SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF CHLORAMPHENICOL DERIVATIVES BASED ON N-[2- HYDROXYPROPYL]METHACRYLAMIDE COPOLYMERS HAVING OLIGOPEPTIDES SPACERS

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With its braod range of antimicrobial activity, chloramphenicol (CAP) is one of the most commonly-used antibiotices in medical practice [1-3]. As has been reported in previous communications [4, 6, 7, 12], several polymeric CAP derivatives have been obtained with a view to prolonging the effect and reducing the toxicity of the agent, and increasing its solubility in water. These include N-vinylpyrrolidone, acrylamide, methacrylic acid and 2-(dimethylamino)ethylmethacrylate copolymers containing CAP ester chains formed from maleic, acrylic, methacrylic and crotonic acids [4, 6, 12], CAP-modified polyacrylic anhydride [4] and xanthane polysaccharide [7], and poly-1-chloramphenicol-(2-methacryloylhydroxyethyl)dimethylammonium chloride [12]. Cross-linked polymeric CAP derivatives have also been synthesized [10].

The antimicrobial activity of the low-molecular CAP ester derivatives is dictated by their ability to release antibiotic in the free form [2, 3], the same phenomenon being observed for the known CAP polymeric conjugates. Their in vitro activity increases with the degree of hydrolysis of the polymer–antibiotic ester bond [4] and does not exceed the activity of the original antibiotic [4, 12].

Polymeric CAP conjugates have prolonged antibiotic activity for both in vitro and in vivo applications (tests on mice and dogs) [4, 6, 7, 12]. The derivatives based on a copolymer of N-vinylpyrrolidone and crotonic acid exhibited a therapeutic effect in vivo, specifically with respect to colon bacillus sepsis [6], although here too in vivo activity did not exceed that of the original antibiotic.

In recent years conjugates based on N-(2-hydroxypropyl)methacryl-amide (HPMA) copolymers, in which the drug (bacteriostatic) is linked with the polymer chain through oligopeptide spacers, have found wide application in providing the requisite transfer of medicines in the organism. HPMA copolymers are water-soluble and biocompatible. The presence of an oligopeptide bridge ensures the cleavage of the active base inside the cells, which the conjugates enter as a result of pinocytosis in the presence of lysosomal enzymes [8, 11, 14]. As the cleavage rate and the biological activity of the conjugates are dictated by the structure of the oligopeptide spacer, the way is opened up to synthesizing derivatives in which the rate of release of the drug inside the cells of the living organism can be modified [8, 11, 14]. The biological activity of such antimicrobial substance conjugates has not previously been investigated.

In the current work we have endeavoured to synthesize chloramphenicol derivatives based on these HMPA copolymers and examine their antimicrobial properties.

The polymeric CAP derivatives based on HMPA copolymers were synthesized by reacting the hydrochloride of a CAP glycine derivative with reactive chains of N-methacryloylpeptide p-nitrophenyl ester copolymers in the presence of triethylamine by the following route:

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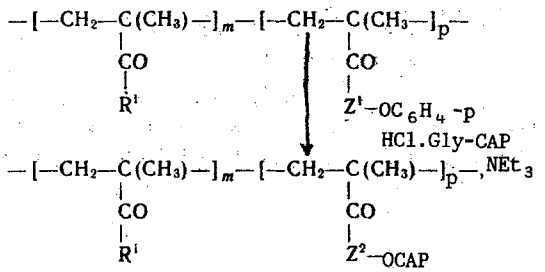
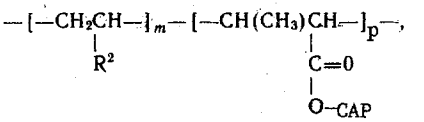
TABLE 1. Polymeric Derivatives of Chloramphenicol (CAP) Antibiotic

