ROLE OF CHITOSAN OLIGOMERS IN REGULATION OF EHRLICH ASCITES TUMOR CELLS PROLIFERATION *IN VITRO*

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Abstract
Preliminary studies of proliferation of Ehrlich ascites tumor (EAT) cells and normal mammary gland epithelial cells have demonstrated the process to be inhibited by degradation products of microcrystalline chitosan, i.e. oligomers. Inhibition of proliferation has been also accompanied by a decreased activity of the M2 pyruvate kinase (PK) isoenzyme in nucleoplasm, what may indicate the role of this enzyme in regulation of tumor cell proliferation. Determinations of nitrogen oxide in tumor and normal cells point to a higher level of this endogenous effector in normal cells. An increase of nitrogen oxide levels in Ehrlich ascites tumor cells effected by chitosan oligomers may indicate increased nitrosylation, and particularly an increased amount of compounds containing sulfhydryl groups and their participation in regulation of nucleoplasm M2 PK isoenzyme activity. Chitosan oligomers have smaller molecules as compared to microcrystalline chitosan and for this reason appear to be more effective than the latter in acting upon the negatively charged cell membrane surfaces, thus contributing to proliferation inhibition.

Key words: *chitosan oligomers, EAT cells proliferation, pyruvate kinase M2 isoenzyme, L-S-nitrosocysteine.*

1. Introduction

Cells with increased proliferation, including tumor cells, switch over from oxygen metabolism to glycolytic metabolism. This happens due to the necessity of maintaining a high reduction potential in these cells that divide rapidly and intensely synthesize DNA, RNA and proteins [1]. Tumor cells are characterized by an increased glycolytic activity that is evoked - among other factors - by increased expression of genes encoding glycolytic enzymes, including the M2 pyruvate kinase isoenzyme [2]. Pyruvate kinase (EC 2.7.1.40) (PK) is a regulatory glycolytic enzyme that is directly associated with ATP synthesis. PK activity has been demonstrated both in the cytoplasm and nucleoplasm of tumor cells [3, 4]. Tumor cells contain the M2 PK isoenzyme, the activity of which is regulated in a different manner as compared to that of the M1 isoenzyme originating from mouse mammary epithelium [5].

In the presence of L-cysteine, the M2 PK isoenzyme demonstrates histone kinase activity, catalyzes the reaction of transferring phosphate residue from 2-phosphoenolopyruvate (2-PEP) to H1 histone [5]. Phosphorylated H1 histone is not capable of inhibiting cell proliferation.

Chitosan, a polymer of β-D-2-deoxy-2-aminoglucose - a product of partial hydrolysis and deacetylation of chitin [6]. Chitosan demonstrates numerous unique properties, such as low toxicity [7], immunostimulatory properties [8], biodegradability [9], as well as anti-tumor activity [10, 11]. In Ehrlich ascites tumor (EAT) cells [12] and bladder cancer cells (T[24]) [13], microcrystalline chitosan inhibited glycolytic activity; at the same time, decreased cell elasticity was observed [13, 14].

Tumor cells demonstrate a lower level of nitrogen oxide synthesis as compared to normal cells. An increased synthesis of NO in EAT cells as the effect of microcrystalline chitosan points to a possible nitrosylation of L-cysteine [15] and in consequence, lack of phosphorylation of H1 histone by the M2 PK isoenzyme [5].

To study the effect of degradation products of microcrystalline chitosan of various degrees of deacetylation oligomers) on formation of L-S-nitrosocysteine in EAT cells and in normal mouse mammary epithelial cells (CRL 1636).

2. Material and methods

2.1. Cell cultures

The studies were carried out on normal mouse mammary epithelial cell line CRL 1636 and Ehrlich ascites tumor (EAT) cells (American Type Culture Collection). Normal cells were cultured in the DME medium (Sigma Chemical Co.) (pH 7.4) supplemented with 10% fetal calf serum (FCS - Gibco), 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml insulin. EAT cells were suspended in the NCTC-135 (Sigma Chemical Co.) enriched with 10% fetal calf serum (FCS – Gibco), 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. The cell cultures were maintained at 37 °C in a humidified atmosphere - the normal cells in 10% CO2 and neoplastic cells in 5% CO2.

