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Original article

Synthesis and in vitro antitumor effect of diclofenac and fenoprofen thiolated and nonthiolated polyaspartamide-drug conjugates

M. Barbarić [a], M. Kralj [b], M. Marjanović [b], I. Husnjak [a], K. Pavelić [b], J. Filipović-Grčić [a], D. Zorc [c], B. Zorc [a,*]

[a] *Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, HR-10000 Zagreb, Croatia*
[b] *Laboratory of Functional Genomics, Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia*
[c] *Faculty of Mechanical Engineering and Naval Architecture, I. Lučića 5, HR-10000 Zagreb, Croatia*

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Abstract

This paper reports the synthesis and antiproliferative effects of new thiomer–diclofenac and fenoprofen conjugates, hydrophilic, bioadhesive, polymeric prodrugs, as well as antiproliferative effects of diclofenac, fenoprofen and a series of previously described polymer–fenoprofen conjugates on five tumor cell lines. Thiolated and nonthiolated polyaspartamides were the chosen polymeric components. Drug-loading ranged from 5.6 to 22.4%, and the amount of SH groups ranged from 6.9 to 45.6 μmol g^{-1}. Tensile studies demonstrated a clear correlation between the amount of thiol and the mucoadhesive properties of the conjugates. The growth-inhibitory activity of the tested polymer–drug conjugates demonstrates that polyaspartamide-type polymers, especially thiolated polymers, enable inhibition of tumor cell growth with significantly lower doses of the active substance.

Keywords: Diclofenac; Fenoprofen; Polyaspartamide; Thiomer; Polymer–drug conjugate; Human tumor cell lines; Antiproliferative effect

1. Introduction

Numerous experimental, epidemiologic and clinical studies suggest that non-steroidal anti-inflammatory drugs (NSAIDs) are promising anticancer drugs [1]. For example, regular consumption of NSAIDs has been shown to reduce colon cancer risk by approximately 50%. Besides, many studies have shown that NSAIDs (e.g., acetylsalicylic acid, sulindac, piroxicam, ibuprofen and indomethacin) are effective chemopreventive agents against carcinogen-induced and genetically manipulated animal models of colon carcinogenesis [1–4]. Moreover, several studies have provided evidence that NSAIDs may also be associated with reduced risk of cancers of the bladder, breast, esophagus, lung, ovary, prostate, stomach, liver, pancreas, tongue and glioblastoma multiforme [5].

The mechanism responsible for the antitumor activity of NSAIDs is still unknown. It is commonly attributed to the inhibition of prostaglandin synthesis, that is, inhibiting the

Abbreviations: Bt, 1-benzotriazolyl; Btc, benzotriazolecarbonyl; BtH, benzotriazole; Dic, diclofenac residue; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; Fen, fenoprofen residue; IC_{50}, concentration that causes 50% growth inhibition; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; NSAIDs, non-steroidal anti-inflammatory drugs; OD, optical density; PG, percentage of growth; PAHA, poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)]-poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)] copolymer; PAHTA, poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)]-poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-2-thioethyl-DL-aspartamide)] copolymer; PAHMA, poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)]-poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-3-mercapto-1-methoxycarbonyl-propyl-DL-aspartamide)] copolymer; PBS, phosphate buffer saline; PDT, cell population doubling time; PHEA, poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]; PHTA, poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-2-thioethyl-DL-aspartamide)]; PSI, poly-DL-(2,5-dioxo-1,3-pyrrolidinediyl); TEA, triethylamine; TWA, total work of bioadhesion.

* Corresponding author. Tel.: +385 1 4856202; fax: +385 1 4856201.
E-mail address: bzbz@pharma.hr (B. Zorc).

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inducible cyclooxygenase isoenzyme COX-2, which is overexpressed in many epithelial tumors (e.g., in colon tumors) [4]. But, antineoplastic effects of NSAIDs may also include activation of apoptosis, inhibition of angiogenesis, or direct inhibition of cancer cell growth by blocking signal transduction pathways responsible for cell proliferation [4,6].

Although prostaglandins are involved in inflammation and pain recognition, they are also a fundamental part of the mechanism that protects the gastric mucosa from gut contents. Hence, inhibition of prostaglandin synthesis causes gastrointestinal (GI) toxicity, which is the most frequently encountered side effect associated with NSAIDs and which causes considerable concern.

Several possibilities of reducing this toxic side effect have been proposed, such as using COX-2 selective NSAIDs, or modified release dosage forms of NSAIDs such as enteric-coating or sustained release formulations. Unfortunately, some of these were also shown to have either toxic side effects or have not been shown to reduce risk [7]. Novel routes of administration have therefore been proposed, such as transdermal administration or drug-delivery systems through buccal mucosa, which should avoid the GI toxicity [8,9]. Besides, topical cancer chemoprevention by NSAIDs has become a promising approach to reduce toxicity [10].

Therefore, despite the enthusiasm about the potential usefulness of NSAIDs, notably selective COX-2 inhibitors, such as anticancer agents, fundamental questions about their safety, efficacy, mechanisms of action, optimal treatment regimens and contraindications for preventive and/or chronic therapy still remain [1]. Consequently, there is still a need for in vitro and/or in vivo studies on the antitumor activity of the various NSAIDs. Special emphasis should also be laid on the design and synthesis of new delivery systems that could diminish the toxic side effects of chronic therapies.

Polymer–drug conjugates may offer many advantages compared to other drug-delivery systems, such as increased drug solubility, prolonged drug release, increased stability and decreased toxicity [11–15]. Thus, binding of NSAIDs to polymer carriers could provide sustained release and activity of lower doses.

Mucoadhesion has been a topic of interest in the design of drug-delivery systems with an aim to prolong the contact of the drug at the site of application and thus enhance drug bioavailability. Mucoadhesive drug-delivery systems in the form of tablets, films, patches, and gels for oral, buccal, nasal, ocular, and topical routes have been described. Thiolated polymers (thiomers) constitute a promising new generation of mucoadhesive polymers. They could provide prolonged residence time of drug-delivery systems on various mucosal tissues, improved cohesive properties, show enzyme inhibitory capabilities and a permeation enhancing effect [16–18]. Since 1999, various thiomers, thiolated derivatives of polycarbophil, carboxymethylcellulose, alginate and chitosan, have been synthesized and evaluated (see for example Refs. [19–22]). Two thiomers of polyaspartamide-type have been developed by our research group [23,24]. In thiomer–drug conjugates, both thiomer and conjugate concepts are combined into one.

