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Short Communication

Characterisation of a novel conjugate of ibuprofen with 3-hydroxybutyric acid oligomers

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Abstract

Objectives A conjugate of ibuprofen with 3-hydroxybutyric acid oligomers has been evaluated as a novel drug delivery model system.
Methods This paper focuses on the synthesis and the characterisation of the physicochemical properties of this conjugate, and on hydrolysis studies in aqueous buffers and simulated intestinal fluid. We also describe the development of an analytical method (HPLC) for hydrolysis studies of this compound.
Key findings The conjugate had high stability in aqueous solutions of pH 6–8 and underwent slow enzymatic hydrolysis.
Conclusions This conjugate is not well suited for oral administration but might be considered a candidate for development of prodrug preparations for parenteral or topical sustained release.
Keywords drug conjugates; HPLC; hydrolysis; ibuprofen; oligo(3-hydroxybutyric acid); solubility

Introduction

Non-toxic biocompatible polymers allow for sustained release of drug molecules in the gastrointestinal tract as well as prolonged release after topical or parenteral administration.[1] Among the countless methods developed in recent years, the technology of polymer–drug conjugation offers several beneficial treatment options. The strategy of drug conjugation involves the reversible attachment of a drug molecule to a (water-soluble) macromolecular carrier. The obtained conjugates show distinctly different pharmacokinetic pathways compared with the non-conjugated drugs. The conjugate thus acts as a prodrug, providing safe transport of the bioactive agent to the target tissue.[2]

Several conjugates of non-steroidal anti-inflammatory drugs with polymers have been designed in order to change the biodistribution and rate of cellular uptake, increase solubility, prolong drug release and decrease acute toxicity.[3–8]

A novel system of this type of conjugate using ibuprofen together with oligomers of 3-hydroxybutyric acid (OHBs) has recently been synthesised and patented in the Centre of Polymer and Carbon Materials of the Polish Academy of Sciences in Zabrze, Poland (Patent PL 196384).[9] The synthesis of the ibuprofen–OHB conjugate, using methods similar to those previously described for conjugation of acetylsalicylic acid,[10] resulted in an amorphous, viscous, semisolid, transparent substance. Its chemical structure, illustrated in Figure 1, has been confirmed by gel-permeation chromatography, ^{1}H NMR, electrospray ionisation mass spectrometry (ESI-MS) and infrared spectroscopy. It has previously been demonstrated that the non-conjugated oligomer is biocompatible and non-toxic *in vitro*.[11]

The aim of the present study was to evaluate the possibility of using OHB conjugates in oral or parenteral formulations. We have determined the solubility in water, aqueous-buffered solutions and organic solvents, and assessed hydrolysis of the conjugate at different pH values and in the presence of hydrolytic enzymes (pancreatin).

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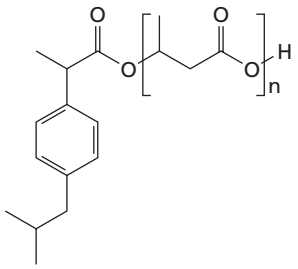

Figure 1 Chemical structure of the conjugate of ibuprofen with oligomers of 3-hydroxybutyric acid (n = 3–7)

Materials and Methods

Synthesis and chemical characterisation of ibuprofen–OHB

(R,S)-β-Butyrolactone (3-hydroxybutyric acid) (Sigma-Aldrich, St Louis, MO, USA) was dried and then distilled under reduced pressure. Ibuprofen ((S)-(+)-2-(4-isobutylphenyl)-propionic acid; Fluka, Buchs, Switzerland) and potassium hydroxide (POCh, Gliwice, Poland) were used without additional purification. Dimethylsulfoxide (DMSO; Sigma-Aldrich) was dried over 5-Å molecular sieves prior to use. (R,S)-β-Butyrolactone was polymerised in DMSO solution under stirring (initial concentration 1 mol/l) in a previously flamed and argon-purged glass reactor. β-Butyrolactone was added to the reactor containing the required amount of a DMSO solution of ibuprofen potassium salt (0.2 mol/l, obtained from the reaction of DMSO solution of (S)-(+)-2-(4-sobutylphenyl)-propionic acid with potassium hydroxide (patent PL 196384).[9] When the polymerisation was complete, the DMSO was stripped off and the residue was re-dissolved in chloroform. Next, an ethyl ether solution of hydrochloric acid was added to the reactor; after 30 min, the reaction mixture was washed with distilled water to remove alkali metal chlorides and residual DMSO. The conjugate was then precipitated in hexane and dried under vacuum for 48 h. All steps of the polymerisation process were performed at room temperature. Polymerisation progress was measured by Fourier transform infrared (FT-IR) spectroscopy based on the intensity of the carbonyl group signal of β-butyrolactone at 1820 cm^{-1}. The yield of the reaction was approximately 80%. The purity of the obtained material was assessed by FT-IR and ^1H NMR spectroscopy.

