Toxicity Evaluation of Choline Ionic Liquid-Based Nanocarriers of Pharmaceutical Agent For Lung Treatment With Extra Antitumor Activity

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Abstract

*In vitro* cytotoxicity evaluation of linear copolymer (LC) containing choline ionic liquid units and its conjugates with an antibacterial drug in anionic form, i.e. *p*-aminosalicylate (LC_PAS), clavulanate (LC_CLV), or piperacillin (LC_PIP) was carried out. These systems were tested against normal: human bronchial epithelial cells (BEAS-2B), and cancers: adenocarcinoma human alveolar basal epithelial cells (A549), and human non-small cell lung carcinoma cell line (H1299). Cells viability, after linear copolymer LC and their conjugates addition for 72 hours, was measured at concentrations range of 3.125-100 μg/mL. The MTT test allowed the designation of IC$_{50}$ index, which was higher for BEAS-2B, and significantly lower in the case of cancer cell lines. The cytometric analyzes, i.e. Annexin-V FITC apoptosis assay and cell cycle analysis as well as gene expression measurements for interleukins IL6 and IL8 were carried out, and showed pro-inflammatory activity of tested compounds towards cancer cells, while it was not observed against normal cell line. In summary, the anticancer property of linear copolymers with antibacterial drugs was shown.

1. Introduction

Polymer-drug conjugates are a common form of drug delivery systems, which are designed to improve drug solubility, control, and targeting therapy$^1$. The other advantages have been reported for adjustment of drug release kinetics profile by the strength of chemical bonding as the conjugation sites and the type/length of the linker connecting drug with the polymer matrix. The presence of reactive carboxylic or hydroxyl and amine groups in polyelectrolytes is convenient for conjugation reactions$^2,3$. The specific type of conjugates are corresponding to the ionic polymers with structures of complexes that use non-covalent bonds, which gain much consideration because of a variety of actions, e.g. electrical conductivity$^4$, electrochemical$^5$, anti-electrostatic$^6$, energetic$^7$, and optical$^8$ properties, as well as herbicidal$^9$, or biological activity$^{10,11}$. The last property of ionic conjugates can be generated by ionic conjugation of the pharmaceutical compounds to provide drug delivery polymer systems. The main requirements for polymeric carriers are related to biocompatibility and non-toxicity. Therefore, among the vehicles, the ionic conjugates can be especially distinguished, notably those based on ionic liquids (ILs), due to their unique benefits, including the concept of *green chemistry* and their ability to ion exchange, which is a strategic way for the introduction of the active pharmaceutical ingredients (API) as the counter-ions$^{11–13}$. Some of ILs are the well-known antibiotics (ampicillin, penicillin G), anti-inflammatory (pravadoline, ibuprofen, naproxen, salicylate), antiviral (trifluoridine), or antifungal (clioquinol) compounds, whereas the others have been used to obtain pharmaceutical precursors, such as lactam, pyrazolone, thiazole, imidazole, and thiazolidine$^{14}$. Generally, API-ILs display enhanced solubility in water or dissolution rates of pharmaceutics$^{15}$ increasing their bioavailability$^{16}$ when compared with the original APIs. For medical purposes, it is highly desirable when the IL polymer matrix offers biological activity, namely antioxidant, anti-tumoral and antimicrobial activity$^{13}$ as it has been proved for tetrabutylammonium-, phosphonium-, imidazolium-, pyridinium-, piperidinium-, pyrroldinium- or choline-based cations$^{17–21}$. Especially, the polymerizable ILs, e.g. 1-(4-vinylbenzyl)-3-methyl imidazolium and 1-
(4-vinylbenzyl)-4-(dimethylamino)-pyridinium hexafluorophosphate, 2,2-diallyl-1,1,3,3-tetraethylguanidinium chloride, vinyl benzyl trimethylammonium chloride, [2-(methacryloyloxy)ethyl]trimethylammonium chloride is also known as choline methacrylate seem to be attractive in the polymer synthesis for drug delivery. The latter one has been also applied to obtain the graft copolymers with polyIL side chains carrying API anions with anti-inflammatory and anti-coagulant properties.