2.1.1. Reagents

A – oligomer (deacetylation degree – DD: 97.7%) (no cytotoxicity after 72 h) and B – oligomer (DD: 70.3%) (no cytotoxicity after 72 h) was provided by the Institute Biopolymers and Chemical Fibres in Łódź. The degree of deacetylation oligomers was measured by the potentiometric titration [16]. C – L-S-nitrosocysteine (L-CSNO) was obtained in the reaction of L-cysteine with sodium nitrate (II) (Sigma-Aldrich) [17, 18] D – L-cysteine (L-Cys) (Sigma-Aldrich).

2.2. Incubation procedure

The stock solution of reagent (A and B 0.5%, C and D 10 mM) was prepared in the medium in accordance with the type of cells – for CRL 1636 normal cells, in the DME medium, while EAT cells were suspended in the NCTC-135 before each experiment and stored at 37 °C. The medium containing (final concentration - A and B 0.05%, C and D 1 mM) reagent was replaced every 24 hours throughout the experiments. The cells were incubated for 72 hours.

2.2.1. Detection of cytotoxicity

The cells were seeded in triplicates into 96-microwell plates at the density of 1 - 8 × 10^3 cells per well and incubated without or with different factors for 24, 48 or 72 hours. Afterwards, the cells were mixed with the reaction mixture from the Cytotoxicity Detection Kit (LDH)(Roche applied science, Germany). The reaction was stopped with 1 M HCl. The colorimetric assay for the quantification of cell death was based on the measurement of lactate dehydrogenase activity released from the damaged cells into the supernatant. The absorbance of the coloured product - formazane - was measured at 490 nm by an ELISA reader.

2.2.2. The L-S-nitrosocysteine level in EAT cells and in normal CRL-1636 cells was determined by RP-HPLC (reversed-phase high performance liquid chromatography). The LC system (Shimadzu Corporation Kyoto Japan) consisted of two solvent delivery module LC 10 AT vp and DGU-14 A degasser, a CTO-10 ASvp column oven, a SIL-10 ADVp autosampler, a SPD-M 10 Avp Diodearray detector. The CLASS-VP 7.2.1. was used for data collection and processing. The samples were separated at 20 °C on a Phenomenex Luna C18 (4 × 2.0 nm i.d.) guard column (4 × 3 mm i.d.).

The standards L-S-nitrosocysteine (1 mM and 2 mM) as well as supernatants of tumor and normal cells were used in derivatization. Derivatization consists in preparation of nitrophenyl derivatives, including N-DNP-CSNO (*N-DNP-L-S-nitrosocysteine*). Samples to be derivatized contained: 100 µl of supernatant, 60 µl of 10% PCA/1 mM BPDS, 96 µl of 2 M KOH-2,4 M KHCO3 and 200 µl of 1% DNFB. Following 24-hour derivatization at ambient temperature in dark, the samples were acidified by addition of 35 µl 70% PCA and centrifuged at 5600 g for 2 min. The resulting supernatants were filtered through a PTFE 0.2 µm Supelco filter and a 70-minute procedure of separation of the analyzed compounds was carried out on HPLC. The chromatographs were analyzed and the separated compounds were identified according to their retention times compared to sample solutions.

Samples were eluted like method [19] with simple modification, a mobile phase consisting of solvent A (water/0.1% trifluoroacetic acid – TFA) and solvent B (acetonitrile/ 0.1% TFA). After injection the samples were eluted with 45% B followed with a 35 min linear gradient to 55% B, then a 5 min linear gradient to 100% B and a 5 min isocratic period. The column was then re-equilibrated to the initial conditions for 15 min. All HPLC solvents were HPLC gradient grade. Analyses of 20 µl of samples were performed at a flow rate of 1.0 ml/min with UV-VIS detection at 365 nm.

2.2.3. Mass spectrometry conditions

N-DNP-CSNO was prepared by known procedure [19]. The examined compound were collected from HPLC and identified by ESI/MS, molecular ion of m/z = 331.3 (positive ionization M^+) corresponding to N-DNP-CSNO. Mass spectrometry was done using Esquire 3000 ESI-MS (Bruker-Daltonics, Bremen Germany) in positive ion mode. Flow rate was set to 3 µl/min using KD 100 Syringe pump (KD scientific, Holliston USA). Basic parameters of ion source were us fallows; heated capillary temperature; 280 °C, capillary voltage 4.7 KV. Resulting were analyzed using Brukers Data Analysis software (ver 3.0).

