Multiple functions of the cholinesterase inhibiting poly-alkylpyridinium salts extracted from the marine sponge, *Haliclona sarai.*

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Abstract:

The interest on the Mediterranean sponge *Haliclona sarai* was raised some years ago by the fact that this sponge appears free from parasites and fouling organisms present in its environment. The study of such a feature was at the beginning due to the interest in finding new and efficient anti-fouling substances for applicative purposes. The characteristic was related to the expression of poly-alkylpyridinium salts (poly-APs), a mixture of two of 3-octylpyridinium polymers, including 29 and 99 monomeric units. The main effect of this compound was represented by the strong specific and non-toxic acetylcholinesterase inhibition *in vitro*. The substance was first tested for its effect on larval development and settling of incrusting organisms, such as *Amphibalanus amphitrite.* The experiments confirmed the ability of Poly-APs to prevent settlement of sessile organism, by impinging on the AChE activity. Acetylcholinesterase is an enzyme associated to the cholinergic signal system, but 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, and is present in some tumour cells and biopsies. Cytotoxicity tests on immortalized and primary cell lines derived from lung tumour (NSCLC) showed a poly-APS dose-dependent selective reduction of cell viability, statistically significant. The same cells, exposed to the poly-APS salts exhibited a loss in the mitochondrial potential, and positive response to apoptosis assays. What makes the poly-APS salts interesting as anticancer therapy adjuvant is that they, at the concentrations inducing apoptosis in tumour cells, seem to scarcely affect the viability of lymphocytes isolated from healthy patients. In this promising frame, the need emerges for the isolation of synthetic homologs of poly-APS molecules, in order to start a study for the therapeutical application of the drug.

Key words: Lung Cancer, Acetylcholinesterase; Apoptosis; Cell Proliferation; Poly-APS

1 Introduction

Nature has supplied several active anticancer agents (vinca alkaloids, anthracyclines, epipodo-phyllotoxin, and taxanes), which have significant-ly 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. 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 [4].

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 [5,6,7]. In particular, the ability to inhibit acetylcholine esterase activity has

been focused by several authors, for its possible regulatory effects on cancer progression.
Since the first '80s, some tumour 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 [8].
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 tumour, 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 alkyl-pyridinium 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 [6].
In this review, an excursus of the potentiality of this natural substance in several field of basic and applied research is shown.

2 Presence of AChE activity in non-neuromuscular cells and tissues

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 tumourigenesis**.** 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 [9]. 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 [15]. The induction of AChE expression was found by cytochemical

staining, immunological 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, found a relationship between AChE and intracellular Ca^{2+} concentration [15,16], 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 Acetylcholinesterase and inflammation

Inflammation is the first step that leads mutagenesis, cause of the stress potentials of cells. The stress potential is generally due to calcium entrance/release into the intracellular compartments, that changes the resting membrane potential from -70 mV up to 10-20 mV.
Such a stress is generally followed by an increase of heavy and very active forms of acetylcholinesterase [17] and by increase or appearance of acetylcholine (ACh) [8] in human tissues. This in turn again raises the presence or the amount of AChE, whose physiological function is to remove acetylcholine from its receptors. In this light, we can explain how AChE might be responsible for the regulation of apoptosis through the modulation of ACh cleavage. Actually, according to the amount of ACh present at the receptorial sites, ACh receptors may be more or less activated. This explains the presence of AChE activity in non cholinergic tumours, that was demonstrated since a number of years [18]. Actually, this enzyme according all the authors, might be responsible for the shift of inflamed tissues between carcinogenesis and apoptosis [19].

3 Poly-APS discovery and research.

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 Sepčič 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 [20].
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 [7]. 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 facilitated their purification by ultrafiltration and size-exclusion chromatography but complicated the calculation of inhibitory constants and other parameters due to the unknown size and molecular weight of the aggregates in solution [7].

Figure 1: The sponge, *Haliclona (Reniera) sarai* (Pulitzer –Finali, 1969) (photo by Tom Turk)

3.1- 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 [21,22]. In addition, the group of Tom Turk and Kristina Sepčić also studied hemolytic and antibacterial activity of poly-APS [23,24], as well as their cytotoxicity which turned out to be important in application of poly-APS as transfecting agents [25,26] due to their ability to form pores in the cell membrane.

3.2 Anti-fouling activity

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 the Authors 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 [6]. 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 [27,28].