Depending on the type of IL or API used, the obtained systems can be used in miscellaneous therapies. Due to the deterioration of the general immune system in the body as a result of a disease caused by various pathogens, the weakened organs are particularly vulnerable to cancer. For this reason, the drug delivery systems with extra anticancer activity are highly desirable. Recently, the ILs with ampicillin, taurine, or p-aminosalicylate and clavulanate have been investigated as potential systems with additional antitumor activity.

Lung cancer as a common and deadly disease is often caused by various external factors, such as pollution of the environment or smoking, as well as by health factors and history of untreated lung diseases. Therefore, in this paper, we report the cytotoxicity evaluation against cancer cell lines, i.e. adenocarcinoma human alveolar basal epithelial cells (A549) and human non-small cell lung carcinoma cell line (H1299), and normal one, namely human bronchial epithelial cells (BEAS-2B), of the ionic drug-polymer carrier. The previously synthesized well-defined linear copolymers of (2-trimethylammonium)ethyl methacrylate and methyl methacrylate (LC) have been investigated successfully by their ability to self-assembling and anion exchange. The biological activity of the ionic copolymers has been generated by the formation of its conjugates with antibacterial drugs in ionic form, i.e. p-aminosalicylate, (PAS), clavulanate (CLV), piperacillin (PIP), which are used in lung diseases treatment, especially tuberculosis. Satisfactory physicochemical properties of the nanocarriers and their drug release facility are sufficient for improvement of the system characteristics with the cytotoxicity evaluation, including colorimetric tests with the use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cytometric analyses by flow cytometry, i.e. apoptosis assay and cell cycle analysis, to confirm their beneficial application as drug delivery systems.

2. Results And Discussion

Amphiphilic linear copolymer (LC) based on [2(methacryloyloxy)ethyl]trimethyl-ammonium chloride as the ionic liquid and methyl methacrylate as non-ionic component, i.e. P(TMAMA-co-MMA) was received as a nanocarrier for delivery of ionic pharmaceutical compounds with antibacterial activity, i.e. p-aminosalicylate (PAS), clavulanate (CLV) and piperacillin (PIP). The previous studies on physicochemical characterization, as well as the drug introduction, and release process have proved that the polymer obtained by ATRP at 40°C, where the ratio of TMAMA/MMA units was equal to 25/75, is a promising drug vehicle. The polymer ability to self-organization was confirmed by critical micelle concentration.
(CMC = 0.055 mg/mL), whereas the formed nanoparticles including those with therapeutic counterions reached sizes ranging in 170-290 nm. The characteristics of conjugate systems varied with drug type and content are presented in Table 1.

The chloride-containing poly(ionic liquid) and its conjugates with PAS, CLV, and PIP were screened for their in vitro cytotoxic activity. PAS is an inhibitor of mycobactin biosynthesis and used as a second-line antituberculosis drug, usually applied to support other agents. CLV is a compound that has a similar structure to penicillin and exhibits inhibitory properties of β-lactamase. It is usually used in combination with other drugs such as β-lactam antibiotics (amoxicillin). Penicillinase enzyme, which attacks the β-lactam antibiotic, is blocked by β-lactamase inhibitor, i.e. CLV. The action of CLV is thoroughly described. PIP is a β-lactam antibiotic with a high activity degree in comparison to other penicillin derivatives. It is a drug with a broad spectrum of action. These agents (PAS, CLV, and PIP) are generally used in respiratory tract infections that are caused by various microorganisms, which may promote cancer. Therefore, an additional effect on early-stage or yet undetected cancer is highly anticipated.

The drug delivery systems were tested against a human bronchial epithelial (BEAS-2B) to exclude the poisonous effect on normal cell lines, and a contrary panel of human cancer cell lines, namely adenocarcinoma human alveolar basal epithelial cells (A549) and human non-small cell lung carcinoma cell line (H1299) to evaluate the anticancer activity as an additional property of the given drug nanocarriers, which can be used in the treatment of lung disease with co-existing cancer.

| Characteristics of linear TMAMA copolymer (LC) and its conjugates with PAS, CLV, and PIP anions
| DPₙ | F_TMAMA | Dₜ (nm) | WCA | DC (%) |
|-----|---------|---------|------|--------|
| LC  | 370     | 0.24    | 239  | 46.1   | -      |
| LC_PAS |       |         | 274  | 63.5   | 75.8   |
| LC_CLV |       |         | 169  | 47.4   | 65.6   |
| LC_PIP |       |         | 291  | 51.4   | 42.6   |

LC: P(TMAMA-co-MMA) with the average molecular weight Mₙ = 46900 g/mol, and dispersity index D = 1.74; DPₙ is the polymerization degree; F_TMAMA is the content of TMAMA units; Dₜ is hydrodynamic diameter by dynamic light scattering (DLS); WCA is water contact angle via goniometry using the sessile drop method; DC is drug content evaluated by UV-Vis.

