Studies on a promising anticancer molecule of marine origin. Results of an interdisciplinary study.

A. ZOVKO[1], T. TURK[1], M. FAIMALI[2], F. GARAVENTA[2], E. CHELOSSI [2], C. FALUGI, MG. ALUIGI[3], C. ANGELINI[3], S. TROMBINO[3], L. GALLUS[3], S. FERRANDO[3], P. RUSSO[4], K. SEPCIC[1], L PALEARI[4]

1University of Ljubljana; 2National Council of Researches (ISMAR, Genova); 4National Institute for Cancer Research (IST, Genova) 3University of Genova, Department of Biology (DIBIO) Viale Benedetto XV, 5, I-16132 Genova, ITALY
falugi@unige.it; http://www.unige.it

Abstract: Poly-APS, a mixture of two of 3-octylpyridinium polymers, including 29 and 99 monomeric units, extracted from the Mediterranean sponge, *Haliclona (Reniera) sarai*, was demonstrated to exert strong specific and non-toxic acetylcholinesterase inhibition in vitro. Since the first '80s, we found that some tumour types, and in particular lung tumours present overexpression of acetylcholinesterase 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, showed positive response to the annexin V assay, and to the T-terminal assay, that are specific markers of the apoptotic event. What makes the poly-APS salts promising as anticancer therapy adjuvant is that they, at the concentrations inducing apoptosis in tumour cells, do not affect the viability of lymphocytes isolated from healthy patients. Moreover, three-dimensional cell cultures (spheroids) of tumour cells, on exposure to poly-APS show a decrease in the membrane-linked oligosaccharides, that are responsible for the adhesivity of the metastatic cells. Moreover, no effects were demonstrated on healthy organs, such as heart, liver, kidney of mice treated by poly-APS, and *in vivo* tumours showed a mass and cellular density significantly reduced. 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

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 [1] and by increase or appearance of acetylcholine (ACh) [2] 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. Recently, AChE was found responsible for the regulation of apoptosis [3] 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.
This explains the presence of AChE activity in non cholinergic tumours, that was demonstrated since a number of years [4]. Actually, this enzyme according all the authors, might be responsible for the shift of inflamed tissues between carcinogenesis and apoptosis [5].
AChE is an enzyme associated to the cholinergic signal system, whose classic role is to remove acetylcholine (ACh) from the receptors at cholinergic synapses. synapses of the cholinergic neurotransmission. In addition, the enzyme is involved both in cell-to-cell communication driving embryonic development, and adult cell functions driven by mechanisms possibly related to the intracellular dynamics evoked by ACh signalling [6]. Moreover, cell migration is also related to AChE that may play the role of a cell-substrate adhesion molecule, through the affinity for laminin. AChE is affected by a number of

natural and synthetic inhibitors, including environmental contaminants.
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 [7].
On the whole, AChE localized in non-neuromuscular tissues of adults should be considered a good response against tumours through the rise of apoptosis and preventing cells migration that in adult organisms is a good feature I for the prevention of metastases spreading.
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, cholineacetyltransferase, and ACh receptors [8] .

2- Non-Neuromuscular localisation of molecules related to the cholinergic neurotransmission system

Cholinergic 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 [10,11]; 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 and are often co-distributed with special extracellular matrix molecules such as fibronectin [9]; and laminin [14]. 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 biological systems undergoing apoptosis [15]. The AChE protein was found 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 [15].

1.2 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 suggested by Karnovsky and Roots, showing the active sites by brown-magenta staining. [20].
The nuclear localization of the enzyme activity has been recently correlated with tumour progression [21],as an homeostatic response to cell proliferation..
Nowaday, a larger awareness takes place about the involvement of AChE in the regulation of carcinogenesis [22], through its role in the control of apoptosis, cell movement, and in inflammatory processes. These mechanisms are related to the AChE role in the hydrolysis of acetylcholine at its nicotinic receptors (AChRs), that are well expressed in lung tumour tissues. AChRs activation causes a loss in apoptosis, thus addressing suffering and inflamed cells to proliferation [23].
This was the state of our studies, when we met the poly-APS molecules.

3. Poly-APS, an anti-AChE natural drug as marine organism defence:

A natural anti-AChE molecule, belonging to the class of polymeric alkylpyridinium salts (poly-APS), produced by the Mediterranean sponge, *Haliclona (Reniera) sarai*, was discovered and characterized in recent years. In this organism, it prevents 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 when used at high concentrations. Colleagues at the CNR-ISMARdemonstrated the non toxic and reversible anti-AChE activity of poly-APS.