Colleagues at the CNR-ISMAR demonstrated the non toxic and reversible anti-AChE activity of poly-APS, and its ability to prevent the metamorphosis and settlement of *Amphibalanus amphitrites* (balanus) (Fig. 3).

The nervous system of the larva of balanus is largely cholinergic, as it is demonstrated by the histochemical AChE reaction (Fig. 2)

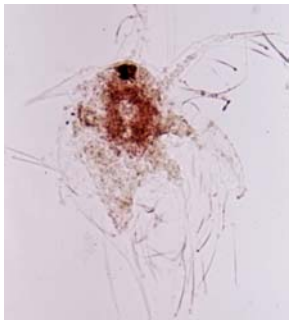
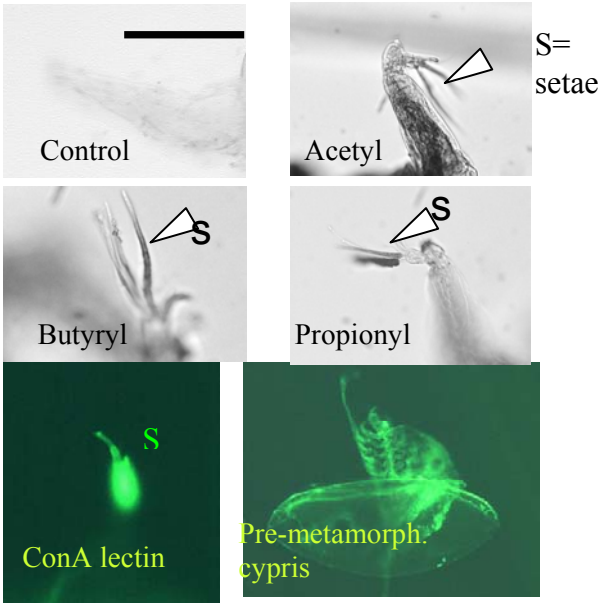


Figure 2. Amphibalanus larva: the brown staining is due to the precipitates of [49] histochemical reaction products, underlining the presence of AChE activity in the protocerebrum and suboesophageal ganglia.

In addition the pre-metamorphosis stage (cypris) of this organism presents cholinesterase (AChE, BChE and PChE)) activities in the setae performing the attachment to the substrate. On the same setae, adhesive molecules with affinity sites for concanavalin A (ConA) are present, shown by green fluorescence..

.3.4 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 [24].



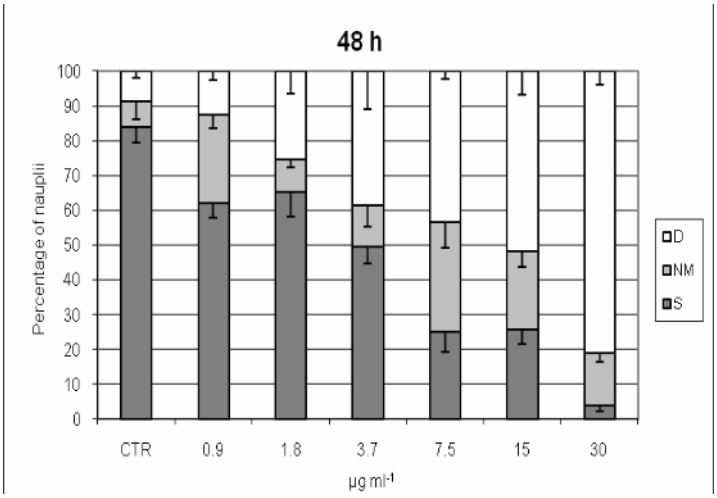


Figure 3. Exposure of 48 hours Amphibalanus nauplii to different doses of poly-APS (shown in the X axis). The Y axis shows the percentages of dead (white), non-metamorphosed (light grey) and metamorphosed (dark grey) larvae for each drug concentration. Original

data performed by the ISMAR group. The figures at the top show the presence of ChE activities co-localised with the conA affinity sites in the setae of pre-metamorphosing cypris. ChE activity sites are evidentiated by the dark reaction product [49]. The photos re from [50].

4 Anti-tumour activity

Lately, poly-APS were also tested as potential chemotherapeutic agents against transformed tumour cells that express molecules related to the 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*. [29]. As reported above, poly-APS exhibit strong anti-acetylcholinesterase, hemolytic, and moderate cytotoxic activity [21,22]. As to the ability of inhibiting (AChE) [22], 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].