Diclofenac and fenoprofen are well-known NSAIDs. It was demonstrated that diclofenac inhibited the growth of several tumor cells in vitro and in vivo [25,26], while antitumor potential of fenoprofen has not been described to date. Therefore, the here-presented study has multiple aims: (i) extension of the current knowledge about the antitumor potential of NSAIDs by investigating the antitumor effect of diclofenac on several tumor cell lines, (ii) evaluation of the possible antitumor effect of fenoprofen, (iii) synthesis and characterization of novel thiomer–diclofenac and thiomer–fenoprofen conjugates, and (iv) investigation of potential benefits of tumor cell growth inhibition with diclofenac/fenoprofen conjugates with thiolated and nonthiolated polyaspartamides.

2. Materials and methods

2.1. Synthesis

2.1.1. Materials and general methods

Melting points were determined on a Boëtius Micro-heating Stage and were uncorrected. IR spectra were recorded on an FT-IR Paragon 500 Spectrometer (Perkin–Elmer, UK) and UV spectra were taken on a Hewlett Packard 8452A Diode Array Instrument (Hewlett Packard, Germany). ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 on a BruAvanse DRX 500, DRX 300 (Bruker, Germany). TMS was used as an internal standard. Dialysis was performed with Visking Dialysis Tubing (Serva, Germany) with a cut-off of 8000–12 000. Precoated Merck silica gel 60 F_{254} plates were used for thin-layer chromatography. Solvent systems were dichloromethane/methanol (9:1), hexane/acetone (4:1) and butanol/acetic acid/water (8:1:1). Spots were visualized by shortwave UV-light and iodine vapour. Column chromatography was performed on silica gel (0.063–0.200 mm), with methanol/dichloromethane (3:1) as eluent.

Diclofenac was purchased from Pliva (Croatia), fenoprofen from Eli Lilly Company (USA), benzotriazole, ethylenediamine, and ethanolamine from Merck (Germany), and cysteamine hydrochloride and DL-homocysteine thiolactone hydrochloride from Aldrich (Germany). Free base from the cysteamine hydrochloride was prepared by the addition of a sodium methoxide/methanol solution. The amines were distilled and dried prior to use. All solvents were of analytical grade purity and dry.

2.1.2. Benzotriazolides of diclofenac (**2a**) and fenoprofen (**2b**)

Compounds **2a** and **2b** were prepared by the reaction of 1-benzotriazolecarboxylic acid chloride (BtcCl, **1**) [27] with diclofenac [28] and fenoprofen [29], respectively. All analytical and spectral data were in agreement with the published results.

2.1.3. 2-Aminoethyl diclofenacamide (**3a**)

A solution of **2a** (3.973 g, 0.010 mol) in toluene (180 ml) was added dropwise to a solution of ethylenediamine (20 ml, 0.300 mol) in toluene (20 ml) over a period of 2 h. Reaction mixture was stirred for 24 h at room temperature and then

extracted several times with water. The organic layer was dried over sodium sulfate, filtered and evaporated under reduced pressure. The obtained crude residue (3.268 g, 97%) was recrystallized from dichloromethane/cyclohexane. M.p. 144–145 °C, [30] 149 °C; CHN analysis for $C_{16}H_{17}Cl_2N_3O$ (338.23): calcd. C 56.82, H 5.07, N 12.42, found: C 56.67, H 4.93, N 11.97; IR (KBr): ν_{max} 3352, 3209, 3022, 2915, 1640, 1575, 1508, 1449, 1416, 1353, 1299, 1271, 1195, 1091, 1023, 950, 898, 845, 766, 741, 711, 669, 617, 568 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.44 (s, 1H, 1″), 8.34 (t, 2H, 4″, $J = 5.4$ Hz), 7.52–6.28 (m, 8H, 9 and arom.), 3.58 (s, 2H, 2), 3.11–3.04 (m, 2H, 2″) 2.59 (t, 2H, 3″, $J = 6.4$ Hz) ppm; ^{13}C NMR (DMSO-d_6) δ: 171.55 (1), 142.88 (8), 137.10 (10), 130.30 (4), 129.28 (11), 129.09 (12, 14), 127.06 (6), 125.49 (3), 124.88 (15), 120.54 (13), 115.83 (5, 7), 42.40 (2″), 41.07 (3″), 37.64 (2) ppm.

2.1.4. 2-Aminoethyl fenoprofenamide (*3b*)

Compound **3b** was prepared following the published procedure [31].

2.1.5. Poly-DL-(2,5-dioxo-1,3-pyrrolidinediyl) (PSI) (*4*)

PSI was prepared by thermal polycondensation of L-aspartic acid in the presence of *o*-phosphoric acid (molar ratio 1.5:1, reduced pressure, 2.5 h at 160 °C) [32].

2.1.6. Poly[α,β-(N-2-aminoethyl-DL-aspartamide)]-poly[α,β-(N-2-hydroxyethyl-DL-aspartamide)] copolymer diclofenac conjugate (PAHA–Dic, *5a*)

To a solution of 0.699 g PSI (0.0072 mol, calculated as monomer units) in 35 ml DMF, a solution of 0.812 g (0.0024 mol) 2-aminoethyl diclofenacamide (**3a**) in 13 ml DMF was added dropwise. The reaction mixture was stirred at room temperature for 72 h and then a solution of 2.2 ml (0.036 mol) ethanolamine in 10 ml DMF was added very slowly. The reaction mixture was stirred for an additional 24 h at room temperature, acidified with 10% hydrochloric acid to pH 4, diluted with water, dialyzed against several changes of deionized water over a period of 3 days and lyophilized. Yield: 1.087 g (60%) of product **5a**; drug-loading: 13.1%; IR (KBr): ν_{max} 3303, 3083, 2938, 2882, 1661, 1548, 1532, 1446, 1366, 1280, 1063, 668 cm^{-1}; UV: $\lambda_{max} = 281$ nm, $A = 1.005$, $\gamma = 240$ μg ml^{-1}, H_2O.

2.1.7. Poly[α,β-(N-2-aminoethyl-DL-aspartamide]-poly[α,β-(N-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(N-2-thioethyl-DL-aspartamide)] copolymer diclofenac conjugate (PAHTA–Dic, *5b*, *5c*)

To a solution of 0.699 g PSI (0.0072 mol, calculated as monomer units) in 35 ml DMF, a solution of 0.812 g (0.0024 mol) 2-aminoethyl diclofenacamide (**3a**) in 13 ml DMF was slowly added. The reaction mixture was stirred at room temperature for 48 h and then divided into two equal parts.