Polmer–carrier			Polymer conjugate			
Polymer	p-nitrophenyl ester chain content, mole %	$\bar{M}_w/\bar{M}_n$	spacer	CAP content		percentage conversion
				wt. %	mol. %	
HPMA-MA-Gly-Gly-p-NPE	11,2	$\frac{17\,000}{13\,000}=1,3$	Gly-Gly-Gly	11,6	6,7	60
HPMA-MA-Gly-Leu-p-NPE	7,6	$\frac{19\,000}{13\,000}=1,5$	Gly-Leu-Gly	5,6	2,9	38
HPMA-MA-Gly-Pal-p-NPE	10,1	$\frac{17\,000}{13\,000}=1,3$	Gly-Pal-Gly	8,1	4,4	44
VP-CA	11,5	$\frac{25\,000}{15\,000}=1,7$	—	1,3	3,8	11



where R = $NHCH_2CH(OH)CH_3$; Z^1 = Gly-Gly, Gly-Leu or Gly-Pal; Z^2 = Gly-Gly-Gly, Gly-Leu-Gly or Gly-Pal-Gly.

As a result, conjugates were obtained in which CAP was separated from the main polymer chain by tripeptide bridges of varying structure. In addition the synthesis was performed of a CAP derivative based on a copolymer of N-vinylpyrrolidone-2 (VP) and crotonic acid (CA), in which the link with the main chain was made without an oligopeptide spacer:



where R^2 = –N-α-pyrrolidone.

The characteristics of the initial polymer–carriers and CAP polymeric conjugates are shown in Table 1. Some 40-60% of all the HPMA copolymer p-nitrophenyl ester groups reacted with CAP. Molecular weight parameters, namely the low molecular weights ($\bar{M}_w$ and $\bar{M}_n$) and the narrow molecular-weight distribution ($\bar{M}_w/\bar{M}_n$) of the polymers, met the requirements for biologically active polymers.

EXPERIMENTAL (CHEMICAL)

Hydrochloride of CAP Glicine Derivative (HCl·Gly-CAP). The CAP N-tert-butoxyglycine derivative (BOX-Gly-CAP) was obtained by reacting 3.6 g (0.2 mmoles) of N-tert-butoxycarbonylglycine (Fluka) with 6.4 g (0.2 mmoles) of CAP in 60 ml of tetrahydrofuran solution in the presence of 4.2 g (0.2 mmoles) of dicyclohexylcarbodimide. The mixture was stirred for 2 h at –5°C and for 8 h at 20°C. After the resultant precipitate for dicyclohexylurea had been removed, the solvent was distilled off. The oily product was purified in a column (d 1 cm, 18 cm) filled with Kieselgel-60 gel (Fluka) using a 2:1 acetone–hexane mixture (R_f of 0.8) as the elutriating agent. Elemental analysis data for the product was in line with calculated values for BOX-Gly-CAP. (Yield 50%.)

To eliminate the protective BOX-group 4 g (0.084 mmoles) of BOX-Gly-CAP were dissolved in 80 ml of a 20% solution of hydrogen chloride in methanol. After the reaction mixture had been stirred for 1 h at room temperature, the solvent was driven off and the product was dried in vacuum over P_2O_5. The oily substance (50% yield) was used for binding CAP with the polymers.

TABLE 2. In Vivo Antistaphylococcus Activity of CAP and Its Polymeric Derivatives for Intraperitoneal Injection

Compound	Suppression Index (SI)		
	CAP dose, μg/mouse		
	10	100	1000
CAP	0	0	57
VP-CA-CAP	0	0	33
HMPA-MA-Gly-Gly-Gly-CAP	0	0	53
HPMA-MA-Gly-Leu-Gly-CAP	0	38	77

HPMA was synthesized using the method described in [13].

p-Nitrophenyl Esters (p-NPE) of N-Methacryloylglycylglycine (MA-Gly-Gly), N-Methacryloylglycylleucine (MA-Gly-Leu), and N-Methacryloylglycylphenylalanine (MA-Gly-Pal) were obtained using the techniques outlined in [9].

