities, including cytotoxicity, hemolysis, inhibition of acetylcholinesterase enzyme activity. and binding to different receptors, antibacterial, antifungal, insecticidal, and microfouling activity [6,7,8].

In particular, the ability to inhibit acetylcholinesterase activity has been focused by several authors, for its possible regulatory effects on cancer progression.

Since the first '80s, some tumor varieties were found to show an overexpression of some cholinesterase activities, especially of acetylcholinesterase (AChE, E.C: 3.1.17).

AChE is an enzyme associated to the cholinergic signal system, whose classic role is to remove acetylcholine (ACh) from the receptors. Nevertheless, the protein is also involved both in cell-to-cell communication driving embryonic development, by mechanisms yet largely unknown, but possibly related to the intracellular dynamics evoked by ACh signaling, and in morphogenetic cell movements, related to AChE function as a cell-substrate adhesion molecule (SAM), through the affinity for laminin. Moreover, this molecule is affected by a number of natural and synthetic inhibitors, including environmental contaminants. For this reason, the activity of AChE was found to be a good biomarker for environmental toxicity, related to the role played by ACh in inflammation and in the control of apoptosis.

During studies conducted on environmental toxicants, such as organophosphate pesticides, we found that a low AChE inhibition promotes apoptosis in human cultured cells, and decreases cell movements, causing embryonic anomalies such as cardia bifida in chick embryos [9]. Apoptosis is one of the good cell responses against tumor, and preventing cells migration is a bad feature for embryonic development, but a good feature for metastasis spreading prevention.

In particular, Non Small Cell Lung Cancer (NSCLC) biopsies and cultured cell lines show enhanced AChE activity, and possess a complete set of molecules related to cholinergic signal system, including vesicular ACh transporter, ACh biosynthetic enzyme, and their receptors. Thus, our hypothesis was that down-regulation of this signalling system, in a natural-like and non-toxic way would help in enhancing anti-cancer cell features.

A natural anti-AChE complex compound, belonging to the class of polymeric alkylpyridinium salts (poly-APS), is produced by the Mediterranean sponge, *Haliclona (Reniera) sarai*, to avoid infestation from other marine organisms. Poly-APS were found to be a mixture of two of 3-octylpyridinium polymers, including 29 and 99 monomeric units, and were demonstrated to exert strong AChE-inhibitory activity *in vitro*. Colleagues at the CNR-ISMAR demonstrated the neurotransmitter/neuromodulator role of AChE in living organisms and the non toxic and reversible activity of poly-APS. This confirmed the requested features of the compound, of both low toxicity and AChE inhibition. In this review, an excursus of the potentiality of this natural substance in several field of basic and applied research is shown.

# 2 Non-neuromuscular localisations of cholinesterase

## 2.1 Localisation in structures associated to cell-to cell communication

Acetylcholinesterase (AChE) plays a key role in terminating neurotransmission at cholinergic synapses. AChE is also found in tissues devoid of cholinergic responses, indicating potential functions beyond neurotransmission. It has been suggested that AChE may participate in development, differentiation, and pathogenic processes such as Alzheimer's disease [10] and tumorigenesis**.** Particularly, during embryonic development, AChE activity was found localized in cells and tissues engaged in cell-to cell communication mediated by ion fluxes, in all the organisms and tissues where it was investigated. Data on the non-nervous location of cholinergic molecules were reviewed to give a general indication of their possible functions [11]. Cholinergic or immunologically related molecules were detected and localized mainly in three classes of differentiative events supported by intracellular ion concentration changes. I: during gamete maturation, activation and interaction [12,13]; II: during the early development of invertebrate and vertebrate embryos. In this case cholinergic molecules are located mainly in moving cells and tissues engaged in relevant morphogenetic events, such as gastrulation and limb bud differentiation, and are often codistributed with special extracellular matrix molecules such as fibronectin [9]; and laminin [14].

III: during inductive communications between mesenchyme and other tissues such as the limb bud development [15]. The cholinergic system thus seems to be a multifunctional cell communication system. It appeared early during evolution as a regulator of intercellular communications mediated by ion dynamics, before becoming involved in highly specialized communication structures, such as synapses and nerve endings.

Non-neuromuscular AChE expression was also found in a number of cell lines upon induction of apoptosis by various stimuli [16]. The induction of AChE expression was determined by cytochemical staining, immunol-ogical analysis, affinity chromatography purification, and molecular cloning. The authors found the AChE protein in the cytoplasm at the initiation of apoptosis and then in the nucleus or apoptotic bodies upon commitment to cell death. Sequence analysis revealed

that AChE expressed in apoptotic cells is identical to the synapse type AChE. Pharmacological inhibitors of AChE prevented apoptosis. Furthermore, blocking the expression of AChE with antisense inhibited apoptosis. So that the authors considered AChE as a potential marker and a regulator of apoptosis. The same authors, more recently, found a relationship between AChE and intracellular $Ca^{2+}$ concentration [17], thus presenting a hypothesis about the mechanisms of apoptosis driven by AChE. This is not surprising, because the cholinergic system is involved in the regulation of cell-to cell communication mediated by ion changes, as above described.