The cells confluence was live-observed (from the MTT plates) and expressed as the percentage of the culture covered the bottom of the well (96-well plates) after 72h of incubation with the compound (LC and its conjugates at a concentration of 100 µg/mL, presented as % of control, Figure 1). It was reported that the polymer with Cl and CLV counterions caused an increase of the confluence in comparison to untreated control (CTR) of normal and cancer cells. Especially in the case of BEAS-2B (LC: 171%; LC_CLV: 175% of the control). The conjugates with PAS and PIP induced the decrease of confluence, but these systems provoked a smaller decline in BEAS-2B cells confluence compared to the CTR (~60% decrease)
than the cancer cells, where the confluence decreased up to 8-30% of CTR. Microscopic images of cells treated by tested systems are shown in Figures 1-4.

The colorimetric MTT test was carried out to evaluate the IC\textsubscript{50} index as the value of a compound concentration corresponding to a 50% growth inhibition. This parameter was determined only for compounds, for which a linear relationship between cell viability and the concentration was observed. The IC\textsubscript{50} index was not determined in the case of chloride-based LC, which did not cause the proliferation of 50% of the normal or cancer cells population. However, it was confirmed for the conjugates with PAS, CLV, or PIP, which selectively affected cells (Table 2). In the case of BEAS-2B cells, the IC\textsubscript{50} index was higher than 100 µg/mL, which means that only a high concentration of these compounds would have a toxic effect on normal cells. In turn, these systems have a higher toxic effect on cancerous cells, due to the lower IC\textsubscript{50} values (49-73 µg/mL). The lowest IC\textsubscript{50} was detected for LC_CLV in the treatment of A549 and H1299 cell lines, and the LC_PIP treated H1299 cell line, indicating the most toxic effect.

| Compound     | IC\textsubscript{50} (µg/mL) | Normal cells | Cancer cells |
|--------------|-----------------------------|--------------|--------------|
|              |                             | BEAS-2B      | A549         | H1299        |
| LC           | -                           | -            | -            |
| LC_PAS       | >100                        | 69.6         | 60.7         |
| LC_CLV       | >100                        | 57.8         | 53.2         |
| LC_PIP       | >100                        | 73.4         | 49.2         |

Cell viabilities were achieved at a series of concentrations (3.125-100 µg/mL) of nanocarrier LC and its conjugates with PAS\textsuperscript{56}, CLV\textsuperscript{57}, and PIP\textsuperscript{58} (Figure 5). The drug-free LC showed non-toxic activity against BEAS-2B cell line, where it was noticed, that higher concentration caused a significant increase of cell viability in comparison to the CTR (145% vs 100%, respectively), and it was smaller at lower concentrations (50–3.125 µg/mL = 135–106%). A similar effect was observed in the treatment of A549 and H1299 cell lines by LC, but the cell viability was slightly reduced in comparison to CTR and the lower concentration evoked the cell death pathway (1003.125 µg/mL = 116-9 %). The introduction of pharmaceutical anions influenced the opposite cytotoxicity behavior of conjugates, which caused a decrease in BEAS-2B cell viabilities compared to untreated cells, although the toxic impact was not remarked in the lower concentrations (PAS: 53-10 %; CLV: 70-102%; PIP: 56-10 % for 100-3.125 µg/mL, respectively). The conjugate systems, in a comparable way acted on the cancer cell lines, where the highest concentration triggered the decrease up to 20% of cell viabilities of A549 and H1299, which did
not show any significant response on the lowest portion of conjugates maintaining the cytotoxicity level like in CTR.