The NMR spectrum was recorded using a Varian VXR-300 multinuclear spectrometer. Standard ^1H NMR spectra were run in $CDCl_3$ using tetramethylsilane as an internal standard. The molecular weight and molecular distributions of the conjugate were determined by gel-permeation chromatography performed at 30°C, using a Spectra Physics 8800 gel-permeation chromatograph (San Jose, USA) with two PL-gel packed columns (1000 Å and 500 Å). The mobile phase was tetrahydrofuran at a flow rate of 1 ml/min. Monodispersed polystyrene standards (PL-Lab, Amherst, MA, USA) were used to generate a calibration curve. ESI-MS was carried out using an LCQ ion trap mass spectrometer (Finningan, San Jose, USA).

Conjugate samples were dissolved in chloroform at a concentration of 0.5 mg/ml, and the solution was introduced into the ESI source by continuous infusion using 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 negative-ion mode were acquired over the range m/z 100–1200. FT-IR spectra were acquired on a Bio-Rad FTS-40A FT-IR spectrometer in the range of 2000–1400 cm^{-1}.

Differential scanning calorimetry (DSC) was performed with a TA DSC 2010 apparatus under nitrogen using a heating/cooling rate of 20°/min.

Quantitative analysis of conjugates

Quantitative analysis was carried out using HPLC (Merck-Hitachi, Darmstadt, Germany). The stationary phase was a LiChrospher 100 RP-18 column (5 μm, Merck) and the mobile phase was a 66 : 34 (v/v) mixture of 10 mM phosphate buffer (pH 7.0) and acetonitrile, delivered at a flow rate of 1 ml/min. Detection was at 220 nm using a UV/Vis detector. The detection limit for ibuprofen was 0.5 μg/ml. Limits of quantification for ibuprofen and ibuprofen–OHB were 1 μg/ml and 50 μg/ml, respectively. Concentrations of unbound ibuprofen and ibuprofen–OHB were calculated from the calibration curves of standard solutions of ibuprofen or ibuprofen–OHB in a 1 : 1 mixture of phosphate buffer (pH 7.5) and methanol. The calibration curves were linear within the analysed range of concentrations (R^2 = 0.9996).

Solubility studies

In order to determine the solubility of ibuprofen–OHB and compare it with that of non-conjugated ibuprofen, 100 mg samples of ibuprofen–OHB and 50 mg samples of ibuprofen were placed in test tubes with 2 ml 50 mM phosphate buffers (pH 6–8), 0.1 M hydrochloric acid or distilled water. The suspensions were stirred continuously at 37°C for 24 h, after which time a considerable amount of the substance remained undissolved. Samples (1 ml) of the liquids were withdrawn and centrifuged (4000 rpm for 10 min) and 0.5 ml of each supernatant was diluted with methanol (1 : 1, v/v) and analysed by HPLC. The experiment was repeated three times and means ± SD calculated.

Hydrolysis studies

Ibuprofen–OHB hydrolysis studies were carried out in triplicate in 50 mM phosphate buffers of pH 6.0, 7.0, 7.5 and 8.0 and in solutions of pancreatin (5 g/l; USP 30 simulated intestinal fluid, pH 6.8) containing monobasic potassium phosphate (6.8 g/l). Pancreatin was purchased from Sigma-Aldrich, reagents for buffered solutions and HPLC were purchased from POCh.