### 2.2 AChE localization in some cancer cell cultures and in lung cancer

The first reports about the presence of AChE in cancer cells begun to appear in the 70's, and in particular related to tumors of nervous-tissue origin, such as neuroblastoma, or NTera2 teratocarcinoma cells, committed to neurogenesis [18]. Since 1980, the presence of AChE activity was reported in blood tumors, such as leukaemia [19, 20, 21], but the scientific community was not keen to acknowledge the presence of molecules related to neurotransmission systems outside the neuromuscular structures. In the 80's it begun to appear evident that in cultured cells belonging to a number of solid tumors, such as HT1080 human fibro sarcoma and RD-2 human rhabdomyosarcoma cells, active molecules of AChE were produced in the perinuclear envelope, processed in the Golgi, and exposed to the cell membrane in 20-40% of the cell population [22, 23], while in normal human fibroblasts, no AChE activity could be demonstrated by the histochemical technique. The specificity of the reaction was shown through the use of specific cholinesterase inhibitors (Fig. 1)

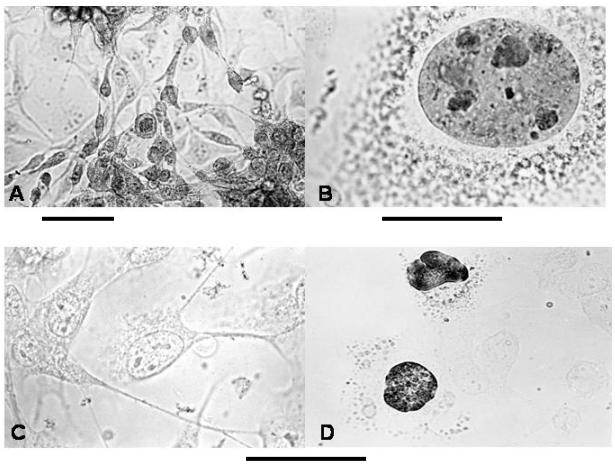



*Figure 1:* Acetylcholinesterase activity, revealed by the histochemical reaction of Karnovsky and Roots (1964). The reaction product is a dark precipitation in the sites of enzyme activity. A: HT1080 fibrosarcoma cells; B: RD2 rhabdomyosarcoma cell; C: control cells, pre-incubated with 10-5 M BW284c51 (specific AChE inhibitor); D: a panoramic view of the rhabdomyosarcoma huge cells. All the bars equal 50 μm.

In the figure, a nuclear localization of the enzyme is shown, that has been recently correlated with tumor progression [24]. Nowaday, a larger awareness takes place about the involvement of AChE in the regulation of cancerogenesis [25], through its role driving towards apoptosis suppression, cell movement, and in inflammatory processes. These mechanisms are mediated through the role of AChE in hydrolysis of acetylcholine at synapses and generally at receptorial sites in non-neural cells and tissue. Actually, the overflow of ACh at receptors, due to inhibition of AChE, over-activates ACh receptors (AChRs), both muscarinic and nicotinic, whose activation causes a loss in apoptosis, thus addressing suffering and inflamed cells to proliferation [26].

On the other hand, in the course of studies on whole organisms and in cultured cells we found that AChE inhibition by neurotoxic compounds, such as organophosphate pesticides, promotes apoptosis in human cultured cells, and decreases cell movements, causing embryonic anomalies such as cardia bifida in chick embryos [9]. Apoptosis is one of the good cell responses against tumour, and preventing cells migration is a bad feature for embryonic development, but a good feature for metastasis spreading prevention.

This contradictory effect may be due to the general toxicity of neurotoxic compounds, whose primary target is AChE activity, but have a number of secondary targets, such as muscarinic receptors [27], whose blockade actually should interfere with the normal modulation of apoptosis.

This was the state of our studies, when we met the poly-APS molecules.

# 3 A brief history of poly-APS discovery and research.

### 2.1 Poly-APS as marine organism defence: discovery and characterisation

3-alkylpyridinium polymers (poly-APS) were discovered about 15 years ago during a student's project of routine screening of marine sponge extracts obtained from the Mediterranean marine sponges. The Slovenian group of researchers lead by Tom Turk and Kristina Sepcic performed extracts of about 25 species, which were tested for acetylcholinesterase (AChE)-inhibitory activity and one of them showed strong, apparently irreversible inhibition of the enzyme.

The most active sample belonged to *Haliclona (Reniera) sarai*, a creamy tuberculated marine sponge that prefers shaded habitats and is common in the holes and ledges of steep underwater walls in the depths between 15 and 50 m. (fig.2, bar equals 50 mm).

The Slovenian Group purified the active molecules which turned out to be polymers composed of head-to-

tail linked 3-octylpyridinium units. The scientists named this compound polymeric alkylpyridinium salts or simply poly-APS. MALDI-TOF spectroscopy, which was at the beginning of its use at the time of poly-APS discovery, revealed that the active compound is actually a mixture of two polymers with respective molecular weights (MW) of 5500 and 18.900 Da. These MW would correspond to the compounds composed of 29-30 and 99-100 monomeric units, respectively [28, 29]. However, recent MALDI-TOF measurements revealed that poly-APS are in fact a single compound with MW of 5500 Da. Therefore, high molecular weight peak observed in the first MALDI measurements was probably due to aggregation (Ines Mancini, personal communication). Poly-APS are structurally similar to cationic detergents, therefore it is not surprising that above critical micellar concentration (0.23 mg/ml) they form aggregates or micellar structures in aqueous solution. This feature facilitates their purification by ultrafiltration and size-exclusion chromatography but complicate the calculation of inhibitory constants and other parameters due to the unknown size and molecular weight of the aggregates in solution.