Further investigations focused on cell death were achieved by flow cytometry, where the Annexin V/PI assay allows to distinguish the variety of cell responses to compound treatment. If the cells survive and stay as living cells, they do not let any of the dyes – Annexin-V (A) or propidium iodide (PI), pass through (A−/PI−). If A or PI are detectable, they indicate through a stage of early (A+/PI−) or late apoptosis (A+/PI+), respectively. If the A is negative, and PI is positive, the cells die by necrosis (A−/PI+). The Annexin V/PI apoptosis assay was carried out by using normal (BEAS-2B) and cancer (A549 and H1299) cells. These lines were treated by the dose (50 µg/mL) of LC or conjugates with pharmaceutical ions, i.e. LC_PAS, LC_CLV, and LC_PIP. The effects after treatment are presented on cell population plots in Figure 6.

The treatment of LC did not cause a large difference in comparison to CTR in normal BEAS2B cells (A−/PI−CTR = 55.6%; A−/PI−LC = 49.5%) (Figure 7a), according to the literature, choline derivatives are characterized by biocompatibility and low cytotoxicity to normal cells21. In turn, this compound provoked the increase of apoptotic state in cancer cells, thus, the number of viable cells decreased significantly, especially for the A549 cell line, parallel to untreated cells (A549: A−/PI−CTR = 84.9; A−/PI−LC = 44.3%; H1299: A−/PI−CTR = 80.2%; A−/PI−LC = 65.7%) (Figure 7b, c).

The type of conjugated pharmaceutical anion had a meaningful influence on the type of cell death. LC_PAS adding induced the decrease of BEAS-2B live cells as compared to CTR up to 47% (A−/PI−LC_PAS = 29.4%) (Figure 7a). In the case of tumor cells their decrease was higher, about 65% (A549: A−/PI−LC_PAS = 32.1%; H1299: A−/PI−LC_PAS = 27.4%) (Figure 7b, c). Similarly, LC_PIP activated the decrease in every cell line, but 91% of living H1299 cells died concerning CTR (A−/PI−LC_PIP = 7.1%). Therefore these systems acted selectively on cells, due to the increase in death of cancer cells. LC_CLV system had a toxic activity for both normal and tumor cells, after treatment the cells survived only in 510% (BEAS-2B: A−/PI−LC_CLV = 2.9%; A549: A−/PI−LC_CLV = 9.3%; H1299: A−/PI−LC_CLV = 7.3%) (Figure 7).

Flow cytometry allowed us to analyze the cell cycle. One of the dyes used is PI, which penetrates the cell membrane of permeabilized and dying cells and leads to DNA binding by intercalation between complementary bases41. It allows providing a piece of information about the PI reaction with DNA and individual phases of the cycle. The cell cycle goes through four phases, for which results are presented as the following parts: sub-G1, G0-G1, S, and G2/M (Figure 8). These measurements were performed both, on normal and cancer cell lines. The cell lines were treated by drug-free carrier LC or its conjugate systems with antibacterial ionic drugs in the dosage of 50 µg/mL and incubated for 72 h. Typical cytometric histograms of the cell cycle are presented in Supplementary Figure S1-3.

In the case of BEAS-2B, the decrease of sub-G1 phase was observed comparing to the CTR (subG1CTR: 57.0% vs sub-G1LC: 36.9%; sub-G1LC_PAS: 7.5%; sub-G1LC_CLV: 29.8%; sub-G1LC_PIP: 9.0%). This phase represents the apoptotic state of cells. In the cancer cells, an increase of apoptotic cells was detected
after the addition of polymer systems, especially for the LC_CLV system (A549 subG1_CTR: 0.7% vs subG1 LC_CLV: 25.8% and H1299 sub-G1_CTR: 7.7% vs sub-G1 LC_CLV: 76.8%). what means lethal effects and visible more dead cells. Significant changes were also noticed in G2/M phase, where the arrest of BEAS-2B cell lines, in comparison to the CTR, was observed (G2/M_CTR: 9.1% vs G2/M_LC: 14.9%; G2/M_LC_PAS: 45.2%; G2/M_LC_CLV: 33.9%; G2/M_LC_PIP: 40.1%), whereas in A549 and H1299 the contrary dependency was remarked after drug treatment (A549 G2/M_CTR: 55.0% vs G2/M_LC: 31.4%; G2/M_LC_PAS: 25.3%; G2/M_LC_CLV: 16.8%; G2/M_LC_PIP: 32.8%, H1299 G2/M_CTR: 32.5% vs G2/M_LC_PAS: 25.9%; G2/M_LC_CLV: 4.8%; G2/M_LC_PIP: 21.7%). That findings confirm the cytostatic effects of tested compounds on the G2/M phase of the cell cycle in normal and cancer cells.