Copolymers of HPMA and N-Methacryloyldipeptide p-Nitrophenyl Esters were obtained by radical copolymerization in acetone at 50°C [9]. The concentration of the monomers was 13 wt.% and that of the initiator, azoisobutyric acid dinitrile, 4.5% by weight of the monomers. After precipitation the copolymer formed in the polymerization reaction was collected, washed several times with acetone and diethyl ether and dried to constant weight.

Synthesis of CAP Polymeric Derivatives. 1. To a solution of 2.4 g of the copolymer formed from HPMA and MA-Gly-Gly-p-NPE, containing 0.528 g (0.0153 mmoles) of p-NPE chains, in 9 ml of dimethylsulfoxide were added 0.7 g (0.0168 mmoles) of HCl·Gly-CAP and 0.17 g (0.0168 mmoles) of triethylamine. The mixture was stirred for 24 h at 25°C, then 1-aminopropanol-2 was added to remove unreacted p-NPE groups. The polymer was precipitated by pouring into acetone and repricipitated from methanol into acetone. Subsequently the low-molecular components were removed from the polymeric CAP derivative in a column packed with Sephadex H-20 (d 3 cm, l 40 cm) using methanol as elutriator. After the polymer fractions had been collected and the solvent had been evaporated in vacuum, the polymer was dissolved in water and freeze-dried. A yield of 2.3 g (85%) of polymeric derivative was obtained. The other CAP derivatives based on HPMA copolymers were synthesized under the same conditions in yields of 82-87%.

2. A sample of 2 g of the copolymer formed from N-vinylpyrrolidone (VP) and crotonic acid (CA), containing 0.183 g (0.021 mmoles) of acid chains, was dissolved in 10 ml of dimethylformamide. To this was added 0.678 g (0.021 mmoles) of CAP and 0.438 g (0.021 mmoles) of dicyclohexylcarbodiimide. The mixture was stirred for 4 h at −5°C and for 20 h at room temperature. After the resultant precipitate of dicyclohexylurea had been removed, the polymer was precipitated by pouring it into diethyl ether. It was then reprecipitated from dimethylformamide into ether and purified in a column packed with Sephadex G-50 (3 × 85 cm) using water as the elutriating agent. The polymer fractions were collected and freeze-dried, a yield of 1.85 g (89%) of polymeric derivative being obtained.

The composition of the polymeric CAP conjugates was determined by spectrophotometry in dimethylsulfoxide solution, with λ_{max} of 278 nm and ε of 8900 liters·mole^{-1}·cm^{-1}, and from chlorine content using elemental analysis.

Molecular weight characteristics of the HPMA copolymers were determined by means of gel permeation chromatography in a column (16 × 1000 mm) packed with a 1:1 mixture of Sepharose 4B and 6B gels at 37°C. The column was calibrated from narrow poly-HPMA fractions, with tris-buffer (pH 8.0) elutriator. A Knauer-205 differential refractometer was employed in the experiments. The p-NPE reactive groups of the copolymers were removed beforehand by aminolysis using 1-aminopropanol-2 in dimethylsulfoxide solution.

Molecular weight parameters of the VP-CA copolymer were determined with a GPC II chromatograph (Millipore Waters). A series of linked columns packed with Gel G 6000-PW and G 3000-PW (7.5 × 600 mm) were used with a 0.1 M sodium acetate solution as elutriator. Calibration was performed from narrow polyvinylpyrrolidone fractions. Copolymer carboxyl groups were preconverted into methylcrotonate groups by reacting them with diazomethane.

EXPERIMENTAL (BIOLOGICAL)

The in vitro activity of the preparations was examined using the serial dilution method. One-day broth (beef extract/peptone broth, BPB) cultures of Staphylococcus aureus 209P were added to test tubes containing the diluted preparation (0.1 ml of microbial suspension, 500,000 microorganisms per ml). The test tubes were kept in a thermostat at 37°C for 24 h, then seeding in sectors was carried out in Petri dishes containing a beef extract/peptone agar (BPA). After the dishes had been kept in a thermostat at 37°C for 24 h, the presence or absence of growth in the sectors was noted. Test tubes containing the culture and BPB as the control.