Figure 2: The sponge*, Haliclona (Reniera) sarai* (Pulitzer –Finali, 1969) (photo by Tom Turk)

## 2.2- AChE inhibitory activity

Because of the strong AChE-inhibitory activity, in the initial years a substantial part of poly-APS research was devoted to the mechanism of inhibition of this enzyme [30, 31] (Fig. 3).

In addition, the group of Tom Turk and Kristina Sepčić had also studied hemolytic activity of poly-APS and their cytotoxicity [29, 32], which turned out to be important in the application of poly-APS as transfecting agents [6, 33, 34] due to their ability to pass through the cell membrane.

## 2.3 Application of poly-APS as non-toxic anti-fouling agent

Another important part of research was initiated during the collection of *H. sarai*, when it was observed that this sponge is never fouled by other organisms. Its soapy surface coating led us to the speculation that poly-APS might play an important role in the sponge protection. Indeed, experiments conducted in collaboration with colleagues in Genova (the ISMAR group, lead by Marco Faimali and Francesca Garaventa), revealed a strong but non-toxic antifouling activity against common fouling organisms like barnacles and mussels and to somewhat lesser extent to microalgae [7]. The AChE-inhibitory activity might play an important role in antifouling activity induced by poly-APS. In fact, AChE activity was detected in the setae of the antennules of barnacle cyprid larvae, which take part in the process of substratum recognition and subsequent settlement [35,36].

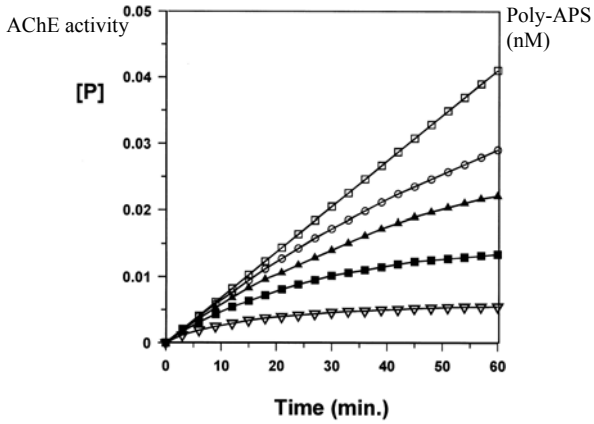



*Figure 3:* Time course of AChE inhibition, at different poly-APS concentrations. The figure is re-drawn from [30].

## 2.4 Antibacterial activity

In addition, poly-APS were also tested for antibacterial activity against terrestrial and marine bacteria. The latter are important for creating the first living layer in the fouling cascade, therefore antibacterial activity of poly-APS might be useful to protect submerged structures in the very first stage of the fouling processes [37].

# 3 Anti-tumour activity

Lately, poly-APS were also tested as potential chemotherapeutic agents against transformed tumor cells that express cholinergic system on their cell membranes. Initial testing revealed some promising results, especially on non-small cell lung cancer both *in vitro* and *in vivo*. [38]. As reported above, poly-APS exhibit strong anti-acetylcholinesterase, hemolytic, and moderate cytotoxic activity [29, 8]. As to the ability of inhibiting (AChE) [8], they can act as competitive inhibitors, at the same way as the organophosphosus compounds, by binding the serine in the catalytic anionic site in the enzyme gorge, or as non-competitive inhibitors, binding at the peripheral anionic site at the rime of the gorge, thus slowing or preventing the entrance of acetylcholine into the catalytic site [30].

AChE is the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) when it is linked to receptors (AChRs) in the nervous system synapses, but also in other localizations. In humans, ACh and the synthesizing enzyme, cholino-acetyltransferase, have been found in epithelial cells, mesothelial, endothelial, muscle and immune cells (mononuclear cells, granulocytes, alveolar macrophages, and mast cells) [39, 40, 41, 42, 43].
The cholinergic molecules present in non-neuromuscular structures play a functional role different from those expressed at neuromuscular sites. Actually, in these sites AChE was found involved in the regulation of cell migration [44], due to the ability to bind laminin [14] and to regulate apoptosis, through the activation of ACh receptors [16]. In particular, [16] found the presence of AChE protein in the cytoplasm at the initiation of apoptosis and then in the nucleus or apoptotic bodies upon commitment to cell death and suggested that AChE is potentially a marker and a regulator of apoptosis. Sequence analysis revealed that AChE expressed in apoptotic cells is identical to the synapse type AChE. Another regulatory pathway on apoptosis is exerted by AChE through the modulation of ACh cleavage. According to the amount of ACh present at the receptorial sites, ACh receptors may be more or less activated. Zhang et al also showed that pharmacological inhibition of AChE activity or the block of AChE expression with antisense inhibited apoptosis.
In some tumor types, following activation of nicotinic and/or muscarinic receptors, the MAPKinase cascade is activated, driving cell proliferation [45, 46]; MAPK are important signal molecules, leading to cell growth an proliferation. At the same way, in the lung cancers following iperactivation of nicotinic receptors, cell death regulation is compromised, thus causing the enhancement of cell proliferation [47]. This can explain why tumor progression is enhanced by tobacco smoking [48].
The therapeutical approach against cancer is to use drugs enhancing cell death and blocking cell proliferation. Some chemotherapeutic agents act through the inhibition of AChE activity; e.g the Irinotecan, a camptothecin derivative, is used in lung cancer treatment and therapy [49, 50].
Thus, the demonstrated non toxic AChE inhibition exerted by the poly-APS salts led us to verify the possibility of anti-neoplastic potential of poly-APS in selected tumor cells.