Preparation of ***5b***: to the first half, a solution of 0.093 g (0.0012 mol) cysteamine in 16 ml DMF was added (ice bath). The reaction mixture was stirred at room temperature for 4 h. A solution of 1.1 ml (0.018 mol) ethanolamine in 10 ml DMF was added dropwise (ice bath). The reaction mixture was stirred for an additional 18 h at room temperature, acidified with 10% hydrochloric acid to pH 4, diluted with water, dialyzed against several changes of cold 5 mmol l^{-1} HCl solution over a period of 3 days and lyophilized. The reaction mixture was light protected throughout the experiment. Yield: 0.583 g (63%); drug-loading: 11.9%; content of SH groups: 9.4 μmol g^{-1}; IR (KBr): ν_{max} 3303, 3085, 2942, 1660, 1547, 1444, 1382, 1293, 1604, 668 cm^{-1}; UV: $\lambda_{max} = 281$ nm, $A = 0.812$, $\gamma = 213$ μg ml^{-1}, H_2O.

Preparation of ***5c***: analogous procedure as for **5b**, but different amounts of cysteamine (0.463 g, 0.006 mol) and ethanolamine (0.72 ml, 0.012 mol) were used. Yield: 0.620 g (66%); drug-loading: 12.1%; content of SH groups: 19.9 μmol g^{-1}; IR (KBr): ν_{max} 3299, 3085, 2942, 1659, 1548, 1532, 1010, 1296, 1065, 668 cm^{-1}; UV: $\lambda_{max} = 281$ nm, $A = 0.976$, $\gamma = 253$ μg ml^{-1}, H_2O.

2.1.8. Poly[α,β-(N-2-aminoethyl-DL-aspartamide)]-poly[α,β-(N-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(N-3-mercapto-1-methoxycarbonyl-propyl-DL-aspartamide)] copolymer diclofenac conjugate (PAHMA–Dic, *5d*)

To a solution of 0.699 g PSI (0.0072 mol, calculated as monomer units) in 35 ml DMF, a solution of 0.812 g (0.0024 mol) 2-aminoethyl diclofenacamide (**3a**) in 10 ml DMF was slowly added. The reaction mixture was stirred at room temperature for 48 h and then a solution of 3.581 g (0.024 mol) methyl-(2-amino-4-mercapto)-butyrate in 15 ml DMF was added (ice bath). The thiol used was obtained from DL-homocysteine thiolactone hydrochloride in a sodium methoxide/methanol solution. The reaction mixture was stirred at room temperature for 24 h. A solution of 0.72 ml (0.012 mol) ethanolamine in 8 ml DMF was added dropwise (ice bath). The reaction mixture was stirred for an additional 10 h at room temperature, acidified with 10% hydrochloric acid to pH 4, diluted with water, dialyzed against several changes of cold 5 mM HCl solution over 4 days and lyophilized. The reaction mixture was light protected throughout the experiment. Yield: 0.749 g (34%); drug-loading: 22.4%; content of SH groups: 45.6 μmol g^{-1}; IR (KBr): ν_{max} 3309, 3075, 2941, 1722, 1663, 1547, 1531, 1446, 1408, 1235, 1063, 749, 668 cm^{-1}; UV: $\lambda_{max} = 281$ nm, $A = 0.831$, $\gamma = 116$ μg ml^{-1}, H_2O.

2.1.9. Poly[α,β-(N-2-aminoethyl-DL-aspartamide)]-poly[α,β-(N-2-hydroxyethyl-DL-aspartamide)] copolymer fenoprofen conjugate (PAHA–Fen, *5e*)

Conjugate **5e** was prepared following the published procedure [31]. Yield: 0.566 g (58%); drug-loading: 7.7%; IR (KBr): ν_{max} 3303, 3084, 2940, 2882, 1709, 1662, 1644, 1566, 1549, 1532, 1428, 1410, 1382, 1244, 1063, 927, 668 cm^{-1}; UV: $\lambda_{max} = 271$ nm, $A = 0.504$, $\gamma = 935$ μg ml^{-1}, H_2O.

*2.1.10. Poly[α,β-(N-2-aminoethyl-DL-aspartamide)]-poly[α,β-(N-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(N-2-thioethyl-DL-aspartamide)] copolymer fenoprofen conjugate (PAHTA–Fen, **5f**)*

To a solution of 0.350 g PSI (0.0036 mol, calculated as monomer units) in 18 ml DMF, a solution of 0.341 g (0.0012 mol) 2-aminoethyl fenoprofenamide (**3b**) in 8 ml DMF was slowly added. The reaction mixture was stirred at room temperature for 48 h and then a solution of 0.926 g (0.012 mol) cysteamine in 15 ml DMF was added (ice bath, nitrogen atmosphere). The reaction mixture was stirred at room temperature for 24 h. A solution of 0.72 ml (0.012 mol) ethanolamine in 10 ml DMF was added dropwise (ice bath). The reaction mixture was stirred for an additional 4 h at room temperature, acidified with 10% hydrochloric acid to pH 4, diluted with water, dialyzed against several changes of cold 5 mM HCl solution over 5 days and lyophilized. The reaction mixture was light protected throughout the experiment. Yield: 0.415 g (47%); drug-loading: 5.6%; content of SH groups: 6.9 μmol g^{-1}; IR (KBr): ν_{max} 3299, 3085, 2939, 1665, 1546, 1531, 1410, 1380, 1244, 1063, 888, 668 cm^{-1}; UV: $\lambda_{max} = 271$ nm, $A = 0.563$, $\gamma = 1440$ μg ml^{-1}, H_2O.

2.2. Determination of the thiol group content

The degree of thiolation was determined by iodimetric titration [20]. A solution of 20 mg of the conjugate in 2 ml buffer solution, pH 3 ($NaHCO_3$/HCl), and 0.2 ml starch solution (1%) was titrated with 1 mM iodine solution until permanent light-blue colour.

2.3. Molecular weight determination

Average molecular weights of conjugates **5a–l** and polymers **6a–d** were determined by size exclusion chromatography (UV detector, $\lambda = 200 \pm 10$ nm). The column set was composed of a precolumn and a column BioSep-SEC-S 3000, 290 Å pore size (Phenomenex, USA). The experimental conditions were mobile phase buffer solution pH 6.7 (50 mM KH_2PO_4 + 50 mM KCl), flow rate 0.35 ml min^{-1} and injection volume 5 μl. The column was calibrated by protein molecular weight standards: thyroglobulin, γ globulin, ovalbumin, myoglobin and vitamin B-12. The column set, ionic strength and pH of the aqueous mobile phase were optimized prior to molecular weight determination. Average molecular weights were between 63 and 65 kDa.