4.1 Localisation of AChE in cultured cancer cells and in lung cancer

The first reports about the presence of AChE in cancer cells begun to be available in the 70's, related to tumours of nervous-tissue origin, such as neuroblastoma, or NTera2 teratocarcinoma cells, committed to neurogenesis [16]. Since 1980, the presence of AChE activity was reported in blood tumours, such as leukaemia [17, 18], 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 obtained
from a number of solid tumours, 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 [19], while in normal human fibroblasts, no AChE activity could be demonstrated by the histochemical technique [49], showing the active sites by brown-magenta staining.
The nuclear localisation of the enzyme activity has been recently correlated with tumour progression [21], as an homeostatic response to cell proliferation.

Nowaday, awareness is increasing about the involvement of AChE in the regulation of cancerogenesis [25], through its role in driving 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].

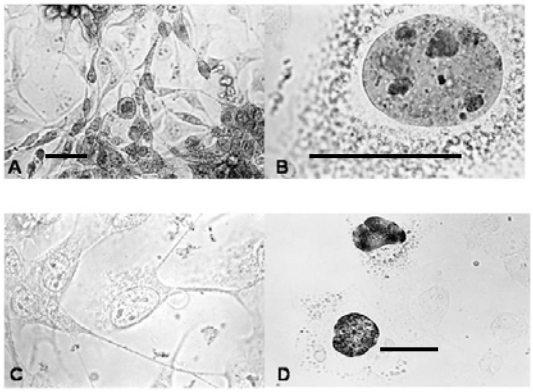


Figure 4: Acetylcholinesterase activity, revealed by the histochemical reaction of [53]. The reaction product is a dark precipitation in the sites of enzyme activity. A: HT1080 fibrosarcoma cells; B: RD2 rhabdomyo-sarcoma cell; C: control cells, pre-incubated with 10-5 M BW284c51 (specific AChE inhibitor); D: a panoramic view of the rhabdomyo-sarcoma huge cells. All the bars equal 20 μm. In the figure, a nuclear localisation of the enzyme is shown, that has been recently correlated with tumour progression [24].

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, extracted and purified from the sponge by the Slovenian researchers
In the new light, poly-APS were tested as potential therapeutic-coadjutant agents against transformed tumour cells that express cholinergic system on their cell membranes. As reported above, poly-APS exhibit strong anti-AChE activity [33] exerted in a competitive way, by irreversibly binding to the serine in the catalytic anionic site of the enzyme gorge [30], or in a non-competitive way, by binding at the peripheral anionic site at the rime of the gorge, thus slowing or preventing the entrance of acetylcholine into the catalytic site.
These first tests were based on a conceptual error. Actually, the fact that in tumours AChE activity was often increased lead to think that a forced decrease of such activity could somehow help the tissues to recover their integrity. On the contrary, in some tumour types, following activation of nicotinic and/or muscarinic receptors, by acetylcholine, the MAPKinase cascade is activated, driving cell proliferation [31, 32]. As a consequence, in the lung cancers following iperactivation of nicotinic receptors, cell death regulation is compromised, thus causing the enhancement of cell proliferation [33]. This can explain why tumour progression is enhanced by tobacco smoking [33]. Actually, the current therapeutical approach against cancer is to use drugs enhancing cell death and blocking cell proliferation.
On the other hand, some chemotherapeutic agents act through the inhibition of AChE activity; e.g. Irinotecan, a camptothecin derivative, is used in lung cancer treatment and therapy [34].
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 tumour cells.

4.2 Experiments on anti-cancer activity of poly-APS salts

Recently, lung cancer was found to express active AChE forms, and to possess a complete set of molecules related to the cholinergic signal system, including vesicular ACh transporter, choline-acetyl transferase, and ACh receptors [35] .
In vitro experiments were performed by [29] 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.

4.3 First phase: in vitro experiments.

4.3.1 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, used as controls in order to compare the effects on cell death of normal and cancer cells.

4.3.2 AChE expression in tumour 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 immunofluorescence, by use of a specific antibody against human AChE). The expression of the molecule was also confirmed by RT-PCR, specific primers of the neural isoform of AChE in lung tumour cell lines (human lung adenocarcinoma epithelial A549, squamous lung carcinoma SKMES, malignant human epidermoid lung carcinoma CALU-1 and primary lines. Surgical biopsies of NSCLC were analyzed by western blot, by the antibody anti AChE. All the tumour lines and homogenates showed the presence of high AChE amounts, statistically confirmed as significant.
In all these studies, the A549 cells were chosen as a preferential model, cause of their features of strong aggressivity and resistance to anti-neoplastic agents, including retinoids [29].