# 4 Experiments on anti-cancer activity of poly-APS salts

This section reports in detail the experiments reported by [38] together with unpublished data, aimed at establishing the balance between apoptosis induction and toxicity in lung cancer (*in vitro* and *in vivo*) and in normal lymphocytes, in order to investigate the reliability of the poly-APS salts as anti-cancer or adjuvant drug.

## 4.1 First phase: in vitro experiments.

### 4.1.1 Anti-cancer activity of poly-APS in cultured cell lines

*The Models* were: A549 cell line (human lung adenocarcinoma epithelial cell line), primary Non Small Cell Lung Cancer (NSCLC) lines, and human normal lymphocytes, in order to compare the effects on cell death between normal and cancer cells.

### 4.1.2 AChE expression in tumor tissues

The presence of AChE (the target molecule of poly-APS in all the immortalized an primary cell lines derived from NSCLC was identified by the method of indirect immuno-fluorescence, by use of a specific antibody against human AChE (Santa Cruz) (Fig. 4)
The presence of the molecule was also confirmed by RT-PCR, by specific primers of the neural isoform of AChE in several lung tumor cell lines (human lung adenocarcinoma epithelial A549, squamous lung carcinoma SKMES, malignant human epidermoid lung carcinoma CALU-1 and primary lines) (Fig. 5a, b). Surgical biopsies of NSCLC were homogenated and analyzed by western blot, by the antibody anti AChE.,
*Figure 4*: immunohistochemical revelation of AChE protein (green fluorescence) in different tumor cell lines.
All the tumor lines and homogenates showed the presence of high AChE amounts (Fig. 5c). Every data points were statistically significative ($P$<0.05) according to one-way ANOVA group comparison and BOnferronni's multiple comparison test. In all these studies, the A549 were used as a preferential model for the successive studies, cause of their features of strong aggressivity and resistance to anti-neoplastic agents, including retinoids.

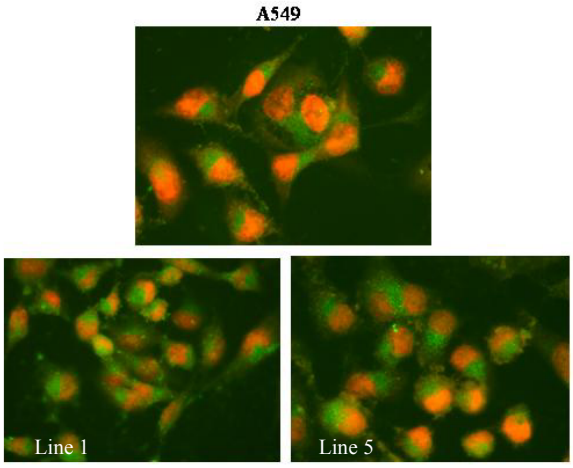



*Figure 4*: immunohistochemical revelation of AChE protein in different lung tumour cell lines. The green fluorochrome indicates the protein localisation.

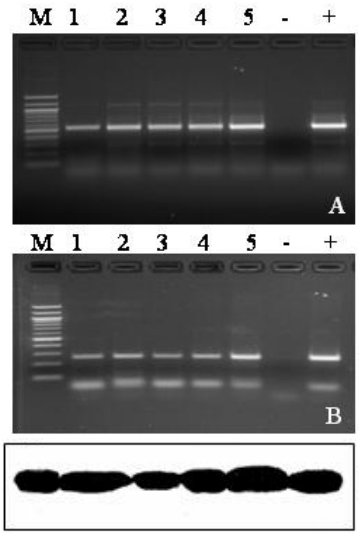



*Figure 5*: Westen blot and RT-PCR for AChE: detection A) RT-PCR with specific primers for β-actin; B) RT-PCR with specific primers for AChE; C) Western blot with anti-AChE antibody on primary NSCLC obtained form surgical intervene. M: 100 bp marker; 1: Calu 1; 2: SKMES; 3 line 1; 4: line 5; 5: A549; 6: control without RT; 7: NTera2/D1

**4.1.3- Activation of second messengers.**

Poly-APS exposure of A459 cells also caused increase in Inositol-trisphosphate (IP3) formation. IP3 is a critical second messenger that, in the frame of acetylcholine receptors, is formed by the activation of a number of G-protein coupled receptors, including the m1-type muscarinic ACh receptors [51]. When it is released in the intracytoplasmic domain, IP3 generally causes $Ca^{2+}$ release from intracellular stores, and consequent increase of intracellular [$Ca^{2+}$] concentration. (Fig. 6c).