2.4. Tensile studies

For tensile studies, samples (50 mg) of lyophilized nonthiolated or thiolated polymer–drug conjugates were compressed into flat-faced test discs ($d = 5$ mm), which were attached to a precise torsion balance. A piece of porcine mucosa (2 cm^2) was mounted on the glass dish and placed on a mobile platform. The discs and the mucosal surfaces were brought in contact in phosphate buffer saline (PBS, pH 7.4) at 22 °C. The force of detachment was measured as a function of displacement, by lowering the mobile platform at a constant rate of 2 mm min^{-1} until complete separation of the components was achieved. The work of fracture, equivalent to the total work of bioadhesion (TWA), was calculated as the area under the force/distance curve.

2.5. Biological studies

2.5.1. Materials

Cell lines were purchased from ATCC-LGC Promochem. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin and trypsin were purchased from Gibco/Invitrogen (USA). DMSO was purchased from Eurobio (France) and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma (USA).

2.5.2. Cell culturing

The HeLa (cervical carcinoma), MCF-7 (breast carcinoma), SW 620 (colon carcinoma), MiaPaCa-2 (pancreatic carcinoma), Hep-2 (laryngeal carcinoma) and WI 38 (diploid fibroblast) cells were cultured as monolayers and maintained in DMEM supplemented with 10% FCS, 2 mmol l^{-1} L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

2.5.3. Proliferation assays

The growth inhibition activity was assessed according to the slightly modified procedure performed at the National Cancer Institute, Developmental Therapeutics Program [33]. The cells were inoculated onto standard 96-well microtiter plates on day 0. Cell concentrations were adjusted according to the cell population doubling time (PDT): 1×10^4 ml^{-1} for HeLa, Hep-2, MiaPaCa-2 and SW 620 cell lines (PDT = 20–24 h), 2×10^4 ml^{-1} for MCF-7 cell lines (PDT = 33 h) and 3×10^4 ml^{-1} for WI 38 (PDT = 47 h). Test agents were then added in five dilutions (100, 75, 50, 25 and 1 μg ml^{-1} for compounds **5a–d** and diclofenac, or 160, 120, 80, 40 and 1 μg ml^{-1} for compounds **5e–l**, **6a–d** and fenoprofen) and incubated over further 72 h. Working dilutions were freshly prepared on the day of testing. The solvent (DMSO) was also tested for possible inhibitory activity at the same concentration as in tested solutions. After 72 h of incubation, the cell growth rate was evaluated by the MTT assay, which detects dehydrogenase activity in viable cells [34]. The absorbance (OD, optical density) was measured on a microplate reader at 570 nm. Percentage of growth (PG) of the cell lines was calculated using one of the following two expressions:

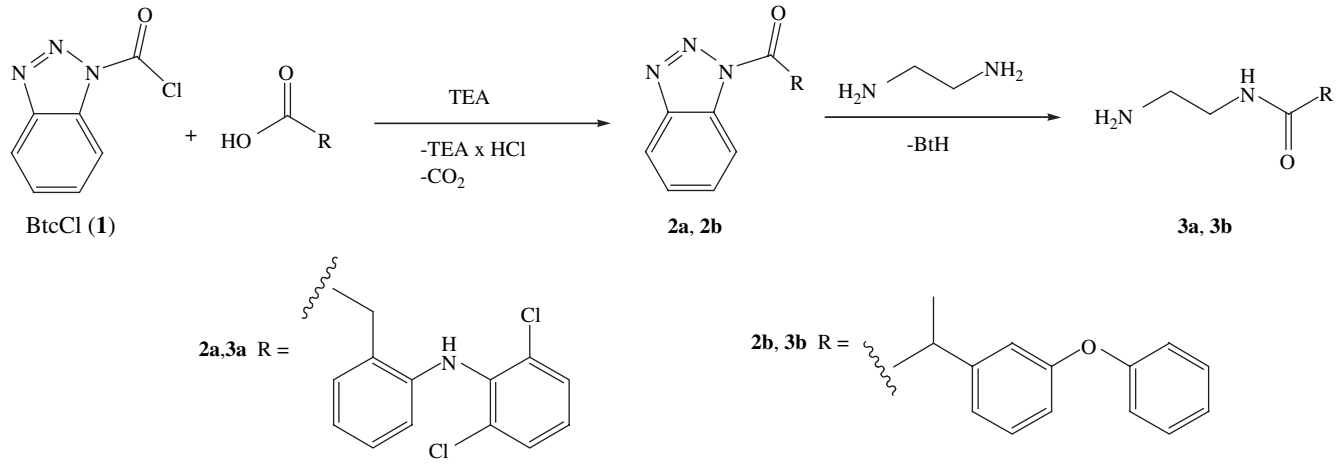
If $(\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}}) \geq 0$, then:

$$\text{PG} = 100 \times (\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}}) / (\text{mean OD}_{\text{ctrl}} - \text{mean OD}_{\text{tzero}}).$$

If $(\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}}) < 0$, then:

$$\text{PG} = 100 \times (\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}}) / \text{OD}_{\text{tzero}}.$$

where mean OD_{tzero} = the average of optical density measurements before exposure of cells to the test compound; mean OD_{test} = the average of optical density measurements after



Scheme 1. Synthesis of 2-aminoethylamides **3a** and **3b**.

the desired period of time; mean OD_{ctrl} = the average of optical density measurements after the desired period of time without exposure of cells to the test compound.

Each test point was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% inhibition. The IC_{50} values for each compound are calculated from dose–response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the reference value (i.e., 50%). If, however, for a given cell line all of the tested concentrations produce PGs exceeding the respective reference level of effect (e.g., PG value of 50), then the highest tested concentration is assigned as the default value, which is preceded by a sign >. Each result is the mean value from three separate experiments.