Long-term incubation with LC_CLV showed the strongest pro-apoptotic and lethal effects in normal and cancer cells. For the interleukins gene expression studies the LC, LC_PAS, LC_CLV, and LC_PIP treated cells were chosen. The 72h long-term treated cells with LC_CLV, responded with the worse condition for a gene of pro-inflammatory cytokines IL6 and IL8 expression evaluation. In normal BEAS-2B cells elevated level of IL6 was observed only after incubation with LC_CLV and LC_PIP, whereas nor LC nor LC_PAS could induce gene expression (Figure 9). The acute and late phase inflammation marker IL6 in BEAS-2B showed on the physiological feature of epithelial cells from the respiratory system – the protection through the inflammation process against damaging agents and pathogens\(^42\). The IL8 gene expression in BEAS-2B cells was elevated above the control level, but similar for tested copolymers and relatively low (Figure 9).

In cancer A549 and H1299 cells after 72 h of incubation, the marker gene IL6 for the late phase of acute inflammation was not expressed (Figure 10 and 11), similar to the low control level. Although the IL8 gene was activated, the cancer cell lines responded with different elevated expression levels. The A549 cells were more sensitive to stimulation of inflammatory cytokines production observed on the transcriptional level. The expression level in A549 cells after 72 h of incubation with LC resulted in lowered level in comparison to the untreated control, whereas LC_PAS was almost 4-times higher (Figure 10). The LC_CLV and LC_PIP did not much influence the pro-inflammatory gens expression.

Modification of copolymers and addition to the linear form (LC) additional drugs (LC_PAS, LC_CLV, and LC_PIP) showed increased potential in inflammatory pathway activation, especially in cancer cells. Both IL6 and IL8 cytokines are important chemokines for TNF\(_\alpha\) (tumor necrosis factor \(\alpha\)) pathway regulation\(^43\) and tested copolymers overstimulated IL8 expression better than LC in H1299 cells (Figure 11). In the more aggressive type of lung cancers, such as adenocarcinoma H1299, IL8 activation promotes also inflammation burst accelerating cell death (pathway activated also in epithelial cells of the pulmonary system upon viral and microbial pathogen infection)\(^44\). Considering possibilities to stimulation of such a mechanism of action, it could be a promising application for copolymers as antimicrobial and anticancer drugs in the future.

### 3. Conclusions
Cytotoxicity evaluation of nanocarriers based on choline linear copolymer (LC) and its ionic conjugates with PAS, CLV, and PIP (LC_PAS, LC_CLV, LC_PIP) as respiratory disease treatment systems, was carried. Due to the purpose of these agents, studies have been conducted against normal BEAS-2B cell lines. However the weakened immune system or often latent, early-stage form of tumor, is the cause of cancer promotion and development, so the extra antitumor activity of such systems is desirable. Therefore the toxicity against cancer cell lines, i.e. A549 and H1299 were also indicated.

Test MTT, cytometric analyses (apoptosis assay and cell cycle analysis), and gene expression measurements for interleukins IL6 and IL8 demonstrated the selectivity of action, wherein the systems: LC, and conjugates with PAS and PIP, did not harm BEAS-2B cell line, while they induced the proliferation of cancer cells. The most cytotoxic activity was shown by the LC_CLV system.

The mechanism of action profile towards tested cell lines of investigated compounds is desirable. The selective activity offers the possibility of future use of ionic copolymer-based carrier systems as antibacterial/antimicrobial drugs in lung diseases or upper respiratory tract treatment with the co-existing or developing cancer.