**4.1.4 Poly-APs cytotoxicity on cancer cells and healthy lymphocytes**

Cytotoxicity tests performed by two methods: the MTS method, and colony forming tests method, on soft-agar (Fig. 7a and 7b) showed that poly-APS salts strongly inhibited cell proliferation with progressive dose- and time-dependent depletion on A549 and on primary Non Small Cell Lung Cancer (NSCLC) lines, while in normal lymphocytes the depletion was sensitively minor, suggesting the possibility of a specific action of the polymer on tumor cells. The concentration of the polymer was ranging between $10^{-5}$ g/ml and $10^{-7}$ g/ml, and the test lasted up to 72 h.

**4.1.5- Apoptosis**

The analysis of apoptotic endpoints confirmed that poly-APS exposure of in vitro models was able to cause cell death in a dose-dependent way in cancer cells, but not in healthy lymphocytes, when used at the doses ranging between $10^{-5}$ g/ml and $10^{-6}$ g/ml

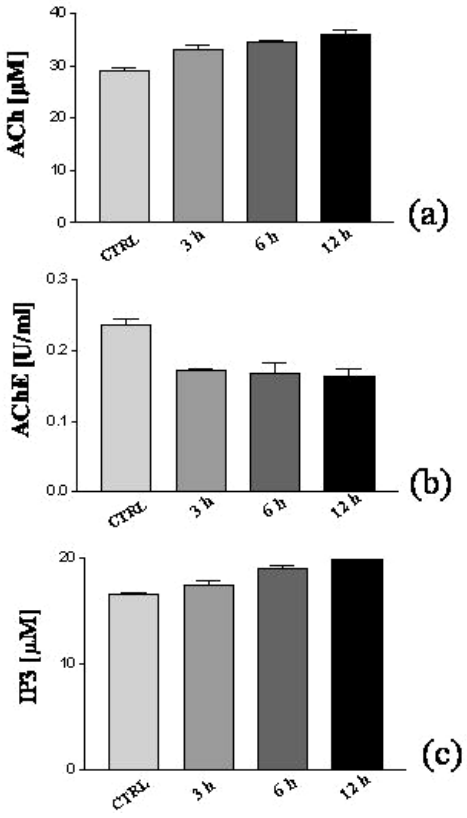



*Figure 6*: Modulation of ACh, AChE and IP3 after poly-APS salts exposure. AChE activity is strongly inhibited by poly-APS salts in A549 cells (b) and as a consequence the ACh lcontent is enhanced (a) and in turn increases intracellular IP3 concentrations (c: HitHunter Inositol-trisphosphate (IP3) FP Assay (*Discoverx, Fremon-CA*).). A549 cell line was treated with $10^{-5}$ g/ml poly-APS at different times (3 h, 6 h, 12 h). The measures were statistically significant at every evaluated times ($P$<0.05) according to one-way ANOVA group comparison and Bonferroni's multiple comparison test.

**4.1.5.1 Early phases**

*Membrane potential*: - One of the early events is represented by calcium dynamics. Cells undergoing apoptosis show high membrane depolarization, due to to high intracellular [$Ca^{2+}$]. Intracellular calcium dynamics are driven by IP3 release, and in turn IP3 receptors in mitochondria drive the events of mitochondrial polarization / depolarization [52].

Mitochondrial depolarization is a general good indicator and cause of apoptosis early events [53, 54]. The loss of mitochondrial potential is made evident by a vital staining, the *MitoCapture Mitochondrial Apoptosis* (MBL international). This reagent enters the mitochondria and fluoresces in red when their membrane potential is normal, while the cytoplasm presents a green autofluorescence. The red fluorescence fades away when the mitochondrial potential decreases. A549 cells exposed to $5x10^{-5}$ g/ml poly-APS showed

scarce or no red fluorescence while non-exposed cells presented red fluorescent mitochondria (Fig.8)