Poly-APS molecules were discovered about 15 years ago during a student's project of routine screening of extracts obtained from the Mediterranean marine sponges. The Slovenian group of researchers lead by Tom Turk and Kristina Sepcic showed a strong, apparently irreversible inhibition of AChE enzyme activity.
The Slovenian Group purified the active molecules which turned out to be polymers composed of head-to-tail linked 3-octylpyridinium units. MALDI-TOF spectroscopy revealed that the most active compound is a molecule with MW of 5500 Da. (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.

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 [24, 25]. In addition, the group of Tom Turk and Kristina Sepčić also studied hemolytic activity of poly-APS and their cytotoxicity [26, 27], which turned out to be important in application of poly-APS as transfecting agents [28, 29] due to their ability to pass through the cell membrane.

4 Anti-tumour activity

Lately, poly-APS were also tested as potential chemotherapeutic 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, 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 [35, 36]; MAPK are important signal molecules, leading to cell growth and proliferation (Davis et al., 1993). Thus, in the lung cancers following iperactivation of nicotinic receptors, cell death regulation is compromised, thus causing the enhancement of cell proliferation [37]. This can explain why tumour progression is enhanced by tobacco smoking [38]. 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 [39].
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.

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

In vitro experiments were performed by [33] 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.

5.1 First phase: *in vitro* experiments.

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

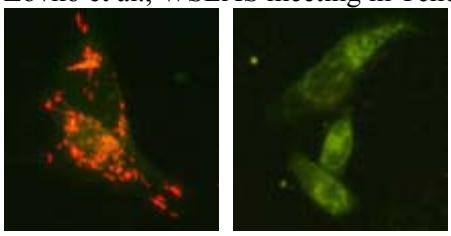
5.1.2 AChE expression in tumour tissues

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

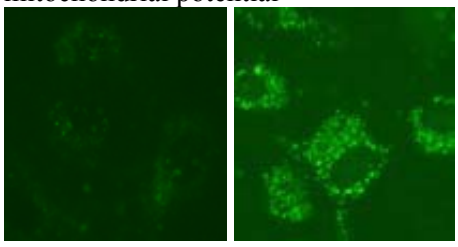
strong aggressivity and resistance to anti-neoplastic agents, including retinoids.

5.1.3 Activation of second messengers.

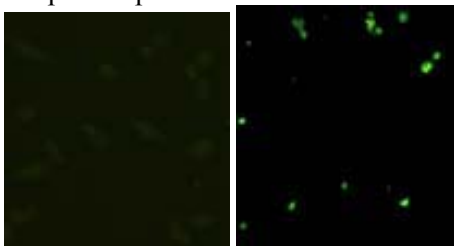
Poly-APS exposure of A459 cells also caused increase in Inositol-trisphosphate (IP3) formation. IP3 is a critical second messenger that, in the frame of acetylcholine receptors, is formed by the activation of a number of G-protein coupled receptors, including the m1-type muscarinic ACh receptors [40]. When it is released in the intracytoplasmic domain, IP3 generally causes calcium ions release from intracellular stores, and consequent increase of intracellular [Ca2+] concentration. (Fig. 6, from Zovko et al., WSEAS meeting in Tenerife, 2009).



A= control; B= upon exposure mitochondrial potential



Caspase expression



Tunel (DNA fragmentation test

The relative changes of ACh, AChE and IP3 were shown in the paper presented by Zovko et al at the WSEAS meeting in Tenerife, 2009.

5.1.4 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. Both the experimental procedures showed that poly-APS salts strongly inhibited cell proliferation with progressive dose- and timedependent 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 the polymer was from 10-5 to 10-7 g/ml, and the test lasted up to 72 h.

5.1.5 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

5.1.5.1 Early phases

Membrane potential:

one of the early events is represented by calcium dynamics. Cells undergoing apoptosis showed high membrane depolarization, due to high intracellular [Ca2+]. Intracellular calcium dynamics are driven by IP3 release, and in turn IP3 receptors in mitochondria drive the events of mitochondrial polarization/ depolarization [41]. Mitochondrial depolarization, is a general good marker of apoptosis early events [42,43]. 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.6 shows the results in A549 cells exposed to poly-APS as previously described.

Figure 6 (see above): After 24 hours of exposure to 10-5 g/ml of Poly-APS salts, cells show an activation of mitochondrial apoptotic pathway

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. [44]) 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 [33]).

5.1.5.2 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

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 [33]. As 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.

5.1.6 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 [45]. 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). CAMs exhibiting these glycan terminals are typically expressed by metastasizing tumours [46, 47, 33].