4. Materials And Methods

4.1. Materials

DMEM-F12 medium, trypsin, sodium phosphate buffer saline (PBS, pH=7.4) were bought from Aldrich (Poznań, Poland). Annexin-V apoptosis assay (BioLegend, San Diego, CA, USA). Propidium iodide solution (PI, BD Biosciences, San Jose, CA, USA). Fetal bovine serum (FBS, EURx, Gdańsk, Poland), physiological saline (PBS without Ca and Mg, PAN-Biotech Gmbh, Aidenbach, Germany), and Annexin-V binding buffer (BD Biosciences, San Jose, CA, USA) were used without earlier preparation. Human bronchial epithelial cells (BEAS-2B), adenocarcinoma human alveolar basal epithelial cells (A549), and human non-small cell lung carcinoma cell line (H1299) were received from ATCC (Cat# ATCC®CRL-9609; Manassas, VA, USA). LC with chloride counterions was synthesized by atom transfer radical polymerization (ATRP), whereas the anion exchange reaction was required to obtain conjugates with pharmaceutical anions, such as p-aminosalicylic (LC_PAS), clavulanic (LC_CLV), and piperacillin (LC_PIP), according to previously described procedures\textsuperscript{45}.

4.2. Characterization

Live Cell Analyzer (JuLi™ Br, NanoEnTek Inc., Seoul, Korea) was used to measure confluence, cell density, and viability monitoring analysis. Cell viability assay was performed by measuring the absorbance of the MTT reaction product (formazan) at 570 nm in a microplate reader (Epoch, BioTek, Winooski, VT, USA). The results of apoptosis and cell cycle analysis were obtained with the use of an Aria III flow cytometer (Becton Dickinson; Franklin Lakes, NJ, USA).

4.3. Cell culture
The cells were grown in a DMEM-F12 medium in sterile culture bottles (75 cm² of culture area) with 10% (v/v) FBS addition and kept at 37°C in the incubator with humidified atmosphere with 5% CO₂. For the MTT tests, the cultures were plated in 96-well plates. For apoptosis and cell cycle assays the cell cultures were plated in 6-well plates.

4.4. MTT Cytotoxicity Assay

10,000 cells were placed into 96-well plates in 0.2 mL of DMEM-F12 medium and incubated. After 24 h the polymer systems were added to each cell, whereas the first row and outer columns were prepared as control cells. The remaining wells were filled with prepared dilution series of the tested polymers (3.125-100 µg/mL). Treated cells were incubated for 72 h in standard conditions, then the solution was removed from wells. The 50 µL of MTT solution were placed into each well (0.5 mg/mL in RPMI 1640 without phenol red) and incubated for 1-2 h. After the MTT solution removal, formed formazan crystals were dissolved in 75 µL of isopropanol/HCl mixture (v/v 1-0.04), and then used for measuring the absorbance at 570 nm. Readings were performed in triplicate (six technical readings for each concentration). The results were expressed as a percentage fraction of the control.

4.5. Flow cytometry

10,000 cells were placed into 6-well plates in 2 mL of medium for 24 h. After treatment, each well was filled with 2 mL of polymer or conjugate sample. The incubation was carried out for 72 h. After that, the solution from the wells was divided into three parts.

The first part was placed into sterile vials and centrifuged for 3 min (0.6 × g, RT). 50 µL of cold Annexin-V labeling buffer and 2.5 µL of FITC-labeled Annexin-V antibody were added to the pellet. Suspended cells were incubated for 30 min in darkness. Then, 250 µL of Annexin-V labeling buffer was added. Samples were tested immediately after preparation using an Aria III flow cytometer.

The second part of the solution was centrifuged, and then the supernatant was removed. After the addition of 250 µL of hypotonic buffer (hypotonic buffer comprised from PI 100 µg/mL in PBS; 5 mg/L of citric acid; 1:9 Triton-X solution; RNase 100 µg/mL in PBS from Sigma, Poznań, Poland) the samples were incubated for 15 min in cold and dark area. The DNA states were determined by fluorescence measurements via BD FACS Aria™ III sorter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) using PE configuration.