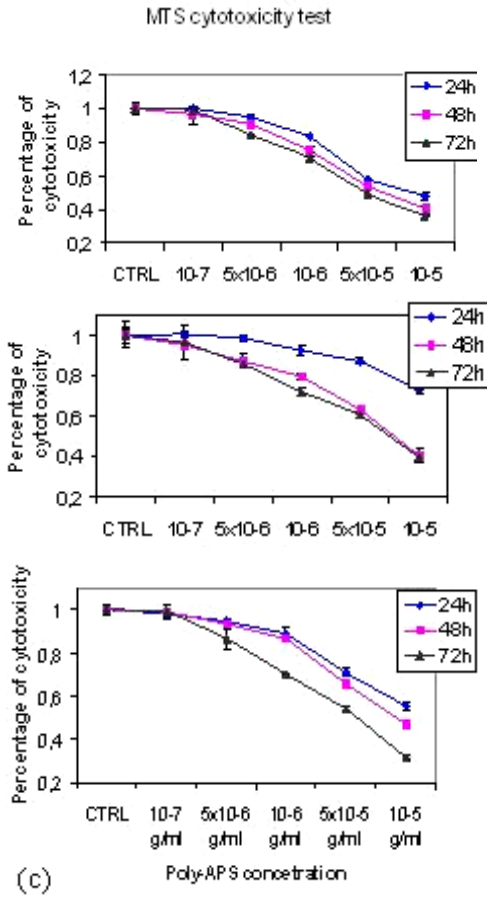



*Figure 7*: Cytotoxic effect of Poly-APS salts evaluated by MTS test. A: A549 cell line. B and C: primary NSCLC cell lines. Tests were carried out for 24, 48 and 72 hours at the indicate concentrations. Bars indicates data ± Standard Deviation.

*Membrane stability:* - Another feature of cells undergoing apoptosis is represented by the loss of membrane stability: the membrane phospholipids loose their symmetry, so that phosphatidyl serine is moved from the inner to the outer surface. This supplies a good biomarker for apoptosis, because annexin V binds with high affinity to phosphatidyl serine. Annexin V conjugated with fluorescein (FITC) is used to identify the destabilized cell membranes of the cells undergoing apoptosis. By flux cytometry, in A549 cells exposed to $5x10^{-5}$ g/ml poly-APS 63.13% apoptosis was measured, while spontaneous apoptosis was 14.13 % and the apoptosis provoked by exposure to vincristine, a powerful apoptosis inducing molecule, routinely used in the laboratory practice as a positive control of apoptosis (e.g. [55]) was 17.2%. The primary cultures also presented apoptosis enhancement on exposure to $5x10^{-5}$ g/ml poly-APS, from 20.49% spontaneous to 83.1% induced by the exposure (figures shown by [38].

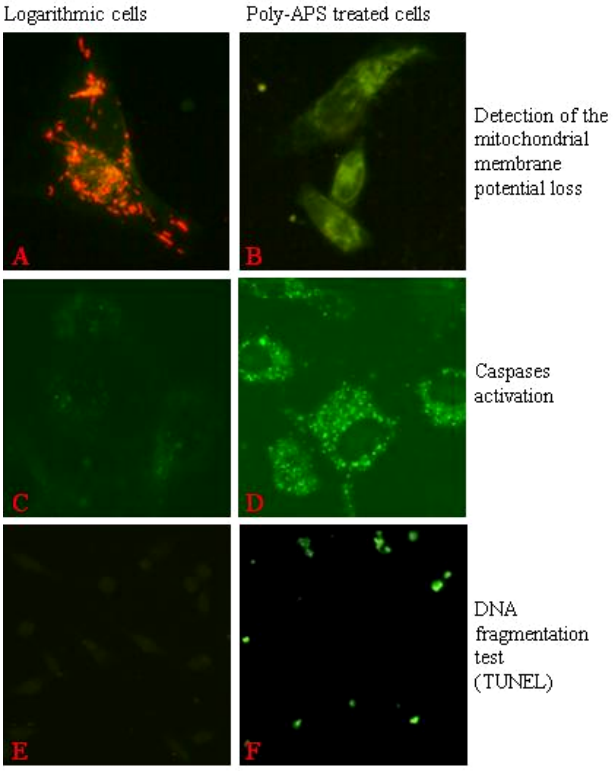



*Figure 8*: Analysis of the main steps of Poly-APS salts induced-apoptosis. A & B: detection of the mitochondrial membrane potential in logarithmic (A) and Poly-APS treated cells (B). C & D: analysis of the activation of caspases in logarithmic (C) and Poly-APS treated cells (D). E & F: DNA fragmentation test in logarithmic (A) and Poly-APS treated cells (B). After 24 hours of exposure to 10-5 g/ml of Poly-APS salts, cells show an activation of mitochondrial apoptotic pathway

**4.1.5.2- Intermediate phases**

*DNA fragmentation:* - During the intermediate phases of apoptosis, DNA fragmentation takes place. The formed nucleosomes were identified by different techniques, from DNA staining by DAPI or Hoechst, that are DNA-binding molecules able to become fluorescent in blue when bound to the A-T complexes, to more sophisticated techniques, such as the *In Situ* Cell Death Detection Kit, Fluorescin, (*Roche*). This latter uses the enzyme Terminal-transferase, able to bind fluorescein (FITC) to the DNA terminals. Thus the cells are labelled when DNA is fragmented. By these techniques, the exposure A549 cell line exposure to $10^{-5}$ e $10^{-6}$ g/ml poly-APS for 24 h revealed a dose dependent positive staining enhanced respect to the control cells (Fig. 9).