4.3.3 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 [36]. When it is released in the intracytoplasmic domain, IP3 generally causes calcium ions release from intracellular stores, and consequent increase of intracellular [Ca2+] concentration. The relative changes of ACh,

AChE and IP3 were shown in the paper (Fig. 5, [20]).

4.3.4 Poly-APs cytotoxicity on cancer cells and healthy lymphocytes [

Cytotoxicity tests were performed on both A549 and cell lines previously described, by two methods: the MTS method, and colony forming tests method on soft-agar [29]. Both the experimental procedures showed that poly-APS salts strongly inhibited cell proliferation with progressive dose- and time-dependent depletion, while in normal lymphocytes the depletion was sensitively minor, suggesting the possibility of a specific action of the polymer on tumour cells. The concentration of poly-APS was from 10^{-5} to 10^{-7} g/ml, and the test lasted up to 72 h.

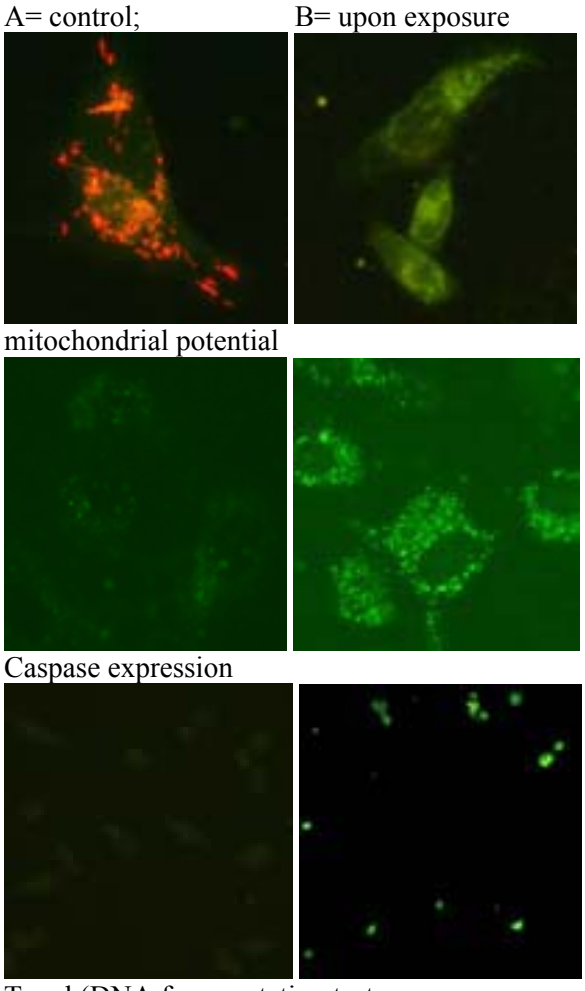


Figure 5- Effects of exposure to 10^{-5} poly-APS on apoptosis-related mechanisms [20].

4.3.5 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

4.3.5.1 Early phases of apoptosis induction

Membrane potential:
one of the early events is represented by calcium dynamics. Cells undergoing apoptosis showed membrane depolarisation, due 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 polarisation/depolarisation [37]. Mitochondrial depolarisation, is a general good marker of apoptosis early events [38,39]. 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. Fig. 5 shows the results in A549 cells exposed to poly-APS as previously described [20].
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 [40] 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 (These findings were shown by [29]).

4.3.5.2 Intermediate phases

DNA fragmentation in A49 cells
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). By these techniques, the 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 [29]
Apoptosis induction in healthy 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 showed that the cell population non exposed to poly-APS presented 93,42% viable cells, while the exposed one presented 89,14% viable cells [29]. As compared with the effects on cancer cells viability, these data show a significant difference, and show that healthy lymphocytes are at a lesser extent affected by the exposure.

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

Tridimensional cultures are routinely used for testing classic chemotherapeutical agents, because they are characterized by a higher resistance to anti-cancer drugs as compared to the monolayer cultures [41]. At sub-lethal concentrations (10^{-7} g/ml) poly-APS salts can inhibit cell aggregation by reducing CAMs. Exposure caused dramatic reduction of the histochemical binding of the wheat germ agglutinine (WGA), of α-D-mannose and α-D-glucose residues, identified by binding of the lectin concanavalin A (ConA) [29]. CAMs exhibiting these glycan terminals are typically expressed by metastasising tumours [42, 43].

4.4 Second phase: Toxicity directed to organs

The following results were obtained 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.