2.2.4. Isolation of nuclei from normal and tumor cells

The Ehrlich ascites tumor cells and reference cells were homogenized in a glass Potter-Elvehjm homogenizer with A buffer (containing 0.25 M saccharose, 3 mM $CaCl_2$, 10 mM Tris) (pH = 7.4), and subsequently centrifuged for 10 min. at 4 °C and 1000 g. (nuclear sediments were washed twice in A buffer). The nuclear sediments from the investigated cells (at the ratio of 1 : 9) were then suspended in B buffer (containing 2.2 M saccharose, 3 mM $CaCl_2$, 10 mM Tris) (pH = 7.4) and centrifuged for 60 min. at 4 °C and 40 000 g. The pellets obtained were washed twice in buffer A and centrifuged at 1000 × g for 5 min. Purified nuclei were lysed according to Bloom and Anderson [20].

2.2.5. Pyruvate kinase activity determination

The activity of pyruvate kinase was determined by the spectrophotometric method according to Bucher and Pfleiderer [21].

2.2.6. Protein concentration levels were determined using the method of Lowry et al. [22].

3. Results and discussion

Nitric oxide is produced from L-arginine in a reaction that is catalyzed by nitric oxide synthase that has four isoforms - neuronal (nNOS), induced (iNOS), endothelial (eNOS) and mitochondrial (mtNOS) [23 - 25]. These isoenzymes differ in their molecular weight, amino acid sequence, as well as expression regulation at the transcriptional level. Under physiological conditions, cells produce a small amount of nitric oxide, while a significant increase in its synthesis is observed in various pathologies, in which the induced isoform of nitric oxide synthase is synthesized.

The activity of nitric oxide is that of an intracellular molecule that changes expression of specific genes through nitrosylation of transcription factors [26].

S-nitrosylation of cysteine residues in the polypeptide chain, as well as of L-cysteine itself, leads to production of L-S-nitrosocysteine that plays a significant role in cell functioning [27].

Regulation of protein function through post-translational modification of thiol groups of protein cysteine as a result of S-nitrosylation is analogous to regulation of protein activity through protein phosphorylation [28].

In view of poor nitric oxide stability and closely defined site of its synthesis, the mode of its transport gains in importance. Nitric oxide carriers include low molecular weight S-nitrosothiols (L-S-nitrosocysteine or S-nitrosoglutathione) and S-nitrosylated proteins, such as S-nitroso-albumin and S-nitroso-hemoglobin [29 - 31].

Production of L-S-nitrosocysteine in both EAT tumor cells and normal CRL 1636 cells was demonstrated by RP-HPLC (***Figure 1***). Contrary to normal cells, EAT tumor cells