In vivo activity of the preparations was tested on CBA line mice weighing 18-20 g (15 animals per group), which received intraperitoneal injections of 10^9 microorganisms of Staphylococcus aureus (209P strain). Three hours after infection the animals were given an intraperitoneal injection of CAP or its polymeric derivative in isotonic sodium chloride solution. The control group received the same amount of isotonic solution. Twenty four hours later a peritoneal lavage was carried out using 5 ml of a sterile isotonic solution. Then 1 ml of the lavage solution was centrifuged for 15 min at 3000 rev/min. The precipitate was washed twice and resuspended in 0.1 ml of isotonic sodium chloride solution. This suspension was then applied evenly to a solid nutrient medium (BPA) and following careful drying at 37°C liquefied BPA was poured over it. After solidification the dishes were kept at 37°C for 48 h, then the number of cultivated colonies was counted.

In order to assess the activity of the preparations a suppression index (SI) was calculated from the equation:

$$SI = \frac{N_C - N_T}{N_T} \cdot 100,$$

where N_C and N_T are the number of microorganisms in the retrieved lavage solution of the control and test groups respectively.

The original CAP registered a bacteriological effect in vitro in concentrations of between 1 and 10 μg/ml and a bactericidal effect in doses 10-15 times the bacteriostatic ones. All the synthesized polymeric CAP derivatives possessed low antimicrobial activity in vitro and inhibited the growth of microorganisms in concentrations of over 2000 μg/ml reckoned against CAP. After a twenty-four-hour preliminary hydrolysis at 37°C and pH 7.5 the activity of the compound in which CAP is linked to the copolymer via a Gly-Pal-Gly bridge underwent a significant increase, and approached that of the original antibiotic. The results obtained were in agreement with literature data, underlining the fact that the activity of CAP ester derivatives is dependent on the level of hydrolysis.

As Table 2 shows, CAP produced a pronounced antimicrobial effect in vivo in dosage of 1000 μg/mouse. The compound in which CAP is attached directly to the polymer chain without an oligopeptide spacer (VP-CA-CAP) possessed a lower activity than the unmodified CAP, while the activity of the derivative with the Gly-Gly-Gly spacer was comparable to that of the low-molecular CAP. However, the most interesting result was obtained for the derivative with the Gly-Leu-Gly spacer. The activity of this compound was greater than that of the original CAP and was registered at a lower antibiotic dose, namely 100 μg/mouse.

In a previous report [15] Ulbrich and co-authors investigated the enzymatic and noncatalytic hydrolysis of polymeric CAP conjugates. It was found that, as in the case of bacteriostatic derivatives linked to oligopeptide spacers via an amide bond [8, 11, 14], for CAP ester conjugates the hydrolysis rate in the presence of one of the most important lysosomal enzymes (cathepsin B) is determined by the nature of the oligopeptide bridge. In the case of the Gly-Leu-Gly spacer conjugate the enzyme hydrolysis rate was considerably greater than both the noncatalytic hydrolysis rate with physiological pH values, and the hydrolysis rate of the Gly-Gly-Gly spacer CAP derivative when cathepsin B was present.

To sum up, the investigation gave rise to a CAP derivative, whose antimicrobial (antistaphylococcus) activity was exhibited in vivo after intraperitoneal administration, exceeding that of the original antibiotic under similar conditions. It may be suggested that the resultant in vivo activity of the Gly-Leu-Gly spacer compound arose from the favorable spacer structure, which ensures effective and gradual removal of CAP from the polymer–carrier directly

in the peritoneal cavity. It would be of interest to study the mechanism of this process with regard to data on staphylococcus phagocytosis and the permeation of the test compound into cells.

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