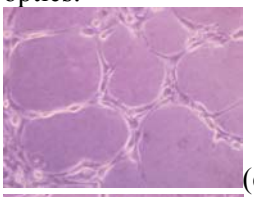
Last unpublished experiments show the exposure effect on angiogenesis

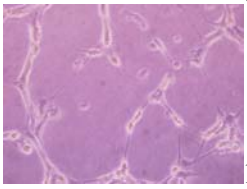
It is well known that malignant tumours express angiogenesis factors, through which they enrich themselves of nutritive and respiratory elements through the increased mass of blood vessels. The last experiments on HUVEC cells show the anti-angiogenetic apoptosis in healthy cells.

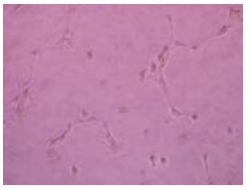
To measure any enrichment of cytoplasmic histone-associated DNA fragments after poly-APS- induced cell death, a commercially available kit was used (Cell Death Detection ELISA, Roche, Mannheim, Germany) using 24-well plates seeded with 30,000 (HUVEC) cells per well and grown in complete medium with various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

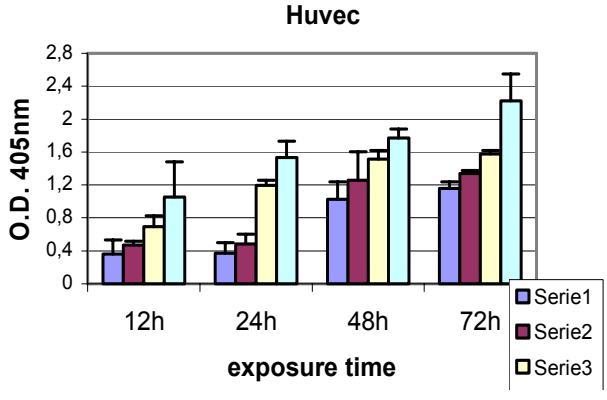
(ref) **Morphogenesis assay**

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 (ref). Matrigel (300 µl/well) thawed at 4°C was added with a cold pipette to a prechilled 24-microwell plate. After polymerization 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. 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.


(control)


10^{-6} 10%FCS


10-5 1%FCS



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. 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 a papers describing their organic synthesis and biological activities was published [51,52], 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 severalnew compounds. Further testing is currently in progress.

7 References

[1]. De La Porte S, Vigny M, Massoulié J, Koenig J. Action of veratridine on acetyl-cholinesterase in cultures of rat muscle cells. Dev Biol. 1984;106(2):450-6.

[2]. Wessler I, Kirkpatrick CJ. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Br J Pharmacol. 2008 154(8):1558-71.

[3]. Ye W, Gong X, Xie J, Wu J, Zhang X, Ouyang Q, Zhao X, Shi Y, Zhang X.AChE deficiency or inhibition decreases apoptosis and p53 expression and protects renal function after ischemia/reperfusion. Apoptosis. 2010 Jan 7.

[4]. Falugi C, Castellani P, Raffanti S, Borsi L, Zardi L. Acetylcholinesterase in normal and malignant human cells. Basic and Applied Histochemistry, Vol.30, No 4, 1986, pp 433-437.

[5] Ofek K, Krabbe KS, Evron T, Debecco M, Nielsen AR, Brunnsgaad H, Yirmiya R, Soreq H, Pedersen BK. Cholinergic status modulations in human volunteers under acute inflammation. Journal of Molecular Medicine. 2007;85(11):1239-51

[6]. Schwartsmann G, Rocha AB, Berlinck R, Jimeno J., Marine organisms as a source of new anticancer drugs.Lancet Oncology Vol.2, 2001, pp 221-230.

[7] Aluigi MG, Angelini C, Falugi C, Fossa R, et al. S.Interaction between organophosphate compounds and cholinergic functions during development. Chemico Biological Interaction. Vol.15, No 157-58, 2005, pp 305-316.

[8]. Trombino S, Cesario A, Margaritora S, Granone P, Motta G, Falugi C, Russo P.Alpha7-nicotinic acetylcholine receptors affect growth regulation of human mesothelioma cells: role of mitogen-activated protein kinase pathway. Cancer Res. 2004 Jan 1;64(1):135-45.