Solutions of ibuprofen–OHB (1 mg/ml) in each of the media described above were prepared and stirred at 37°C for 72 h. Samples (1 ml) of the 20 ml total volume were withdrawn after 6, 24, 48 and 72 h (without replacement). After diluting with methanol (1 : 1 v/v) the samples were analysed by HPLC.

Drug formulation studies

Different formulations (i.e. oily solutions and oil–water emulsions) containing ibuprofen–OHB were prepared for

stability and drug-release studies. The triglyceride was chosen because of the conjugate's good solubility in Miglyol 812 (Caesar & Loretz, Hilden, Germany). A solution of ibuprofen–OHB at a concentration of 25% (w/w) was prepared and sterilised by filtration through a 0.2 μm pore size filter under aseptic conditions. An oil–water emulsion containing 30% of a solution of ibuprofen–OHB in Miglyol 812 (33.3% w/w) was prepared by high-speed homogenisation with an Ultra-Turrax T-25 (Janke & Kundel, Staufen, Germany). Tween 80 (Fluka), 2% (w/w), was used as an emulsifier. The emulsion was sterilised by autoclaving (15 min at 121°C).

Concentrations of ibuprofen–OHB in the formulations were measured before and directly after sterilisation, and 7, 30 and 90 days after preparation, using the HPLC method described above. The pH and the size of the oil droplets in the emulsion were also measured at these time points using an Orion 350 pH meter and Mastersizer E laser diffractometer (Malvern Instruments, Malvern, UK), respectively. All measurements were performed at least in triplicate.

Drug release from the above preparations and from the pure substance was also evaluated. An appropriate amount of each preparation containing 250 mg ibuprofen–OHB, or 250 mg ibuprofen–OHB alone, was placed in a cellulose membrane dialysis bag (cut-off 12 400 Da; Sigma). The bag was immersed in 50 ml phosphate buffer (pH 7.5) and underwent gentle continuous shaking at 37°C. After 3, 6, 24 and 48 h, 1 ml samples were drawn and replaced with an equal quantity of freshly prepared pre-warmed buffer. Samples were diluted with methanol (1 : 1 v/v) and the concentrations of ibuprofen–OHB measured using the HPLC method described above. All experiments were carried out in triplicate.

Statistical analysis

The influence of pH on solubility and hydrolysis of the ibuprofen–OHB, and the dependence of drug-release rate on the type of formulation were analysed using the Kruskal–Wallis one-way analysis of variance. The same test was also used to investigate whether the concentration of ibuprofen–OHB changed significantly during the course of the stability tests. Individual differences between the formulations and various conditions used in hydrolysis studies were tested using Dunn's post-hoc test. $P < 0.05$ denoted significance in all cases.

Results

Chemical characterisation of ibuprofen–OHB

The analytical methods (HPLC, FT-IR and ^{1}H NMR spectroscopy) enabled characterisation of ibuprofen–OHB. M_n and M_w/M_n values, determined by gel-permeation chromatography with reference to polystyrene standards, were 570 Da and 1.21, respectively. The content of ibuprofen in ibuprofen–OHB was 36.2% (w/w), 2.5% of which remained unconjugated.

^{1}H NMR spectra in $CDCl_3$ exhibited the following signals (δ, ppm): 0.88 (d, 6H, CH($C\underline{H}_3$)$_2$); 1.20, 1.29 (2d, 3H, CH-$C\underline{H}_3$); 1.44, (d, 3H, CH-$C\underline{H}_3$); 1.83 (m, 1H, $C\underline{H}$(CH_3)$_2$); 2.44, 2.58 (2d, 2H, O-$C\underline{H}_2$-CH); 3.65 (m, 1H, Ar-$C\underline{H}$-CH_3); 5.14, 5.25 (2m, 1H, O-$C\underline{H}$-CH_3); 7.06–7.27 (m, 4H, Ar$\underline{H}$).

ESI-MS analysis of ibuprofen–OHB in negative-ion mode indicated the existence of oligomeric 3-hydroxybutyrate chains with ibuprofen and carboxyl end groups. The number of repeating 3-hydroxybutyrate units in the positive ion varied from $n = 3$ to $n = 7$ in the conjugates. The most abundant cation was located at *m/z* 635 Da and was assigned to the conjugate with five 3-hydroxybutyrate units. The observable peaks showed a peak-to-peak mass increment of about 86 Da, which corresponds to the molecular weight of the 3-hydroxybutyrate repeating unit.