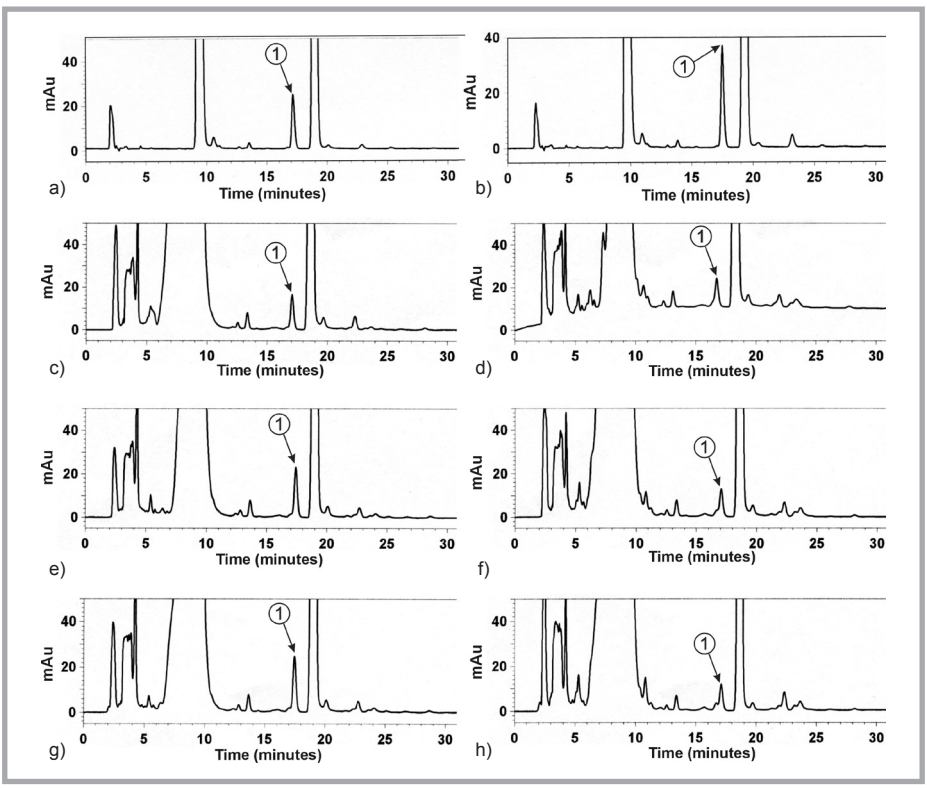

Figure 1. *Chromatograms of RP-HPLC separation of L-S-nitrosocysteine (L-S-CSNO); L-S-CSNO standards - 1 mM (a) and 2 mM (b), L-S-CSNO (controls) contained in EAT tumor cells (c) and normal CRL 1636 cells (d), L-S-CSNO produced in the presence of oligomer A in EAT tumor cells (e) and normal CRL 1636 cells (f) and produced in the presence of oligomer B in EAT tumor cells (g) and normal CRL 1636 cells (h), incubated for 72 h. Peaks: 1 = N-DNP-L-S-nitrosocysteine.*

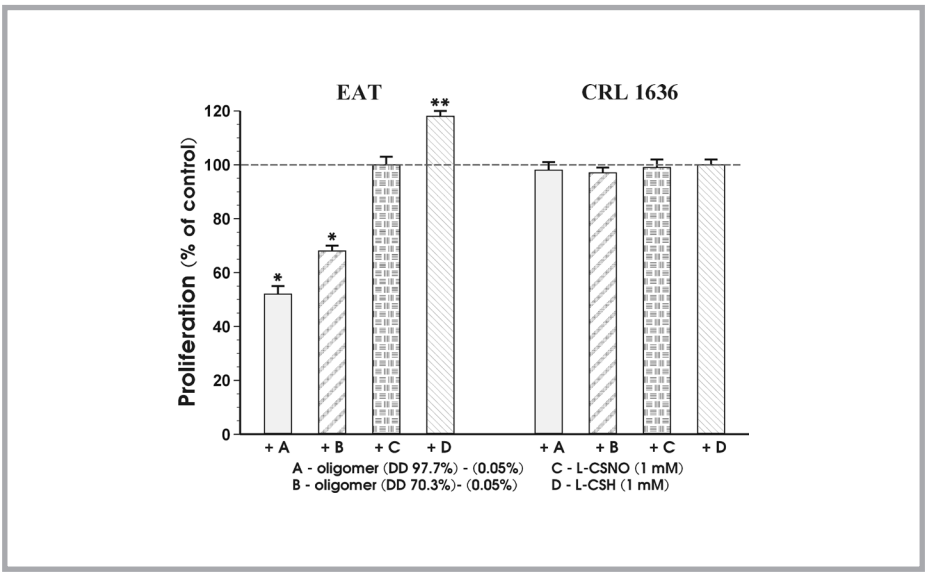

Figure 2. *The effect of oligomers A and B (final concentration 0.05%), L-S-nitrosocysteine (CSNO) C (final concentration 1 mM) and L-cysteine (L-CSH) D (final concentration 1 mM) incubated for 72 h in 37 °C on the proliferation of EAT and CRL 1636 cells. * P < 0.001, ** P < 0.05 (Student's t-test) vs. the control.*