HEALTHY LYMPHOCYTES

*Apoptosis induction in lymphocytes:* - Lymphocytes from healthy donors were exposed for 24 h to the higher concentration of poly-APS found effective for inducing apoptosis in lung cancer cells ($5x10^{-5}$ g/ml). The annexin V essay was carried out as previously described, and the results of fuorescence were analysed

by flux cytometry. The cell population non exposed to poly-APS presented 93,42% viable cells, while the exposed one presented 89,14% viable cells [38]. As

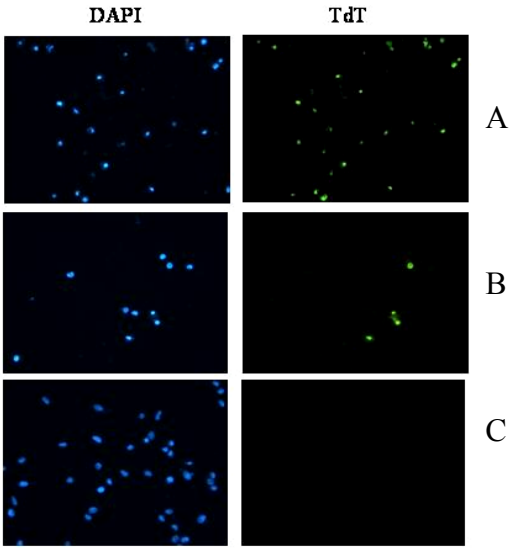



*Figure 9*: DNA fragmentation in A549 cells exposed to differnt concentrations of poly-APS for 24 h. The green staining is due To annexin V fluorescence. The nuclei are stained with DAPI. A: exposure to $10^{-5}$ g/L Poly-APS; B: exposure to $10^{-6}$ g/L; C: control

compared with the effects on cancer cells viability, these data show a significant difference, and show that healthy lymphocytes are scarcely affected by the exposure.

#### 4.1.6 Anti-cancer activity of poly-APS in tridimensional cell cultures (spheroids)

These experiments were carried out by [38] et al (2006) in order to test the effects of poly-APS on the metastasis process. This culture type is routinely used for testing classic chemotherapeutical agents, because it is characterized by a higher resistance to anti-cancer drugs as compared to the monolayer cultures [56]. At sub-lethal concentrations ($10^{-7}$ g/ml) poly-APS salts can inhibit cell aggregation by reducing CAMs. Exposure caused dramatic reduction of the N-acetylglucosamine residues identified by the histochemical binding of the wheat germ agglutinine (WGA) conjugated with TRITC and of α-D-mannose and α-D-glucose residues, identified by binding of the lectin concanavalin A (ConA) conjugated with FITC. CAMs exhibiting these glycan terminals are typically expressed by metastasizing tumors [57, 58, 38].

## 4.2- second phase: Toxicity directed to organs.

Poly-APS was injected in the caudal vein of C57 BL/6N male and female mice at the concentrations of 1 mg/Kg and 0.5 mg/Kg. The treated specimens (n=30, weight= 20±2 gr, 8 weeks aged, 6 specimens/group) were sacrificed at time intervals (0; 30 min; 1h; 2h; 24h) and immediately heart, liver and kidney were fixed in paraformaldehyde 3% PBS, dehydrated in alcohol and sectioned 5 μm thick. The organs did not present anomalies, nor inflammation signs (Fig. 10).

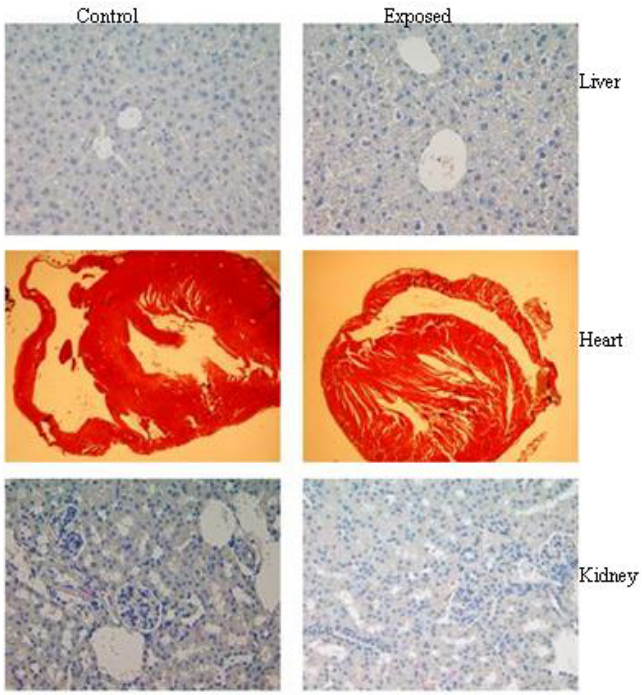



*Figure 10*: Histological aspects of the main organs involved in elaboration, disposal and detoxification of Poly-APS salts, following the caudal vein injection.

## 4.3 Third phase: in vivo Experiments

This phase was performed by Prof. Adriana Albini and her group of Researchers, in the Laboratory of Experimental Oncology in the National Institute for Cancer Research (IST). The reported results are derived from personal communication

In nude mice (60 males and females, 20±2 gr, 8 weeks old) $5x10^6$ cells of (A549) lung tumor were inoculated subcutaneously, forming 6 groups (10 specimens/ group). After 5 days, when the volume of the tumours was measurable, the different groups were injected with 0.5 mg/Kg; 0.25 mg/Kg; 0.125 mg/Kg; 0,05 mg/Kg poly-APS salts for 5 days/week, in the peri-tumor region, while 2 control groups were injected at the same way with sterile water. The experiment was repeated for 3 times.

