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Laboratory note

Oligo(3-hydroxybutanoate) conjugates with acetylsalicylic acid and their antitumour activity

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Abstract

In this paper we discuss the anticancer activity of acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugates, their characteristics and in vitro biological evaluation. Acetylsalicylic acid (aspirin) attached via hydrolysable ester bonds to non-toxic well-defined 3-hydroxybutanoic acid oligomers shows novel method of drug modification.

The resulting conjugates were more effective than aspirin in growth inhibition of human colon adenocarcinoma cells HT-29 and human colon carcinoma cells HCT 116 in vitro. Treatment of rats with doses as high as 2 g of the conjugate (equivalent to 0.6 g of pure aspirin) per kg of body weight did not exhibit toxic effects.

Keywords: Acetylsalicylic acid; Oligo(3-hydroxybutanoate); Polymer–drug conjugate; Antiproliferative effect

1. Introduction

In recent years an extensive research aimed at the design of novel drug delivery systems (DDS) has been observed. The attachment of a drug to polymeric carrier molecules via hydrolysable bonds is one of the most promising approaches. It is expected that the conjugated form of a drug shows modified pharmacokinetics and biodistribution, allowing controlled drug release. Polymeric carriers used for drug modification should be non-toxic, non-immunogenic, biocompatible and biodegradable. Conjugation should not change the original structure of the drug used. The number of polymers proposed in the literature as drug carriers is still growing [1–7]. However, some of the synthetic polymers proposed as drug carriers contain traces of toxic catalysts used for their syntheses [8]. On the other hand, many natural biopolymers as poly(3-hydroxybutanoate), PHB, produced by bacteria cells contain small amount of impurities such as proteins (ca. 2%) and lipids (ca. 0.5%) [9].

In search of polymeric drug carriers we have recently developed novel method of oligo(3-hydroxybutanoate) synthesis, which can be used as non-toxic drug carrier [10,11]. The chemical structure of this oligomer is similar to that of high molecular weight natural PHB, it is pure and does not contain toxic impurities and it can be useful for drug modification.

Aspirin, discovered over 100 years ago, is widely used as non-steroidal anti-inflammatory drug (NSAID) [12]. This drug is also used in antiplatelet therapy [13,14]. Moreover, it was found to reduce risk of colon cancer [15–22]. Although it is well-known that aspirin inactivates both forms of cyclooxygenase, various aspects of its therapeutic activities are still not fully recognized.

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Pharmacological properties of aspirin and other drugs can be improved via formation of biodegradable drug–polymer conjugates (PDCs). In the literature different polymer–drug conjugates with non-steroidal anti-inflammatory drugs were described where a wide range of polymers, such as poly(anhydride-esters), dendrimers, dextran, copolymers of 2-hydroxyethyl methacrylate (HEMA), were used as carriers [23–26]. Conjugates of non-steroidal anti-inflammatory drugs (NSAIDs), including acetylsalicylic acid (aspirin), with polymers are created in the hope of obtaining new forms of drugs, which will possess advantageous properties such as changes of biodistribution and the rate of cellular uptake, increased solubility, prolonged drug release, increased stability and decreased toxicity. In the present work, we report on the synthesis and give the characteristics of the conjugate of non-steroidal anti-inflammatory drug such as acetylsalicylic acid with polymer, using novel anionic ring-opening polymerization, yielding non-toxic oligo(3-hydroxybutyrate). Oligo (3-hydroxybutyrate) is biodegradable and attractive for medical applications including drug release systems [10]. We demonstrate also that the conjugation of acetylsalicylic acid (ASA) with oligo(3-hydroxybutanoate) (OHB) increases significantly the antiproliferative activity against human colon cancer cells in vitro.

2. Materials and methods

2.1. Synthesis

2.1.1. Materials and general methods

(*R*,*S*)-β-Butyrolactone (4-methyl-2-oxetanone) (Aldrich) was purified as described previously [27]. Acetylsalicylic acid (2-acetoxybenzoic acid) (Aldrich) was used without additional purification. Sodium hydride 60% suspension in oil (Riedel-de Haën) was washed with dry tetrahydrofuran (THF) and dried before using; dimethyl sulfoxide (DMSO) 99.9% (Aldrich) was dried over molecular sieves and used without additional purification.