[9] Meshorer E, Soreq H Virtues and woes of AChE alternative splicing in stress-related neuropathologies. Trends in Neuroscience, Vol.29,No 4, 2006, pp 216-24

[10] Falugi C. Localisation and possible role of molecules associated with the cholinergic system during "nonnervous" developmental events. European Journal of Histochemistry, Vol.37,No 4, 1993, pp 287-94

[11] Angelini C, Baccetti B, Piomboni P, et al.
Acetylcholine synthesis and possible functions duringsea urchin development. European Journal of Histochemistry, Vol.48, No 3, 2004, pp 235-243

[14] Johnson G, Moore SW, Human acetylcholine
esterase binds to mouse laminin-1 and human collagen
IV by an electrostatic mechanism at the peripheral
anionic site. Neuroscience Letters, Vol.337, No 1,
2003, pp 37-44.

[15] Zhang XJ, Yang La, Zhao Q, Caen JP et al. Inductionof acetylcholinesterase expression during apoptosis in various cell types. Cell Death and Diferentiation, Vol.9, No8, 2002, pp 790-800

[16] Gearhart JD, Mintz B Contact-mediated myogenesis and increased acetylcholinesterase activity in primary cultures of mouse teratocarcinoma cells. Proceedings of the National Academy of Science, USA, Vol.71, No 5, 1974, pp 1734-1738.

[17] Conscience JF, Meier W. (1980) Coordinateexpression of erythroid marker enzymes during dimethylsulfoxide-induced differ-entiation of Friend erythroleukemia cells. Experimental Cell Research, Vol.125, No 1, 1980, pp 111-119.

[18] Massoulié J, Pezzementi L, Bon S, Krejci E, Vallette FM.Molecular and cellular biology of cholinesterases. Progress Neurobiology, Vol.41, No 1, 1993, pp 31-91.

[20] Zovko et al., WSEAS meeting in Tenerife, 2009

[21] Santos SC, Vala I, Miguel C, Barata JT, et al.. Expression and subcellular localisation of a novel nuclear acetylcholinesterase protein. Journal of Biological Chemistry, Vol.282, No 35, 2007, pp 25597- 25603

[21] Perry C, Soreq H ACHE gene expression in human brain tumours involves alternative splicing and CREBinduced proliferative signals. Israeli Medical Association Journal, Vol.8, No 5, 2006 , pp 364-365.

[22] Pettersson A, Nilsson L, Nylund G, et al.. Is acetylcholine an autocrine/paracrine growth factor via the nicotinic alpha7-receptor subtype in the human colon cancer cell line HT-29)? European Journal of Pharmacology, Vol.609, No 1-3, 2009, pp 27-33.

[23] Casida JE, Quistad GB. 2004. Organophosphate toxicology: safety aspects of non-acetyl-cholinesterase secondary targets. Chemical Research and Toxicology, Vol.17: 2004, pp 983-998.

[24] Sepčić K, Guella G, Mancini I, Pietra F, Serra MD, Menestrina G, Tubbs K, Maček P, Turk T., Characterization of anticholinesterase-active 3-alkylpyridinium polymers from the marine sponge *Reniera sarai* in aqueous solutions. Journal of Natural Products, Vol.60, No 10, 1997b, pp 991-996.

[25] Sepčić K, Marcel V, Klaebe A, Turk T, Šuput D, Fournier D (1998): Inhibition of acetylcholine-sterase by an alkylpyridinium polymer from the marine sponge, *Reniera sarai*. Biochimical and Biophysical Acta, Vol.1387, 1998, pp 217-225.

[26 Sepčić K, Poklar N, Vesnaver G, Fournier D, Turk T, Maček P (1999): Interaction of 3-alkylpyridinium polymers from the sea sponge *Reniera sarai* with insect acetylcholinesterase. Journal of Protein Chemistry, Vol.18, No 3, 1999, pp 251-257.

[27] Malovrh P, Sepčić K, Turk T, Maček P., Characterization of haemolytic activity of 3-alkylpyridinium polymers from the marine sponge *Reniera sarai*. Comparative Biochemistry and Physiology, C: Pharmacol Toxicol Endocrinol., Vol.124, No 2, 1999, pp 221-226.

[28] Scott RH, Tucker SJ, Evans RM, MacEwan DJ, Jaspars M, Sepčić K, Cell membrane permeability by alkylpyridinium sponge toxin preparations and their utility as novel transfection reagents. In Neurotoxicological Targets from Functional Genomics & Proteomics, (SCI), London, 2004..

[29] McLaggan D, Adjimatera N, Sepčić K, et al.. Pore forming polyalkylpyridinium salts from marine sponges versus synthetic lipofection systems: distinct tools for intracellular delivery of cDNA and siRNA. BMC Biotechnology, Internet Publication, 2006, 6, 6.