3. Results and discussion

3.1. Chemistry

Several new polymer–drug conjugates of polyaspartamide-type were prepared. Poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)]-poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)] copolymer (PAHA) and two similar thiolated polymers, namely, poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)]-poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-2-thioethyl-DL-aspartamide)] copolymer (PAHTA) and poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)]-poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-3-mercapto-1-methoxycarbonyl-propyl-DL-aspartamide)] copolymer (PAHMA) were the chosen polymeric components while the chosen drugs were diclofenac and fenoprofen. The drugs were first transformed into 2-aminoethyl amides, compounds bearing free amino groups that permit binding to the appropriate polymeric backbone (Scheme 1). 2-Aminoethyl diclofenacamide (**3a**) was synthesized by aminolysis of diclofenac benzotriazolide with ethylenediamine, by an analogous reaction to that previously published for 2-aminoethyl fenoprofenamide (**3b**) [31]. In this reaction, the excess of amine was crucial to avoid the formation of bis-diclofenac ethylenediamide. The starting benzotriazolide was prepared from 1-benzotriazolecarboxylic acid chloride (**1**) and diclofenac [28]. Compound **3a** was previously described by other authors, without detailed analytical data [30]. Full chemical characterization of **3a** is given in materials and methods and atom enumeration is shown in Fig. 1.

The prepared 2-aminoethyl amides **3a** or **3b** were used in the next reaction step for partial aminolysis of poly-DL-(2,5-dioxo-1,3-pyrrolidinediyl) (PSI, **4**), the reactive polysuccinimide polymer prepared by thermal polycondensation of L-aspartic acid [32]. The reaction was performed in a DMF solution, at amide/PSI molar ratio 1:3 (calculated as monomer units), which enabled substitution of at most one-third of succinimide units. Aminolysis of the remaining units was performed first by means of thiol and then by ethanolamine (Scheme 2). The thiol bearing compounds were cysteamine (products **5b**, **5c** and **5f**) and methyl-(2-amino-4-mercapto)-butyrate (product **5d**). The thiolated step was omitted in the synthesis of conjugates **5a** and **5e**. A minimum of one-third of the succinimide units was opened by ethanolamine to assure hydrosolubility of the final conjugates (all products **5a–f** were freely soluble in water). Completion of aminolysis was checked by IR spectroscopy (absence of succinimide absorption at 1715 cm^{-1}).

The following polymer–drug conjugates were prepared: PAHA–Dic (**5a**), PAHTA–Dic (**5b**, **5c**), PAHMA–Dic (**5d**), PAHA–Fen (**5e**) and PAHTA–Fen (**5f**). PHEA–Fen (**5g**) and the related conjugate with glycine, PHEA–Gly–Fen

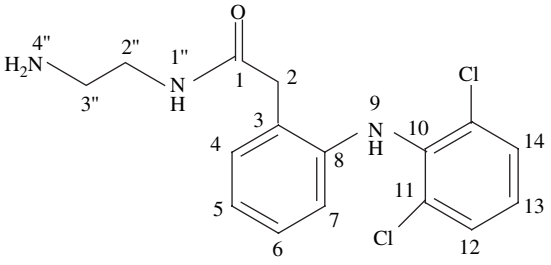
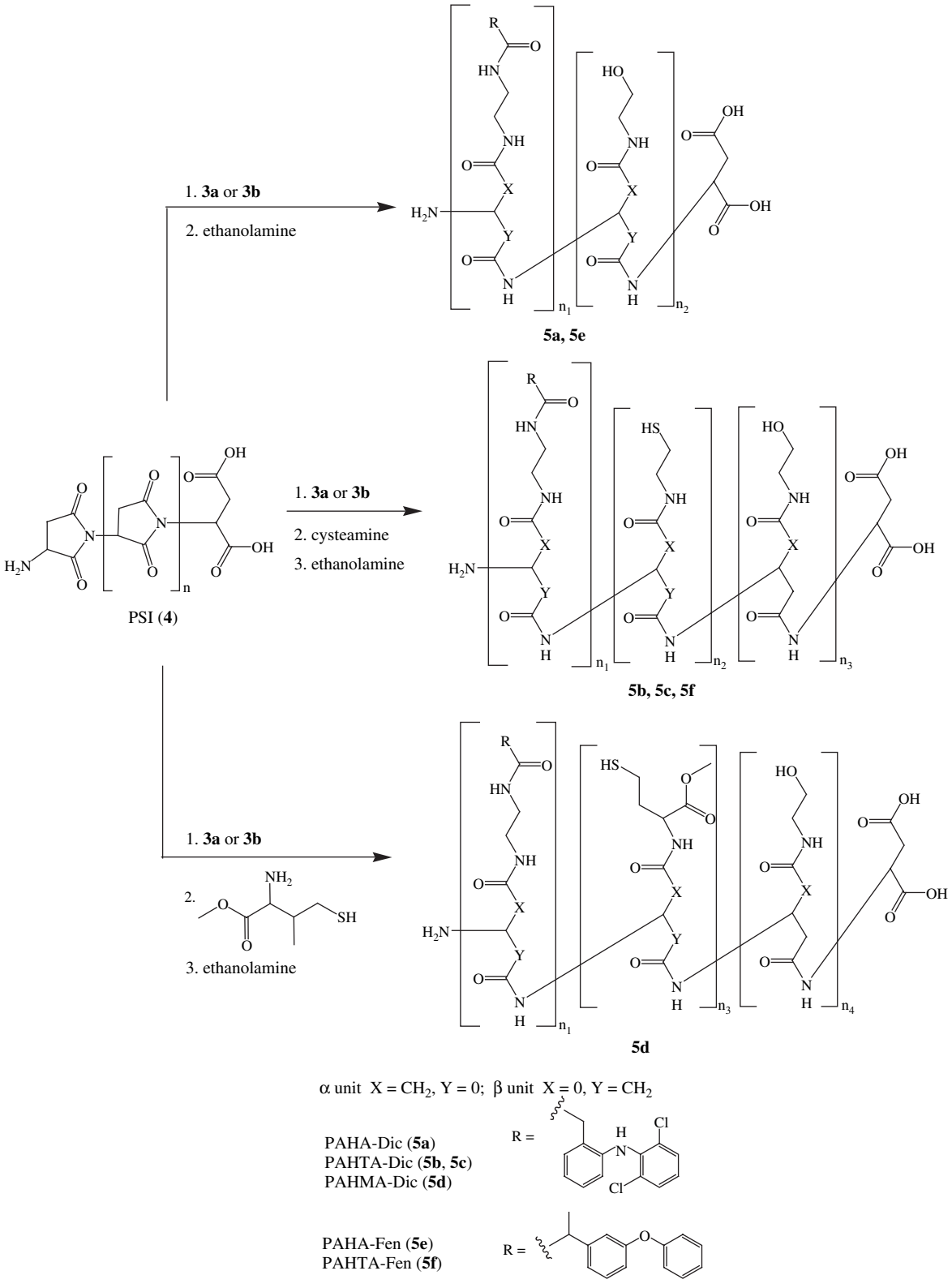


Fig. 1. Chemical structure and atom enumeration of compound **2a**.