The NMR spectra were recorded using a Varian VXR-300 multinuclear spectrometer. ^{1}H and ^{13}C standard NMR spectra were run in $CDCl_3$ by using TMS as an internal standard. The molecular weight and molecular distributions of conjugates were determined by gel-permeation chromatography (GPC). Gel-permeation chromatography was performed at 30 °C, using a Spectra Physics 8800 gel-permeation chromatograph with two PL-gel packed columns (10^3 and 500 Å), THF was used as a mobile phase with a flow rate of 1 mL/min. Monodisperse polystyrene standards (PL-Lab.) were used to generate a calibration curve. Electrospray ionization mass spectrometric (ESI-MS) experiments were carried out using the Finningan LCQ ion trap mass spectrometer (Finningan, San Jose, CA, USA). Conjugate samples were dissolved in methanol or in $CHCl_3$ at a concentration of 0.5 mg/mL, and such solutions were introduced into the ESI source by continuous infusion with the use of a syringe pump at a flow rate of 3 μL/min. The ESI source was operated at 4.5 kV, with the capillary heater at 200 °C, and sheath gas pressure of 40 psi. Mass spectra in positive-ion mode were acquired over the range of $m/z = 100/1200$. Infrared spectra FT-IR were acquired on a BIO-RAD FTS-40A Fourier transform infrared spectrometer in the range of 2000–1400 cm^{-1}.

2.1.2. Synthesis of conjugates: general procedure

(*R*,*S*)-β-Butyrolactone was polymerized in dimethyl sulfoxide (DMSO) solution under stirring in a previously flamed and argon-purged glass reactor. Acetylsalicylic acid sodium obtained by the reaction of acetylsalicylic acid in DMSO with sodium hydride (molar ratio of reagents 1:1), respectively, was used as the initiator. Into the reactor containing the required amount of the solution of the initiator, β-butyrolactone monomer was added as described previously [28]. When the polymerization was completed, the solvent was stripped-off and the residue was re-dissolved in $CHCl_3$. Next, ethyl ether solution of HCl was added into the reactor. After 30 min the reaction mixture was washed six times with distilled water in order to remove alkali metal chloride and residual DMSO. Then the conjugate was precipitated in hexane and dried under vacuum for 48 h. All polymerization experiments were performed at room temperature. The initial concentration of β-butyrolactone was 1.0 mol/dm^3 in each experiment and the initial concentration of the initiator was changed in the range from 2.5×10^{-1} to 1.25×10^{-1} mol/dm^3. The polymerization progress was measured by Fourier transform infrared spectroscopy (FT-IR) based on the intensity of carbonyl group of monomer β-butyrolactone at 1820 cm^{-1}. The purity of the obtained material was controlled by FT-IR and ^{1}H NMR spectroscopy.

2.2. Biological studies

2.2.1. Cell lines and treatment

Human colon adenocarcinoma cells HT-29 and human colon carcinoma cells HCT 116 were obtained from American Type Cell Collection [ATCC]. HT-29 were maintained in RPMI 1640 (SIGMA–Aldrich) and HCT 116 were grown in McCoy medium (Sigma–Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (ICN Pharmaceuticals) and 100 μg/mL gentamicin (Sigma–Aldrich) at 37 °C in humidified atmosphere with 5% CO_2. The cells used for experiments were grown in 25 mL flasks and subcultured every 2–3 days. For experiments, cells up to 15 passages were used.

Conjugates of acetylsalicylic acid with oligo(3-hydroxybutanoate) (ASA–OHB) and oligo(3-hydroxybutanoate) (OHB) were prepared as stock solutions at the concentration of 200 mg/mL in dimethyl sulfoxide (DMSO) and added into culture medium immediately before use. Final concentration of DMSO in the medium did not exceeded 0.6%. Control cells were treated with the same concentration of DMSO.

2.2.2. Cytotoxicity assay

For 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays cells were seeded at the density 1.000 cells/well in 96-well plates and incubated for 24 h to attach. The medium was aspirated and replaced with

a medium containing different concentrations of tested oligomers. After the treatment, the medium was aspirated and the cells were incubated for 3 h with 50 μl of MTT (0.5 mg/mL) solution in DMEM without phenol red at 37 °C in humidified atmosphere with 5% CO_2. After aspiration of the medium, 100 μL of 0.04 N HCl–isopropanol was added into each well in order to dissolve formazan crystals. The absorbance of the samples was measured with El_x800 Microplate Reader (Biotek Instruments Inc.) at wavelength 570 nm. All the experiments were performed independently at least three times. Experimental results are expressed as percentages of control cell viability.