The glass transition temperature of ibuprofen–OHB, determined using DSC under nitrogen atmosphere, was –35.44°C.

Interpretation of chromatograms

Figure 2 shows a representative HPLC chromatogram of Ibuprofen–OHB. The peak at approximately 3.2 min represents unconjugated ibuprofen (Figure 2a). Since the oligomer does not give any peaks in the chromatogram (Figure 2b), it was concluded that all other peaks shown in Figure 2a were caused by conjugates of different molecular weights. Peaks at later retention times represent long-chain conjugates, whereas the peak at retention time 7.9 min resulted from a conjugate of ibuprofen with monomers or dimers of 3-hydroxybutyric acid.

The areas of the peak of ibuprofen (t_R 3.2 min) and the conjugate (t_R 7.9 min) were investigated for solubility and hydrolysis studies and linear correlations between areas of these peaks and concentrations of both ibuprofen and conjugate were found.

Solubility of ibuprofen–OHB in selected media

Ibuprofen–OHB was freely soluble in methanol, ethanol and chloroform and slightly soluble in water. In buffered aqueous solutions the solubility increased with increasing pH

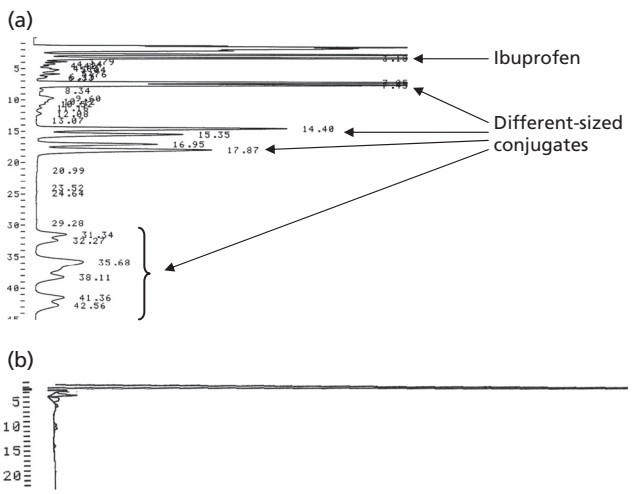

Figure 2 Representative HLPC chromatograms of: (a) the conjugate of ibuprofen and oligomers of 3-hydroxybutyric acid and (b) the non-conjugated oligomer of 3-hydroxybutyric acid. The concentration of both solutions was 1 mg/ml.

Table 1 Solubility of ibuprofen and its conjugate with oligomers of 3-hydroxybutyric acid (Ibuprofen–OHB) at different pH values (50 mM phosphate buffers) and in selected organic solvents

Solvent	Ibuprofen	Ibuprofen–OHB
0.1 M HCl	0.07 ± 0.001	0.036 ± 0.06
Water	0.196 ± 0.03	0.1 ± 0.01
pH 6.0 buffer	0.723 ± 0.06	4.2 ± 0.2
pH 7.0 buffer	2.77 ± 0.05	21.0 ± 0.1
pH 7.5 buffer	5.53 ± 0.14	31.1 ± 0.1
pH 8.0 buffer	5.9 ± 0.13	38.5 ± 0.05
Methanol, ethanol, chloroform	>100	>100
Miglyol 812, castor oil	>50	>500

Values are mean solubility (in mg/ml) ± SD of at least three independent sets of experiments.

(Table 1). This was also observed for ibuprofen, although its solubility in buffered solutions was significantly (6-fold) lower than the solubility of ibuprofen–OHB. The solubility of ibuprofen–OHB in 0.1 M HCl as a gastric fluid surrogate was 36 ± 6 μg/ml. Solvent pH had a significant influence on solubility ($P < 0.05$, Kruskal–Wallis test). In all cases, Dunn's test confirmed differences between solubility in all tested media and water, 0.01 M HCl and pH 8.0 buffer. Only the differences between buffers of similar pH values (6.0 and 7.0; 7.0 and 7.5) were not significantly different.