Scheme 2. Synthesis of polymer-drug conjugated **5a–f**.

(**5h**) or β-alanine spacer, PHEA–β-Ala–Fen (**5i**) as well as analogous conjugate PHPA–Fen (**5j**), PHPA–Gly–Fen (**5k**) and PHPA–β-Ala–Fen (**5l**) were prepared according to the previously published method [29,31]. Structures of conjugates **5g–l** and the drug-loading are given in Table 2. Blank polymers PHEA (**6a**) and PHPA (**6b**) were prepared by aminolysis of PSI (**4**) with ethanolamine [32] or propanolamine [31,32]. PHTA (**6c**) and PAHA (**6d**) were prepared by aminolysis of

Table 1
Preparation and characterization of new polymer–drug conjugates **5a–f**

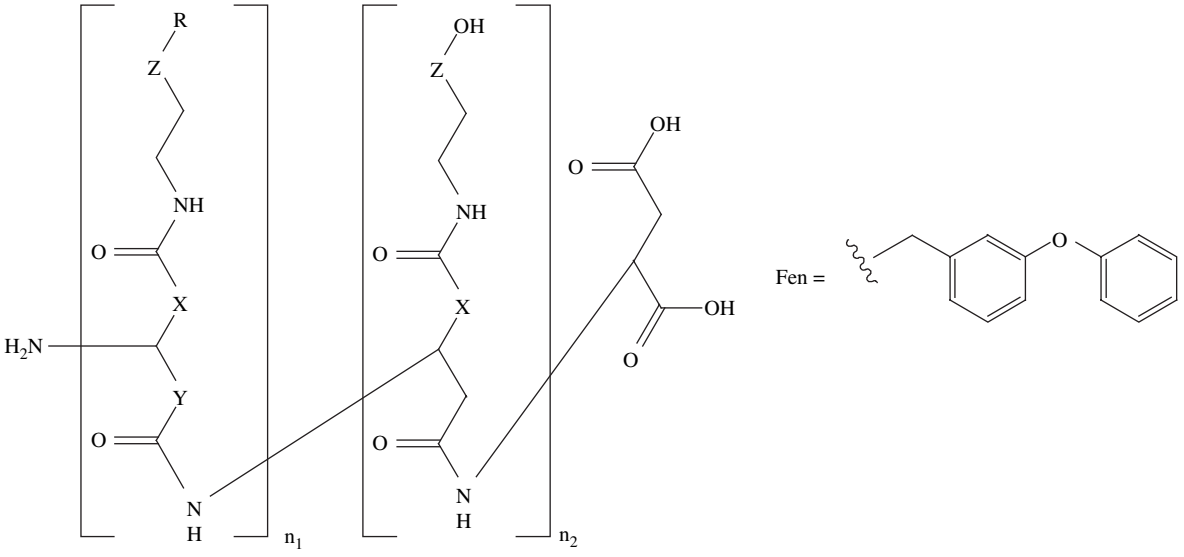
Polymer–drug conjugate	PSI/amide[a,b]/thiol[c,d]/ ethanolamine molar ratio	Time[e] (h)	Yield (%)	Drug-loading (%)	Amount of SH (μmol g^{-1})[f]	TWA (μJ ± SD)
PAHA–Dic (**5a**)	3:1:0:15[a]	90	60	13.1	–	2.87 ± 0.11
PAHTA–Dic (**5b**)	3:1:1:15[a,c]	65	63	11.9	9.4	5.06 ± 1.73
PAHTA–Dic (**5c**)	3:1:5:10[a,c]	65	66	12.1	19.9	5.81 ± 0.80
PAHMA–Dic (**5d**)	3:1:10:5[a,d]	61	34	22.4	45.6	8.51 ± 0.87
PAHA–Fen (**5e**)	3:1:0:20[b]	51	58	7.7	–	0.66 ± 0.16
PAHTA–Fen (**5f**)	3:1:10:10[b,c]	71	47	5.6	6.9	1.43 ± 0.41

[a] Diclofenacamide.
[b] Fenoprofenamide.
[c] Cysteamine.
[d] Methyl-(2-amino-4-mercapto)-butyrate.
[e] Room temperature.
[f] Average of four determinations.

PSI with two amines, cysteamine/ethanolamine or ethanolamine/ethylenediamine [24,35].

The prepared polymer–drug conjugates differed in the bound drug, drug-loading, thiolated fragment and the amount of SH groups. Products **5a–d** were diclofenac-bearing conjugates, while **5e–l** were conjugates of fenoprofen. PAHTA–Dic (**5b** and **5c**) had practically the same drug-loading, but different amounts of thiol groups. Thiolated moiety was missing in two new conjugates, **5a** and **5e**. Diclofenac loading in **5a** was similar as in **5b** and **5c**. In conjugate **5a**, as well as in **5e**, drugs were linked to the polymeric backbone by the amide bond. Fenoprofen loading in **5e** was similar as in **5f**, but **5e** was not thiolated (Table 1). Products **5g–i** were PHEA and **5j–l** PHPA derivatives, in which fenoprofen was bound to

Table 2
Structure of previously described polymer–drug conjugates (**5g–l**) and polymers (**6a–d**)



α unit X = CH_2, Y = 0; β unit X = 0, Y = CH_2

Polymer or polymer–drug conjugate	Z	R	Drug-loading (%)
PHEA–Fen (**5g**)	0	OCOFen	31.9
PHEA–Gly–Fen (**5h**)	0	$OCOCH_2NHCOFen$	20.4
PHEA–β-Ala–Fen (**5i**)	0	$OCO(CH_2)_2NHCOFen$	46.9
PHPA–Fen (**5j**)	CH_2	OCOFen	20.1
PHPA–Gly–Fen (**5k**)	CH_2	$OCOCH_2NHCOFen$	21.5
PHPA–β-Ala–Fen (**5l**)	CH_2	$OCO(CH_2)_2NHCOFen$	26.9
PHEA (**6a**)	0	OH	–
PHPA (**6b**)	CH_2	OH	–
PHTA (**6c**)	0	SH	–
PAHA (**6d**)	0	NH_2	–

the polymeric carrier by ester bonds. These products differed in the spacer length and drug-loading.