In vivo toxicity of free and conjugated acetylsalicylic acid (aspirin) was performed at Institute of Industrial Organic Chemistry in Pszczyna. The acute oral toxicity test was performed according to the GLP and the requirements of OECD Guidelines (OECD 420, fixed dose procedure) in rats.

3. Results and discussion

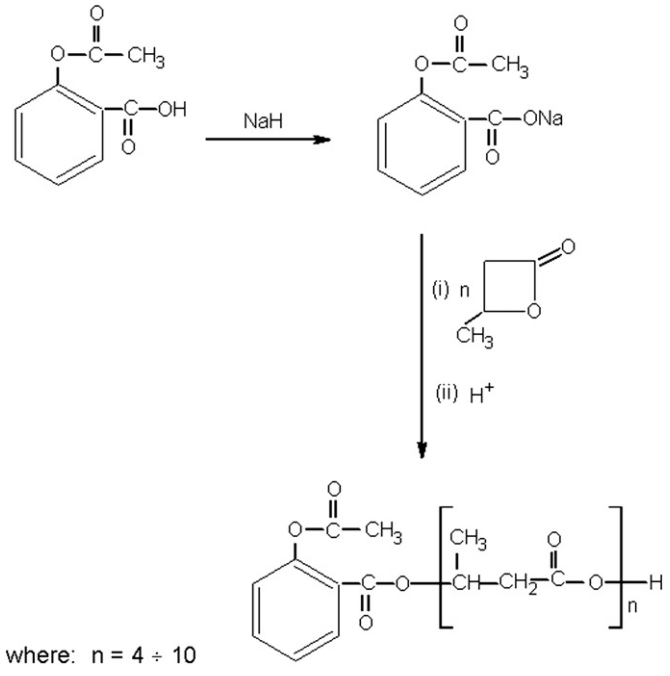
3.1. Chemistry

In the present paper we provide data on the synthesis of acetylsalicylic acid (aspirin) conjugates with well-defined non-toxic oligo([*R*,*S*]-3-hydroxybutanoate) [10,11]. This oligomer has been covalently ligated to acetylsalicylic acid. Recently we have showed that oligomeric (*R*,*S*)-3-hydroxybutanoic acid can be obtained in anionic polymerization of (*R*,*S*)-β-butyrolactone, when dimethyl sulfoxide (DMSO) is used as polar solvent activating anionic polymerization species. First, the initiator acetylsalicylic acid sodium salt was obtained in the reaction of acetylsalicylic acid with sodium hydride. The carboxylate anion of the acetylsalicylic acid initiator attacks the chiral carbon atom of the monomer causing alkyl-oxygen bond scission. The synthesis of the conjugate is outlined in Scheme 1.

The polymer chain growth proceeds entirely via carboxylate anions and the polymer formed bear acetylsalicylic acid and carboxylic end groups. The characteristics of the acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugates prepared are shown in Table 1.

The GPC curves were symmetrically monomodal with molecular weight distribution of 1.13–1.15. These results indicate that oligomerization reaction of β-butyrolactone proceeds quantitatively and the resulting conjugates posses predicted molecular weight and low molecular weight distribution. The molecular weight of the resulting linear polymers depends on the monomer (β-butyrolactone) to initiator (acetylsalicylic acid sodium salt) molar ratio. The molecular weight distribution is relatively narrow ($M_n/M_w = 1.13–1.15$), which indicates the uniformity of conjugates obtained.

The results indicate that anionic ring-opening polymerization of β-butyrolactone initiated with alkali metal salt of acetylsalicylic acid constitutes the convenient method of synthesis of novel conjugates of acetylsalicylic acid (aspirin) and non-toxic oligo(3-hydroxybutanoate).



Scheme 1. Synthesis of acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugate.

The proposed structure of conjugates was confirmed by ^1H NMR and ^{13}C NMR spectroscopies as well as ESI-MS spectrometry. Typical ^1H NMR spectrum of acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugate (see sample 2, Table 1) is presented in Fig. 1.