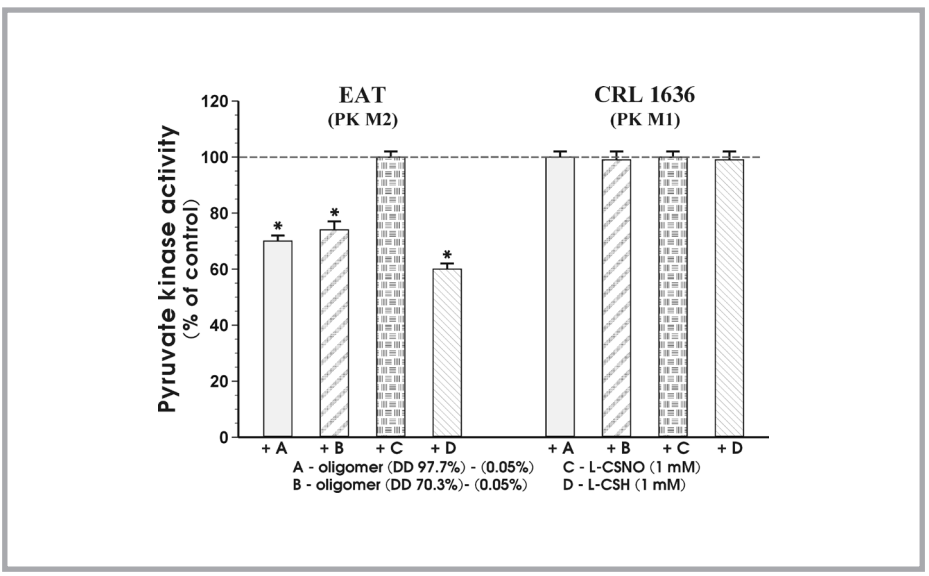

Figure 3. *The effect of oligomers A and B (final concentration 0.05%), L-S-nitrosocysteine (CSNO) C (final concentration 1 mM) and L-cysteine (L-CSH) D (final concentration 1 mM) incubated for 72 h in 37 °C on the activity pyruvate kinase of EAT (izoenzyme M2) and CRL 1636 (izoenzyme M1). * P < 0.05 (Student's t-test) vs. the control.*

showed an increased synthesis of L-S-nitrosocysteine in the presence of chitosan oligomers. This phenomenon indicates the effect of iNOS expression induction in the presence of chitosan oligomers, what leads to an increased NO synthesis NO [4, 15], and in consequence to increased nitrosylation of L-cysteine and production of L-S-nitrosocysteine.

Chitosan oligomers A and B resulted in inhibition of proliferation of tumor cells only (***Figure 2***), with the effect being slightly stronger in case of chitosan oligomer with a higher degree of deacetylation. L-S-nitrosocysteine did not affect proliferation of both tumor and normal cells. In the presence of 1 mM L-cysteine, EAT tumor cells demonstrated increased proliferation.

The effect of inhibition of the activity of the M2 PK isoenzyme from tumor cell nucleosole was observed in the presence of the investigated A and B chitosan oligomers (***Figure 3***). Inhibition of the M2 PK isoenzyme activity was stronger when the employed oligomer was characterized by a higher degree of deacetylation. Contrary to tumor cells, normal cells did not show the effect of inhibition of the activity of the M1 PK isoenzyme as the effect of the investigated chitosan oligomers. L-S-nitrosocysteine did not affect the activity of both the M1 and M2 PK isoenzyme; only L-cysteine in tumor cells contributed to a decrease of kinase activity of the M2 PK isoenzyme, what was observed in previous investigations [3].

Among possible mechanisms of the effect of chitosan oligomers there is activation of the iNOS isoform gene, what leads to an increased nitric oxide synthesis [4]. In turn, an increased synthesis of nitric oxide leads to nitrosylation of L-cysteine, what may decrease its concentration in a cell and in consequence evoke lack of activation of the activity of M2 PK isoenzyme histone kinase. When the said activity is absent, histone H1 phosphorylation by the M2 PK isoenzyme is decreased. Non-phosphorylated histone H1 is a potent inhibitor of cell proliferation [32]. The problem of the effect of chitosan oligomers on inhibition of tumor cell proliferation requires further investigations.

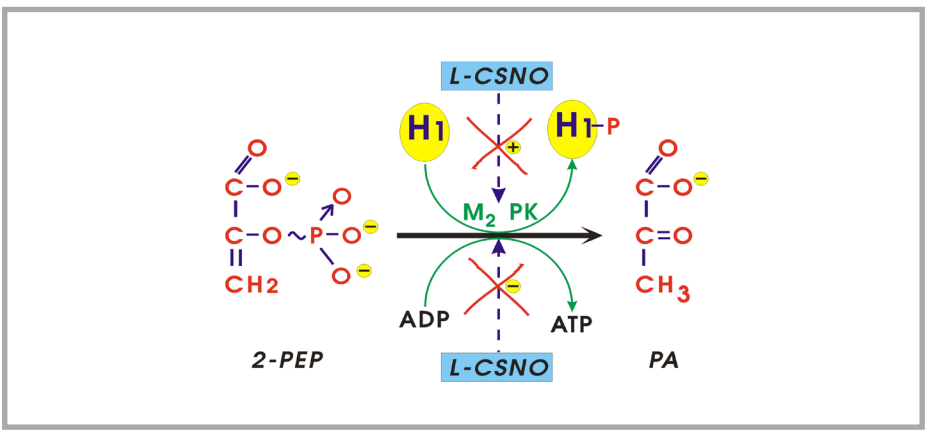

Figure 4. *Postulated control of nucleus activity of M2 pyruvate kinase (M2 PK) by L-S-nitrosocysteine (L-CSNO). 2-PEP, 2-phosphoenolpyruvate; PA, pyruvate; H_1, histone H_1; H_1-P, phosphohistone H_1; (+) activation; (–) inhibition.*