The proof that diclofenac and fenoprofen were covalently bound in the prepared polymer–drug conjugates was found in the UV spectra. The conjugates absorbed UV-light in the same absorption ranges as diclofenac and fenoprofen, whereas PAHA, PHTA, PAHTA and PAHMA had no UV-absorption at these wavelengths. The absence of nonconjugated drug was confirmed by TLC using solvent systems in which polymer derivatives remained at the start and diclofenac, fenoprofen, benzotriazolides **2**, or aminoamides **3** moved with the mobile phase. In IR spectra of conjugates **5a–f**, strong amide carbonyl absorptions at 1653 (amide I) and 1540 cm^{-1} (amide II) were present. IR spectra of conjugate **5d** bearing ester functionality in the thiolated fragment showed an additional carbonyl absorption peak at 1722 cm^{-1}. Ester carbonyls were also present in products **5g–l**.

Drug-loading in polymer–drug conjugates was estimated by UV-spectroscopy at $\lambda = 281$ nm for diclofenac and $\lambda = 271$ nm for fenoprofen. Percentage of diclofenac ranged from 11.9 to 22.4% and the percentage of fenoprofen ranged from 5.6 to 46.9%. Drug-loading in the newly prepared conjugates depended on the molar ratio of reactants **3a** or **3b** and monomer units of PSI, but was not strictly stoichiometric. The values of experimentally determined drug-loading were always lower than the expected ones, due to the incomplete coupling reactions.

The degree of thiolation was determined by iodimetric titration. Amounts of free SH groups immobilized on the polyaspartamide backbone ranged from 6.9 to 45.6 μmol g^{-1}.

Mucoadhesive properties of the conjugates were determined in vitro by performing tensile studies, which demonstrated a clear correlation between the amounts of free SH groups and their mucoadhesive properties. The observed TWA was higher for conjugates with more free SH groups (Table 1). The TWA of thiolated conjugates was more than twice higher compared to the nonthiolated conjugates of both drugs. TWA of nonthiolated conjugate PAHA–Dic (**5a**) was more than four times higher than the TWA of nonthiolated PAHA–Fen (**5f**), indicating that the type of the bound drug and drug-loading affect the mucoadhesive properties of conjugates as well.

3.2. Biological results

Diclofenac, fenoprofen and their conjugates **5a–l** were tested for their potential antiproliferative effect on a panel of six human cell lines, five of which were derived from five cancer types (HeLa, MCF-7, SW 620, MiaPaCa-2, Hep-2) and one from diploid fibroblasts (WI 38). The concentrations used correspond to approximately $1–7 \times 10^{-4}$ mol l^{-1} of diclofenac and fenoprofen, which is in agreement with the tumor cell growth-inhibitory effective concentrations of diclofenac, and other NSAIDs in various tumor cell types published so far [6,25,26,36]. Lower doses (0.01–1 μg ml^{-1}) were also tested, but they did not produce any antiproliferative effect (data not shown).

The tested compounds showed different antiproliferative effects on the presented cell line panel (Table 3). Diclofenac noticeably inhibited the growth of all tested cell lines (Table 3 and Fig. 2A), with the IC_{50} concentrations ranging between 26 and 67 μg ml^{-1} (corresponding to approximately 1×10^{-4} mol l^{-1}), while fenoprofen was less effective ($IC_{50} \geq 160$ μg ml^{-1} (corresponding to 6.6×10^{-4} mol l^{-1}). Compounds **5a**, **5b**, **5c**, **5e** and **5f** slightly and dose-dependently inhibited the growth of some of

Table 3
Growth inhibition of tumor cells and normal human fibroblasts (WI 38) in vitro

IC_{50} (μg ml^{-1})[a]						
Compound	Cell lines					
	Hep-2	HeLa	MiaPaCa-2	SW 620	MCF-7	WI 38
Diclofenac	43 ± 11	26 ± 17	55 ± 3	51 ± 14	60 ± 10	67 ± 34
Fenoprofen	>160	86 ± 62	>160	>160	≥160	n.d.[b]
PAHA–Dic (**5a**)	>160	>160	>160	≥160	>160	>160
PAHTA–Dic (**5b**)	>100	>100	75 ± 11	75 ± 30	>100	>100
PAHTA–Dic (**5c**)	>100	>100	≥100	>100	>100	>100
PAHMA–Dic (**5d**)	75 ± 5	18 ± 8	34 ± 2	61 ± 3	64 ± 3	28 ± 27
PAHA–Fen (**5e**)	>160	≥160	>160	>160	>160	n.d.
PAHTA–Fen (**5f**)	>160	≥160	>160	>160	>160	n.d.
PHEA–Fen (5**g**)	≥160	>160	≥160	≥160	≥160	n.d.
PHEA–Gly–Fen (**5h**)	>160	>160	>160	≥160	≥160	n.d.
PHEA–β-Ala–Fen (**5i**)	>160	>160	>160	>160	≥160	n.d.
PHPA–Fen (**5j**)	>160	109 ± 57	>160	>160	>160	n.d.
PHPA–Gly–Fen (**5k**)	>160	>100	≥160	≥160	88 ± 57	n.d.
PHPA–β-Ala–Fen (**5l**)	>160	>100	>160	≥160	≥160	n.d.
PHEA (**6a**)	>160	>160	>160	>160	>160	>160
PHPA (**6b**)	>160	>160	>160	>160	>160	>160
PHTA (**6c**)	>160	>160	>160	>160	>160	>160
PAHA (**6d**)	>160	>160	>160	>160	>160	>160

[a] IC_{50} – the concentration that causes 50% growth inhibition.
[b] n.d. – Not determined.