In the ^1H NMR spectrum are presented beside the signals characteristic of oligo(3-hydroxybutanoate) chain (peaks 10–11), the signals attributed to end group of acetylsalicylic acid (peaks 1–5). Moreover, signals at $\delta = 5.4$ ppm and $\delta = 1.4$ ppm ascribed to C*H* (6) and CH_3 (7) groups,

Table 1
Preparation and characterization of acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugates

Sample	Initiator[a]	$M_{n,th}$[b] (g/mol)	$M_{n,GPC}$[c] (g/mol)	M_w/M_n[d]	Time[e] (h)	Average number of mers (*n*)	Yields (%)
1.	ASANa	524	480	1.15	64	4	81
2.	ASANa	610	540	1.15	72	5	82
3.	ASANa	782	740	1.13	80	7	82
4.	ASANa	868	820	1.14	80	8	82

[a] Initial concentration of ASANa acetylsalicylic acid sodium salt was changed in the range from 2.5×10^{-1} to 1.25×10^{-1} mol/dm^3 and of β-butyrolactone was 1.0 mol/dm^3 in each experiment; conversion in each experiment was equal to 100%.

[b] $M_{n,th}$ is the theoretical molecular weight calculated from the following formula: $M_{n,th} = [M]_0/I_0 \times 86 + 180$, where $[M]_0$ and $[I]_0$ are the initial concentrations of monomer and initiator, respectively; 86 is the molecular weight of butyrolactone monomer and 180 is the molecular weight of end group (acetylsalicylic acid).

[c] Determined by gel-permeation chromatography (GPC) according to polystyrene standards with a low polydispersity.

[d] Estimated on the basis of GPC measurements.

[e] Room temperature.

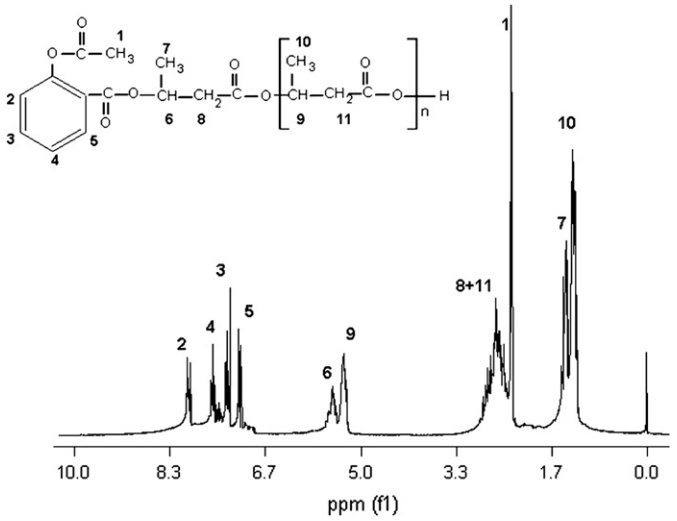


Fig. 1. ^{1}H NMR spectra (in $CDCl_3$) of acetylsalicylic acid with oligo (3-hydroxybutanoate) conjugate $M_{n,GPC} = 540$.

respectively, of 3-hydroxybutyrate unit bonded directly with acetylsalicylic acid end group were found.

Typical ^{13}C NMR spectrum of acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugate (see sample 2, Table 1) is presented in Fig. 2.

The ^{13}C NMR spectrum of acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugate shows characteristic signals of acetylsalicylic acid, peaks 1–9, and the observed groups are characteristic of polymer chain, peaks 10–13.

In the ESI-MS spectrum of acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugate (see sample 2, Table 1) is presented in Fig. 3, signals ascribed to molecular ions of oligomer chains with acetylsalicylic acid and carboxylic end groups are present.

These results indicate that in the reaction of β-butyrolactone with sodium salt of acetylsalicylic acid in the presence

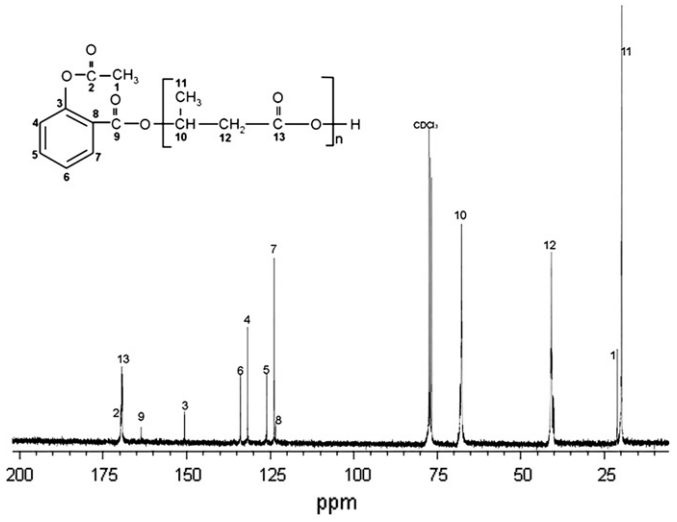


Fig. 2. ^{13}C NMR spectra (in $CDCl_3$) of acetylsalicylic acid with oligo (3-hydroxybutanoate) conjugate $M_{n,GPC} = 540$.