Hydrolysis studies

Figure 3 illustrates the rate of hydrolysis of ibuprofen–OHB in buffers of pH 6.0, 7.0, 7.5 and 8.0 and in simulated intestinal fluid containing pancreatin (pH 6.8). The initial amount of ibuprofen–OHB in each solution was 20 mg, corresponding to 7.24 mg ibuprofen. The average rate of ibuprofen release in different media varied from 0.54 μg/h at pH 6 to 1.7 μg/h at pH 8. The average ibuprofen release rate was significantly higher (13.5 μg/h) in the presence of pancreatin.

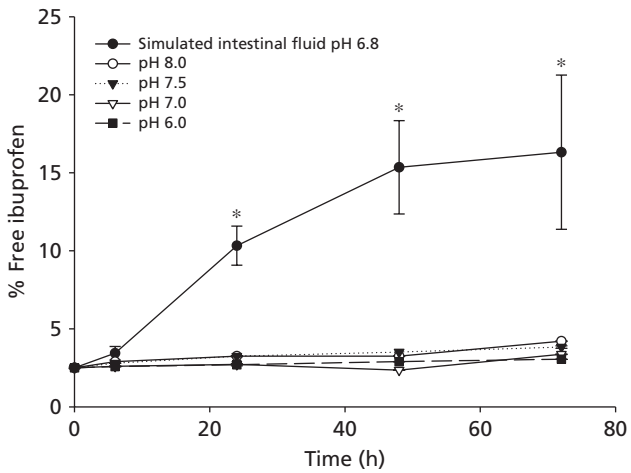

Figure 3 Hydrolysis profile of the conjugate of ibuprofen with oligomers of 3-hydroxybutyric acid in buffered aqueous solutions (pH 6–8) and in pancreatin solution (5 g/l; pH 6.8). Values are means ± SD of at least three independent sets of experiments (*$P < 0.05$; Kruskal–Wallis one-way analysis of variance followed by Dunn's post-hoc test).

In the absence of enzymes, virtually no hydrolysis occurred (Figure 3). The Kruskal–Wallis test revealed statistically significant differences at each time point; however, only the differences in hydrolysis profiles between buffered solutions and simulated intestinal fluid were unambiguously confirmed by Dunn's test.

Properties of ibuprofen–OHB formulations

Table 2 shows the result of stability studies. Concentrations of ibuprofen–OHB, size of the oil droplets in emulsion and pH values did not change significantly during the course of the stability test. In addition, no optical signs of physical instability were noticed.

Ibuprofen-OHB release profiles from the prepared formulations and the pure substance are presented in Figure 4, revealing significant differences between the release profiles of the pure substance and the investigated formulations at 24 and 48 h.

Discussion

The structure of ibuprofen–OHB was confirmed using ESI-MS, HPLC, FT-IR and ^{1}H NMR spectroscopy. The low molecular weight of the compound (n = 3–7) makes it suitable for relatively fast degradation *in vivo*. The developed HPLC method allowed determination of the unconjugated (released) ibuprofen as well as that in the ibuprofen-OHB conjugate. The method was used for determination of ibuprofen-OHB solubility, hydrolysis and stability.

Because of the low solubility of ibuprofen–OHB in aqueous solutions and high solubility in castor oil and Miglyol 812, the conjugate could be suggested as a candidate for emulsion and oily solution formulations. Stability of ibuprofen–OHB in the prepared formulations during thermal sterilisation as well as at storage conditions has been confirmed. The results also indicated that ibuprofen–OHB undergoes enzymatic hydrolysis, but the process is slow, with only 13.8% of the total conjugated ibuprofen being released after 72 h' incubation. A similar trend in peak area change was observed in chromatograms of the samples incubated at 37°C with and without pancreatin. Increased peak area of ibuprofen and ibuprofen–OHB of low molecular weight (peak 7.9 min, Figure 2a) coincides with the decrease of a peak area representing longer chains of oligomer (t_R 16.9 min and 41.3/42.5 min), suggesting that the enzymatic- or pH-dependent hydrolysis of the oligomer chain starts at the far end from the ibuprofen moiety of the molecule.