4. Conclusion

Degradation products of microcrystalline chitosan – oligomers (soluble fraction), similarly as microcrystalline chitosan, contribute to increasing the synthesis of L-S-nitrosocysteine. L-S-nitrosocysteine is a product of L-cysteine nitrosylation, what results in a drop of L-cysteine level in the cell and at the same time decreases the M2 PK isoenzyme histone 1 kinase activity. This has a contributory effect in inhibiting EAT cell proliferation (Fig. 4).

The effect of cell proliferation inhibition by chitosan oligomers is higher in case of higher charge oligomers, i.e. those obtained from chitosan with a higher degree of deacetylation.

5. References

1. *Wojtczak L., Teplova V.V., Bogucka K., Czyż A., Makowska A., Więckowski M.R., Duszyński J., Evtodienko Yu.V.: (1999) Effect of glucose and deoxyglucose on the redistribution of calcium in Ehrlich ascites tumour and Zajdela hepatoma cells and its consequences for mitochondrial energetics. Further arguments for the role of Ca2+ in the mehanism of the crabtree effect. Eur. J. Biochem. 263, pp. 495-501.*
2. *Weber G; (1996) Gann Monograph 1, pp. 151-178.*
3. *Gumińska M., Stachurska M.B., Ignacak J.: (1988) Pyruvate kinase isoenzymes in chromatin extracts of Ehrlich ascites tumour, Morris hepatoma 7777 and normal mouse and rat livers. Biochim. Biophys. Acta, 966, pp. 207-213.*
4. *Ignacak J., Dulińska J., Pałka I., Wrona-Wiśniewska M., Niekraszewicz A.: (2009) The effect of microcrystalline chitosan on the activity of pyruvate kinase M2 isoenzyme involved in regulating proliferationof Ehrlich ascites tumor (EAT) cellsin vitro. In: Jaworska M. (ed.) Progress on Chemistry and Application of Chitin and its Derivatives. Polish Chitin Society, Monograph, vol. XIV, pp. 111-120.*
5. *Ignacak J., and Stachurska M.B.: (2003) The dual activity of pyruvate kinase type M2 from chromatin extracts of neoplastic cells. Comp. Biochem. Phys. 134, pp. 425-433.*
6. *Struszczyk H, (ed.); (1995) New Aspects of Chemistry and Application of Chitin and its Deriwatives. Pol. Chitin. Soc., Łódź.*
7. *Knapczyk J., Krówczyński L.: (1987) Utylisation of chitin and Chitozan properties in drug formulaction. Bull. Sea. Fisheries Inst. 18, pp. 46-47.*
8. *Nishimura K., Nishimura S., Nishi N., Saiki I., Tokura S., Mizukoshi N., Azuma I.: (1984) Immunological activity of chitin derivatives. Vacine, 2, pp. 93-99.*
9. *Munday V.K., Cox P.J.: (2000) Compressed xanthan and karaya gum matrices: Hydration, erosion and drug relase mechanism. Inter. J. of Parmaceutics, 203, pp. 179-192.*
10. *Nishimura K., Nishimura S., Nishi N., Tone Y., Azuma I.: (1987) Adjuvant activity of chitin derivatives in mice and guinea pigs. Vacine 3, pp. 379-384.*
11. *Sirica A.E., Woodman R.J.: (1971) Selective aggregation of L 1210 leukemia cells by the polycation chitosan. J. Nat. Cancer. Inst. 47, pp. 377-388.*
12. *Gumińska M., Ignacak J., Wójcik E.: (1996) In vitro inhibitory effect of chitosan and its degradation products on energy metabolism in Ehrlich ascites tumour cells (EAT) Pol. J. Parmacol. 48, pp. 495-501.*
13. *Lekka M., Laidler P., Ignacak J., Łabędź M., Lekki J., Struszczyk H., Stachura Z., Hrynkiewicz A.Z.: (2001) The effect of chitosan on stiffness and glycolitic activity of human blader cells. Biochim. Biophys. Acta, 1540, pp. 127-136.*
14. *Ignacak J., Lekka M., Struszczyk H., Laidler P.: (2001) The influence microcrystalline chitosan of various deacetylation degree on energetic metabolism and elasticity of Ehrlich ascitestumour*