[33] Paleari L, Trombino S, Falugi C, Gallus L, et al. Marine sponge-derived polymeric alkylpyridinium salts as a novel tumour chemotherapeutic targeting the cholinergic system in lung tumours. International Journal of Oncology, Vol.29, No 6, 2006, pp 1381-1388.

[34] Drews U Cholinesterase in embryonic development. Progress in Histochemistry and Cytochemistry, Vol.7, No 3, 1975, pp 1-52.

[35] Ukegawa JI, Takeuchi Y, Kusayanagi S, Mitamura K, Growth-promoting effect of muscarinic acetylcholine receptors in colon cancer cells. Journal of Cancer Research. Clinical Oncology, Vol.129, No 5, 2003, pp . 272-278.

[37] Cooke JP, Bitterman H. Nicotine and angiogenesis: a new paradigm for tobacco related diseases. Annals of Medicine, Vol.36, No 1, 2004, pp 33-40.

[38] Minna JD. Nicotine exposure and bronchial epithelial cell nicotinic acetylcholine receptor expression in the pathogenesis of lung cancer. Journal of Clinical Investigation, Vol.111, No 1, 2003, pp 81- 90.

[39] Dodds HM, Hanrhan J, Rivory LR. The inhibition of acetylcholinesterase by irinotecan and related camptothecins: key structural properties and experimental variables. Anticancer Drugs Research, Vol.16, No 4-5, 2001, pp 239-246.

[40] Xia HJ, Yang G. Inositol 1,4,5-trisphosphate 3-kinases: functions and regulations. Cell Research, Vol.15, No 2, 2005, 83-91.

[41] Hajnoczky G, Csordas G, Krishnamurthy R, Szalai G. Mitochondrial calcium signalling driven by the IP3 receptor. Journal of Bioenergetics and Biomembranes, Vol.32, No 1, 2000, pp 15-25.

[42] Duchen, M.R. Mitochondria and calcium: from cell signalling to cell death. Journal of Physiology, Vol.1, 2000, pp 57-68.

[43] Mattson MMP, Chan SL. Calcium orchestrates apoptosis. Nature Cell Biology, Vol.12, 2003, pp 10041-1043.

[44] Zhu BK, Wang P, Zhang XD, Jiang CC, et al. Activation of Jun N-terminal kinase is a mediator of vincristine-induced apoptosis of melanoma cells.Anticancer Drugs, Vol.19, No 2, 2008, pp 189-200.

[45] Mueller-Klieser W. Tumour biology and experimental therapeutics. Critical Reviews in oncology/Hematology, Vol 36, No 2-3, 2000, pp 123-139

[46] De Albuquerque Garcia Redondo P, Nakamura CV et al. Differential Expression of Sialic Acid and Nacetylgalactosamine residues on the cell surface of intestinal epithelial cells according to normal or metastatic potential. The journal of Histochemistry and Cytochemistry, Vol.52, No 5, 2004, pp 629-640.metastatic potential.

[47] Powlesland AS, Hitchen PG, Parry S, Graham SA et al. Targeted glycoproteomic identification of cancer cell glycosylation. Glycobiology, Vol.19, No 8, 2009, pp 899-909.

[48] Thunnissen FB. Acetylcholine receptor pathway and lung cancer. Journal of Thoracic Oncology, Vol.4, No 8, 2009, pp 943-946.

[50] Chernyavsky AI, Arredondo J, Galitovskiy V, et al. Structure and function of the nicotinic arm of acetylcholine regulatory axis in human leukemic T cells. International Journal of Immunopathology and Pharmacology, Vol.22, No 2, 2009, pp 461-472.

[51] Mancini I, Sicurelli A, Guella G, Turk T**,** Maček P**,**Sepčić K (2004): Synthesis and bioactivity of linearolygomers related to polymeric alkylpyridinium metabolites from the Mediterranean sponge *Reniera sarai*. Organic & Biomolecular Chemistry, Vol.2, No 9, 2004, pp 1368-1375.

[52] Turk T, Sepčić K, Mancini I, Guella G (2008): 3-Aklypyridinium and 3-alkylpyridine compounds from marine sponges, their synthesis, biological activities, and potential use. In: Studies In Natural Products Chemistry, 35, Elsevier, 960 p.

.

[53] Karnovsky MJ, Roots L. 1964 A "direct-coloring" thiocholine method for cholinesterases The journal of Histochemistry and Cytochemistry, Vol.12, No 3, 1964, pp 219-221.

[54] Fassina, G., Vené, R., Morini, M., Minghelli, S., Benelli, R., Noonan, D. M., and Albini, A. (2004) Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by Epigallocatechin-3-gallate. *Clin. Cancer Res.* **10,** 4865–4873