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 did not show damage to the main organs targeted by chemotherapeutic agents, nor presented inflammation signs.

5. Exposure effects on angiogenesis

It is well known that malignant tumours express angiogenesis factors, through which they enrich themselves of nutritive and respiratory elements through the increased mass of blood vessels. The last experiments on HUVEC cells show the anti-angiogenetic apoptosis in healthy cells.

To measure any enrichment of cytoplasmic histone-associated DNA fragments after poly-APS- induced cell death, a commercially available kit was used (Cell Death Detection ELISA, Roche, Mannheim, Germany) using 24-well plates seeded with 30,000 (HUVEC) cells per well and grown in complete medium with various concentrations of poly-APS.

5.1 Morphogenesis assay

The effects of poly-APS on the ability of endothelial cells to reorganize and differentiate into networks were assessed in the Matrigel morphogenesis assay as described previously [44] (ref). Matrigel (300 µl/well) thawed at 4°C was added with a cold pipette to a prechilled 24-microwell plate. After polymerisation of Matrigel at 37 °C, 7 × 104 cells/well were layered in endothelial cell growth medium without serum on top of the polymerized gel in the presence or absence of poly-APS at the indicated concentrations.

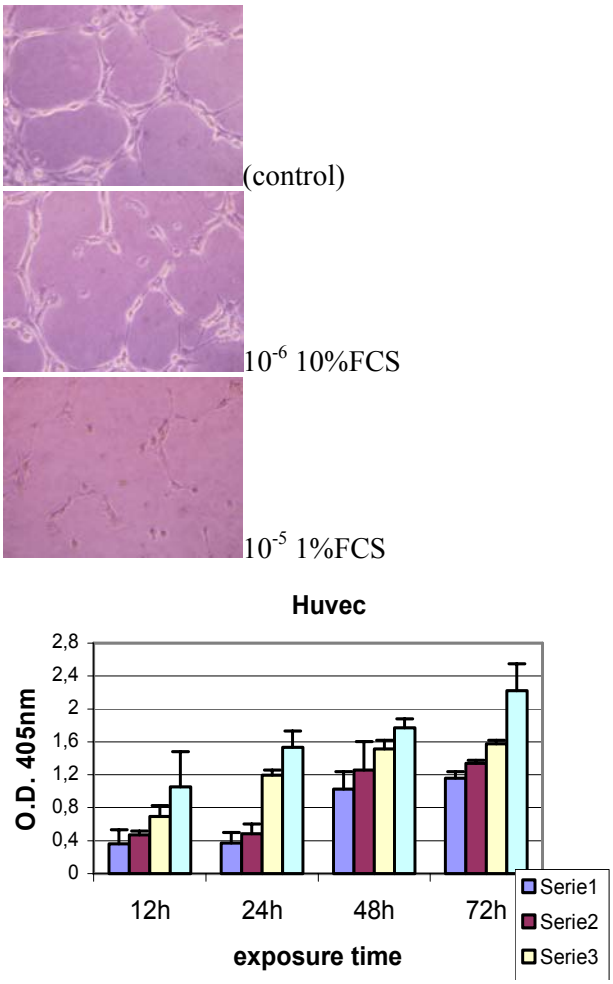


Figure 6 and 7. Exposure to poly-APS. The effects on morphogenesis were evident after a few hours of incubation at 37°C in humidified atmosphere. Wells were photographed at 7 h with a Leitz DR-IMB microscope with charge-coupled device (CCD) optics.

6 Discussion

These reports suggest that poly-APS salts may represent a promising anti-AChE, retaining pro-apoptotic biological activity. In addition, the ability to be used as a transfection agent amplifies the possibility to vector other anti-cancer drugs. The main effect may be exerted by enhancing the permanence of ACh at the receptorial sites. ACh, not removed from the receptor by the impaired AChE activity, should prevent its responses to new inputs. This would block or slower the receptors' effect, that in some tumours is to enhance cell proliferation and block apoptotic pathways via opening the ion channels associated to nicotinic receptors [45]. This should increase the apoptotic rate, as we have shown.

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 as carcinogenetic diseases [46]. Actually 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 [46].

The reported biological activities of natural poly-APS was somewhat hindered by limited quantities from natural sources. Therefore, attempts were made to obtain synthetic analogues with the same or even improved biological activities as compared to the natural compounds. The first series of poly-APS analogues was successfully synthesized and papers describing their organic synthesis and biological activities was published [47,48], 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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