Consistent with the results obtained by Zhao and colleagues[12] suggesting that ester prodrugs of ibuprofen with glucopyranoside undergo slow hydrolysis at physiological pH, we demonstrated that stability of ibuprofen–OHB may be suboptimal for the oral delivery of the conjugate because of the presence of a strong ester bond between the active molecule and polymer chain. According to Davies,[13] the maximum concentration of ibuprofen in plasma following administration of a typical dose (200–600 mg) via different routes exceeds 20 mg/l and occurs after 1–2 h. In order to achieve similar concentrations, approximately

Table 2 Stability of the conjugate of ibuprofen with oligomers of 3-hydroxybutyric acid (OHB) formulations over 90 days

Preparation	Time of measurement (days)		Ibuprofen–OHB concentration (mg/ml)	pH	Size of oil droplets (μm)*	
					d(0.5)	d(0.9)
Solution in Miglyol 812	0	BS	248.7 ± 6.50	ND	ND	ND
(250 mg/ml)	0	AS	246.2 ± 6.35	ND	ND	ND
	7		242.9 ± 10.15	ND	ND	ND
	30		243.0 ± 8.50	ND	ND	ND
	90		239.9 ± 9.95	ND	ND	ND
Oil–water emulsion	0	BS	100.3 ± 0.90	6.12 ± 0.05	1.46 ± 0.09	2.59 ± 0.18
(100 mg/ml)	0	AS	100.1 ± 1.05	6.10 ± 0.06	1.35 ± 0.05	2.5 ± 0.2
	7		99.1 ± 1.00	6.05 ± 0.08	1.72 ± 0.15	2.75 ± 0.25
	30		88.6 ± 8.05	6.11 ± 0.07	1.35 ± 0.14	2.82 ± 0.21
	90		97.0 ± 7.5	6.10 ± 0.09	1.38 ± 0.14	2.98 ± 0.25

Values are means ± SD ($n \geq 3$ independent sets of experiments). d(0.5), d(0.9), upper limits of diameter for 50% and 90% of droplets, respectively. AS, after sterilisation; BS, before sterilisation; ND, not determined.

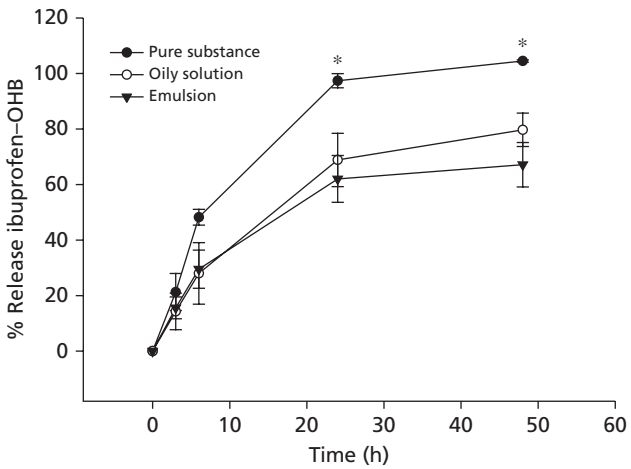

Figure 4 Release profiles of ibuprofen conjugated with oligomers of 3-hydroxybutyric acid from the pure substance, triglyceride solution (Miglyol 812) and oil–water emulsion. Values represent means ± SD of at least three independent sets of experiments (*$P < 0.05$; Kruskal–Wallis one-way analysis of variance followed by Dunn's post-hoc test).

100 mg of ibuprofen would have to be released from ibuprofen–OHB directly after administration, which would require a large dose of the conjugate. A possible solution may be to insert a spacer group between the drug molecule and the OHB moiety. Several groups have demonstrated that this strategy accelerates the hydrolysis, and hence the amount of available drug increases to a level that allows for effective treatment.[7,14] However, the approach proposed for ibuprofen-OHB might be suitable for other drugs for which a lower therapeutic dose released in a sustained manner is required. The evaluated compound may be a model substance, particularly for parenteral or ocular administration.

Conclusions

Slow hydrolysis of ibuprofen–OHB at physiological conditions disqualifies it from use in oral dosage forms. However, drug–OHB conjugates may be potential prodrug candidates for parenteral administration or topical applications that require sustained release. Good solubility in pharmaceutically acceptable oils allows for preparation of solutions and emulsions. Stability and drug-release profiles of these are currently being investigated in in-vivo studies in a rabbit model.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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