cells. In: Struszczyk H (ed.) Progress on Chemistry and Application of Chitin and its Derivatives. Polish Chitin Society, Monograph, Vol. VII, pp. 117-123.
15. Ignacak J., Pałka I., Struszczyk H.: (2004) The effect of microcrystalline chitosan on the in vitro proliferation of Ehrlich ascites tumor (EAT) cells and mouse mammary epithelium. In: Struszczyk H (ed.) Progress on Chemistry and Application of Chitin and its Derivatives. Polish Chitin Society, Monograph, Vol. X, pp. 114-119.
16. Struszczyk H.: (1987) Microcrystalline Chitozan. I. Properties and preparation. J. Appl. Polym. Sci. 33, pp. 177-187.
17. Tsikas D., Gutzki F.M., Rossan S., Bauer H., Neumann Ch., Dockendorff K., Sandmann J., Frolich J.C.: (1997) Measurement of nitrite and nitrate in biological fluids by gas chromatography – mass spectrometry and by the Griess assay : problems with the Griess assay – solutions by gas chromatography – mass spectrometry. Anal. Biochem. 244, pp. 208-220.
18. Tsikas D., Sandmann J., Rossa S., Gutzki F.M., Frolich J.C.: (1999) Investigations of S-transnitrosylation reactions reactions between low- and high-molecular-weight S-nitroso compounds and their tiols by high-performance liquid chromatography and gas chromatography – mass spectrometry. Anal. Biochem. 270, pp. 231-241.
19. Dominick P.K., Cassidy P.B., Roberts J.C.: (2001) A new and versatile method for determination of thiolamines of biological importance. J. Chromatogr. B, 761, pp. 1-12.
20. Bloom K.S., Anderson J.N.: (1978) Fractionation and characterization of chromosomal proteins by the hydroxyapatite dissociationmethod. J. Biol. Chem. 253, pp. 4446-4450.
21. Bucher I., Pfleiderer G.: (1955) Protein kinase from muscle. In: Collowick S P, Kaplan N D (eds), Methods in Enzymology, Acad. Press. New York 1, pp. 435-440.
22. Lowry O.H., Rosebrouggh N.J., Farr A.L., Randal R.J.: (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, pp. 265-273.
23. Alderton W.K., Cooper C.e., KnowlesR.G.: (2001) Nitric oxide synthasestructure, function and inhibition. Biochem. J., 357, pp. 593-615.
24. Brokes P.S.: (2004) Mitochondrial nitric oxide synthase. Mitochondrion, 3, pp. 187-204.
25. Ghafourifar P., Cadenas E.: (2005) Mitochondrial nitric oxide synthase. Trends Pharmacol. Sci., 26, pp. 190-195.
26. Rahman I., MacNee W.: (2000) Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeltic approaches. Free Rad. Biol. Med. 28, pp. 1405-1420.
27. Lindermayr C., Saalbach G., Durner J.: (2005) Proteomic identification of S-nitrosylated proteins in Arabidopsis. Plant. Physiol. 132, pp. 921-930.
28. Hart C.M.: (1999) Nitric oxide in adult lung disease. CHEST 115, pp. 1407-1417.
29. Bogdan C.: (2001) Nitric oxide and the regulation of gene expression. Trends. Cell Biol. 11, pp. 66-75.
30. Ueno T., Yoshimura T.: (2000) The physiological activity and in vivo distribution of dinitrosyl dithiolato iron complex. Jpn. J. Pharmacol. 82, pp. 95-101.
31. Vanin A.F., Sanina N.A., Serezhenkov V.A., Burbaev D.S., Lozinsky V.I., Aldoshin S.M.: (2007) Dinitrosyl-iron complexes with thiol-containing ligands: spatial and electronic structures. Nitric Oxide 16, pp. 82-93.
32. Jerzmanowski A., Cole R.D.: (1993) Histone H1 in modulation of chromatintranscriptional activity. Acta Biochem. Pol. 40, pp. 17-22.