After 25 days, all the tumors treated with poly-APS presented size significantly minor than the tumours of control specimens (Fig. 11). No one of the treated specimens presented either collateral disease or reduced weight.

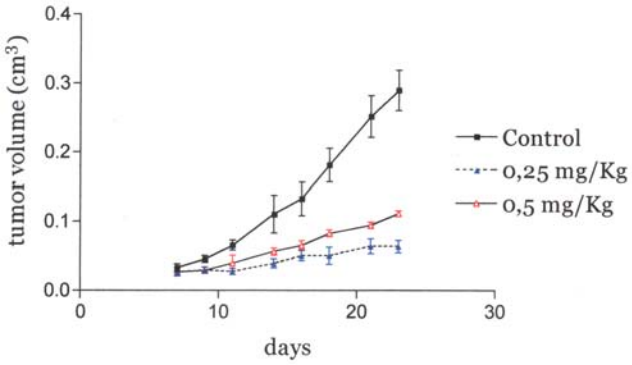



*Figure 11*: Tumour volume (Y axis) in control and exposed to different poly-APS concentrations in nude mice

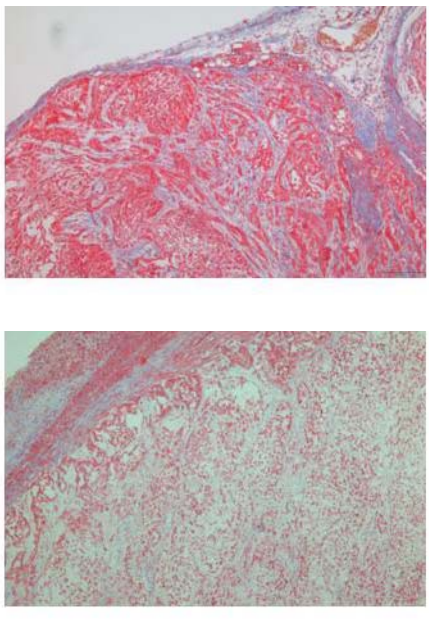
Histological analyses of tumor sections stained by the trichromic Masson medium showed a different organization of the neoplastic tissues between exposed and control mice. Samples non-exposed to poly-APS showed an encapsulated multilobated structure, with connective tissue (stained in blue color) homogeneously distributed inside the tumor mass. The tumor cells of epithelial origin were densely packed in the inner part of the tumor. On the contrary, in the samples injected with 0.25 mg/Kg poly-APS not infiltrated connective tissue was present inside the mass, and the number of epithelioid tumor cells was lower than in controls (Fig. 12).

# 5 Discussion

These listed reports suggest that poly-APS salts may represent very promising anti-cancer agents, for their biological activity against tumor, and almost complete safety of non-cancer cells, such as lymphocytes and organs such as liver, heart, kidney [38]. Moreover the ability to be used as a transfection agent amplifies the possibility to vector other anti-cancer drugs and facilitate their action in a selective way.

The main anti-cancer effects may be exerted through AChE activity inhibition, because such an inhibition would enhance the permanence of ACh at the receptorial sites, with two opposite effects: high amounts of ACh, not removed from the receptor, should block its responses to new inputs. This would prevent their effect of enhancing cell proliferation via opening ion channels, such as the $Na^+$ channels associated to nicotinic receptors, that in turn trigger the pathway involved in lung cancer progression, recently described by [59]. Actually. A strong awareness is taking place about the therapeutical use of AChE inhibitors in a number of diseases where ACh receptors may play a role in inflammatory as well carcinogenetic diseases [60].

On the other hand, the *in vivo* protective effects may also be exerted by enhancing ACh amount at receptorial sites, because ACh, besides its function of signal molecule, also functions as a local humoral factor translating environmental stimuli into alterations in T cell development and function [61,62].



(a)

(b)

*Figure 12*: Histological aspects of tumours isolated from unexposed (a) and exposed (b) nude mice to 0.23 mg/Kg poly-APS.

On the other hand, the reported biological activities of natural poly-APS, that might find application in medicine as transfecting or chemotherapeutic agents, and in environment protection as new non-toxic and environmentally friendly antifouling agents, was somewhat hindered by limited quantities from natural sources and by difficulties associated with undisclosed size and possible size heterogeneity of these molecules. Therefore, attempts were made to obtain synthetic analogues of more defined size, with the same or even improved biological activities as compared to the natural compounds. Furthermore, this approach enables us to obtain larger quantities of poly-APS analogues absolutely required for some experiments, like *in situ* antifouling tests. The first series of poly-APS analogues was successfully synthesized and a paper describing their organic synthesis and biological activities was published in 2004 [63]. The synthesis, activities and potential use of poly-APS was recently also described in a review paper by [64]. In the last couple of years further attempts to obtain new analogues and improve the organic synthesis were made mainly by the group of organic chemists in Aberdeen (Scotland) and Trento, Italy. This new approach was quite successful and promising results were obtained by initial testing of several new compounds. Further testing is currently in progress.

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