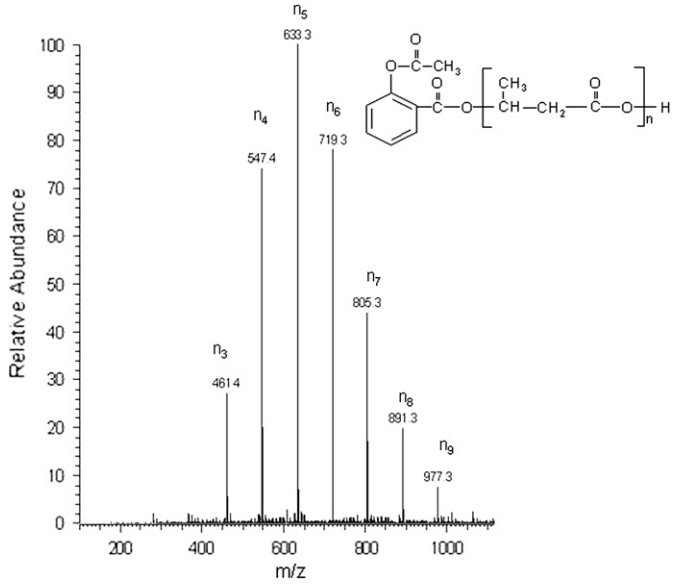


Fig. 3. ESI-MS (positive-ion mode) spectrum of acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugate $M_{n,GPC} = 540$, chains of conjugate adducts $[M + Na]^+$ with acetylsalicylic acid and carboxylic end groups. Index n corresponds to the number of 3-hydroxybutanoate repeating unit.

of dimethyl sulfoxide (DMSO), ring-opening oligomerization of β-butyrolactone is induced yielding the oligomers of 3-hydroxybutanoic acid having acetylsalicylic acid as the end group and polymer with narrow molecular weight distribution is formed. This results show that the oligo(3-hydroxybutanoate) is covalently attached to acetylsalicylic acid through hydrolysable ester group.

3.2. Biological results

In order to characterize the toxicity of acetylsalicylic acid with oligo(3-hydroxybutanoate) conjugates (ASA–OHB) the acute oral toxicity test (OECD 420, fixed dose procedure) was performed. The application of 2000 mg/kg body weight of conjugate (equivalent to 0.6 g of free acetylsalicylic acid (aspirin) per kg body weight) caused no clinical symptoms or mortality during the test. Our preliminary investigation of anti-inflammatory action of conjugates in rats did not show significant differences between equivalent doses of acetylsalicylic acid (ASA) and conjugate acetylsalicylic acid–oligo (3-hydroxybutanoate) (ASA–OHB), as determined by hind paw plethysometry (data unpublished).

However, interestingly we found that conjugation of acetylsalicylic acid (ASA) with oligo(3-hydroxybutanoate) (OHB) highly increased cytostatic effect on cancer cells. The effectiveness of acetylsalicylic acid (ASA) and acetylsalicylic acid with oligo(3-hydroxybutanoate) (ASA–OHB) conjugate $M_{n,GPC} = 540$ (see sample 2, Table 1) in killing cancer cells was performed MTT survival assay on two cell lines: HT-29 and HCT 116 and calculated IC_{50}. Figs. 4 and 5 exemplify this study, while IC_{50} values are shown in Table 2.

Inhibition of proliferation of cells treated with acetylsalicylic acid (ASA) and acetylsalicylic acid with oligo

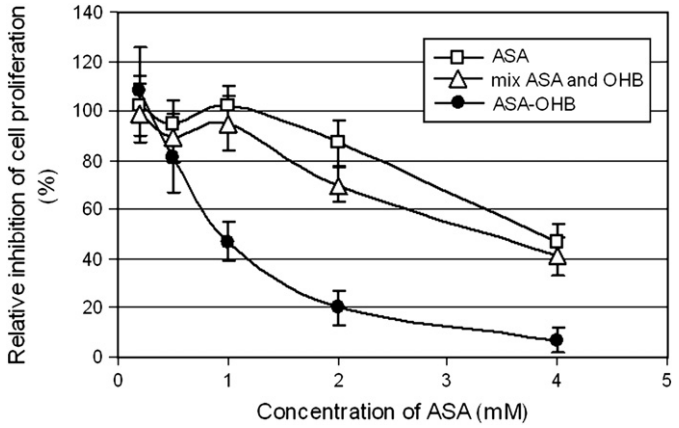


Fig. 4. Cytotoxicity of different doses of ASA, ASA bound to oligomer (ASA-OHB) and ASA mixed with oligomer (mix ASA and OHB) (in this case the corresponding doses of oligomer were equal to the mass of oligomer in the conjugate). MTT assay in HT-29 cells treated with the tested substances for 72h.