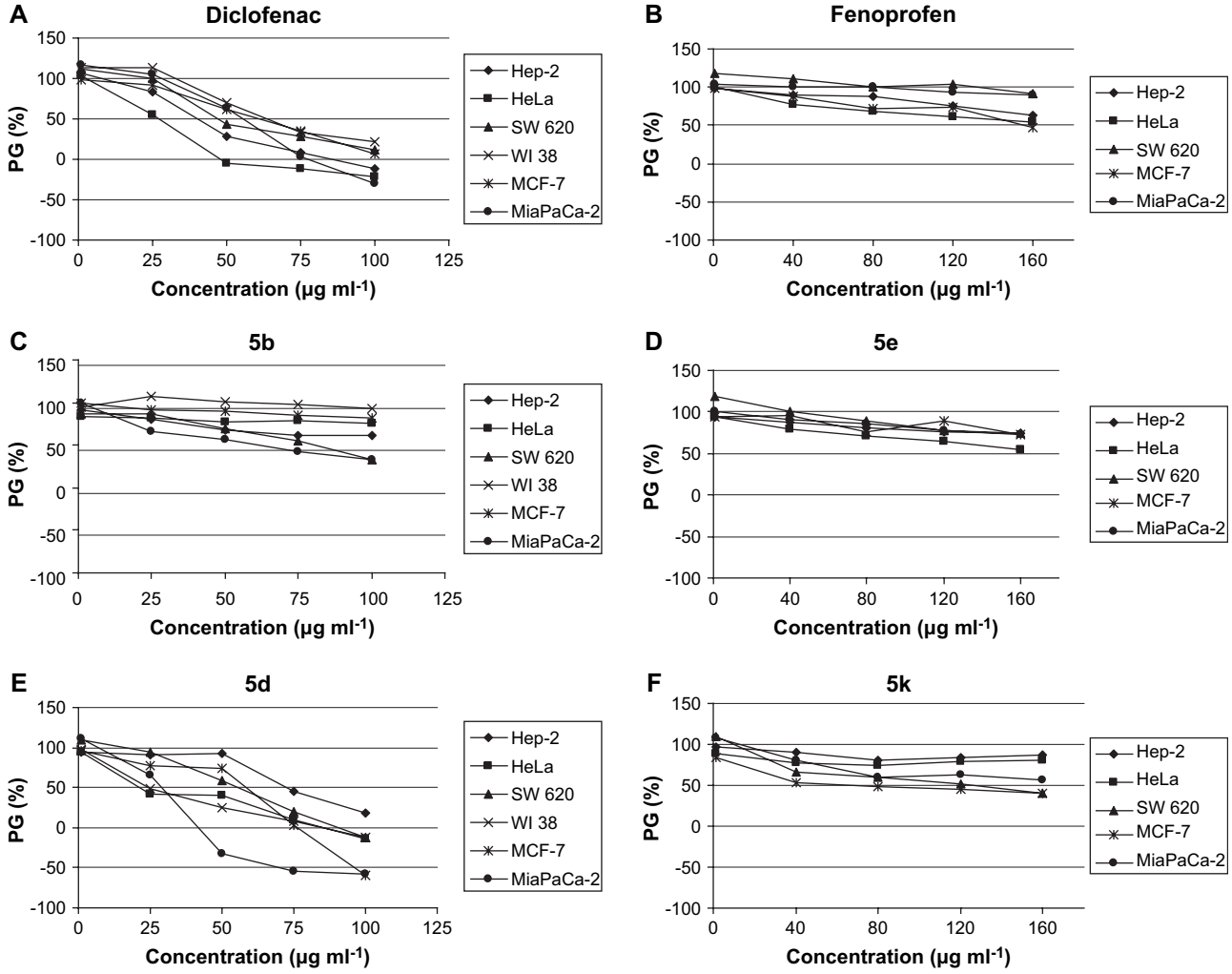


Fig. 2. Dose–response profiles for diclofenac (A), fenoprofen (B), polymer–diclofenac conjugates **5b** (C), **5d** (E), and polymer–fenoprofen conjugates **5e** (D) and **5k** (F) tested on various human cell lines in vitro. The cells were treated with the compounds at different concentrations, and percentage of growth (PG) was calculated. Each point represents a mean value of four parallel samples in three individual experiments.

the cell lines, while compounds **5g–l** produced no apparent dose-dependent effect in the tested concentration range (they similarly inhibited growth in the concentration range of 40–160 μg ml^{-1}), although the inhibitory effect was more pronounced compared to fenoprofen conjugates **5e** and **5f** (Fig. 2D and F). Besides, the IC_{50} values mostly exceeded the highest tested concentration (100 or 160 μg ml^{-1}) (Table 3 and Fig. 2). However, if the ratio of active substances in these conjugates is taken into account, it can be seen that the activity of conjugated drugs is significantly higher than the activity of free drugs. For example, the IC_{50} value for compound **5b** on MiaPaCa-2 cells, 75 ± 11 μg ml^{-1}, correlates with 8.9 μg ml^{-1} of free drug, which is approximately 6-fold lower than the IC_{50} of diclofenac (55 ± 3 μg ml^{-1}). Furthermore, **5d** strikingly and differentially inhibited the growth of all tested cell lines (Fig. 2D), with special selectivity towards MiaPaCa-2 and HeLa cells.

Considering the diclofenac loading in the **5d** conjugate, one can see that the conjugated drug is three to approximately ten times (depending on the cell line) more active than the free one. However, a comparison of IC_{50} values for tumor cells and normal fibroblasts (WI38) indicates that both diclofenac and **5d** showed no selectivity.

The best antiproliferative activity of **5d** among all the tested diclofenac conjugates could not be fully explained by the highest drug-loading (22.4%). As the inhibitory effect of **5d** varies differently between the cell lines compared to the inhibitory effect of diclofenac, one can assume that the structure of the polymeric chain and the amount of free SH groups (highest for **5d**) may have a different impact on different cell lines.

Fenoprofen and its conjugates show modest inhibitory activity (Fig. 2B, D, and F), with IC_{50} concentrations ≤ 1 mmol l^{-1}. However, the fenoprofen conjugates **5e** and **5f** inhibit growth equally or even more strongly than fenoprofen alone, despite the low drug-loading (6–7%). It can be taken that approximately 18 times less active substance is necessary for the same inhibitory activity when the drug is conjugated. All other nonthiolated conjugates **5g–k** had somewhat stronger inhibitory effects, most probably due to much higher drug-loadings (20–47%). It is important to emphasize that the parent polymers

6a–d with no bound drug had no inhibitory effect on the cell lines tested.

4. Conclusions

A series of thiolated and nonthiolated polymer–drug conjugates of diclofenac and fenoprofen were prepared and tested for antiproliferative activity in vitro. The polymer–drug conjugates differed in the polymer type, bound drug, drug-loading, thiolated fragment and the amount of SH groups.

Diclofenac noticeably inhibited the growth of all tested cell lines, while fenoprofen showed modest antiproliferative activity in the tested concentration range. However, the growth-inhibitory activity of the tested polymer–drug conjugates clearly demonstrates that using polyaspartamide-type polymers, notably thiolated polymers, enables inhibition of tumor cell growth with significantly lower doses of the active substance, which is extremely important for potential chemopreventive and/or antitumor treatment regimens. Additional studies should be performed to test the activities of polymer–drug conjugates in vivo, especially as topical (transdermal, transmucosal) drug-delivery systems.

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