4.6. RT-qPCR genes expressions for pro-inflammatory interleukins IL6 and IL8

The third part of the cells solution was collected by trypsinization. From the pellet, RNA was extracted using a phenol-chloroform method, with the procedure from the Total RNA Isolation kit (A&A Biotechnology). The RNA concentration and quality were estimated by UV-Vis spectrophotometer (Nanodrop, Thermofisher). The commercial set of reagents (Real-Time 2xPCR Master Mix SYBR A; A&A Biotechnology) and following Genomed pairs: IL6 (AGATCACCTAGTCCACCC) and
(GTCTGCCAAACCAGCCTTG); IL8 (GGTGCAGTTTTGCCAAGGAG) and IL-8 reverse (ACCAAGGCACAGTGGAACAA); reference RPL41 (ACGGTGCAACAAGCTAGCGG) and (TCCTGCGTTGGGATTCCTTG) was used to gene expression assays for pro-inflammatory (IL6, IL8). The thermocycler CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) was used to execute the quantitative PCR reaction, followed by reverse transcription (NG dART RT kit, EURx). For qRT-PCR reaction the following thermal profile was used: I) 50°C, 2 min, II) 95°C, 4 min, III) 54 cycles ended with fluorescence reading: 95°C, 45 s.; 52,3°C, 30 s.; IV) 72°C, 5 min, V) melting curve in the temperature range 5292 °C (every 0,5°C at 5 s), VI) incubation carried at 4°C. The thermal profile of the reaction was described previously in\textsuperscript{45}. The standardized value calculation of relative gene expression level in an unknown sample was performed concerning control expression by the following formula \( R = 2^{-\Delta\Delta CT}\). 

### Declarations

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7. Author contributions

K.N. and D.N. planned and designed the research; K.N. synthesized co-polymers and performed chemical and \textit{in vitro} studies; M.S. planned and designed biological evaluation; analyzed and interpreted results of the biological tests; M.A-O. performed RT-PCR experiments and data analysis; K.N., M.S., and D.N. wrote the article. All authors reviewed the manuscript.

8. Additional Information

Competing Interests: The authors declare no competing interests.

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**Figures**

**Figure 1**

The confluence of treated for 72 h cells by linear polymer or its conjugates at concentration of 100 µg/mL. Results presented as % in comparison to the untreated controls (CTR=100%) of each cell line.

**Figure 2**

Microscopic images by Live Cell Analyzer for untreated control cells vs. treated BEAS-2B cells by LC and LC conjugates. The scale bar represents 500 µm.
Figure 3

Microscopic images by Live Cell Analyzer for untreated control cells vs. treated A549 cells by LC and LC conjugates. The scale bar represents 500 µm.

**Figure 4**

Microscopic images by Live Cell Analyzer for untreated control cells vs. treated H1299 cells by LC and LC conjugates. The scale bar represents 500 µm.

**Figure 5**

Cell viability of linear copolymer LC and their conjugates with PAS, CLV, and PIP at different concentrations for the treatment of a) BEAS-2B b) A549, and c) H1299 cell lines, after 72 h of incubation in comparison to the CTR (not treated) cells.

**Figure 6**
Typical dot plots of cell populations after treatment with LC, LC_PAS, LC_CLV, LC_PIP compared to control untreated cells by flow cytometry in BEAS-2B, A549, and H1299 cell lines.

Figure 7

Annexin-V apoptosis results for normal (a) BEAS-2B, and cancer (b) A549 and (c) H1299 cell lines after 72 h of incubation.

Figure 8

Cell cycle analysis of normal BEAS-2B, and tumor A549 and H1299 cell lines treated by carrier LC or conjugates with PAS, CLV or PIP.
Figure 9

Relative gene expression level, for pro-inflammatory IL6 and IL8 cytokines in BEAS-2B cells after 72 h of incubation [], results from 3 repeats with +/- 5% error bar. Cells were treated with a dose of 0 (CTR) or 50 μg/mL; LC-CLV – not analyzed.

A549 inflammatory cytokine gene's expression

Relative gene expression

CTR      LC      LC_PAS    LC_CLV    LC_PIP
Sample [72h of incubation]
Figure 10
Relative gene expression level, for pro-inflammatory IL6 and IL8 cytokines in A549 cells after 72 h of incubation [], results from 3 repeats with +/- 5% error bar. Cells were treated with a dose of 0 (CTR) or 50 μg/mL; LC-CLV – not analyzed.

Figure 11
Relative gene expression level, for pro-inflammatory IL6 and IL8 cytokines in H1299 cells after 72 h of incubation [], results from 3 repeats with +/- 5% error bar. Cells were treated with a dose of 0 (CTR) or 50 μg/mL; LC-CLV – not analyzed.

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