(3-hydroxybutanoate) conjugates (ASA−OHB) was dose dependent as can be seen from MTT assays (Figs. 4 and 5).

The inhibition of proliferation of cells treated with acetylsalicylic acid with oligo(3-hydroxybutanoate) (ASA−OHB) conjugate was much more pronounced than after treatment with acetylsalicylic acid. The addition of oligomer of 3-hydroxybutanoate $M_{n,GPC} = 400$ (sample 1, Table 1 [11]) to acetylsalicylic acid (mix ASA and OHB) did not influence the proliferation of cells, which implies that chemical binding of oligo(3-hydroxybutanoate) (OHB) to acetylsalicylic acid (ASA) is necessary for the enhancement of biological activity of this system.

Overall growth inhibition of cells expressed as IC_{50} was 5−7 times higher for ASA−OHB conjugate as compared to acetylsalicylic acid (free aspirin, Table 2) for HT-29 and HCT 116 cells, respectively. Results from light microscopic observation

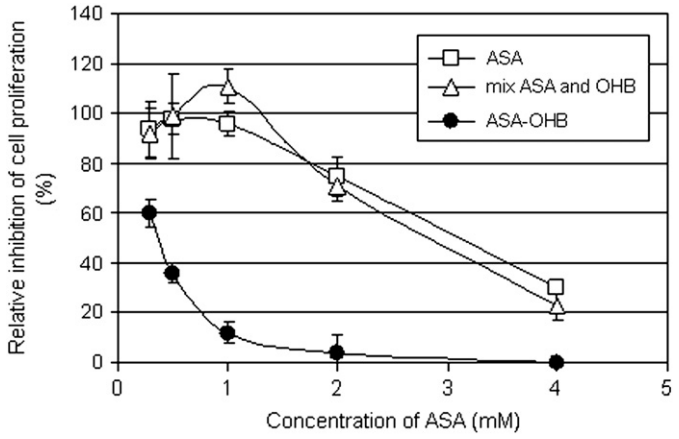


Fig. 5. Cytotoxicity of different doses of ASA, ASA bound to oligomer (ASA−OHB) and ASA mixed with oligomer (mix ASA and OHB) (in this case the corresponding doses of oligomer was equal to the mass of oligomer in the conjugate). MTT assay in HCT 116 cells treated with the tested substances for 72 h.

Table 2
Comparison of the equivalent concentration of ASA or ASA bound to OHB (ASA−OHB), necessary to reduce cell proliferation by 50% compared to control (IC_{50})

Cell line	Compound IC_{50} (mM)	
	ASA	ASA−OHB
HT-29	3.8 ± 0.18	0.74 ± 0.19
HCT116	2.8 ± 0.13	0.4 ± 0.12

The data represent mean from three different experiments ± standard deviation. Cells were treated for 72 h.

and MTT proliferation assay showed unambiguous cytotoxic effects of acetylsalicylic acid with oligo(3-hydroxybutanoate) (ASA−OHB) conjugates against tumor cells in vitro. The conjugates evoked changes of shape of the examined cancer cells, which began to round up, with vacuolization of the cytoplasm. Many cells detached easily from the plastic flasks and moved into medium. Cell morphology alterations could be observed 24 h after addition of a drug (data not shown).

4. Conclusions

We describe the synthesis and properties of conjugates of the polymers with acetylsalicylic acid. The results of the present study revealed that in the reaction of β-butyrolactone with acetylsalicylic acid regioselective ring-opening oligomerization of β-butyrolactone takes place and drug−polymer conjugates are prepared. Based on the obtained results, further studies concerned with preparation and perspective biomedical applications of the novel pharmaceutical material that combine both acetylsalicylic acid and oligo(3-hydroxybutanoate) structural units are underway in our laboratory.

In conclusion, conjugates of acetylsalicylic acid with oligo(3-hydroxybutanoate) seem to be less toxic than free aspirin when administrated to rats and the conjugates show antiproliferative properties against the tested cancer cell lines.

Acknowledgments

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